Decreasing methane yield with increasing food intake keeps daily methane emissions constant in two foregut fermenting marsupials, the western grey kangaroo and red kangaroo

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Abstract
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ABSTRACT

Fundamental differences in methane (CH₄) production between macropods (kangaroos) and ruminants have been suggested and linked to differences in the composition of the forestomach microbiome. Using six western grey kangaroos (Macropus fuliginosus) and four red kangaroos (Macropus rufus), we measured daily absolute CH₄ production in vivo as well as CH₄ yield (CH₄ per unit of intake of dry matter, gross energy or digestible fibre) by open-circuit respirometry. Two food intake levels were tested using a chopped lucerne hay (alfalfa) diet. Body mass-specific absolute CH₄ production resembled values previously reported in wallabies and non-ruminant herbivores such as horses, and did not differ with food intake level, although there was no concomitant proportionate decrease in fibre digestibility with higher food intake. In contrast, CH₄ yield decreased with increasing intake, and was intermediate between values reported for ruminants and non-ruminant herbivores. These results correspond to those in ruminants and other non-ruminant species where increased intake (and hence a shorter digesta retention in the gut) leads to a lower CH₄ yield. We hypothesize that rather than harbouring a fundamentally different microbiome in their foregut, the microbiome of macropods is in a particular metabolic state more tuned towards growth (i.e. biomass production) rather than CH₄ production. This is due to the short digesta retention time in macropods and the known distinct ‘digesta washing’ in the gut of macropods, where fluids move faster than particles and hence most likely wash out microbes from the forestomach. Although our data suggest that kangaroos only produce about 27% of the body mass-specific CH₄ of ruminants, it remains to be modelled our data suggest that kangaroos only produce about 27% of the body mass-specific CH₄ of ruminants, it remains to be modelled in CH₄ emission found among different groups of herbivores even when standardized by body mass (Franz et al., 2010, 2011b).

The complex foregut of macropods consists of a colon-like tubular morphology and is divided into a saccoform and a larger tubiform region (Hume, 1984; Langer et al., 1980). Microbial fermentation occurs in both regions (Hume, 1984). Although several studies suggested that macropods produce very little CH₄ in comparison to ruminants (Dellow et al., 1988; Kempton et al., 1976; Madsen and Bertelsen, 2012; von Engelhardt et al., 1978), the variety of the methodologies used complicates comparison of data. von Engelhardt et al. (1978) and Hume (1999) mainly attributed the presumably low CH₄ emissions of macropods to their comparatively short digesta passage time. Other groups investigated the foregut microbiome in order to find an explanation. Ouwerkerk et al. (2005) and Gulino et al. (2013) identified a diverse and complex bacterial ecosystem consisting of several known, but also of approximately 50% novel genera with still unknown function. Ciliate protozoa and fungi were also found in similar density levels to those in the rumen (Dellow et al., 1988). Reductive acetogens that reduce hydrogen to acetate were found to be the main hydrogen sink in macropods, supporting the assumed low CH₄ emissions (Gagen et al., 2010; Godwin et al., 2014; Klieve, 2009; Ouwerkerk et al., 2009). Methanogenic archaea were also present, but in much lower density than in the rumen – that is, up to 1000-fold less (Evans et al., 2009; Klieve et al., 2012). The density of archaea in the foregut seems to be highly dependent on the individual animal and its


KEY WORDS: Macropod, CH₄, Forestomach, Digesta washing, Digestion, Fermentation, Methanogenesis

INTRODUCTION

Methane (CH₄) is a potent greenhouse gas (GHG), accounting for 16% of total anthropogenic GHG emissions in 2010, second only to CO₂ (IPCC, 2014). From total emissions, 28% originates from ruminant livestock as the largest source (Klieve, 2009). Enteric CH₄ is generated by archaea through reducing hydrogen, which is a by-product of microbial fermentation of plant material, especially fibre, in the main fermentation chamber of the forestomach (i.e. the rumen), and which must be removed in order to maintain efficient fermentation (Stevens and Hume, 1998). As a consequence of their high global relevance, enteric CH₄ emissions are well studied for domestic ruminants such as cattle and sheep as well as, on a smaller scale, for hindgut fermenters such as equids and pigs. Ruminants produce the highest amounts of CH₄ in relation to their body mass (Franz et al., 2010; Hironaka et al., 1996; McCaughey et al., 1999). Although other pathways exist for the utilization of enteric hydrogen (Morvan et al., 1996; Pope et al., 2011), methanogenesis is the main hydrogen sink in ruminants. However, drivers determining the dominating type of enteric hydrogen sink are still poorly understood (Klieve, 2009; Morvan et al., 1996). In this respect, the presence of a complex foregut such as the reticulorumen may be beneficial for Archaea. Thus, as they share this anatomical feature, non-ruminating foregut fermenters such as hippopotamids, peccaries, sloths, macropods and colobine monkeys are interesting target species to investigate biological drivers causing the large variation in CH₄ emission found among different groups of herbivores even when standardized by body mass (Franz et al., 2010, 2011b).
species, with *Macropus rufus* harbouring densities below detectable limits (Klieve et al., 2012). Furthermore, the detected archaea appeared to be novel with some presumably not being methanogenic, as PCR assays used to target the functional mcrA gene, known to be associated with methanogenesis in ruminants, failed (Klieve et al., 2012). However, it appears also possible that the low CH₄ emission of macropods is simply the result of their generally lower metabolism (McNab, 1986; Munn and Dawson, 2003) and lower food intake (Munn et al., 2008) compared with ruminants. This phenomenon has been demonstrated before for animals with a lower metabolic rate and hence lower food intake, which emit correspondingly less CH₄, namely, for tortoises compared with mammalian hindgut fermenters (Franz et al., 2011a; with the probable additional effect of a lower body temperature in the reptiles reducing microbial activity) and for camelids compared with ruminants (Dittmann et al., 2014a,b).

In order to enlarge the database on macropod methane emission and to address the open questions, we experimentally investigated in vivo CH₄ production as the target variable and metabolic rate, food and energy intake and fibre digestibility as explanatory variables. This was assessed on two levels of food intake and in two different species. In particular, we expected absolute CH₄ production as well as CH₄ production per unit food or energy intake to be within the range of non-ruminant mammals when corrected for body mass. Additionally, because an increase in food intake typically reduces the time digesta is retained in the gut and hence is subjected to microbial digestion, we expected a lower CH₄ production per unit food intake at the higher food intake level.

**MATERIALS AND METHODS**

The experiment was conducted under University of New South Wales (UNSW) animal care and ethics committee (ACEC) permit no. 11/118A and 14/97B. Studies were undertaken at the UNSW Ardil Zone Research Station at Fowlers Gap (31°05’S, 141°43’E), western New South Wales, Australia, from late June to September 2014 (i.e. austral winter–early spring). Six mature female western grey kangaroos (*Macropus fuliginosus Desmarest 1817*) and three mature female and one immature female red kangaroo (*Macropus rufus Desmarest 1822*) were used. Five of the *M. fuliginosus* were caught from the wild 4 months in advance to allow them to acclimatize to human handling. One *M. fuliginosus* and all four *M. rufus* were hand-reared and thus used to be handled by humans. The kangaroos were kept as a group in an enclosure of about 4 hectares. Two study animals at a time were transferred to individual outdoor pens (1.40 m×1.20 m=1.68 m²) for a 2 week acclimation period. During this period the animals were fed *ad libitum* exclusively on chopped lucerne hay. Four days prior to the measurements, feed allowance was set to a level covering 75% of maintenance energy requirement (MER) of 385 kJ digestible energy kg⁻⁰.⁷⁵ day⁻¹ (Munn and Dawson, 2003). Animals had *ad libitum* access to drinking water at all times.

After the acclimation period, animals were transferred to indoor metabolism cages. Before the kangaroos were moved, they were anaesthetized by an intramuscular injection of a mixture of Zoletil (50% tiletamine and 50% zolazepam, 100 mg ml⁻¹; used dosage: 1 ml per 20 kg body mass, *M. f*; Virbac Animal Health, Milperra, Australia) and Pamlan (diazepam, 5 mg ml⁻¹; used dosage: 2 ml per 20 kg, *M. r*; Ceva Animal Health Pty Ltd, Glenorie, Australia). This was also done when transferring the kangaroos back to their enclosure at the end of the measurements. These two periods of immobilization were also used for weighing.

Respirometry was conducted in two separate metabolism cages (dimensions: 3.06 m³ and 2.66 m³) placed in a temperature-regulated room (25–30°C). The metal mesh cages were sealed with walls consisting of corrugated plastic panels. The cages were large enough for the animals to lie, stand and stand upright, and to turn around freely but not to leap. The floor of the cages consisted of metal mesh covered by a rubber mat with holes of a diameter of 4.5 cm, allowing urine and faeces to pass through for collection. Faeces and urine were collected every 12 h from slide trays under each cage. Faeces were caught on a mesh grid, and urine was collected underneath the grid in the trays and funnelled to collection tubes. Lucerne hay and fresh water were provided in food hoppers that could be replenished from the outside via a lid without opening the whole front of the chambers; the slits around the lid were sealed with insulation tape after refilling the hoppers. The respiration chambers were fitted with air inlets of 4 cm diameter on the bottom front and air outlets on the top to ensure a constant airflow generated by a pump (Flowkit 100; Flowkit 500, Sable Systems, Las Vegas, NV, USA). Out-flowing air was ducted via flexible hoses to a gas multiplier, which allowed the simultaneous measurement of two individuals and recording of baseline values from ambient air, at intervals of 600 s per chamber and 300 s for the baseline data. Gas concentrations were measured by O₂ and CO₂ analysers (Foxbox, Sable Systems). Methane was measured by a CH₄ analyser (MA-10, Methane Analyzer, Sable Systems). Data were adjusted for temperature, air flow rates and barometric pressure. Air flow rates and barometric pressure were constantly recorded during respirometry (Foxbox, Sable Systems). The airflow produced by the pumps averaged 45–50 l. Gas analysers were manually calibrated with calibration gases (pure nitrogen gas, and a mixture containing 20.90% O₂, 0.50% CO₂, 0.50% CH₄ dissolved in N₂) at the beginning of each measurement period. Data obtained by the respirometry system were analysed with ExpeData software (Sable Systems) for O₂ consumed and CH₄ and CO₂ emitted after correcting for gas input calculated from flow and concentrations of incoming air. The metabolic rate was calculated based on two 23 h measurement periods, therefore accounting for the activity of the animals inside the chamber. Resting metabolic rate (RMR) of the animals was calculated as the average of the 20 lowest O₂ measurements per individual within the entire measurement (adapted from Derno et al., 2005). In order to estimate metabolic rate, we multiplied the amount of O₂ consumed (in 1 h⁻¹) by 20.08 kJ (McNab, 2008). Volume measures of CH₄ were transformed into energy using the conversion factor of 39.57 kJ l⁻¹ (Brouwer, 1965). Methane production was expressed in absolute values, as body mass-specific values, and as CH₄ yield in relation to dry matter intake (DMI), gross energy intake (GEI), digestible energy intake (DEI) and intake of digestible neutral detergent fibre (dNDF).

Once in the respiratory chamber, each animal was successively fed at two different food intake levels: 75% of MER and *ad libitum*. During the 75% food intake level, the animals were fed at 09:00 h and 19:00 h. During the period of *ad libitum* food intake, food hoppers were checked every 2 h and opened and refilled, if required. Animals were allowed to acclimatize to the cages for 3 days before the start of measurements, and in all cases faecal output had stabilized prior to the beginning of data collection. This allowed the animals to return to a normal food intake level despite the unfamiliar housing conditions, and resulted in a stable faecal output to food intake ratio. Another 3 days of adaptation were included when switching from the restricted to the *ad libitum* food intake level. After each 3 day adaptation, 3 days of measuring food intake and faecal production including two cycles of 23 h respirometry were performed per food intake level. Although the
typical number of days for subsequent measurements of intake and defaecation is 5 for herbivores, a shorter 3 day period was accepted to reduce the amount of time animals had to spend in the metabolism cages, and was considered acceptable because of the comparatively short mean retention time (MRT) in macropods (e.g. 30 h for particles in adult M. rufus in Munn and Dawson, 2006). At 09:00 h the measurements were always stopped for a break of 1 h to allow the opening of the chambers for feeding, collecting faeces and urine, and removing residual uneaten lucerne hay.

Food intake, faecal and urinary output and residual hay were measured on a daily basis. Samples of chopped lucerne hay, leftovers and faeces were immediately dried at 60°C and ground to 0.75 mm with a centrifuge mill (Dayton Electric Manufacturing Co., Niles, IL, USA). Standard nutrient analyses (AOAC, 1995) were carried out, including the determination of content of dry matter and total ash (AOAC no. 942.05), crude protein (CP, AOAC no. 977.02), neutral detergent fibre (NDF, AOAC no. 2002.04), acid detergent fibre and acid detergent lignin (ADF and ADL, AOAC no. 973.18). For NDF analysis, α-amylase was used. Fibre data were expressed without residual ash. Gross energy (GE) was determined by bomb calorimetry (IKA-Calorimeter C4000, Ika, Stauffen, Germany). All analyses were performed in duplicate. Diet composition and nutrient analysis (AOAC, 1995) were carried out, including the determination of crude protein (N x 6.25), crude ash, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), carbohydrates (XY), starch and crude fat. All nutrients were calculated as the percentage of the respective intake not recovered in the leftovers, and the corresponding amounts offered or recovered, respectively. The apparent digestibility for dry matter, nutrients and energy was calculated as the percentage of the respective intake not eliminated via faeces (Robbins, 1993). Results were compared between macropod species and intake levels by two-way ANOVA that always included the interaction between the two factors (species × intake level). Because there was never a significant two-way ANOVA that always included the interaction between the two factors and energy was calculated as the percentage of the respective intake not recovered in the leftovers, and the corresponding amounts offered or recovered, respectively. The apparent digestibility for dry matter, nutrients and energy was calculated as the percentage of the respective intake not eliminated via faeces (Robbins, 1993).

RESULTS

The nutrient composition of the lucerne hay as ingested is shown in Table 1. Hay fed at 75% MER was completely consumed by all kangaroos except when offered the ad libitum diet (at amounts where 72±12% of the amount offered was ingested), kangaroos tended to select lucerne hay particles with lower NDF concentration (NDF in hay offered: 48.5±0.7% versus NDF in hay leftover: 50.7±2.3%; Wilcoxon test, P=0.059). When changing from the restricted food to ad libitum offer, food intake and DEI were significantly increased in both species to more than 1.5-fold (Table 2). Daily faecal excretion doubled with the increased feeding level. Faecal dry matter concentration was not significantly affected by food intake level, consistently accounting for 37–44% of faecal wet mass. Also, no significant difference in apparent digestibility of dry matter, crude protein or NDF was noticed (Table 2). The RMR was higher on the ad libitum intake level, as was also true for CO₂ production and the respiratory quotient (Table 3).

Macropus fuliginosus had a significantly higher body mass than M. rufus, but there were no species differences in feed intake and digestion variables measured (Table 2). In addition, the absolute O₂ consumption and CO₂ production were significantly higher in M. fuliginosus than in M. rufus (Table 3).

Examples of diurnal patterns of O₂ consumption and CO₂ and CH₄ emission in a M. fuliginosus (Fig. 1A,B) and a M. rufus (Fig. 1C,D) specimen during the ad libitum regimen illustrate the fluctuations in the level of metabolic energy use and in CH₄ production over the day. In some cases, parallel peaks in O₂ consumption/CO₂ emission and CH₄ emission were evident (Fig. 1). No significant relationship was found between food intake level and absolute daily CH₄ production (Table 2, Fig. 2A). In contrast, there were significant differences in CH₄ yield (CH₄ per unit of DMI, GEI and dNDFi) between intake levels (Table 3), and significant negative correlations between intake level and CH₄ yield expressed as CH₄ production either per unit of DMI (σ=−0.48, P=0.032; Fig. 2B) or per unit of GEI (σ=−0.48, P=0.032). CH₄ produced per unit dNDFi, however, did not differ significantly between intake levels (Table 3), although there was a trend for a negative correlation between intake level and CH₄ produced per unit dNDFi (σ=−0.42, P=0.064; Fig. 2C). At ad libitum food intake, CH₄ yield of macropods was similar to that reported for horses (Fig. 2B,C).

### Table 1. Mean (±s.d.) nutrient concentrations found in the lucerne hay as actually ingested for the 75% MER and ad libitum food intake periods

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>75% MER</th>
<th>Ad libitum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (g kg⁻¹ as fed)</td>
<td>832±29</td>
<td>832±29</td>
</tr>
<tr>
<td>Total ash (g kg⁻¹ DM)</td>
<td>97±2</td>
<td>98±3</td>
</tr>
<tr>
<td>Crude protein (g kg⁻¹ DM)</td>
<td>202±8</td>
<td>210±11</td>
</tr>
<tr>
<td>Neutral detergent fibre (g kg⁻¹ DM)</td>
<td>502±20</td>
<td>477±15</td>
</tr>
<tr>
<td>Acid detergent fibre (g kg⁻¹ DM)</td>
<td>32±2</td>
<td>–</td>
</tr>
<tr>
<td>Acid detergent lignin (g kg⁻¹ DM)</td>
<td>83±6</td>
<td>–</td>
</tr>
<tr>
<td>Gross energy (kJ g⁻¹ DM)</td>
<td>18.9±0.2</td>
<td>18.8±0.1</td>
</tr>
</tbody>
</table>

MER, maintenance energy requirement; DM, dry matter.

### Table 2. Mean (±s.d.) body mass, intake and digestibility of two kangaroo species when subjected to 75% MER and to ad libitum food intake

<table>
<thead>
<tr>
<th>Species</th>
<th>75% MER</th>
<th>Ad lib.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter intake (g kg⁻¹ day⁻¹)</td>
<td>239±16</td>
<td>408±16</td>
</tr>
<tr>
<td>Relative (g kg⁻⁰·⁵ g⁻¹ day⁻¹)</td>
<td>24±1</td>
<td>41±10</td>
</tr>
<tr>
<td>Dry matter excretion (g kg⁻¹ day⁻¹)</td>
<td>92±12</td>
<td>180±46</td>
</tr>
<tr>
<td>Relative (g kg⁻⁰·⁵ g⁻¹ day⁻¹)</td>
<td>9±1</td>
<td>16±4</td>
</tr>
<tr>
<td>Faecal dry matter (g kg⁻¹ day⁻¹)</td>
<td>437±127</td>
<td>372±76</td>
</tr>
<tr>
<td>Apparent digestibility (%)</td>
<td>61±4</td>
<td>63±8</td>
</tr>
<tr>
<td>NDF</td>
<td>52±6</td>
<td>53±10</td>
</tr>
<tr>
<td>Crude protein</td>
<td>74±3</td>
<td>74±5</td>
</tr>
<tr>
<td>DEI (kJ kg⁻⁰·⁷ g⁻¹ day⁻¹)</td>
<td>266±13</td>
<td>458±133</td>
</tr>
<tr>
<td>MEI (kJ kg⁻⁰·⁷ g⁻¹ day⁻¹)</td>
<td>228±11</td>
<td>390±11</td>
</tr>
</tbody>
</table>

MER, maintenance energy requirement; NDF, neutral detergent fibre; DEI, digestible energy intake; MEI, metabolizable energy intake (calculated as 0.85 DEI). Data are for N=6 M. fuliginosus and N=4 Macropus rufus.
Table 3. Mean±s.d. gaseous exchange of two kangaroo species when subjected to 75% MER and to ad libitum food intake

<table>
<thead>
<tr>
<th>Units</th>
<th>Macropus fuliginosus</th>
<th>Macropus rufus</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75% MER</td>
<td>Ad lib.</td>
<td>75% MER</td>
</tr>
<tr>
<td>O₂ consumption</td>
<td>1 day⁻¹</td>
<td>192±34</td>
<td>192±24</td>
</tr>
<tr>
<td>Metabolic rate</td>
<td>kJ kg⁻⁰.⁷⁵ day⁻¹</td>
<td>386±63</td>
<td>380±46</td>
</tr>
<tr>
<td>RMR</td>
<td>kJ kg⁻⁰.⁷⁵ day⁻¹</td>
<td>306±32</td>
<td>320±22</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>1 day⁻¹</td>
<td>154±25</td>
<td>168±27</td>
</tr>
<tr>
<td>CO₂/O₂ (RQ)</td>
<td></td>
<td>0.81±0.08</td>
<td>0.88±0.08</td>
</tr>
<tr>
<td>CH₄ production</td>
<td>1 day⁻¹</td>
<td>3.05±1.38</td>
<td>3.09±1.31</td>
</tr>
<tr>
<td>CH₄/O₂ (RQ)</td>
<td>1 kg⁻¹</td>
<td>0.14±0.05</td>
<td>0.14±0.05</td>
</tr>
<tr>
<td>%GEI</td>
<td>1 DMI</td>
<td>12±6.99</td>
<td>7.5±2.12</td>
</tr>
<tr>
<td>%DEI</td>
<td>1 day</td>
<td>2.6±1.02</td>
<td>1.6±0.44</td>
</tr>
<tr>
<td>%dNDFi</td>
<td>1 kg⁻¹</td>
<td>4.5±1.92</td>
<td>2.6±0.51</td>
</tr>
<tr>
<td>dNDFi</td>
<td>1 day</td>
<td>48.8±21.5</td>
<td>30.6±7.3</td>
</tr>
</tbody>
</table>

RMR, resting metabolic rate; RQ, respiratory quotient; Mᵦ, body mass; DMI, dry matter intake; GEI, gross energy intake; DEI, digestible energy intake; dNDFi, digestible neutral detergent fibre NDF intake.

Data are for N=6 Macropus fuliginosus and N=4 Macropus rufus. Bold indicates values considered as trends.

The CH₄/CO₂ ratios found in M. fuliginosus and M. rufus (Table 3) were similar to values reported for wallabies (Macropus rufogriseus) and horses but notably lower than for ruminants and camels (Fig. 3A). Additionally, in relation to body mass, the absolute CH₄ production of M. fuliginosus and M. rufus was very similar to that previously reported for M. rufogriseus and hindgut fermenting mammals in general. However, CH₄ yield of M. fuliginosus and M. rufus per unit of DMI, GEI and dNDFi as well as related to body mass shows a different picture (Fig. 4A–C): the macropod measurements were higher than expected for non-ruminants but lower than in ruminants.

**DISCUSSION**

The present study relates in vivo measurements of CH₄ production in two macropod species to their body mass, food intake level and the intake of energy and fibre digestibility. The results facilitate a comparison with other herbivores that suggests that CH₄ production in macropods is not fundamentally different from that in other mammals, but is similarly low to that in, for example, horses when compared with ruminants, and lead to a hypothetical explanation for why physiological characteristics of macropods could be responsible for these low values. Here, we will first compare our findings on intake, digestion and metabolism with literature data, and then put our CH₄ measurements in a comparative context.

**Effect of feeding regimen and kangaroo species on intake, digestion and metabolic rate**

The ad libitum DMI of M. rufus in the present study (44 g kg⁻⁰.⁷⁵ day⁻¹) was in the range of data reported in the literature for this species on a lucerne hay diet (35–53 g kg⁻⁰.⁷⁵ day⁻¹) (Hume, 1974; Munn and Dawson, 2003, 2006). However, the corresponding DEI of M. rufus was actually higher in the present study (458 kJ kg⁻⁰.⁷⁵ day⁻¹) than that reported by Munn and Dawson (2003) (385 kJ kg⁻⁰.⁷⁵ day⁻¹).

The apparent digestibility of dry matter found for M. rufus in the present study (56% apparent digestibility) closely resembled literature values (55–57%; Hume, 1974; Munn and Dawson, 2003, 2006). Although we found no statistically significant differences between the apparent digestibility of dry matter by M. fuliginosus compared with M. rufus, the somewhat higher
average apparent digestibility of dry matter by M. fuliginosus (ca. 63%) has been found in other studies (e.g. Munn et al., 2014). These data add further support to the idea that M. fuliginosus digests dry matter more efficiently than M. rufus.

The RMR estimated for M. rufus in the present study (301 kJ kg$^{-0.75}$ day$^{-1}$) is higher than literature data of basal metabolic rate (BMR) (197 and 210 kJ kg$^{-0.75}$ day$^{-1}$; Dawson and Hulbert, 1970; Dawson et al., 2000) for this species. This could reflect seasonal effects on the basal metabolism of the species, but field research on seasonal metabolic changes in large marsupials generally is lacking. Moreover, BMR data for M. fuliginosus are lacking. However, M. fuliginosus is closely related to Macropus giganteus, which shows slightly higher BMR (233 kJ kg$^{-0.75}$ day$^{-1}$; Dawson et al., 2000) than M. rufus; it may be assumed therefore that the metabolic rate of M. fuliginosus also exceeds that of M. rufus and our findings support this assumption (RMR of M. fuliginosus: 320 kJ kg$^{-0.75}$ day$^{-1}$; Table 3).

The comparison of metabolizable energy intake (MEI, calculated as 85% of DEI) and metabolic rate of the kangaroo species studied at the 75% MER feeding level confirmed the intended status of energy deficiency both for M. fuliginosus with 226 kJ kg$^{-0.75}$ day$^{-1}$ MEI versus 386 kJ kg$^{-0.75}$ day$^{-1}$ metabolic rate (paired t-test P=0.002) and for M. rufus with 297 kJ kg$^{-0.75}$ day$^{-1}$ MEI versus 316 kJ kg$^{-0.75}$ day$^{-1}$ metabolic rate (P=0.719). In line with this,

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Fig. 2. Relationship of methane production to relative dry matter intake (rDMI) in M. fuliginosus and M. rufus, and in Equus caballus. Data for E. caballus are from Dansen et al. (2015) and Franz et al. (2010). (A) Absolute amounts per day. (B) Yield per unit of dry matter intake (DMI), (C) Yield per unit of digestible neutral detergent fibre intake (dNDFi).

Fig. 3. Comparison of methane production in M. fuliginosus and M. rufus to other mammalian herbivores. (A) CH4/CO2 ratio in M. fuliginosus and M. rufus (present study), Macropus rufogriseus (Madsen and Bertelsen, 2012), E. caballus (Dansen et al., 2015), Choloepus didactylus (Vendl et al., 2015), Vicugna pacos, Lama glama and Camelus bactrianus (Dittmann et al., 2014b) in comparison to ruminants (grey-shaded area; data from Hellwing et al., 2013; Lassen et al., 2012; Madsen et al., 2010; Sauer et al., 1998). (B) Methane production of M. fuliginosus and M. rufus (at 75% MER and when fed ad libitum) related to body mass as absolute amounts per day in comparison to ruminants (dark regression line with 95% confidence intervals; Franz et al., 2010) and non-ruminant mammalian herbivores (light regression line with 95% confidence intervals; Franz et al., 2011b). Comparative data for Macropus eugenii from von Engelhardt et al. (1978) and for M. rufogriseus from Madsen and Bertelsen (2012).
the respiratory quotient measured at 75% MER reflects a more intensive fat metabolism (M. fuliginosus: 0.81; M. rufus: 0.74), whereas the higher respiratory quotient determined for animals fed ad libitum indicates that primarily carbohydrate metabolism was used to fuel metabolic rate (M. fuliginosus: 0.88; M. rufus: 0.85). During ad libitum feeding, MEI closely corresponded to energy requirements in both M. fuliginosus (390 kJ kg\(^{-0.75}\) day\(^{-1}\) MEI versus 380 kJ kg\(^{-0.75}\) day\(^{-1}\) metabolic rate, \(P=0.843\)) and M. rufus (399 kJ kg\(^{-0.75}\) day\(^{-1}\) MEI versus 388 kJ kg\(^{-0.75}\) day\(^{-1}\) metabolic rate, \(P=0.901\)).

**Effect of feeding regimen and kangaroo species on methane emission**

The absolute CH\(_4\) production measured in the kangaroos of the present study confirmed previous findings in wallabies (hay or hay with concentrates in Madsen and Bertelsen, 2012; chopped or pelletled roughage in von Engelhardt et al., 1978), and also confirmed the general similarity of macropods to roughage-fed hindgut fermenters like horses as suggested by Franz et al. (2011b). However, the measurements of CH\(_4\) yield, presented here for the first time for macropods, are intermediate to values measured in hindgut fermenters and ruminants. Varying DMI had little influence on absolute CH\(_4\) production but influenced CH\(_4\) yield, indicating that DMI – and hence most likely digesta retention time – is an important factor influencing CH\(_4\) production. These relationships indicate the presence of clear differences in CH\(_4\) production between macropods and ruminants and hypotheses on the origin of these differences are needed. Although variation in digesta retention is associated with variation in fibre digestibility in kangaroos (Munn and Dawson, 2006; Munn et al., 2008), it was surprising that we found no effect of food intake level on digestibility measurements.

For a comparative understanding of in vivo CH\(_4\) production, a combined evaluation of the absolute amounts of CH\(_4\) emitted and the CH\(_4\) yield (CH\(_4\) in relation to parameters like DMI, GEI or dNDFi) is required. Absolute CH\(_4\) production and CH\(_4\) yield need not automatically co-vary in the same direction. In the present study, absolute CH\(_4\) production did not change with increasing DMI, which means that CH\(_4\) yield necessarily decreased. A similar pattern was observed across ratite species (Frei et al., 2015b), where large differences in ad libitum DMI and CH\(_4\) yield led to comparable mass-specific absolute daily CH\(_4\) production. In a study with two-toed sloths (Choloepus didactylus), one specimen with an exceptionally high DMI did not have an outstanding body mass-corrected absolute daily CH\(_4\) emission, but did have a distinctively lower CH\(_4\) yield than its conspecifics (Vendl et al., 2015). By reviewing 48 studies on CH\(_4\) production in ruminants, Blaxter and Clapperton (1965) found a negative correlation between DMI and CH\(_4\) yield. However, it has to be pointed out that CH\(_4\) production and yield do not always correlate in the same way: even though a negative correlation between DMI and CH\(_4\) yield was displayed by sheep, absolute CH\(_4\) production increased with higher DMI, as is typical for ruminants (Hammond et al., 2014). Such findings suggest that, within an organism, a change in DMI can have an influence on CH\(_4\) production and hence the activity of the microbiome, and that it is the combination of that activity and the amount of material on which the microbiome can act (the DMI) that determines absolute CH\(_4\).

The most probable explanation for a decrease in CH\(_4\) yield with intake is via the MRT of the digesta in the digestive tract. A number of studies have demonstrated the negative correlation between DMI and MRT in ratites (Frei et al., 2015c), sloths (Vendl et al., 2015), ruminants (Clauss et al., 2007; Hammond et al., 2014) and macropods (Munn et al., 2008). In essence, a higher food intake leads to a faster passage of digesta through the digestive tract, mostly

![Image](https://example.com/image1.png)

**Fig. 4.** Various measures of methane yield in *M. fuliginosus* and *M. rufus* compared with other mammalian herbivores. Methane production is given (A) per unit of dry matter intake (DMI), (B) as a proportion of gross energy intake (GEI) and (C) per unit of digestible fibre intake (dNDFi) (at 75% MER and when fed ad libitum ) in comparison to ruminants (dark regression line; Franz et al., 2010) and non-ruminant mammalian herbivores (light regression line; Franz et al., 2011b). Thin lines indicate the 95% confidence interval of regression lines.
because of the limited capacity of the gut to expand. A shorter MRT, in turn, was correlated with a lower CH4 yield in various studies with sheep (Barnett et al., 2013, 2015; Goopy et al., 2013; Hammond et al., 2014; Pinares-Patino et al., 2003). Janssen (2010) summarized these findings, creating a model for the prediction of CH4 yield that used a range of factors, including MRT.

With respect to this effect of MRT on CH4 production, Shi et al. (2014) found no difference in microbe counts and the composition of the microbiome of sheep at different MRT, but a difference in the expression of methanogenesis pathway genes in rumen Archaea along with varying MRT. This indicates that changes in MRT might influence microbe species composition or their number less than the metabolic state of these microbes. A difference in the metabolic state of microbes might thus lead to differences in the production of CH4.

In vitro studies with inoculum from ruminants indicated that foods vary in their ‘partitioning factor’, i.e. in the degree to which they trigger energy transfer into microbial growth or into short-chain fatty acid and hence also CH4 production (Blümmel et al., 1997; Moss and Newbold, 2000). As a result, microbial synthesis is negatively correlated with methane production. By feeding diets of different concentrations of water-soluble carbohydrates, Moss et al. (2001) confirmed this finding in sheep in vivo: a low level of water-soluble carbohydrates resulted in less microbial matter and a higher CH4 yield, whereas higher levels of water-soluble carbohydrates led to a parallel increase of microbial biomass and a reduction of CH4 yield. Generally, MRT most likely is one factor that influences the microbiome’s metabolic state in such a way (Janssen, 2010; Shi et al., 2014), and hence the generally lower MRT of macropods compared with ruminants (Munn et al., 2008) may well explain lower CH4 yields in the former (Fig. 5, top).

Additionally, the MRT that the microbiome is specifically exposed to might differ from the general MRT of particulate digesta as a result of a process termed ‘digesta washing’ (Dittmann et al., 2015; Müller et al., 2011): if fluids move through a gut compartment...
faster than the particles, they ‘wash out’ very fine particles from the digesta bulk and thus, most particularly, microbes. A high fluid throughput can thus create conditions of reduced MRT for microbes while retaining longer MRT for digesta particles. Using combinations of solute and particulate digesta markers, a very distinct digesta washing has been demonstrated in macropods (Dellow, 1982; Munn and Dawson, 2006; Munn et al., 2012; Schwarm et al., 2009), which probably differ in this characteristic from many ruminant species (cf. second row of Fig. 5). In an in vitro study with rumen inoculum maintained at different dilution rates, Isaacsen et al. (1975) demonstrated that increasing the dilution (i.e. the ‘wash out’ of microbes) led to a concomitant decrease in CH4 yield and an increase in microbial mass yield (see Fig. 5, bottom). Hence, we hypothesize that the macropod microbiome, because of a generally shorter MRT than in ruminants and a distinct digesta washing, is in a metabolic state that minimizes CH4 losses and maximizes microbial yield (Fig. 5).

The nevertheless higher-than-expected CH4 yield in macropods of the present study seemingly contradicts literature findings of extremely low in vivo CH4 emission (Kempton et al., 1976; Dellow et al., 1988; Madsen and Bertelsen, 2012; also previously contradicted by von Engelhardt et al., 1978), very small populations of foregut Archaea (Evans et al., 2009; Kliève et al., 2012; Ouwerkerk et al., 2009) and the assumed dominance of reductive acetogens as hydrogen sinks in macropods (Godwin et al., 2014). However, as pointed out by Ouwerkerk et al. (2005) and Gulino et al. (2013), macropods generally seem to harbour rather unique microbe communities with many as yet undescribed species. This may explain why it was not possible to sufficiently detect a resident Archaea population in the kangaroos with common PCR primers. Furthermore, a lack of detection of enteric Archaea or of methane production in vitro does not necessarily prove that species are low or non-producers. Similar to this line of reasoning in macropods, Fievez et al. (2001) and Miramontes-Carillo et al. (2008) suggested that ostriches (Struthio camelus) produce very little or no CH4 based on in vitro measurements and molecular studies. However, Frei et al. (2015a) nevertheless found significant amounts of methane produced by ostriches in vivo, at a magnitude expected for similar-sized non-ruminant mammals.

The site of CH4 production in macropods is under debate. Kempton et al. (1976) measured CH4 emitted via breathing and (anally) via flatulence separately, and could only detect anal CH4 emission. These authors therefore suggested that CH4 is only formed in the hindgut of macropods. Madsen and Bertelsen (2012) supported this hypothesis based on the daily fluctuations of CH4 emissions they detected during chamber respirometry (similar to the irregular CH4 emission patterns found in the present study in Fig. 1). These authors concluded that such fluctuations indicated emission by flatulence, in contrast to ruminants where 95% of ruminal CH4 is emitted via breathing or eructation (Murray et al., 1976). However, two arguments contradict this interpretation in our view. First, CH4 produced in the hindgut may basically also be emitted via breathing, as evidenced by studies in humans and horses – both species where CH4 is produced in the hindgut and recovered in the breath via a face mask (McKay et al., 1985; Sasaki et al., 1999). Second, the daily CH4 emission pattern can be irregular in ruminants also (Crompton et al., 2011; Hironaka et al., 1996; Kinsman et al., 1995). Crompton et al. (2011) demonstrated that CH4 emission peaks concurred with feeding events in sheep. In animals with access to food in the respiration chamber, as in the present study and in the study of Madsen and Bertelsen (2012), irregular CH4 emission peaks might therefore simply indicate an irregular spacing of feeding bouts of the experimental animals. For future studies, a parallel recording of respiration measurements and behavioural observations would therefore be interesting. While the hindgut cannot be ruled out as a site of CH4 production in macropods, and the hindgut microbiome of macropods still remains to be explored, we consider the evidence currently available not sufficient to assume that CH4 production does not occur in the macropod forestomach.

Our data suggest that a kangaroo produces about 27% of body mass-specific volume of CH4 compared with ruminants. This corresponds to an annual amount of some 1000 l CH4 per kangaroo of an assumed body mass of 20 kg. According to the Australian Department of Environment (2011), Australia currently harbours a wild kangaroo population of about 34 million individuals belonging to either of the four largest macropod species (M. fuliginosus, M. giganteus, M. robustus and M. rufus). We assume an average body mass of 20 kg, aware that estimating a realistic mean body mass for either of the four mentioned species is challenging and depends on factors such as sex, regional differences, intensity of harvest and proportion of juvenile individuals (Grigg, 2002). The total assumed number of kangaroos is probably an underestimate because populations of remote areas are not included and no reliable numbers on smaller macropod species are available. Under these rough assumptions, the four large macropod species produce a volume of about 38 billion litres of CH4 per year. In comparison, according to the Australian Bureau of Statistics (2013), the domestic ruminant livestock in Australia includes 29.3 million cattle (mean body mass: 496±155 kg; mean CH4 production: 0.63±0.11 kg CH4 day−1 and 75.5 million sheep (mean body mass: 59±21 kg; mean CH4 production: 0.49±0.13 kg CH4 day−1 (based on the data collection from Dittmann et al., 2014b), producing about 4138 billion litres of CH4 per year. There is also a large population of feral camels consisting of about 1 million individuals (Saalfeld and Edwards, 2010) causing CH4 emissions estimated to account for 66 billion litres per year (Dittmann et al., 2014b). Therefore, CH4 emissions of 34 million kangaroos only account for less than 1% of that of domestic ruminant livestock and for about 56% of feral camels. Wilson and Edwards (2008) based their calculations of the Australian CH4 budget on much lower emission levels for kangaroos, which were derived from Kempton et al. (1976), and suggested a change of attitude towards kangaroo meat in Australia as a means to reduce meat production-associated GHG emissions. Evidently, such comparison must be made with caution, and should consider factors such as growth rates (which are mostly lower in marsupials than in eutherian mammals; Case, 1978) and then relate CH4 emitted to units of produced muscle food (emission intensity).

Conclusions
The absolute CH4 emissions of kangaroos in this study were similar to literature results and closely resembled those of similar-sized hindgut fermenters. However, their CH4 yield was higher than expected, being of a magnitude in between that of hindgut fermenters and ruminants. We suggest that the apparent difference between macropods and ruminants, resembling that between many other non-ruminants (such as horses) and ruminants, is not due to a unique composition of the microbiome but rather to differences in the metabolic state of this microbiome. In order to confirm this hypothesis, microbial yield and growth rates should be investigated with metabolomics and transcriptomics approaches and compared in relation to differing MRT and DMI levels in macropods versus those in sheep and other herbivores such as horses. Expectations linked to transfaunation, i.e. transfer of the macropods’ microbiome to ruminants (Kliève, 2009; Wilson and Edwards, 2008), would only be realistic if this assumption is wrong, or if it can be
demonstrated that the effect of this microbiome is stable under the conditions of DMI, MRT and digesta washing present in the target ruminants’ forestomach.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.M. and M.C. designed the experiments; A.M. and K.L. ensured the physical and logistical prerequisites for the experiments; C.V., M.S., K.L. and A.M. performed the experiments; C.V. and M.C. performed statistical analyses; C.V., M.C., M.K., J.H. and A.M. drafted the manuscript; all authors commented on the final version of the manuscript.

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