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RESEARCH ARTICLE

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

Fundamental differences in methane (CH_4) 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 CH_4 production *in vivo* as well as CH_4 yield (CH_4 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 CH_4 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, CH_4 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 CH_4 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 CH_4 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 volume of CH_4 of ruminants, it remains to be modelled with species-specific growth rates and production conditions whether or not significantly lower CH_4 amounts are emitted per kg of meat in kangaroo than in beef or mutton production.

KEY WORDS: Macropod, CH_4, Forestomach, Digesta washing, Digestion, Fermentation, Methanogenesis

INTRODUCTION

Methane (CH_4) is a potent greenhouse gas (GHG), accounting for 16% of total anthropogenic GHG emissions in 2010, second only to CO_2 (IPCC, 2014). From total emissions, 28% originates from ruminant livestock as the largest source (Klieve, 2009). Enteric CH_4 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 CH_4 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 CH_4 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 CH_4 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 sacciform 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 CH_4 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 CH_4 emissions of macropods to their comparably 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 CH_4 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

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List of symbols and abbreviations

ADF	acid detergent fibre
ADL	acid detergent lignin
BMR	basal metabolic rate
DEI	digestible energy intake
DMI	dry matter intake
dNDFi	intake of digestible neutral detergent fibre
GE	gross energy
GEI	gross energy intake
GHG	greenhouse gases
M_b	body mass
MER	maintenance energy requirement
MRT	mean retention time
NDF	neutral detergent fibre
rDMI	relative dry matter intake
RMR	resting metabolic rate

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 CH_4 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 CH_4; 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* CH_4 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 CH_4 production as well as CH_4 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 CH_4 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 Arid 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 acclimate to human handling. One *M. fuliginosus* and all four *M. rufus* were hand-reared and thus used to being 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 m^2) 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_b; Virbac Animal Health, Milperra, Australia) and Pamlin (diazepam, 5 mg ml^{-1}; used dosage: 2 ml per 20 kg M_b; 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 m^3 and 2.66 m^3) 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 multiplexer, 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 O_2 and CO_2 analysers (Foxbox, Sable Systems). Methane was measured by a CH_4 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% O_2, 0.50% CO_2, 0.50% CH_4 dissolved in N_2) at the beginning of each measurement period. Data obtained by the respirometry system were analysed with ExpeData software (Sable Sytems) for O_2 consumed and CH_4 and CO_2 emitted after correcting for gas input calculated from flow and concentrations of incoming air. The mean 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 O_2 measurements per individual within the entire measurement (adapted from Derno et al., 2005). In order to estimate metabolic rate, we multiplied the amount of O_2 consumed (in 1 h^{-1}) by 20.08 kJ (McNab, 2008). Volume measures of CH_4 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 CH_4 yield in relation to dry matter intake (DMI), gross energy intake (GEI), digestible energy intake (DEI) and intake of digestible neutral detergent fibre (dNDFi).

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 acclimate 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

Journal of Experimental Biology

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

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 urine 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 intake were calculated from the nutrient composition of the hay offered and that 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 interaction between species and intake level, the corresponding *P*-values are not displayed. The significance of simple correlations was tested by Spearman's σ. Analyses were performed in SPSS 21.0 (SPSS Inc., Chicago, IL, USA). The significance level was set to $\alpha=0.05$, with values of up to 0.10 considered as trends.

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 by one. 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 levels (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 CO_2 production and the respiratory quotient (Table 3).

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

Nutrient	75% MER	*Ad libitum*
Dry matter (g kg^{-1} as fed)	833±22	832±29
Total ash (g kg^{-1} DM)	97±2	98±3
Crude protein (g kg^{-1} DM)	202±8	210±11
Neutral detergent fibre (g kg^{-1} DM)	503±20	477±15
Acid detergent fibre (g kg^{-1} DM)	322±7	–
Acid detergent lignin (g kg^{-1} DM)	83±6	–
Gross energy (kJ g^{-1} DM)	18.9±0.2	18.8±0.1

MER, maintenance energy requirement; DM, dry matter.

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 O_2 consumption and CO_2 production were significantly higher in *M. fuliginosus* than in *M. rufus* (Table 3).

Examples of diurnal patterns of O_2 consumption and CO_2 and CH_4 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 CH_4 production over the day. In some cases, parallel peaks in O_2 consumption/CO_2 emission and CH_4 emission were evident (Fig. 1). No significant relationship was found between food intake level and absolute daily CH_4 production (Table 3, Fig. 2A). In contrast, there were significant differences in CH_4 yield (CH_4 per unit of DMI, GEI and dNDFi) between intake levels (Table 3), and significant negative correlations between intake level and CH_4 yield expressed as CH_4 production either per unit of DMI ($\sigma=-0.48$, $P=0.032$; Fig. 2B) or per unit of GEI ($\sigma=-0.48$, $P=0.032$). CH_4 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 CH_4 produced per unit dNDFi ($\sigma=-0.42$, $P=0.064$; Fig. 2C). At *ad libitum* food intake, CH_4 yield of macropods was similar to that reported for horses (Fig. 2B,C).

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

	Macropus fuliginosus		*Macropus rufus*		*P*-level	
	75% MER	*Ad lib.*	75% MER	*Ad lib.*	Species	Intake level
Body mass (kg)	21.7±2.9	21.8±3.0	17.3±4.6	17.7±4.6	0.020	n.s.
Dry matter intake						
Absolute (g day^{-1})	239±16	408±16	228±23	385±159	n.s.	0.002
Relative (g $kg^{-0.75}$ day^{-1})	24±1	41±10	28±8	44±12	n.s.	0.001
Dry matter excretion						
Absolute (g day^{-1})	92±12	180±46	79±9	165±53	n.s.	<0.001
Relative (g $kg^{-0.75}$ day^{-1})	9±1	16±4	10±1	19±3	n.s.	<0.001
Faecal dry matter (g kg^{-1})	437±127	372±76	408±131	396±105	n.s.	n.s.
Apparent digestibility (%)						
Dry matter	61±4	63±8	65±5	56±7	n.s.	n.s.
NDF	52±6	53±10	57±9	44±10	n.s.	n.s.
Crude protein	74±3	74±5	77±3	72±4	n.s.	n.s.
DEI (kJ $kg^{-0.75}$ day^{-1})	266±13	458±133	350±136	469±186	n.s.	0.015
MEI (kJ $kg^{-0.75}$ day^{-1})	226±11	390±113	297±116	399±158	n.s.	0.015

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*.

Journal of Experimental Biology

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

Table 3. Mean(±s.d.) gaseous exchange of two kangaroo species when subjected to 75% MER and to *ad libitum* food intake

		Macropus fuliginosus		*Macropus rufus*		*P*-level	
	Units	75% MER	*Ad lib.*	75% MER	*Ad lib.*	Species	Intake level
O_2 consumption	1 day^{-1}	192±34	192±24	133±27	162±48	0.010	n.s.
Metabolic rate	kJ $kg^{-0.75}$ day^{-1}	386±63	380±46	316±50	388±69	n.s.	n.s.
RMR	kJ $kg^{-0.75}$ day^{-1}	306±32	320±22	252±39	301±44	0.027	**0.055**
CO_2 production	1 day^{-1}	154±25	168±27	94±14	140±38	0.003	0.025
CO_2/O_2 (RQ)		0.81±0.08	0.88±0.08	0.74±0.05	0.85±0.05	n.s.	0.011
CH_4/CO_2 ratio		0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	n.s.	**0.071**
CH_4 production	1 day^{-1}	3.05±1.38	3.09±1.31	2.98±0.91	2.60±0.61	n.s.	n.s.
	1 kg^{-1} day^{-1}	0.14±0.05	0.14±0.05	0.19±0.10	0.16±0.08	n.s.	n.s.
	1 kg^{-1} DMI	12.68±4.99	7.54±2.12	12.87±2.91	7.78±3.57	n.s.	0.007
	%GEI	2.65±1.02	1.60±0.44	2.70±0.60	1.63±0.76	n.s.	0.007
	%DEI	4.54±1.92	2.62±0.51	4.17±0.88	3.04±1.41	n.s.	0.023
	1 kg^{-1} dNDFi	48.8±21.5	30.6±7.3	44.5±8.1	40.9±19.7	n.s.	n.s.

RMR, resting metabolic rate; RQ, respiratory quotient; M_b, 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 *M. fuliginosus* and *N*=4 *Macropus rufus*. Bold indicates values considered as trends.

The CH_4/CO_2 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 camelids (Fig. 3A). Additionally, in relation to body mass, the absolute CH_4 production of *M. fuliginosus* and *M. rufus* was very similar to that previously reported for *M. rufogriseus* and hindgut fermenting mammals in general. However, CH_4 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 CH_4 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 CH_4 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 CH_4 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

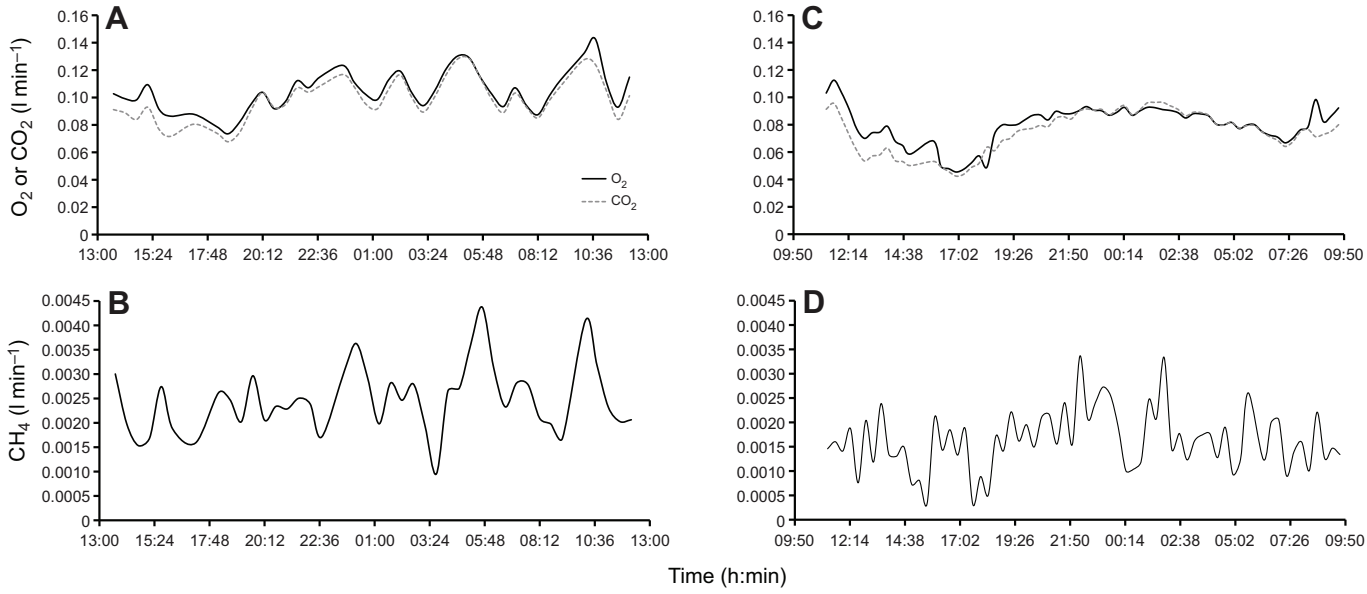

Fig. 1. Diurnal patterns of gas consumption and emission in *Macropus fuliginosus* and *Macropus rufus*. (A,C) O_2 consumption and CO_2 emission, and (B,D) CH_4 emission data for a *M. fuliginosus* (A,B) and a *M. rufus* (C,D) specimen during *ad libitum* feeding.

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

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,

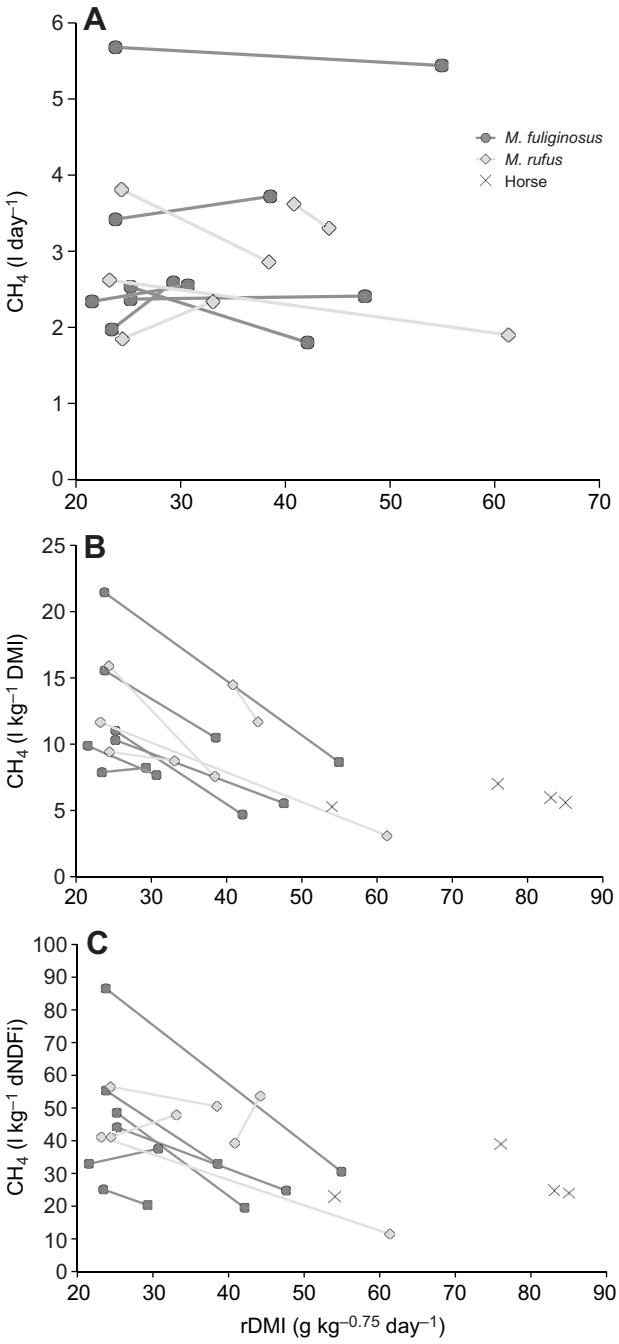

Fig. 2. Relationship of methane production to relative dry matter intake (rDMI) in *M. fuliginosus* and *M. rufus*, and in *Equus caballus*. Data for *E. caballus* are from Dansen et al. (2015) and Franz et al. (2010). (A) Absolute amounts per day. (B) Yield per unit of dry matter intake (DMI). (C) Yield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

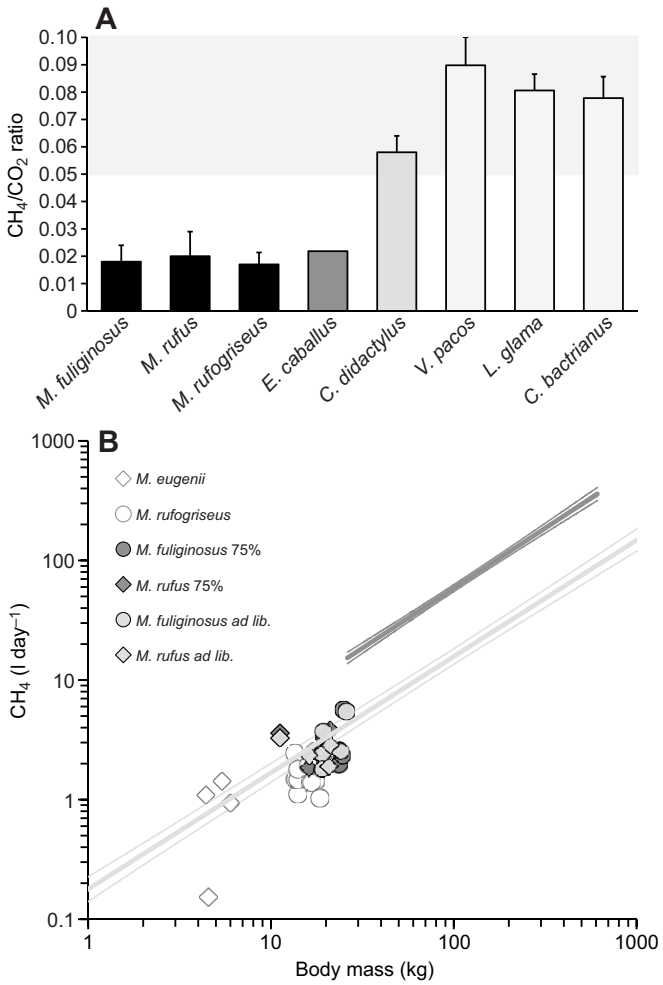

Fig. 3. Comparison of methane production in *M. fuliginosus* and *M. rufus* to other mammalian herbivores. (A) CH_4/CO_2 ratio in *M. fuliginosus* and *M. rufus* (present study), *Macropus rufogriseus* (Madsen and Bertelsen, 2012), *E. caballus* (Dansen et al., 2015), *Choloepus didactylus* (Vendl et 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).

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

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).

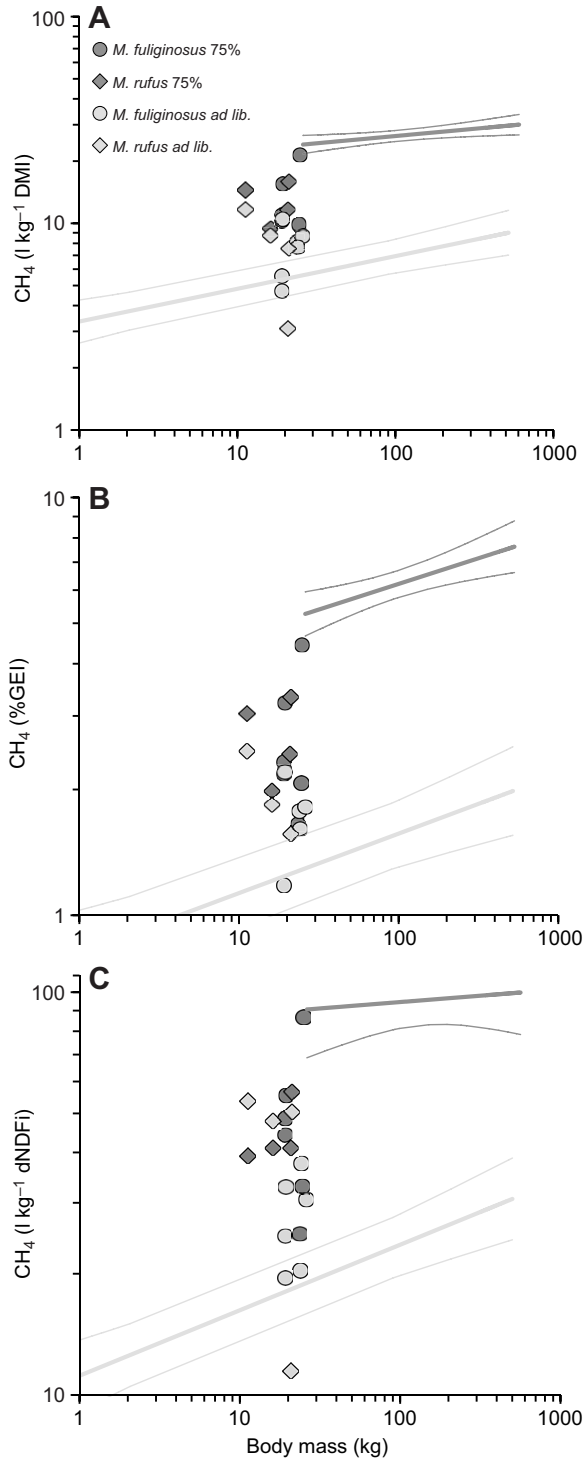

Fig. 4. Various measures of methane yield in *M. fuliginosus* and *M. rufus* compared with other mammalian herbivores. Methane production is given (A) per unit of dry matter intake (DMI), (B) as a proportion of gross energy intake (GEI) and (C) per unit of digestible fibre intake (dNDFi) (at 75% MER 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.

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 only or hay with concentrates in Madsen and Bertelsen, 2012; chopped or pelleted 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

Journal of Experimental Biology

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

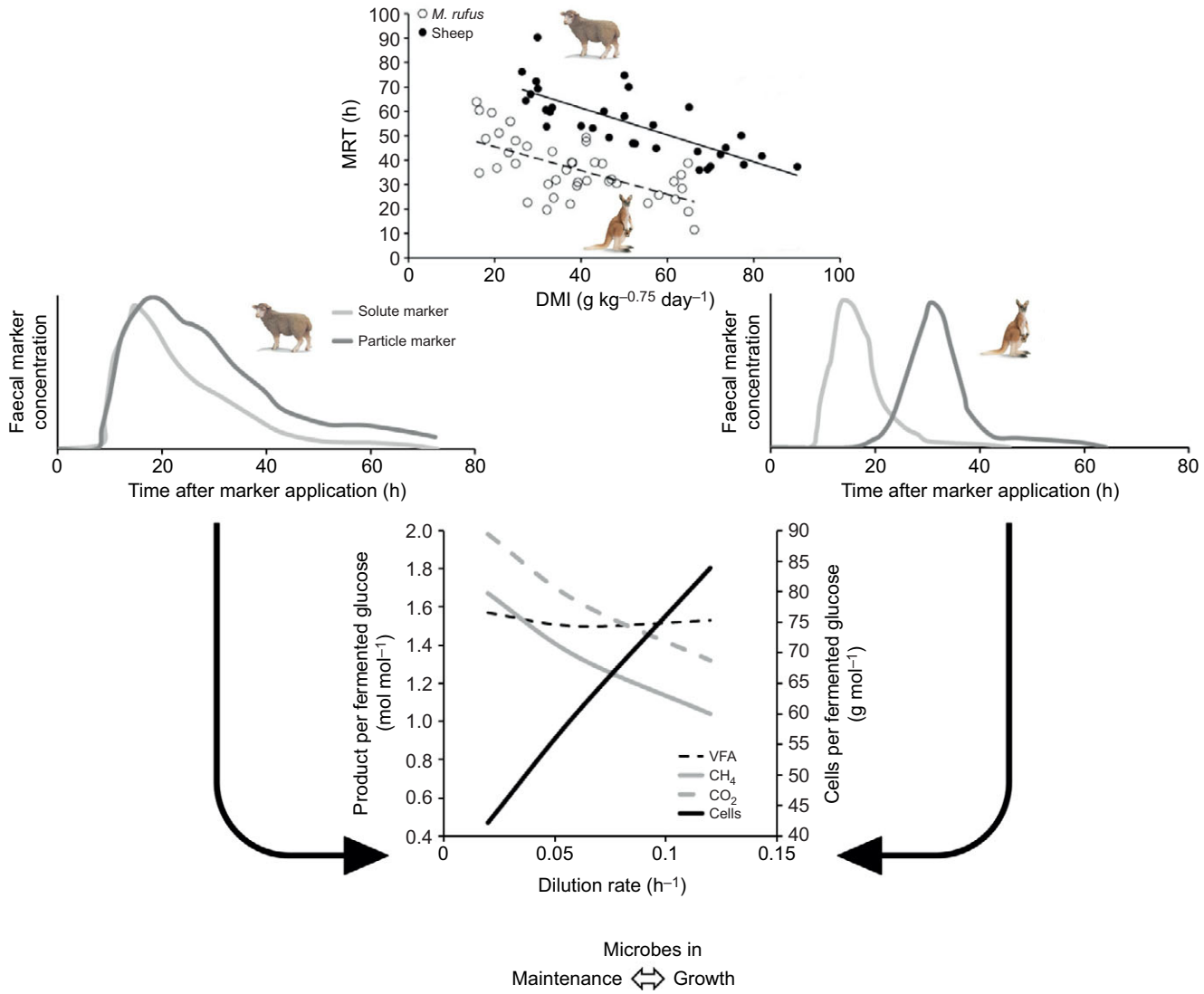

Fig. 5. Schematic representation of differences between sheep and macropods, suggested to explain differences in CH_4 production between these herbivore groups. Top, general difference in mean retention time (MRT) independent from dry matter intake (DMI) between macropods and sheep (from Munn et al., 2008), where a shorter MRT triggers growth of foregut microbes and a decrease in CH_4 yield, and the macropod foregut contains more microbes in the growth state and therefore yields less CH_4. Middle, general difference in 'digesta washing' (the difference in the passage of fluids and particles through the gut) between sheep and macropods (data from Dellow, 1982), leading to even more microbes being in the growth stage in macropod foreguts. Bottom, an increased dilution in an *in vitro* system leading to a higher yield of microbial biomass (growth) and a lower CH_4 yield (from Isaacson et al., 1975). VFA , volatile fatty acids.

because of the limited capacity of the gut to expand. A shorter MRT, in turn, was correlated with a lower CH_4 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 CH_4 yield that used a range of factors, including MRT.

With respect to this effect of MRT on CH_4 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 CH_4. *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 CH_4 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 CH_4 yield, whereas higher levels of water-soluble carbohydrates led to a parallel increase of microbial biomass and a reduction of CH_4 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 CH_4 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

Journal of Experimental Biology (2015) 218, 3425-3434 doi:10.1242/jeb.128165

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, Isaacson et al. (1975) demonstrated that increasing the dilution (i.e. the 'wash out' of microbes) led to a concomitant decrease in CH_4 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 CH_4 losses and maximizes microbial yield (Fig. 5).

The nevertheless higher-than-expected CH_4 yield in macropods of the present study seemingly contradicts literature findings of extremely low *in vivo* CH_4 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; Klieve 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 CH_4 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 CH_4 production in macropods is under debate. Kempton et al. (1976) measured CH_4 emitted via breathing and (anally) via flatulence separately, and could only detect anal CH_4 emission. These authors therefore suggested that CH_4 is only formed in the hindgut of macropods. Madsen and Bertelsen (2012) supported this hypothesis based on the daily fluctuations of CH_4 emissions they detected during chamber respirometry (similar to the irregular CH_4 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 CH_4 is emitted via breathing or eructation (Murray et al., 1976). However, two arguments contradict this interpretation in our view. First, CH_4 produced in the hindgut may basically also be emitted via breathing, as evidenced by studies in humans and horses – both species where CH_4 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 CH_4 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 CH_4 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 CH_4 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 CH_4 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 CH_4 production does not occur in the macropod forestomach.

Our data suggest that a kangaroo produces about 27% of body mass-specific volume of CH_4 compared with ruminants. This corresponds to an annual amount of some 1000 l CH_4 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 CH_4 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 CH_4 production: 0.63±0.11 l kg^{-1} day^{-1} and 75.5 million sheep (mean body mass: 59±21 kg; mean CH_4 production: 0.49±0.13 l kg^{-1} day^{-1} (based on the data collection from Dittmann et al., 2014b), producing about 4138 billion litres of CH_4 per year. There is also a large population of feral camels consisting of about 1 million individuals (Saalfeld and Edwards, 2010) causing CH_4 emissions estimated to account for 66 billion litres per year (Dittmann et al., 2014b). Therefore, CH_4 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 CH_4 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 CH_4 emitted to units of produced muscle food (emission intensity).

Conclusions

The absolute CH_4 emissions of kangaroos in this study were similar to literature results and closely resembled those of similar-sized hindgut fermenters. However, their CH_4 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 (Klieve, 2009; Wilson and Edwards, 2008), would only be realistic if this assumption is wrong, or if it can be

Journal of Experimental Biology

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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References

AOAC (1995). *Official Methods of Analysis of AOAC International*. Arlington, VA: Association of Official Analytical Chemists.
Australian Bureau of Statistics (2013). Agricultural Commodities, Australia, 2012–2013. Cat. no. 7121.0. http://www.abs.gov.au. Accessed 28 May 2015.
Australian Department of the Environment (2011). http://www.environment.gov.au. Accessed 28 May 2015.
Barnett, M. C., Goopy, J. P., McFarlane, J. R., Godwin, I. R., Nolan, J. V. and Hegarty, R. S. (2013). Triiodothyronine influences digesta kinetics and methane yield in sheep. *Anim. Prod. Sci.* **52**, 572-577.
Barnett, M. C., McFarlane, J. R. and Hegarty, R. S. (2015). Low ambient temperature elevates plasma triiodothyronine concentrations while reducing digesta mean retention time and methane yield in sheep. *J. Anim. Physiol. Anim. Nutr.* **99**, 483-491.
Blaxter, K. L. and Clapperton, J. L. (1965). Prediction of the amount of methane produced by ruminants. *Br. J. Nutr.* **19**, 511-522.
Blümmel, M., Steingass, H. and Becker, K. (1997). The relationship between in vitro gas production, in vitro microbial biomass yield and N incorporation and its implications for the prediction of voluntary feed intake of roughages. *Br. J. Nutr.* **77**, 911-921.
Brouwer, E. (1965). Report of sub-committee on constants and factors. In *Energy Metabolism* (ed. K. Blaxter), pp. 441-443. London: Academic Press.
Case, T. J. (1978). On the evolution and adaptive significance of postnatal growth rates in the terrestrial vertebrates. *Q. Rev. Biol.* **53**, 243-282.
Clauss, M., Streich, W. J., Schwarm, A., Ortmann, S. and Hummel, J. (2007). The relationship of food intake and ingesta passage predicts feeding ecology in two different megaherbivore groups. *Oikos* **116**, 209-216.
Crompton, L. A., Mills, J. A. N., Reynolds, C. K. and France, J. (2011). Fluctuations in methane emission in response to feeding pattern in lactating dairy cows. In *Modelling Nutrient Digestion and Utilisation in Farm Animals* (ed. D. Sauvant), pp. 176-180. Wageningen: Wageningen Academic Publishers.
Dansen, O., Pellikaan, W. F., Hendriks, W. H., Dijkstra, J., Jacobs, M. P. T., Everts, H. and van Doorn, D. A. (2015). Daily methane production pattern of Welsh ponies fed a roughage diet with or without a cereal mixture. *J. Anim. Sci.* **93**, 1916-1922.
Dawson, T. and Hulbert, A. J. (1970). Standard metabolism, body temperature, and surface areas of Australian marsupials. *Am. J. Physiol.* **218**, 1233-1238.
Dawson, T. J., Munn, A. J., Blaney, C. E., Krockenberger, A. and Maloney, S. K. (2000). Ventilatory accommodation of oxygen demand and respiratory water loss in kangaroos from mesic and arid environments, the eastern grey kangaroo (*Macropus giganteus*) and the red kangaroo (*Macropus rufus*). *Physiol. Biochem. Zool.* **73**, 382-388.
Dellow, D. W. (1982). Studies on the nutrition of macropodine marsupials. III. The flow of digesta through the stomach and intestine of macropodines and sheep. *Aust. J. Zool.* **30**, 751-765.
Dellow, D. W., Hume, I. D., Clarke, R. T. J. and Bauchop, T. (1988). Microbial activity in the forestomach of free-living macropodid marsupials: comparisons with laboratory studies. *Aust. J. Zool.* **36**, 383-395.
Derno, M., Jentsch, W., Schweigel, M., Kuhla, S., Metges, C. C. and Matthes, H. D. (2005). Measurements of heat production for estimation of maintenance energy requirements of Hereford steers. *J. Anim. Sci.* **83**, 2590-2597.
Dittmann, M. T., Hummel, J., Runge, U., Galeffi, C., Kreuzer, M. and Clauss, M. (2014a). Characterising an artiodactyl family inhabiting arid habitats by its metabolism: low metabolism and maintenance requirements in camelids. *J. Arid Environ.* **107**, 41-48.
Dittmann, M. T., Runge, U., Lang, R. A., Moser, D., Galeffi, C., Kreuzer, M. and Clauss, M. (2014b). Methane emission by camelids. *PLoS ONE* **9**, e94363.
Dittmann, M. T., Hummel, J., Hammer, S., Arif, A., Hebel, C., Müller, D. W. H., Fritz, J., Steuer, P., Schwarm, A., Kreuzer, M. et al. (2015). Digesta kinetics in gazelles in comparison to other ruminants: evidence for taxon-specific rumen fluid throughput to adjust digesta washing to the natural diet. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **185**, 58-68.
Evans, P. N., Hinds, L. A., Sly, L. I., McSweeney, C. S., Morrison, M. and Wright, A.-D. G. (2009). Community composition and density of methanogens in the foregut of the tammar wallaby (*Macropus eugenii*). *Appl. Environ. Microbiol.* **75**, 2598-2602.
Fievez, V., Mbanzamihigo, L., Piattoni, F. and Demeyer, D. (2001). Evidence for reductive acetogenesis and its nutritional significance in ostrich hindgut as estimated from in vitro incubations. *J. Anim. Physiol. Anim. Nutr.* **85**, 271-280.
Franz, R., Soliva, C. R., Kreuzer, M., Steuer, P., Hummel, J. and Clauss, M. (2010). Methane production in relation to body mass of ruminants and equids. *Evol. Ecol. Res.* **1**, 727-738.
Franz, R., Soliva, C. R., Kreuzer, M., Hatt, J.-M., Furrer, S., Hummel, J. and Clauss, M. (2011a). Methane output of tortoises: its contribution to energy loss related to herbivore body mass. *PLoS ONE* **6**, e17628.
Franz, R., Soliva, C. R., Kreuzer, M., Hummel, J. and Clauss, M. (2011b). Methane output of rabbits (*Oryctolagus cuniculus)* and guinea pigs *(Cavia porcellus)* fed a hay-only diet: implications for the scaling of methane production with body mass in non-ruminant mammalian herbivores. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **158**, 177-181.
Frei, S., Dittmann, M. T., Reutlinger, C., Ortmann, S., Hatt, J.-M., Kreuzer, M. and Clauss, M. (2015a). Methane emission by adult ostriches (*Struthio camelus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **180**, 1-5.
Frei, S., Hatt, J.-M., Ortmann, S., Kreuzer, M. and Clauss, M. (2015b). Comparative methane emission by ratites: differences in food intake and digesta retention level out methane production. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **188**, 70-75.
Frei, S., Ortmann, S., Reutlinger, C., Kreuzer, M., Hatt, J.-M. and Clauss, M. (2015c). Comparative digesta retention patterns in ratites. *Auk Ornithol. Adv.* **132**, 119-131.
Gagen, E. J., Denman, S. E., Padmanabha, J., Zadbuke, S., Al Jassim, R., Morrison, M. and McSweeney, C. S. (2010). Functional gene analysis suggests different acetogen populations in the bovine rumen and tammar wallaby forestomach. *Appl. Environ. Microbiol.* **76**, 7785-7795.
Godwin, S., Kang, A., Gulino, L.-M., Manefield, M., Gutierrez-Zamora, M.-L., Kienzle, M., Ouwerkerk, D., Dawson, K. and Klieve, A. V. (2014). Investigation of the microbial metabolism of carbon dioxide and hydrogen in the kangaroo foregut by stable isotope probing. *Int. Soc. Microb. Ecol.* **8**, 1855-1865.
Goopy, J. P., Donaldson, A., Hegarty, R., Vercoe, P. E., Haynes, F., Barnett, M. and Oddy, V. H. (2013). Low-methane yield sheep have smaller rumens and shorter rumen retention time. *Br. J. Nutr.* **111**, 578-585.
Grigg, G. C. (2002). Conservation benefit from harvesting kangaroos: status report at the start of a new millenium - a paper to stimulate discussion and research. In *A Zoological Revolution. Using Native Fauna to Assist in its Own Survival* (ed. D. Lunney and C. Dickman), pp. 53-76. Mosman: Royal Society of New South Wales and Australian Museum.
Gulino, L.-M., Ouwerkerk, D., Kang, A. Y. H., Maguire, A. J. and Kienzle, M. (2013). Shedding light on the microbial community of the macropod foregut using 454-amplicon pyrosequencing. *PLoS ONE* **8**, e61463.
Hammond, K. J., Pacheco, D., Burke, J. L., Koolaard, J. P., Muetzel, S. and Waghorn, G. C. (2014). The effects of fresh forages and feed intake level on digesta kinetics and enteric methane emissions from sheep. *Anim. Feed Sci. Technol.* **193**, 32-43.
Hellwing, A. L. F., Sørensen, M. T., Weisbjerg, M. R., Vestergaard, M. and Lund, P. (2013). Can rapeseed lower methane emission from heifers? *Acta Agric. Scand. A* **62**, 259-262.
Hironaka, R., Mathison, G. W., Kerrigan, B. K. and Vlach, I. (1996). The effect of pelleting of alfalfa hay on methane production and digestibility by steers. *Sci. Tot. Environ.* **180**, 221-227.
Hume, I. D. (1974). Nitrogen and sulphur retention and fibre digestion by euros, red kangaroos and sheep. *Aust. J. Zool.* **22**, 13-23.

Hume, I. D. (1984). Principal features of digestion in kangaroos. *Proc. Nutr. Soc. Austr.* **9**, 76-81.
Hume, I. D. (1999). *Marsupial Nutrition*. Cambridge: Cambridge University Press.
IPCC (2014). Summary for policymakers. Summary for policymakers. In Climate change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (ed. C. B. Field, V. R. Barros, D. J. Dokken, K. J. Mach, M. D. Mastrandrea, T. E. Bilir, M. Chatterjee, K. L. Ebi, Y. O. Estrada, R. C. Genova, B. Girma, E. S. Kissel, A. N. Levy, S. MacCracken, P. R. Mastrandrea and L. L. White), pp. 1-32. Cambridge: Cambridge University Press.
Isaacson, H. R., Hinds, F. C., Bryant, M. P. and Owens, F. N. (1975). Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *J. Dairy Sci.* **58**, 1645-1659.
Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* **160**, 1-22.
Kempton, T. J., Murray, R. M. and Leng, R. A. (1976). Methane production and digestibility in the grey kangaroo and sheep. *Aust. J. Biol. Sci.* **29**, 209-214.
Kinsman, R., Sauer, F. D., Jackson, H. A. and Wolynetz, M. S. (1995). Methane and carbon dioxide emissions from dairy cows in full lactation monitored over a six-month period. *J. Dairy Sci.* **78**, 2760-2766.
Klieve, A. V. (2009). Microbial contribution to and amelioration of enteric methane emissions from domestic herbivores. *Microbiol. Austr.* **30**, 82-84.
Klieve, A. V., Ouwerkerk, D. and Maguire, A. J. (2012). Archaea in the foregut of macropod marsupials: PCR and amplicon sequence-based observations. *J. Appl. Microbiol.* **113**, 1065-1075.
Langer, P., Dellow, D. W. and Hume, I. D. (1980). Stomach structure and function in three species of macropodine marsupials. *Aust. J. Zool.* **28**, 1-18.
Lassen, J., Løvendahl, P. and Madsen, J. (2012). Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. *J. Dairy Sci.* **95**, 890-898.
Madsen, J. and Bertelsen, M. F. (2012). Methane production by red-necked wallabies (*Macropus rufogriseus*). *J. Anim. Sci.* **90**, 1364-1370.
Madsen, J., Bjerg, B. S., Hvelplund, T., Weisbjerg, M. R. and Lund, P. (2010). Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. *Livestock Sci.* **129**, 223-227.
McCaughey, W. P., Wittenberg, K. and Corrigan, D. (1999). Impact of pasture type on methane production by lactating beef cows. *Can. J. Anim. Sci.* **79**, 221-226.
McKay, L. F., Eastwood, M. A. and Brydon, W. G. (1985). Methane excretion in man - a study of breath, flatus, and faeces. *Gut* **26**, 69-74.
McNab, B. K. (1986). Food habits, energetics, and the reproduction of marsupials. *J. Zool.* **208**, 595-614.
McNab, B. K. (2008). An analysis of the factors that influence the level and scaling of mammalian BMR. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **151**, 5-28.
Miramontes-Carillo, J. M., Ibarra, A. J., Ramírez, R. M., Ibarra, A. F. J., Miramontes, V. A. L. and Lezama, G. R. (2008). Poblaciones bacterianas utilizadoras de hidrógeno presentes en el tracto gastrointestinal del avestruz (*Struthio camelus var. domesticus*). *Av. Invest. Agropec.* **12**, 43-54.
Morvan, B., Bonnemoy, F., Fonty, G. and Gouet, P. (1996). Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals. *Curr. Microbiol.* **32**, 129-133.
Moss, A. R. and Newbold, C. J. (2000). The impact of hexose partitioning on methane production in vitro. *Reprod. Nutr. Dev.* **40**, 211-212.
Moss, A. R., Newbold, C. J. and Givens, D. I. (2001). The impact of hexose partitioning in sheep in vivo. *Proc. Br. Soc. Anim. Sci.*, 157. http://www.bsas.org.uk/wp-content/themes/bsas/proceedings/Pdf2001/157.pdf
Müller, D. W. H., Caton, J., Codron, D., Schwarm, A., Lentle, R., Streich, W. J., Hummel, J. and Clauss, M. (2011). Phylogenetic constraints on digesta separation: variation in fluid throughput in the digestive tract in mammalian herbivores. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **160**, 207-220.
Munn, A. J. and Dawson, T. J. (2003). Energy requirements of the red kangaroo (*Macropus rufus*): impacts of age, growth and body size in a large desert-dwelling herbivore. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **173**, 575-582.
Munn, A. J. and Dawson, T. J. (2006). Forage fibre digestion, rates of feed passage and gut fill in juvenile and adult red kangaroos (*Macropus rufus*): why body size matters. *J. Exp. Biol.* **209**, 1535-1547.
Munn, A. J., Streich, W. J., Hummel, J. and Clauss, M. (2008). Modelling digestive constraints in non-ruminant and ruminant foregut-fermenting mammals. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **151**, 78-84.
Munn, A. J., Tomlinson, S., Savage, T. and Clauss, M. (2012). Retention of different-sized particles and derived gut fill estimate in tammar wallabies (*Macropus eugenii*): physiological and methodological considerations. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **161**, 243-249.
Munn, A. J., Skeers, P., Kalkman, L., McLeod, S. R. and Dawson, T. J. (2014). Water use and feeding patterns of the marsupial western grey kangaroo (*Macropus fuliginosus melanops*) grazing at the edge of its range in arid Australia, as compared with the dominant local livestock, the Merino sheep (*Ovis aries*). *Mamm. Biol.* **79**, 1-8.
Murray, R. M., Bryant, M. P. and Leng, R. A. (1976). Rates of production of methane in the rumen and large intestine of sheep. *Br. J. Nutr.* **36**, 1-14.
Ouwerkerk, D., Klieve, A. V., Forster, R. J., Templeton, J. M. and Maguire, A. J. (2005). Characterization of culturable anaerobic bacteria from the forestomach of an eastern grey kangaroo, *Macropus giganteus*. *Lett. Appl. Microbiol.* **41**, 327-333.
Ouwerkerk, D., Maguire, A. J., McMillen, L. and Klieve, A. V. (2009). Hydrogen utilising bacteria from the forestomach of eastern grey (*Macropus giganteus*) and red (*Macropus rufus*) kangaroos. *Anim. Prod. Sci.* **49**, 1043-1051.
Pinares-Patino, C. S., Ulyatt, M. J., Lassey, K. R., Barry, T. N. and Holmes, C. W. (2003). Rumen function and digestion parameters associated with differences between sheep in methane emissions when fed chaffed lucerne hay. *J. Agric. Sci.* **140**, 205-214.
Pope, P. B., Smith, W., Denman, S. E., Tringe, S. G., Barry, K., Hugenholtz, P., McSweeney, C. S., McHardy, A. C. and Morrison, M. (2011). Isolation of *Succinivibrionaceae* implicated in low methane emissions from Tammar wallabies. *Science* **333**, 646-648.
Robbins, C. T. (1993). *Wildlife Feeding and Nutrition*. San Diego: Academic Press.
Saalfeld, W. K. and Edwards, G. P. (2010). Distribution and abundance of the feral camel (*Camelus dromedarius*) in Australia. *Rangeland J.* **32**, 1-9.
Sasaki, N., Hobo, S. and Yoshihara, T. (1999). Measurement for breath concentration of hydrogen and methane in horses. *J. Vet. Med. Sci.* **61**, 1059-1062.
Sauer, F. D., Fellner, V., Kinsman, R., Kramer, J. K., Jackson, H. A., Lee, A. J. and Chen, S. (1998). Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. *J. Anim. Sci.* **76**, 906-914.
Schwarm, A., Ortmann, S., Wolf, C., Streich, W. J. and Clauss, M. (2009). Passage marker excretion in red kangaroo (*Macropus rufus*), collared peccary (*Pecari tajacu*) and colobine monkeys (*Colobus angolensis, C.* polykomos, Trachypithecus johnii). *J. Exp. Zool. A Ecol. Genet. Physiol.* **311A**, 647-661.
Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H. et al. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome Res.* **24**, 1517-1525.
Stevens, C. E. and Hume, I. D. (1998). Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiol. Rev.* **78**, 393-427.
Vendl, C., Frei, S., Dittmann, M. T., Furrer, S., Osmann, C., Ortmann, S., Munn, A., Kreuzer, M. and Clauss, M. (2015). Digestive physiology, metabolism and methane production of captive Linné's two-toed sloths (*Choloepus didactylus*). *J. Anim. Physiol. Anim. Nutr.* (online). doi:10.1111/jpn.12356.
von Engelhardt, W., Wolter, S., Lawrenz, H. and Hemsley, J. A. (1978). Production of methane in two non-ruminant herbivores. *Comp. Biochem. Physiol. A. Physiol.* **60**, 309-311.
Wilson, G. R. and Edwards, M. J. (2008). Native wildlife on rangelands to minimize methane and produce lower-emission meat: kangaroos versus livestock. *Conserv. Lett.* **1**, 119-128.

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