

Reduced enteric methane emissions alters carbon isotope ratio of animal-derived products

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Abstract

Numerous strategies to reduce methane emission from ruminant livestock are being developed, but evaluating their effectiveness is limited by the lack of widely available methods to quantify emissions. Here, we explore stable carbon isotope ratios of animal-derived products as a potential biomarker for inferring changes in enteric methane emission. Methanogenesis in the rumen preferentially uses the light isotope of carbon, ^{12}C , concentrating the heavy isotope, ^{13}C , in the animal. It is hypothesised that reducing methane production changes the carbon isotope ratios ($\delta^{13}\text{C}$) of the animal-derived products relative to diet.

To test this hypothesis, a randomised controlled feeding trial was conducted on sheep with variable levels of methane suppression. The trial involved a baseline period (no suppression) and an experimental period with four treatment groups with different levels of supplementation with the methane inhibitor 3-NOP (Bovaer®). Methane yields measured in respiration chambers decreased by 22.2%, 33.5% and 52.9% for low, mid and high doses, respectively. Biomatter (i.e. wool, blood plasma, and rumen fluid) $\delta^{13}\text{C}$ values were compared to diet to establish the biomatter-diet $\delta^{13}\text{C}$ offset ($D_{\text{biomatter}}$). The $D_{\text{biomatter}}$ values differed significantly for the 3-NOP treatment groups versus control groups for wool and plasma, providing support for this novel biomarker approach.

1. Introduction

Enteric emissions from ruminant livestock constitute roughly 25% of direct anthropogenic global methane emissions^{1,2}, stimulating extensive efforts to develop ways to reduce enteric methane through diet composition, inhibitors, probiotics, genomics, and breeding³. Development of novel strategies and verification of actualised reductions in livestock requires methods to assess changes in enteric methane emissions from individual animals. Currently, livestock emission measurements are limited by the significant infrastructural investment required to build and maintain complex equipment^{4,5}. The dearth of readily accessible methods restricts the global capacity to develop and evaluate methane reducing technology, and to verify actualised effects in production systems, ultimately slowing efforts to reduce greenhouse gas emissions from agriculture. Here we explore whether stable carbon isotope ratios ($\delta^{13}\text{C}$) of animal-derived products might provide an avenue for assessing enteric methane emissions for individual ruminant animals.

The difference in the masses of the stable isotopes of carbon, ^{12}C and ^{13}C , leads to fractionation (the selective partitioning of isotopes in different materials) during biological and physical reactions. Fractionations in animal-derived products (e.g. tissues, hair, biofluids, gases and waste) are typically presented relative to the $\delta^{13}\text{C}$ values of the diet because it is the source of carbon for all growth and metabolism^{6,7}. (See methods for explanation of isotopic nomenclature). One of the largest biological fractionations of carbon isotopes occurs during the formation of methane by archaea^{8,9}. Methanogenesis by archaea preferentially incorporates ^{12}C into the CH_4 causing enteric methane $\delta^{13}\text{C}$

values to be more negative than the diet by 40‰ to 50‰^{10,11}. Therefore, it is hypothesized that the selective loss of ¹²C as methane concentrates ¹³C in the animal. This process has been proposed to explain the differences in ruminants as compared to non-ruminants in terms of observed fractionations between animal-derived products (e.g. tooth enamel and breath) and diet^{6,7}. Establishing these fractionations in animals with different dietary physiologies (e.g. ruminants and non-ruminant foregut fermenters, coprophagous and non-coprophagous hindgut fermenters) is important in ecology, paleoecology and agriculture to support the interpretation of carbon isotope ratios of animal-derived products as proxies for proportional dietary intake of C₃ and C₄ vegetation^{6,7,12,13}.

To date, studies on how methane production affects carbon isotope ratios of animal-derived products have focused on comparisons of digestive physiologies and have not yet considered whether carbon isotope ratios can be used to detect changes in enteric methane emissions within a single species of ruminant. In theory, the concentration of ¹³C in the animal due to the preferential loss of ¹²C in methane should vary in proportion to the fraction of carbon lost as methane if other factors, such as species, are controlled. Simple mass balance calculations¹⁴ can be employed to quantify the magnitude of isotopic effect in animal-derived products that is expected as a result of reduced methane production by ruminants. Cattle and sheep lose at least 5% of the digestible carbon in their diet as methane¹⁵. Using this as a starting point, a hypothetical comparison of two ruminant animals can be calculated as follows: a control animal who loses 5% of the digestible dietary carbon as methane and an abated animal whose emissions have been reduced by half and loses only 2.5% of digestible carbon as methane (Fig. 1; Supplementary Table S1). The methane d¹³C value estimated in this example of -69‰ illustrates the preferential incorporation of ¹²C relative to ¹³C relative to the diet d¹³C value of -28‰. In the methane abated animal, less ¹²C is lost, meaning more is retained for growth and metabolism and able to be incorporated into the animal-derived products (e.g. hair, wool, plasma). In this example, the mass balance prediction is that a reduction in methane production by 50% should lead to a shift of 1‰ in carbon isotope ratios of animal-derived products.

To test the hypothesis that changes in methane production in ruminant livestock leave a discernible isotopic fingerprint in the carbon isotope ratios of animal-derived products, a controlled animal trial was conducted with 24 sheep split into four experimental groups: a control and three different levels of methane suppression. Methane suppression was achieved by inclusion of three different dosages of the inhibitor 3-nitrooxypropanol (3-NOP) (Bovaer®, DSM-Firmenich Nutrition Products Ltd.) in fodder cubes. The resulting methane abatements were measured using respiration chambers. Carbon isotope ratios of the diet, wool, blood plasma, rumen fluid and faeces were measured to determine whether discernible carbon isotope differences exist between methane-abated and control animals. The aim of the study is to provide a proof-of-concept for how variation in carbon isotope ratios due to methane production could be leveraged as a method for detecting changes in methane production in ruminant livestock.

2. Methods

2.1 Animal trial

The experiment was performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (eighth edition, 2013), complied with ARRIVE guidelines and was approved by CSIRO Livestock Animal Ethics Committee (Approval no.24 – 06).

A power analysis determined that a minimum of six animals per group was required to detect significant differences in methane yield between treatments. Thirty-two healthy Merino sheep (body condition score of at least 3) were selected by body weight (BW: 46–50 Kg on May 20, 2024) and gender (wethers only) from the CSIRO Chiswick commercial Merino flock born in 2022. The final group of 24 Merino wethers (average BW = 57.4 ± 2.7 kg, s.d.) were selected based on their temperament and suitability following a period of acclimatisation and training to animal house and respiration chamber conditions (July 22 – September 1, 2024). Animals were blocked into groups of similar weight and randomly assigned to one of the four treatments ($n = 6$ per treatment) termed control, low, mid and high in reference to the level of the methane inhibiting supplement 3-nitrooxypropanol (3-NOP) (Bovaer®, DSM-Firmenich Nutrition Products Ltd.) delivered during the experimental phase. No formal allocation concealment was employed. Average weights were not significantly different between treatment groups prior the commencement of the study.

The trial was conducted in two phases: a baseline and an experimental phase (Fig. 2). During the 10-week baseline phase, all animals in all groups were fed *ad libitum* a constant and uniform baseline diet of lucerne and oaten hay formulated into fodder cubes (MultiCube, Yarrowonga, Vic.) with no 3-NOP. During the 10-week experimental phase, the four treatment groups were each fed *ad libitum* a custom made lucerne and oaten hay fodder cube diet produced by MultiCube with one of four different levels of the methane inhibiting supplement 3-NOP: none (control), low, mid and high (55, 130 and 256 mg/kg DM, respectively). The nutritional profile of feed, analysed by DPI NSW (Wagga Wagga Agricultural Institute), is shown in Supplementary Table S2. Safety and welfare considerations prevented blinding of animals' treatment groups to ensure the rapid and effective management of potential adverse effects. The risk of potential selection or information bias was reduced by having objective outcome measures quantified using standardised protocols.

The baseline diet was a commercially available batch of lucerne and oaten hay fodder cube from MultiCube that was subsampled for isotope analysis. The four experimental diets, with either no (control) or varying amounts of 3-NOP (low, mid and high), were manufactured at one time from the same batch of lucerne and oaten hay to minimise variation in carbon isotope ratios among diets. The 3-NOP was provided already mixed with millrun (Bovaer® 2%) for feed manufacturing purposes and thus an equivalent amount of millrun without any 3-NOP was added to the control diet (0.5% of total ingredients). Feed samples were analysed by ALS Global (Melbourne, Australia) to determine the 3-NOP concentration. The control diet contained no 3-NOP, while the low-, mid- and high-dose diets contained 3-NOP at concentrations of 55, 130 and 256 mg/kg DM, respectively.

The trial commenced July 22, 2024. Animals were housed in group pens (weeks 1–3) and then in individual pens (week 4) in an animal house at the CSIRO Chiswick Research Property. Then animals were transferred to the Centre for Animal Research and Teaching (CART) sheep unit at the University of New England (UNE) where they were housed in individual pens in association with measurement in respiration chambers for the baseline phase (weeks 5–9). Following completion of baseline methane measurements, animals were returned to CSIRO Chiswick Research Property and housed in the group pens to begin the experimental phase of the trial (week 10–14). The animals were returned the CART sheep unit at UNE for methane measurements for the experimental phase and were housed in individual pens (weeks 15–18) before returning to be housed group pens at Chiswick Research Property for the remainder of the trial (weeks 19–20).

At the start of the trial, wool was clipped from a patch on the mid side of each animal. All animals were then fed the control diet for three weeks to enable wool within the skin, but formed prior to the trial, to grow to the surface and to a suitable length for trimming. After the 3 weeks, the ‘transitional’ wool was removed from the patch and discarded to ensure that the wool that was subsequently collected was grown entirely during the baseline period. After 7 weeks on the baseline diet, the patch of wool was clipped again and collected as the baseline wool sample.

For the experimental phase, animals were gradually introduced to the new diet that contained 3-NOP over a period of five days. After 3 weeks on the experimental diets, the transitional patch of wool was once again removed and discarded, and the experimental wool growth period began. After another 7 weeks on the experimental diet, the wool was collected as the experimental wool sample.

After at least 50 days on either the baseline or experimental diet, animals had their methane emissions measured in respiration chambers at the University of New England, Armidale NSW. Prior to entering the respiration chambers, feed dry matter intake (DMI) was measured for a minimum of 13 days. Animals were measured for at least two days (consecutively) and up to three days (non-consecutively), with a day consisting of at least 23 hours in the chamber to obtain methane production (CH_4 g/day) and methane yield (CH_4 g/ kg DMI / day) values for individual animals. While in the respiration chambers, feed intake was measured.

Samples of fodder cube feed were collected throughout the trial for analysis of isotope ratios and, for the experimental period for measurement of the concentration of 3-NOP. During the baseline and experimental phases, blood and faeces were sampled 3 h post-feeding on the day that the animals completed two days of measurement in respiration chambers. Blood samples from all animals were collected by jugular venipuncture using a 10 ml blood Vacutainer tube (BD Vacutainer® tubes containing lithium heparin). Blood was stored at room temperature for 30 minutes then refrigerated until being centrifuged for 15 minutes at $2000 \times g$ at 4°C to isolate the plasma. Plasma was then frozen at -80°C and freeze-dried prior to analysis. Faeces were oven dried (72hr at 60°C) and ground. In addition, during the experimental phase, rumen fluid was collected by esophageal intubation at 3 h post feeding following the second day in the respiration chamber. The pH of the rumen fluid was measured

immediately after collection and samples were immediately put on ice and then stored at – 80°C until analysed for ruminal fermentation metabolites.

2.2 Gas measurements

Methane (CH₄) emission was assessed using 8 open-circuit polycarbonate respiration chambers¹⁸ with animals measured in three cohorts of eight animals in individual respiration chambers for 48h. The chambers were opened for approximately 10 to 15 min towards the end of the 24 h period to facilitate the cleaning of the respiration chambers, collection of refusals, and to provision of fresh feed for the animals. The concentrations (ppm) of CH₄, ammonia, carbon dioxide, dinitrogen-oxide, and air pressure (Kpa) from each chamber were quantified using an INNOVA multipoint sampler gas analyser (INNOVA 1409; 24 Channel Multipoint Sampler, LumaSense Technologies, Raunheim, Germany) at regular intervals of approximately 9.0 min. The concentration of H₂ (ppm) per chamber was measured using Dräger X-am 5,000 (Draeger Safety Pacific Pty. Ltd., Notting Hill, VIC, Australia). Assessment of CH₄ recovery in the chambers was conducted by injecting pure CH₄ at a predetermined rate using a mass-flow controller (Sierra Instruments, Monterey, USA). At the start and end of the chamber session, the chamber airflow rate (in and outflow) was recorded with time. The temperature within the chamber was continuously monitored and recorded by a data logger, and the average recovery of CH₄ was calculated (Average mean ± SD: 104.8 ± 4.84%). Using the atmospheric data and flow rate of the chamber, the chamber volume was converted to volume at standard temperature and pressure (Vstp), allowing for CH₄ ppm to be converted to CH₄ g. Grams of CH₄ was further corrected for each chamber based on the calculated recovery value for the chamber. CH₄ and H₂ production (g) were calculated for each 9-minute interval throughout the 24 h period and totalled to provide a daily methane production value (g/day).

2.3 Rumen fermentation metabolites

Concentrations of volatile fatty acids (VFAs) (acetate, propionate, n-butyrate, iso-butyrate, iso-valerate and n-valerate) were measured by gas chromatography (GC) as described by Gagen et al. (2014)¹⁹. Iso-valerate (3-methyl butyrate) includes 2-methyl butyrate, which co-elutes. The NH₃-N concentration was determined by a colorimetric method following Chaney and Marbach²⁰. An UltiMate® 3000 HPLC system (Dionex, Sunnyvale, CA, USA) with a dedicated Photodiode Array Detector and an Autosampler was used to measure organic acids in the supernatants of rumen fluid samples as described by Gagen et al. (2014)¹⁹.

2.4 Isotopic measurements

Carbon isotope ratios are expressed in delta notation (δ¹³C) as deviations from unity in the ratio of the number of atoms of ¹³C to ¹²C in a sample relative to that in a standard²¹.

$$\delta^{13}C = \frac{[^{13}C/^{12}C]_{sample}}{[^{13}C/^{12}C]_{standard}} - 1 \text{ (Eq. 1)}$$

The standard used for reporting $\delta^{13}\text{C}$ values is the Vienna PeeDee Belemnite (VPDB)²². Natural abundance carbon isotope ratios are small and thus are multiplied by a factor of 1000 and expressed in parts per thousand or permil (‰). A $\delta^{13}\text{C}$ value of zero indicates that the ratio of the number of ^{13}C to ^{12}C atoms in the sample is the same as that in the standard. Negative values indicate that the sample has less ^{13}C relative to ^{12}C than the standard, and positive values indicate that the sample has more ^{13}C relative to ^{12}C than the standard. Similarly, $\delta^{13}\text{C}$ values of two samples can be compared to determine whether one has less ($\delta^{13}\text{C}$ is more negative or smaller), or more ($\delta^{13}\text{C}$ less negative or larger) ^{13}C relative to ^{12}C than another. Inter-sample comparisons can be quantified as differences between carbon isotope ratios of two materials, termed here offsets. For simplicity, the term biomatter is used here to refer to all animal-derived materials. In this study, carbon isotope ratios of biomatter are compared to carbon isotope ratios of the diet and expressed as biomatter-diet offsets ($D_{\text{biomatter}}$).

$$\Delta_{\text{biomatter}} = \delta^{13}\text{C}_{\text{biomatter}} - \delta^{13}\text{C}_{\text{diet}} \text{ (Eq. 2)}$$

The specific types of biomatter are identified in the postscript and are wool, plasma, faeces, fluid and pellet, with the last two referring to the fluid and pellet subsamples of the rumen fluid, respectively. When comparing among values of $D_{\text{biomatter}}$, a smaller value indicates less ^{13}C relative to ^{12}C , or said another way, more ^{12}C relative to ^{13}C . Thus, the hypothesis tested here is that reducing methane emissions will reduce $D_{\text{biomatter}}$ values in materials synthesized by the animal.

The baseline diet was collected at the start of the trial, and the four experimental diets (control, low, mid and high) were subsampled throughout the 10 weeks, with carbon isotope ratios measured on seven subsamples from each of the four diets. Fodder cubes and faeces samples were oven dried at 60°C and ground to a fine powder in a puck mill prior to analysis. The wool was untreated, and measurements were conducted on entire strands of wool to ensure they were representative of the entire growth period. Plasma, rumen fluid and rumen pellet samples were freeze dried prior to analysis.

The $\delta^{13}\text{C}$ values of the diet, wool, plasma, faeces, rumen fluid and rumen pellet were measured using an elemental analyser (EA3000, EuroVector, Pavia, Italy) coupled to a continuous flow isotope ratio mass spectrometer (Nu Horizon, Wrexham, UK) at Mawson Analytical Spectrometry Services at the University of Adelaide. Diet, wool, plasma, and faeces were analysed in duplicate or triplicate and averaged, while rumen fluid and pellet samples were analysed once each. Both isotopic drift and linearity (peak area effects)²² were monitored throughout and corrections were applied as required, leading to small and uniform adjustments to values, due to the uniform sample weights (standard deviation of corrections applied within a run < 0.06‰). Measured $\delta^{13}\text{C}$ values were normalised to VPDB using reference materials run in the same analytical sequence (N = 18 standards). These included three in-house standards, glycine ($\delta^{13}\text{C} = -31.2\text{‰}$), glutamic acid ($\delta^{13}\text{C} = -16.72\text{‰}$) and triphenylamine (TPA, $\delta^{13}\text{C} = -29.2\text{‰}$) and USGS88 as a certified reference material ($\delta^{13}\text{C} = -16.06\text{‰}$). The in-house standards were calibrated against USGS and IAEA certified reference materials (USGS40, USGS41, IAEA-2). To evaluate precision for each analytical sequence, the standard deviation of the differences between the measured and

expected values was first calculated for each reference material. The overall precision for that sequence was then calculated as the average of the standard deviations for all three standards weighted by the proportional representation of each standard in the sequence. Precision was $\pm 0.10\%$ or better for diet, wool, plasma, rumen fluid and rumen pellet, and $\pm 0.16\%$ or better for the faeces.

2.5 Data analysis

The effect of 3-NOP inclusion into the fodder cubes (control, low, mid, high) was analysed for body weight, DMI, rumen fermentation metabolites and methane production. Data was analysed as a univariate model using the GLM procedure of SPSS (IBM Corp., version 21.0, Armonk, NY, USA), with the animal as the experimental unit. The treatment level was considered the fixed effect. Linear, cubic and quadratic components of the response to incremental levels of 3-NOP in the diet were evaluated using polynomial contrasts. Effects were declared significant at $p < 0.05$ and p -values between 0.05 and 0.10 were considered as a trend. When significant differences were detected, differences among means were tested by pairwise comparisons (Tukey-Kramer).

Some animals had reduced feed intake while in the respiration chambers leading to unrepresentative emissions measurements. Thus, methane and hydrogen emissions data were excluded for days when dry matter intake in the chamber was $< 60\%$ of normal intake. The difference in averaged dry matter intake detected (p -value < 0.001) was 24% lower in respiration chambers than in pens, which was similar to other studies that reported 15–25% reduction in DMI when animals were in respiration chambers^{23,24} (experimental period total $N = 11$, control (3), low (3), mid (2) and high (3)). Rumen fluid was collected on the day following chamber measurements three hours after feeding, and to gain a representative rumen fluid sample, individuals who consumed $< 80\%$ of normal DMI on the morning of sampling were excluded from the analyses of rumen metabolites and rumen fluid isotope ratios (Total $N = 18$, control (4), low (4), mid (5) and high (5)). However, analyses of the isotope data for wool, plasma and faeces, included all animals ($N = 24$) because the brief reduction in intakes while in respiration chambers (2–3 days), would have had a minimal effect on longer-term (up to 49 day) isotope signatures of these materials.

Carbon isotope ratios of feed and biomatter offsets for the different treatment groups were analysed for both the baseline and experimental phases separately using anova (aov) in R followed by Tukey-Kramer post-hoc tests of significance to determine significance of pairwise comparisons, which allows for unequal sample sizes. Within-group comparisons of $D_{\text{biomatter}}$ for the baseline versus the experimental phases were conducted in R using paired t -tests of significance. To avoid conflating temporal changes due to growth with actual changes due to methane abatement, comparisons between the baseline and experimental phases were restricted to animal-derived products for which the control group $D_{\text{biomatter}}$ were statistically indistinguishable for the two phases. For the experimental phase, results for individual animal's $D_{\text{biomatter}}$ values were compared to their methane yield using linear regression in R, with evaluation of significance for non-zero slopes. Differences in $D_{\text{biomatter}}$ from the control group were regressed against group averaged methane for the experimental phase with coefficients of

determination (R^2 values) reported for regressions using all individual animals $D_{\text{biomatter}}$ values, although, for simplicity, group-averaged $D_{\text{biomatter}}$ values are plotted.

2.5 Mass balance model of isotopic effects of methane abatement

To assess the observed reductions in $D_{\text{biomatter}}$ associated with reduced methane emissions, a mass balance model¹⁴ illustrated in Fig. 1 was quantitatively evaluated using the measurements from the trial (calculations illustrated in Supplementary Table S1). The daily dry matter feed intake (DMI), dry matter digestibility of the diet, and measured C:N ratios were used to calculate the daily digestible carbon intake (g C/day) for each group. Daily methane emissions (g CH_4 /day) were converted to daily C loss as methane (g C/day) that enabled the calculation of the percentage of digestible carbon lost as methane for each treatment group. By assuming that the $\delta^{13}\text{C}$ value measured for faeces provided a good estimate of the $\delta^{13}\text{C}$ non-digestible portion of the diet, the $\delta^{13}\text{C}$ of the digestible C was calculated by rearranging the following simple mass balance equation in which F represents the fraction of carbon intake that is digestible C.

$$\delta^{13}\text{C}_{\text{diet}} = F \times \delta^{13}\text{C}_{\text{digestible}} + (1 - F) \times \delta^{13}\text{C}_{\text{faeces}} \quad (\text{Eq. 3})$$

The digestible carbon was then divided into a portion lost as methane (M) and a portion retained. The $\delta^{13}\text{C}$ for enteric methane was estimated from the $\delta^{13}\text{C}$ of diet using a calibration based on a published meta-analysis of enteric methane $\delta^{13}\text{C}$ offsets relative to diet¹¹. Rearranging the following mass balance equation enabled calculation of the carbon isotope ratio of the carbon retained in the animal, $\delta^{13}\text{C}_{\text{retained}}$.

$$\delta^{13}\text{C}_{\text{digestible}} = M \times \delta^{13}\text{C}_{\text{methane}} + (1 - M) \times \delta^{13}\text{C}_{\text{retained}} \quad (\text{Eq. 4})$$

The carbon retained represents what is available for biosynthesis of animal-derived products. Thus, the difference between the methane abated groups and the control in $\delta^{13}\text{C}_{\text{retained}}$ provided a prediction of the magnitude of expected decreases in $D_{\text{biomatter}}$ in the experimental phase relative to the control. The predicted decreases in $D_{\text{biomatter}}$ with methane abatement was compared to observations for each group.

3. Results

3.1 Methane emission measurements

For the baseline period, methane production (g CH_4 /day) and methane yield (g CH_4 /kg DMI) did not differ between experimental groups, all of whom received the same feed, indicating that there were no inherent group differences (p -values = 0.95 and 0.759 for methane production and yield, respectively) (Fig. 3 and Supplementary Table S3). In contrast, during the experimental phase, when animals received different dosages of the methane inhibitor 3-NOP, a significant linear effect was detected on methane

production and yield among groups (p -value < 0.001; with R^2 = 0.823 and 0.811 respectively), showing a dose-dependent response (Fig. 3 and Table 1). There was a significant effect (p -value < 0.001) on methane production (g CH₄/day) at the low, mid and highest dose levels, which produced 22.0 ± 4.1 (s.d.), 18.2 ± 0.42 and 13.3 ± 2.7 g CH₄/day of methane respectively, representing a 24.7, 37.7 and 54.5% decrease in methane production compared to control animals (29.2 ± 1.6 g CH₄/day). Methane yield showed a similar pattern, with a significant decrease (p -value = 0.003) for the three levels compared to control. Results showed a 22.2%, 33.5% and 52.9% decrease in methane yield for low, mid and highest dose respectively (17.2 ± 2.7 , 14.7 ± 2.9 and 10.4 ± 2.3 g CH₄/ kg DMI) compared to 22.1 ± 1.0 g CH₄/ kg DMI for the control group. Emissions from all animals during the baseline phase averaged 19.6 ± 2.1 g CH₄/Kg DMI (n = 19) which is similar to the control group during the experimental phase that averaged 22.1 ± 1.0 g CH₄/Kg DMI (n = 3). Conversely, H₂ expelled by 3-NOP supplemented animals showed a dose-dependent linear increase when CH₄ was inhibited, for H₂ production and yield (p -value $\leq$ 0.002; R^2 = 0.945 and 0.949 respectively) (Table 1).

3.2 Rumen metabolites and animal performance

The level of 3-NOP inclusion effects on rumen fermentation metabolites, dry matter intake and body weight in sheep during the experimental phase are shown in Table 1 and Supplementary Table S4. No significant differences (p -value > 0.05) were observed on body weight, average daily weight gain or dry matter intake between the control and 3-NOP treated animals. Rumen fermentation metabolite measurements showed significant changes in rumen function for animals supplemented with 3-NOP. There was a shift in volatile fatty acid (VFAs) pathways towards higher propionate (trend, p -value = 0.058) and increased branched chain VFAs with a subsequent decrease (p -value < 0.05) in acetate and Acetate:Propionate ratio. Rumen organic acids were also significantly (p -value < 0.05) affected by the level of 3-NOP, with increases in rumen succinate and fumarate concentrations observed.

Table 1

Treatment effect on performance, DMI, methane emissions, methane yield, and rumen fermentation metabolites of sheep fed with fodder cubes containing different levels of 3-NOP. Results reported as a group mean $\pm$ standard error of the mean

	Control	Low	Mid	High	p-value	Polynomial contrast
BW (kg) end trial	68.0 $\pm$ 1.23	68.2 $\pm$ 2.04	67.4 $\pm$ 2.79	67.2 $\pm$ 1.86	0.986	n.s.
ADWG (Kg) 70 d	0.092 $\pm$ 0.01	0.108 $\pm$ 0.01	0.091 $\pm$ 0.01	0.097 $\pm$ 0.01	0.769	n.s.
DMI (Kg) pen	1.75 $\pm$ 0.05	1.68 $\pm$ 0.12	1.69 $\pm$ 0.08	1.66 $\pm$ 0.11	0.910	n.s.
DMI(Kg) chamber	1.33 $\pm$ 0.09	1.27 $\pm$ 0.06	1.26 $\pm$ 0.15	1.29 $\pm$ 0.08	0.948	n.s.
CH ₄ g/day	29.2 ^a $\pm$ 0.95	22.0 ^{ab} $\pm$ 2.40	18.2 ^{bc} $\pm$ 0.30	13.3 ^c $\pm$ 1.53	0.001	<i>L</i>
CH ₄ g/kg DMI	22.1 ^a $\pm$ 1.00	17.2 ^{ab} $\pm$ 1.53	14.7 ^{bc} $\pm$ 2.03	10.4 ^c $\pm$ 1.35	0.003	<i>L</i>
H ₂ g/day	0.00 ^c $\pm$ 0.00	0.35 ^b $\pm$ 0.07	0.54 ^b $\pm$ 0.03	0.89 ^a $\pm$ 0.07	0.001	<i>L</i>
H ₂ g/kg DMI	0.00 ^c $\pm$ 0.00	0.27 ^b $\pm$ 0.07	0.44 ^b $\pm$ 0.03	0.73 ^a $\pm$ 0.06	0.002	<i>L</i>
Succinate (mM)	0.15 ^b $\pm$ 0.03	0.31 ^{ab} $\pm$ 0.08	0.38 ^a $\pm$ 0.04	0.28 ^{ab} $\pm$ 0.01	0.029	<i>L,Q</i>
Fumarate (mM)	0.05 ^b $\pm$ 0.01	0.09 ^{ab} $\pm$ 0.02	0.13 ^a $\pm$ 0.02	0.04 ^b $\pm$ 0.03	0.042	<i>Q</i>
VFA (%)						
Acetate	67.0 ^a $\pm$ 0.22	58.6 ^b $\pm$ 1.25	60.7 ^b $\pm$ 0.86	61.2 ^b $\pm$ 1.05	0.001	<i>L,Q,C</i>
Propionate	22.6 $\pm$ 0.97	28.8 $\pm$ 2.10	28.0 $\pm$ 1.37	25.9 $\pm$ 1.37	0.058	<i>Q</i>
Iso-Valerate	0.52 ^b $\pm$ 0.07	0.66 ^{ab} $\pm$ 0.10	0.90 ^{ab} $\pm$ 0.13	0.99 ^a $\pm$ 0.08	0.025	<i>L</i>
N-Valerate	0.97 $\pm$ 0.05	1.26 $\pm$ 0.14	1.22 $\pm$ 0.06	1.25 $\pm$ 0.05	0.070	<i>L</i>
Ratio A:P	2.98 ^a $\pm$ 0.13	2.07 ^b $\pm$ 0.18	2.19 ^b $\pm$ 0.14	2.40 ^{ab} $\pm$ 0.18	0.009	<i>L,Q</i>

^{abc}Within each row, treatment mean values with different superscript letters indicate a significant difference ($p < 0.05$).

Contrast: Significant ($P < 0.05$) linear (L), Cubic (C), or quadratic (Q) effects of the response to level of 3-NOP estimated by polynomial contrast. n.s.: not significant

Treatments: Control, Low, Mid and High (0, 55, 130 and 256 mg 3-NOP/kg DM, respectively).

3.3 Carbon isotope ratios

3.3.1 Carbon isotope ratios of fodder cube diets

The $d^{13}C$ value for the baseline diet was -29.0‰ (Supplementary Table S5) and this value was used for comparison with biomatter samples from the baseline phase. No significant differences were found in $d^{13}C$ value of the four experimental diets (single-factor anova, $p > 0.05$). Thus, to obtain a representative value for the dietary input throughout the experimental phase, all experimental diet measurements ($n = 28$) were averaged giving an experimental diet $d^{13}C$ value of -28.33 ± 0.44 (s.d.) ‰ (Supplementary Table S5), that was used for comparison with the biomatter $d^{13}C$ values for all groups during the experimental phase.

3.3.2 Wool carbon isotope offsets from diet (D_{wool})

The D_{wool} values did not differ among groups for the baseline phase (p -value = 0.08) but did differ significantly among groups during the experimental phase with active methane inhibition (p -value = $2E-5$) (Figs. 4a and 4b, Table 2). Tukey's post-hoc test indicated that the D_{wool} for the control group was significantly larger than all other groups during the experimental phase (p -values < 0.01 for all pairwise comparisons). In addition, the high group was significantly smaller than all other groups (p -value < 0.05). The low and mid groups did not differ significantly from one another. (Data for biomatter $d^{13}C$ values for all individuals available in Supplementary Table S6 and group mean $d^{13}C$ values presented in Supplementary Table S7).

Paired comparisons of D_{wool} for the same individuals during the baseline and experimental phases showed no statistical difference within the control group, suggesting that differences in the methane-abated groups can be attributed to methane suppression, rather than changes over time (e.g. due to growth or changes in metabolism). The paired comparisons of trial phases showed that D_{wool} values that were significantly lower (p -value < 0.05) during the experimental phase than the baseline phase for all the methane-abated groups (low, mid, and high) (Supplementary Figure S1).

3.3.3 Plasma carbon isotope offsets from diet (D_{plasma})

The D_{plasma} values during the baseline phase showed no significant differences among groups (p -value = 0.70), whereas the experimental phase showed strongly significant differences among groups (p -value

= 3 E-11) (Figs. 4c and 4d, Table 2; Supplementary Tables S6 and S7). During the experimental phase, all groups differed significantly from one another.

Paired comparisons of the baseline and experimental phases for individuals in the control group indicated that D_{plasma} values differed significantly between the phases (Table 2), and thus other paired baseline–experimental comparisons were not conducted because they could not be interpreted in relation to methane abatement.

3.3.4 Faeces carbon isotope offsets from diet (D_{faeces})

The $D^{13}\text{C}_{\text{faeces}}$ values were generally very similar among groups within the baseline and experimental periods, with the only significant difference being during the baseline phase between the control and the other groups (p -value = 0.01) (Figs. 4e and 4f; Supplementary Tables S6 and S7). Notably, there were no significant differences in D_{faeces} among any of the groups during the experimental phase (p -value = 0.44).

Paired comparisons of D_{faeces} from the baseline and experimental phases for individual control animals group differed significantly (p -value = $7\text{e-}4$), and thus other paired baseline–experimental comparisons were not considered indicative of the effects of methane abatement.

3.3.5 Rumen fluid and pellet offsets from diet (D_{fluid} and D_{pellet})

For the experimental phase, no significant differences were seen among groups in D_{pellet} (p -value = 0.12) (Table 2, Supplementary Figure S2; Supplementary Tables S6 and S7) but a trend in D_{fluid} (p = 0.05) was observed with the methane-abated groups exhibiting generally lower D_{fluid} values than the control group (Fig. 4g, Table 2).

Table 2
Carbon isotope biomatter-diet offsets ($D_{\text{biomatter}}$) for wool, plasma and rumen fluid for the baseline and experimental phases for each treatment group (control, low, mid and high). Results reported as a group mean $\pm$ standard error of the mean in ‰.

	Control	Low	Mid	High	<i>p</i> -value
Baseline					
D_{wool}	2.47 ± 0.15	2.73 ± 0.07	2.68 ± 0.13	2.35 ± 0.08	0.08
D_{plasma}	3.76 ± 0.04	3.77 ± 0.04	3.75 ± 0.03	3.68 ± 0.05	0.70
D_{faeces}	0.44 ± 0.05	0.20 ± 0.07	0.23 ± 0.05	0.16 ± 0.05	0.01
Experimental					
D_{wool}	$2.77^a \pm 0.07$	$2.24^b \pm 0.09$	$2.24^b \pm 0.09$	$1.83^c \pm 0.13$	2E-05
D_{plasma}	$3.58^a \pm 0.03$	$3.16^b \pm 0.04$	$3.31^c \pm 0.02$	$2.94^d \pm 0.03$	3E-11
D_{faeces}	-1.01 ± 0.08	-1.06 ± 0.03	-1.03 ± 0.03	-1.11 ± 0.02	0.44
D_{fluid}	0.28 ± 0.15	-0.32 ± 0.15	-0.32 ± 0.20	-0.38 ± 0.14	0.05
D_{pellet}	0.54 ± 0.18	-0.15 ± 0.35	0.59 ± 0.21	0.16 ± 0.12	0.12

^{abcd}Within each row, treatment mean values with different superscript letters indicate a significant difference ($p < 0.05$).

Treatments: Control, Low, Mid and High (0, 55, 130 and 256 mg 3-NOP/kg DM, respectively).

3.4 Relationship between carbons isotope offsets and methane

Carbon isotope offsets for wool (D_{wool}), plasma (D_{plasma}) and rumen pellets (D_{pellet}), were all significantly correlated (p -value < 0.05 for non-zero slope) with the individual animal's methane yields, with a fit to the linear model of $R^2 = 0.6024$, $R^2 = 0.6706$ and $R^2 = 0.3895$ for wool, plasma and rumen pellet, respectively (Figure S4). These positive correlations indicate that animals with lowered methane yields also had lower carbon isotope offsets for wool, plasma and rumen fluid pellets. Decreased carbon isotope offsets represent that the animal-derived products contained increasing ^{12}C relative to ^{13}C with decreasing methane emissions.

Carbon isotope offsets for rumen fluid supernatant (D_{fluid}) and faeces (D_{faeces}) were not significantly correlated with individual animals' methane yields ($p > 0.05$ for a non-zero slope). The lack of correlation

with faeces suggests that D_{faeces} is insensitive to variations in methane production and instead is reflective of the diet carbon isotope ratio.

To assess the effect of methane suppression on carbon isotope offsets from diet, group means were analysed (Table 2), which allowed utilisation of all isotope data ($n = 24$) in spite of having only a subset of animals with reliable methane measurements ($n = 11$). The justification for this comparison is that 3-NOP is known to provide inhibition in sheep^{25,26} and thus the group means provide a reasonable representation of group-level inhibition. To enable comparisons across biological materials, isotope offsets were differenced from the control group means and compared group mean methane yields with a fit to the linear model of $R^2 = 0.665$ for wool, $R^2 = 0.758$ for plasma, and $R^2 = 0.096$ for faeces, respectively (Fig. 5).

3.5 Model predicted versus observed decreases in $D_{\text{biomatter}}$

Mass balance model calculations were made for each treatment group using equations 3 and 4 and data from the experimental phase for daily feed intake (Table 1), measured dry matter digestibility (DMD) of the diet of 65% (Supplementary Table S2), weight percent C of the diet (Supplementary Table S5), and methane emissions (Table 1). Group mean $d^{13}\text{C}$ values were used for diet and faeces (Supplementary Tables S5 and S7). The $d^{13}\text{C}$ value for enteric methane of -69.3‰ was calculated relative to the diet¹¹. The resulting predicted decreases in $D_{\text{biomatter}}$ relative to the control were -0.41‰ , -0.70‰ and -0.98‰ for the low, mid and high groups, respectively (Supplementary Table S1, Supplementary Figure S3). Comparison of the observed and predicted decreases in $D_{\text{biomatter}}$ showed that the best match is in wool (slope of 0.88 and $R^2 = 0.640$), with moderately good correspondence in plasma (slope = 0.57 and $R^2 = 0.705$) and rumen fluid (slope of 0.64 and $R^2 = 0.312$) (Fig. 6, Supplementary Table S1).

4. Discussion

Greater methane production by ruminants relative to non-ruminants has been hypothesized to underlie differences in their respective biomatter-diet carbon isotope offsets^{6,7} due to the preferential incorporation of ^{12}C in methane^{8,9}. To our knowledge, no study has yet considered the effect of variable methane production on the biomatter obtained from a single species of ruminants. Here, a controlled feeding trial in sheep with variable methane inhibition was conducted to test whether methane abatement creates a discernible isotopic difference in animal-derived products. The specific hypothesis being tested was that reduced methane emissions will cause reduced loss of ^{12}C in methane and greater incorporation of ^{12}C into the animal, leading to discernible reductions in carbon isotope offsets between animal biomatter and diet ($D_{\text{biomatter}}$).

Supplementation with 3-NOP during the experimental phase reduced methane yield by 22%, 33% and 53% on average relative to the control for the low, mid and high groups, respectively. This methane abatement is consistent with previous studies using 3-NOP as a methane inhibitor with levels between 80–114 mg 3-NOP / Kg DMI, detecting between 24–27% reduction in methane yield in sheep^{25,26}. The H₂ expelled by the animal increased when CH₄ was inhibited during 3-NOP supplementation. An inverse relationship between enteric CH₄ decreases and H₂ loss through eructation has been reported previously when anti-methanogenic supplements were supplemented to ruminants^{27–31}. These results indicate that a proportion of the unutilized metabolic hydrogen accumulates when CH₄ is inhibited and is eructated as H₂ gas, which still represents an energy loss to the animal. Dietary supplementation with 3-NOP significantly altered rumen fermentation function. A shift in organic acids and VFA fermentation profiles were observed in the current trial, characterized by an increase in the concentration of fumarate and succinate and the proportion of propionate and branched-chain fatty acids, alongside a decrease in acetate. This pattern in microbial fermentation is accepted as an indication of methanogenesis inhibition and has been reported in studies using various inhibitors, including 3-NOP^{26,32–34}, bromoform^{31,35,36}, and other halogenated methane analogues^{30,37,38}.

The baseline phase of the trial confirmed that methane emissions and carbon isotope offsets for wool (D_{wool}) and plasma (D_{plasma}) were not significantly different among the treatment groups, increasing confidence in the results from the experimental phase where significant differences were observed in methane emissions, and in D_{wool} and D_{plasma} . For all three abated groups (low, mid and high), D_{wool} and D_{plasma} values were significantly reduced relative to the control group. When comparing methane yield and $D_{\text{biomatter}}$ values for individual animals, D_{wool} , D_{plasma} and D_{pellet} each exhibited significant positive correlations with methane yield, with lower emissions correlated to lower $D_{\text{biomatter}}$ values. Together, these results support the hypothesis that reduced methane yield causes discernible reductions in biomatter-diet carbon isotope offsets.

A simple mass balance model enabled examination of the magnitude of change in $D_{\text{biomatter}}$ expected as a result of reducing the amount of carbon lost as methane. For a 53% reduction in methane yield, as was observed for the high group, the predicted change in carbon isotope ratios was 0.98‰, which matched closely with the decrease observed in wool of 0.94‰. Blood plasma and rumen fluid exhibited smaller decreases in the high group of only 0.65‰ and 0.66‰, respectively, suggesting that not all materials record the same magnitude of isotopic change during methane abatement. The similarity in the predicted and observed change in wool suggests that this conceptual model and simple mass balance calculations provides a viable explanation for the processes underpinning the wool observations.

There was no significant change in D_{faeces} among treatment groups during the experimental phase, which indicates that faecal isotope ratios more directly reflect the isotope ratio of the diet and are not significantly affected by changes in methane emissions. Methane production in the intestine accounts for only 10–15% of total ruminant methane production of which the majority of intestinal methane is

absorbed and expelled through the lungs³⁹. The methane inhibitor 3-NOP is not expected to inhibit intestinal methanogenesis as it rapidly binds to its target enzyme, MCR in the rumen and also easily diffuses through membranes and is denitrated in the liver, meaning that little to no 3-NOP is expected to reach the intestine especially as data is available to show that it is rapidly metabolised and broken down in the rumen to its constituent parts, namely propanediol and nitrate/nitrite^{40,41}. Furthermore, previous studies have demonstrated that carbon isotope ratios of digesta remain relatively stable during passage through the gastrointestinal tract of ruminants, potentially due to the significant contribution of undigested dietary carbon to the faeces. As a result, sheep faecal carbon isotope ratios can be used to infer dietary intake in terms of proportion of C₃ and C₄ vegetation because these plant types differ in their $\delta^{13}\text{C}$ ratios^{12,13}. The observed D_{faeces} values for the experimental phase match closely with literature values of approximately -1‰ for ruminant^{16,17}. The consistency of the isotopic offset of faeces from diet in this highly controlled study suggests also that $\delta^{13}\text{C}_{\text{faeces}}$ could potentially provide a viable approach to estimating $\delta^{13}\text{C}_{\text{diet}}$ in livestock production systems where dietary selection can be variable, such as in mixed C₃-C₄ grazing environments.

The results of this study clearly demonstrate carbon isotopic changes in animal-derived products in response to reductions in methane emission, suggesting that isotopic ratios of biomatter could provide a useful biomarker for methane abatement. To employ carbon isotope ratios to verify livestock methane abatements would require knowledge of the carbon isotope ratio of the diet either by direct measurement (e.g. $\delta^{13}\text{C}$ of feedlot diets or pasture samples) or by proxy estimation (e.g. $\delta^{13}\text{C}_{\text{faeces}}$ or vegetation mapping) or large enough number of samples to adjust for nutrition by proxy, e.g. paddock. Even with this limitation regarding the $\delta^{13}\text{C}$ values of the diet, the prospect for an isotope-based biomarker approach for evaluating methane reductions in ruminant livestock is promising because it could reduce reliance on costly infrastructure, making it eminently scalable for widespread use.

Although promising, additional research is required to further validate the use of carbon isotopic changes in animal-derived products to monitor methane reduction in livestock, due to the magnitude of the observed changes in the carbon isotope ratio (-0.53‰ and -0.94‰) for a 22–53% methane reduction with a uniform experimental diet and due to the limited animal numbers used in the current trial. Future research should address the effect of different diets, seasonality, breed and varying livestock production conditions (such as grazing, feedlot, and dairy systems), commercial practices to reduce methane levels (feed additives, improved feed sources and animal genetics) and incorporate a larger number of animals to better account for individual differences in feed selection, intake, methane production and response to intervention strategies.

5. Conclusions

This proof-of-concept trial aimed to explore whether enteric methane production's natural and inherent isotopic features could be leveraged to detect changes in enteric emissions and potentially lead to a novel approach for assessing methane abatement in livestock. The tested-hypothesis was that reduced

methane emissions would correspond to reduced biomatter–diet carbon isotope offsets. A controlled feeding trial with four levels of methane inhibition (control, low, mid and high) demonstrated that decreased methane emissions were associated with measurable decreases in carbon isotope offsets in wool and plasma, and with a trend in rumen fluid. Mass balance isotope modelling provides a conceptual framework and a quantitative prediction tool of the expected magnitude of change that matches well with the observations in wool.

An isotopic method for inferring methane abatement would have the advantage over existing measurement methods by removing infrastructural barriers because the requirement would be for physical samples to be sent to a laboratory along with contextual metadata about diet or associated samples of diet or faeces. As a result, with more research, this approach has the potential to be used at scale and around the globe, including in developing countries where access to methane measurement infrastructure is limited. Additionally, an isotopic method has the potential to provide a long-term measure of abatement that integrates over the entire period that the materials are being formed (e.g. in wool it could be a year), rather than being a short-term spot measurement (e.g. 1–2 days). As a result, more reliable and meaningful assessments over production-relevant timescales could be obtained. With further development, such a technique could have the potential to catalyse global efforts to reduce methane emissions from livestock production.

Declarations

Competing Interests

This research relates to International Patent Application No.: PCT/AU2024/050596 filed on June 06, 2024, in the name of Commonwealth Scientific and Industrial Research Organisation.

Additional information

This research relates to International Patent Application No.: PCT/AU2024/050596 filed on June 06, 2024, in the name of Commonwealth Scientific and Industrial Research Organisation. The patent is held solely by the organisation and the authors do not have any ownership or potential financial interest in it.

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declaration

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Author Contribution

FA McNerney – Conceptualization (co-lead), writing – original draft preparation (lead), methodology, investigation, formal analysis, visualisation, funding acquisition. G Martinez Fernandez – Writing – original draft preparation (supporting), methodology, investigation, formal analysis. S Denman – Writing – review & editing, methodology, investigation, formal analysis. J McNally – Writing – review & editing, methodology, investigation. R Gomez Exposito – Writing – review & editing, formal analysis, visualisation. C Gerber – Conceptualisation (supporting), writing – review & editing. S Dominik – Conceptualisation (supporting), writing – review & editing, methodology, funding acquisition. N Welti – Conceptualisation (co-lead), writing – review & editing, funding acquisition.

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Data Availability

All carbon isotope data have been deposited into the CSIRO Data Access Portal and are available at the following URL: <https://data.csiro.au/collection/csiro:70043>.

References

1. Saunois, M. et al. Global Methane Budget 2000–2020. *Earth Syst. Sci. Data*. **17**, 1873–1958. 10.5194/essd-17-1873-2025 (2025).
2. Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P. & Tricarico, J. M. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. *J. Dairy Sci.* **97**, 3231–3261. <https://doi.org/10.3168/jds.2013-7234> (2014).
3. Roques, S. et al. Recent Advances in Enteric Methane Mitigation and the Long Road to Sustainable Ruminant Production. *Annu. Rev. Anim. Biosci.* **12**, 321–343. <https://doi.org/10.1146/annurev-animal-021022-024931> (2024).

4. Tedeschi, L. O. et al. Quantification of methane emitted by ruminants: a review of methods. *J. Anim. Sci.* **100** 10.1093/jas/skac197 (2022).
5. Hill, J., McSweeney, C., Wright, A. G., Bishop-Hurley, G. & Kalantar-Zadeh, K. Measuring Methane Production from Ruminants. *Trends Biotechnol.* **34**, 26–35. 10.1016/j.tibtech.2015.10.004 (2016).
6. Passey, B. H. et al. Carbon isotope fractionation between diet, breath CO₂, and bioapatite in different mammals. *J. Archaeol. Sci.* **32**, 1459–1470. <https://doi.org/10.1016/j.jas.2005.03.015> (2005).
7. Cerling, T. E. et al. CH₄/CO₂ Ratios and Carbon Isotope Enrichment Between Diet and Breath in Herbivorous Mammals. *Front. Ecol. Evol.* **9** 10.3389/fevo.2021.638568 (2021).
8. Heyer, J., Maaß, I. & H., H. & Isotopenfraktionierung des Kohlenstoffs bei der mikrobiellen Methanbildung. *Isotopenpraxis Isot. Environ. Health Stud.* **12**, 202–205. 10.1080/10256017608543912 (1976).
9. Botz, R., Pokojski, H. D., Schmitt, M. & Thomm, M. Carbon isotope fractionation during bacterial methanogenesis by CO₂ reduction. *Org. Geochem.* **25**, 255–262. [https://doi.org/10.1016/S0146-6380\(96\)00129-5](https://doi.org/10.1016/S0146-6380(96)00129-5) (1996).
10. Metges, C., Kempe, K. & Schmidt, H. L. Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the $\delta^{13}\text{C}$ value of food in dairy cows. *Br. J. Nutr.* **63**, 187–196. 10.1079/BJN19900106 (1990).
11. Chang, J. et al. Revisiting enteric methane emissions from domestic ruminants and their $\delta^{13}\text{CCH}_4$ source signature. *Nat. Commun.* **10**, 3420. 10.1038/s41467-019-11066-3 (2019).
12. Norman, H. C., Wilmot, M. G., Thomas, D. T., Masters, D. G. & Revell, D. K. Stable carbon isotopes accurately predict diet selection by sheep fed mixtures of C₃ annual pastures and saltbush or C₄ perennial grasses. *Livest. Sci.* **121**, 162–172. 10.1016/j.livsci.2008.06.005 (2009).
13. Witt, G. B., Moll, E. J., Beeton, R. J. S., Murray, P. J. & Isotopes Wool, and Rangeland Monitoring: Let the Sheep Do the Sampling. *Environ. Manage.* **22**, 145–152. 10.1007/s002679900091 (1998).
14. Hayes, J. M. Fractionation of Carbon and Hydrogen Isotopes in Biosynthetic Processes*. *Rev. Mineral. Geochem.* **43**, 225–277. 10.2138/gsrmg.43.1.225 (2001).
15. Clauss, M. et al. Comparative methane production in mammalian herbivores. *Animal* **14**, s113–s123. <https://doi.org/10.1017/S1751731119003161> (2020). Review.
16. Codron, D. et al. Tracking the fate of digesta C-13 and N-15 compositions along the ruminant gastrointestinal tract: Does digestion influence the relationship between diet and faeces? *Eur. J. Wildl. Res.* **58**, 303–313. 10.1007/s10344-011-0581-3 (2012).
17. Reid, R. E. B., Crowley, B. E. & Haupt, R. J. The prospects of poop: a review of past achievements and future possibilities in faecal isotope analysis. *Biological Reviews* n/a, (2023). <https://doi.org/10.1111/brv.12996>
18. Goopy, J. P. et al. Low-methane yield sheep have smaller rumens and shorter rumen retention time. *Br. J. Nutr.* **111**, 578–585. 10.1017/S0007114513002936 (2014).

19. Gagen, E. J. et al. Investigation of a new acetogen isolated from an enrichment of the tammar wallaby forestomach. *BMC Microbiol.* **14**, 314. 10.1186/s12866-014-0314-3 (2014).
20. Chaney, A. L. & Marbach, E. P. Modified Reagents for Determination of Urea and Ammonia. *Clin. Chem.* **8**, 130–132. 10.1093/clinchem/8.2.130 (1962).
21. Skrzypek, G. et al. Minimum requirements for publishing hydrogen, carbon, nitrogen, oxygen and sulfur stable-isotope delta results (IUPAC Technical Report). *Pure and Applied Chemistry* **94**, 1249–1255, (2022). doi:10.1515/pac-2021-1108
22. Werner, R. A. & Brand, W. A. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun. Mass. Spectrom.* **15**, 501–519. 10.1002/rcm.258 (2001).
23. Bickell, S. L., Revell, D. K., Toovey, A. F. & Vercoe, P. E. Feed intake of sheep when allowed ad libitum access to feed in methane respiration chambers. *J. Anim. Sci.* **92**, 2259–2264. 10.2527/jas.2013-7192 (2014).
24. Robinson, D. L. et al. Sire and liveweight affect feed intake and methane emissions of sheep confined in respiration chambers. *Animal* **8**, 1935–1944. <https://doi.org/10.1017/S1751731114001773> (2014).
25. Rivelli, I. et al. Efficacy of 3-NOP applied in drinking water on enteric methane reduction in sheep. *Anim. Feed Sci. Technol.* **327**, 116400. <https://doi.org/10.1016/j.anifeedsci.2025.116400> (2025).
26. Martínez-Fernández, G. et al. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. *J. Dairy Sci.* **97**, 3790–3799. <https://doi.org/10.3168/jds.2013-7398> (2014).
27. Mitsumori, M. et al. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *Br. J. Nutr.* **108**, 482–491. 10.1017/s0007114511005794 (2012).
28. Hristov, A. N. et al. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proceedings of the National Academy of Sciences* **112**, 10663–10668, doi: (2015). 10.1073/pnas.1504124112
29. Veneman, J. B. et al. Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and Animal Productivity in Dairy Cows? *PLOS ONE*. **10**, e0140282. 10.1371/journal.pone.0140282 (2015).
30. Martinez-Fernandez, G. et al. Methane Inhibition Alters the Microbial Community, Hydrogen Flow, and Fermentation Response in the Rumen of Cattle. *Front. Microbiol.* **7** 10.3389/fmicb.2016.01122 (2016).
31. Kinley, R. D. et al. Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. *J. Clean. Prod.* **259**, 120836. <https://doi.org/10.1016/j.jclepro.2020.120836> (2020).
32. Haisan, J. et al. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. *J. Dairy Sci.* **97**, 3110–3119. <https://doi.org/10.3168/jds.2013-7834> (2014).

33. Haisan, J. et al. The effects of feeding 3-nitrooxypropanol at two doses on milk production, rumen fermentation, plasma metabolites, nutrient digestibility, and methane emissions in lactating Holstein cows. *Anim. Prod. Sci.* **57**, 282–289. <https://doi.org/10.1071/AN15219> (2017).
34. Vyas, D., McGinn, S. M., Duval, S. M., Kindermann, M. & Beauchemin, K. A. Effects of sustained reduction of enteric methane emissions with dietary supplementation of 3-nitrooxypropanol on growth performance of growing and finishing beef cattle¹. *J. Anim. Sci.* **94**, 2024–2034. [10.2527/jas.2015-0268](https://doi.org/10.2527/jas.2015-0268) (2016).
35. Roque, B. M., Salwen, J. K., Kinley, R. & Kebreab, E. Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *J. Clean. Prod.* **234**, 132–138. <https://doi.org/10.1016/j.jclepro.2019.06.193> (2019).
36. Roque, B. M. et al. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. *PLOS ONE*. **16**, e0247820. [10.1371/journal.pone.0247820](https://doi.org/10.1371/journal.pone.0247820) (2021).
37. Mitsumori, M. et al. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *Br. J. Nutr.* **108**, 482–491. [10.1017/S0007114511005794](https://doi.org/10.1017/S0007114511005794) (2012).
38. Abecia, L. et al. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. *J. Dairy Sci.* **95**, 2027–2036. <https://doi.org/10.3168/jds.2011-4831> (2012).
39. Murray, R. M., Bryant, A. M. & Leng, R. A. Rates of production of methane in the rumen and large intestine of sheep. *Br. J. Nutr.* **36**, 1–14. [10.1079/BJN19760053](https://doi.org/10.1079/BJN19760053) (1976).
40. Duin, E. C. et al. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proceedings of the National Academy of Sciences* **113**, 6172–6177, doi: (2016). [10.1073/pnas.1600298113](https://doi.org/10.1073/pnas.1600298113)
41. Govoni, M., Tocchetti, P. & Lundberg, J. O. Metabolism and Pathways for Denitration of Organic Nitrates in the Human Liver. *J. Pharmacol. Exp. Ther.* **346**, 96–104. <https://doi.org/10.1124/jpet.113.203356> (2013).

Figures

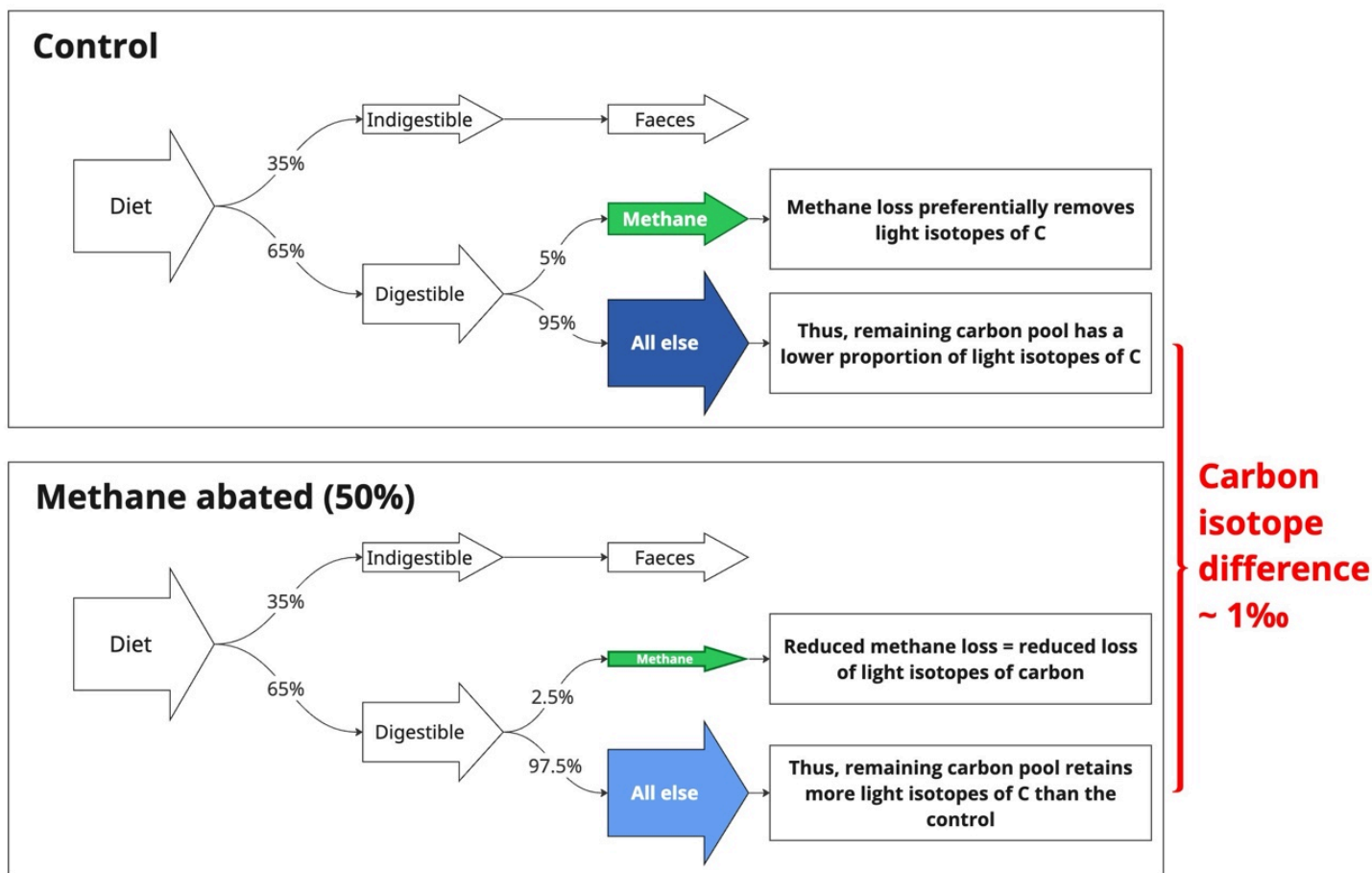


Figure 1

Schematic illustrating the isotopic effect of methane reduction on animal-derived products using a mass balance approach. Arrows indicate the division of dietary carbon into indigestible and digestible portions, with the digestible portion further divided into that lost as methane and the remainder (all else) that is available for growth and metabolism. Simple mass balance calculations enable estimation of the expected isotopic effect of a 50% reduction in methane emissions by assuming a constant $\delta^{13}\text{C}$ value for diet and assuming faeces is 1‰ more negative than diet, as has been observed for ruminants^{16,17}. The $\delta^{13}\text{C}$ value of methane can be calculated relative to the $\delta^{13}\text{C}$ of the diet following Chang et al. (2019)¹¹. The outcome is that the $\delta^{13}\text{C}$ values for carbon available for growth and metabolism is predicted to differ by ~1‰ between a control animal and an animal with a reduction in enteric methane of 50% (Example calculations in Supplementary Table S1).

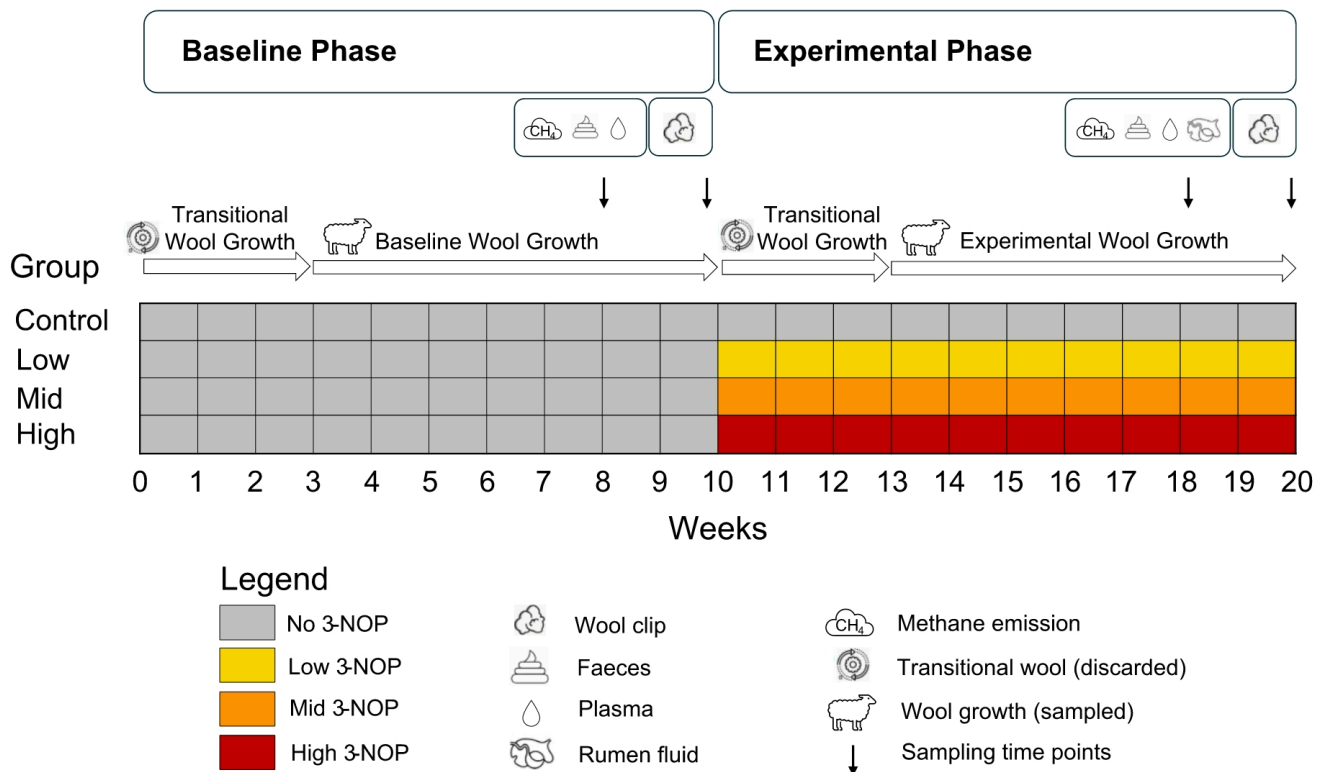


Figure 2

Schematic illustration of animal feeding trial design and sample collection timeline.

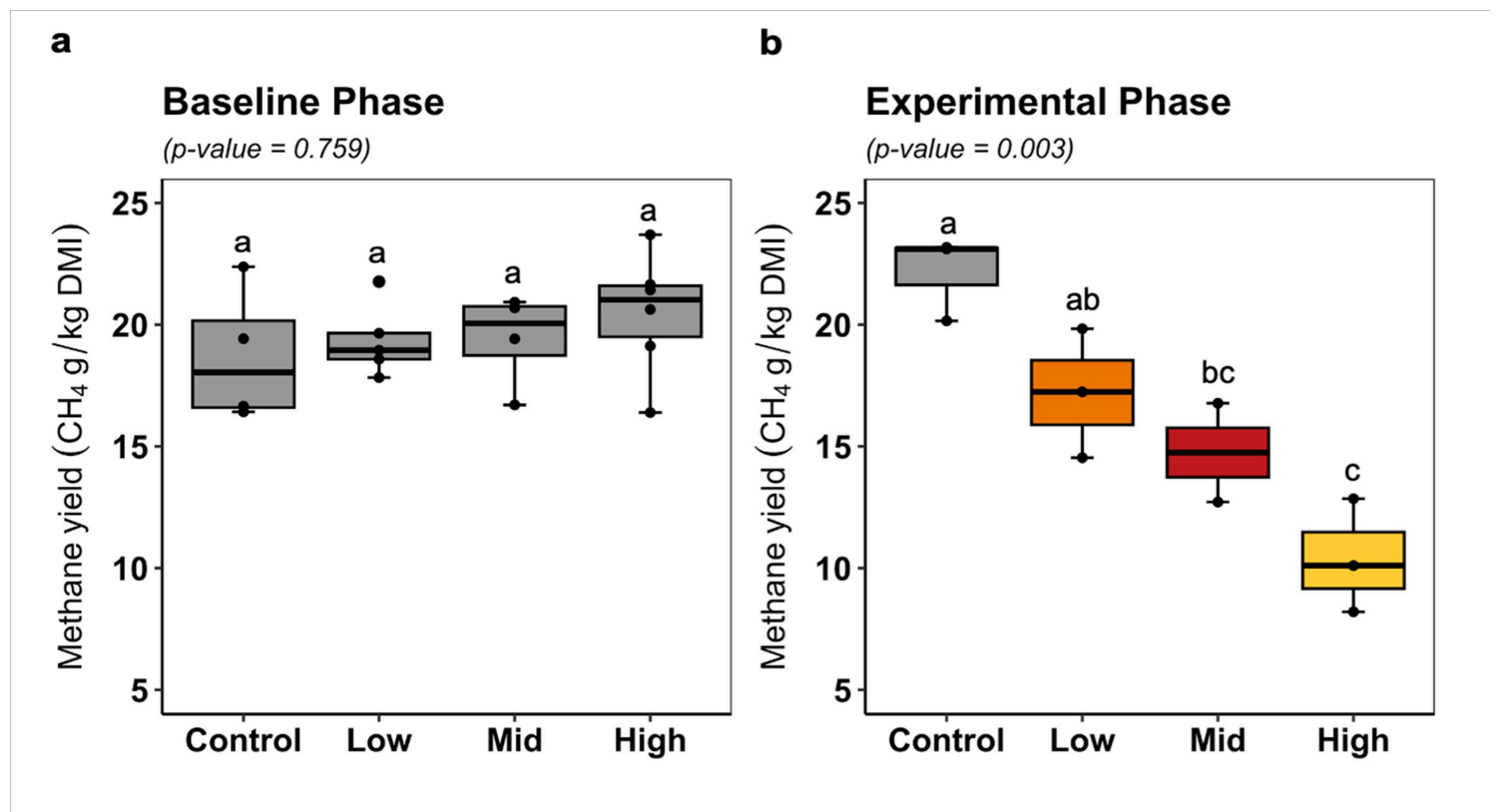


Figure 3

Methane yield (g CH₄/kg DMI) for the baseline phase (left) and the experimental phase (right) grouped by treatment group. Adjusted p-value from Anova shown, and results of Tukey-Kramer post-hoc pairwise comparisons indicated with different letters for groups that differ significantly ($p < 0.05$). For each box-and-whisker plot, the box represents the interquartile range with the median shown as a horizontal line; whiskers reach to the maximum and minimum values, with outliers shown as individual data points. Treatments: Control, Low, Mid and High (0, 55, 130 and 256 mg 3-NOP/kg DM, respectively)

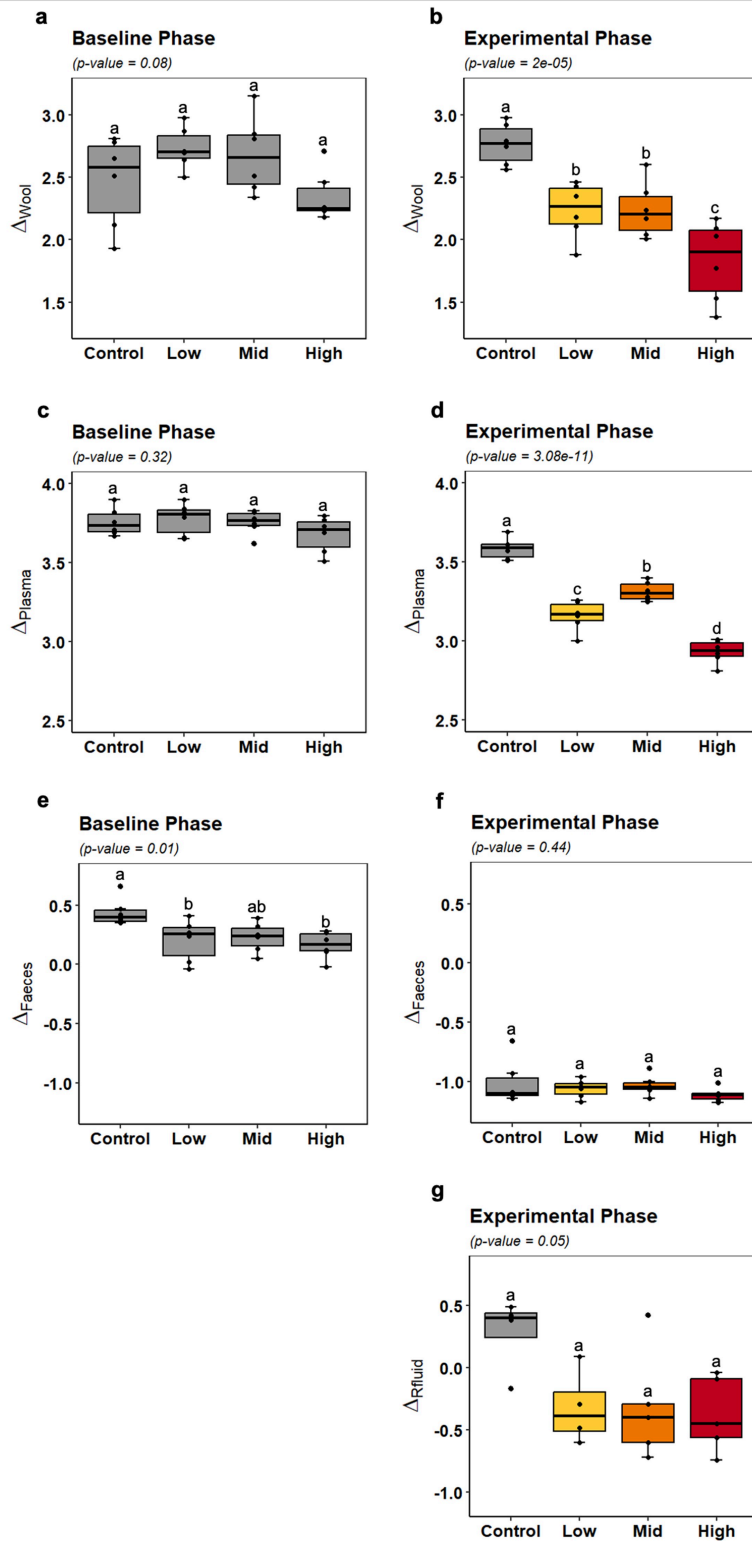


Figure 4

Carbon isotope offsets relative to diet for wool (D_{wool}) during the baseline (a) and experimental (b) phases; for blood plasma (D_{plasma}) during the baseline (c) and experimental (d) phases; for faeces (D_{faeces}) during the baseline (e) and experimental (f) phases; and for the rumen fluid for the experimental phase (g). Note for panels a-f, all animals (6 per group) were included, whereas panel g includes only those with greater than 80% dry matter intake, to ensure representative rumen fluid. Adjusted *p*-value

from Anova shown. Results of Tukey-Kramer post-hoc pairwise comparisons indicated with different letters for groups that differ significantly ($p < 0.05$). For each box-and-whisker plot, the box represents the interquartile range (IQR) with the median shown as a horizontal line; whiskers reach to the maximum and minimum values, with outliers shown as individual data points. Treatments: Control, Low, Mid and High (0, 55, 130 and 256 mg 3-NOP/kg DM, respectively).

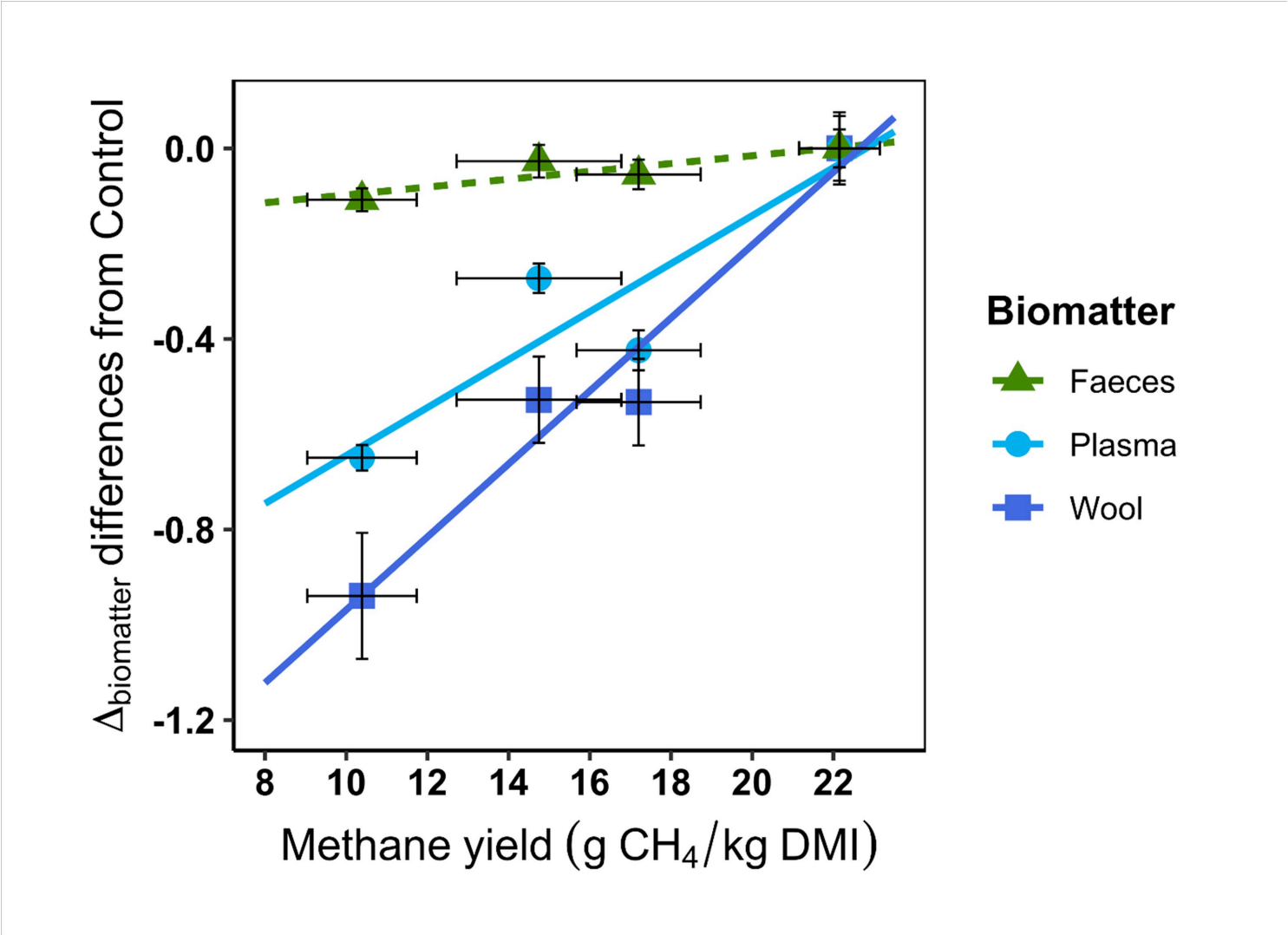


Figure 5

Difference in carbon isotope ratio offsets relative to the control for wool, plasma, faeces as a function of methane yield for each treatment group. Error bars indicate standard error for each group. Linear regressions for each sample type are also shown, with solid lines for those materials with significant differences among groups and dotted lines where differences among all four groups were not significant. Treatments: Control, Low, Mid and High (0, 55, 130 and 256 mg 3-NOP/kg DM, respectively).

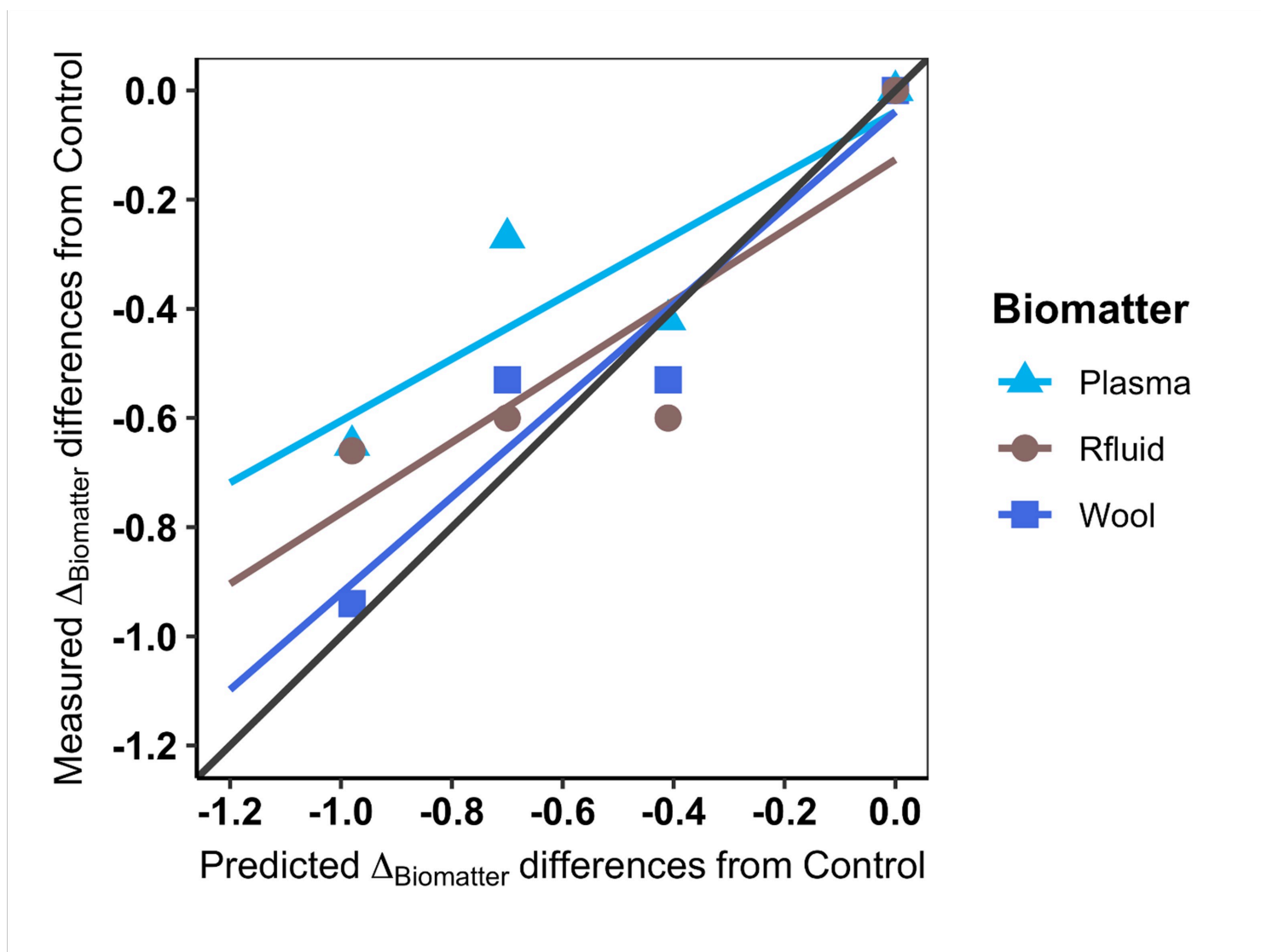


Figure 6

Predicted versus measured decreases in carbon isotope offsets relative to control for wool, plasma, and rumen pellet as a result of methane abatement. Black line represents a 1:1 line. (For mass balance calculations, see Supplementary Table S1.)

Supplementary Files

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- [SupplementaryV1McInerneyetalSciReports.docx](#)
- [SupplementaryTableS1.xlsx](#)
- [SupplementaryTableS6.xlsx](#)