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Repeatability of carbon dioxide and methane emissions and oxygen consumption by forage-consuming beef heifers

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ABSTRACT

Gas exchange by ruminant livestock is deemed as a contributor to climate change. As such, research to reduce the production of greenhouse gasses is an emerging need. It is important that these studies reach definitive outcomes. Because the majority of cows are sustained by grazing forages, the objective of this study was to assess the variation in methane (**CH_4**) and carbon dioxide (**CO_2**) emissions and oxygen (**O_2**) consumption among forage-consuming beef cattle. Recommendations as to the number of days over which data need to be collected and the numbers of experimental units that need to be used in comparing treatments can be inferred from this information. Heifers (n = 30) from a three-breed composite population of beef cattle were used in this research. They were introduced to a portable automated head-chamber system in January 2023 and had continuous access to the system through February 2024 (414 d). Data were uploaded to a server, and proprietary algorithms were used to calculate CH_4 and CO_2 emission and O_2 consumption of the heifers. Repeatability of the daily average values was calculated, and those estimates were used to infer the accuracy of measurements obtained over varying numbers of days. The numbers of heifers that are required to achieve a specified power-of-the-test for an experiment comparing two treatments and for the comparison of an observed mean against a threshold were calculated. Methane required 45 days of measurements to obtain a mean with 80% accuracy. Carbon dioxide required data from only 28 days. Given the cost of the technology to measure gasses in the grazing situation and the recommended number of animals to utilize it at one time, some care will be required in designing appropriate experiments and to properly interpret the observed effects particularly when they are not statistically significant.

Implications

Research that investigates the contribution of respiratory gasses to global warming must be fundamentally and statistically accurate to allow mitigation procedures to be derived and implemented by livestock managers. In the current study, 45 days of measurements were required to characterize the methane phenotype of an individual animal with an 80% accuracy. The carbon dioxide phenotype required data from only 28 days of measurement. Given the cost of the technology to measure gasses in the grazing situation and the recommended number of animals to utilize it at one time, some care will be required in designing appropriate experiments and to properly interpret the observed effects particularly when they are not statistically significant.

Introduction

Livestock are major contributors to food security worldwide and utilize upwards of 70% of agricultural land (Sakadevan and Nguyen, 2017). Measurements of gasses produced by livestock are useful in studies of rumen microbiology, animal metabolism, as well as greenhouse gas production (Ellis et al., 2008; Mohamed and Chaudhry, 2008; Suriyapha et al., 2024). The extent of methane (**CH_4**) emissions quantifying them is of current interest. This quantification is essential to assess the tradeoffs between strategies to mitigate climate change and technical approaches for increasing production. The sulfur hexafluoride tracer technique has been used to measure methane production from grazing and

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non-housed animals (Johnson et al., 1994). More recently, less invasive systems have been developed for the same purpose (Garnsworthy et al., 2012; Huhtanen et al., 2015). The GreenFeed portable automated head-chamber system (**AHCS**; C-Lock, Inc., Rapid City, South Dakota) has also been found useful to measure CH_4 and carbon dioxide (**CO_2**) emission and oxygen (**O_2**) consumption by grazing ruminants. Previous studies have used this technology in pen-fed settings (Arbre et al., 2016; Manafiazar et al., 2017; Ryan et al., 2022) and in these confined environments, the repeatability of daily CH_4 and CO_2 production was found to be high. However, to the best of our knowledge, there are few (e.g., Raynor et al., 2024) evaluations of animals with AHCS when their diet was almost entirely composed of rangeland grasses or harvested forages. Regardless of the technology that is used, experiments to examine strategies for mitigation of greenhouse gas (**GHG**) emission require the use of an appropriate number of animals for ethical reasons.

It is essential that before starting actual data collection, the number of experimental units to be observed in the experiment is established. Pilot studies are useful to provide estimates of the means and SDs of the response variables, which are useful for determining sample sizes of future experiments (Festing and Altman, 2002). The number of experimental units is a function of the size of a biologically meaningful effect that is to be detected, the variation among subjects, and the *P* level with which the null hypothesis is to be rejected. Any experiment has three possible outcomes. The null hypothesis can be rejected at a predetermined significance level (**α**) indicating the *P* that it is in fact true. Alternatively, the null hypothesis can be accepted with a *P* of rejecting the null hypothesis when, in fact, it is false (**β**). Finally, in an experiment with an insufficient number of experimental units, the outcome can give rise to the situation whereby no decision can be made with respect to the null hypothesis. It is this latter situation that should be avoided in the conduct of research. Unfortunately, there are numerous reports in the literature where α exceeds the desired threshold and the investigators conclude there was no effect without any evaluation of statistical power (e.g., Pereira et al., 2021). The objective of this pilot study was to assess the variation in CH_4 and CO_2 emissions and O_2 consumption among forage-consuming beef cattle. With this information, recommendations as to the number of days over which data need to be collected and the numbers of experimental units that need to be used in comparing treatments will be inferred. These criteria can be used to determine differences between experimental treatments (e.g. diet) or to the comparison of estimated breeding values for two animals (Pickering et al., 2015).

Material and methods

This pilot study was conducted in the extensive environment of the Northern Great Plains at the USDA- Agricultural Research Service, Fort Keogh Livestock and Range Research Laboratory near Miles City, Montana, USA (46° 24′30 N, 105° 50′24 W, elevation 722 m) covering a period from 5 January 2023, through 23 February 2024. The Fort Keogh Livestock and Range Research Laboratory covers 22 500 ha of rangeland, which includes rolling hills and barren land with small intersecting creeks that drain into established bordering rivers. Predominant grass genera at the study sites include crested wheatgrass (*Agropyron cristatum*), and blue grama (*Bouteloua gracilis*) within a mixed grass-dominated landscape (Küchler, 1964; Waterman et al., 2021).

Heifers (n = 30) were sampled from a composite population of beef cattle that was originally designed to contain 50% Red Angus, 25% Charolais, and 25% Tarentaise germplasm (Newman et al., 1993). However, a recent evaluation of the breed composition of this population indicates the current representation of germplasm to be approximately 57% Tarentaise, 38% Red Angus, and 5% Charolais (Hay et al., 2022). The population was stratified by winter supplementation treatments that had been applied to the grandams during mid to late gestation (beginning approximately 80 d before parturition) and the heifers used in this study were randomly selected from within each strata. Briefly, supplemental alfalfa hay or pellets were provided at rates of 1.8 kg/d or 1.2 kg/d. During days when access to pasture forage was limited due to snow cover, heifers were fed at a rate equivalent to 10.9 or 9.1 kg alfalfa hay/d. An in-depth description of protocols has previously been reported (Roberts et al., 2010; Waterman et al., 2011; Waterman et al., 2017).

The heifers (n = 30) born in 2022 (average birth date 4/21/2022 ± 2.28d; birth weight 66.4 ± 1.5 kg; weaning weight 194.4 ± 3.6 kg) were introduced to the AHCS in January 2023 (BW 244.8 ± 5.1 kg) and continually had access to the AHCS through February 2024 (414 d). However, mechanical issues (17 d) and feeding behavior whereby heifers only used the AHCS on 71.5% of the days during the time they had access to the system precluded a complete record of daily observations for the entire period. Heifers received a 100% forage (80% silage and 20% grass hay) diet in a dry lot from 5 January 2023 through 11 June 2023 (proximate analysis 56.2% DM, 12.4% CP, 71.6% TDN) when they were taken to native range pasture (Average proximate analysis of five forage samples 90.4% DM, 12.1% CP, 59.5% TDN) along with the AHCS and remained on pasture until late fall/early winter when heifers were brought back into the dry lot and received a 100% grass hay diet (proximate analysis 81.0% DM, 11.1% CP, 53.1% TDN) through the end of this study on 23 February 2024 just prior to heifers calving (BW 470.2 ± 6.1 kg).

Bait was used to attract animals to the AHCS, and its consumption was measured for each animal using an automated system (C-Lock, Inc., Rapid City, South Dakota, USA). At each visit to the AHCS, an individual cow was identified by an electronic identification tag in her ear as bait was dispensed. Bait is delivered at the programmed intervals while an animal's head is in the prescribed location of the AHCS to encourage the animal to remain in the system for a minimum of 3 min (Velazco et al., 2016). Bait was dispensed at 30-s intervals up to eight drops/ feeding event and allowing a maximum of eight feeding events/d. Animals that complete a sampling interval were locked out of the system for a minimum of 3 h before another sampling was allowed to account for diurnal variation by distributing sampling throughout the day. From 5 January 2023 through 24 January 2024, dispensed bait averaged 38.62 ± 1.34 g of wheat middling pellets (proximate analysis 87.6% DM, 18.3% CP, 69.5% TDN), and from 25 January 2024 through 23 February 2024, dispensed bait averaged 62.71 ± 11.7 9 g whole corn/ drop (proximate analysis 84.5% DM, 7.1% CP, 74% TDN) when averaged over 10 drops. Completion of a single feeding event activated the system to delay subsequent events for 3 h to allow for a more complete sampling of respiratory gasses throughout the day. Ideal manifold flow rate through the AHCS is reported to be 26 L/s or greater (Gunter et al., 2017). The current data set included 25 319 observations with an average of 34.75 ± 0.05 L/s , with a maximum of 72.19 L/s and a minimum of 6.22 L/s. Respiratory gas fluxes measured in the AHCS were only measured when the animal's head was in proper location within the AHCS as indicated by an infrared sensor. Data from the AHCS sensors were uploaded to a server, and proprietary algorithms (C-Lock, Inc., Rapid City, South Dakota) were used to calculate hourly CH_4 and CO_2 emissions and O_2 consumption.

The data that were generated in this pilot project were analyzed using PROC MIXED of SAS (SAS Institute Inc, 2015). The model contained a fixed effect for the two winter supplementation treatments applied to the granddams of these heifers (15 heifers from each grand-maternal background) and a random effect of the heifers themselves. The dependent variables were estimates of daily CH_4, CO_2 and O_2 emissions. Repeatability was calculated as the ratio of the variance between animals to the sum of the between- and within-animal variance components. Accuracy (**ACC**) of the mean over n days was calculated as: $ACC = \frac{nr}{(1+(n-1)r}$ wherein r is the estimated repeatability. The numbers of heifers required to achieve a specified power-of-the-test were calculated using one of the many online calculators that are available for this purpose (Faul et al., 2007). With this calculator, the sample size is computed as a function of the *P* of accepting a false hypothesis (β), the *P* of rejecting a true hypothesis (α, often 0.05 or 0.01), and the effect size deemed to be important and therefore to be detected with *P* (1-β, typically referred to as power-of-the-test). Two basic experiments were considered: one wherein the null hypothesis pertained to the difference between two treatments and a second wherein the observed level of the response variable was tested against a threshold. A priori chosen levels of significance (α = 0.10, 0.05 and 0.01), power-of-the-test (1-β = 0.80, 0.90 and 0.95) and magnitude of potential treatment effects that might be deemed to be relevant (Δ = 2, 4, 6, 8, 10, 15 and 20% difference between two treatments) were examined.

Results and Discussion

Estimates of the between- and within-heifer variance components are presented in Table 1 along with the overall means and CV for O_2, CH_4 and CO_2. Estimates of repeatability over days, calculated from these parameter estimates, were 0.089, 0.081 and 0.125 for O_2 consumption and CH_4 and CO_2 emissions, respectively.

All of the heifers were subject to the same feeding regime throughout the course of the study. However, the different feed management strategies for the heifers over the study period (i.e., fed forage in dry lot, pastured, and again fed forage in dry lot) could lead to questions about the heterogeneity of the estimates of repeatability over time. The corresponding estimates of repeatability for O_2 consumption and CH_4 and CO_2 emissions during the times when the heifers were fed forage in a dry lot, pastured, and again fed forage in the dry lot were 0.068, 0.110 and 0.106; 0.140, 0.060 and 0.167; and 0.111, 0.135, and 0.154, respectively. While somewhat variable, these estimates are consistently lower than those from the published literature. Arbre et al. (2016) estimated repeatability of 0.62 for CH_4 emissions (g/day) and 0.77 for CO_2 emissions (g/day). They state that these estimates were within the range of those from other studies (Huhtanen et al., 2013; Huhtanen et al., 2015). In the aforementioned experiments, animals were fed rations that contained significant amounts of concentrates in controlled environments, whereas in the present study, the free-ranging heifers were able to select diets of their own choosing. The degree to which the more fibrous diets of these heifers contributed to the increased temporal variability observed in the present study is not known. It is suggested that a degree of caution is advised in the extrapolation of results pertaining to gas production by cattle across divergent environments. In addition, all of these studies are based on relatively few animals and random variation in the results due to sampling is to be expected. Estimates of the accuracy of the mean over a number of days, from which valid data were obtained, based on the estimates of repeatability over the duration of the trial, are illustrated in Fig. 1. Methane (CH_4) required the greatest number of days to obtain a mean with an accuracy of 80% (45 days). Carbon dioxide (CO_2) required data from fewer days (28 days). Note that these are days with valid records and because of system malfunctions and because the heifers did not use the system daily, the actual length of a test period would necessarily be longer. Arthur et al. (2018) found that 30 visits to the Greenfeed system, each > 3 min in length, were needed to generate an accurate phenotype for methane production for individual animals based on a study of 616 young Angus cattle.

Shown in Table 2 are the numbers of animals that would be required to achieve a given power-of-the test for differences in methane production between any two arbitrary treatments, given a priori established effects size that was deemed by the investigators to be of biological importance. The values in Table 2 were generated for an experiment in which two treatments were compared. These values tend to be conservative for experiments with greater numbers of treatments (Gold et al., 1975). The values depend on the assumption of homogeneity of within–treatment variances and independence of the errors in the observations. If similar values are generated for experiments with more than two treatments, the assumptions that comparisons among treatments are independent and planned in advance of the data collection are also implied. As an example of the use of Table 2, if an investigator wanted to conduct an experiment in which there was a 90% chance of detecting a 6% difference between two treatments with *P* α = 0.10, then, the experiment should utilize 38 animals per treatment.

Raynor et al. (2024) indicate that further research addressing grazing animal enteric CH_4 emissions in extensive rangelands is needed. Further, efforts should be context–specific for comparative efforts across rangeland ecosystems and animal origin to inform more accurate assessments of the sustainability of grazing beef cattle related to greenhouse gas mitigation strategies. In many practical situations wherein the number of experimental units is limited, declarations of no treatment effect should only be cautiously considered unless very large differences between treatments could be anticipated. Reporting how the number of experimental units that were allocated to each treatment was determined would result in more robust publications (Festing and Altman, 2002). Transparency in scientific publications allows readers an increased opportunity to interpret the results and repeat the experiments (Kilkenny et al., 2009). Presentation of the power-of-the test for the main hypothesis of interest facilitates proper interpretation of the treatment effects that do not rise to the threshold necessary to declare them significant.

In the context of greenhouse gas production, assuming the null hypothesis that "the treatment has no effect and does not reduce the CH_4 emission by cattle" is true. If after analyzing the data, a Type I error occurs, it would be concluded that the treatments indeed have an effect. However, in reality, the treatment is useless and advocating its use is wasteful. Alternatively, assume the null hypothesis that "the treatment has no effect and does not reduce the CH_4 emission by cattle" is false, but the null hypothesis is not rejected after analyzing the data. As a consequence, a useful intervention could be wrongly ignored. Presentation of power-of-the-

Table 1
Estimates of the variances between and within animals and the means and CV for oxygen consumption, and carbon dioxide and methane production by forage-consuming beef heifers.

Dependent variable	Between animal variance	Within animal variance	Mean	Coefficient of variation, %
Oxygen, g/d	87 539	900 229	4 187	24
Carbon dioxide, g/d	207 787	1 459 282	5 285	24
Methane, g/d	252	2 853	180	31

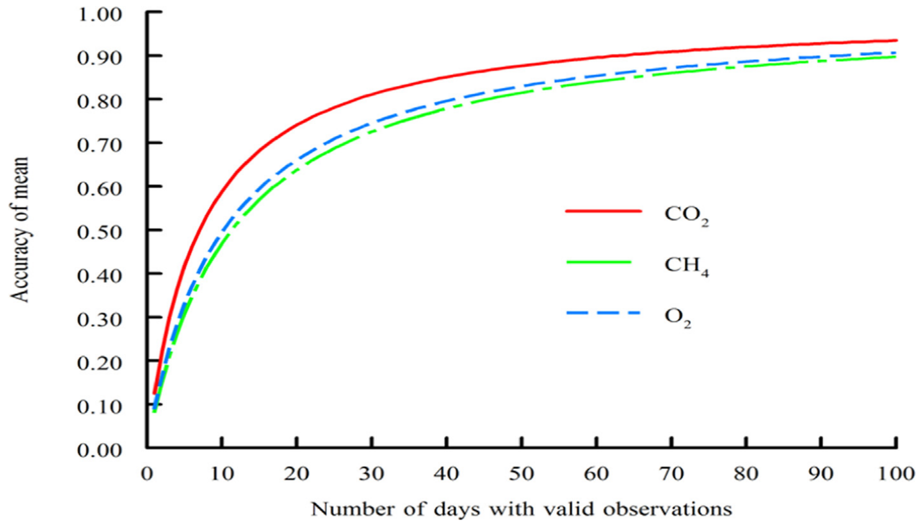

Fig. 1. Functional relationships between the number of days over which valid observations of gasses were recorded by the C-Lock system and the accuracy of their means for beef heifers that primarily consumed forages.

Table 2
Number of beef heifers at grazing required per treatment to achieve the specified power-of-the-test for differences in methane production in the comparison of two treatments to be detected with probabilities α = 0.10, 0.05 and 0.01.

Power (1-β)	α	% difference between treatments						
		2	4	6	8	10	15	20
0.80								
	0.10	240	61	28	16	11	6	4
	0.05	304	77	35	20	14	7	5
	0.01	453	115	52	30	20	10	7
0.90								
	0.10	331	84	38	22	14	7	5
	0.05	407	103	47	27	18	9	6
	0.01	576	146	66	38	25	12	8
0.95								
	0.10	419	106	48	27	18	9	6
	0.05	503	127	57	33	22	10	7
	0.01	689	174	79	45	30	14	9

test calculations provides some evidence for the conclusion that the null hypothesis is in fact true. For the beef industry, knowledge of GHG production and the performance of individual animals would be valuable in selecting animals that fit the environment by being ideally both low emitters and high performers (Raynor et al., 2024). This study provides information that is needed in the design of conclusive experiments wherein GHG emissions of grazing beef cattle are important response variables.

Shown in Table 3 are the numbers of animals that would be required to achieve a given power-of-the test for the observed level

Table 3
Number of beef heifers at grazing required to achieve a given power-of-the-test for the equivalence of μ and a prespecified threshold (T) level of methane production, given the between-animal SD (σ) when the null hypothesis is to be rejected with probabilities α = 0.10, 0.05 and 0.01.

Power (1-β)	α	Effect size ((μ − T)/σ)						
		0.227	0.454	0.680	0.907	1.134	1.701	2.268
0.80								
	0.10	89	23	11	7	5	3	3
	0.05	122	32	15	10	7	4	4
	0.01	198	52	25	16	11	7	5
0.90								
	0.10	129	33	16	9	7	4	3
	0.05	168	43	20	12	9	5	4
	0.01	256	66	31	19	13	8	6
0.95								
	0.10	168	43	20	12	8	5	3
	0.05	212	54	25	15	10	6	4
	0.01	309	80	37	22	16	9	7

of methane production being equivalent to a constant threshold. In testing an observed level of response against a threshold, power might be of particular relevance if, for example, tax policy were based on the attainment of a certain level of GHG production (Bistline et al., 2024) or for the validation of predictive models of greenhouse gas emissions (Gavrilova et al., 2019). In this context, it would seem that power-of-the-test should be high and fairly modest effect sizes would seem to be of interest. Cohen (1969) provides widely accepted values for small, medium and large effects as 0.2, 0.5 and 0.8, respectively. Thus, if a true effect on methane production of size 0.227 were to have a 95% *P* of being detected at a significance level of 0.05, then, data should be recorded from 212 animals.

Conclusion

This technical note provides guidance for the use of an AHCS instrument that is suitable for future research in the study of emissions of major greenhouse gasses by cattle. Given the capacity of an AHCS to evaluate approximately 30 animals simultaneously and a length of test of 45 days as determined herein, having adequate power-of-the-test for an effect of moderate size in the evaluation of a single breed or technology appears feasible. Powerful examinations of even smaller effect sizes could be obtained through replication. Proper experimental design, including length of the test period and number of animals tested, can produce greater confidence in the results of future research into gas emissions by livestock.

Ethics approval

All of the animals that were used in this research received the standard of care outlined in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (4th edition) (Tucker et al., 2020), and protocols were reviewed and approved by the Fort Keogh Animal Care and Use Committee (IACUC No. 11223-1).

Data and model availability statement

The data/models were not deposited in an official repository, and the data/models are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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CRediT authorship contribution statement

M.D. MacNeil: Writing – original draft, Methodology, Formal analysis, Conceptualization. **R.C. Waterman:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of interest

The authors declare no conflict of interest.

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