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Changes in feed intake during isolation stress in respiration chambers may impact methane emissions assessment

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Running head

Stress in isolation to record CH_4 decreases intake

Summary text

Methane, a major greenhouse gas emitted by livestock, requires robust methods of measurement in order to identify new and appropriate mitigation strategies. This study demonstrates that isolation within respiration chambers, the current most precise method of methane measurement in livestock, could underestimate emissions due to a reduction in feed intake. If changes in behaviour and physiology due to isolation stress are modelled, this would refine estimations of livestock GHG emissions that will help to find the most appropriate measures to mitigate climate change.

Abstract

Respiration chambers are considered the 'gold standard' technique for measuring *in vivo* methane (CH_4) emissions in live animals. However, the imposed isolation required may alter feeding behaviour and intake which ultimately impact CH_4 emissions. The aim of this study was to assess the impact of isolation within respiration chambers on feed intake and CH_4 emissions with two different diets and breeds of beef cattle. In addition, a routine stressor (transport) was used to examine the relationship between individual stress responsiveness and changes in feed intake during isolation. Eighty-four steers (castrated males) (569 ± 5.7 kg body weight, BW) were divided into two groups and each group fed with one of two basal diets consisting of (g /kg dry matter, DM) either 50:50 (Mixed) or 8:92 (Concentrate) forage to concentrate ratios. Within each basal diet there were 3 supplementation treatments: (i) control (ii) calcium nitrate and (iii) rapeseed cake. The stress biomarkers plasma cortisol, creatine kinase (CK), and free fatty acids (FFA) were determined before (0h) and after (30 min, 3h, 6h and 9h) a 30 min journey, when steers were transported to the respiration chamber facilities. Methane emissions were measured over a 3-day period using individual respiration chambers. Dry matter intake was assessed within the group-housed pens (4 weeks before entry to training pen), in the training pens and the chambers. Cortisol, FFA and CK increased ($P < 0.05$) after transport confirming a stress response. Dry matter intake (g /kg BW) decreased ($P < 0.001$) during isolation in the training pens (14.7 ± 0.28) and the chambers (14.3 ± 0.26) compared to that of the same animals in the group pens (16.8 ± 0.23).

Dry matter intake during isolation decreased more in those animals which had an increased ($P < 0.05$) stress response during transport as measured by cortisol, FFA and CK. With the Mixed diet, the decline in DMI was estimated to result in an increase in CH_4 (g/kg DMI) ($R = 0.25$, $P = 0.001$) which did not occur with the Concentrate diet. According to the results of this experiment, the stress associated with isolation reduces the DMI resulting in an increase in g CH_4/kg DMI in fibrous diets. Habituation to isolation needs refinement in order to reduce the impact of stress on intake and therefore achieve more accurate estimates of methane emissions. Alternatively, modelling CH_4 estimations according to behavioural and physiological changes associated with isolation stress would improve accuracy of CH_4 estimations.

Additional keywords: beef cattle, feeding behaviour, methane, stress physiology.

Introduction

Livestock production is a major contributor to anthropogenic greenhouse gas emissions (GHG) (Gerber *et al*., 2013). One of the most prominent agricultural GHG is methane (CH_4), produced by ruminants due to enteric fermentation (IPCC, 2013). To assess the exact contribution of livestock to CH_4 emissions and to understand the causes of variation in emissions resulting from factors such as diet or breed, a variety of measurement techniques have been developed. These include the laser CH_4 detector (Ricci *et al*., 2014), the sulphur hexafluoride (SF_6) tracer technique and respiration chambers (Grainger *et al*., 2007). Respiration chamber measurements are based on the continuous

measurement of target gases (e.g. CH_4) excreted from animals housed in individual chambers and are considered the 'gold standard' for measuring enteric CH_4 emissions in ruminants as they can provide a continuous and precise analysis of the CH_4 emitted during a given period of time.

However, in respiration chambers animals need to be individually housed in an artificial environment which inevitably changes their behaviour and motivation for social interactions. Ruminants are gregarious animals and isolation from the group provokes anxiety and stress (Boissy and Le Neindre, 1997). The stress response can cause changes in behaviour, the endocrine system and metabolism, amongst other responses (Sapolsky, 2002), which can affect rumen fermentation (Hutcheson and Cole, 1986). The first aim of this paper was to estimate the effect of isolation on intake and subsequent production of CH4 emissions in cattle.

Transport is a common commercially relevant stressor that all production animals experience at least once in their lives. The potential of transport to cause stress in cattle has been well studied (Grandin, 1997, Palme *et al*., 2000) and the magnitude of its physiological response might be used as a proxy measure of the response to other sources of stress. The second aim of the study was to correlate the magnitude of physiological changes in feed intake caused by isolation with individual differences in stress responsiveness to a routine stressor (i.e. transport) in order to estimate the impact of isolation and subsequent production of CH_4 emissions with two different diets and breeds of beef cattle.

Materials and methods

Eighty-four steers (castrated males) were allocated across 6 pens (12 m x 6 m) balanced for breed, sire and body weight (BW). Pens were provided with sawdust bedding and were equipped with a total of 32 automated feeding stations (HOKO feeders, INSENTEC B.V., Markenesse, The Netherlands). *Ad libitum* access to water and food was available. Cattle used in this experiment were part of a larger project to investigate the effect of cattle breed types, diets and dietary CH_4 mitigation strategies on performance, efficiency and CH_4 (Duthie et al., 2015; Troy et al., 2015). The experiment followed a balanced 2 (breed) x 2 (basal diet) x 3 (treatment) factorial design. Feeding consisted of one of two basal diets (g/kg DM basis, forage:concentrate): either 500:500 (Mixed) or 80:920 (Concentrate), respectively; and three treatments (no treatment (control), calcium nitrate and rapeseed cake). The breeds tested were either Charolais or Luing. Each combination of diet*treatment (6 different combinations) was allocated to a different pen. Additional information about dietary treatments can be found in Troy *et al*. (2015).

All steers were given eight weeks to adapt to the group-housed environment, electronic feeding system and diets. After full adaptation to the group-pen environment and experimental diets, steers remained in the group-pens for a minimum of eight weeks before CH_4 measurements to record performance, feed efficiency and methane emissions, data from which were published in Troy et al. (2015) and Duthie et al. (2016). Within the group-pens, DMI was individually measured daily (DMI_{Group}) and BW weekly for the four-week period immediately prior to entry to the respiration chamber facility. Thereafter, 76 out of 84 steers (balanced for diet, treatment and breed) were

125 transported to respiration chamber facilities (complete with training pens and
126 chambers) using a randomised block design (six chambers x twelve weeks).

127 Steers were transported in groups of six in a trailer towed by a tractor for
128 approximately 30 min at a stocking density of 1.2 m^2 / steer. As animals from
129 this study had either never been transported or only once in their lives, this was
130 assumed to constitute a stressor that was used to assess how animals coped
131 with an acute stress challenge.

132 Immediately after transportation steers were moved to single training
133 pens for a 6-day training period to acclimatise them to individual penning. The
134 design of the training pens was identical to the chambers with the exception that
135 visual and tactile contact between animals was possible between five of the six
136 adjacent training pens, whilst tactile contact was not possible between adjacent
137 respiration chambers. Subsequently, steers were moved to the respiration
138 chambers for 72 hours to sample the respiratory gases. CH_4 was measured in
139 six individual indirect open-circuit respiration chambers. Details of the
140 methodology used to measure CH_4 can be found in Troy *et al*. (2015). One
141 chamber malfunctioned during week 6 and 7, which resulted in the requirement
142 for a 13^{th} week of chamber analysis.

143 For each steer, individual DMI was measured in both the training pen
144 ($DMI_{Training}$) and the respiration chamber ($DMI_{Chamber}$), although DMI recordings
145 during the first 24 hours in the training pens were not used for analysis. In all
146 locations (group, training and chamber), DMI was expressed as g/kg BW for
147 each corresponding period (i.e. group pens, training pens and chambers).

148

149 *Assessment of stress biomarkers during transport*

Five blood samples were taken from each steer at the following time points relative to the transportation: immediately before the start of transport (-30 min) and 0, 3, 6 and 9 hours after the end of the transport. Blood samples were collected when animals were restrained in the weigh crate by jugular venepuncture using a 10 ml blood collection tube (Vacutainer®; BD Inc.) containing sodium heparin. Blood samples were immediately centrifuged (2,000G for 20 min at 4°C) to separate the blood plasma, which was stored at -21°C until further analysis. Plasma cortisol, free fatty acids (FFA) and creatine kinase (CK) were analysed as biomarkers of the stress response. Cortisol reflects the hypothalamic–pituitary–adrenal axis, which coordinates the physiological stress response. Free fatty acids are an indicator of lipid metabolism which increases during a stress response and CK measures muscle tissue damage. Plasma cortisol was measured in all samples by colorimetric ELISA using an automatic analyser (Bio-Plex, Bio-Rad, Hercules, USA) according to a previously described method (Al-Dujaili et al., 2012). Plasma free fatty acids (FFA) and creatine kinase (CK) activity were analysed on samples -30 min, 3h and 9h, with an Olympus analyser using a FFA Quantification Kit (Sigma-Aldrich, Merck KGaA, St Louis, Missouri, USA; Catalogue number MAK044 SIGMA) and a Multiskan (Thermo Scientific) using a CK Activity Colorimetric Assay Kit (BioVision, San Francisco, California, USA; Catalogue Number K777-100) respectively.

The physiological response of all biomarkers was calculated as the area under the curve (AUC) of all sampling times after transport (Mialon *et al*., 2012).

Statistical analyses

Analyses were carried out with the Statistical Analysis System (SAS Software; SAS Institute Inc, Cary, NC, USA; 2002–2008). The effect of transport on the stress biomarkers (cortisol, FFA and CK) was calculated by linear mixed models (Proc Mixed) of the samples through time fitting 'time' as a fixed effect, 'animal' as repeated measure and 'group pen', representing the pen where steers were housed in social groups, and 'methane cohort', indicating the week that steers were transported to the chamber facilities, as random effects. When ANOVA showed significant differences ($P < 0.05$), a least square means comparison test (LSMEANS), including the Tukey multiple comparison test, was performed to determine at which times the concentrations significantly differed.

Proc Mixed was used to assess the contribution of the AUC of cortisol, FFA and CK to DMI at all locations (group pen, training pen and respiration chamber) and differences observed between locations. The model was as follows:

$$Yi = \alpha + \beta i_{breed} + \beta i_{diet} + \beta i_{treatment} + \mu i_{biomarker} + \gamma i_{pen} + \gamma i_{methane\text{-}cohort} + \varepsilon i$$

Where Yi is the expected daily DMI of the ith animal, α is the regression intercept, β are the fixed variables (breed, diet and treatment), μ is the covariable (cortisol, FFA or CK), γ represents the random effects (pen and methane cohort) and εii is the residual error of the i^{th} animal. The effect of DMI on CH_4 emissions was also assessed using a Proc Mixed model with the same variables and random effects but replacing the stress biomarker with DMI as a covariable.

An 'extreme groups' analysis was also carried out in which animals were divided *retrospectively* into groups that differed with respect to each stress covariable using quartile splits. In this analysis, animals that scored in the

highest quartile (Q1) with respect to the stress biomarker were classified as High extreme and animals that scored in the lowest quartile (Q4) were regarded as Low extreme. This group splitting was made to produce distinct populations of animals based on physiological stress responses. The contribution of cortisol, FFA and CK to DMI at all locations was again performed with the population extremes using Proc Mixed with the previously used fixed and random effects plus the grouping factor ('High' or 'Low'). Statistical significance was assumed at $P \leq 0.05$ and tendencies at $P \leq 0.1$ for all analyses.

Results

Stress biomarkers

Plasma concentrations of cortisol, FFA and CK increased ($P < 0.05$) at least in one sample after transport compared to basal concentrations as represented in Table 1. The AUC of all stress biomarkers were significantly correlated ($P < 0.05$) (Llonch *et al*., unpublished data). The number of steers with a quartile 1 or quartile 4 measurement for each biomarker is shown in Table 2 according to breed, diet and treatment.

Associations between DMI and stress biomarkers

DMI (g /kg BW) was higher in Luing compared to Charolais in the group pens (DMI_{Group} 17.2 ± 0.35 *vs.* 16.5 ± 0.29; $P = 0.056$), training pens (15.3 ± 0.37 vs. 14.1±0.40; $P = 0.009$) and chambers (14.6 ± 0.38 *vs.* 13.8 ± 0.33; $P = 0.055$). The $DMI_{Group,}$ did not vary between weeks (Figure 1) and showed an average value of 16.9 ± 0.23 g DMI/kg BW. When steers were isolated in the training pens, $DMI_{Training}$ significantly decreased to 14.7 ± 0.28 g DMI/kg BW ($P <$

225 0.001). $DMI_{Training}$ was related to the FFA concentration (r = -0.074, *P* = 0.060).
226 The association between $DMI_{Training}$ with stress biomarkers was also found with
227 regard to FFA and CK when the extreme group analysis was performed. The
228 high extreme group of FFA and CK showed lower $DMI_{Training}$ than the low group
229 (r = -2.43, *P* = 0.0098; r = -1.42, *P* = 0.083 respectively).

230 $DMI_{chamber}$ (14.3 ± 0.26 g DMI/kg BW) was similar to $DMI_{training}$ (P >
231 0.05) but was lower than DMI_{group} (P < 0.001), as represented in Figure 1. The
232 $DMI_{Chamber}$ of each steer was also associated with their FFA concentration (r = -
233 0.081, *P* = 0.029). This was confirmed with the extreme group analysis where
234 high extremes of FFA were associated with reduced DMI per kg of BW (r = -
235 2.18; *P* = 0.004). Intake while in the chamber tended (r = -1.42, P = 0.059) to be
236 related to CK for those animals in the High extreme group.

237

238 *Associations between the magnitude of DMI reduction during isolation and*
239 *stress biomarkers*

240 The magnitude of depression in DMI between group housing and training
241 isolation (DMI_{Group} – $DMI_{Training}$) compared to that during chamber isolation
242 (DMI_{Group} - $DMI_{Chamber}$) was associated with some of the stress biomarkers.
243 Animals in the High extreme group for FFA had a 1.08 fold greater reduction in
244 DMI in the training pens compared to low extremes (*P* = 0.077). In the
245 respiration chambers, the linear models showed a correlation between FFA (r =
246 0.18; *P* = 0.027) and CK (r = 0.024; *P* = 0.038) with the $DMI_{Chamber}$ reduction.
247 The extreme group analysis confirmed this association as the $DMI_{Chamber}$
248 reduction was 1.46 times greater in the high FFA than the low FFA group (*P* =
249 0.089).

Estimation of the impact of DMI changes during isolation on methane emissions

CH_4 emissions (g /kg DMI) varied according to diet as concentrate-fed steers emitted 8.12 fold less CH_4 than forage-fed animals ($P < 0.0001$). As depicted in Figure 2, CH_4 emissions (kg/DMI) decreased as DMI increased (g CH_4/kg DMI = 38.82 - 1.092*g DMI/kg BW; $R^2 = 0.26$; $P = 0.001$) whereas in the concentrate diet we found no association between $DMI_{Chamber}$ and CH_4 (g /kg DMI). This means that for this diet, CH_4 emissions per kg of DMI fell as DMI increased. Troy et al. (2015) also found that the genotype had no mitigation effect whereas adding nitrate or increasing the oil content of the mixed diet reduced CH_4 emissions, similar to those expected from previous reports. However, these mitigation strategies did not work when used with high concentrate diets.

Discussion

The objectives of this paper were to determine changes in feed intake during isolation and whether these changes are associated with the stress sensitivity of each animal, measured using a routine stressor (transport). Both the behavioural and the physiological response to stress show stressor specificity (Matter et al., 2000) and any comparison between different sources of stress should be taken with care. For instance, adrenocortical responses (i.e. cortisol release) are sensitive toward the degree of stress but can reach a ceiling-effect at the higher end of the response spectrum (Harbutz and Lightman, 1992). The statistical analysis chosen in this experiment (i.e. extreme group analysis) allows the possible ceiling effect on cortisol release to be minimised by

considering the relative response of animals compared to their conspecifics instead of the absolute response.

Cattle are gregarious and isolation from the herd induces stress which results in changes in their behaviour and physiology (Boissy and Le Neindre, 1997). In response to some stressful situations such as transport or painful castration (i.e. burdizzo), evidence suggests that cattle reduce feed intake (Galyean and Hubbert, 1995; Fisher *et al*., 1996). Thus, if isolation causes stress to cattle it is likely that it will adversely affect feed intake. The results of this experiment confirm this hypothesis as when cattle were isolated, with (at the training pens) or without (at the chambers) tactile contact with conspecifics, feed intake significantly decreased compared to prior group housing. It is arguable that the effects of stress due to transportation could last and that the reduction in feed intake could be the result of transportation. However, as stated by Palme *et al*. (2000), physiological evidence of stress after transport lasts no longer than 48 h which confirms the fact that intake decline in training pens (six days) and chambers (three days) was a result of isolation.

The results of the stress biomarkers show that the plasma concentration of cortisol, FFA and CK increased immediately after transport which confirms that the transport-induced stress response could be detected using the stress biomarkers.

These stress biomarkers were used to monitor the association between intake reduction during isolation and sensitivity to a routine stressor at the individual animal level. To make such a comparison we hypothesised that the physiological stress response to transport would be associated with behavioural changes (feed intake) in response to isolation. Confirming our hypothesis,

299 results showed that some stress biomarkers were associated with the quantity
300 of feed intake. For example, the $DMI_{training}$ was negatively correlated with FFA
301 and CK indicating that higher responders ate less feed in the training pens. The
302 extreme group analysis showed that reduced intake in the respiration chambers
303 was correlated with higher concentrations of FFA and CK. Although the
304 statistical power was sometimes weak, probably due to the effects of diets and
305 breeds which increased the number of degrees of freedom, the results suggest
306 that the stress response during isolation is negatively correlated with feed
307 intake. Similarly, animals which exhibited higher stress responses were
308 associated with a greater decline in DMI during isolation. According to our
309 results, the association between the stress biomarkers after transport and the
310 decline in feed intake during isolation (DMI_{Group} compared to both $DMI_{Training}$ and
311 $DMI_{Chamber}$) was moderate. For instance, the model of the extreme group
312 analysis showed that animals of the high FFA extreme decreased 33% more
313 with respect to $DMI_{Training}$ and 40% more with respect to $DMI_{Chamber}$ relative to
314 DMI_{Group} compared to the low FFA extreme animals. These results suggest that
315 changes in the plasma concentration of stress biomarkers are associated with
316 variation in feed intake during isolation.

317 There is an additional behavioural change during isolation that is not
318 associated with the stress response. In cattle, group housing encourages more
319 feeding bouts and feed consumption compared to isolated animals; a
320 behavioural pattern usually referred as social facilitation (Albright, 1992). Due to
321 this, cattle in isolation are expected to decrease the number of feeding visits
322 and the quantity of feed intake which adds to the stress-derived decrease in
323 feed intake. In addition, as it is not a result of stress, it is very likely that training

will not have an effect on the change of this behavioural pattern during isolation. The reason to house steers in individual training pens before being allocated to the respiration chambers is to habituate them to isolation and to reduce the impact of isolation stress subsequently. However, according to Figure 1, habituation was not observed as the decrease in feed intake during training did not recover in the respiration chamber.

Emissions of enteric CH_4 in cattle are profoundly influenced by feed intake. To account for the effects of differences in feed intake between animals, enteric CH_4 emissions are usually expressed as g CH_4/kg DMI. This allows comparison of estimates of the emissions from animals of different ages, breeds and stages of production with different energy requirements. However, as Buddle *et al*. (2011) showed, CH_4 emissions, expressed as g CH_4/kg DMI, proportionally increase when feed intake is lower probably due to an increase in rumen retention time and fermentation. Our results partially confirm this finding as such an effect occurred in the forage rich diet whereas it could not be demonstrated with the high concentrate diet. This disparity between different diets might be due to the fact that with the concentrate diet, a decrease in intake may also increase the retention time but the lower quantity of fibre attenuates fibre fermentation compared to a mixed diet and therefore the reduces methanogenesis. Figure 2 depicts the magnitude of response in CH_4 as a consequence of a decrease in DMI. Considering the estimated reduction of DMI during isolation in the respiration chambers, an estimated scenario of approximately 2 g DMI/kg BW reduction (from 16.5 to 14.5 g DMI/kg BW) in high responding animals (Q1 according to FFA), when on the mixed diet, would result in CH_4 emissions increasing from 14.2 to 16.9 g CH_4/ kg DMI. This

estimated 16% CH_4 variation due to stress sensitivity during isolation represents a significant impact on enteric CH_4 recordings that has never been previously reported.

Using a 6-day habituation period, the effects of isolation on feed intake are still significant. Therefore, in order to reduce the impact of isolation on individual methane assessment a refinement of the training procedure would be desirable. In this regard, future studies should try to ascertain the duration and methodology for training in order to minimise the effect of isolation. On the other hand, if the effects of isolation can be monitored, data could be used to adjust CH_4 calculation equations according to sensitivity to isolation and refine CH_4 assessment.

Conclusions

Isolation of beef cattle either with or without tactile contact between conspecifics decreases feed intake. Based on the results of this experiment, a 6-day period of habituation does not significantly improve the reduction of feed intake during isolation in respiration chambers. Road transport for 30 min increases plasma cortisol, FFA and CK showing evidence of an acute stress response. The variation in individual stress response is moderately associated with the decrease in feed intake during isolation which exacerbates the lack of social facilitation at feeding. Developing improved habituation methods or building the capacity to refine methane estimations based on individual animal stress responsiveness would result in more precise assessments of enteric CH_4 in cattle.

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