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

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13

14 **Running head**

15 Stress in isolation to record CH₄ decreases intake

16

17 **Summary text**

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

26

27 **Abstract**

28 Respiration chambers are considered the 'gold standard' technique for
29 measuring *in vivo* methane (CH₄) emissions in live animals. However, the
30 imposed isolation required may alter feeding behaviour and intake which
31 ultimately impact CH₄ emissions. The aim of this study was to assess the
32 impact of isolation within respiration chambers on feed intake and CH₄
33 emissions with two different diets and breeds of beef cattle. In addition, a
34 routine stressor (transport) was used to examine the relationship between
35 individual stress responsiveness and changes in feed intake during isolation.
36 Eighty-four steers (castrated males) (569 ± 5.7 kg body weight, BW) were
37 divided into two groups and each group fed with one of two basal diets
38 consisting of (g /kg dry matter, DM) either 50:50 (Mixed) or 8:92 (Concentrate)
39 forage to concentrate ratios. Within each basal diet there were 3
40 supplementation treatments: (i) control (ii) calcium nitrate and (iii) rapeseed
41 cake. The stress biomarkers plasma cortisol, creatine kinase (CK), and free
42 fatty acids (FFA) were determined before (0h) and after (30 min, 3h, 6h and 9h)
43 a 30 min journey, when steers were transported to the respiration chamber
44 facilities. Methane emissions were measured over a 3-day period using
45 individual respiration chambers. Dry matter intake was assessed within the
46 group-housed pens (4 weeks before entry to training pen), in the training pens
47 and the chambers. Cortisol, FFA and CK increased ($P < 0.05$) after transport
48 confirming a stress response. Dry matter intake (g /kg BW) decreased ($P <$
49 0.001) during isolation in the training pens (14.7 ± 0.28) and the chambers (14.3
50 ± 0.26) compared to that of the same animals in the group pens (16.8 ± 0.23).

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

62

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

65

66 **Introduction**

67 Livestock production is a major contributor to anthropogenic greenhouse gas
68 emissions (GHG) (Gerber *et al.*, 2013). One of the most prominent agricultural
69 GHG is methane (CH_4), produced by ruminants due to enteric fermentation
70 (IPCC, 2013). To assess the exact contribution of livestock to CH_4 emissions
71 and to understand the causes of variation in emissions resulting from factors
72 such as diet or breed, a variety of measurement techniques have been
73 developed. These include the laser CH_4 detector (Ricci *et al.*, 2014), the sulphur
74 hexafluoride (SF_6) tracer technique and respiration chambers (Grainger *et al.*,
75 2007). Respiration chamber measurements are based on the continuous

76 measurement of target gases (e.g. CH₄) excreted from animals housed in
77 individual chambers and are considered the 'gold standard' for measuring
78 enteric CH₄ emissions in ruminants as they can provide a continuous and
79 precise analysis of the CH₄ emitted during a given period of time.

80 However, in respiration chambers animals need to be individually
81 housed in an artificial environment which inevitably changes their behaviour and
82 motivation for social interactions. Ruminants are gregarious animals and
83 isolation from the group provokes anxiety and stress (Boissy and Le Neindre,
84 1997). The stress response can cause changes in behaviour, the endocrine
85 system and metabolism, amongst other responses (Sapolsky, 2002), which can
86 affect rumen fermentation (Hutcheson and Cole, 1986). The first aim of this
87 paper was to estimate the effect of isolation on intake and subsequent
88 production of CH₄ emissions in cattle.

89 Transport is a common commercially relevant stressor that all
90 production animals experience at least once in their lives. The potential of
91 transport to cause stress in cattle has been well studied (Grandin, 1997, Palme
92 *et al.*, 2000) and the magnitude of its physiological response might be used as a
93 proxy measure of the response to other sources of stress. The second aim of
94 the study was to correlate the magnitude of physiological changes in feed intake
95 caused by isolation with individual differences in stress responsiveness to a
96 routine stressor (i.e. transport) in order to estimate the impact of isolation and
97 subsequent production of CH₄ emissions with two different diets and breeds of
98 beef cattle.

99

100 **Materials and methods**

101 Eighty-four steers (castrated males) were allocated across 6 pens (12 m x 6 m)
102 balanced for breed, sire and body weight (BW). Pens were provided with
103 sawdust bedding and were equipped with a total of 32 automated feeding
104 stations (HOKO feeders, INSENTEC B.V., Marknesse, The Netherlands). *Ad*
105 *libitum* access to water and food was available. Cattle used in this experiment
106 were part of a larger project to investigate the effect of cattle breed types, diets
107 and dietary CH₄ mitigation strategies on performance, efficiency and CH₄
108 (Duthie et al., 2015; Troy et al., 2015). The experiment followed a balanced 2
109 (breed) x 2 (basal diet) x 3 (treatment) factorial design. Feeding consisted of
110 one of two basal diets (g/kg DM basis, forage:concentrate): either 500:500
111 (Mixed) or 80:920 (Concentrate), respectively; and three treatments (no
112 treatment (control), calcium nitrate and rapeseed cake). The breeds tested were
113 either Charolais or Luing. Each combination of diet*treatment (6 different
114 combinations) was allocated to a different pen. Additional information about
115 dietary treatments can be found in Troy *et al.* (2015).

116 All steers were given eight weeks to adapt to the group-housed
117 environment, electronic feeding system and diets. After full adaptation to the
118 group-pen environment and experimental diets, steers remained in the group-
119 pens for a minimum of eight weeks before CH₄ measurements to record
120 performance, feed efficiency and methane emissions, data from which were
121 published in Troy et al. (2015) and Duthie et al. (2016). Within the group-pens,
122 DMI was individually measured daily (DMI_{Group}) and BW weekly for the four-
123 week period immediately prior to entry to the respiration chamber facility.
124 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 m² / 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. CH₄ was measured in
139 six individual indirect open-circuit respiration chambers. Details of the
140 methodology used to measure CH₄ 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 13th 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*

150 Five blood samples were taken from each steer at the following time points
151 relative to the transportation: immediately before the start of transport (-30 min)
152 and 0, 3, 6 and 9 hours after the end of the transport. Blood samples were
153 collected when animals were restrained in the weigh crate by jugular
154 venepuncture using a 10 ml blood collection tube (Vacutainer[®]; BD Inc.)
155 containing sodium heparin. Blood samples were immediately centrifuged
156 (2,000G for 20 min at 4°C) to separate the blood plasma, which was stored at -
157 21°C until further analysis. Plasma cortisol, free fatty acids (FFA) and creatine
158 kinase (CK) were analysed as biomarkers of the stress response. Cortisol
159 reflects the hypothalamic–pituitary–adrenal axis, which coordinates the
160 physiological stress response. Free fatty acids are an indicator of lipid
161 metabolism which increases during a stress response and CK measures
162 muscle tissue damage. Plasma cortisol was measured in all samples by
163 colorimetric ELISA using an automatic analyser (Bio-Plex, Bio-Rad, Hercules,
164 USA) according to a previously described method (Al-Dujaili et al., 2012).
165 Plasma free fatty acids (FFA) and creatine kinase (CK) activity were analysed
166 on samples -30 min, 3h and 9h, with an Olympus analyser using a FFA
167 Quantification Kit (Sigma-Aldrich, Merck KGaA, St Louis, Missouri, USA;
168 Catalogue number MAK044 SIGMA) and a Multiskan (Thermo Scientific) using
169 a CK Activity Colorimetric Assay Kit (BioVision, San Francisco, California, USA;
170 Catalogue Number K777-100) respectively.

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

173

174 *Statistical analyses*

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

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

$$189 \quad Y_i = \alpha + \beta_{i_{\text{breed}}} + \beta_{i_{\text{diet}}} + \beta_{i_{\text{treatment}}} + \mu_{i_{\text{biomarker}}} + \gamma_{i_{\text{pen}}} + \gamma_{i_{\text{methane-cohort}}} + \epsilon_i$$

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

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

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

208

209 **Results**

210 *Stress biomarkers*

211 Plasma concentrations of cortisol, FFA and CK increased ($P < 0.05$) at least in
212 one sample after transport compared to basal concentrations as represented in
213 Table 1. The AUC of all stress biomarkers were significantly correlated ($P <$
214 0.05) (Llonch *et al.*, unpublished data). The number of steers with a quartile 1 or
215 quartile 4 measurement for each biomarker is shown in Table 2 according to
216 breed, diet and treatment.

217

218 *Associations between DMI and stress biomarkers*

219 DMI (g /kg BW) was higher in Luing compared to Charolais in the group pens
220 (DMI_{Group} 17.2 ± 0.35 vs. 16.5 ± 0.29 ; $P = 0.056$), training pens (15.3 ± 0.37 vs.
221 14.1 ± 0.40 ; $P = 0.009$) and chambers (14.6 ± 0.38 vs. 13.8 ± 0.33 ; $P = 0.055$).
222 The DMI_{Group} , did not vary between weeks (Figure 1) and showed an average
223 value of 16.9 ± 0.23 g DMI/kg BW. When steers were isolated in the training
224 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 ($DMI_{\text{Group}} - DMI_{\text{Training}}$) compared to that during chamber isolation
242 ($DMI_{\text{Group}} - DMI_{\text{Chamber}}$) 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).

250

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

252 CH₄ emissions (g /kg DMI) varied according to diet as concentrate-fed steers
253 emitted 8.12 fold less CH₄ than forage-fed animals ($P < 0.0001$). As depicted in
254 Figure 2, CH₄ emissions (kg/DMI) decreased as DMI increased (g CH₄/kg DMI
255 = $38.82 - 1.092 \cdot \text{g DMI/kg BW}$; $R^2 = 0.26$; $P = 0.001$) whereas in the concentrate
256 diet we found no association between DMI_{Chamber} and CH₄ (g /kg DMI). This
257 means that for this diet, CH₄ emissions per kg of DMI fell as DMI increased.
258 Troy et al. (2015) also found that the genotype had no mitigation effect whereas
259 adding nitrate or increasing the oil content of the mixed diet reduced CH₄
260 emissions, similar to those expected from previous reports. However, these
261 mitigation strategies did not work when used with high concentrate diets.

262

263 **Discussion**

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

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

276 Cattle are gregarious and isolation from the herd induces stress which results in
277 changes in their behaviour and physiology (Boissy and Le Neindre, 1997). In
278 response to some stressful situations such as transport or painful castration (i.e.
279 burdizzo), evidence suggests that cattle reduce feed intake (Galyean and
280 Hubbert, 1995; Fisher *et al.*, 1996). Thus, if isolation causes stress to cattle it is
281 likely that it will adversely affect feed intake. The results of this experiment
282 confirm this hypothesis as when cattle were isolated, with (at the training pens)
283 or without (at the chambers) tactile contact with conspecifics, feed intake
284 significantly decreased compared to prior group housing. It is arguable that the
285 effects of stress due to transportation could last and that the reduction in feed
286 intake could be the result of transportation. However, as stated by Palme *et al.*
287 (2000), physiological evidence of stress after transport lasts no longer than 48 h
288 which confirms the fact that intake decline in training pens (six days) and
289 chambers (three days) was a result of isolation.

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

294 These stress biomarkers were used to monitor the association between
295 intake reduction during isolation and sensitivity to a routine stressor at the
296 individual animal level. To make such a comparison we hypothesised that the
297 physiological stress response to transport would be associated with behavioural
298 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

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

330 Emissions of enteric CH₄ in cattle are profoundly influenced by feed
331 intake. To account for the effects of differences in feed intake between animals,
332 enteric CH₄ emissions are usually expressed as g CH₄/kg DMI. This allows
333 comparison of estimates of the emissions from animals of different ages, breeds
334 and stages of production with different energy requirements. However, as
335 Buddle *et al.* (2011) showed, CH₄ emissions, expressed as g CH₄/kg DMI,
336 proportionally increase when feed intake is lower probably due to an increase in
337 rumen retention time and fermentation. Our results partially confirm this finding
338 as such an effect occurred in the forage rich diet whereas it could not be
339 demonstrated with the high concentrate diet. This disparity between different
340 diets might be due to the fact that with the concentrate diet, a decrease in intake
341 may also increase the retention time but the lower quantity of fibre attenuates
342 fibre fermentation compared to a mixed diet and therefore the reduces
343 methanogenesis. Figure 2 depicts the magnitude of response in CH₄ as a
344 consequence of a decrease in DMI. Considering the estimated reduction of DMI
345 during isolation in the respiration chambers, an estimated scenario of
346 approximately 2 g DMI/kg BW reduction (from 16.5 to 14.5 g DMI/kg BW) in
347 high responding animals (Q1 according to FFA), when on the mixed diet, would
348 result in CH₄ emissions increasing from 14.2 to 16.9 g CH₄/ kg DMI. This

349 estimated 16% CH₄ variation due to stress sensitivity during isolation represents
350 a significant impact on enteric CH₄ recordings that has never been previously
351 reported.

352 Using a 6-day habituation period, the effects of isolation on feed intake
353 are still significant. Therefore, in order to reduce the impact of isolation on
354 individual methane assessment a refinement of the training procedure would be
355 desirable. In this regard, future studies should try to ascertain the duration and
356 methodology for training in order to minimise the effect of isolation. On the other
357 hand, if the effects of isolation can be monitored, data could be used to adjust
358 CH₄ calculation equations according to sensitivity to isolation and refine CH₄
359 assessment.

360

361 **Conclusions**

362 Isolation of beef cattle either with or without tactile contact between conspecifics
363 decreases feed intake. Based on the results of this experiment, a 6-day period
364 of habituation does not significantly improve the reduction of feed intake during
365 isolation in respiration chambers. Road transport for 30 min increases plasma
366 cortisol, FFA and CK showing evidence of an acute stress response. The
367 variation in individual stress response is moderately associated with the
368 decrease in feed intake during isolation which exacerbates the lack of social
369 facilitation at feeding. Developing improved habituation methods or building the
370 capacity to refine methane estimations based on individual animal stress
371 responsiveness would result in more precise assessments of enteric CH₄ in
372 cattle.

373

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381

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