

Final report

Project Title: Reduction in ruminal methane-producing activity by oral administration of select nitrocompounds.

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SUMMARY

The production of methane within the rumen is an inefficient digestive process resulting in the conversion of potentially energy-yielding substrates into a form that can not be conserved by the host. Results from the present experiments demonstrate that nitroethane inhibits ruminal methane-producing activity by as much as 70% and whole animal methane emissions by as much as 30%. By comparison, monensin typically causes a transitory reduction in ruminal methane production of approximately 30%. At the highest level of nitroethane administration, the reductions in methane emissions were maintained for the duration of the experiment (8 days). The inhibitory activity of nitroethane appeared to be transitory with the lower treatment levels, most likely due to an increased rate of nitroethane degradation. At least one ruminal microbe (*Denitrobacterium detoxificans*) is known to metabolize nitroethane, coupling its reduction to the oxidation of hydrogen or formate. Unlike that which occurs with the use of monensin, reductant (electrons) that otherwise would have been consumed during the reduction of carbon dioxide to methane were not redirected to the production of the more reduced volatile fatty acids, propionate and butyrate. The fate of these electrons is not yet known but it is attractive to hypothesize that they may have been consumed by anabolic cell processes (i.e., microbial cell growth) or via other reductive processes that may benefit the host. Thus, whereas nitroethane may initially inhibit ruminal methanogenesis via a direct chemical mechanism, it could also reduce ruminal methane production, at least in part, via consumption of electrons that otherwise could be used to reduce carbon dioxide to methane. These results support the concept that “nitro-reduction” may be a viable approach to reduce economic losses associated with ruminal methane production and warrant future research aimed at developing thermodynamically favorable nitrocompounds (i.e., 2-nitropropionate, 2-nitro-4-methylsulfanyl-butanoic, etc.) that when reduced will yield compounds (i.e., alanine, methionine, etc.) readily usable by the host animal.

INTRODUCTION

Ruminal methane production is a digestive inefficiency resulting in the loss 2 to 4% of gross energy intake for ruminants consuming high concentrate diets and up to 15% gross energy intake for ruminants consuming high forage diets (Johnson and Johnson, 1995; Van Nevel and Demeyer, 1996). Economically, this digestive inefficiency is calculated to cost the United States cattle feeding industry more than \$900,000/day for cattle losing 2 to 4% of gross energy (GE) intake as methane (Van Kessel, personal communication). Methane is also considered an important greenhouse gas (Johnson and Johnson, 1995; Moss et al., 2000). In the United States, about 20% of the total methane output is produced from enteric fermentation, of which ruminants are the major producers (EPA, 2004).

Numerous chemical inhibitors have been shown to reduce methanogenesis but many of these also inhibited the oxidation of hydrogen produced during digestion and fermentation of feedstuffs (Van Nevel and Demeyer, 1995; Van Nevel and Demeyer, 1996). Metabolic processes that consume hydrogen are

thought to be beneficial to the animal and the microbial population because they prevent the accumulation of hydrogen to levels potentially inhibitory to ruminal fermentation (Miller, 1995). Moreover, the effects of many of these early interventions on methane production were often transient in nature, likely due to the rumen microflora's ability to adapt to ecological challenges (Van Soest and Demeyer, 1995). Some more recent attempts to develop interventions to reduce ruminal methanogenesis have been successful either in prolonging the methane reducing potential *in vivo* (Kung et al., 2003; Machmüller and Kreuzer, 2004; Wright et al., 2004) or in conserving the energetic efficiencies associated with hydrogen-transfer reactions *in vitro* (Anderson et al., 1998; Asanuma et al., 1999; Iwamoto et al., 2002; Sar et al., 2005). Other recent research efforts to inhibit methanogenesis have yielded varying degrees of success (Dohme et al., 2001; Fievez et al., 2003; Lee et al., 2002; Miller and Wolin, 2001; Ungerfeld et al., 2003).

Nitroethane and 2-nitropropanol have been shown to inhibit ruminal methanogenesis *in vitro* without markedly increasing hydrogen accumulations ($< 3 \mu\text{mol/ml}$ rumen fluid) or adversely affecting the ratio of acetate to propionate produced in mixed culture incubations (Anderson et al., 2003). A particular attractive feature of these nitrocompounds is that they inhibit the growth of the zoonotic pathogens, *Listeria monocytogenes*, *Campylobacter* and *Salmonella* (Dimitrijevic et al., 2005; Jung et al., 2003; Jung et al., 2004a, b) and thus have the potential to be developed into a feed supplement to enhance preharvest safety. The effects of oral administration of nitroethane and 2-nitropropanol on ruminal methane-producing activity, volatile fatty acid production were evaluated *in vivo*. The effects of nitroethane on select gastrointestinal bacterial populations were also evaluated.

MATERIALS AND METHODS

EXPERIMENT 1: Thirty mature ewes averaging 66.3 ± 13.1 (SD) kg body weight (BW) and maintained on a Bermuda grass hay:cracked corn diet (9:1) with *ad libitum* access to mineral supplementation were randomly allocated ($n = 5$ per treatment) to 0, 24 or 72 mg nitroethane/kg body weight per day or to 0, 40 or 120 mg 2-nitropropanol/kg body weight per day. Treatments were administered individually via oral gavage of a sodium salt solution of nitroethane (Majak et al., 1986) diluted with water to 120 mg nitroethane/ml, or an aqueous solution containing 400 mg 2-nitropropanol/ml. These solutions were further diluted with water in order to administer approximately equal volumes to all sheep. Each day's treatments were administered in two equal sized portions given at the morning (08:00) and afternoon (16:00) meals. Rumen fluid collected via stomach tube 2 h after administration of the morning meal 3 days before initiation of treatment (pretreatment) and on days 1, 2 and 5 of treatment was transferred immediately upon collection into serum vials which were then capped, returned to the lab and apportioned for determinations of volatile fatty acid concentrations and methane-producing activity. Each serum vial was filled completely (approximately 60 ml) before capping to minimize contact of air with the fluid.

EXPERIMENT 2: Two ruminally cannulated Holstein cows maintained on rye grass pasture were administered 0.12 g nitroethane/kg body weight per day in two equal sized portions (08:00 and 16:00) over an 8 day period. Ruminal fluid collected before and at indicated intervals during the treatment period was analyzed for volatile fatty acid concentrations. Ruminal fluid and feces collected likewise were also used for *in vitro* determination of methane-producing activity.

EXPERIMENT 3: Twenty-four Holstein steers averaging 319 ± 6.5 (SD) kg BW were randomly allocated in two replicates (3 steers/treatment per replicate) to treatments of 0, 30, 60 and 120 mg nitroethane/kg BW per day. Treatments were orally administered as the sodium salt immediately before individual offering of the morning (08:00) and afternoon (16:00) meals (40% alfalfa, 30% bermuda grass, 11% cottonseed hulls, 11.5% corn, 7% molasses and 0.5% vitamin premix). Ruminal fluid was collected from each steer via a stomach tube 2 h after the morning feeding and put into separate 60 ml serum vials which were immediately capped, returned to the lab and apportioned for measurement of VFA concentrations or for *in vitro* measurement of methane-producing or nitroethane-reducing activity. All data obtained from 1

steer allocated to control (0 mg nitroethane/kg BW per day) treatment was excluded from the study because of an accidental dosing with nitroethane.

ANALYTICAL

Volatile fatty acid concentrations were measured by gas chromatography (Hinton et al., 1990). Methane-producing activity was determined via *in vitro* incubation (39°C for 3 h) of rumen fluid or feces (5 ml or 2 g, respectively) with anaerobic buffer (5 ml or 10 ml, respectively) containing 60 mM sodium formate, 0.2 g finely ground alfalfa and a H₂:CO₂ (1:1) atmosphere. At the end of the incubation period, methane concentrations present in the headspace of the incubations were determined by gas chromatography (Allison et al., 1992). Nitroethane-reducing activity was determined via similar *in vitro* incubation except with additions of 10 mM nitroethane. Nitroethane concentrations in samples collected at 0, 3, 6 and 24 h of incubation were measured colorimetrically (Anderson et al., 2003). Whole animal methane emissions were measured using the sulfur hexafluoride tracer gas technique (Ulyatt et al., 1999).

STATISTICS

In experiments 1 and 3, each animal served as an experimental unit. Tests for effects of treatment, replicate (where applicable), day of treatment or their interaction on methane-producing activity, nitroethane-reducing activity and volatile fatty acid concentrations were performed by a repeated measures analysis of variance (Statistix[®] 8 Analytical Software, Tallahassee, FL, USA). Means were further separated using a Tukey's multiple range test. Log transformations of generic *E. coli* and *Campylobacter* concentrations or the net change in concentrations were analyzed likewise by a repeated measures analysis of variance. Tests for effects of treatment or day of treatment on proportions of *Salmonella* culture-positive steers were done using a Fishers Exact test (Statistix[®] 8 Analytical Software). Beginning and ending body weights and whole animal methane emissions in experiment 3 were analyzed by analysis of variance using SAS. Whole animal methane measurements collected on day -4, -2 and 0 prior to NE administration were used to establish baseline methane emissions for each steer. The data was analyzed using repeated measures with treatment group as the main effect. In experiment 2, each animal served as its own pretreatment control and the data from this experiment are presented descriptively without statistical analysis.

RESULTS

EXPERIMENT 1: Daily oral doses of 24 and 72 mg nitroethane/kg body weight caused reductions in methane-producing activity, which is an indirect measure of numbers of viable methane-producing bacteria, by as much as 42 and 69% respectively, when compared to control animals given no nitroethane (Table 1). By comparison, a daily oral dose of 120 mg 2-nitropropanol/kg BW was needed to reduce methane-producing activity by 38% from that of untreated control animals (Table 2). Reductions in the methane-producing activity observed on day 2 of nitrocompound treatment indicate that the effect of nitrocompounds was dose dependent and may have been diminished by day 5 of treatment (Tables 1 and 2).

Oral nitroethane treatment had no effect ($P > 0.05$) on accumulations or molar proportions of volatile fatty acids in rumen contents collected from the sheep (Figure 1). Oral nitropropanol treatment reduced ($P < 0.01$) ruminal accumulations of acetate but not propionate or butyrate ($P > 0.05$; Figure 2). Molar proportions of volatile fatty acids produced were not affected by nitropropanol treatment ($P > 0.05$).

EXPERIMENT 2: A follow-up study with two ruminally cannulated Holstein cows was conducted and results support those obtained with sheep as intraruminal administration of 120 mg nitroethane/kg body weight reduced methane-producing activity in these cattle by as much as 72% compared to pre-dose activities (Figure 3). Again, the effect of nitroethane on ruminal methane-producing activity was greatest within the first four days of treatment and was diminished by day 8 of treatment. Along with the reductions in ruminal methane-producing activity, there were marked increases in fecal methane-

producing activity. Volatile fatty acid concentrations fluctuated during the course of the study (Figure 4) but molar proportions of acetate to the sum of propionate and butyrate differed little throughout the study again indicating that electrons produced during digestion of feeds were not disposed of via production of more reduced fermentation acids (Figure 4).

EXPERIMENT 3: Ruminal nitroethane-reducing activity (Figure 5) and methane-producing activity (Figure 6) differed ($P < 0.05$) markedly between the first and second replicates; therefore, the data from each replicate were analyzed separately. In the first replicate, an effect of day of treatment was observed on nitroethane-reducing activity as *in vitro* incubation of ruminal fluid collected before and on day 8 of treatment revealed that rates of nitroethane degradation increased ($P < 0.05$) from 0.022 ± 0.04 to 0.185 ± 0.12 μmol nitroethane/ml per h. Incubation of ruminal fluid collected before and on day 2, 4 and 8 of treatment revealed a main effect of treatment on methane-producing activity, with rates from animals administered 60 or 120 mg nitroethane/kg BW at least 25% lower ($P < 0.05$) than those measured from animals administered 0 or 30 mg nitroethane/kg BW (7.87 ± 2.14 and 8.00 ± 1.82 μmol CH_4 /ml per h, respectively)(Figure 6). A main effect of day of treatment was also observed as methane-producing activities were reduced ($P < 0.05$) 23, 18 and 39% on days 2, 4 and 8 of treatment, respectively, when compared to pretreatment measurements (8.60 ± 1.51 μmol CH_4 /ml per h)(Figure 6).

In the second replicate, which immediately followed the first, pretreatment ruminal nitroethane-reducing activity (0.412 ± 0.04 μmol nitroethane/ml per h) was higher ($P < 0.05$) and methane-producing activity (3.32 ± 0.86 μmol CH_4 /ml per h) was lower ($P < 0.05$) than in the first replicate (Figures 5 and 6). Consequently, an effect of treatment ($P = 0.09$) or time of treatment ($P = 0.53$) was not observed on methane-producing activity; however, activities measured in rumen contents from steers administered 30 and 60 mg nitroethane/kg BW were 22 and 27% lower ($P < 0.05$), respectively, than that measured in control steers administered no nitroethane (3.71 ± 0.82 μmol CH_4 /ml per h). Methane-producing activity from steers administered 120 mg nitroethane/kg BW was 19% lower, but not significantly, than that of the controls. An effect of replicate was not observed ($P > 0.05$) on ruminal volatile fatty acid concentrations, consequently data from both replicates were analyzed together. Nitroethane treatment did not cause reductant to be redirected to the production of propionate and butyrate (Figure 7).

Campylobacter were not recovered from rumen contents of any of the cattle at any time. Pretreatment fecal *Campylobacter* concentrations varied considerably and were not uniformly distributed across treatments (0, 1.88 ± 1.7 , 2.05 ± 0.9 and 2.24 ± 2.1 \log_{10} CFU/g for cattle allocated to 0, 30, 60 or 120 mg nitroethane/kg body weight, respectively). A repeated measures analysis of variance of the change in *Campylobacter* concentrations from pretreatment to day 8 revealed an increase of 0.48 ± 1.36 \log_{10} cfu/g feces in control animals and nearly a 10-fold reduction in fecal *Campylobacter* concentrations in the animals administered nitroethane (decrease of 0.99 ± 2.03 , 0.93 ± 2.47 and 0.95 ± 1.86 \log_{10} cfu/g feces for 30, 60 or 120 mg nitroethane/kg body weight treatments, respectively), however, this was not significant ($P = 0.33$).

Quantitative recovery of *Salmonella* from the cattle was negative in all cases thus indicating that concentrations of *Salmonella* harbored in these cattle were below our quantitative level of detection (10 colony forming units per g gut content). Qualitative recovery (theoretical level of detection = 1 cell per 2 g of gut contents) of this bacterium was also quite variable, with the average frequency of cattle in the respective treatment groups culturing positive for *Salmonella* ranging positive ranging from 0 to 83% and a median recovery across all treatments of 33%. Tests for effects of treatment or day of treatment by a Fishers Exact test were nonsignificant ($P \geq 0.50$).

Fecal *E. coli* concentrations were unaffected ($P > 0.65$) by treatment (5.55 ± 0.84 , 5.66 ± 0.44 , 5.68 ± 0.71 and 5.63 ± 0.72 \log_{10} cfu/g feces for 0, 30, 60 or 120 mg nitroethane/kg body weight treatments, respectively) or by day of treatment 5.67 ± 0.76 and 5.59 ± 0.58 \log_{10} cfu/g feces for measurements made pretreatment and on day 8, respectively. Upon feeding a terminal chlorate supplement (fed after

sampling on day 8) at one half the known efficacious dose to all the steers, fecal *E. coli* concentrations were reduced ($P < 0.02$) the next day by approximately 10-fold to $4.68 \pm 1.37 \log_{10}$ cfu/g feces; the chlorate effect was not enhanced ($P > 0.05$) by prior nitroethane treatment.

In order to assess the effect of nitroethane on whole animal methane emissions, experiment 3 was conducted in collaboration with Dr. Gordon Carstens at Texas A&M University. In this case, beginning and ending body weights and whole animal methane emissions in both replicates 1 and 2 were analyzed together due to replicate or the interaction term of replicate by treatment not being significant ($P > 0.05$) in the model. During the 8-d study period, dry matter intake averaged 8.5, 8.8, 7.2, and 7.8 ± 0.5 kg/d for 0, 30, 60 and 120 mg nitroethane/kg body weight treatments, respectively. Beginning and ending (on day 8 post treatment) body weights were not affected ($P > 0.10$) by nitroethane treatment and averaged 319 ± 6.5 and 327 ± 7.2 kg, respectively. Pretreatment whole animal methane emissions were not affected by treatment and averaged 6.35 ± 0.4 % of gross energy intake (GEI; Figure 8). On days 2 and 4, steers administered 30 (5.8 ± 0.35 and 4.9 ± 0.45 % GEI on d 2 and 4, respectively) and 120 (5.6 ± 0.54 and 5.2 ± 0.69 % GEI on d 2 and 4, respectively) mg nitroethane/kg body weight had lower ($P < 0.02$) methane emissions than control steers (7.4 ± 0.39 and 7.1 ± 0.55 % GEI on d 2 and 4, respectively) with steers administered 60 mg nitroethane/kg BW being intermediate (5.8 ± 0.35 and 5.73 ± 0.45 % GEI on d 2 and 4, respectively). Methane emissions of steers administered 120 mg nitroethane/kg body weight continued to be lower than control steers on d 8 (4.6 vs 6.4 ± 0.69 % GEI) with steers administered 30 or 60 mg nitroethane/kg body weight being intermediate.

DISCUSSION AND IMPLICATIONS

Consistent with earlier *in vitro* observations (Anderson et al., 2003), results presented here confirm the methane-inhibiting properties of both nitroethane and 2-nitropropanol, although results showed that nitroethane was the superior methane-inhibiting compound. The effects of the nitrocompounds on methane-producing activity were dose dependent. As observed with a variety of earlier developed methane-inhibiting compounds (McAllister et al., 1996; Van Nevel and Demeyer, 1996), evidence obtained here suggests a potential adaptation of the rumen microflora to the nitrocompounds. In the present case, ruminal adaptation to the nitrocompounds was likely due to an *in vivo* enrichment of nitrocompound-reducing bacteria, as evidenced by increased nitroethane-reducing activity, capable of depleting effective concentrations of the inhibitors. At present, only one ruminal microbe, *Denitrobacterium detoxificans*, an obligate respiring anaerobe, is known to metabolize nitroethane and a variety of other nitrocompounds, coupling their reduction to the oxidation of hydrogen or formate (Anderson et al., 2000). While typically present in the rumen at low concentrations ($\leq 10^3$ organisms/ml), populations of this bacterium were enriched during growth with increasing concentrations of nitrocompound (Anderson et al., 1996). Our observations that these and other related nitrocompounds (Anderson et al., 1998; 2003) exerted an immediate effect on methanogenesis in nonadapted populations indicates that they act primarily to directly inhibit methanogenesis rather than solely serving as alternative electron acceptors. In contrast to what occurs in nonadapted populations, competitive consumption of reductant by nitro-reducing bacteria in adapted populations could possibly play a role in reducing enteric methanogenesis if sufficient amounts of acceptor could be safely administered. For instance, addition of *D. detoxificans* and an appropriate amount of a suitable electron acceptor (20 mM nitrate) to *in vitro* incubations of unadapted rumen microflora resulted in > 94 % reduction in methane production and significant increases in rates of nitrate disappearance (Anderson et al., 1998). Whereas we observed no adverse effect of the nitrocompounds on the sheep or cattle in this study, upper limits able to be safely administered have yet to be determined.

Our findings that nitroethane-reducing activity increased in control steers during the treatment period in the first replicate of experiment 3 and that competent nitroethane-reducing bacterial populations were established in the rumens of steers during the pretreatment period of the second replicate of experiment 3 suggests the horizontal transfer of bacteria such as *D. detoxificans* between treated and untreated

animals. Once acquired, it is reasonable to hypothesize that the competent nitro-respiring populations could be maintained, at least temporarily, on endogenous acceptors other than nitroethane that may be present in the rumen. At least one strain of *D. detoxificans* is known to respire on nitrate (Anderson *et al.*, 2000), but whether it was nitrate or other undefined acceptors that were present is unknown. Transmission of high ruminal nitrate-reducing activity from cattle adapted to high levels of nitrate to unadapted cattle kept in separate pens has been reported (Cheng *et al.*, 1985). Considering that *D. detoxificans* can consume reductant at the expense of methanogenesis (Anderson *et al.*, 1998), it is reasonable to suspect that the presence of this bacterium may have contributed to the low methane-producing activity observed during the pretreatment period of the second replicate in experiment 3. Because our *in vitro* procedure for measuring methane-reducing activity does not contain nitroethane, it would not detect the potential of *D. detoxificans* to consume reductant at the expense of methanogenesis. This may explain why we see that the nitroethane-caused inhibition of methane-producing activity diminishes over time whereas we do not see a diminishing effect of the highest nitroethane dose on whole animal methane emissions.

In agreement with earlier *in vitro* results (Anderson *et al.*, 2003), nitroethane had no effect on amounts or molar proportions of volatile fatty acids produced. Whereas 2-nitropropanol caused slight reductions in ruminal acetate accumulations, it did not increase accumulations of propionate or butyrate. Thus, neither nitrocompound caused a redirection of reductant (electrons) that otherwise would have been consumed during the reduction of carbon dioxide to methane to be used for the production of the more reduced volatile fatty acids, propionate and butyrate. Some other methane inhibiting chemicals, such as monensin (Mbanzamihiigo *et al.*, 1996) and 9,10 anthraquinone (Kung *et al.*, 2003), typically cause decreased production of acetate and increased production of propionate within rumen contents. The fate of the unused electrons in the present study is not yet known but further studies are warranted to determine if these may have been consumed by anabolic cell processes (i.e., microbial cell growth) or by other reductive processes that may benefit the host.

Early *in vitro* evidence had indicated that chlorate by itself was sufficient to induce optimal expression of the targeted respiratory nitrate reductase enzyme in *E. coli* but not in *Salmonella* (Anderson *et al.*, 2000). Thus we were not surprised to find that prior nitroethane treatment did not enhance the effect of the chlorate product against *E. coli* but had hypothesized that nitroethane administration would enhance the bactericidal activity of chlorate against *Salmonella* in cattle as it had in swine (Jung and Anderson, 2004). Unfortunately, the level of *Salmonella*, as well as *Campylobacter*, in the steers in the present study was too low to allow for an adequate test of this hypothesis.

5. Conclusions

Results presented here are the first to confirm the methane-inhibiting activity of nitroethane and 2-nitropropanol *in vivo* and showed that nitroethane had greater methane-inhibiting activity than 2-nitropropanol. The methane-inhibiting activity of the nitrocompounds appeared to be transient, probably due to an adaptation occurring within the rumen, which possibly could be resolved by increasing the amount of nitrocompound administered. Our findings that the nitrocompounds had little, if any, effect on amounts or molar proportions of volatile fatty acids produced within the rumen indicates that fermentative efficiencies associated with microbial interspecies hydrogen transfer were not compromised. Unlike earlier studies, we were unable to demonstrate in this study that nitroethane inhibited the growth of certain zoonotic pathogens. Nevertheless, these results support the concept that nitro-inhibition may be a viable approach to reduce economic and environmental costs associated with ruminal methane production.

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ABSTRACTS and PROCEEDINGS RESULTING FROM THIS WORK

Anderson, R.C., G.E. Carstens, T.R. Callaway, C.L. Schultz, T.S. Edrington and D.J. Nisbet 2005. Effect of intraruminal nitroethane administration on methane-producing activity and volatile fatty acid production *in vivo*. 2005 Conf. Gastrointestinal Function, pp. 23.

Anderson, R.C., G.E. Carstens, R.K. Miller, T.R. Callaway, C.L. Schultz, T.S. Edrington, R.B. Harvey and D.J. Nisbet. 2004. Effect of nitroethane administration on ruminal VFA production and specific activity of methane production. *J. Anim. Feed Sci.* 13 (Suppl. 1):23-26.

Brown, E.G., G.E. Carstens, L.J. Slay, S.A. Woods, M.J. Quinn, J.L. McReynolds, R.C. Anderson and D.J. Nisbet. 2005. Effects of nitroethane administration on methane production in growing steers. *Southern Section Amer. Soc. Anim. Sci.* (In press).

Table 1. Effect of oral nitroethane administration on methane-producing activity in ovine rumen contents *in vivo*

Treatment (per kg body weight d ⁻¹) ^a	Mean (± SD) methane-producing activity (µmol CH ₄ /g rumen fluid per h)			
	Pretreatment	Day 1	Day 2	Day 5
0 mg nitroethane	6.76 ± 1.0 ^b	5.66 ± 1.1 ^{b,c}	6.61 ± 1.0 ^b	6.16 ± 0.6 ^b
24 mg nitroethane	6.79 ± 0.3 ^b	4.25 ± 0.8 ^{c,d}	3.60 ± 0.9 ^{d,e}	5.10 ± 0.6 ^{b,c}
72 mg nitroethane	6.82 ± 0.3 ^b	3.25 ± 0.6 ^{d,e}	2.00 ± 0.5 ^e	4.24 ± 0.7 ^{c,d}
Treatment effect	<i>P</i> = 0.0001			
Day effect	<i>P</i> < 0.0001			
Interaction	<i>P</i> < 0.0001			

^aAdministered via oral gavage (*n* = 5 per treatment) of the sodium salt of nitroethane in two equal sized portions at 08:00 and 16:00. Data were analyzed by a repeated measures analysis of variance and means were further separated using a Tukey's multiple range test. ^{b, c, d, e}Means with unlike superscripts differ (*P* < 0.05).

Table 2. Effect of oral 2-nitropropanol administration on methane-producing activity in ovine rumen contents *in vivo*

Treatment (per kg body weight d ⁻¹) ^a	Mean (± SD) methane-producing activity (µmol CH ₄ /g rumen fluid per h)			
	Pretreatment	Day 1	Day 2	Day 5
0 mg 2-nitropropanol	6.18 ± 1.0 ^{b,c}	6.95 ± 1.4 ^b	7.00 ± 0.8 ^b	6.40 ± 0.9 ^{b,c}
40 mg 2-nitropropanol	7.29 ± 1.7 ^b	6.12 ± 0.7 ^{b,c}	6.03 ± 0.9 ^b	6.61 ± 1.5 ^{b,c}
120 mg 2-nitropropanol	6.02 ± 1.3 ^{b,c}	6.24 ± 0.8 ^{b,c}	4.37 ± 0.9 ^c	5.77 ± 0.7 ^{b,c}
Treatment effect	<i>P</i> = 0.1432			
Day effect	<i>P</i> = 0.0942			
Interaction	<i>P</i> = 0.0086			

^aAdministered via oral gavage (*n* = 5 per treatment) of an aqueous solution of 2-nitropropanol in two equal sized portions at 08:00 and 16:00. Data were analyzed by a repeated measures analysis of variance and means were further separated using a Tukey's multiple range test.

^{b, c}Means with unlike superscripts differ (*P* < 0.05).

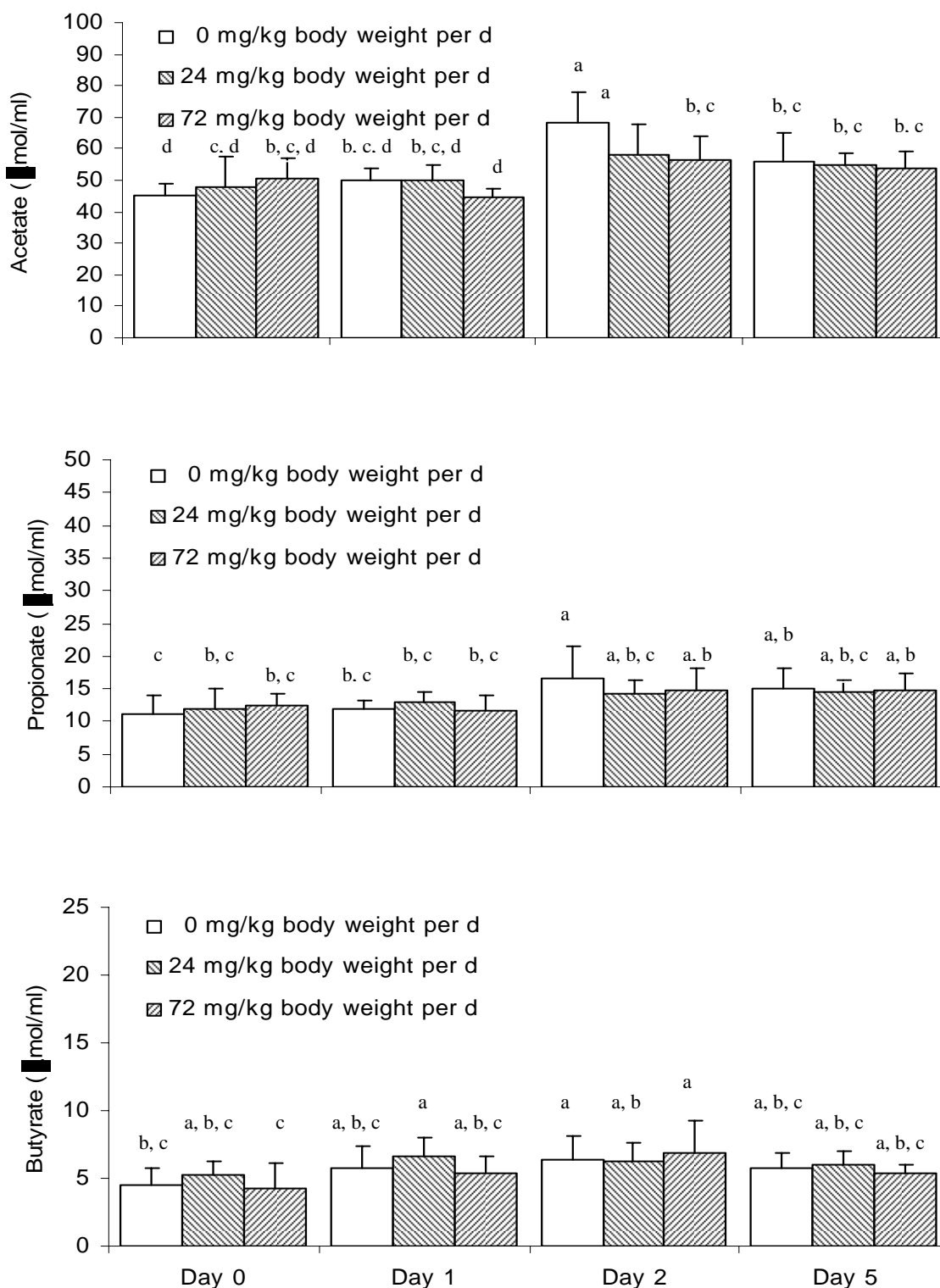


Figure 1. Effect of oral nitroethane administration ($n = 5/\text{treatment}$) on volatile fatty acid accumulation in ovine ruminal contents. Data were analyzed by a repeated measures analysis of variance. For acetate, main effects were $P = 0.40$, $P < 0.0001$ and $P = 0.14$ for treatment, day of treatment and their interaction, respectively. For propionate, main effects were $P = 0.96$, $P = 0.001$ and $P = 0.73$ for treatment, day of treatment and their interaction, respectively. For butyrate, main effects were $P = 0.73$, $P < 0.005$ and $P = 0.69$ for treatment, day of treatment and their interaction, respectively. Means were further separated using a Tukey's multiple range test and values with unlike superscripts differ, $P < 0.05$.

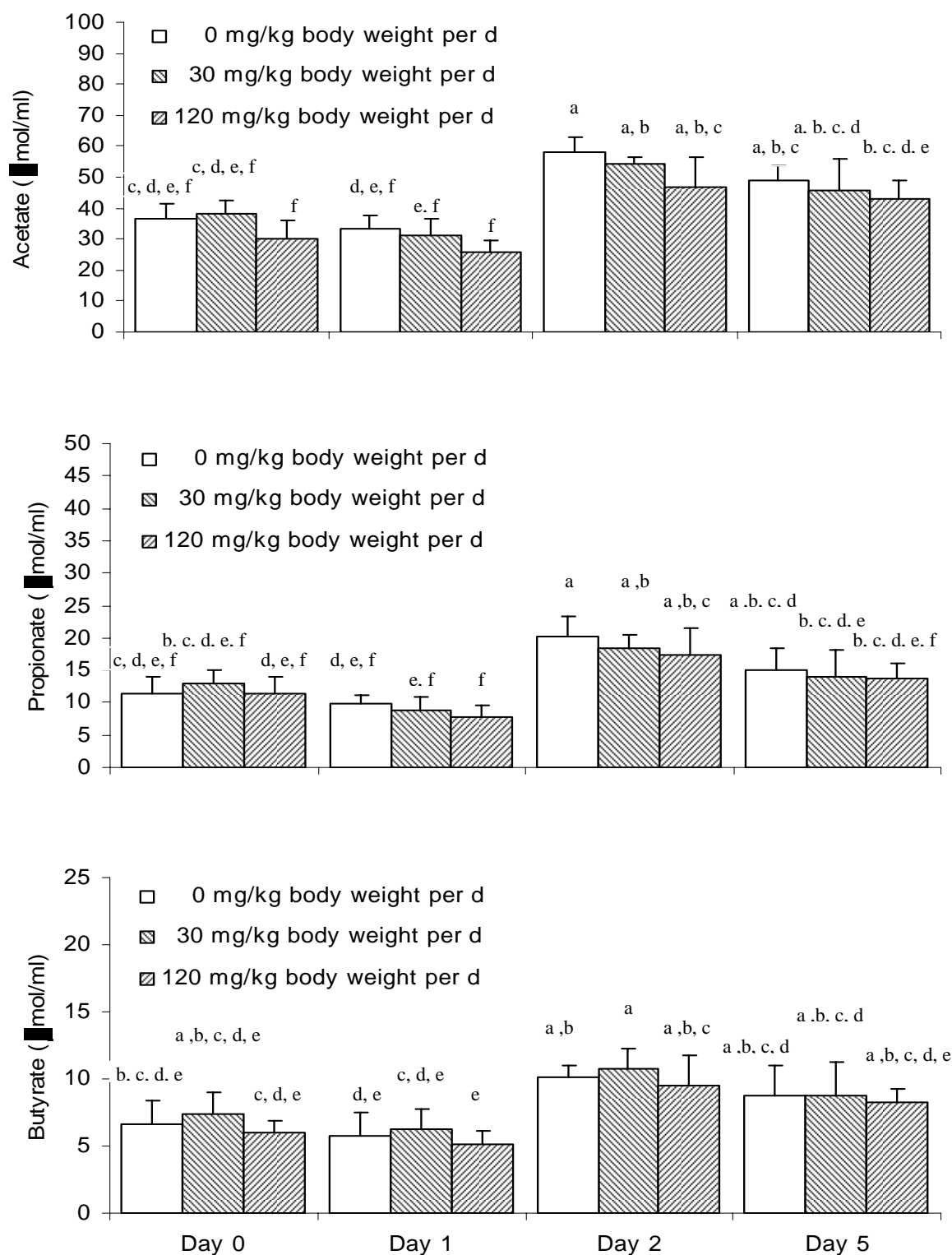


Figure 2. Effect of oral nitroethane administration ($n = 5/\text{treatment}$) on volatile fatty acid accumulation in ovine ruminal contents. Data were analyzed by a repeated measures analysis of variance. For acetate, main effects were $P < 0.01$, $P < 0.0001$ and $P = 0.82$ for treatment, day of treatment and their interaction, respectively. For propionate, main effects were $P = 0.46$, $P < 0.0001$ and $P = 0.71$ for treatment, day of treatment and their interaction, respectively. For butyrate, main effects were $P = 0.36$, $P < 0.0001$ and $P = 0.99$ for treatment, day of treatment and their interaction, respectively. Means were further separated using a Tukey's multiple range test and values with unlike superscripts differ, $P < 0.05$.

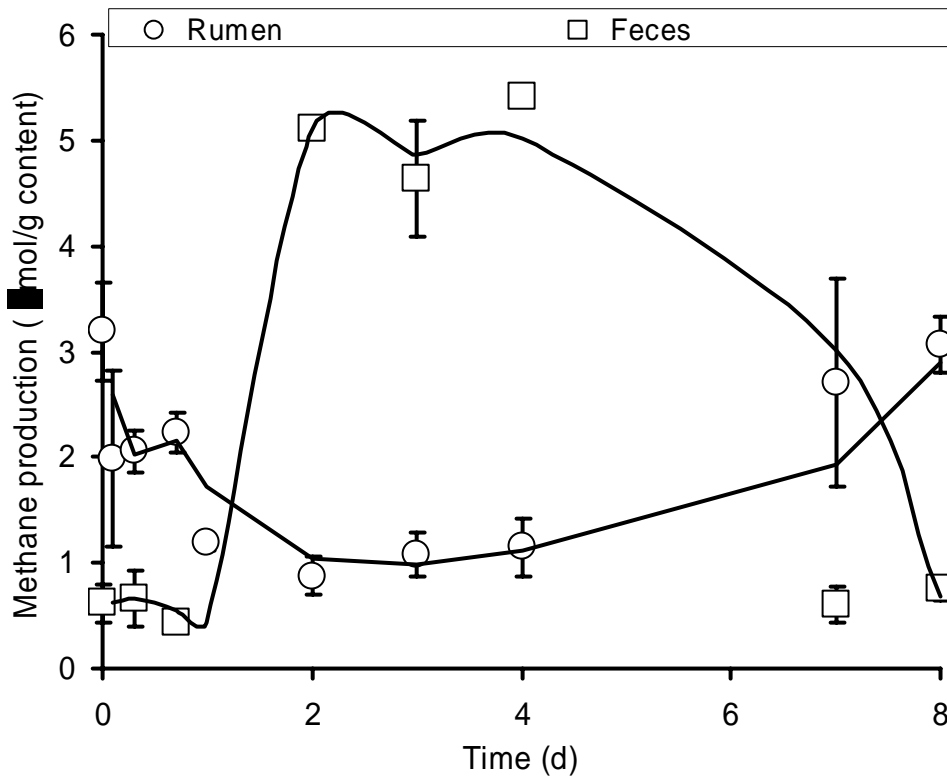


Figure 3. Effect of intraruminal nitroethane administration on ruminal methane-producing activity in bovine ruminal contents. Nitroethane was administered to each of two cattle, with each animal serving as its own control, thus the data are presented descriptively only.

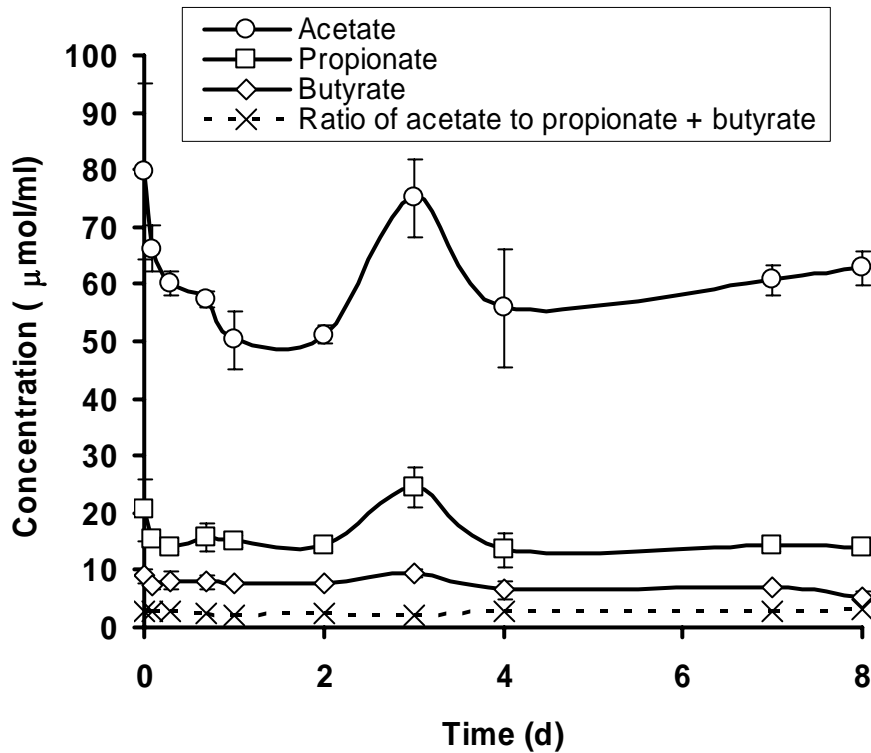


Figure 4. Effect of intraruminal nitroethane administration on accumulations of volatile fatty acids in bovine ruminal contents. Nitroethane was administered to each of two cattle, with each animal serving as its own control, thus the data are presented descriptively only.

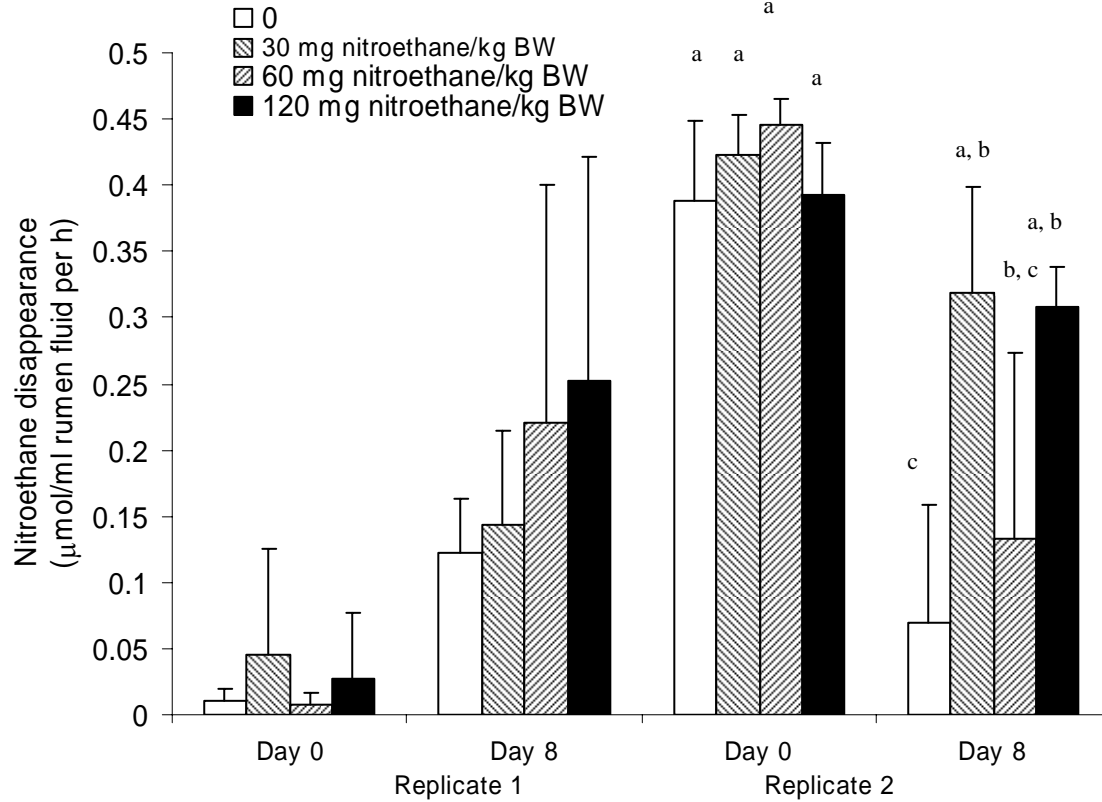


Figure 5. Effect of oral nitroethane administration ($n = 3$ /treatment per replicate) on bovine ruminal nitroethane-reducing activity. Data were analyzed by a repeated measures analysis of variance. Main effects in replicate 1 were $P = 0.65$, $P < 0.004$ and $P = 0.58$ for treatment, day of treatment and their interaction, respectively. Main effects in replicate 2 were $P < 0.04$, $P = 0.0001$ and $P = 0.04$ for treatment, day of treatment and their interaction, respectively. Means within each replicate were further separated using a Tukey's multiple range test and values with unlike superscripts differ, $P < 0.05$.

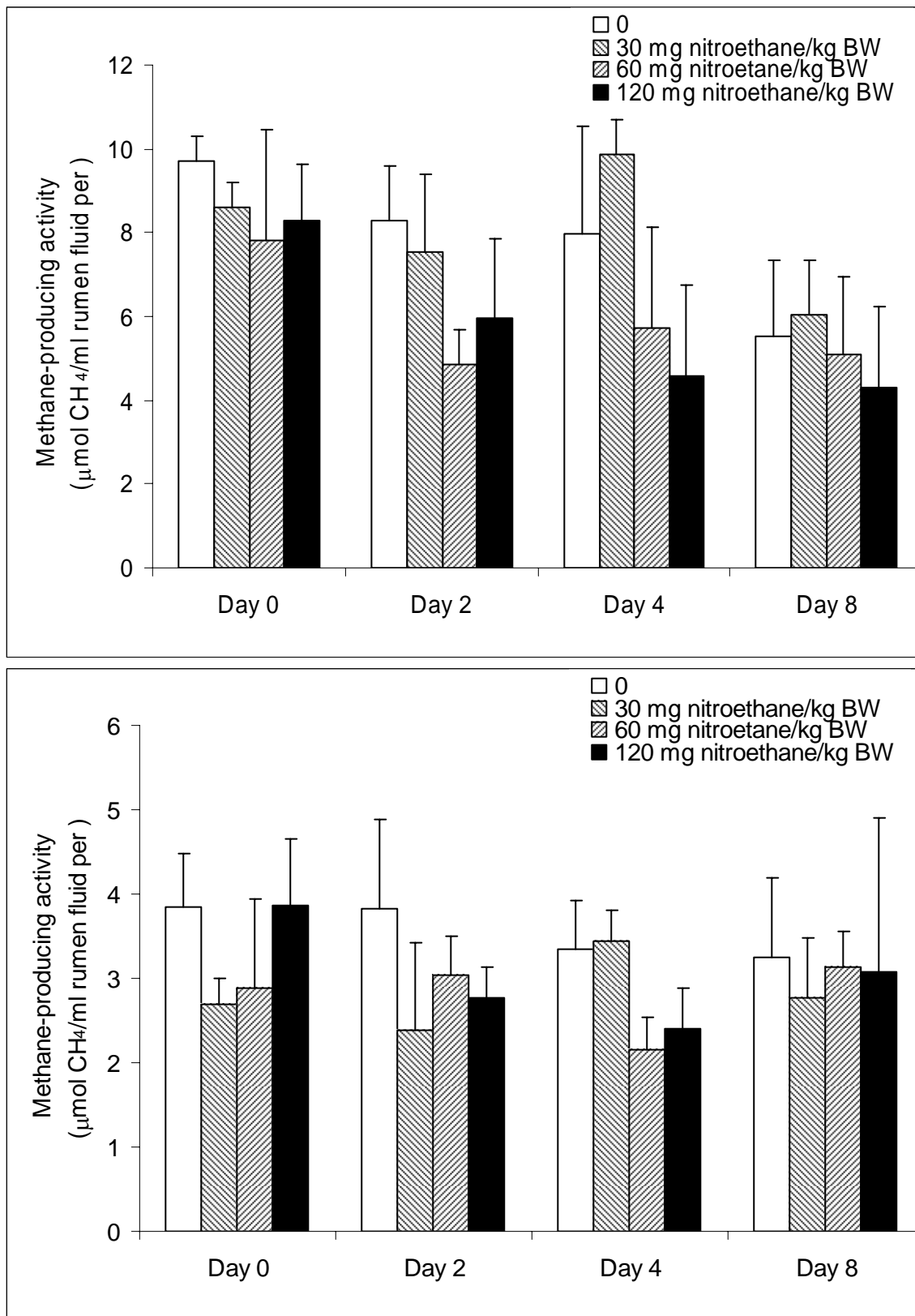


Figure 6. Effect of oral nitroethane administration ($n = 3/\text{treatment per replicate}$) on bovine ruminal methane-producing activity. Data were analyzed by a repeated measures analysis of variance. Main effects in replicate 1 (top figure) were $P < 0.02$, $P < 0.002$ and $P = 0.46$ for treatment, day of treatment and their interaction, respectively. Main effects in replicate 2 (bottom figure) were $P = 0.09$, $P = 0.53$ and $P = 0.51$ for treatment, day of treatment and their interaction, respectively. Means within each replicate were further separated using a Tukey's multiple range test and values with unlike superscripts differ, $P < 0.05$.

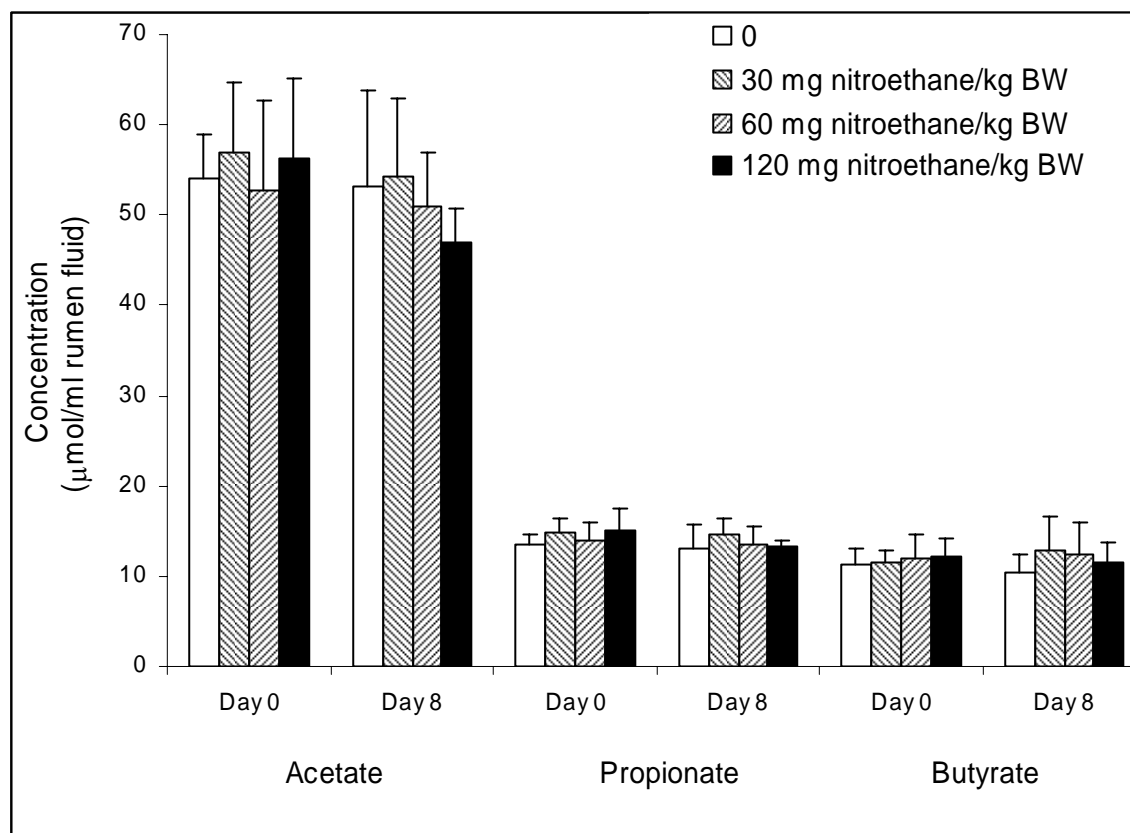


Figure 7. Effect of oral nitroethane administration ($n = 6/\text{treatment}$) on volatile fatty acid accumulation in bovine ruminal contents. Data were analyzed by a repeated measures analysis of variance. For acetate, main effects were $P = 0.69$, $P = 0.06$ and $P = 0.41$ for treatment, day of treatment and their interaction, respectively. For propionate, main effects were $P = 0.41$, $P = 0.15$ and $P = 0.59$ for treatment, day of treatment and their interaction, respectively. For butyrate, main effects were $P = 0.61$, $P = 0.84$ and $P = 0.63$ for treatment, day of treatment and their interaction, respectively.

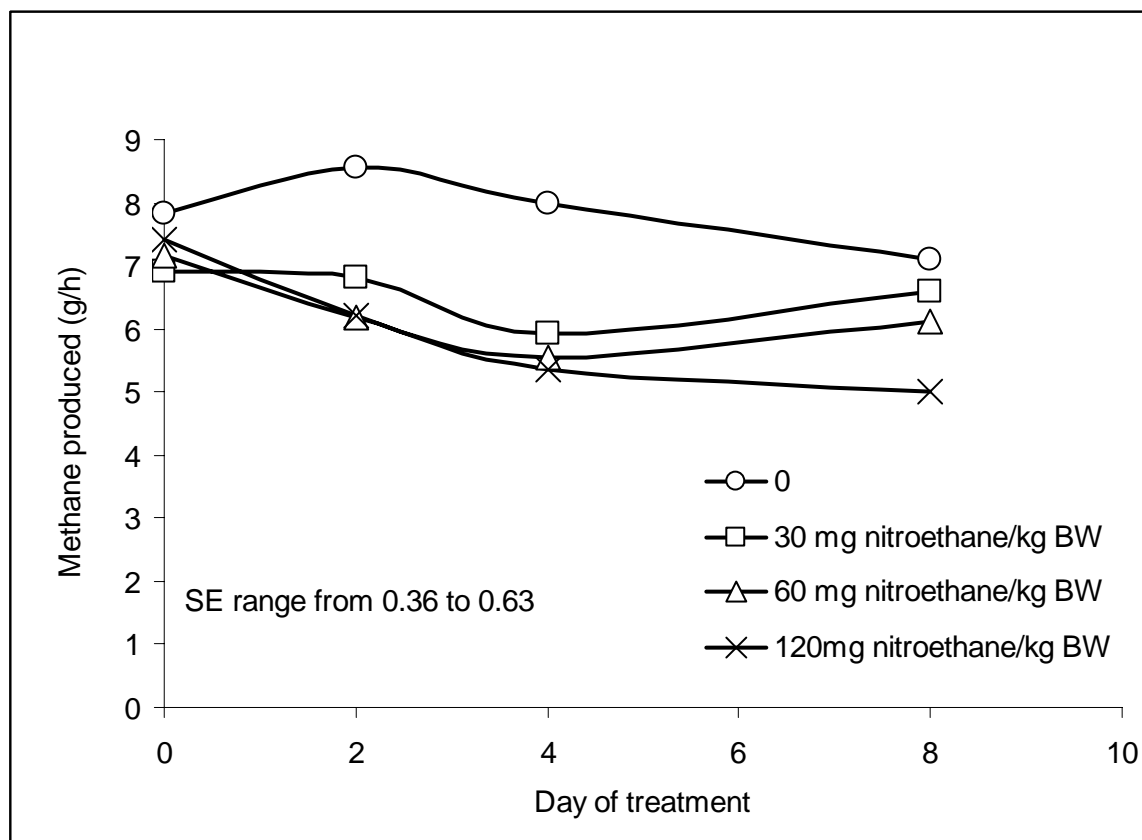
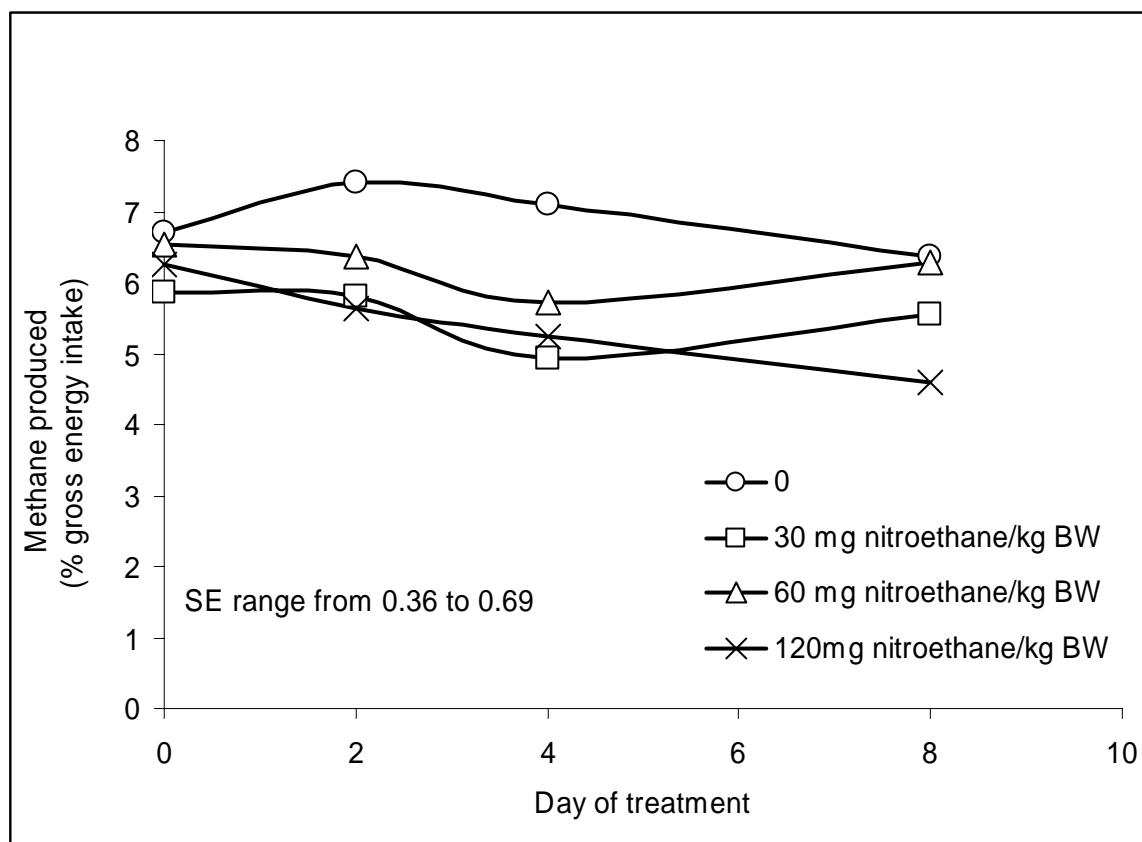


Figure 8. Effect of oral nitroethane administration ($n = 6/\text{treatment}$) on whole animal methane emissions as express as methane lost as a percent of gross energy intake (top figure) or as g methane produced/h (bottom figure)(data from Brown et al., 2005).