Effects of 3-nitrooxypropanol on enteric methane production, rumen fermentation, and feeding behavior in beef cattle fed a high-forage or high-grain diet¹

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ABSTRACT: The objective of the study was to determine whether feeding a diet supplemented with 3-nitrooxypropanol (3-NOP) affects feeding behavior altering intake and rumen fermentation. Two experiments were conducted with 9 rumen-cannulated beef steers in a replicated 3 × 3 Latin square design where animals received a high-forage or high-grain diet. Treatments were 1) a basal diet (CON), the CON diet supplemented with 3-NOP (dNOP; 100 mg/kg in dietary DM or 1 g/d), or the CON diet with 3-NOP (1 g/d) infused into the rumen (infNOP). Each experimental period consisted of 14-d diet adaptation and 7-d sample collection. A 7-d washout period was provided between experiment periods. All data were analyzed as a Latin square design using Mixed Procedure of SAS. In Exp. 1 (high-forage diet), methane yield (measured by the Greenfeed system) was lowered by 18% (18.6 vs. 22.7 g/kg DMI; $P < 0.01$) by dNOP compared with CON. Rumen fermentation was altered similarly by both NOP treatments compared with CON where dNOP and infNOP increased ($P < 0.01$) rumen pH at 3 h and decreased ($P < 0.01$) proportion of acetate in total VFA. However, DMI, feed consumption rate (0 to 3, 3 to 6, 6 to 12, and 12 to 24 h after feeding), particle size distribution of

orts, and feeding behavior (videotaped for individual animals over 48 h) were not affected by dNOP and infNOP compared with CON. In Exp. 2 (high-grain diet), methane production was not affected by dNOP or infNOP compared with CON. Dry matter intake, feed consumption rate, particle size distribution of orts, and feeding behavior were not altered by dNOP and infNOP compared with CON. However, both dNOP and infNOP affected rumen fermentation where total VFA decreased ($P = 0.04$) and acetate proportion in total VFA tended to decrease ($P = 0.07$) compared with CON. In conclusion, dietary supplementation of 3-NOP did not affect feeding behavior of beef steers fed a high-forage or high-grain diet. However, rumen fermentation was similarly changed when 3-NOP was provided in the diet or directly infused in the rumen. Thus, observed changes in rumen fermentation with 3-NOP were not due to changes in feeding behavior indicating no effects on the organoleptic property of the diets. In addition, according to small or no changes in DMI in both experiments and relatively small changes in rumen fermentation in Exp. 2, a greater dosage level of 3-NOP than 100 mg/kg (dietary DM) may need further examination of its effects on feeding behavior of beef cattle.

Key words: 3-nitrooxypropanol, beef cattle, feeding behavior, methane production, rumen fermentation

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INTRODUCTION

The feed additive 3-nitrooxypropanol (3-NOP) has been shown to be an effective enteric methane mitigant with consistent effects across studies regardless of animal species and diet composition (Dijkstra et al., 2018). Although not reported in dairy cattle, the decrease in methane with 3-NOP in beef cattle fed high-forage or high-grain diets was often associated with a decrease in DMI (Romero-Perez et al., 2014; Vyas et al., 2016). The possible explanation for decreased DMI is the concomitant increase in rumen propionate concentration, which potentially leads to reduced feed intake (Allen et al., 2009; Vyas et al., 2016). In addition, an increase in rumen pH and decrease in total VFA concentration has often been observed in animals fed 3-NOP (Reynolds et al., 2014; Romero-Perez et al., 2015a; Vyas et al., 2018a). An increase in rumen pH is assumed to occur due to decreased total VFA concentration and changes in proportion of acetate and propionate were often explained by increases in H_2 production in the rumen (Lopes et al., 2016; Vyas et al., 2018a).

Changes in rumen fermentation similar to those frequently observed when animals are fed 3-NOP can also occur due to changes in feed consumption rate or feeding behavior of animals due to a change in the organoleptic properties of the diet. For example, changes in organoleptic properties by inclusion of nitrate in a ration altered DMI, feed consumption, and rumen fermentation (Lee et al., 2015b). In addition, feeding preference altered by adding supplemental flavors to concentrates was observed, potentially affecting feed intake (Harper et al., 2016). Organoleptic properties are the experience of food through the senses (e.g., taste, sight, smell, and texture; Chauhan and Sharma, 2003). However, few studies have reported the effects of 3-NOP on feeding behavior of beef cattle (Vyas et al., 2018a), thus possible changes in DMI and rumen fermentation due to altered feeding behavior of animals fed 3-NOP cannot be ignored. The hypothesis of the current studies, therefore, was that if inclusion of 3-NOP in beef cattle diets (high-forage and high-grain diets) changed the organoleptic properties of diets, feeding behavior of cattle would be affected, which could contribute to changes in feed intake and rumen fermentation. However, if infusion of 3-NOP in the rumen changed feed intake and rumen fermentation, this would provide evidence that 3-NOP directly affects rumen fermentation.

MATERIALS AND METHODS

Animals used in the experiments were cared for according to the guidelines of the Institute of Animal Care Use Committee at the Ohio State University, which reviewed and approved all procedures.

Experiment 1: High-Forage Diet

Animals, diets, and experimental design. The experiment was performed at the Beef Research Center, Ohio Agricultural Research and Development Center, the Ohio State University (Wooster, OH). Nine rumen-cannulated Angus beef steers (about 11 mo in age; average $\pm$ SD at the beginning of Exp. 1; 451 ± 21 kg BW;) were used in a replicated 3×3 Latin square design. Animals were grouped into 3 squares according to BW and the experiment consisted of 3 periods with 14-d dietary adaptation and 7-d sampling. Between periods, a washout of 7 d was provided to all animals to minimize potential carryover effects of dietary treatments. Two treatment diets were formulated (Table 1) and randomly assigned to animals in each square and period: 1) CON, a high-forage diet; 2) dNOP, CON diet supplemented with 3-NOP (DSM Nutritional Products, Animal Nutrition and Health, Basel, Switzerland; 100 mg/kg of DM equivalent to 1.06 g/d); 3) infNOP, CON diet with 3-NOP (1.08 g/d) infused into the rumen. The dosage level of 3-NOP (100 mg/kg DM) in the current study was selected in terms of methane mitigating effectiveness (see the detail in Discussion) and economic application rates in practice (manufacturer's information). The amount of 3-NOP infused daily was determined based on DMI of individual animals. Tap water was mixed with NOP for the ruminal infusion and the solution was prepared twice a day. For animals on the infNOP treatment, the infusion of 3-NOP mimicked the typical pattern of 3-NOP ingestion of cattle when offered a diet supplemented with 3-NOP based on the intake patterns reported by Lee et al. (2015b). Thus, 80% of the daily amount of 3-NOP was infused during the first 12 h after feeding and 20% during the last 12 h. For the infusion, the solution of 3-NOP in a bucket (20-Liter capacity) was continuously stirred with a magnetic stir bar and pumped (Ismatec ISM943A, IDEX Health & Science, Wertheim-Mondfeld, Germany) into the rumen via Tygon tubing (3.18 mm inner diameter $\times$ 6.35 mm outer diameter $\times$ 1.59 mm). The CON and dNOP animals received an infusion of water (10 L/d) without 3-NOP. No infusion was

Table 1. Ingredients and chemical composition of the high-forage diet¹

Items	High-forage diet	
	CON	dNOP
Ingredients (DM, %)		
Corn silage	64.4	64.7
Distillers grain	10.2	10.2
Corn grain, ground	12.9	12.6
Soybean meal	8.5	8.4
Trace mineral mix ²	1.5	1.5
Copper sulfate	0.004	0.004
Zinc sulfate	0.003	0.003
Limestone	1.86	1.83
Sodium phosphate	0.51	0.50
Vitamin A	0.001	0.001
Vitamin D	0.001	0.001
Vitamin E	0.07	0.07
3-NOP product ^{3,4}	0.00	0.10
Chemical composition (% of DM)		
DM, % as-fed basis	48.2	48.1
OM	92.7	92.9
CP	13.6	13.9
NDF	28.3	28.9
ADF	17.2	17.1
Ca	0.67	0.63
P	0.42	0.38

¹CON, control; dNOP, the control diet supplemented with NOP.

²The premix contained (as-is basis) 39.3% of sodium, 53.2% of chloride, 70 mg/kg of cobalt, 400 mg/kg of copper, 70 mg/kg of iodine, 1,750 mg/kg of iron, 2,800 mg/kg of manganese, and 3,500 mg/kg of zinc (Morton Salt Inc., Chicago, IL).

³DSM Nutrition and Health, Saint Louis, France.

⁴Active 3-NOP concentration in the dNOP diet, 100 mg/kg DM.

conducted during washout periods. The CON diet was formulated for growing beef cattle to meet the requirements of all nutrients for 1.5 kg/d ADG according to [BCNRM \(2016\)](#).

All animals were housed in individual tie stalls and the diets were prepared as total mixed rations (TMR) every morning and fed once daily for ad libitum intake (target refusal, 5% of feed offered) with free access to water.

Sampling procedures. During the entire experiment, feed offered and refused were recorded daily for individual animals. Samples of corn silage were collected weekly to determine DM and proportion of corn silage in the diet was adjusted according to DM content (100 °C overnight). Individual feed ingredients were also sampled weekly and composited by treatment. Ort samples were collected from individual animals twice weekly and composited by animal and period. The composited ort samples were subsampled and frozen until analysis of particle size distribution and the rest was used for

chemical analysis. Composite feed ingredients and ort samples were dried (55 °C for 72 h), ground to pass a 1-mm screen (Wiley Mill; Arthur A. Thomas Co., Philadelphia, PA), and stored in the lab until analyses. Body weights of individual animals were measured on 2 consecutive days at the beginning and end of each period.

On day 15 and 16 in each period, 5 color wireless Internet protocol (IP) video cameras (model F19805P; Amcrest Technologies, Houston, TX) were installed in the barn (2 animals per camera) and behaviors of feeding, drinking, standing, and lying were continuously recorded. Feed consumption rates (0 to 3 h, 3 to 6 h, 6 to 12 h, and 12 to 24 h) were also measured for individual animals on day 15 and 16 by manually weighing amounts of feed remaining in bunks at each time point ([Lee et al., 2015b](#)).

Enteric methane and carbon dioxide production was measured for individual animals on day 17, 18, and 19 using the Greenfeed system (C-Lock Inc., Rapid City, SD) according to the procedure (calibration and measurements) published in [Hristov et al. \(2015a\)](#). Briefly, before starting the experiment the animals were trained to the Greenfeed system. The bait feed used in this study was a pellet feed (23% of corn grain, 15% of corn distillers grain solubles, 50% of soyhulls, 8% of soybean meal, 3% of vegetable fat, 0.5% of urea, and 0.5% limestone on an as-is basis) produced at the feed mill, Ohio Agricultural Research and Development Center, the Ohio State University (Wooster, OH). Methane and carbon dioxide measured at each time point (8 time points per animal and period over 3 d) was calculated as average daily production (g/d) by animal within period. Methane yield (g/kg DMI) was calculated as methane production divided by average DMI on day 17, 18, and 19.

Rumen samples were collected 0, 3, and 6 h after feeding on day 20 and 21 from individual animals. Whole rumen contents were taken from 5 locations in the rumen (cranial dorsal, cranial ventral, central, caudal dorsal, and caudal ventral), mixed thoroughly and then a subsample was collected. The subsample was strained through 2 layers of a mesh screen (0.5 mm) and pH (AE150 pH Benchtop meter; Thermo Fisher Scientific Inc., Waltham, MA) was measured immediately. An aliquot of rumen fluid (5 mL) was preserved with 0.5 M H₂SO₄ (1 mL) for analysis of ammonia and another aliquot (5 mL) was preserved with 25% metaphosphoric acid (1 mL) to determine VFA and D/L-lactate. All samples were frozen at -20 °C and thawed to make a composite of these by animal and period before analyses.

Laboratory analyses. The ground feed ingredients were submitted to Rock River Laboratory (Watertown, WI) for standard chemical analyses (<https://www.rockriverlab.com/pages/Animal-Nutrition.php>; Table 1). Particle size distribution of orts was determined using a Penn State Particle Separator with 2 screens (18.4 and 7.9 mm; Kononoff et al., 2003).

All samples of rumen fluid were centrifuged at $12,000 \times g$ (4 °C) for 10 min and supernatant was collected and analyzed for ammonia (Chaney and Marbach, 1962). Rumen VFA and D/L-lactate concentrations were determined using gas chromatography (model 5890; Hewlett-Packard, Wilmington, DE) with a polar capillary column (30 m $\times$ 0.32 mm $\times$ 1 μ m; ZB-FFAP; Phenomenex Inc., Torrance, CA) where crotonic and malonic acid were used as internal standards for VFA and lactate, respectively (Guyader et al., 2017).

Behavior of individual animals recorded using cameras was downloaded and analyzed using the Observer software (version 5.0.25; Noldus Information Technology B.V., Wageningen, The Netherlands). Activities monitored were frequency and time spent feeding, oral manipulations (biting, chain chewing, and licking), tongue rolling, oral activity not visible (NV; oral manipulation or tongue rolling was not determined because heads were not visible), and drinking when animals were standing. These observations were not made when animals were lying down. Time spent standing was also observed. Feeding behavior data for individual animals were further processed for meal information. The fixed meal criterion of 300 s was used to calculate meal activities (Dong et al., 2018). A meal, therefore, was considered as all feeding events occurring with the interval less than 5 min between feeding events. When meal events were identified, total duration and frequency of meals within 24 h were calculated. Mean DM consumed per meal was calculated by average DMI (day 15 and 16) divided by meal frequency. Mean meal duration was calculated by total meal duration divided by meal frequency.

Statistical Analysis

The data for DMI observed for the 7-d sampling phase in each period were statistically analyzed using Proc Mixed of SAS (SAS 9.4; SAS Institute, Cary, NC). The model included the fixed effect of treatment, period, day, and their 2- and 3-way interactions and the random effect of square and animal within square. Repeated

measures with day were included with the covariance structure of ar(1) according to lowest Bayesian information criterion. Overall rumen pH was analyzed using the same model above except that hour after feeding (0, 3, and 6 h) was used as a repeated measure. Data for BW, feed consumption rate, particle size distribution of orts, rumen fermentation, methane production and yield, and behavior activities were analyzed using the same model except that day, interactions that included day, and repeated measures were removed from the model. Statistical differences were declared at $P \leq 0.05$. Differences between treatments with $0.05 < P \leq 0.10$ were considered as a tendency toward significance. When the main effect of treatment was significant, means were separated by pairwise *t*-test (pdiff option). Data are presented as least square means.

Experiment 2: High-Grain Diet

The experimental design and procedures were the same as used in Exp. 1 except for the following. The animals used in Exp. 1 were used in Exp. 2, but they were re-grouped into squares due to changes in BW. During Exp. 2, the infusion of 3-NOP for infNOP was at a constant rate throughout the day (about 0.042 g/h or 1 g/d). Therefore, the solution of 3-NOP was prepared every morning. During Exp. 2, a high-grain diet supplemented with 3-NOP (100 mg/kg DM) was fed to animals that received the dNOP treatment. The diet was formulated for finishing cattle (Table 2) to meet their requirements of all nutrients for 1.9 kg/d ADG (BCNRM, 2016). Between Exp. 1 and 2, animals were fed transition diets over 4 wk where proportion of concentrate and forage increased and decreased, respectively, in a stepwise manner. Sample collection, laboratory analyses, and statistical analyses were conducted as described in Exp. 1.

RESULTS

Experiment 1: High-Forage Diet

The amount of 3-NOP provided to animals on dNOP and infNOP treatments was similar as intended (Table 3). Furthermore, DMI and BW of animals were not different ($P \geq 0.11$) among treatments. As expected, beef steers consumed about 50% and 80% of feed offered during the first 6 and 12 h, respectively, after feeding. However, feed consumption rates did not differ ($P \geq 0.28$) among treatments. Supplemental or infused 3-NOP did

Table 2. Dietary ingredients and chemical composition of the high-grain diet¹

Items	High-grain diet	
	CON	dNOP
Ingredients (DM, %)		
Corn silage	9.8	9.7
Whole shelled corn	72.8	72.8
Distillers grain	10.0	10.1
Corn grain	1.0	1.0
Soybean meal	2.1	2.1
Urea	0.1	0.1
Trace mineral mix ²	1.5	1.5
Limestone	1.5	1.5
Calcium phosphate	0.5	0.5
Magnesium sulfate	0.003	0.003
Potassium chloride	0.15	0.15
Zinc sulfate	0.002	0.002
Copper sulfate	0.001	0.001
Vitamin A	0.001	0.001
Vitamin D	0.001	0.001
Vitamin E	0.05	0.05
3-NOP product ^{3,4}	0.0	0.10
Chemical composition (% of DM)		
DM, % (as-fed basis)	80.0	80.0
OM	94.3	94.2
CP	12.7	12.6
NDF	14.6	14.8
ADF	5.0	5.7
Ca	0.7	0.8
P	0.5	0.5

¹CON, control; dNOP, the control diet supplemented with NOP.

²The premix contained (as-is basis) 39.3% of sodium, 53.2% of chloride, 70 mg/kg of cobalt, 400 mg/kg of copper, 70 mg/kg of iodine, 1,750 mg/kg of iron, 2,800 mg/kg of manganese, and 3,500 mg/kg of zinc (Morton Salt Inc., Chicago, IL).

³DSM Nutrition and Health, Saint Louis, France.

⁴Active 3-NOP concentration in the dNOP diet, 100 mg/kg DM.

not affect particle size distribution of orts compared with CON (Table 4).

Rumen pH (overall) tended to be different ($P = 0.059$) among treatments (Table 5) and no treatment by hour interaction was observed. The difference in overall rumen pH among treatments occurred because the decrease in rumen pH at 3 and 6 h after feeding was lower when 3-NOP was provided to animals (i.e., dNOP and infNOP) compared with CON. At 3 h after feeding, rumen pH was greater ($P < 0.01$) for dNOP followed by infNOP and CON. The same trend was observed at 6 h after feeding, but the difference was not statistically different ($P = 0.21$). Rumen NH_3 and total VFA concentrations were not different among treatments. However, proportion of acetate in total VFA was lowest ($P < 0.01$) for dNOP followed by infNOP and CON. Conversely, proportion of propionate was greater ($P < 0.01$) for dNOP vs. CON and infNOP, with no difference between infNOP and CON. As a result, the ratio of acetate (C2) to propionate (C3) was lowest ($P < 0.01$) for dNOP followed by infNOP and then CON. Iso-butyrate, butyrate, and iso-valerate tended to differ ($P \geq 0.052$) among treatments where proportions were greater for dNOP vs. CON. No treatment differences in D/L-lactate concentration were observed.

Methane production was decreased (181 vs. 220 g/d; $P < 0.01$) by 18% for dNOP compared with CON (Table 6). Although infNOP lowered methane production by 10% compared with CON, this was not statistically different. Methane yield (g/kg DMI) was also lower (18%; $P < 0.01$) for dNOP compared with CON, but no difference was observed between CON and infNOP.

Table 3. Effects of 3-NOP fed or infused on DMI and BW in beef steers ($n = 9$ per treatment) fed a high-forage (Exp. 1) or high-grain diet (Exp. 2)

	Treatments ¹			SEM	P-value
	CON	dNOP	infNOP		
Experiment 1					
Water infused, L/d	10	10	10		
3-NOP consumed or infused, g/d	0	1.06	1.08		
DMI, kg/d	10.3	10.6	10.3	0.43	0.27
Initial BW, kg	494	489	493	9.4	0.11
Final BW, kg	517	522	519	11.3	0.69
Experiment 2					
Water infused, L/d	10	10	10		
3-NOP consumed or infused, g/d	0	0.98	1.02		
DMI, kg/d	10.2	9.6	9.8	0.60	0.27
Initial BW, kg	675	682	678	29.9	0.34
Final BW, kg	699	697	693	30.8	0.53

¹CON, control; dNOP, the control diet supplemented with 3-NOP (active compound, 100 mg/kg dietary DM); infNOP, animals were fed the control diet and 3-NOP was ruminally infused.

During behavior observation, DMI was not different among treatments (Table 7). No differences in meal events (criterion, duration, frequency) or any other behaviors ($P > 0.39$) were observed except that the duration and frequency of oral manipulation (biting, chain chewing, and licking) was lower ($P = 0.03$) for dNOP compared with CON. However, these were not different between CON and infNOP.

Experiment 2: High-Grain Diet

Dry matter intake and BW of animals were not affected ($P \geq 0.27$) by treatment. Animals consumed about 66% of feed offered within 6 h after feeding and about 90% of feed offered within 12 h after feeding (Table 4). However, feed consumption rates were not different ($P \geq 0.70$) among treatments. Particle size distribution of orts was not altered ($P \geq 0.39$) when 3-NOP was fed or infused into the rumen compared to CON.

Rumen pH (overall or at individual time points) was not different ($P \geq 0.14$) among treatments (Table 5). Total VFA concentration was less ($P = 0.04$) for infNOP compared with CON without

a difference between dNOP and infNOP. Proportion of acetate tended to decrease ($P = 0.07$) and that of butyrate tended to increase ($P = 0.10$) for dNOP and infNOP compared with CON. Proportions of other VFA were not affected by treatment. However, D/L-lactate concentrations tended to be greatest ($P = 0.08$) for infNOP followed by dNOP and CON.

Methane production and yields were not different ($P \geq 0.23$) among treatments (Table 6). During methane measurements, DMI was also not affected by treatments. Dry matter intake of animals was also not different among treatments during behavior measurements (Table 7). Meal events (criterion, duration, frequency) and other behaviors were not affected ($P \geq 0.17$) by either dNOP or infNOP compared with CON.

DISCUSSION

Experiment 1: High-Forage Diet

3-Nitrooxypropanol is an effective feed additive that lowers enteric methane emissions from ruminant animals, with consistent effects across species of ruminant and type of diets

Table 4. Effects of 3-NOP fed or infused on feed consumption rates and particle size distribution of orts in beef steers ($n = 9$ per treatment) fed a high-forage (Exp. 1) or high-grain diet (Exp. 2)

	Treatments ¹			SEM	P-value
	CON	dNOP	infNOP		
Experiment 1					
Feed intake, kg (as-fed)	21.6	22.5	21.8	0.85	0.41
% of feed consumed					
0 to 3 h	32.2	31.7	31.0	3.82	0.80
3 to 6 h	15.0	17.0	15.0	2.38	0.35
6 to 12 h	34.0	31.1	34.5	2.03	0.28
12 to 24 h	18.9	20.3	19.5	4.30	0.85
Particle size of orts (% of as-is)					
Large (>1.84 cm)	10.3	9.1	9.5	2.57	0.88
Medium (0.79 to 1.84 cm)	53.5	51.4	54.6	2.28	0.35
Small (<0.79 cm)	36.2	39.5	38.9	4.31	0.61
Experiment 2					
Feed intake, kg (as-fed)	13.8	13.6	13.6	0.90	0.91
% of feed consumed					
0 to 3 h	47.7	47.0	46.6	4.12	0.97
3 to 6 h	18.0	18.0	18.1	2.07	0.99
6 to 12 h	25.5	26.8	27.7	3.13	0.70
12 to 24 h	8.8	8.2	7.8	1.56	0.87
Particle size of orts (% of as-is)					
Large (1.84 cm)	0.89	0.35	0.47	0.280	0.39
Medium (0.79 to 1.84 cm)	41.7	41.4	38.2	4.64	0.61
Small (0.12 to 0.79 cm)	57.4	58.2	61.4	4.76	0.60

¹CON, control; dNOP, control diet supplemented with 3-NOP added to the ration (active compound, 100 mg/kg dietary DM); infNOP, control diet with 3-NOP ruminally infused.

Table 5. Effects of 3-NOP fed or infused on rumen fermentation characteristics in beef steers ($n = 9$ per treatment) fed a high-forage (Exp. 1) or high-grain diet (Exp. 2)

	Treatments ¹			SEM	<i>P</i> -value
	CON	dNOP	infNOP		
Experiment 1					
Overall rumen pH	6.19	6.34	6.27	0.043	0.059
0 h	6.65	6.69	6.67	0.092	0.87
3 h	6.03 ^c	6.29 ^a	6.15 ^b	0.041	<0.01
6 h	5.91	6.05	5.98	0.056	0.21
NH ₃ , mg/dL	8.6	7.4	7.2	0.67	0.27
Total VFA, mmol/L	118.6	113.4	111.0	3.53	0.15
(molar % of total VFA)					
Acetate	63.7 ^a	58.7 ^c	61.6 ^b	0.87	<0.01
Propionate	20.0 ^b	22.7 ^a	21.1 ^b	0.99	<0.01
Iso-butyrate	0.88	0.99	0.90	0.031	0.052
Butyrate	12.2	13.7	12.8	0.48	0.091
Iso-valerate	1.63	1.95	1.72	0.092	0.057
Valerate	1.23 ^b	1.44 ^a	1.34 ^{ab}	0.052	<0.01
C2/C3	3.26 ^a	2.65 ^c	2.98 ^b	0.168	<0.01
D/L-Lactic acid, mmol/L	0.17	0.15	0.14	0.015	0.14
Experiment 2					
Overall rumen pH	6.13	6.21	6.27	0.081	0.22
0 h	6.16	6.27	6.39	0.101	0.14
3 h	6.15	6.20	6.25	0.080	0.42
6 h	6.10	6.16	6.19	0.077	0.38
NH ₃ , mg/dL	9.3	6.10	6.9	2.98	0.29
Total VFA, mmol/L	106.6 ^a	101.8 ^{ab}	94.7 ^b	4.58	0.04
(molar % of total VFA)					
Acetate	52.7	48.3	47.3	2.17	0.07
Propionate	28.2	27.8	28.1	3.05	0.99
Iso-butyrate	1.28	1.44	1.52	0.123	0.34
Butyrate	13.2	16.7	17.8	2.19	0.10
Iso-valerate	2.55	3.18	3.02	0.315	0.29
Valerate	2.00	2.59	2.31	0.283	0.24
C2/C3	2.04	1.95	1.96	0.307	0.95
D/L-Lactic acid, mmol/L	0.14	0.16	0.19	0.016	0.08

^{abc}Within a row, means without a common superscript letter differs ($P < 0.05$).

¹CON, control; dNOP, the control diet supplemented with 3-NOP (active compound, 100 mg/kg dietary DM); infNOP, animals were fed the control diet and 3-NOP was ruminally infused.

Table 6. Effects of 3-NOP fed or infused on methane production in beef steers ($n = 9$ per treatment) fed a high-forage (Exp. 1) or high-grain diet (Exp. 2)

	Treatments ¹			SEM	<i>P</i> -value
	CON	dNOP	infNOP		
Experiment 1					
DMI, ² kg/d	9.7	9.9	9.6	0.47	0.66
CH ₄ , g/d	219.6 ^a	181.2 ^b	200.2 ^{ab}	11.27	<0.01
CH ₄ , g/kg DMI	22.7 ^a	18.6 ^b	21.3 ^a	1.93	<0.01
CO ₂ , kg/d	10.3	10.7	10.1	0.69	0.12
Experiment 2					
DMI, ² kg/d	9.1	8.5	8.6	0.64	0.49
CH ₄ , g/d	119.0	106.5	97.6	17.1	0.24
CH ₄ , g/kg DMI	13.4	12.8	11.2	2.43	0.23
CO ₂ , kg/d	10.5	10.4	10.4	0.68	0.98

^{abc}Within a row, means without a common superscript letter differs ($P < 0.05$).

¹CON, control; dNOP, the control diet supplemented with 3-NOP (active compound, 100 mg/kg dietary DM); infNOP, animals were fed the control diet and 3-NOP was ruminally infused.

²Average DMI during 3-d methane measurement.

Table 7. Effects of 3-NOP fed or infused on behaviors of beef steers ($n = 9$ per treatment) fed a high-forage (Exp. 1) or high-grain diet (Exp. 2)

	Treatments ¹			SEM	P-value
	CON	dNOP	infNOP		
Experiment 1					
DMI, ² kg/d	10.4	10.8	10.4	0.52	0.64
Meal					
Meal criterion, min ³	5.0	5.0	5.0	—	—
Total meal duration, min/d	226.8	228.5	215.1	17.24	0.71
Meal frequency	18.3	17.9	18.2	0.90	0.95
Mean DM, kg per meal	0.59	0.61	0.58	0.039	0.82
Mean duration, min per meal	13.4	12.9	12.0	1.40	0.61
Standing, min/d	494.9	464.5	483.4	21.54	0.39
Lying, ⁴ min/d	945.2	975.5	956.6		
Drinking, min/d	7.4	6.8	6.9	1.22	0.75
Oral manipulation, ⁵ min/d	27.5 ^a	18.4 ^b	21.7 ^{ab}	3.21	0.03
Tongue rolling, min/d	8.1	8.9	10.2	2.35	0.81
Oral + tongue, min/d	35.6	27.4	32.0	4.36	0.14
NV, ⁶ min/d	16.1	17.6	13.9	3.67	0.84
Experiment 2					
DMI, ² kg/d	11.2	11.0	11.1	0.72	0.92
Meal					
Meal criterion, min ³	5.0	5.0	5.0		
Total meal duration, min/d	199.5	201.6	219.5	12.52	0.17
Meal frequency	15.5	15.4	16.7	1.03	0.48
Mean DM, kg per meal	0.73	0.73	0.67	0.446	0.38
Mean duration, min per meal	13.8	13.6	14.0	1.54	0.98
Standing, min/d	480.3	465.8	475.2	22.71	0.74
Lying, ⁴ min/d	959.7	974.2	964.8		
Drinking, min/d	5.2	4.3	6.8	1.21	0.62
Oral manipulation, ⁵ min/d	22.8	25.9	26.7	7.09	0.69
Tongue rolling, min/d	10.2	9.5	10.4	2.08	0.87
Oral + tongue, min/d	33.1	35.4	37.1	7.67	0.76
NV time, ⁶ min/d	6.4	8.0	6.8	3.67	0.83

^{abc}Within a row, means without a common superscript letter differs ($P < 0.05$).

¹CON, control; dNOP, the control diet supplemented with 3-NOP (active compound, 100 mg/kg dietary DM); infNOP, animals were fed the control diet and 3-NOP was ruminally infused.

²Average DMI during 2-d behavior observation.

³Meal criterion of 300 s (5 min) was used for all animals to calculate meal events.

⁴Lying time (min/d) = 1,440 – standing time.

⁵Biting, chain chewing, and licking.

⁶Animal heads were not visible to determine oral manipulation and tongue rolling.

(Dijkstra et al., 2018; Jayanegara et al., 2018). Although the mode of action of 3-NOP for methane mitigation is known (Duin et al., 2016), other effects of 3-NOP on changes in DMI, rumen pH, and VFA composition are not clearly understood.

In the current study, methane production and yield decreased when a high-forage diet supplemented with 3-NOP at 100 mg/kg DM was fed to animals compared with CON. The degree of methane mitigation was similar to a study by Vyas et al. (2016) when beef cattle were fed a high-forage diet with 3-NOP at 100 mg/kg DM. Interestingly, when 3-NOP was directly infused into the rumen,

methane mitigation was small compared with dNOP (only a numerical decrease), which is difficult to explain. The 3-NOP was infused continuously (80% within the first 12 h and 20% at 12 to 24 h after feeding) throughout the day in Exp. 1. It is possible that providing 3-NOP via an infusion line resulted in it residing on the top surface of the digesta mass in the rumen, which may have delayed 3-NOP from being fully mixed with rumen contents. Also, the 3-NOP product includes silicon dioxide as a carrier (90%) and silicon dioxide is not soluble in water. Stirring the solution of 3-NOP during the infusion increased the viscosity of the solution, which

sometimes (about 2 to 5 cases per period) clogged the infusion line during the first 12 h of infusion (i.e., 80% of the daily dosage). Depending on the severity of clogging, it took about 30 to 90 min per case to unclog the line during which time an unknown amount of 3-NOP was lost. This may have, at least in part, contributed to the minimal effect of infNOP on methane mitigation compared with dNOP.

The amount of 3-NOP supplied (i.e., 1 g/d or 100 mg/kg dietary DM) was similar between dNOP and infNOP (when the unknown loss of 3-NOP for infNOP is ignored), which did not affect DMI of animals compared with CON. When 3-NOP was provided to dairy cows, DMI was usually unaffected (Haisan et al., 2014; Reynolds et al., 2014; Hristov et al., 2015b; Lopes et al., 2016). However, decreases in DMI of beef cattle fed a high-forage diet were often observed (Romero-Perez et al., 2014; Vyas et al., 2016, 2018a) although not always (Vyas et al., 2018b). The discrepancy in DMI responses to 3-NOP among studies might be related to length of the feeding period and the dietary concentration of 3-NOP. For example, to our knowledge, most long-term studies with beef cattle fed a high-forage diet observed decreased DMI with 3-NOP supplementation (Vyas et al., 2016, 2018a) while no difference in DMI was observed in most short-term studies, i.e., Latin square design (except Romero-Perez et al., 2014). In studies with dairy cows, almost all studies were conducted in short-term Latin square designs except that of Hristov et al. (2015b), which used a relatively long-term continuous design (i.e., 12-wk observation). In this study by Hristov et al. (2015b), however, DMI was not affected by 3-NOP. Thus, the lack of effect of feeding 3-NOP on DMI in the current study might be, at least in part, related with the experimental design (i.e., short-term Latin square design). In addition, DMI of beef cattle is decreased by 3-NOP supplementation in a dose-dependent manner (Vyas et al., 2016), suggesting that the degree of changes in DMI are also dependent upon dosage level of 3-NOP. The dosage level of NOP (0.1% in or 100 mg/kg of dietary DM) in the current study was likely not high enough to affect DMI. In agreement, Vyas et al. (2016) fed a high-forage diet with 2 levels of 3-NOP (100 and 200 mg/kg dietary DM) where the low level of 3-NOP did not affect DMI of beef cattle, whereas 3-NOP at 200 mg/kg dietary DM significantly decreased DMI.

Lack of effect of dNOP and infNOP on DMI suggests that supplementation of the high-forage diet with 3-NOP at 100 mg/kg dietary DM had

minimal or no effects on organoleptic properties of the diet. This is supported by the lack of differences in feed consumption rates among treatments. Feed consumption rate is a good indicator of negative changes in organoleptic properties of a diet that cause a decrease in DMI (Lee et al., 2015b). Another indicator is particle size distribution of orts; a feed additive that negatively affects organoleptic properties often causes sorting of feed by animals (Lee et al., 2015a). The lack of difference in particle size distribution of orts among treatments in the current study supports the conclusion that dietary supplementation with 3-NOP at 100 mg/kg DM did not alter organoleptic properties of a high-forage diet.

Rumen fermentation, however, was altered by the dietary and ruminal supply of 3-NOP as expected. Increases in rumen pH for dNOP and infNOP compared with CON are in agreement with previous in vitro and in vivo studies in the literature. In a study by Romero-Perez et al. (2014), a high-forage diet supplemented with increasing levels of 3-NOP (about 0, 40, 120, and 250 mg/kg dietary DM) linearly increased rumen pH. In a RUSITEC study by Romero-Perez et al. (2015a), rumen pH increased with increasing inclusion rate of 3-NOP in a high-forage diet (about 0, 83, 165, 333 mg/kg dietary DM). One study with dairy cows observed increased rumen pH when 3-NOP was pulse-dosed into the rumen at about 120 mg/kg dietary DM (Reynolds et al., 2014). In these studies, increases in rumen pH when 3-NOP was provided were explained by decreases in total VFA concentration in the rumen. As part of our hypothesis, we expected that changes in feeding behavior and feed consumption rates of animals might affect rumen pH when 3-NOP was provided to animals. However, this was not the case in the current study given the lack of effect of 3-NOP on feeding behavior and feed consumption rate (see more discussion about feeding behavior below). This leads us to the conclusion that 3-NOP has a direct effect in the rumen by altering rumen fermentation, including rumen pH. In addition, numerical decreases in total VFA concentrations may, at least in part, be attributed to the increases in rumen pH for dNOP and infNOP in the current study. A decrease in proportion of acetate and increase in propionate and butyrate proportions in total VFA for dNOP and infNOP compared with CON was not surprising. Changes in proportion of these VFA by 3-NOP have been quite consistent across studies with animals fed a high-forage diet (Haisan et al., 2014; Romero-Perez et al., 2014, 2015b). As a result, 3-NOP supply often

decreases the ratio of C2 to C3, as was observed in the current study. When enteric methane production is inhibited, rumen fermentation shifts from acetate to propionate production for $2H^+$ disposal. Valerate is another sink for $2H^+$ in the rumen, which accounts for its increase for dNOP and infNOP in the current study.

The information about how 3-NOP in a diet influences feeding behavior of beef cattle is lacking. The durations of standing and lying of animals were similar to that of cattle in confined conditions (Dong et al., 2018). Meal frequency and duration were slightly greater for animals in the current study compared with other studies (Holtshausen et al., 2011; He et al., 2015; Dong et al., 2018), probably because DMI of animals in the current study was greater. In addition, meal events (e.g., frequency and duration) can differ depending upon the measurement method of determining feeding behavior (e.g., GrowSafe system or video recording) and method of analyzing behavior data (fixed meal criterion or meal criterion analyzed using models; Bailey et al., 2012). In the current study, we did not observe any treatment differences for most behavior activities including meal events; therefore, we reject our hypothesis that 3-NOP changes the organoleptic properties of diets when offered to beef cattle at 100 mg/kg DM. Interestingly, the duration of oral manipulation (i.e., biting, chain chewing, and licking) was less for dNOP compared with CON, which is difficult to explain. However, it cannot be ignored that oral manipulation may have been hidden during periods of NV activity.

The primary purpose of the current study was to identify the cause of changes in DMI and rumen fermentation in animals offered 3-NOP and we hypothesized that altered feeding behavior might be a factor causing the changes. However, because no changes in feeding behavior occurred, despite significant changes in rumen fermentation, we conclude that 3-NOP at 100 mg/kg dietary DM directly influenced rumen fermentation regardless of method of supplementation. Effects of 3-NOP on rumen bacterial populations appear to be inconsistent (Haisan et al., 2014; Romero-Perez et al., 2014, 2016; Lopes et al., 2016). However, Lopes et al. (2016) observed in dairy cows that 3-NOP at 60 mg/kg dietary DM decreased the population of *Ruminococcus* spp. and increased *Butyrivibrio* spp., which is in line with the decrease in acetate proportion and increase in butyrate proportion, respectively, observed in the current study. Further studies are needed to understand the interaction between 3-NOP and rumen microbes in terms of feed

fermentation. It is worth noting that the effectiveness of 3-NOP as a methane mitigation approach increases as dosage levels of 3-NOP increase, and the dosage level of 3-NOP in the current study was relatively low (100 vs. 200 mg/kg DM) compared with other studies with beef cattle fed a high-forage diet (Vyas et al., 2016). Therefore, determining feeding behavior and feed consumption rates of beef cattle at higher dosage levels of 3-NOP (e.g., 200 mg/kg DM) is needed to confirm the result of the current study that reports no effects of 3-NOP on organoleptic properties of a high-forage diet in addition to changes in rumen fermentation and feeding behavior.

Experiment 2: A High-Grain Diet

Compared to a high-forage diet, feeding a high-grain diet produces less enteric methane production because more $2H^+$ sinks (propionate) are available in the rumen and the condition of low rumen pH inhibits methanogens and protozoa (Hristov et al., 2013). When compared at the same dosage, the efficacy of 3-NOP in mitigating methane has been shown previously to be greater when a high-grain diet is fed compared with a high-forage diet (Vyas et al., 2016). Vyas et al. (2016) speculated that the concentration of methyl coenzyme M may be lower in the rumen for a high-grain vs. high-forage diet, resulting in greater efficacy of 3-NOP in methane mitigation. In the current study, however, no differences in methane production and yield were found when a high-grain diet containing 3-NOP at 100 mg/kg dietary DM was fed to animals. This is in agreement with a study by Vyas et al. (2016) in which 3-NOP at 100 mg/kg dietary DM did not decrease methane production and yield in beef cattle fed a high-grain diet, while 3-NOP at 200 mg/kg decreased methane production and yield up to 80% compared with control. Another study from the same group found a significant decrease by about 50% in methane production and yield at 125 mg 3-NOP in dietary DM when a high-grain diet was fed (Vyas et al., 2018a). Vyas et al. (2018b) examined various dosage levels of 3-NOP with a high-grain diet and concluded that 3-NOP at 100, 150, and 200 mg/kg dietary DM was effective for beef cattle. Reasons for the discrepancy of 3-NOP in methane mitigating efficacy between studies are not clear. Experimental design (short vs. long term), dietary composition, animal variation, and methane measurement technique (although reliable measurements of daily methane production by Greenfeed has been reported; Huhtanen et al., 2019) may have, at least

in part, attributed to the variability. Although a discrepancy of 3-NOP in methane mitigating efficacy among studies exists, results from the current study and some others indicate that the dosage level of 3-NOP at 100 mg/kg dietary DM was insufficient to effectively mitigate methane production in beef cattle fed a high-grain diet.

Lack of difference in DMI and feed consumption rates among treatments is in line with those in Exp. 1. The dosage of 3-NOP at 100 mg/kg dietary DM in the high-grain diet did not appear to alter the organoleptic properties of the diet, as evidenced by no treatment effects on DMI, feed consumption rates, and particle size distribution of orts. This conclusion is further supported by the lack of treatment effects on animal behavior. The duration of standing and lying activities were similar to other studies in which beef cattle were fed a high-grain diet (González et al., 2008; Dong et al., 2018). The meal events of animals (meal frequency, total meal duration, meal size) were also similar with those in the literature (González et al., 2009; Bailey et al., 2012; Dong et al., 2018). In the current study, dNOP and infNOP did not affect meal events or any other behaviors, confirming that 3-NOP at 100 mg/kg dietary DM did not alter organoleptic properties of the high-grain diet.

Studies on the effects of 3-NOP for high-grain diets on rumen fermentation are limited. One in vitro RUSITEC study (Romero-Perez et al., 2017) and 1 feedlot study (Vyas et al., 2018b) have examined effects of 3-NOP on rumen fermentation using a high-grain diet. In the current study, changes in rumen fermentation due to dNOP and infNOP were relatively small compared with the changes in Exp. 1. No decrease in total VFA was observed in a RUSITEC study that used a high-grain diet (Romero-Perez et al., 2017), which is in agreement with the current study, i.e., CON vs. dNOP. However, the significant decrease in total VFA concentration for infNOP vs. CON in addition to the numerical decrease for dNOP vs. CON suggests that continuous supply of 3-NOP throughout the day was more effective for 3-NOP to influence rumen fermentation compared with 3-NOP being provided via the diet. When 3-NOP was provided with the diet (i.e., dNOP), about 65% and 90% of the daily dosage of 3-NOP was consumed within 6 and 12 h after feeding, respectively, in a day. It has been suggested that the efficacy of 3-NOP in methane mitigation is greater when provided via the feed compared with a pulse dose into the rumen at the same dosage level (Reynolds et al., 2014; Haisan et al., 2017). It is assumed that dietary supplementation of 3-NOP

provides the compound to the rumen as the diet is consumed, resulting in a constant supply of 3-NOP to the rumen accompanying the feed, unlike a pulse dose of 3-NOP in the rumen. In the current study, the supply of 3-NOP to animals was more constant for infNOP vs. dNOP.

A tendency for decreased proportion of acetate in total VFA for dNOP and infNOP vs. CON is consistent with the findings of a RUSITEC study and a feedlot study that also used a high-grain diet (Romero-Perez et al., 2017; Vyas et al., 2018b). As indicated for Exp. 1, a decrease in acetate proportion in total VFA probably occurred because increases in $2H^+$ accumulation in the rumen (due to methane mitigation) shifted rumen fermentation from acetate to propionate production (Reynolds et al., 2014; Romero-Perez et al., 2015b; Haisan et al., 2017). The proportion of propionate, however, was not altered by dNOP and infNOP, which is in agreement with a RUSITEC study (Romero-Perez et al., 2017). However, Vyas et al. (2018b) observed an increase in propionate proportion in feedlot cattle fed a high-grain diet. The lack of difference in propionate proportion in the current study can be explained, at least in part, by the relatively low dosage level of 3-NOP (100 mg/kg dietary DM) and a tendency for increased lactate concentration for dNOP and infNOP compared with CON (conversion between propionate and lactate). The concentration of lactate in the rumen is an important factor affecting rumen pH. In the current study, however, lactate concentration was small and its contribution to propionate in the rumen would be trivial.

Although the effects of 3-NOP on rumen fermentation were relatively small (compared with Exp. 1), the changes are generally in agreement with those observed in Exp. 1 and in the literature. The lack of effect of 3-NOP on feeding behavior and changes in rumen fermentation when 3-NOP was infused in the rumen are also consistent with Exp. 1, indicating that changes in rumen fermentation were not caused by changes in organoleptic properties of the high-grain diet when supplemented with 3-NOP at 100 mg/kg dietary DM. The results suggest that 3-NOP directly affected feed fermentation in the rumen. The RUSITEC study by Romero-Perez et al. (2017) is the only study with a high-grain diet that examined effects of 3-NOP on methanogen population, which decreased with supplementation with 3-NOP. Further studies are needed to understand the effects of 3-NOP on the microbial population and activity in relation to feed fermentation in beef cattle fed a high-grain diet. It appears that

3-NOP at 100 mg/kg dietary DM was insufficient to reduce the methane production of cattle fed a high-grain diet, unlike the results for Exp. 1. Therefore, more studies with a high-grain diet supplemented with 3-NOP at greater levels (e.g., 150 or 200 mg/kg dietary DM) are needed to confirm our results.

In conclusion, adding 3-NOP to the diet at 100 mg/kg DM decreased methane yield by 18% when beef steers were fed a high-forage diet but no reduction was observed when a high-grain diet was fed. Dry matter intake, feed consumption rates, particle size distribution of orts, and feeding behavior were not affected by 3-NOP supplementation of a high-forage or high-grain diet, suggesting that the inclusion of 3-NOP did not alter organoleptic properties of the diet. Rumen fermentation was altered similarly by dNOP and infNOP compared with CON (e.g., rumen pH and VFA composition) although the degree of change differed between a high-forage diet and high-grain diet. This is clear evidence that 3-NOP directly affected rumen fermentation when 3-NOP was present in the rumen, regardless of method of provision of 3-NOP. Further studies are needed to confirm the lack of effect of 3-NOP on DMI and feeding behavior when it is provided at higher dosage levels (i.e., 200 mg/kg DM). Studies are also needed to better understand the interactions between rumen microbes and 3-NOP in relation to rumen fermentation.

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