



Short communication: Short-term effect of 3-nitrooxypropanol on feed dry matter intake in lactating dairy cows

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Short-term effect of 3-nitrooxypropanol on feed dry matter intake in lactating dairy cows

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1 **INTERPRETIVE SUMMARY: Short communication: *Short-term effect of 3-***
2 ***nitrooxypropanol on feed dry matter intake in lactating dairy cows. By Melgar et al., page 000.***

3 A diet containing 3-nitrooxypropanol (3-NOP), an enteric methane inhibitor under investigation,
4 administered at concentrations from 30 to 120 mg/kg feed dry matter and a control diet were
5 offered simultaneously to dairy cows to evaluate the effect of 3-NOP on short-term dry matter
6 intake. Compared with control, diet dry matter intake during the test period was quadratically
7 increased by 3-NOP. Data from this study suggest that a diet containing 3-NOP does not have a
8 negative effect on short-term dry matter intake in lactating dairy cows.

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10 **RUNNING HEAD: 3-NITROOXYPROPANOL IN DAIRY COWS**

11
12 ***Short communication: Short-term effect of 3-nitrooxypropanol on feed dry matter intake in***
13 ***lactating dairy cows***

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ABSTRACT

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31 The objective of this study was to investigate the effect of 3-nitrooxypropanol (3-NOP),
32 an enteric methane inhibitor under investigation, on short-term dry matter intake (DMI) in
33 lactating dairy cows. Following a 1-wk adaptation period, 12 multiparous Holstein cows were
34 fed a basal TMR containing increasing levels of 3-NOP during 5 consecutive, 6-d periods. The
35 experiment was conducted in a tie-stall barn. Feed bins were split in half by a solid divider and
36 cows simultaneously received the basal TMR supplemented with: (1) a placebo, without 3-NOP,
37 or (2) 3-NOP included in the TMR at 30, 60, 90, or 120 mg/kg feed DM (experimental periods 2,
38 3, 4, and 5, respectively). Cows received the control diet (basal TMR plus placebo premix)
39 during experimental period 1. A premix containing ground corn grain, soybean oil, and molasses
40 was used to incorporate 3-NOP in the ration. Cows were fed twice daily: 60% of the daily feed
41 allowance at 0800 h and 40% at 1800 h. Feed offered and refused was recorded at each feeding.
42 During the morning feedings, each cow was offered either control or 3-NOP-treated TMR at
43 150% of her average intake during the previous 3 d. After collection of the evening refusals,
44 cows received only the basal TMR without the premix until the next morning feeding. The test
45 period for the short-term DMI data collection was defined from morning feeding to afternoon
46 refusals collection during each day of each experimental period. Location (left or right) of the
47 control and 3-NOP diets within a feed bin was switched every day during each period to avoid
48 feed location bias. Dry matter intake of TMR during the test period was quadratically increased
49 by 3-NOP, compared with the control. Inclusion of 3-NOP at 120 mg/kg feed DM resulted in
50 decreased 10-h DMI, compared with the lower 3-NOP doses, but was similar to the control.
51 There was no effect of feed location (left or right) within feed bin on DMI. Data from this short-
52 term study suggest that 3-NOP does not have a negative effect on DMI in lactating dairy cows.

53 **Keywords:** 3-nitrooxypropanol, short-term dry matter intake, dairy cattle

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Short Communication

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57 The enteric methane inhibitor under investigation 3-nitrooxypropanol (**3-NOP**) was
58 developed by Duval and Kindermann (2012). Data from studies with beef cattle indicated
59 decreased DMI when 3-NOP was included at 53, 161, and 345 mg/kg feed DM (Romero-Pérez
60 et al., 2014) and at 100 and 200 mg/kg feed DM (Vyas et al., 2016). In beef animals that had not
61 been previously exposed to 3-NOP, Lee et al. (2020) observed less preference for a diet with 3-
62 NOP compared to a diet without 3-NOP when offered a choice. These authors also observed that
63 within 7 d, the animals were accustomed to the 3-NOP diet. Overall, long-term studies with 3-
64 NOP have shown no negative effects of the inhibitor on DMI and lactation performance in dairy
65 cows (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2019). Melgar et al.
66 (2020a), however, observed approximately 5% decrease in DMI of early-lactation cows
67 receiving 60 mg 3-NOP/kg feed DM (although there was no effect on DMI when expressed on a
68 BW basis). The decreased DMI in that study did not affect milk or energy-corrected milk yields,
69 but the 3-NOP cows appeared to gain less BW than the control cows. Although this may not be a
70 significant concern in practical dairy farming as cows will recover body condition in late
71 lactation and the dry period, it is important to understand if 3-NOP does affect DMI or if the data
72 by Melgar et al. (2020a) were an artifact of the experimental design in that study. Feed intake in
73 dairy cows can be affected by multiple factors (Allen, 1996, 2000), with palatability being one of
74 them (Baumont, 1996). Anecdotal observations by researchers and barn staff involved in
75 experiments with 3-NOP conducted at the Pennsylvania State University suggested that TMR

76 containing 3-NOP may have had a distinct odor, which could potentially affect DMI (Goatcher
77 and Church, 1970; Albright, 1993). According to Goatcher and Church (1970), cows use their
78 senses to discriminate between feeds. Along with taste and chemosensory irritation, odor is one
79 important chemical factor that can determine palatability and affect appetite (Baumont, 1996).
80 With dairy cows, odoriferous compounds increased grass silage intake by about 8% over an 8-
81 wk period (Weller and Phipps, 1989). However, Dohi et al. (1991) reported that odor from cattle
82 feces deterred cows from consuming the feed. Moreover, Spence (2015) suggested that the sense
83 of smell (or olfaction) contributes most of the information to the chemical fragment of the feed
84 palatability complex. Therefore, the objective of the current experiment was to investigate if
85 organoleptic characteristics (smell/odor or taste) of a TMR containing 3-NOP would have any
86 adverse effect on short-term DMI in lactating dairy cows. We hypothesized that, when offered
87 simultaneously, short-term DMI would be similar between TMR with and without 3-NOP.

88 Animals involved in the experiment were cared for according to the guidelines of The
89 Pennsylvania State University Institutional Animal Care and Use Committee. The committee
90 reviewed and approved the experiment and all procedures involving animals. The study was
91 conducted with 12 multiparous Holstein cows, averaging (\pm SD) 74 ± 22 DIM, 53 ± 12 kg milk
92 yield, and 630 ± 146 kg BW, at The Pennsylvania State University's Dairy Teaching and
93 Research Center's tie-stall barn (University Park, PA). Feed bins were split in half by a solid
94 divider and, following a 1-wk adaptation to the barn environment, cows received a control diet
95 (without 3-NOP) on both sides of the feed bin for 6 d (period 1). Starting with period 2, all 12
96 cows simultaneously received 2 diets, one without 3-NOP (control) and another with 3-NOP
97 included at 30 (period 2), 60 (period 3), 90 (period 4), or 120 (period 5) mg/kg feed DM basis
98 (treatments 30NOP, 60NOP, 90NOP, and 120NOP, respectively). Each treatment was offered

99 sequentially to all cows for 6 d; thus, the experiment consisted of 5, 6-d experimental periods.

100 The 3-NOP inclusion rates were based on previous long-term experiments conducted in our
101 laboratory (40 to 80 mg 3-NOP/kg feed DM; Hristov et al., 2015; Melgar et al., 2020a) and
102 higher rates (90 and 120 mg/kg DM) were included to exaggerate the effect of treatment on the
103 organoleptic characteristics of the TMR as related to short-term DMI. Location (left or right) of
104 the control and 3-NOP TMR within a feed bin were identified with a color band (i.e., yellow for
105 control and blue for 3-NOP TMR) and sides were switched daily before the morning feeding to
106 avoid feed location bias; thus, each treatment TMR was located at each side of the feed bin for a
107 total of 3 d during each experimental period. The basal TMR contained (% DM basis): corn
108 silage, 39; alfalfa haylage, 11; grass hay, 4; corn grain ground, 12; corn grain cracked, 2;
109 soybean seed roasted, 8; canola meal, 7; candy by-product meal, 7; whole cottonseed, 3;
110 molasses, 5; and a mineral-vitamin premix, 2. Chemical composition of the diet was (% DM
111 basis or as indicated): CP, 16.5; NDF, 29.4; ADF, 19.1; NE_L, 1.76 Mcal/kg; NFC, 48; ash, 6.7;
112 Ca, 0.82; and P, 0.44. Supplementation of 3-NOP to the basal TMR was through a premix
113 containing (% DM basis): ground corn grain, 60; soybean oil, 5; dry molasses, 15; and an active
114 or placebo supplement, 20 (both from DSM Nutritional Products, Basel, Switzerland). The active
115 supplement contained 10.9% 3-NOP on SiO₂ and propylene glycol and the placebo supplement
116 contained SiO₂ and propylene glycol only. Both control and 3-NOP premixes were prepared and
117 properly labeled the day before the start of each experimental period, kept at 4°C in sealed
118 containers with no headspace, and were mixed with the basal TMR every morning replacing an
119 equivalent amount of TMR. The inclusion rate of the premix was adjusted according to the
120 targeted 3-NOP concentration for each experimental period and DM of the basal TMR. Cows
121 were fed ad libitum twice daily (at approximately 0800 and 1800 h) and had free access to

122 drinking water. The basal TMR was prepared using a stationary mixer (Electra-Mix, model 1062,
123 I. H Rissler; Mohnton, PA) and separate mixers (Rissler Mobile TMR Mixer Model 1050; I. H.
124 Rissler) were used to mix the control and 3-NOP TMR. The daily DM allowance was split at
125 60%, fed at the morning feeding, and 40% fed at the evening feeding. Feed offered and refused
126 was recorded at each feeding. During the morning feedings, each cow was offered either control
127 or 3-NOP TMR at 150% of her normal intake during the previous 3 d. One third of each TMR
128 (i.e., control and 3-NOP) allocated for the morning feeding was stored in 20-kg plastic containers
129 and fed around 1300 h due to limited space in the feed bins. The plastic containers were color-
130 coded to match the color on the feed bin side assigned to the each TMR for that day. The basal
131 TMR without the premix was stored in one Rissler mixer until fed after collection of the
132 afternoon feeding refusals. **Cows received only the basal TMR (i.e., without the 3-NOP or
133 placebo premixes) until the next morning feeding and feed was pushed up to the cows 4 to 6
134 times daily. The intention of this interrupted offering of 3-NOP TMR was to avoid adaptation
135 and thus being able to evaluate the effect of short-term exposure to 3-NOP-supplemented feed on
136 DMI.**

137 The amount of feed offered and refused was weighed individually and recorded for each
138 cow, from each location side (left or right of the feed bin), at the morning and evening feeding to
139 measure daily as-fed intake during the entire experiment. The test period for short-term DMI
140 data was defined from 0800 to 1800 h (i.e., from morning feeding to afternoon refusals
141 collection) during each day of each experimental period. Samples of the TMR and refusals were
142 collected every 3 d and stored at -20°C. Dry matter content of the TMR and refusals was
143 determined by drying at 55°C for 72 h in a forced-air oven and used to calculate DMI from the
144 as-fed TMR intake data. Samples of the feed ingredients were collected throughout the

145 experiment, composited, dried as for the TMR samples, and ground in a Wiley Mill (Thomas
146 Scientific; Swedesboro, NJ) through a 1-mm sieve. Samples were submitted to Cumberland
147 Valley Analytical Services (Waynesboro, PA) for chemical composition analyses. Analyzed
148 chemical composition of the individual feed ingredients and their inclusion rate in the TMR were
149 used to calculate chemical composition of the basal TMR.

150 Cows were milked twice daily at 0600 and 1800 h and milk production was recorded at
151 each milking. Milk samples were collected during day 4 (p.m. milking) and day 5 (a.m. milking)
152 of the adaptation and experimental periods. An aliquot of each milk sample was placed in tubes
153 with a preservative (2-bromo-2-nitropropane-1, 3-diol) and submitted to Dairy One Cooperative,
154 Inc. (Ithaca, NY) for analysis of milk fat, true protein, and lactose using Milkoscan models 6000,
155 FT+ (Foss Electric A/S, Hillerød, Denmark). Milk from 3-NOP fed cows was discarded for the
156 duration of the study and for an additional 7-d after the study was completed.

157 All data were analyzed using SAS, version 9.4 (SAS Institute Inc., Cary, NC). Data were
158 tested for normality using the UNIVARIATE procedure. Short-term (10-h) DMI data were
159 analyzed using the MIXED procedure. A total of 576 observations were used in the 10-h DMI
160 analysis [12 cows \times 4 periods (period 1 data were not included in the analysis) \times 6 days \times 2 sides
161 of the feed bin]. The model included treatment (control and 3-NOP), side (left and right side of
162 the feed bin), and treatment \times side interaction. Overall, 24-h DMI was analyzed with treatment,
163 day of TMR offering, and the interaction treatment \times day of TMR offering. A total of 360
164 observations were used in the 24-h DMI analysis [12 cows \times 5 periods (period 1 data were
165 included in the analysis) \times 6 days]. In both models, cow was random effect and all others were
166 fixed. Data were also analyzed using orthogonal and polynomial contrasts to evaluate 3-NOP
167 treatments vs. control and linear and quadratic effects of 3-NOP inclusion rate. Data are

168 presented as least squares means. Statistical differences were considered significant at $P \leq 0.05$
169 and a trend toward significance at $0.05 < P \leq 0.10$. Descriptive statistics for the production data
170 (milk yield and milk composition) were computed using the MEANS procedure.

171 Due to the nature of the experimental design of the study (short-term and partial treatment
172 of the TMR offered to the cows), milk yield and milk composition data are not reported in tables
173 and are presented here only as a reference. Average milk yield was 50.9, 51.0, 48.6, 49.6, and
174 49.9 kg/d (SEM = 2.91 kg/d) for control, 30NOP, 60NOP, 90NOP, and 120NOP, respectively.
175 During the experiment, milk fat, milk true protein, and lactose averaged (\pm SEM) $3.55 \pm 0.154\%$,
176 $2.78 \pm 0.035\%$, and $4.86 \pm 0.033\%$, respectively.

177 Samples of TMR were analyzed for 3-NOP concentration by DSM Nutritional Products
178 (Global R&D Analytics, Kaiseraugst, Switzerland). Analyzed concentrations of 3-NOP in the
179 TMR were 0, 30.6, 60.0, 92.8, and 120.5 mg/kg feed DM for control, 30NOP, 60NOP, 90NOP,
180 and 120NOP, respectively. Relative SD was below 3.1% for each 3-NOP level set of samples.

181 Table 1 contains both 10-h test period and overall 24-h DMI data. During the 10-h test
182 period, DMI was increased ($P < 0.001$; quadratic effect) by 3-NOP. Compared with control, 3-
183 NOP increased ($P < 0.001$) 10-h DMI by 26, 27, and 35% (30, 60 and 90 mg 3-NOP/kg feed
184 DM, respectively). The 10-h test period DMI for the highest dose of 3-NOP was not different (P
185 = 0.35; not shown in Table 1) from the control. Overall, 24-h DMI, which included 3-
186 NOP/control TMR followed by basal TMR offerings, was not affected ($P = 0.33$) by 3-NOP
187 compared with the control. Rate of 3-NOP inclusion had no effect ($P \geq 0.14$) on 24-h DMI of the
188 cows. Both effect of day (of treatment) and treatment \times day interaction were significant ($P <$
189 0.001 ; Table 1, footnote 4) for 24-h DMI. Plotting the 24-h DMI data showed variability in the
190 day-to-day DMI, but no visible trends over the course of treatment (6 d). The interaction

191 treatment \times day appeared to be caused by differences in DMI among treatments during the first 3
192 d of the experimental periods, similar DMI on d 4, and lower DMI for 30NOP on d 5 (data not
193 shown). Kim et al. (2019) fed 100 mg 3-NOP/kg feed DM with a high-forage or a high-grain
194 diets and observed no effect of 3-NOP on DMI in beef cattle. **These authors reported no effect of**
195 **3-NOP on feeding behavior or feed sorting in both experiments (i.e., high-forage or high-grain**
196 **diets).** Supplementation with 3-NOP at 100 mg/kg feed DM in a short-term eating preference
197 study in beef cattle fed high-forage or high-grain diets showed no effect of 3-NOP
198 supplementation on DMI (Lee et al., 2020). Similarly, a short-term dose-response study by
199 Melgar et al. (2020b) with mid-lactation dairy cows, suggested that inclusion of 3-NOP up to
200 200 mg/kg feed DM, administered via the TMR, had no effect on DMI, when compared with the
201 control; however a linear tendency for decreased DMI was observed with increasing 3-NOP
202 dose. Vyas et al. (2018) also reported that increased inclusion rate of 3-NOP decreased DMI by 7
203 and 5% during the backgrounding and finishing phase in beef cattle, respectively. **A recent 3-**
204 **NOP meta-analysis of beef and dairy data (Kim et al., 2020) also reported an overall decrease in**
205 **DMI with 3-NOP.**

206 **As indicated earlier, the basis for the current study was: (1) clearly distinct odor of the 3-**
207 **NOP TMR, compared with the control TMR, sensed by project staff in several experiments**
208 **conducted at our dairy facility, and (2) the lower absolute DMI by 3-NOP cows reported in**
209 **Melgar et al. (2020a). Dry matter intake in dairy cows is affected by several important factors,**
210 **including gut/rumen fill and chemostatic mechanisms (Allen, 2000). In the current preference**
211 **study, however, we focused on the short-term effects of 3-NOP on DMI. Clearly, gut fill did not**
212 **play a role in the current experiment as cows were fed the same basal diet and there are no data**
213 **indicating that feed passage rate is directly affected by 3-NOP. We also believe chemostatic**

214 regulation of DMI was unlikely due to the short-term nature of our experiment. In addition, we
215 have not seen an increase in the absolute concentration of propionate in ruminal fluid of cows
216 receiving 3-NOP (Lopes et al., 2016; Melgar et al., 2020a), although molar proportion of
217 propionate may increase (Lopes et al., 2016; Kim et al., 2019 - beef steers fed a high-forage
218 diet). It has to be also pointed out that in the current experiment: (1) cows always had access to
219 and a choice between 3-NOP and control TMR during the 10-h test period, and (2) 3-NOP and
220 control TMR were offered for 10 h only and then cows were offered the control TMR for the
221 remaining 14 h of the feeding cycle. Thus, it appears that the lack of effect of 3-NOP on short-
222 term DMI in the current experiment was most likely a result of lack of effect of the compound on
223 the organoleptic properties of the feed. Kim et al. (2019) arrived at a similar conclusion in beef
224 cattle. Although cows in the current experiment had access to 3-NOP TMR for only 10 h/d, the
225 possibility of carry-over effects of residual sensations from previous sensory experiences
226 (Lawless and Heymann, 1998) on short-term DMI cannot be eliminated. Our assumption
227 however, is that these potential carry-over effects were minimized due to the longer (14 h)
228 exposure to untreated, control TMR. In sensory evaluation studies, techniques such as washout
229 periods and using of palate cleansers have been proposed to minimize carry-over effects during
230 tasting (Johnson and Vickers, 2004).

231 Digestible feed energy not converted into enteric methane, due to inhibition of
232 methanogenesis as the case with 3-NOP, has the potential to increase energy availability for
233 productive purposes. Milk production or BW gain responses to 3-NOP, however, have been
234 inconsistent (see discussion in Melgar et al., 2020a). More recently, we performed a meta-
235 analysis of long-term 3-NOP studies conducted at The Pennsylvania State University and
236 reported an overall, moderate increase in milk fat concentration with 3-NOP compared to

237 untreated control diets (Melgar et al., 2019b). If confirmed, this response can be a stimulus for
238 dairy producers to adopt the use of 3-NOP in their operations once it becomes available

239 Overall, there was no effect ($P = 0.51$) of feed location side on the 10-h test period DMI
240 in the current study (Table 1, footnote 4). There was, however, treatment \times feed location side
241 interaction ($P = 0.002$) for 10-h DMI. Analysis of this interaction (Figure 1) indicated that during
242 the 30NOP treatment period, cows had higher ($P < 0.001$) 10-h DMI from the right side than the
243 left side of the feed bin. After this initial 3-NOP inclusion period, no feed location side effect on
244 DMI was observed, suggesting that the interaction may have been a result of an initial
245 adjustment of the cows to the experimental setup.

246 Data from this short-term study suggest that 3-NOP does not have a negative effect on
247 TMR DMI, which was our original concern based on anecdotal observations by research staff
248 involved in 3-NOP studies conducted at The Pennsylvania State University. In fact, we observed
249 a quadratic increase in the 10-h TMR DMI with 3-NOP vs. the control. Long-term studies have
250 reported no effect or decreased DMI by lower doses of 3-NOP in dairy cattle (Hristov et al.,
251 2015; Melgar et al., 2019; Van Wesemael et al., 2019; 3-NOP included up to 80 mg/kg DM).
252 The current data suggest that within the maximum effectiveness range of 3-NOP inclusion (up to
253 100 mg/kg feed DM; Melgar et al., 2020b), organoleptic properties are not likely to affect short-
254 term DMI of a diet containing 3-NOP in lactating dairy cows. It has to be noted that our data
255 pertain to the effect of 3-NOP only and not the supplement used to deliver 3-NOP (which also
256 contains SiO₂ and propylene glycol). Propylene glycol, for example, may not be palatable to
257 dairy cows and may decrease DMI (Nielsen and Ingvarsten, 2004). There are some indications,
258 however, that SiO₂ may increase feed intake in some livestock species (Martel-Kennes et al.,
259 2016; Ikusika et al., 2019).

260

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391 Table 1. Dry matter intake during 10-h test period and overall, 24-h period of a total mixed ration
 392 309 containing increasing levels of 3-nitrooxypropanol (3-NOP) in dairy cows

Item ¹	Treatment ²					SEM ³	P value ⁴		
	Control	30NOP	60NOP	90NOP	120NOP		Overall	C vs. Trt	L
10-h DMI	6.90	8.72	8.78	9.31	7.23	0.370	<0.001	0.14	<0.001
24-h DMI	26.7	26.9	25.6	27.4	25.7	0.94	<0.001	0.33	0.62

393 ¹Feed with or without 3-NOP was offered during a 10-h test period, after which only feed without 3-NOP or placebo supplements (i.e.,
 394 basal TMR) was offered to the cows for the remaining 14 h

395 ²Treatments were control (no 3-NOP) and 3-NOP included at (mg/kg feed DM): 30, 60, 90, and 120 (30NOP, 60NOP, 90NOP, and
 396 120NOP, respectively). Cows received the control diet on both sides of the feed bin in period 1; starting with period 2, cows
 397 simultaneously received 2 diets, one without 3-NOP (control) and 30NOP (period 2), 60NOP (period 3), 90NOP (period 4), or
 398 120NOP (period 5). Measured concentration of 3-NOP in TMR were 0, 30.6, 60.0, 92.8, and 120.5 mg/kg feed DM, for each dose
 399 level, respectively. Data are presented as LSM.

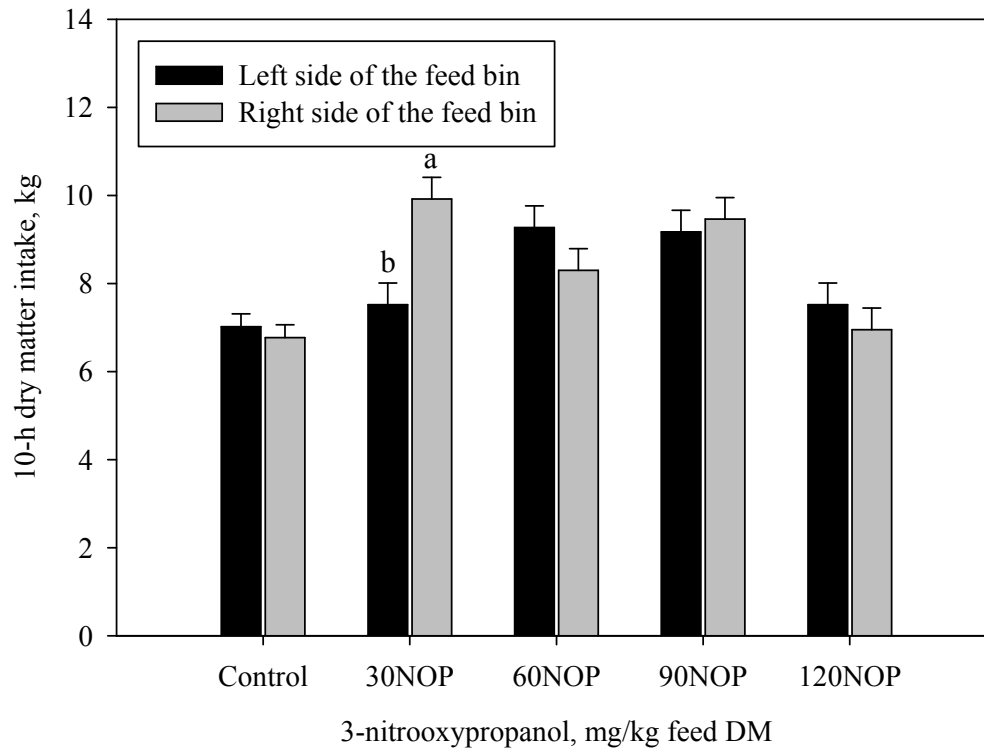
400 ³Largest SEM published in table; n = 576 for 10-h DMI and n = 360 for 24-h DMI (n represents number of observations used in the
 401 statistical analysis).

402 ⁴P-values for the overall treatment effect and contrasts (C vs. Trt, control vs. all 3-NOP treatments; L, linear effect of 3-NOP dose; Q,
 403 quadratic effect of 3-NOP dose). For 10-h DMI: effect of feed location side (left or right), $P = 0.51$; treatment \times feed location side
 404 interaction, $P = 0.002$. For 24-h DMI: Effect of day, $P < 0.001$; treatment \times day interaction, $P < 0.001$.

405 Figure 1. Treatment by feed location side (left or right location in the feed bin) interaction for dry
406 matter intake during a 10-h test period of a total mix ration containing 3-nitrooxypropanol (3-
407 NOP) in dairy cows. Treatments were control, and 3-NOP (mg/kg feed DM): 30, 60, 90, and 120
408 for 30NOP, 60NOP, 90NOP, and 120NOP, respectively. Data are presented as least square
409 means and bars represent SEM; $n = 72$ (number of independent data points for each mean value).
410 Overall, treatment \times feed location side interaction, $P = 0.002$. Means with different letters (a,b)
411 within feed location side differ at $P < 0.05$.

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412 Figure 1. Melgar et al., 2020



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