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Intravenous Glucagon Like Peptide-1 Infusion Does Not Affect Dry Matter Intake or Hypothalamic mRNA Expression of Neuropeptide Y, Agouti Related Peptide and Proopiomelanocortin in Wethers.

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Complete List of Authors:	Relling, Alejandro; CONICET, IGEVET CCT la Plata; Veterinary College, Animal Sciences Loerch, Steve; The Ohio State University, Reynolds, Christopher; University of Reading, School of Agriculture, Policy and Development
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Manuscripts

1   **Intravenous Glucagon Like Peptide-1 Infusion Does Not Affect Dry Matter Intake or**

2   **Hypothalamic mRNA Expression of Neuropeptide Y, Agouti Related Peptide and**

3   **Proopiomelanocortin in Wethers.**

4   Alejandro E. Relling[1,2,†], Steven C. Loerch[2], Christopher K. Reynolds[3]

5   [1] Ohio State University Interdisciplinary Nutrition Program (OSUN), and

6   [2] Department of Animal Sciences, The Ohio State University, OARDC, 1680 Madison Ave.,

7   Wooster 44691-4096

8   [3] School of Agriculture, Policy and Development, University of Reading, Earley Gate, Reading

9   RG6 6AR, United Kingdom.

10   Correspondence: Alejandro Relling, arelling@fcv.unlp.edu.ar

11   [†] Current address: IGEVET, CCT-La Plata, CONICET; Laboratorio de Nutrición Animal,

12   Facultad de Ciencias Veterinarias. Universidad Nacional de La Plata. Calle 60 y 118, CP

13   B1900AVW. La Plata, Buenos Aires, Argentina.

14

15   **ABSTRACT**

16   The objectives of the present study were to determine effects of jugular vein infusions of

17   glucagon like peptide-1 (GLP-1) and dietary fat inclusion on dry matter intake, nutrient

18   digestibility and hypothalamic mRNA concentration of neuropeptide Y, agouti related peptide,

19   and proopiomelanocortin in growing sheep. Thirty six wethers were used. Treatments were a

20   control diet (n = 11), dietary addition (6 % of dry matter) of Ca salts of palm oil fatty acids (n =

21   12), or 6-d jugular vein infusions of 0.155 µg/kg body weight/day of GLP-1 (n = 11). Hormone

22   concentrations were measured in jugular vein plasma from samples taken on d 1, 4 and 6. On d

23   7, the wethers were slaughtered for hypothalamus collection to measure mRNA concentration.

24   The dietary addition of 6 % of Ca salts of palm oil increased plasma GLP-1 concentration ($P <$

25   0.01) and decreased dry matter intake on d1, but not on d6 (time x treatment interaction, $P <$

26   0.05). The infusion of GLP-1 did not change dry matter intake ($P > 0.20$), but increased neutral

27   detergent fiber digestibility ($P < 0.01$). In conclusion, glucagon like peptide-1 infusion or feeding

28   fat did not decrease dry matter intake or affect hypothalamic neuropeptide mRNA concentrations

29   of sheep.

30   **RÉSUMÉ**

31   Les objectifs de cette étude étaient d'évaluer l'effet des infusions dans la veine jugulaire

32   du glucagon-like peptide-1 (GLP-1) et de l'addition alimentaire de matières grasses sur

33   l'ingestion de matière sèche (IMS), la digestibilité des nutriments et la concentration de l'ARNm

34   dans l'hypothalamus du neuropeptide Y (NPY), de la protéine agoutie (AgRP), et de la pro-

35   opiomélanocortine (POMC). Trente-six béliers ont été utilisés. Les traitements ont été un régime

36   témoin (n 11), addition alimentaire (6% de la MS) de sels de Ca d'acides palmitiques (n12), ou 6

37   jours (j) d'infusion dans la veine jugulaire de 0.155 µg/kg PC/j de GLP-1 (n 11). Les

38   concentrations d'hormones ont été mesurées dans le plasma de la veine jugulaire des échantillons

39   prélevés le jour 1, 4 et 6. Le jour 7, les béliers ont été abattus pour la collecte de l'hypothalamus

40   pour mesurer la concentration de l'ARNm de NPY, AgRP et POMC. L'ajout de 6% de sels de Ca

41   d'acides palmitiques a augmenté la concentration plasmatique de GLP-1 (P <0,01) et diminué

42   l'lMS du j 1, mais pas du j 6 (interaction de temps x traitement, P <0,05). L'infusion de GLP-1

43   n'a pas changé l'IMS (P> 0,20), mais a augmenté la digestibilité des fibres au détergent neutre (P

44   <0,01). Il n'y avait aucune différence dans la concentration de l'ARNm de NPY, AgRP ou

45   POMC en raison de l'infusion de GLP-1 ou de l'addition alimentaire de matières grasses. En

46   conclusion, la seule perfusion intraveineuse de GLP-1 n'a pas diminué l'IMS chez les ovins en

47   croissance.

48   Mots-clés: glucagon-like peptide-1, ingestion de matière sèche, mouton, neuropeptides

49   hypothalamiques

50

51   *Running head:* Relling et al*.* Glucagon like peptide 1 infusion in sheep

52   Keywords*:* glucagon-like peptide-1, dry matter intake, sheep, hypothalamic neuropeptides

53

54   **Abreviations: AgRP**, agouti-related peptide; CP, crude protein; **DM**, dry matter; **DMI**, dry

55   matter intake**; FA,** fatty acids; **GLP-1,** glucagon-like peptide-1(7, 36) amide; **ICV,**

56   intracerebroventricular; NDF, neutral detergent fiber; **NPY**, neuropeptide Y; **OM**, organic

57   matter; **POMC**, proopiomelanocortin.

58

59   **INTRODUCTION**

60   In nonruminants, increasing plasma glucagon-like peptide-1(7, 36) amide (GLP-1)

61   concentration decreases feed intake (Turton et al., 1996). In ruminants, an increase in plasma

62   GLP-1 concentration has been associated with a decrease in dry matter intake (DMI) when fat

63   was added to the diet (Relling and Reynolds, 2007; Bradford et al., 2008). Also, intrajugular

64   infusion of GLP-1 in wethers decreased DMI in a similar manner as feeding fat (Relling et al.,

65   2011). However, the central mechanism of how GLP-1 regulates feed intake is not certain. In

66   fasted rats, intracerebroventricular (ICV) infusion of GLP-1 did not change mRNA concentration

67   for neuropeptide Y (NPY) (Turton et al., 1996). In contrast, Seo et al. (2008) reported that ICV

68 infusion of GLP-1 decreased NPY and agouti-related peptide (AgRP) and increased

69 proopiomelanocortin (POMC) mRNA concentration in the hypothalamus of fasted rats. In

70 ruminants, *in vitro* culture of sheep hypothalamus in media containing GLP-1 did not change the

71 relative concentration of NPY, AgRP or POMC mRNA (Relling et al., 2012). However, an

72 increase in NPY and AgRP was associated with an increase in plasma GLP-1 concentration and

73 a decrease in DMI when fat was fed to growing lambs (Relling et al., 2010). There is a paucity of

74 information on the effect of intravenous infusion of GLP-1 on the mRNA concentration for

75 hypothalamic neuropeptides associated with DMI regulation. Therefore the objective of our

76 study was to determine the effect of jugular vein infusion of GLP-1 on DMI and mRNA

77 concentration of the neuropeptides NPY, AgRP and POMC in growing wethers.

78

79   **MATERIALS AND METHODS**

80   Animal care followed guidelines recommended in the *Guide for the Care and Use of*

81 *Agricultural Animals in Agricultural Research and Teaching* (FASS, 1998) and procedures used

82 were approved by the Animal Care Committee of the Ohio Agricultural Research and

83 Development Center.

84   Three weeks before the start of the experiment, 36 Targhee x Hampshire wethers (40.7 ±

85 3.3 kg BW) were fed a pelleted control diet (Table 1) formulated to meet nutrient requirements

86 of growing lambs according to the National Research Council (NRC, 1985). The wethers were

87 grouped by weight and housed in three pens with 12 wethers each. Daily rations were provided

88 at 0800 h, and wethers were fed for ad libitum intake of dry matter (DM, 10 % refusal)

89 throughout the study. Treatments were 7 d of: 1) control diet (CONT); 2) supplemental dietary

90 fat (Ca-salts of palm oil) at 6% of ration DM (FAT); 3) control diet with intravenous GLP-1

91   (GLP-1; 0.155 µg/kg BW/d of GLP-1(7-36) amide H6795, Bachem California Inc, CA) in 1 L of

92   saline (0.9% NaCl). The wethers on the CONT and FAT treatments received a control

93   intravenous infusion of 1 L of sterile saline solution daily. The hormone solutions and the saline

94   solutions were made and infused as described in Relling et al., (2011). The amount of GLP-1

95   infused was estimated to increase plasma concentration to a level similar to that observed when

96   supplemental fat is fed (Relling et al., 2011).

97   The experiment was conducted as a completely randomized block design. Each of the

98   three groups of wethers was considered as a block. Within each block, the 12 wethers were

99   allocated randomly to one of the three treatments (n = 4/treatment). Two weeks before the

100   experiment began the wethers were housed in individual pens. Wethers fed supplemented fat

101   were adapted to fat supplementation for 2 wk before sampling began, with an amount equal to 2

102   % of ration DM fed on d 1 of the adaptation period, 4 % on d 2 and 3, and 6 % from d 4

103   onwards. One week before the experiment started, the lambs were moved into metabolic crates

104   described previously (Murphy et al., 1994) and adapted to procedures used during the sampling

105   week, including feeding, removal of orts, and changing of fecal collection containers. Forty five

106   hours before the experiment started, jugular vein catheters were established as described

107   previously (Relling et al., 2011). Two animals experienced a drop in DMI to less than 50 % of

108   the previous day's intake when lambs were moved into the metabolic crates. Therefore, before

109   the infusions started, one wether on the control treatment and one wether on the GLP-1 treatment

110   were removed from the experiment. The infusions started at 1000 h on d 1 of the experimental

111   period. The bottles with sterile saline solution and those with hormones in solution were kept on

112   ice during the infusion. The infusion line from the bottle to the animal was sterilized using an

113   ethylene oxide (EtO) gas (Cole-Parmer, Vernon Hills, IL). The connection between the bottle

114   with the infusion and the infusion line included a sterile 0.45 μm syringe filter (Whatman

115   International Ltd, Florham Park, NJ).

116   Between the end of each day's infusion and the start of the following day, the infusion

117   lines were flushed with sterile saline solution (9 g/L of NaCl) and the filters were changed. Feed

118   was offered daily at 1300 h and the refusals were removed and weighed 23 h later at 1200 h. For

119   digestibility and plasma samples, samples were collected and processed as described previously

120   (Relling et al., 2011). Briefly, to measure digestibility, total fecal collection was performed daily

121   during the last 5 d of each experimental period. Five percent of the total daily feces was collected

122   and composited for analysis of DM, neutral detergent fiber (NDF), crude protein (CP), fatty

123   acids (FA), and ash concentration as described previously (Beckman and Weiss, 2005). Blood

124   samples (10 ml) were taken 6 and 8 h after feed was offered on d 1, 4, and 6 of each

125   experimental period. Blood samples were immediately transferred into polypropylene tubes

126   containing solutions of disodium EDTA and benzamidine HCl (1.6 mg and 4.7 mg/ml blood,

127   respectively) and placed on ice. After centrifugation for 25 min at 1800 x g and 4°C, plasma was

128   partitioned into individual polypropylene tubes for each analysis to be performed, flash frozen

129   using liquid N_2 within 40 min of sample collection, and stored at -80°C until analyzed. Samples

130   from the infusate were taken after the in line filters during the first sampling time on d 4 to

131   confirm that the infusate contained the correct concentration of GLP-1. Concentrations of

132   insulin and GLP-1 were measured using radioimmuno assays as described previously (Reynolds

133   et al., 1989; Benson and Reynolds 2001). The intra-assay CV averaged less than 12.5 % for

134   insulin and less than 11% for GLP-1. Minimum sensitivities (90% of zero standard binding) of

135   the insulin and GLP-1 assays were 0.0027 and 0.001 ng/tube, respectively. Plasma glucose

136   concentration was measured using a colorimetric assay (#1070 Glucose Trinder, Stanbio

137   Laboratory, Boerne, TX). Plasma NEFA concentration was measured using microtiter plates and

138   a plate reader in a two-reaction, enzyme based assay (Wako Chemicals USA, Richmond, VA) as

139   described by Johnson and Peters (1993).

140   The morning of the seventh day of infusions, the lambs were transported 165 km

141   (transport time was 100 min) to an abattoir for hypothalamus collection. It has been previously

142   reported (Relling et al., 2010) that there were no effects of the same transportation routine on the

143   mRNA concentration in the hypothalamus of similar lambs (Relling et al., 2010). The

144   hypothalamus was collected as described by Relling et al., (2010). During hypothalamus

145   collection, one sample from a lamb on the GLP-1 treatment was lost due to damage of the brain

146   caused by the captive bolt used at slaughter.

147   To measure mRNA concentration for NPY, AgRP and POMC, the protocol and primers

148   used were as described by Relling et al., (2010). Briefly, RNA was extracted with TRIzol®

149   (Invitrogen Carlsbad, CA) using procedures recommended by the manufacturer. Concentration

150   of RNA was determined by measuring absorbance at 260 nm. Reverse transcription (RT) PCR

151   was performed as described by Ndiaye et al. (2008). The relative mRNA concentration of NPY,

152   AgRP, and POMC were determined by quantitative RT PCR using the DNA Engine Monitor 2

153   (BioRad Laboratories, Hercules, CA). Primers for NPY, AgRP and POMC were validated in

154   sheep hypothalamic tissue. Oligonucleotide primers for NPY, AgRP and POMC were obtained

155   from Qiagen Operon Biotechnologies (Alameda, CA). The primer sequences used are described

156   on Table 2. The quantitative RT PCR was run for a maximum of 35 cycles, under the following

157   conditions: denaturing at 94° C for 30 s, annealing at 60° C for 60 s, and extension at 72° C for

158   60 s. Concentrations of NPY, AgRP and POMC were normalized to cyclophilin B mRNA

159   expression in the same sample to determine the relative mRNA concentrations of NPY, AgRP,

160   and POMC. Homologous standard curves were prepared from purified NPY, AgRP, and POMC

161   cDNA PCR products were used to calculate the steady-state concentration of NPY, AgRP, and

162   POMC mRNA in triplicate wells for each sample. The PCR amplification products were

163   electrophoretically separated on 1.5 % agarose gels and visualized with ethidium bromide. For

164   initial validation, the specific band corresponding to the size of the expected NPY, AgRP, and

165   POMC cDNA fragment was cut and purified using the QIAquick Gel Extraction Kit (Qiagen

166   Sciences) for sequence confirmation.

167   The data were statistically analyzed as a complete randomized block design with repeated

168   measurements in time using the MIXED procedure of SAS (Version 9.1, SAS Institute, Cary,

169   NC) and a model testing the random effects of wether and block, and the fixed effect of

170   treatment and time and their interaction. For digestibility and mRNA concentration data, a

171   similar statistical model was used without the effect of time. The two daily plasma samples for

172   hormones and metabolites from the three days of sampling in each experimental period were

173   analyzed in the lab individually but the average for each day was used in the statistical analysis.

174   Fisher's protected LSD test was used to compare treatments at a P value of 0.10.

175

176   **RESULTS**

177   There was a time by treatment interaction for DMI ($P < 0.05$; Figure 1), due to a greater

178   DMI for GLP-1 and control-fed wethers compared with the fat-fed wethers on d 1, but no

179   difference in DMI on d 6. Metabolizable energy intake and digestibility of DM, CP, FA and

180   organic matter (OM) was not different among the treatments ($P > 0.15$; Table 3). The addition of

181   dietary fat decreased ($P < 0.05$) and there was a trend for GLP-1 infusion to increase ($P < 0.10$)

182   NDF digestibility compared with control fed wethers. Feeding fat or GLP-1 infusion did not

183   change plasma concentrations of insulin and glucose ($P > 0.30$; Table 4) compared with the

184   control wethers. Compared with control wethers, plasma GLP-1 and NEFA concentrations

185   increased due to additional dietary fat ($P < 0.05$), but were not affected ($P > 0.10$) by GLP-1

186   infusion. Hypothalamic mRNA concentrations of NPY, AgRP and POMC were not affected by

187   treatments ($P > 0.25$; Table 5).

<div align="center">**DISCUSSION**</div>

189   In the present study there was no overall effect of treatments on DMI, although similar

190   amounts of fat or GLP-1 infusion tended to decrease DMI in sheep in previous studies (Reynolds

191   et al., 2006; Relling et al., 2010; Relling et al., 2011). As observed in the present study, the

192   inclusion of 6 % fat in the diet numerically decreased NDF digestibility in sheep (Relling et al.,

193   2011). Harvatine and Allen (2006) reported that the inclusion of fat in the diet decreased ruminal

194   digestibility, but not total tract digestibility of NDF. A possible reason for the decrease in NDF

195   digestibility observed in the present experiment could be due to an increased rate of passage, as

196   observed in sheep fed a similar fat supplemented diet (Relling et al., 2011); however, rate of

197   passage was not measured in the current experiment. The infusion of GLP-1 tended to increase

198   NDF digestibility compared with control-fed wethers. Our assumption was that an increase in

199   NDF digestibility with GLP-1 infusion would be due to a decrease in gut motility and increased

200   retention time of nutrients in the rumen and/or hindgut, allowing more time for NDF

201   fermentation by gut microbes. Results of the present study could be explained by an increase in

202   digesta retention time, but in a previous study (Relling et al., 2011) GLP-1 infusion at the same

203   rate had no effect on rate of passage or NDF digestibility. In addition, the effect of fat on NDF

204   digestibility observed in the present study was opposite to the effect of GLP-1 infusion, but

205   feeding fat increased plasma concentration of GLP-1. These observations suggest that the effects

206   of fat on NDF digestibility were not due to the increase in GLP-1 concentration observed.

207   Feeding fat increased plasma GLP-1 concentration, but the infusion of GLP-1 did not

208   change plasma GLP-1 concentration compared with control-fed wethers. The lack of response of

209   plasma GLP-1 concentration to GLP-1 infusion could be due to a decrease in endogenous

210   secretion into blood, an increased clearance rate, or both. This lack of response of plasma GLP-1

211   concentration was unexpected and may in part explain the lack of effects of GLP-1 infusion on

212   DMI. However, this does not explain the observed increase in NDF digestibility during GLP-1

213   infusion. As has been observed previously, feeding supplemental fat increased plasma NEFA

214   concentration (Gagliostro and Chilliard, 1991; Relling and Reynolds, 2007), perhaps due to a

215   higher plasma concentration of lipoproteins (Gagliostro and Chilliard, 1991).

216   In the present study, there were no differences in hypothalamic mRNA concentrations for

217   the neuropeptides NPY, AgRP and POMC due to feeding fat or GLP-1 infusion. It is been

218   observed that feeding the same amount of supplemental fat increases NPY and AgRP (Relling et

219   al., 2011) in growing wethers. The reason for the lack of response of mRNA concentrations for

220   hypothalamic neuropeptides to supplemental fat in the present experiment is not certain. In the

221   case of the GLP-1 infusion treatment, it may have been due to the inability to achieve a sustained

222   increase in plasma concentrations with the dose infused. However, the lack of response on

223   hypothalamic neuropeptide mRNA concentration is also reflected by the lack of differences on

224   DMI observed on day 6

225   In conclusion, a 7 day intravenous infusion of GLP-1 did not decrease DMI or affect

226   hypothalamic neuropeptide mRNA concentrations in growing lambs, however, elevated plasma

227   concentration of GLP-1 was not achieved. These results suggest that the reduced DMI often

228   observed in ruminants fed supplemental fat is not attributable solely to increased plasma

229   concentrations of GLP-1.

230

231   **ACKNOWLEDGMENTS**

237

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295   Table 1

296   Formulation and chemical composition of the control diet and fat supplemented diets

	Diet (% of DM)	
	Control and	
Item	GLP-1[z]	Fat
Ingredients		
Alfalfa meal, 17% CP	20.00	20.00
Soy hulls	20.00	20.00
Ground corn	48.59	43.90
Ca salts of palm oil[y]	-	6.00
Soybean meal, 48% CP	8.00	8.09
Urea	0.50	0.60
Limestone	1.50	-
Monosodium phosphate	0.05	0.05
Trace mineral salts	0.50	0.50
Vitamin A (30,000 IU/g)	0.01	0.01
Vitamin D (3,000 IU/g)	0.01	0.01
Vitamin E (44 IU/g)	0.05	0.05
Selenium (200 mg/g)	0.09	0.09
Animal-vegetable fat	0.30	0.30
Ammonium chloride	0.40	0.40

Chemical composition

NDF	28.01	24.68
CP	14.96	15.75
Ash	5.92	5.16
Total fatty acids	2.88	7.25

297

298   [z] Intravenous GLP-1infused

299   [y] Megalac®, Church and Dwight Co., Inc., Princeton, NJ.

300 Table 2. Primer sequences used for the reverse transcriptase quantitative PCR

301

Item	Forward Sequence, 5' to 3'	Reverse Sequence, 5' to 3'
NPY[z]	tcagcgctgcgacactacat	gcagagactggagagcaagt
AgRP[z]	cctgaggaagccttattcct	caggattcatgcagccttac
POMC[z]	agtgtcaggacctcaccacg	gctgctgctaccattccga

302 [z] NPY = Neuropeptide Y; AgRP = Aguti-related peptide; POMC = Proopiomelanocortin.

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316   Table 3

317   Dry matter intake (DMI), metabolizable energy intake (MEI) and digestibility of diet

318   components in growing wethers fed a control diet, the control diet plus 6 % Ca salts of palm oil

319   (6% Fat), or infused intravenously with 0.155 µg/kg BW/day of GLP-1.

320

	Treatments				
Item	Control	6% Fat	GLP-1	S.E.	P
Lambs per treatment	11	12	11		
DMI (kg/d)	1.33	1.29	1.35	0.07	0.83
MEI (Mcal/d)	3.44	3.73	3.58	0.19	0.68
Digestibility (%)					
Dry matter	69.51	69.26	71.51	0.96	0.21
Organic matter	53.34	50.79	49.74	1.45	0.22
Neutral detergent fiber	48.38	40.31[*]	52.39[†]	1.64	0.01
Crude protein	65.44	67.76	67.30	1.00	0.24
Fatty acids	82.09	85.70	80.02	2.00	0.17

321

322   [*] Differs from control, $P < 0.05$.

323   [†] Differs from control, $P < 0.10$.

324

325 Table 4

326 Plasma hormone and metabolite concentration in growing wethers fed a control diet, the control

327 diet plus 6 % Ca salts of palm oil (6% Fat), or infused intravenously with 0.155 µg/kg BW/day

328 of GLP-1.

329

	Treatments				
Item	Control	6% Fat	GLP-1	S.E.	*P*
Insulin (pM)	312	270	270	23	0.34
GLP-1[z] (pM)	23	34[*]	25	2	0.01
Glucose (mM)	3.63	3.55	3.57	0.09	0.73
NEFA[z] (mM)	49.54	77.54[*]	58.58	8.81	0.08

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332 [z] GLP-1= glucagon-like peptide-1 (7-36) amide; NEFA= non esterified fatty acid.

333 [*] Differs from control, $P < 0.05$.

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336   Table 5

337   Hypothalamic concentrations of mRNA in growing wethers fed a control diet, the control diet

338   plus 6 % Ca salts of palm oil (6% Fat), or infused intravenously with 0.155 µg/kg BW/day of

339   GLP-1.

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Item^z	Treatments			S.E.	*P*
	Control	6% Fat	GLP-1		
NPY	0.786	0.216	0.137	0.33	0.37
AgRP	0.200	0.0314	0.0458	0.09	0.40
POMC	0.311	0.168	0.0843	0.09	0.25

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343   ^{z} Concentrations of mRNA (relative to cyclophilin B) for neuropeptide Y (NPY), agouti related

344   peptide (AgRP), and proopiomelanocortin (POMC).
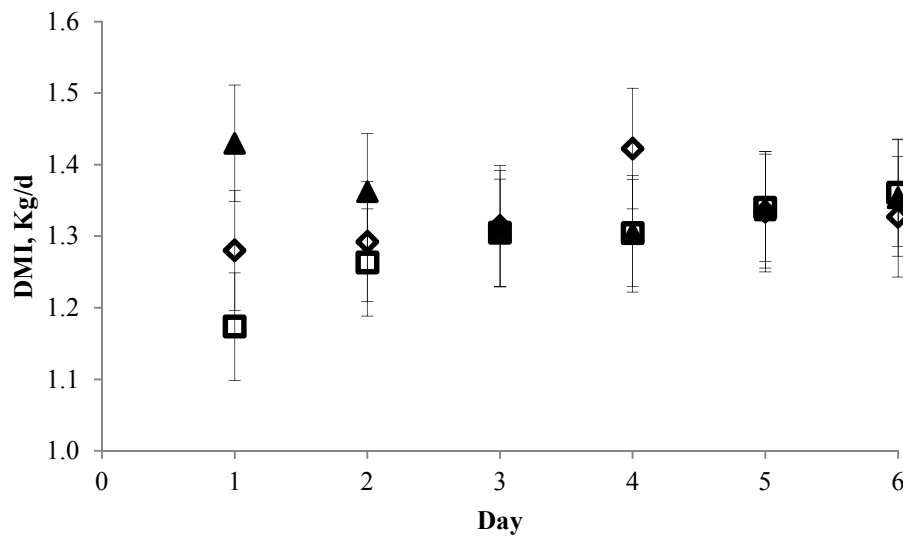
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350   Figure 1

351   Dry matter intake (DMI) over 6 days in wethers fed a diet without supplemental fat (◊) a diet

352   with the addition of 6 % Ca salts of palm oil (□) or the control diet and infused with 0.155 µg/kg

353   BW/day of GLP-1 (7-36) amide (▲). Time by treatment interaction ($P < 0.05$).