Effect of feed restriction and supplemental dietary fat on gut peptide and hypothalamic neuropeptide messenger ribonucleic acid concentrations in growing wethers

A. E. Relling, J. L. Pate, C. K. Reynolds and S. C. Loerch

J Anim Sci 2010.88:737-748.
doi: 10.2527/jas.2009-2316 originally published online Nov 6, 2009;

Effect of feed restriction and supplemental dietary fat on gut peptide and hypothalamic neuropeptide messenger ribonucleic acid concentrations in growing wethers[1]

A. E. Relling,*†[2] J. L. Pate,‡ C. K. Reynolds,§ and S. C. Loerch†[3]

*The Ohio State University Interdisciplinary Nutrition Program (OSUN), and †Department of Animal Sciences, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster 44691-4096; ‡Department of Dairy and Animal Science, The Pennsylvania State University, University Park 16802; and §School of Agriculture, Policy and Development, University of Reading, Earley Gate, Reading RG6 6AR, United Kingdom

ABSTRACT: The objectives of the present study were 1) to evaluate the effects of supplemental fat and ME intake on plasma concentrations of glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide, ghrelin, and oxyntomodulin; and 2) to determine the association of these peptides with DMI and the hypothalamic concentration of mRNA for the following neuropeptides: neuropeptide Y (NPY), agouti-related peptide (AgRP), and proopiomelanocortin (POMC). In a completely randomized block design with a 2 × 2 factorial arrangement of treatments, 32 pens with 2 wethers each were restricted-fed (2.45 Mcal/lamb per day) or offered diets ad libitum (n = 16) with or without 6% supplemental fat (n = 16) for a period of 30 d. Dry matter intake was measured daily. On d 8, 15, 22, and 29, BW was measured before feeding, and 6 h after feeding, blood samples were collected for plasma measurement of insulin, GLP-1, CCK, ghrelin, glucose-dependent insulinotropic polypeptide, oxyntomodulin, glucose, and NEFA concentrations. On d 29, blood was collected 30 min before feeding for the same hormone and metabolite analyses. At the end of the experiment, wethers were slaughtered and the hypothalami were collected to measure concentrations of NPY, AgRP, and POMC mRNA. Offering feed ad libitum (resulting in greater ME intake) increased plasma insulin and NEFA concentrations ($P = 0.02$ and 0.02, respectively) and decreased hypothalamic mRNA expression of NPY and AgRP ($P = 0.07$ and 0.02, respectively) compared with the restricted-fed wethers. There was a trend for the addition of dietary fat to decrease DMI ($P = 0.12$). Addition of dietary fat decreased insulin and glucose concentrations ($P < 0.05$ and 0.01, respectively) and tended to increase hypothalamic mRNA concentrations for NPY and AgRP ($P = 0.07$ and 0.11, respectively). Plasma GLP-1 and CCK concentrations increased in wethers offered feed ad libitum compared with restricted-fed wethers, but the response was greater when wethers were offered feed ad libitum and had supplemental fat in the diet (fat × intake interaction, $P = 0.04$). The prefeeding plasma ghrelin concentration was greater in restricted-fed wethers compared with those offered feed ad libitum, but the concentrations were similar 6 h after feeding (intake × time interaction, $P < 0.01$). Supplemental dietary fat did not affect ($P = 0.22$) plasma ghrelin concentration. We conclude that insulin, ghrelin, CCK, and GLP-1 may regulate DMI in sheep by regulating the hypothalamic gene expression of NPY, AgRP, and POMC.

Key words: agouti-related peptide, gut peptide, insulin, intake, neuropeptide Y, sheep

J. Anim. Sci. 2010. 88:737–748
doi:10.2527/jas.2009-2316

[1]We are grateful to D. Clevenger, P. Tirabasso, and G. Lowe (Animal Sciences Department, The Ohio State University, Wooster) for their assistance with animal care and feeding, to H. Zerby, D. O'Diam, and graduate students working in The Ohio State University abattoir (Wooster), and to K. Lee (Animal Sciences Department, The Ohio State University) and K. Ndiaye (Department of Anatomy and Physiology, Kansas State University) for their help in the validation of the PCR work. Supported in part by a grant from the Ohio Agricultural Research and Development Center Research Enhancement Competitive Grants Program. Salaries provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

[2]Current address: National Research Council (CONICET), Departamento de Producción Animal, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina.

[3]Corresponding author: loerch.1@osu.edu

Received July 20, 2009.
Accepted October 16, 2009.

INTRODUCTION

The gut peptides glucagon-like peptide-1(7,36) amide (**GLP-1**), oxyntomodulin (**OXM**), and cholecystokinin-8 (**CCK**) can reduce, and ghrelin can increase DMI in nonruminants (Dakin et al., 2004; Gale et al., 2004). In ruminants, supplemental dietary fat (Relling and Reynolds, 2007a; Bradford et al., 2008) increased plasma concentrations of GLP-1 and CCK and decreased prefeeding concentration of ghrelin; these changes were associated with a decrease in DMI in lactating dairy cows. Plasma glucose-dependent insulinotropic polypeptide (**GIP**) concentration increased with supplemental dietary fat (Relling and Reynolds, 2007a), but not when fat was postruminally infused (Relling and Reynolds, 2008), and increased with an increase in ME intake (**MEI**) when starch and casein were postruminally infused. In all these studies, the effect of supplemental dietary fat on gut peptides was confounded with the amount of DMI because all animals were offered feed ad libitum. Therefore, it is not known if the gut peptides acted as mediators of the decrease in DMI observed, or if the concentration increase was simply a response to the presence of extra dietary fat. Hypothalamic mRNA concentration of neuropeptide Y (**NPY**) and agouti-related peptide (**AgRP**) increased in fasted sheep (Adam et al., 2002). These neuropeptides are potent stimulators of DMI (Valassi et al., 2008). However, fasting had no effect on hypothalamic mRNA for proopiomelanocortin (**POMC**), a peptide that decreases DMI. The effect of amount of feed intake and its interaction with supplemental dietary fat on GLP-1, CCK, OXM, ghrelin, and GIP has not been reported in ruminants. In addition, the association of plasma concentrations of gut hormones with concentrations of hypothalamic neuropeptides that regulate DMI has not been reported for ruminants. The objectives of the present study were to determine the effect of supplemental dietary fat and amounts of DMI and MEI on plasma concentrations of GLP-1, CCK, GIP, ghrelin, and OXM in wethers and their associated effect on hypothalamic concentrations of the neuropeptides NPY, AgRP, and POMC.

MATERIALS AND METHODS

All animal procedures were approved by the Agricultural Animal Care and Use Committee of The Ohio State University and followed guidelines recommended in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1998).

Animals and Diets

Sixty-four Targhee × Hampshire wethers (initial average BW = 36.8 ± 2.3 kg) were used. The wethers were divided into 2 groups based on initial BW. Each group was used in 2 consecutive periods, with the group of larger lambs being used in the first period. In each period, 32 wethers were housed in 16 pens with 2 wethers per pen. Pens were 1.5 × 4.9 m and were on an expanded metal floor in a barn that was not temperature controlled. Wethers within each period were blocked again by BW into a heavy and a light group. Wethers were fed a pelleted mixed diet containing 40% forage and 60% concentrate (Table 1). The diet was formulated to meet nutrient requirements for finishing wethers according to the NRC (1985). The diet was provided once daily at 0800 h. Daily DMI was measured based on DM content of the mixed diet and daily feed refusals. Metabolizable energy intake was estimated daily based on the estimated ME concentration of the feed (NRC, 1985, 2001) and DMI.

Table 1. Formulation and chemical composition (%, DM basis) of the control diet and diet supplemented with 6% Ca salts of palm oil[1]

	Diet[2]	
Item	Control	Palm oil
Ingredient		
Alfalfa meal, 17% CP	20.00	20.00
Soy hulls	20.00	20.00
Ground corn	48.59	43.90
Megalac	—	6.00
Soybean meal	8.00	8.09
Urea	0.50	0.60
Limestone	1.50	—
Monosodium phosphate	0.05	0.05
Trace mineral salt	0.50	0.50
Vitamin A, 30 kIU/g	0.01	0.01
Vitamin D, 3 kIU/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		
CP	13.88	14.65
NDF	28.01	24.68
Ash	0.13	0.10
Total fatty acids	3.31	7.26

[1]Ca salts of palm oil (Megalac, Church and Dwight Co. Inc., Princeton, NJ).
[2]Control = control diet; palm oil = control diet with the addition of 6% of Ca salts of palm oil.

Treatments

A completely randomized block design with a 2 × 2 factorial arrangement of treatments was used. The treatments were the amount of feed offered (intake effect; restricted vs. ad libitum DMI) and supplemental dietary fat [fat effect; control diet vs. 6% of Ca salts of palm oil on a DM basis (palm oil diet)] as main effects during the 30-d treatment periods. The amount of feed provided for the restricted-fed control wethers was 2.5% of the pen average BW measured the day before the experiment started, and wethers were offered the same amount of feed daily until the end of the experi-

Table 2. Primer sequences used for quantitative reverse transcription PCR

Item[1]	Forward sequence, 5′ to 3′	Reverse sequence, 5′ to 3′
NPY	tcagcgctgcgacactacat	gcagagactggagagcaagt
AgRP	cctgaggaagccttattcct	caggattcatgcagccttac
POMC	agtgtcaggacctcaccacg	gctgctgctaccattccga

[1]NPY = neuropeptide Y; AgRP = agouti-related peptide; POMC = proopiomelanocortin.

ment. This intake level was set to ensure that wethers were in positive energy balance but that they would have less MEI than those offered feed ad libitum. For the wethers restricted-fed palm oil, the amount of DM fed was isocaloric on an estimated ME basis with the restricted-fed control wethers; therefore, they received 2.265% of BW. For the wethers offered feed ad libitum (ad libitum-fed), the diet was provided at 110% of the anticipated ad libitum intake. The amount of fat fed for the palm oil treatment was selected because a similar amount of supplemented fat has resulted in reduced DMI in sheep (Appeddu et al., 2004; Reynolds et al., 2006) and increased plasma concentrations of gut peptides in dairy cattle (Relling and Reynolds, 2007a). The control diet was fed for ad libitum intake for 15 d before the beginning of treatments to all the wethers in the study. The restriction in feed for the restricted group was initiated on d 1 of the experiment. The supplemental fat was added to the diet incrementally, with an amount equal to 2% of dietary DM fed on d 1 of the treatment period, 4% on d 2, and the total 6% on d 4. On sampling days, feed delivery was staggered at 3-min intervals per pen to allow blood sampling to be at the same relative times postfeeding.

Sampling

Feed refusals were collected and weighed daily at 0700 h. Body weight was measured on d 1, 8, 15, 22, and 29 at 0730 h. Feed samples were taken weekly and pooled for each period and analyzed for DM (100°C), ash (600°C), Kjeldahl N, NDF, and total fatty acids (Table 2), as described by Beckman and Weiss (2005). On d 8, 15, 22, and 29, feed in the bunks was weighed 6 h after feeding to determine the amount of DMI from feeding to the time blood was collected.

Blood samples (10 mL) were collected from a jugular vein on d 8, 15, 22, and 29, beginning 6 h after feeding, and on d 29 at 0730 h, after the wethers were weighed and 30 min before they were fed. Blood samples were immediately transferred to tubes containing solutions of disodium EDTA and benzamidine hydrochloride (1.6 and 4.7 mg/mL of blood, respectively) and placed on ice. After centrifugation for 25 min (1,800 × *g* at 4°C), plasma was aliquoted into individual polypropylene tubes for each hormone analysis to be performed, flash-frozen using liquid N_2 within 60 min after collection, and stored at −80°C until analyzed. For CCK analysis, plasma from both wethers in each pen was composited in a tube containing 500 Kallikrein-inhibitor units of dried aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) per milliliter of plasma added (Benson and Reynolds, 2001) and flash-frozen using liquid N_2 after each plasma addition.

For measurement of the mRNA for the neuropeptides NPY, AgRP, and POMC, the wethers were slaughtered by captive bolt and exsanguination beginning at 0600 h. For removal of the hypothalamus for the first group (block), wethers in each pen were randomly slaughtered in 1 of 2 facilities on d 30 and 31. On d 30, lambs were slaughtered in proximity to where they were housed. On d 31, the remaining wether in each pen was slaughtered in the Animal Sciences Meat Laboratory (The Ohio State University, Columbus), approximately 165 km from where it was housed. Because no differences in gene expression were due to the travel distance before slaughter, for the second period, wethers were all slaughtered on d 30 in the Animal Sciences Meat Laboratory.

The hypothalamus was removed after slaughter, as reported previously (Glass et al., 1984). In brief, the frontal landmark for the hypothalamus was the optic chiasm. Caudal to the optic chiasm was the third ventricle. The first incision was a 1.2-cm lateral-lateral cut behind the optic chiasm. Two frontal-caudal cuts of 1.5 cm were made parallel to the third ventricle. The fourth cut closed that rectangular area. A final cut was made at 0.6 cm in depth to provide a tissue sample as a cube of 1.2 × 1.5 × 0.6 cm. The hypothalamic samples were flash-frozen in liquid N_2 within 10 min after slaughter.

Hormone and Metabolite Analysis

Concentrations of insulin, GLP-1, and CCK were measured using RIA, as described previously (Benson and Reynolds, 2001), and were validated via parallel displacement of labeled hormone binding by serial dilution of ovine plasma samples. Plasma GIP concentration was measured as described by Relling and Reynolds (2007b), using a different primary antibody (G-27-07, Phoenix Pharmaceuticals Inc., Belmont, CA), and was validated by displacement of labeled human GIP (T-027-02, Phoenix Pharmaceuticals Inc.) binding by serial additions of ovine plasma, which was parallel to the displacement by serial additions of human GIP standards (Sigma-Aldrich, St. Louis, MO). Plasma OXM concentration was measured in a pooled sample of plasma from both wethers in each pen taken 6 h after feeding on d 29. The assay used a commercial kit (Oxyntomodulin RIA Kit RKU-028-22, Phoenix Pharmaceuticals Inc.)

and was validated as described by Relling (2009) based on displacement of OXM binding caused by incremental additions of sheep plasma compared with an OXM standard curve. The average recovery of human OXM in sheep plasma was 105 ± 8%. Plasma ghrelin concentration was measured using an octanolyated ghrelin kit (Active Ghrelin Kit GHRA-88HK, Linco Research, St. Charles, MO), which also was validated by measuring parallel displacement of labeled ghrelin binding caused by serial addition of sheep plasma compared with the ghrelin standard curve. Immediately after thawing, the plasma samples (500 μL) used for ghrelin analysis were acidified with 25 μL of 1 *M* HCl and 5 μL of phenylmethylsulfonyl fluoride (10 mg/mL), as recommended in the kit protocol to decrease the breakdown of active ghrelin. Sample analysis was performed as described by Bradford et al. (2008). The intraassay CV averaged less than 7.9, 13.4, 11.0, 10.4, 10.3, and 7.2% for insulin, GLP-1, OXM, GIP, CCK, and ghrelin assays, respectively. Minimum sensitivities (90% of zero standard binding) of the insulin, GLP-1, OXM, GIP, CCK, and ghrelin assays were 0.0027, 0.001, 0.0045, 0.003, 0.0006, and 0.00022 pmol/tube, respectively. Plasma glucose concentration was measured using a colorimetric assay (1070 Glucose Trinder, Stanbio Laboratory, Boerne, TX). Plasma NEFA concentration was measured using microtiter plates and a plate reader in a 2-reaction, enzyme-based assay (Wako Chemicals USA, Richmond, VA), as described by Johnson and Peters (1993).

Hypothalamic Concentration of Neuropeptide mRNA

For RNA extraction, the TRIzol procedure (Invitrogen, Carlsbad, CA) was used. Concentration of RNA was determined by measuring absorbance at 260 nm. Reverse transcription (**RT**) PCR was performed as described in Ndiaye et al. (2008). Relative mRNA concentrations of NPY, AgRP, and POMC were determined by quantitative RT PCR using the DNA Engine Monitor 2 (Bio-Rad Laboratories, Hercules, CA). Primers for NPY, AgRP, and POMC were validated in sheep hypothalamic tissue. Oligonucleotide primers for NPY, AgRP, and POMC were obtained from Qiagen (Qiagen Operon Biotechnologies, Alameda, CA). The primer sequences used are described in Table 2. Primers were diluted to a working concentration of 15 μ*M* with nuclease-free water (Sigma-Aldrich Corp.). The quantitative RT PCR was run and validated as described previously (Ndiaye et al., 2008) for a maximum of 35 cycles under the following conditions: denaturing at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. Concentrations of NPY, AgRP, and POMC were normalized to peptidylprolyl isomerase B (cyclophilin B) mRNA expression in the same sample to determine the relative mRNA concentrations of NPY, AgRP, and POMC. Homologous standard curves prepared from purified NPY, AgRP, and POMC cDNA PCR products were used to calculate the steady-state concentrations of NPY, AgRP, and POMC mRNA in triplicate wells for each sample. The PCR amplification products were separated electrophoretically on 1.5% agarose gels and visualized with ethidium bromide. For initial validation, the specific band corresponding to the size of the expected NPY, AgRP, and POMC cDNA fragment was cut and purified using a QIAquick Gel Extraction Kit (Qiagen Sciences, Valencia, CA) for sequence confirmation. A control sample that was not reverse transcribed was used to confirm that the product obtained was not amplified from genomic DNA.

Statistical Analysis

For the overall treatment effects on postfeeding plasma insulin, GLP-1, CCK, GIP, and metabolite concentrations, BW on d 8, 15, 22, and 29, and daily DMI and MEI, pen averages were statistically analyzed as repeated measures using mixed models procedures (SAS Inst. Inc., Cary, NC), to test the random effects of pen and blocks (period and BW) and the fixed effects of fat, intake, time (day of sampling), the fat × intake interaction, and the fat × intake × time interaction. The restricted intake treatments were not included in the analysis for DMI and MEI because these were dictated by the experimental design and were not dependent variables. Because plasma OXM concentration was measured only on d 29 of the study, the repeated statement, the time effect, and its interaction were removed from the model. To account for any nonhomogeneous variance caused by the restricted intake for all variables, intake was included in the model as a group effect, and the SEM are presented independently in the tables. The covariance structures compared included unstructured, spatial power, compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, and variance components. Dependent variables were analyzed using the structure giving the best fit based on the Akaike information criterion (Littell et al., 1996). For the changes in plasma hormone concentrations over time on d 29 of sampling, the concentrations at 0730 h (prefeeding) were compared only with the concentrations 6 h after feeding (postfeeding) for that day, using the same model as for the previous analysis but with the pre- and postfeeding concentrations as repeated measurements. In all analyses, if the 3-way interaction of fat × intake × time was not significant ($P > 0.10$), it was removed from the model. For the hypothalamic mRNA concentration data, the same model was used but the repeated measurement statement was removed from the model.

The correlation of plasma GLP-1 with OXM was analyzed using correlation procedures of SAS, using the average of each pen on d 29 for both variables. With the same procedure, the correlations of plasma GLP-1 and GIP concentrations with plasma NEFA concentration were measured, using the individual animal concentrations for d 8, 15, 22, and 29 (pre- and postfeeding samples).

Table 3. Mean DMI and ME intake (MEI) of growing wethers ad libitum-fed or restricted-fed diets with or without the addition of 6% Ca salts of palm oil[1,2]

	Restricted		Ad libitum		SEM[3]		*P*-value[4]		
Item	Control	Palm oil	Control	Palm oil	Rest	Adlib	Fat	Intake	F × I
Wethers, n	16	16	16	16					
Pens,[5] n	8	8	8	8					
Daily DMI, kg	0.94	0.85	1.97	1.74	—	0.08	0.12	—	—
Daily MEI,[6] Mcal	2.46	2.46	5.20	5.01	—	0.23	0.20	—	—
DMI at sampling[6]	0.94	0.85	0.64	0.66	0.01	0.025	0.26	0.01	0.02
MEI at sampling[6]	2.46	2.46	1.67	1.91	0.03	0.179	0.02	0.01	0.05

[1]Mean represents the average of 29 d of the experiment for daily DMI and MEI, and the average for d 8, 15, 22, and 29 for DMI and MEI at bleeding.
[2]Control = control diet; palm oil = control diet with the addition of 6% of Ca salts of palm oil.
[3]Rest = restricted-fed wethers; adlib = ad libitum-fed wethers.
[4]Fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F × I = interaction of fat and intake main effects.
[5]Pen was used as the experimental unit.
[6]DMI (kg) and MEI (Mcal) at sampling (time of bleeding) is the amount that the wethers had consumed by 6 h after the feed was offered.

RESULTS

BW, DMI, and MEI

Body weight (fat × intake × time; $P < 0.01$) increased over time for the ad libitum-fed wethers, but for the restricted-fed wethers, BW decreased during the first week of the experiment and increased only slightly thereafter (Figure 1). Supplemental dietary fat did not affect BW change over time ($P = 0.60$; Figure 1). Feeding Ca salts of palm oil numerically decreased daily DMI and daily MEI of ad libitum-fed wethers, but these differences were not statistically significant ($P = 0.12$ and 0.20, respectively; Table 3). Restricting intake changed the pattern of feed consumption. At 6 h postfeeding (at the time of bleeding), DMI was greater for the restricted-fed wethers than for wethers fed ad libitum; within the restricted-fed group, DMI was greater for those fed the control diet than for those fed the palm oil diet (fat × intake, $P = 0.02$; Table 3). Metabolizable energy intake was also greater at 6 h after feeding for restricted-fed wethers compared with wethers fed ad libitum. Within the ad libitum group, wethers fed the control diet had less MEI than those fed the palm oil diet (fat × intake, $P < 0.05$; Table 3).

Overall Changes in Plasma Concentration of Hormones and Metabolites

Restricting DMI and supplementation of fat in growing wethers decreased plasma insulin concentration ($P < 0.05$ and 0.02, respectively; Table 4). A fat × intake interaction ($P < 0.05$) was detected for plasma GLP-1 and CCK concentrations (Table 4). Ad libitum-fed wethers had greater plasma GLP-1 and CCK concentrations than restricted-fed wethers. Feeding fat increased plasma GLP-1 and CCK concentrations in the ad libitum-fed wethers, but not in the restricted-fed wethers. Plasma OXM did not change ($P = 0.30$) because of the intake regimen or the addition of dietary fat (Table 4). For the first 3 wk of the experiment, plasma GIP concentration was greater in the ad libitum-fed wethers compared with those restricted-fed and those fed fat compared with those fed the control diet; however, plasma concentration of GIP was not affected by intake or fat supplementation on d 29 of the experiment (fat × intake × time interaction $P < 0.10$; Figure 2). Plasma glucose concentration was greater in wethers fed the control diet ($P < 0.01$) than in those fed the palm oil diet (Table 4). The amount of feed offered did not change plasma glucose concentration ($P = 0.21$). Plasma NEFA concentration (Table 4) increased because of addition of dietary fat ($P < 0.01$) and also because of the increase in feed offered ($P < 0.02$).

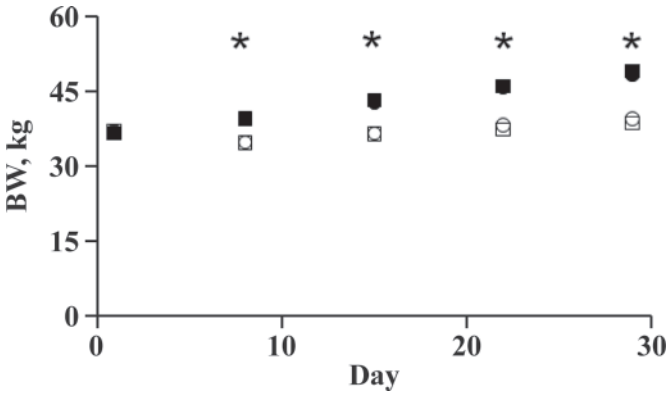

Figure 1. Average BW (kg) of growing lambs restricted-fed with (palm oil, □) or without (control, ○) the addition of 6% Ca salts of palm oil, and ad libitum-fed with (palm oil, ■) or without (control, ●) the addition of 6% Ca salts of palm oil after a 29-d feeding period. $P < 0.01$ for the intake × fat × time interaction. The SEM for restricted and ad libitum were 0.5 and 0.6 kg, respectively, and an asterisk (*) indicates $P < 0.05$ for means separated using the slice option (SAS Inst. Inc., Cary NC).

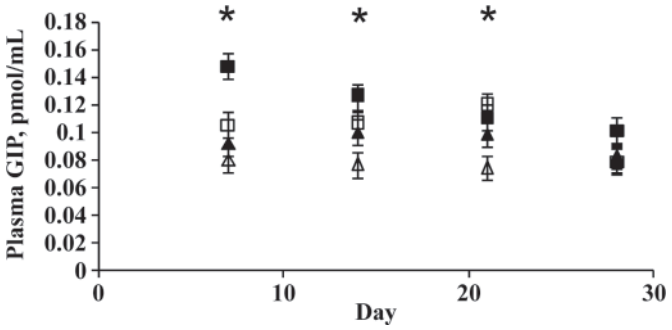

Figure 2. Plasma glucose-dependent insulinotropic polypeptide (GIP) concentration (pmol/mL) of growing lambs restricted-fed with (palm oil, □) or without (control, Δ) the addition of 6% Ca salts of palm oil, and ad libitum fed with (palm oil, ■) or without (control, ▲) the addition of 6% Ca salts of palm oil after a 29-d feeding period. The SEM for restricted and ad libitum were 0.004 and 0.005 pmol/mL, respectively, and an asterisk (*) indicates $P < 0.05$ for means separated using the slice option (SAS Inst. Inc., Cary NC).

Plasma Concentration of Hormones and Metabolites Pre- and Postfeeding

A 3-way interaction ($P = 0.03$) between intake, fat supplementation, and time relative to feeding was detected for plasma insulin concentration (Table 5). For the restricted-fed wethers, plasma insulin concentration was greater at 6 h after feeding than at prefeeding. Ad libitum-fed wethers had greater plasma insulin concentration than those with restricted intake. In addition, plasma insulin concentration did not change after feeding wethers the control diet, but was greater postfeeding for those fed the palm oil diet. Plasma GLP-1 concentration was greater for wethers prefeeding than postfeeding for both the palm oil and control diets when wethers were offered feed ad libitum and for the palm oil diet when intake was restricted, but not for the control diet when intake was restricted (fat × intake and time, $P = 0.07$). Plasma GLP-1 concentration increased in ad libitum-fed wethers compared with those with restricted intakes (Table 5). Plasma CCK concentration was greater prefeeding than postfeeding for wethers fed the palm oil and control diets ad libitum and for those restricted-fed the palm oil diet, but not for those restricted-fed the control diet (fat × intake and time; $P = 0.08$ and 0.07, respectively; Table 5). Plasma CCK concentration was greater in ad libitum-fed than in restricted-fed wethers; this response was greater when fat was added in the diet (Table 5). Plasma GIP concentration was greater ($P < 0.01$) at 6 h after feeding than at the prefeeding sampling. The addition of fat in the diet did not change plasma GIP concentration in restricted-fed wethers, but increased plasma GIP concentration in ad libitum-fed wethers (Table 5). Plasma ghrelin concentration was greater prefeeding only in the restricted-fed wethers (time × intake, $P < 0.01$). The addition of fat did not change plasma ghrelin concentration ($P > 0.20$). Plasma glucose concentration was greater in the ad libitum-fed than in the restricted-fed wethers. The postfeeding plasma glucose concentration was greater than the prefeeding concentration for wethers fed the control and palm oil diets at restricted intake and for those fed the control diet ad libitum, but not when the palm oil diet was offered ad libitum (time × intake and time × fat, $P < 0.01$; Table 5). Plasma NEFA concentration was greater in the prefeeding samples only in the restricted wethers (time × intake, $P < 0.01$). There was a positive correlation for plasma GLP-1 and GIP concentrations with plasma NEFA concentration postfeeding (r = 0.40 and 0.17, $P < 0.01$ and 0.06 for GLP-1 and GIP, respectively). However, plasma GLP-1 and GIP concentrations were negatively correlated with plasma NEFA concentration (r = −0.30 and −0.33, $P < 0.10$ and 0.06 for GLP-1 and GIP, respectively).

Table 4. Mean plasma hormone and metabolite concentrations in growing wethers ad libitum-fed or restricted-fed diets with or without the addition of 6% Ca salts of palm oil[1,2]

	Restricted		Ad libitum		SEM[4]		*P*-value[5]		
Item[3]	Control	Palm oil	Control	Palm oil	Rest	Adlib	Fat	Intake	F × I
Wethers, n	16	16	16	16					
Pens,[6] n	8	8	8	8					
Insulin, pmol/mL	0.287	0.211	0.308	0.295	0.021	0.021	0.04	0.02	0.16
GLP-1,[7] pmol/mL	0.021	0.024	0.029	0.038	0.001	0.002	0.02	0.01	0.04
OXM, pmol/mL	0.177	0.188	0.197	0.186	0.009	0.012	0.98	0.44	0.31
CCK, pM	4.94	6.07	9.14	17.38	0.47	2.18	0.01	0.01	0.04
GIP,[8] pmol/mL	0.079	0.103	0.093	0.122	0.005	0.006	0.01	0.01	0.69
Glucose, m*M*	4.33	4.06	4.35	4.17	0.056	0.056	0.01	0.22	0.51
NEFA,[7] m*M*	0.060	0.074	0.065	0.086	0.003	0.004	0.001	0.01	0.13

[1]Each mean represents blood samples taken on d 8, 15, 22, and 29 of the experiment.
[2]Control = control diet; palm oil = control diet with the addition of 6% of Ca salts of palm oil.
[3]GLP-1 = glucagon-like peptide-1; OXM = oxyntomodulin; CCK = cholecystokinin; GIP = glucose-dependent insulinotropic polypeptide.
[4]Rest = restricted-fed wethers; Adlib = ad libitum-fed wethers.
[5]Fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F × I = interaction of fat and intake main effects.
[6]Pen was used as the experimental unit.
[7]Day effect, $P < 0.05$; data not presented.
[8]Intake × fat × day, $P < 0.10$.

Table 5. Plasma hormone and metabolite concentrations in growing wethers at 30 min prefeeding and at 6 h postfeeding, after 29 d of feeding ad libitum or at a restricted intake, diets with or without the addition of 6% Ca salts of palm oil[1]

	Restricted		Ad libitum		SEM[3]		*P*-value[4]				
Item[2]	Control	Palm oil	Control	Palm oil	Rest	Adlib	Fat	Intake	F × I	Time	F × I × T
Wethers, n	16	16	16	16							
Pens,[5] n	8	8	8	8							
Insulin, pmol/mL					0.022	0.027	0.70	0.01	0.06	0.01	0.03
Prefeeding	0.137	0.121	0.284	0.300							
Postfeeding	0.245	0.190	0.268	0.359							
GLP-1, pmol/mL					0.003	0.003	0.01	0.01	0.37	0.05	0.07
Prefeeding	0.0227	0.0311	0.0354	0.0441							
Postfeeding	0.0226	0.0259	0.0304	0.042							
CCK, p*M*					1.4	3.1	0.03	0.01	0.08	0.07	0.20
Prefeeding	5.75	9.27	12.71	20.45							
Postfeeding	6.09	4.90	8.44	18.59							
GIP, pmol/mL					0.006	0.008	0.01	0.16	0.01	0.01	0.61
Prefeeding	0.044	0.047	0.068	0.087							
Postfeeding	0.082	0.079	0.081	0.10							
Ghrelin,[6] pmol/mL					0.004	0.001	0.22	0.01	0.80	0.01	0.91
Prefeeding	0.049	0.052	0.018	0.023							
Postfeeding	0.017	0.023	0.015	0.024							
Glucose,[6,7] m*M*					0.061	0.073	0.07	0.01	0.06	0.01	0.87
Prefeeding	3.73	3.68	4.14	4.31							
Postfeeding	4.24	3.88	4.37	4.20							
NEFA,[6] m*M*					0.032	0.007	0.16	0.01	0.99	0.01	0.78
Prefeeding	0.168	0.219	0.059	0.094							
Postfeeding	0.066	0.070	0.074	0.089							

[1]Control = control diet; palm oil = control diet with the addition of 6% of Ca salts of palm oil.
[2]GLP-1 = glucagon-like peptide-1; CCK = cholecystokinin; GIP = glucose-dependent insulinotropic polypeptide.
[3]Rest = restricted-fed wethers; Adlib = ad libitum-fed wethers.
[4]Fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F × I = interaction of fat and intake main effects; time = main effect of pre- and postfeeding; F × I × T = interaction of fat, intake, and time main effects.
[5]Pen was used as the experimental unit.
[6]Time × intake, $P < 0.01$.
[7]Time × fat, $P < 0.01$.

Hypothalamic mRNA Concentrations of NPY, AgRP, and POMC

Hypothalamic mRNA concentrations for NPY and AgRP were greater ($P = 0.07$ and 0.02, respectively) for restricted-fed than for ad libitum-fed wethers (Table 6). Supplemental dietary fat tended to increase NPY and AgRP mRNA concentrations ($P = 0.07$ and 0.11, respectively) compared with the control diet. The relative concentration of POMC in the hypothalamus was numerically, but not significantly ($P = 0.15$), greater for ad libitum-fed wethers compared with those fed at the restricted intake. Fat supplementation did not affect ($P = 0.60$) the relative concentration of POMC in the hypothalamus.

DISCUSSION

BW, DMI, and MEI

By design, the restricted wethers consumed less feed than those offered feed ad libitum. This resulted in a decrease in BW during the first week because of the decrease in feed provided and the potential effects of less gut fill. All lambs gained BW after d 8 of the experiment, but the rate of BW gain was greater for the ad libitum-fed wethers, as observed previously (Murphy et al., 1994). In our study, the addition of fat resulted in numerical decreases in DMI and MEI, but these decreases were not statistically significant. The addition of a similar concentration of dietary fat as a combination of vegetable and algal oils decreased DMI in previous experiments on lactating sheep (Reynolds et al., 2006), but in the present study, a rumen-inert fat was fed, which may in part explain the lack of a statistically significant effect of palm oil on DMI. The restricted-fed lambs finished consuming their ration within 1 h after feed delivery at 0800 h. As a result of this eating behavior, at the time of bleeding, the DMI and MEI were actually greater for the restricted-fed wethers compared with those fed ad libitum. This pattern of meal consumption could have influenced plasma concentrations of hormones and metabolites at 6 h postfeeding.

Overall Changes in Plasma Concentration of Hormones and Metabolites

To our knowledge, this study is the first to report the effects of intake and fat supplementation on plasma

Table 6. Relative mRNA concentration for hypothalamic neuropeptides that regulate DMI after 30 d of ad libitum or restricted feeding of diets with or without the addition of 6% Ca salts of palm oil[1]

	Restricted		Ad libitum		SEM[3]		*P*-value[4]		
Item[2]	Control	Palm oil	Control	Palm oil	Rest	Adlib	Fat	Intake	F × I
Wethers, n	16	16	16	16					
Pens,[5] n	8	8	8	8					
NPY/CYC	0.545	1.261	0.189	0.538	0.267	0.261	0.07	0.07	0.52
AgRP/CYC	0.162	0.369	0.054	0.095	0.072	0.071	0.11	0.02	0.28
POMC/CYC	0.067	0.090	0.142	0.161	0.053	0.051	0.68	0.16	0.97

[1]Control = control diet; palm oil = control diet with the addition of 6% of Ca salts of palm oil.

[2]NPY/CYC = relative concentration of neuropeptide Y mRNA using cyclophilin B as a housekeeping gene; AgRP/CYC = relative of agouti-related peptide mRNA concentration using cyclophilin B as a housekeeping gene; POMC/CYC = relative concentration of proopiomelanocortin mRNA using cyclophilin B as a housekeeping gene.

[3]Rest = restricted-fed wethers; Adlib = ad libitum-fed wethers.

[4]Fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F × I = interaction of fat and intake main effects.

[5]Pen was used as the experimental unit.

concentrations of GLP-1, GIP, CCK, OXM, and ghrelin in growing sheep. Similar effects of intake on plasma insulin concentration have been observed previously (Sano et al., 1999). In restricted-fed sheep, plasma insulin concentration decreased compared with that in ad libitum-fed sheep (Sano et al., 1999). A decrease in plasma insulin concentration has been observed with the inclusion of dietary fat in ruminant diets (Relling and Reynolds, 2007a). However, in the present experiment, fat supplementation resulted in decreased insulin for restricted-fed wethers and increased insulin for ad libitum-fed wethers. Differences in plasma insulin concentration attributable to intake and fat supplementation were greater at 6 h postfeeding than at prefeeding.

An increase in plasma GLP-1 and CCK when fat was added to the diet has been observed previously in dairy cows (Choi and Palmquist, 1996; Relling and Reynolds, 2007a). In those studies, the increases in plasma gut peptide concentrations were associated with a decrease in DMI. In the present study, there was a numerical trend for a decrease in ad libitum DMI for wethers consuming the supplemental fat diet, which could have been caused by the increases in GLP-1 and CCK observed. Gut peptides have been reported to be associated with regulation of feed intake in nonruminants (Gale et al., 2004). To our knowledge, this is the first study to measure plasma concentrations of gut peptides in sheep when intake was controlled. Plasma GLP-1 and CCK concentrations were not changed by the addition of supplemental fat in the diet when wethers were fed at a restricted intake. The interaction between dietary fat supplementation and intake could imply that CCK and GLP-1 regulate DMI in ruminants to maintain a similar MEI when they are fed ad libitum. In restricted-fed animals, the addition of fat in the diet did not change the plasma concentrations of CCK and GLP-1, even though the animals consumed almost double the amount of supplemental fat (31.1 g/d in the control diet vs. 61.7 g/d in the palm oil diet). However, when the lambs had free access to a diet with greater energy density, the plasma gut peptide concentration increased, presumably to regulate MEI to a constant amount. Therefore, the presence of fat in the diet per se did not increase plasma CCK and GLP-1 concentrations. This could be seen when we compared the fatty acid intake of the fat-supplemented wethers that were restricted-fed (61.7 g/d) with the fatty acid intake of wethers offered the control diet ad libitum (65.2 g/d), in which an increase in fatty acids of approximately 5% produced approximately 20 and 50% greater responses in plasma GLP-1 and CCK concentrations, respectively. These observations indicate that the secretion of these gut peptides, and their secretion in response to dietary fat, is modulated by intake.

Oxyntomodulin is a peptide derived from the proglucagon gene in the L cells in the small intestine (Holst, 1997). The AA sequence of OXM contains the sequence of glucagon plus 8 additional AA on the C-end of the molecule. The OXM molecule is also included in a larger protein called glicentin. Glicentin and OXM have been called "gut glucagon" because of the cross-reactivity they have with the pancreatic glucagon molecule in some assays (Manns, 1972). In the past, gut glucagon was measured as the difference between the total glucagon, which was measured with the assays that cross-react with pancreatic and the gut glucagon assay, and the one specific for pancreatic glucagon (Manns, 1972; Ghatei et al., 1983). All the molecules of the proglucagon gene, such as GLP-1, GLP-2, and OXM or glicentin, are cosecreted by the L cells (Holst, 1997). In nonruminants, it has been reported that OXM reduces food intake (Cohen et al., 2003; Dakin et al., 2004) by binding the GLP-1 receptor (Small and Bloom, 2004). In the present study with lambs, we did not observe substantial changes in plasma OXM concentrations caused by the amount of feed offered or supplemental dietary fat. However, there was a trend for plasma GLP-1 and OXM concentrations to be correlated ($r = 0.28$; $P = 0.11$). It is not known whether the L cells in

ruminants secrete OXM, glicentin, or other peptides that include glicentin. The metabolic pathway of the peptide in ruminants has not been reported. In a study with multiple-catheterized lactating dairy cows, Benson and Reynolds (2001) showed that gut glucagon was released by the liver, which could indicate that a major peptide secreted by the L cells is metabolized after its release to a peptide that cross-reacted with the total glucagon peptide.

Plasma GIP concentration was increased mainly by an increase in supplemental dietary fat. These differences were diminished by wk 4 of sampling. An increase in plasma GIP concentration was observed when fat was fed in lactating dairy cows (Relling and Reynolds, 2007a) and in preruminant goats (Martin et al., 1993a). However, oil infusion for 7 d did not increase plasma GIP concentration in lactating dairy cows (Relling and Reynolds, 2008), but plasma GIP did increase because of an increase in energy intake from abomasal infusion of casein or starch. In the present study, plasma GIP concentration in restricted-fed wethers supplemented with fat was greater than for ad libitum-fed wethers fed the control diet, even though the average total fat consumption in the latter group was greater (61.7 vs. 65.2 g, respectively). This could be due to a possible role for GIP in regulating energy redistribution, not only as influenced by the total MEI, but also as dependent on the source of that energy. This assumption has not been proven in ruminants; however, the importance of GIP in energy redistribution has been studied in mice (Miyawaki et al., 2002), in which the absence of a GIP receptor caused mice to be leaner than mice with a functional GIP receptor. In lactating dairy cows, plasma GIP concentration was negatively correlated with RQ (Relling et al., 2009), indicating that GIP could play a role in regulating energy utilization in ruminants.

Plasma glucose concentration decreased with the dietary supplementation of fat, but did not differ because of intake. A similar effect was observed previously when fat was fed to ruminants with ad libitum access to feed (Relling and Reynolds, 2007a). The lack of difference in plasma glucose attributable to intake is likely the result of insulin regulation of glucose utilization. The increase in NEFA concentration attributable to supplemental fat and intake observed at 6 h postfeeding was observed previously (Gagliostro and Chilliard, 1991; Relling and Reynolds, 2007a). Increased NEFA concentration in response to fat intake may be due to an increase in the release of NEFA from plasma lipoproteins. A similar increase in plasma NEFA concentration was observed when oil was infused in lactating dairy cows because of a greater plasma concentration of lipoproteins (Gagliostro and Chilliard, 1991). Increases in plasma insulin concentration for the control animals compared with those fed the palm oil diet could cause a decrease in lipolysis in adipose tissue. The gut peptides GLP-1 and GIP decrease lipolysis in ovine adipose tissue (Martin et al., 1993b). In the present study, we found a negative correlation between plasma concentration of GIP and NEFA (r −0.20; $P < 0.02$), but not for GLP-1 and NEFA ($P > 0.30$). The negative correlation between GIP and NEFA concentrations is possibly due to the role of GIP in decreasing lipolysis in ovine adipose tissue (Martin et al., 1993b); however, other factors may be influencing plasma NEFA concentration. As mentioned, some of the circulating plasma NEFA could be derived from a release from plasma lipoproteins (Gagliostro and Chilliard, 1991).

Plasma Concentration of Hormones and Metabolites Pre- and Postfeeding

Plasma insulin concentration was greater in the postfeeding sampling for all treatments except for lambs fed the control diet ad libitum. A postprandial increase in plasma insulin concentration was observed previously in sheep (Matsunaga et al., 1999) and may be due to an increase in the production of insulin secretagogues such as propionate. It is not clear why a postprandial response in plasma insulin concentration was not observed for the control diet fed ad libitum, but differences in the pattern of intake over the course of the day may have been responsible.

For plasma GLP-1 and CCK concentrations, based on previous studies (Relling and Reynolds, 2007a), we expected greater concentrations 6 h after feeding than before feeding. The increase in plasma concentrations of GLP-1 and CCK prefeeding compared with postfeeding in the restricted-fed wethers could have been due to their feeding behavior and rate of feed consumption. The restricted-fed wethers consumed all their daily diet within 1 h, which could have affected the flow of digesta to the small intestine. It is also possible that the daily patterns of secretion for GLP-1 and CCK are different in cattle than in sheep. This could explain why plasma concentrations of GLP-1 and CCK were greater prefeeding than after feeding. However, in 2 consecutive samplings taken at 5 and 7 h after feeding in sheep, there was not a time effect (Relling, 2009). This is similar to what was observed in dairy cattle (Relling and Reynolds, 2007a). As we had expected, plasma GIP concentration was greater after feeding than before feeding. Plasma GIP concentration was observed previously to increase as a result of an increase in nutrient absorption in sheep (McCarthy et al., 1992) and in lactating dairy cows (Relling and Reynolds, 2007a). This increase may be due to a possible role of GIP in nutrient partitioning in ruminants (Martin and Faulkner, 1994).

The intake × time interaction observed in this study for plasma ghrelin concentration was due to an increase in only preprandial ghrelin plasma concentration for the restricted-fed wethers. Plasma ghrelin concentrations were similar for pre- and postprandial sampling times in the ad libitum-fed wethers and at the postprandial sampling time for the restricted-fed wethers. As discussed, differences in eating behavior occurred

such that, at the 6 h sampling time, the DMI and MEI were actually greater for the restricted-fed wethers compared with those fed ad libitum. This pattern of meal consumption could have influenced the plasma ghrelin response. The ad libitum-fed wethers had a more continuous supply of feed and nutrients. Thus, plasma ghrelin did not differ before and after feed was offered. However, the restricted-fed wethers experienced a period of approximately 22 h without feed before the prefeeding blood sampling. Our data indicate ghrelin was serving as a signal for energy insufficiency in the restricted-fed wethers, as was reported for nonruminants (Fernandez-Fernandez et al., 2006). Our result was also similar to that observed by Wertz-Lutz et al. (2008), in which an increase in plasma ghrelin concentration was observed in beef cattle during nutrient restriction. In the restricted-fed steers in the study by Wertz-Lutz et al. (2008), the plasma ghrelin concentration was always greater than that in steers whose intake was not restricted. However, in the present study, postprandial concentrations were similar in the restricted and ad libitum-fed wethers. A possible explanation for this difference is that in the study by Wertz-Lutz et al. (2008), the animals were consuming only 80% of their nutrient requirements and they were losing BW. In the present study, the restriction was smaller and lambs were gaining BW. Ghrelin is secreted in a pulsatile fashion when energy is restricted (Bradford and Allen, 2008; Wertz-Lutz et. al., 2008). Our single blood-sampling schedule at the pre- and postfeeding time points would not allow detection of a pulsatile secretion pattern if it occurred prefeeding in the restricted-fed wethers. In ad libitum-fed dairy cows that were not in a negative energy balance, there were no differences between pre- and postprandial plasma ghrelin concentrations (Bradford and Allen, 2008). The preprandial increase in plasma ghrelin concentration in the present study could have been due to the reduced MEI imposed for the restricted-fed wethers. Preprandial plasma ghrelin may have been stimulating appetite and contributing to the aggressive eating behavior observed for the restricted-fed wethers. In the present study, the wethers finished their ration within 1 h and we did not observe a fat effect on plasma ghrelin concentration. However, in studies using dairy cattle (Bradford et al., 2008; Relling, 2009), an increase in fat intake produced a decrease in plasma ghrelin concentration compared with control cows in samples taken immediately before feeding. Unlike previous experiments (Bradford et al., 2008; Wertz-Lutz et al., 2008), we did not observe a preprandial increase in plasma ghrelin concentration in wethers offered the control diet ad libitum. The lack of ghrelin response prefeeding when wethers were offered feed ad libitum may also have been due to differences in the pattern of intake over the course of the day.

Preprandial plasma glucose concentration was less than postprandial concentration in the restricted wethers. This occurred despite greater plasma insulin postfeeding vs. prefeeding for these lambs. Part of that response may have been influenced by the pattern of meal consumption discussed above. Plasma glucose concentration was also greater postfeeding for lambs fed the control diet ad libitum. Unlike the restricted-fed lambs, this occurred when plasma insulin was less. Lambs fed the palm oil diet ad libitum had less plasma glucose postfeeding than prefeeding. These lambs also had the greatest plasma insulin concentration after feeding.

The increase in preprandial NEFA concentration in the restricted-fed lambs may have been due to an increase in fat mobilization caused by lesser plasma insulin concentrations. A prefeeding increase in NEFA concentration was not observed in lambs offered feed ad libitum. The preprandial concentration of NEFA was inversely correlated with the preprandial plasma concentrations of GIP and GLP-1. This inverse association could have been due to a function of GIP and GLP-1 in decreasing lipolysis from adipose tissue (Martin et al., 1993b). Plasma NEFA concentration followed a similar pattern of response as observed for plasma ghrelin concentration. This association was observed previously (Bradford and Allen, 2008) in early-lactation dairy cows. We cannot confirm if increases in plasma NEFA concentration are a signal to increase plasma ghrelin concentration or if they are due to the decrease in plasma insulin concentration; however, it seems likely that these mechanisms are interacting to increase appetite.

Hypothalamic mRNA Concentrations of NPY, AgRP, and POMC

In the present study, we observed a decrease in mRNA for neuropeptides that stimulate DMI (NPY and AgRP) in lambs offered feed ad libitum compared with those fed at a restricted intake. Hypothalamic POMC mRNA concentration did not change because of the amount of DMI (restricted vs. ad libitum-fed wethers). Similar results have been reported in ad libitum compared with fasted sheep (Adam et al., 2002). Despite our hypothesis that feeding fat would result in a decrease in NPY and AgRP, in the present study, we observed an increase in NPY and AgRP attributable to feeding fat. Results in rat models have shown conflicting data on the effect of fat on hypothalamic neuropeptides (Wang et al., 2002; Dziedzic et al., 2007). In Wang et al. (2002), NPY mRNA concentration decreased for rats consuming saturated fats but increased for rats consuming high-fat diets rich in n-3 or n-6 fatty acids. However, Dziedzic et al. (2007) showed a decrease in rat hypothalamic NPY mRNA concentration attributable to high-fat diets rich in saturated fat, but there were no changes for high-fat diets rich in n-3 and n-6 fatty acids compared with low-fat diets. The role of particular fatty acids in mRNA expression of hypothalamic neuropeptides has not been studied in ruminants. In the present study, the increase in mRNA concentrations for NPY and AgRP was associated with less DMI but similar MEI for wethers fed palm oil compared with wethers fed the control diet. However, the changes in hypo-

thalamic concentrations for the neuropeptides could be associated with changes in hormones and metabolites that occur in these wethers. The reduced mRNA concentrations in the ad libitum-fed lambs are associated with a greater degree of satiety for the ad libitum-fed lambs. The decrease in these neuropeptide concentrations for the restricted-fed and control diets is probably due to the interaction of many peripheral signals that are processed in the hypothalamus. The signals that may influence the decrease in mRNA concentrations for NPY and AgRP for the ad libitum-fed lambs are the increased plasma concentrations of insulin and CCK, or decreased NEFA and prefeeding ghrelin. It has been shown in rat models that central administration of insulin decreases NPY mRNA concentration (Schwartz et al., 1992). In the present study, an inverse association was observed between plasma insulin concentration and NPY mRNA concentration, not only because of differences in DMI, but also because of the additional dietary fat. It has also been found in rats that CCK decreases NPY mRNA concentration (Bi and Moran, 2002). In the current study, we showed a negative association between plasma CCK concentration and NPY and AgRP mRNA concentrations. However, plasma CCK concentration responds to the interaction of the amount of intake and the additional dietary fat. This interaction was not observed in the orexigenic neuropeptides. Therefore, we can infer that CCK is not the principal regulator of NPY and AgRP gene expression, but it is possible that CCK has a secondary role after the action of insulin. It is not known if the increase in plasma GLP-1 concentration could be responsible for the changes in NPY mRNA concentration. There were no changes in rat hypothalamic NPY mRNA concentration after intraventricular infusion of GLP-1 (Turton et al., 1996). Therefore, it is possible that GLP-1 regulates the decrease in DMI when ad libitum-fed wethers are supplemented with fat via a mechanism that does not involve NPY, AgRP, or POMC. However, these are assumptions because the direct effect of GLP-1 in sheep hypothalamus has not been investigated. Ghrelin increases NPY and AgRP mRNA concentrations (Dimaraki and Jaffe, 2006) in nonruminants. To our knowledge, this is the first study to show the association between plasma ghrelin concentration and the hypothalamic mRNA concentrations of NPY, AgRP, and POMC in growing sheep. In our study, there was a positive association between prefeeding plasma ghrelin concentration and mRNA concentrations of the orexigenic peptides. From this, it seems likely that the increase in ghrelin concentration observed prefeeding in this and other studies (Bradford and Allen, 2008; Wertz-Lutz et al., 2008) is in part responsible for an increase in appetite. It has been reported that fatty acids inhibit the expression of NPY and AgRP in nonruminants (Lam et al., 2005). In this study, we also found a positive association between NEFA and NPY and AgRP mRNA concentrations with the additional dietary fat but a negative association attributable to the amount of DMI. The increase in plasma NEFA concentration 6 h after feeding caused by ad libitum feeding could be another factor that contributes to the decrease in NPY and AgRP concentrations. However, the additional dietary fat increases plasma NEFA concentration and also produces an increase in NPY and AgRP. There is not a clear physiological explanation for the positive association between the increase in NEFA when feeding additional fat and the increase in mRNA for the orexigenic neuropeptides. However, it is possible that the changes in neuropeptides are not due to changes solely in a hormone or metabolite, but that they may reflect the interaction of those changes.

In conclusion, the numerical decrease in DMI observed when supplemental fat was provided to sheep offered feed ad libitum was associated with a downregulation of expression of the orexigenic neuropeptides. The data in this study support the assumption that the mechanisms by which an animal senses an increase in energy density in the diet and "tells" the hypothalamus to decrease DMI is probably via endocrine signals such as changes in insulin or gut peptide concentration.

LITERATURE CITED

Adam, C. L., Z. A. Archer, P. A. Findlay, L. Thomas, and M. Marie. 2002. Hypothalamic gene expression in sheep for cocaine- and amphetamine-regulated transcript, pro-opiomelanocortin, neuropeptide Y, agouti-related peptide and leptin receptor and responses to negative energy balance. Neuroendocrinology 75:250–256.

Appeddu, L. A., D. G. Ely, D. K. Aaron, W. P. Deweese, and E. Fink. 2004. Effects of supplementing with calcium salts of palm oil fatty acids or hydrogenated tallow on ewe milk production and twin lamb growth. J. Anim. Sci. 82:2780–2789.

Beckman, J. L., and W. P. Weiss. 2005. Nutrient digestibility of diets with different fiber to starch ratios when fed to lactating dairy cows. J. Dairy Sci. 88:1015–1023.

Benson, J. A., and C. K. Reynolds. 2001. Effects of abomasal infusion of long-chain fatty acids on splanchnic metabolism of pancreatic and gut hormones in lactating dairy cows. J. Dairy Sci. 84:1488–1500.

Bi, S., and T. H. Moran. 2002. Actions of CCK in the controls of food intake and body weight: Lessons from the CCK-A receptor deficient OLETF rat. Neuropeptides 36:171–181.

Bradford, B. J., and M. S. Allen. 2008. Negative energy balance increases periprandial ghrelin and growth hormone concentrations in lactating dairy cows. Domest. Anim. Endocrinol. 34:196–203.

Bradford, B. J., K. J. Harvatine, and M. S. Allen. 2008. Dietary unsaturated fatty acids increase plasma glucagon-like peptide-1 and cholecystokinin and may decrease premeal ghrelin in lactating dairy cows. J. Dairy Sci. 91:1443–1450.

Choi, B. R., and D. L. Palmquist. 1996. High fat diets increase plasma cholecystokinin and pancreatic polypeptide, and decrease plasma insulin and feed intake in lactating cows. J. Nutr. 126:2913–2919.

Cohen, M. A., S. M. Ellis, C. W. Le Roux, R. L. Batterham, A. Park, M. Patterson, G. S. Frost, M. A. Ghatei, and S. R. Bloom. 2003. Oxyntomodulin suppresses appetite and reduces food intake in humans. J. Clin. Endocrinol. Metab. 88:4696–4701.

Dakin, C. L., C. J. Small, R. L. Batterham, N. M. Neary, M. A. Cohen, M. Patterson, M. A. Ghatei, and S. R. Bloom. 2004. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. Endocrinology 145:2687–2695.

Dimaraki, E. V., and C. A. Jaffe. 2006. Role of endogenous ghrelin in growth hormone secretion, appetite regulation and metabolism. Rev. Endocr. Metab. Disord. 7:237–249.

Dziedzic, B., J. Szemraj, J. Bartkowiak, and A. Walczewska. 2007. Various dietary fats differentially change the gene expression of neuropeptides involved in body weight regulation in rats. J. Neuroendocrinol. 19:364–373.

FASS. 1998. Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Savoy, IL.

Fernandez-Fernandez, R., A. C. Martini, V. M. Navarro, J. M. Castellano, C. Dieguez, E. Aguilar, L. Pinilla, and M. Tena-Sempere. 2006. Novel signals for the integration of energy balance and reproduction. Mol. Cell. Endocrinol. 254–255:127–132.

Gagliostro, G., and Y. Chilliard. 1991. Duodenal rapeseed oil infusion in early and midlactation cows. 4. In vivo and in vitro adipose tissue lipolytic responses. J. Dairy Sci. 74:1830–1843.

Gale, S. M., V. D. Castracane, and C. S. Mantzoros. 2004. Energy homeostasis, obesity and eating disorders: Recent advances in endocrinology. J. Nutr. 34:295–298.

Ghatei, M. A., L. O. Uttenthal, N. D. Christofides, M. G. Bryant, and S. R. Bloom. 1983. Molecular forms of human enteroglucagon in tissue and plasma: Plasma responses to nutrient stimuli in health and in disorders of the upper gastrointestinal tract. J. Clin. Endocrinol. Metab. 57:488–495.

Glass, J. D., R. P. Amann, and T. M. Nett. 1984. Effects of season and sex on the distribution of cytosolic estrogen receptors within the brain and the anterior pituitary gland of sheep. Biol. Reprod. 30:894–902.

Holst, J. J. 1997. Enteroglucagon. Annu. Rev. Physiol. 59:257–271.

Johnson, M. M., and J. P. Peters. 1993. Technical note: An improved method to quantify nonesterified fatty acids in bovine plasma. J. Anim. Sci. 71:753–756.

Lam, T. K., G. J. Schwartz, and L. Rossetti. 2005. Hypothalamic sensing of fatty acids. Nat. Neurosci. 8:579–584.

Littell, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. SAS System for Mixed Models. SAS Inst. Inc., Cary, NC.

Manns, J. G. 1972. Separation of pancreatic and gut glucagon-like immunoreactivity (G.L.I.) with observations on plasma concentrations of these hormones during lactation. Can. J. Physiol. Pharmacol. 50:554–560.

Martin, P. A., and A. Faulkner. 1994. Gastric inhibitory polypeptide—Its metabolic effects in sheep. Biochem. Soc. Trans. 22:37S.

Martin, P. A., A. Faulkner, and J. P. McCarthy. 1993a. Effects of consumption of milk and milk constituents on plasma concentrations of gastric inhibitory polypeptide and metabolites in preruminant goat kids. J. Endocrinol. 138:167–173.

Martin, P. A., A. Faulkner, and G. E. Thompson. 1993b. Effects of gut hormones on ovine adipose metabolism in vivo using microdialysis. Biochem. Soc. Trans. 21:443S.

Matsunaga, N., N. T. Arakawa, T. Goka, K. T. Nam, A. Ohneda, Y. Sasaki, and K. Katoh. 1999. Effects of ruminal infusion of volatile fatty acids on plasma concentration of growth hormone and insulin in sheep. Domest. Anim. Endocrinol. 17:17–27.

McCarthy, J. P., A. Faulkner, P. A. Martin, and D. J. Flint. 1992. Changes in the plasma concentration of gastric inhibitory polypeptide and other metabolites in response to feeding in sheep. J. Endocrinol. 134:235–240.

Miyawaki, K., Y. Yamada, N. Ban, Y. Ihara, K. Tsukiyama, H. Zhou, S. Fujimoto, A. Oku, K. Tsuda, S. Toyokuni, H. Hiai, W. Mizunoya, T. Fushiki, J. J. Holst, M. Makino, A. Tashita, Y. Kobara, Y. Tsubamoto, T. Jinnouchi, T. Jomori, and Y. Seino. 2002. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nat. Med. 8:738–742.

Murphy, T. A., S. C. Loerch, K. E. McClure, and M. B. Solomon. 1994. Effects of restricted feeding on growth performance and carcass composition of lambs. J. Anim. Sci. 72:3131–3137.

Ndiaye, K., D. H. Poole, and J. L. Pate. 2008. Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. Biol. Reprod. 78:786–793.

NRC. 1985. Nutrient Requirements of Sheep. 6th ed. Natl. Acad. Press, Washington, DC.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Relling, A. E. 2009. Effect of gut peptides on hypothalamic mRNA concentrations and dry matter intake in ruminants. PhD Diss. The Ohio State Univ., Columbus.

Relling, A. E., L. A. Crompton, S. C. Loerch, and C. K. Reynolds. 2009. Plasma concentration of glucose-dependent insulinotropic polypeptide is negatively correlated with respiratory quotient in lactating dairy cows. J. Anim. Sci. 87(E. Suppl. 2):470–471.

Relling, A. E., and C. K. Reynolds. 2007a. Feeding rumen-inert fats differing in degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. J. Dairy Sci. 90:1506–1515.

Relling, A. E., and C. K. Reynolds. 2007b. Plasma concentrations of gut peptides in dairy cattle increase after calving. J. Dairy Sci. 90:325–330.

Relling, A. E., and C. K. Reynolds. 2008. Abomasal infusion of casein, starch and soybean oil differentially affect plasma concentrations of gut peptides and feed intake in lactating dairy cows. Domest. Anim. Endocrinol. 35:35–45.

Reynolds, C. K., V. L. Cannon, and S. C. Loerch. 2006. Effects of forage source and supplementation with soybean and marine algal oil on milk fatty acid composition of ewes. Anim. Feed Sci. Technol. 131:333–357.

Sano, H., A. Takebayashi, Y. Kodama, K. Nakamura, H. Ito, Y. Arino, T. Fujita, H. Takahashi, and K. Ambo. 1999. Effects of feed restriction and cold exposure on glucose metabolism in response to feeding and insulin in sheep. J. Anim. Sci. 77:2564–2573.

Schwartz, M. W., A. J. Sipols, J. L. Marks, G. Sanacora, J. D. White, A. Scheurink, S. E. Kahn, D. G. Baskin, S. C. Woods, D. P. Figlewicz, and D. Porte. 1992. Inhibition of hypothalamic neuropeptide Y gene expression by insulin. Endocrinology 130:3608–3616.

Small, C. J., and S. R. Bloom. 2004. Gut hormones and the control of appetite. Trends Endocrinol. Metab. 15:259–263.

Turton, M. D., D. O'Shea, I. Gunn, S. A. Beak, C. M. Edwards, K. Meeran, S. J. Choi, G. M. Taylor, M. M. Heath, P. D. Lambert, J. P. Wilding, D. M. Smith, M. A. Ghatei, J. Herbert, and S. R. Bloom. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69–72.

Valassi, E., M. Scacch, and F. Cavagnini. 2008. Neuroendocrine control of food intake. Nutr. Metab. Cardiovasc. Dis. 18:158–168.

Wang, H., L. H. Storlien, and X. F. Huang. 2002. Effects of dietary fat types on body fatness, leptin, and ARC leptin receptor, NPY, and AgRP mRNA expression. Am. J. Physiol. Endocrinol. Metab. 282:E1352–E1359.

Wertz-Lutz, A. E., J. A. Daniel, J. A. Clapper, A. Trenkle, and D. C. Beitz. 2008. Prolonged, moderate nutrient restriction in beef cattle results in persistently elevated circulating ghrelin concentrations. J. Anim. Sci. 86:564–575.