EFFECT OF GUT PEPTIDES ON HYPOTHALAMIC mRNA CONCENTRATION
AND DRY MATTER INTAKE IN RUMINANTS

DISSERTATION

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ABSTRACT

A series of six experiments was conducted to gain a better understanding of mechanisms involved in feed intake regulation by ruminants. Specifically, this dissertation investigated the effect of changes in concentrations of metabolites (i.e., glucose and non-esterified fatty acids (NEFA)), and hormones (i.e., insulin, glucagon like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), ghrelin, cholecystokinin (CCK) and oxyntomodulin (OXM)), on dry matter intake (DMI) and Hypothalamic mRNA concentration of neuropeptide Y (NPY), agouti-related peptide (AgRP) and proopiomelanocortin (POMC) in ruminants.

In Chapter 3, in vitro experiments were performed to determine the effect of metabolites (glucose and propionate), and the splanchnic hormones insulin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and polypeptide YY (PYY), on sheep Hypothalamic mRNA concentration for neuropeptide Y (NPY), agouti-related peptide (AgRP), and proopiomelanocortin (POMC). The incubation of sheep hypothalamus in glucose, propionate, insulin, CCK, GLP-1, or PYY did not affect the mRNA concentration for NPY, AgRP, or POMC compared with incubation of hypothalamus in the control media. Hypothalamus incubated in media containing glucose plus insulin did not result in changes in mRNA for NPY or AgRP, but increased POMC mRNA concentration.
compared with hypothalamus incubated in the control media. This implies that an interaction of factors, i.e hormones and/or metabolites is needed to change the mRNA concentration of neuropeptides that regulate DMI.

In Chapter 4, the effect of feeding fat and restricting DMI on plasma concentrations of insulin, GLP-1, GIP, CCK, ghrelin and oxyntomodulin (OXM) and the hypothalamic concentration of the neuro-peptides NPY, AgRP and POMC in growing lambs were reported. Feeding wethers ad libitum increased plasma insulin, GIP and NEFA concentration and decreased hypothalamic mRNA expression of NPY and AgRP compared with feeding wethers at a restricted intake. The addition of dietary fat decreased DMI, insulin and glucose concentration and increased plasma GIP and hypothalamic mRNA concentration for NPY and AgRP. Plasma GLP-1 and CCK concentration increased for wethers fed ad libitum compared with those restricted-fed, but for those fed ad libitum, the response was greater when they had supplemental fat in the diet. Pre-feeding plasma ghrelin concentration was higher in the restricted compared with the ad libitum-fed wethers, but the concentration was similar after feeding.

In Chapters 5 and 6, the effects of infusion of physiological doses of GLP-1 and CCK on DMI are described. In Chapter 5, infusion of GLP-1, but not CCK, had effects on DMI and initial meal size similar to effects of feeding fat. However, in Chapter 6 the results of GLP-1 infusion do not support the results of Chapter 5. Based on these and other results from these studies, it is apparent that there are many other factors, independent of GLP-1, that regulate DMI. Moreover, it is likely that effects of feeding fat on DMI are due to additive effects of multiple hormones and metabolic signals, not solely to changes in plasma GLP-1 or CCK concentration.
Chapter 7 described the validation of an OXM assay for bovine plasma, and acute (hours) and chronic (1 week) effects of increased abomasal supply of protein (casein), carbohydrate (starch), or fat (soybean oil) to the small intestine on plasma concentrations of ghrelin and OXM on days 1 and 7 of infusion. Oil and casein infusion decreased pre-feeding plasma ghrelin concentration on both days. The infusion of oil, starch or casein did not change plasma OXM concentration. The present data indicate that the decrease in plasma ghrelin concentration due to oil infusion could be associated with a decrease in DMI; however, the decrease in ghrelin concentration due to casein infusion was not associated with a decrease in DMI, and that plasma OXM concentration is not associated with DMI.

From the results in the current dissertation, it can be concluded that GLP-1 has a role in regulating DMI, but GLP-1 is not the sole factor that determines appetite. Results of these experiments suggest that the interaction among metabolic hormones and metabolites, and their combined effects, are more important for the regulation of DMI in ruminants than a single factor such as GLP1.
Dedication

To my family and friends; in particular my parents, brothers, and nieces.
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CHAPTER 1

INTRODUCTION

The understanding of the regulation of dry matter intake (DMI) is very important in ruminant nutrition, due to its importance for milk and meat production. A break point of DMI regulation was theorized by Conrad et al. (1964). In a multiple regression analysis study that considered 114 trials in lactating dairy cows, they showed that feed digestibility has an important role in determining DMI. When feed digestibility is less than 68 %, DMI is regulated directly by gut fill. On the other hand, when feed digestibility was higher than 68 %, the regulation of DMI was called “chemostatic” regulation. However, the understanding of the factors in “chemostatic” regulation was not clear. Based on non-ruminant theories of glucose as a mediator of DMI (Mayer, 1955), some studies were conducted (Manning et al., 1959) to establish the role of glucose in DMI regulation in ruminants; however, the results showed that plasma glucose concentration does not regulate DMI in ewes (Manning et al., 1959). According to Conrad (1963), chemostatic regulation is the regulation of DMI through plasma metabolites (i.e. glucose, acetic acid, propionic acid) only. However, the understanding of the biology of the regulation of DMI showed that not only the metabolites regulate DMI, but also certain hormones.
Some metabolic hormones that are known to influence DMI are insulin, leptin and gastrointestinal hormones. Gastrointestinal hormones, also called gut peptides, were first discovered in the early 1900s, with the discovery of secretin and gastrin; however, it was not until the 1960’s and 1970’s that their relationship with feed intake and metabolism was elucidated (Rehfeld, 1998). Gut peptides known to regulate feed intake in non-ruminants include ghrelin, cholecystokinin (CCK), glucagon-like peptide-1(7-36) amide (GLP-1), oxyntomodulin (OXM), and peptide tyrosine tyrosine (PYY). However, in ruminants there are few data that describe the role of these hormones in the regulation of DMI.

Some studies in cattle and sheep showed a relationship between plasma gut peptide concentration and DMI. Also there are some studies that report the effect of pharmacological doses of some of these gut peptides on DMI. However, there are no studies that report the chronic effect of physiological increases in some of these hormones on DMI. Similarly, there are no studies that have measured the effect of these gut peptides on the gene expression of hypothalamic neuropeptides that regulate feed intake in other species, such as neuropeptide Y, agouti-related peptide, and proopiomelanocortin. Therefore, the objectives of this doctoral dissertation were to determine the effect of the gut peptides on DMI, MEI, and energy partitioning in ruminants; and to associate the changes in gut peptide concentration and DMI with the mRNA concentration of neuropeptides that regulate intake.
1) Role of metabolites in DMI regulation.

Although most of the nutrients per se have the possibility to be regulators of DMI, I will base my discussion mainly on glucose, propionate and lipids.

a) Glucose

The importance of glucose in the regulation of intake in non-ruminants was stated by Mayer (1953), and he proposed the “glucostatic mechanism of regulation of food intake”. He showed that low plasma glucose was associated with appetite. Two years after that, he also explained the importance of glucose as a short term regulator of feed intake “day to day” (Mayer, 1955), but not in a long term regulation of feed intake. At the same time there was a study conducted in rats to evaluate the effect of glucose on different tissues (Forssberg and Larsson, 1954). In that study, Forssberg and Larsson (1954) using radioisotope $^{14}$C glucose showed areas receptive to glucose in the liver and in the feeding area of the hypothalamus. In both tissues the uptake of glucose increased in fasted animals. In the hypothalamus, Forssberg and Larsson (1954) reported that in the fed state there is a change in the areas that were more receptive to glucose compared with the fasted. Form these results they conclude that glucose modulates hypothalamic function in
both fasting and fed states. This was also supported by the findings of Brecher and Waxler (1949) and Marshall et al (1955), in which the absorption of goldthioglucose (GTG) by the hypothalamus produced damage of the axons that connect the ventromedial with the lateral area of the hypothalamus. The axon damage was the consequent cause of obesity in mice. This effect was not observed with other goldthio molecules (Marshall and Mayer, 1956) inferring that the damage produce by the gold was associated with the uptake of glucose by the tissue. Baile et al. (1970) showed no hypothalamic lesions in sheep or goats after carotid infusion of GTG. Even though the studies in non-ruminants showed an important role of glucose in the regulation of food intake, the data on ruminants did not support the same role. The main reason for that difference could be the lower glucose plasma concentration in ruminants compared with non-ruminants.

One of the earliest studies on glucose and DMI regulation in ruminants was conducted in goats (Manning et al. 1959). In that experiment a bolus of glucose was infused intravenously (IV). The amount of glucose infused varied from 150 to 500 g per infusion. The same doses were repeated one hour after the first bolus. In this study they report no differences in DMI due to glucose infusion; however, the experimental design was not completely clear. From that study they conclude that glucose does not decrease DMI in ruminants. However, the type of diet that those sheep were eating is not reported in the paper, and if the diet had a very low digestibility, it is possible that the mechanism that limited feed intake was gut fill and not the increase in plasma glucose. In another study conducted by Dowen and Jacobson (1960) in which Guernsey, Holstein-Friesian and Jersey twin cows where infused with different metabolites, they did not observe a decrease in DMI when glucose was infused. They infused over a period of 8 hours, 4 and
25% of the daily requirements of energy for maintenance. In this report, there was no information presented regarding statistical analysis, physiological state of the animals or the digestibility of the chopped alfalfa hay, which is important in the understanding of the mechanism of regulation of DMI.

A different approach in the understanding of glucose as a regulator of DMI in ruminants was used by Seoane and Baile (1972). In this study they infused glucose and 2 deoxy-D-glucose (2-DG – an intracellular competitive inhibitor of glucose utilization) in the third ventricle during a 5 min period. When 2-DG was infused the results were not consistent; there was a decrease in DMI for the low concentration (265 µmol) when the 2-DG was infused with distilled water, and there was no difference in DMI when the high dose (530 µmol) of 2-DG was infused with distilled water. There was an increase in DMI when 2-DG was infused with synthetic central nervous system fluid. From these results they conclude that the effect of the metabolites on the third ventricle was due to an osmotic effect more than a metabolic effect.

These results are based on the central (hypothalamic) effect of glucose as a regulator of DMI. However, the oxidation of metabolites in the liver as a regulator of DMI is also important (Friedman and Tordoff, 1986). Because of the low amount of glucose absorption and the low glucose oxidation in the liver in ruminants (Reynolds, 2002), liver oxidation of glucose may not be an important physiological mechanism for DMI regulation in ruminants. These mechanisms of regulation of DMI will be discussed in detail later, in the section of the liver as a regulator of DMI.

In some studies in ruminants where insulin metabolism was studied (McGuire et al. 1995; Mackle et al., 1999) via a hyperinsulinemic-euglycemic clamp, in which glucose was
infused in the jugular vein, a decrease in DMI was observed; however, plasma glucose concentration did not change. A deeper explanation of these studies will be explained later in the insulin section of regulation of DMI.

In some studies where propionate was intraruminally infused (Oba and Allen, 2003a b), an increase in plasma concentration of glucose was associated with a decrease in DMI. However, plasma propionate concentration also increased. These studies will be described in more detail in the section of propionate regulation of DMI.

From the above discussion, it seems that at a physiological plasma glucose concentration, glucose does not play a primary role in the regulation of DMI in ruminants. However, the data regarding the effect of glucose per se on the ruminant hypothalamus is quite equivocal.

b) Propionate

The study of propionate as a regulator of DMI was based on the concept that absorption of glucose is limited in ruminants. This is due to the fermentation of the carbohydrates that take place in the rumen. The first experiments to study propionate as a regulator of DMI were done by infusing propionate in the jugular vein (Dowden and Jacobson, 1960) or into the rumen (Baile and Mayer, 1969). In those studies, the authors observed a decrease in DMI, but they did not know the mechanism of how propionate could regulate DMI. An important point to consider is that based on the information reported in those papers, it is difficult to know how much of the energy supply came from the feed and how much came from the infusate. In a more recent study (Leuvenink et al., 1997); Na-propionate was infused for a short period of time (20 min) in the mesenteric and portal vein. In this study a decrease in DMI was observed during the infusion period for the
mesenteric artery infusion, but there was not a decrease in DMI for the portal infusion. Also, Leuvenink et al. (1997) report that the mesenteric vein infusion of Na-propionate produces an increase in plasma concentration of glucose, insulin and propionate in the jugular vein in sheep. The increase in glucose and insulin were similar, independent of the site of propionate infusion, but the concentration of propionate was lower when propionate was infused in the portal vein compared with the mesenteric vein infusion. They postulated that the lack of effect when Na-propionate was infused in the portal vein was due to a non-homogeneous distribution of the propionate through the liver. In a study conducted by Subiyatno et al. (1996), DMI did not change when propionate was infused in the jugular vein. In that study, Na-propionate was infused IV in the jugular vein during 5 minutes and at a dosage of 2.5 mmol/kg. They also reported an increase in jugular plasma concentration of insulin, glucose and propionate. These differences in results due to infusion of propionate have been explained by Allen et al. (2005). In brief, the decrease in DMI caused by propionate depends on the oxidation of this compound by the liver; the oxidation will depend on the rate of infusion and the physiological state of the animal (Allen et al., 2005). When glucose and insulin concentrations are high, propionate is oxidized in the liver, which decreases DMI by stimulating the vagus nerve. On the other hand, DMI is not influenced by propionate when there is a high demand for glucose, because propionate will follow a gluconeogenic pathway, thus decreasing liver oxidation of propionate.

Although the liver oxidation theory of regulation of DMI explains how propionate regulates DMI, there were no studies found that report the hypothalamic effect of
propionate. The main reason for this could be due to the high (up to 95%) removal of propionate by the liver (Reynolds, 2006).

c) Lipids

The dietary addition of various lipid sources is a common practice in ruminant nutrition. This practice is common in the dairy industry to increase energy density of the feed, and thereby hopefully increase energy intake. However, the addition of lipids in the diet is commonly associated with a decrease in DMI and DM digestibility. The degree of decrease in DMI and digestibility depends on the amount and degree of unsaturation of the lipid source. Although the exact mechanism by which lipids decrease DMI is not completely known, the main mechanisms suggested for why fat decreases DMI are by increasing liver fatty acid oxidation and increasing gut peptides secretion. Further, the decrease in DM digestibility may produce a decrease in DMI through an increase in gut fill (Allen, 2005).

The effect of fat supplementation and liver oxidation was well documented in non-ruminants by Langhans and Scharrer (1987). In a study in rats they showed that the infusion of 2-mercaptoacetate, a β-oxidation antagonist, stimulates feed intake in animals with an intact vagus nerve, but there was no increase in feed intake when the rats where vagotomized. These data suggest that the signal of oxidation of fatty acids in the liver is sent to the central nervous system via the vagus nerve. Due to the limited ability of the ruminant liver to oxidize fatty acids (Emery et al. 1992), the lipid liver oxidation theory has been hypothesized in ruminants (Allen et al. 2005) as a possible regulator of DMI. The mechanism of how liver oxidation of metabolites regulates DMI will be discussed in a later section of this review.
The effect of lipids on gut peptides and the regulation of DMI had been well described in non-ruminants (Walsh, 1994). Some gut peptides that decrease intake are glucagon-like peptide 1(7-36) amide (GLP-1), cholecystokinin (CCK), peptide tyrosine tyrosine (PYY), and oxyntomodulin (OXM) (Small and Bloom, 2004). There is also a gut peptide that increases intake called ghrelin (Small and Bloom, 2004). The effect of gut peptides on feed intake is via decreasing gut motility, activating the vagus nerve, and by a direct effect on the hypothalamus (Walsh, 1994). However, the data in ruminants are limited. Some of the hormones that show an association with regulation of DMI in dairy cows when fat is fed are GLP-1, CCK (Relling and Reynolds, 2006b), and ghrelin (Bradford et al., 2008). On the other hand, there is only an association of an increase in plasma GLP-1 concentration when fat is infused into the abomasum, but not for CCK (Litherland et al., 2005; Relling and Reynolds, 2006b). The effect of the different gut peptides on DMI regulation will be discussed in a later section specific for gut peptides.

2) Role of hormones in DMI regulation

The endocrine system includes the study of many hormones; however, in this section the discussion will be based on hormones whose plasma concentrations have a direct association with regulation of DMI and metabolism. Another aspect of these hormones is that their plasma concentration is also associated with the presence and absorption of nutrients by the gastrointestinal tract. The hormones that will be discussed are insulin, ghrelin, CCK, entero glucagon products, glucose-dependent insulinotropic polypeptide (GIP), and PYY.
a) Insulin

Insulin is a pancreatic hormone secreted by the B-cells in the endocrine pancreas. In non-ruminants, the main stimulus for its secretion is plasma glucose concentration; however, amino acids also can stimulate insulin secretion. The uptake of glucose by the B-cells increases its oxidation, producing more ATP. The increase in intracellular ATP stimulates the ATP sensitive K\textsuperscript{+} channels, producing a membrane depolarization opening Ca\textsuperscript{++} channels. The increase in intracytosolic Ca\textsuperscript{++} concentration triggers the secretion of insulin. Also, the gut peptides GIP and GPL-1 stimulate insulin secretion in a glucose dependent manner (Holst, 2004). A well known function of insulin is the regulation of body energy redistribution, by increasing glucose utilization by the peripheral tissues. Insulin is also involved in the regulation of gene expression and enzymatic activity of proteins involved in glycolysis, gluconeogenesis, lipolysis and lipogenesis. However, this review will be focused on the importance of insulin in the differences between non-ruminants and ruminants regarding the regulation of DMI.

In non-ruminants, one of the first articles that report a reduction in DMI by insulin was published by Woods et al. (1979). In that study they did a chronic infusion of different doses of insulin in the cerebrospinal fluid of baboons, and they observed a dose response in feed intake. Similar results have been described in other species (Schwartz et al., 1999; Stanley et al. 2005). The transport of insulin through the blood brain barrier (BBB) is mediated via a transporter (Baura et al., 1993). Once insulin crosses the BBB, it binds to receptors in the hypothalamus (Schwartz et al., 1992; Benoit et al., 2002). Once in the hypothalamus, insulin can inhibit the expression of the orexigenic peptide neuropeptide Y (NPY), in the paraventricular nucleus. This was shown by Schwartz et al. (1992) in rats.
with a central infusion (third ventricle) of insulin. They conducted 3 experiments; two of them utilized a short term infusion with different doses of insulin (0.4 and 4 mU every 12 h for 72 h) and the third experiment was a long term continuous infusion (2 mU/day for 6 days). They compared the mRNA concentration for NPY via in situ hybridization from the fed rats, fasted rats infused with insulin or fasted rats infused with sterile saline solution. Fasted rats that received the higher dose during short term infusion and the rats that received the long term infusion had similar concentrations of NPY mRNA as the fed rats, which was lower than the fasted rats that were infused with sterile saline solution.

Insulin also increases gene expression of the anorexic neuropeptide pro-opiomelanocortin (POMC) in the arcuate nucleus (ARC) (Benoit et al., 2002). In a study conducted in rats, Benoit et al. (2002) showed (via an immunocytochemical technique) a colocalization of insulin receptor and POMC in the ARC. Also, using a similar approach as Schwartz et al. (1992), Benoit et al. (2002) compared the effect of insulin infusion on POMC gene expression. The treatments were 4 mU insulin infusion into the third ventricle in fasted vs. fasted non infused and fed rats. The fasted infused rats had similar concentration of POMC mRNA as the fed rats, which were higher than the fasted non infused rats. The particular function of the hypothalamic peptides NPY and POMC in DMI regulation will be discussed later in the section on the hypothalamus as a regulator of DMI.

In ruminants, the stimulus for insulin secretion differs from non-ruminants. The major difference is that the amount of glucose absorbed by the small intestine is less and there is an increase in VFA absorption in ruminants (Reynolds, 2002) compared with non-ruminants. In Harmon’s (1992) review, he reports different experiments in which propionate and butyrate increase insulin plasma concentration. The mechanisms of how
VFA stimulate insulin secretion are not completely known. Due to the greater liver uptake of VFA in ruminants (Reynolds, 2002), it seems that the secretion of insulin by the pancreas is not only stimulated by metabolite concentration, but also by the autonomic nervous system (Bloom and Edwards, 1984). Another difference is the lack of effect of GIP and GLP-1 in ruminants in regulating insulin secretion. This will be discussed in detail later for each particular hormone with incretin effect. The main explanation for this lack of effect could be the lower plasma glucose concentrations in ruminants (Holst, 1997; Faulkner and Martin, 1999) compared with non-ruminants.

The regulation of DMI via insulin is variable depending on the insulin concentration (Grovum, 1995). A good example of this was shown by Deetz et al. (1980). When insulin was infused 1, 2 or 3 times the plasma concentration (2, 4 or 6 mU insulin/kg BW respectively) during 15 minutes in the portal vein, a decrease in DMI was observed. This decrease in DMI was associated with an increase in insulin jugular plasma concentration and no changes in plasma glucose concentration. However, when insulin was infused during a 24 h period, there was an increase in DMI associated with a decrease in glucose jugular plasma concentration. The dose that produced the increase in DMI was 2mU insulin/kg BW. From these data, I assume that insulin per se decreases DMI in ruminant (Figure 1), as long as the animal does not reach a hypoglycemic state. In a more recent study conducted by Mackle et al. (1999), insulin decreased DMI. Using a hyperinsulinemic-euglycemic clamp for 4 days they observed a decrease in DMI associated with the infusion of insulin in the jugular vein. Glucose concentration was maintained at a basal level by the addition of approximately 3.3 kg of glucose per day.
For that reason the decrease in DMI could be more related to the high amount of glucose infused that a direct effect of insulin.

As mentioned previously in the propionate section, a decrease in DMI was associated with an increase in plasma insulin concentration when Na-propionate was infused in the rumen (Oba and Allen, 2003 a-b). However, in these studies, jugular plasma concentration of propionate and glucose increased. From these data, it is hard to isolate the effect of insulin per se in the regulation of DMI or if the effect is only due to liver oxidation of propionate. For that reason, a different approach in the experimental design needs to be used to isolate the effect of glucose, propionate and insulin per se in the regulation of DMI.

b) Gut Peptides

Gut peptides is the common name used for the gastrointestinal hormones. This group of hormones was first discovered in the early 1900s, with the discovery of secretin and gastrin; however, it was not until the 1960’s and 1970’s that their relationship with feed intake and metabolism was elucidated (Rehfeld, 1998). Even though there are many hormones secreted by the gastrointestinal tract, this review will focus on the ones that I consider more relevant for the regulation of DMI or that are associated with energy metabolism. Gut peptides known to regulate feed intake in non-ruminants include ghrelin, CCK, GLP-1 and PYY. Although GIP does not have a direct effect on DMI regulation, I will discuss it because it has an indirect effect in DMI regulation due to its role in increasing insulin and possibly GLP-1 secretion.
i) Ghrelin

Ghrelin is a gut peptide discovered in 1999 by Kojima et al. (1999) as the natural ligand for growth hormone secretagogue receptor. It is secreted mainly by the stomach; however, some ghrelin immunoreactive cells have been found in the hypothalamus, pancreas, gonads, lung, among other tissues (Perez-Tilve et al., 2006). The cellular mechanism of regulation of ghrelin secretion is not known; however, plasma concentration of ghrelin is higher in the fasting period (Perez-Tilve et al., 2006). This association was also observed in beef cows (Hayashida et al., 2001) and sheep (Sugino et al., 2004). Ghrelin simulates GH secretion and enhancement of appetite in non-ruminants (Kojima and Kangawa, 2005). Also, ghrelin increases adipogenesis in rat adipose tissue in vitro (Choi et al., 2003). The effect of ghrelin in the hypothalamus is opposite to the effect of leptin; ghrelin increases NPY and AgRP gene expression (Kojima and Kangawa, 2005). However, the finding of ghrelin receptors in the vagus nerve suggests that ghrelin could increase feed intake by increasing the vagus nerve activity (Perez-Tilve et al., 2006).

Itoh et al. (2006) showed that plasma glucose concentration increased for 3 h after a jugular bolus infusion of 0.3 nmol/kg BW of ghrelin in lactating dairy cows; however, plasma glucose concentration decreased after the same treatment in non-lactating cows. Neither of these responses was due to insulin, which showed the same change in pattern as observed in non-ruminants. In this study, the author did not report DMI or milk yield, which would help to interpret these results. Plasma ghrelin concentration is inverse to the energy balance in ruminants (Bradford and Allen, 2008; Wertz-Lutz et al, 2008). Dairy cows in early lactation, in which the animals were in a negative energy balance, showed a
pre-feeding surge in plasma ghrelin concentration (Bradford and Allen, 2008). This pre-prandial increase in plasma ghrelin concentration was not observed in mid lactation dairy cows which were in a positive energy balance (Bradford and Allen, 2008). It is worthy to mention that the diet used in that study did not have any type of added supplementary fat. In a different study from the same lab (Bradford et al., 2008), dairy cows supplemented with dietary fat, independent of the degree of saturation, did not show the pre feeding increase in plasma ghrelin concentration; however, the pre feeding surge was observed in the control-fed cows.

Wertz-Lutz et al. (2008) showed in beef cattle that energy intake restriction increased plasma ghrelin concentration compared with animals with a higher energy intake. In that experiment, the steers were fed 80 % or 240 % of their NEm requirements for the low and high energy intake, respectively, for 3 weeks. A higher plasma ghrelin concentration for the low energy intake was observed during sampling throughout each day for the three weeks of the experiment. The daily pattern of ghrelin observed in the study presented by Wertz-Lutz contrasts with other studies (Bradford and Allen, 2008; Bradford et al. 2008) in that plasma concentration was greater concentration was throughout the entire day and not only a pre-feeding surge. A possible explanation for this difference may be the degree of energy restriction.

Another interesting finding related with ghrelin is the secretion of a different hormone that is derived from the same prepropeptide. It is a 23 amino acids (AA) hormone called obestatin (Zhang et al., 2005). Even though its plasma concentration does not vary with meals in rats, the intraperitoneal infusion of human obestatin decreased feed intake in mice (Zhang et al. 2005). There are some disagreements regarding the effect of obestatin
in non-ruminants (Chartrel et al., 2007). The effect of obestatin is not known in dairy cows. However, obestatin infusion in dairy cows did not change milk yield or DMI compared with control cows (Roche et al., 2007).

ii) Cholecystokinin

Cholecystokinin was first discovered in 1928 as a hormone that regulates gallbladder contraction (Rehfeld, 1998). In the 1940s another hormone that stimulates exocrine pancreas secretion was found, and it was called pancreozimin. But in the 1960s, Jorpes and Mutt showed that CCK and pancreozimin were the same hormone (Rehfeld, 1998). Cholecystokinin is a gut peptide secreted by the I cells in the lower part of the small intestine. A particular characteristic of this peptide is that is has six different forms that vary in AA number. All six forms arise from the same prepropeptide, with a common 8-AA active carboxyl end. The 33-AA form is mainly secreted by the gut in humans (Walsh, 1994) but the other forms are CCK-83, CCK-58, CCK-39, CCK-22, and CCK-8. Cholecystokinin-8 is also produced by the hypothalamus (De Fanti et al., 1998). Cholecystokinin is secreted in response to the presence of nutrients in the small intestine (Choi and Palmquist, 1996; Hara et al., 2000). In almost all species, the presence of fat and protein in the lumen of the small intestine stimulate CCK secretion.

For this review, I will group the functions of CCK into the ones that increase overall nutrient digestibility and the ones that decrease feed intake. The former are: increased gallbladder contraction, an increase in pancreatic secretion (Walsh, 1994) and a decrease in stomach and intestinal motility (Walsh, 1994; Kumar et al., 2004). Cholecystokinin decreases feed intake mainly by decreasing meal size, but there is also an increase in the number of daily meals, such that the total intake is not always reduced (Moran, 2004).
The decrease in feed intake may be in part due to the effects of decreasing gut motility (Kumar et al., 2004). The decrease in feed intake is due to a possible hypothalamic modification of NPY expression (Bi et al., 2004), and to a vagal response caused by CCK-8 (Moran et al., 1997; Reidelberger et al., 2004). Circulating CCK-8 seems to be the form that mainly regulates the hypothalamic regulation of feed intake; however, it should be considered that CCK-8 is also synthesized in the hypothalamus (De Fanti et al., 1998). Recent data suggest that CCK-58 reduces intake via receptors on the vagus nerve, and it may be the most important endocrine form in dogs (Reidelberger et al., 2004).

In ruminants the specific role of CCK in DMI regulation is not known. Kumar et al. (2004) showed that after intravenous infusion of devazepide, a CCK receptor antagonist, reticulo-rumen motility increased; however, there were no changes in DMI. The data about plasma CCK concentration and what stimulates its secretion is controversial. The first work that I am aware in which CCK was measured in cows was conducted by Furuse et al. (1991). They did not show significant differences in plasma CCK concentration in three Holstein-Friesian cows during a 6-h period. After that, some research studied the role of CCK in regulating DMI when fat was added to the diet. Those studies show different results. Apparently, there are two different responses: one is when fat is fed, and the other when fat is post-ruminally infused. When fat was added to the diet, there was an increase in jugular plasma CCK concentration associated with a decrease in DMI (Choi and Palmquist, 1996; Choi et al., 2000; Harvatine and Allen, 2005; Relling and Reynolds 2007b). On the other hand, in most of the studies where fat was postruminally infused, there were no changes in arterial plasma CCK concentration (Benson and Reynolds, 2001; Litherland et al., 2005), or even a decrease in plasma CCK concentration.
There is only one study (Chelikani et al., 2004) in which an increase in plasma CCK was observed after abomasal infusion of oil. The reason for that was probably the high amount of oil infused (1 kg/d). However, with the data available I cannot draw conclusions regarding the importance of CCK in the depression of feed intake when fat is added to the diet. It is possible that in ruminants, CCK also decreased meal size, but daily intake is compensated with an increase in meal numbers per day. There are few data regarding the effect of macronutrients on the secretion or plasma concentration of CCK in ruminants. Swanson et al. (2004) observed a decrease in jugular vein CCK-8 concentration after the abomasal infusion of casein in beef steers compared with starch and a mixture of starch and casein, which was unexpected based on results from other species. However, when 800 g/d of casein was infused in the abomasum in lactating dairy cows, jugular plasma CCK concentration increased (Relling and Reynolds 2008). In this last study the amount of casein infused was almost twice that infused by Swanson et al. (2004). Also, post-ruminal infusion of corn starch did not increase plasma CCK concentration (Relling and Reynolds, 2008).

iii) Entero-proglucagon:

I will use the term entero-proglucagon to refer to all the products derived from the glucagon gene in the L cells of the small intestine. The glucagon gene (Figure 2) is expressed in the A-cells in the pancreas, the L-cells in the lower small intestine and part of the large intestine, and in some areas of the brain (Holst, 1997). The products of the gene that are synthesized depend on the cell where the gene is expressed (Figure 2). The products of the L-cells that I will discuss in this review, because of their association with feed intake regulation, are glicentin, OXM, GLP-1, and GLP-2.
Glicentin and Oxyntomodulin.

Glicentin is a 69-AA peptide produced in the L cells in the small intestine (Holst, 1997). The peptide contains the sequence of OXM and glucagon, which are the AA form 33 to 69, and 33 to 61 respectively. Oxyntomodulin is a 37 AA peptide secreted by the L-cells in the small intestine. It has the glucagon sequence plus eight AA on the C-terminal end (Holst, 1997). The small intestine secretes both glicentin and OXM; however, the percentage of each secreted and which are the mechanisms that regulate their secretion are not known.

The first studies analyzed both peptides using a differential assay, in which the plasma concentration was calculated by the difference between the assay for total glucagon (that detects pancreatic glucagon and OXM or glicentin) and the one specific for pancreatic glucagon. The reason for that was because the antibodies in the total glucagon assay cross-react with the 3 peptides (Holst, 1997). This assay was not specific for OXM or glicentin, so it was not clear which molecule was measured, or a clear distinction of the effect of the different peptides.

Some studies conducted in Bloom’s lab, in which OXM was infused, showed that OXM decreased feed intake in humans (Cohen et al., 2003) and in rats (Dakin et al., 2004). Oxyntomodulin effects on the regulation of feed intake seem to be by binding to the GLP-1 receptor; however, the binding of OXM to the receptor has low affinity (Small and Bloom, 2004). The data about OXM in domestic animals is limited; however, it may have the same role as in humans.
In dairy cows plasma entero-glucagon concentration increases due to stage of lactation (Manns, 1972; Benson and Reynolds, 2001) and post ruminal fat infusion (Benson and Reynolds, 2001). In the study conducted by Benson and Reynolds (2001) entero glucagon was measured with a differential assay, using an antibody specific for pancreatic glucagon and the other one that has a 43% of cross-reactivity with OXM, but there is not report if is any cross-reactivity with glicentin. Also in that experiment they measured a release of entero-glucagon from the liver, but not from the intestine. One possible interpretation for this could be that the L-cells in the small intestine secrete glicentin or a larger peptide, which will be metabolized to OXM or a peptide that cross react with the total antibody assay in the liver. However, the metabolism of these peptides is not known in ruminants. In the study conducted by Manns (1972), entero-glucagon was also measured by a differential assay, but in that study they use the same antibody for both assays, but pancreatic glucagon was extracting using an acetone extraction.

(2) Glucagon-like peptide-1

As was mentioned before, GLP-1 is a peptide that is derived from the glucagon gene (Holst, 1997). It is mainly produced by the L cells in the small intestine, and its active form is the 7-36 amide. It is also secreted in response to the presence of nutrients in the lumen of the small intestine. Knapper et al. (1995) reported that a duodenal infusion of fat or a mixture of fat and glucose increased GLP-1 secretion in pigs more than did glucose alone. These results were different from what they had expected from their human data, in which glucose was more potent than fat as a stimulus for GLP-1 secretion. This difference in results implies that the stimulus for GLP-1 secretion varies among species (Knapper et al., 1995; Hansen et al., 2004). Also, the gut peptide GIP increased
GLP-1 secretion (Damholt et al., 1998); however, this secretion is species specific (Deacon, 2005). Once GLP-1 is in the plasma, it has a short half-life (five minutes) because it is rapidly degraded by the enzyme dipeptidyl peptidase IV (Perfetti and Merkel, 2000).

The functions of GLP-1 have been well established because of the feasibility of GLP-1 receptor agonists and antagonists, called exendin-4 and exendin-3 respectively (Holst 2006). Exendin-4, originally from a poisonous lizard, binds and activates the GLP-1 receptor, and it has a longer half life. On the other hand, exendin-3 is a truncated form of exendin, which binds to the GLP-1 receptor but does not activate it (Holst 2006). Glucagon-like peptide-1 has three main functions: (a) incretin effects, which are effects of hormones secreted by the small intestine during the digestive process that stimulate the pancreas to secrete insulin to prepare other tissues (i.e. adipose and muscle) for the utilization of absorbed nutrients, (b) ileal brake effects, and (c) central inhibition of feed intake. All of these effects will directly or indirectly impact the amount of feed that an animal consumes. As an incretin, GLP-1 enhances insulin secretion (Walsh, 1994) and, as already discussed, insulin is a potent regulator of feed intake because of an inhibition of NPY in the hypothalamus (Gale et al., 2004). However, the secretion of insulin due to GLP-1 is glucose concentration dependent (Holst, 1997). Regardless of its function in increasing insulin secretion, GLP-1 also increases insulin gene expression. In that way GLP-1 up-regulates, in the short and long term, insulin secretion. Another function of GLP-1 is the reduction in gastrointestinal motility (Holst, 1997), due to an ileal brake effect. It is called “ileal brake” because the signal (GLP-1 in this case) that stops/decreases gut motility comes from the ileum. The central action of GLP-1 is on the
hypothalamus, where GLP-1 receptors have been found (Shimizu et al., 1987; Turton et al., 1996; Holst, 1997). The mechanism responsible for this central regulation is not known, but the confluence with the functions of leptin, insulin and CCK in the systems NPY/AgRP- POMC/CART are possible. Other functions of GLP-1, which are not related to feed intake include the stimulation of lipogenesis, which is insulin-independent (Martin et al., 1993).

In ruminants, the data for GLP-1 secretion and function is limited. It has been shown that the physiological state of the animal has an important role in the plasma concentration of GLP-1, being higher in lactating ruminants than in dry ones (Faulkner and Martin, 1997; Relling and Reynolds, 2007a). In early lactation, the changes in plasma concentration of GLP-1 seem to be directly related with changes in the physiological state of the animal but not with DMI (Relling and Reynolds, 2007a). The presence of fat in the lumen of the small intestine in dairy cows increases jugular plasma GLP-1 concentration (Benson and Reynolds. 2001; Relling, 2006). On the other hand, there is only one study that I am aware of where the effect of protein and carbohydrates on plasma GLP-1 concentration was reported (Relling and Reynolds, 2008). Long term (7-d) abomasal infusion of casein did not increase plasma GLP-1 concentration, but the infusion of starch for the same period of time decreased plasma GLP-1 concentration (Relling and Reynolds, 2008).

In the first study done in dairy cattle in which GLP-1 was measured (Benson and Reynolds, 2001), a mixed of 400 g/d of soybean and rapeseed oil was continuously infused in the abomasum during 7 d. In that study, a significant decrease of DMI was associated with a significant increase in plasma concentration of GLP-1. Similar results were observed by Litherland et al. (2005) in which increasing concentration (0, 200, 400,
600 g/d) of free fatty acids or soybean oil was infused for 5 days. In this study, they found a linear decrease in DMI associated with a linear increase in plasma GLP-1 concentration. In a study conducted in our lab (Relling and Reynolds 2007b), plasma GLP-1 concentration increased when 3.5% of fat was added to the diet. The sources of fat in that experiment were three types of fat differing in the degree of saturation; the unsaturated sources increased plasma GLP-1 concentration to a greater extent compared with saturated fats. The increase in GLP-1 was associated with a decrease in DMI within the three types of fats. However, there was not an increase in GLP-1 concentration after the first day of abomasal infusion of 500 g/d of soybean oil (Relling and Reynolds, 2008). From these results, I can assume that the increase in plasma GLP-1 plays an important role in decreasing DMI when fat is fed for a long period of time.

Regarding the other functions of GLP-1, Faulkner and Martin (1999) showed that GLP-1 stimulated the secretion of insulin when the plasma levels of glucose were increased by intravenous glucose infusion (3.38 vs. 4.50 mM for dry ewes and 3.24 vs. 4.78 mM for lactating ewes before and after infusion, respectively). This is the only study I am aware of in which GLP-1 has been infused to ruminants. In other studies where GLP-1 was measured in ruminants, the incretin effect of GLP-1 has not been observed (Benson and Reynolds, 2001; Litherland et al. 2005; Relling and Reynolds, 2007b and 2008). My assumption for this is that in those studies the plasma concentration of glucose was not high enough to reach the threshold proposed previously for non-ruminants (Holst, 1997).

(3) Glucagon-like peptide 2

Glucagon-like peptide 2 is another hormone that is derived from the glucagon gene. It is co-secreted with GLP-1 by the L-cells; therefore, the mechanisms that stimulate GLP-2
secretion are the same as for GLP-1 (Holst 1997). Glucagon-like peptide 2 is degraded by the plasma enzyme dipeptidase IV, with a half life of 7 minutes (Burrin et al., 2003). The main function described for GLP-2 is to increase nutrient absorption; it does this by decreasing gut motility, increasing digestive enzyme activity and nutrient transport, enhance intestinal growth, all in coordination with an increase in intestinal blood flow (Burrin et al., 2003). The effect of GLP-2 on feed intake is uncertain. Tsai et al. (1997) did not observe a decrease in feed intake in mice during a 9-d period of GLP-2 infusion; however, the route of infusion of the peptide on that study is not clear. On the other hand, Tang-Christensen et al. (2000) showed that the central infusion of GLP-2 in rats decreased DMI for two hours after infusion.

iv) Peptide YY
Peptide Tyrosine Tyrosine (PYY) is a polypeptide secreted by the L cells in the small intestine in response to the presence of key nutrients in the lumen of the small intestine (Onaga et al., 2000; Onaga et al., 2002; Batterham et al., 2003). The major secreted form of this hormone contains 36 amino acids (PYY$_{1-36}$), but there also is a truncated form (PYY$_{3-36}$) produced by the cleavage of 2 amino acids at the NH$_2$ terminus (Onaga et al., 2002). Both forms decreased feed intake when they were peripherally infused; however, the PYY$_{3-36}$ form seems to be more potent (Perez-Tilve et al., 2006). The L cells are located primarily in the lower part of the small intestine (jejunum and ileum) and upper colon (Onaga et al., 2000; Onaga et al., 2002). When nutrients are absorbed by the L cells, they trigger the secretion of PYY. Although the mechanism is not well defined, the strongest hypothesis is that PYY secretion is signaled by protein kinase A (PKA) (Onaga et al., 2002). In non-ruminants, dietary fat seems to be the most potent nutrient that
regulates PYY secretion, but carbohydrates, amino acids and bile salts also can stimulate its secretion (Onaga et al., 2002).

Polypeptide YY regulates feed intake by two mechanisms: ileal brake mechanisms and a central effect on appetite at the hypothalamus level. The so-called “ileal brake” is a decrease in motility and secretory activity of the stomach and small intestine due to signals originated in the ileum (Spiller et al., 1984), which may not be constant in all species (Onaga et al., 2002). The central effects seem to be constant across species and occur via the inhibition of NPY (Batterham et al., 2003; Gale et al., 2004). However, some researchers have been unable to reproduce this central effect using PYY infusion in rodents (Tschop et al., 2004).

Due to the molecular structure difference from ruminant vs. non-ruminant PYY (Herzog et al., 1994), it is hard to measure and study the role of PYY in ruminants. Some attempts have been made using human, mice and rat commercial kits (Benson, 1999; Relling, 2006), but the results revealed that those antibodies do not cross-react with bovine PYY. On the other hand, Onaga et al. (2000) reported in sheep that plasma PYY concentration did not change over a 2-day period, even after the ingestion of diets based on either forage or concentrate. This is the only report of plasma PYY concentration in ruminants of which I am aware. In that assay they used anti-porcine PYY antibodies. The jugular infusion of porcine PYY produced a decrease in duodenal but not ruminal activity in sheep (Onaga et al. 1997).

v) Glucose-dependent insulinotropic polypeptide

Even though GIP is a gut peptide that does not have a direct effect on feed intake regulation, I will discuss it in this review due to its importance in lipogenesis in the
adipose tissue, and as an insulin, GLP-1 and GLP-2 secretagogue in some species. It was first named gastric inhibitory polypeptide because of its inhibitory effect on gastric acid secretion. It is a 42 AA polypeptide secreted by the K cells, which are located mainly in the duodenum and upper small intestine (Walsh, 1994). As with other gut peptides, GIP secretion is stimulated by the presence of nutrients in the small intestine. The presence of fat in the small intestine is a potent secretagogue for GIP, but carbohydrates also influence GIP secretion in pigs (Knapper et al., 1995) and humans (Walsh, 1994).

This peptide has multiple functions; the first one demonstrated was the effect on gastric acid secretion, but it also has functions in the regulation of metabolism as a component of the entero-insular axis. As for GLP-1, GIP increases insulin secretion in non-ruminants, in a glucose concentration dependent manner. The stimulation of insulin secretion by the B-cells in the pancreas is mediated by an increase in cAMP concentration that produces an increase in intracellular Ca^{++}, which is the final signal responsible for increased insulin secretion (Holst and Gromada, 2004). Another key function of GIP is its role in the regulation of lipid metabolism, where it not only increases the activity of lipoprotein lipase in adipose tissue and reduces the plasma triglycerides concentration (Kieffer, 2003) and increases lipogenesis (Hauner et al., 1988). Another function that seems to be species-specific is the stimulation of GLP-1 secretion (Deacon, 2005). Damholt et al. (1998, 1999) have shown that the secretion of GLP-1 is stimulated by GIP in canine intestinal cell cultures, and the response is dose-dependent. Damholt et al. (1998) postulated that GLP-1 secretion is stimulated not only by glucose, but also by GIP. Similar results have been observed in rat studies, but not in human or porcine (Deacon, 2005). In vitro studies suggest GIP mediates GLP-1 secretion via an increase in PKA in
the L cells. Martin et al. (1993) showed in sheep that GIP and GLP-1 decrease lipolysis. In this study, they measured a decrease in glycerol concentration after the intravenous infusion of either GIP or GLP-1.

Studies conducted in preruminant goats and dairy cows showed that fat increased GIP plasma concentration (Martin et al., 1993; Relling 2007b, respectively). In lactating dairy cows the addition of 3.5% of fat in the diet increased plasma GIP concentration, independent of the degree of unsaturation (Relling and Reynolds, 2007b). Despite that, GIP concentration did not increase in adult (Nilssen et al., 1983) or preruminant (Martin et al., 1993) goats after a duodenal infusion or meal of a glucose solution. Glucose-dependent insulino tropic polypeptide increased after abomasal infusion of starch in lactating dairy cows (Relling and Reynolds, 2008). Reynolds et al. (2002) observed that GIP secretion into the portal vein was not altered by abomasal infusion of casein or a mixture of essential amino acids. However, when 800 g/d of casein were abomasally infused in lactating dairy cows, jugular plasma concentration of GIP increased (Relling and Reynolds, 2008). The effect of GIP on increasing insulin secretion has not been studied in ruminants, but the studies that measured both insulin and GIP in ruminants did not show an association between them. Also, in the studies in which GIP and GLP-1 was measured in dairy cows (Relling and Reynolds, 2007b, 2008), no association was observed between these peptides. This does not support the idea that GIP stimulates GLP-1 secretion.
3) Role of the liver in the regulation of DMI

The idea that the liver is a regulator of appetite started when Russek (1963) showed that intraperitoneal infusion of glucose rapidly increased portal glucose concentration but not arterial glucose concentration. This increase in portal glucose concentration was associated with a decrease in feed intake (Russek, 1963). Based on that observation and correlating the importance of the liver as the first barrier (organ) to receive the post-absorptive blood, many other studies were conducted to discover the role of the liver as a regulator of feed intake. Langhans et al. (1985) showed that not only glucose but also other metabolites (lactate, pyruvate, malate, glycerol, and hydroxyl-butyrate) decrease feed intake via a mechanism that involved the liver. In that study they did a hepatic vagotomy and subcutaneously infused the metabolites. Comparing non-infused (control) with the vagotomized and sham-vagotomized infused rats, they observed a decrease in feed intake in the sham-vagotomized, but the other two were similar. Also, the gastric infusion of drugs that inhibit glucose or fatty acids oxidation, 2-DG and methyl palmoxirate, respectively, also inhibit the effect of glucose and fatty acids oxidation on feed intake (Friedman et al., 1986). The increase in feed intake caused by 2-DG and methyl palmoxirate is additive, showing that it is the oxidation of metabolites per se, independent of the type of metabolite that regulates feed intake via the liver.

The mechanism of how the signal is sent from the liver to the vagus nerve is not known; however, it is known that the cytosol concentration of ATP and/or calcium plays an important role in this regulation (Langhans, 1996; Rawson et al., 2003, respectively). The oxidation of metabolites that takes place in the hepatocyte increases the ratio ATP/ADP, which drives the Na-K pump that affects the membrane potential (Langhans, 1996). This
change in membrane potential is translated by an unknown mechanism in an action potential to the vagus nerve that produces satiety (Langhans, 1996). On the other hand, Rawson et al. (2003) proposed that the intracellular Ca\(^{++}\) concentration was the stimulus to trigger the vagal response. To demonstrate this, they treated hepatocyte cell cultures with 2, 5 anhydro D mannitol, a compound that traps phosphates and decreases ATP concentration. In that experiment, they observed an increase in hepatocyte Ca\(^{++}\) concentration, and concluded that Ca\(^{++}\) has an important role in the regulation of the signaling from the hepatocyte to the vagus nerve.

In ruminants, Allen (2005) proposed that propionate oxidation in the liver causes the same effect as glucose in non-ruminants; this is due to the lower amount of glucose absorbed by ruminants compared with non-ruminants. In some studies conducted in Allen’s lab (Oba and Allen, 2003a, b) they showed that propionate infusion decreased DMI. In a follow up study (Bradford and Allen, 2006), they infused cows with phlorizin (a compound that decreases glucose reabsorption by the kidney) and did not observe changes in DMI. The rationale behind that experiment was that the increase in glucose lost in the urine will decrease propionate oxidation due to an increase in glyconeogenesis, which will increase DMI. One possibility for the lack of response could be that the animals were in early lactation, and the DMI was at that time at a “maximum” due to the high glucose demand by the mammary gland.

Another aspect that needs to be considered is that hepatic binding of CCK stimulates vagal signaling that has been associated also to a decrease in feed intake (Moran et al., 1997). Therefore, it is difficult to separate the effect of absorbed metabolite oxidation and the post-prandial CCK response.
4) Role of the hypothalamus in the regulation of DMI

As described previously in the glucose section of this review, the importance of the hypothalamus as a key center for regulation of appetite and satiety was described in the 1950s, when the damage caused by GTG was observed in the hypothalamus (Brecher and Waxler, 1949; Marshall et al., 1955). In brief, they observed that the thio-molecules produce damage in certain areas of the hypothalamus, which results in obesity. At that time, the mechanism of how this was regulated was not clear.

The first neuropeptide shown to be involved in the increasing feed intake was NPY (Clark et al., 1984). More studies were conducted on neuropeptides as regulators of feed intake when the role of leptin in the hypothalamus was established (Porte, 2002). Peptides known to increase appetite include neuropeptide Y (NPY) and agouti-related peptide (AgRP), while peptides that decrease appetite include proopiomelanocortin gene product (POMC), through its target peptides α-melanocyte-stimulating hormone (α-MSH), and cocaine and amphetamine-regulated transcript (CART). The mechanism of how they interact to regulate feed intake is not completely understood, mainly because of the complexity of interaction that take place in the nucleus of the hypothalamus (Kalra et al., 1999). The secretion of both orexigenic and anorexigenic groups of peptides is regulated by peripheral signals, some of which were discussed previously (Gale et al., 2004). Insulin, leptin, GLP-1, CCK, PYY, and glucose in general decrease the secretion of the orexigenic peptides, and increase the secretion of the anorexigenic peptides (Gale et al. 2004). Ghrelin, on the other hand, increases the secretion of the orexigenic peptides.

Although in non-ruminants there are many studies that support what was previously described, the data in ruminants on the signals that stimulate or depress the secretion of
these neuropeptides is limited. Despite this lack of data, I do not expect that the regulation of the secretion of these peptides differs in ruminants compared with non-ruminants. There are going to be certain differences, which are more related to the difference in physiological parameters, such as the lower plasma glucose concentration. Adam et al. (2002) showed a relative increase in hypothalamic NPY and AgRP mRNA expression in fasted sheep. This increase in NPY and AgRP was associated with a decrease in plasma glucose and leptin concentration, but there was no effect of fasting on the amount of mRNA for POMC and CART in the hypothalamus. Also, a lower NPY and AgRP gene expression was observed in high body condition score sheep compared with low body condition score (Archer et al., 2002). In the study from Archer et al. (2005) the decrease in the orexigenic peptides was associated with an increase in plasma concentrations of glucose, insulin, and leptin, but also their DMI was higher.

The link between the metabolites and hormones in the regulation of the gene expression of the neuropeptides is been described by Minokoshi et al. (2004). In that study they showed how a decrease of activity of the enzyme AMP-activated protein kinase (AMPK) decreased feed intake. The main reasons for the decrease in intake with the decrease in AMPK are: first it is possible that AMPK activates some transcription factors for orexigenic neuropeptides, such as NPY, AgRP or MCH (in the second order neurons). Second, AMPK decreases the activity of acetyl CoA carboxylase. This enzyme catalyzes the reaction from acetyl CoA to Malonyl CoA. Malonyl CoA is a one of the first products in the synthesis of fatty acids, but its synthesis (and when is present in the cell) decreases the activity of the protein CPT I (which transport long chain fatty acids (LCFA_ to the inside of the mitochondria to be oxidized). For that reason, the decrease in AMPK
produce an increase in malonyl CoA concentration in the cell, and subsequently an increase in LCFA. It has been shown (Lam et al. 2005) that hypothalamic increases in cytosolic LCFA concentration decreases feed intake. The mechanism of how this occurs is not known, but it is possible that LCFA have receptors in the hypothalamic cells, that work as transcription factors.

In conclusion, the mechanism of regulation of DMI is quite complex, and the role of metabolites and metabolic hormones and their interactions in DMI is not well known in ruminants.
Figure 1 (from Grovum, 1995). Changes in DMI due to increasing concentrations of plasma insulin.
Figure 2 (adapted from Holst, 1997). Proglucagon gene and its products in A-cells in the pancreas, L-cells in the small intestine and some cells of the brain.
CHAPTER 3

EFFECTS OF GLUCOSE, PROPIONATE AND SPLANCHNIC HORMONES ON NEUROPEPTIDE mRNA CONCENTRATION IN THE OVINE HYPOTHALAMUS

Abstract

The in vitro effect of metabolites and splanchnic hormones on ovine hypothalamic mRNA concentration for neuropeptides that regulate appetite is not known. Therefore, the objective of this study was to measure the in vitro effect of media containing metabolites (glucose, propionate) or hormones (insulin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), polypeptide YY (PYY)) on hypothalamic mRNA concentration for neuropeptide Y (NPY), agouti-related peptide (AgRP), and proopiomelanocortin (POMC). Hypothalamic tissue from market lambs was obtained at slaughter and immediately incubated in culture media for 2 h at 36º C. Treatments included a control Dulbecco's Modified Eagle Medium (DMEM) or DMEM with the following additions: glucose, propionate, insulin, GLP-1, PYY, CCK, or glucose plus insulin. Abundance of mRNA for NPY, AgRP, and POMC was measured using quantitative reverse transcriptase PCR. Fisher's protected LSD test was used to compare changes in relative mRNA concentrations for the hypothalamus incubated in the control
media versus the rest of the treatments. The incubation of sheep hypothalamus in glucose, propionate, insulin, CCK, GLP-1, or PYY did not affect the mRNA concentration for NPY, AgRP, or POMC ($P > 0.20$) compared with incubation of hypothalamus in the control media. Hypothalamus incubated in media containing glucose plus insulin did not result in changes in mRNA for NPY or AgRP, but increased POMC mRNA concentration compared with the control hypothalamus incubation ($P < 0.05$). This interaction suggests both hormones and metabolites are needed to elicit a change in one of the major neuropeptides known to regulate DMI.

Introduction

Despite the importance of DMI in ruminant production, the mechanism of how DMI is regulated is not fully known. Much is known about the role of hypothalamic peptides in the regulation of appetite and feed consumption in non-ruminants (Wilding, 2002; Gale et al., 2004). Neuropeptides known to increase appetite include neuropeptide Y (NPY) and agouti-related peptide (AgRP), while proopiomelanocortin (POMC) is a neuropeptide that decreases appetite (Valassi et al. 2008). An increase in hypothalamic concentration of mRNA for NPY and AgRP was observed in fasting compared with ad libitum-fed sheep (Adam et al., 2002). However, there are few reports regarding the role of metabolites and hormones in the hypothalamus concentration of these neuropeptides in ruminants.

In non-ruminants, the syntheses of these hypothalamic peptides are influenced by plasma metabolites (e.g. glucose; Lee et al. 2005) and hormones (e.g. insulin; Schwartz et al., 2002; Gale et al., 2004). In sheep, an early study using gold thioglucoce (Baile, 1968)
showed that glucose does not regulate DMI, which suggested there was no glucostatic regulation of DMI in ruminants. On the other hand, studies in dairy cattle (Sheperd and Combs, 1998; Oba and Allen, 2003) have observed a decrease in DMI associated with an increased in jugular vein plasma glucose concentration, due to ruminal propionate infusion. Under normal conditions, virtually all the propionate absorbed into the portal vein is removed by the liver (Reynolds, 2006), and the effect of propionate on meal size is believed to be a consequence of propionate oxidation and ATP generation in the liver (Oba and Allen, 2003). A review of the literature revealed no previous studies comparing the effect of glucose, propionate or insulin on the synthesis of hypothalamic peptides in ruminants. Some of the hormones that regulate feed intake in non-ruminants are insulin, cholecystokinin (CCK), glucagon-like peptide-1(7, 36) amide (GLP-1), and peptide YY (PYY) (Valassi et al., 2008). In ruminants, a decrease in DMI has been associated with an increase in plasma concentration of GLP-1 and CCK (Bradford et al. 2008; Relling and Reynolds, 2007), but there are no investigations of the direct effect of these gut peptides on gene expression of the hypothalamic neuropeptides. Therefore, the objective was to measure the effects of increased concentrations of glucose, propionate or hormones (insulin, CCK, GLP-1, PYY) on mRNA abundance for the orexigenic neuropeptides NPY and AgRP, and the anorexic neuropeptide, POMC, in sheep hypothalamic tissue cultured ex-vivo.

Materials and Methods

Hypothalami were removed from 40 market lambs, 5 to 10 minutes after slaughter in the abattoir of The Ohio State University Meat Laboratory (Columbus, OH), and used in an
incomplete block design experiment. The criterion for blocking was the day of slaughter. The wethers were euthanized by captive bolt and exsanguination. The top of the skull was removed with a hand saw and the hypothalamus was removed with a scalpel as described by Glass et al. (1984). In brief, the frontal landmark for the hypothalamus was the optic chiasm. Caudal of the optic chiasm is the 3rd 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 3rd ventricle. The fourth cut closed that rectangular area. A final cut was made at 0.6 cm in depth to provide a cubed shaped tissue sample with dimensions of 1.2 by 1.5 by 0.6 cm. Once the hypothalami were removed they were incubated in 10 ml of Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) with 1% fetal bovine serum and the hormone/metabolite treatment for 2 h at 37°C. After the 2 h incubation, the hypothalami were removed from the media, rinsed with sterile saline solution, flash frozen in liquid N\textsubscript{2}, and stored at –80°C until RNA was extracted.

For the first day of sampling (block 1), the treatments (n=4 per treatment) were: low glucose control, (C; 1mM of glucose DMEM media #11054 Invitrogen, Carlsbad, CA); high glucose (HG; 10 mM of glucose, DMEM media, high glucose media # 10313 diluted in low glucose media #11054 , Invitrogen, Carlsbad, CA); propionate (P; 1 mM of Na propionate and 1mM of glucose in DMEM); insulin (I; 1 nM of bovine insulin and 1mM glucose in DMEM); or glucose-insulin (G x I; 1nM of insulin and 10 mM of glucose in DMEM). For the second day of sampling (block 2), the treatments (n=4 per treatment) were: C; HG; cholecystokinin (CCK; 80 pM of CCK-8 sulfide (C2175 Sigma-Aldrich Inc, St Louis, MO) and 1 mM glucose in DMEM); glucagon-like peptide-1(GLP-1; 120 pM of GLP-1 (7-36) amide (H6795 GLP-1(7-36) amide, Bachem California Inc,
CA). and 1mM of glucose in DMEM); or peptide YY (PYY; 100 pM of bovine PYY 3-36 (SynPep corporation, Dublin, Ca) and 1 mM of glucose DMEM). Bovine PYY was used because the AA sequence of ovine PYY was not known when the experiment was conducted.

The incubation dose used for the HG treatment was chosen because this concentration was shown to increase NPY and AgRP in non-ruminants (Lee et al., 2005), a dose that is approximately three times the normal plasma concentration. For the other treatments, doses were also approximately three times physiological plasma concentrations.

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). The relative mRNA concentration of NPY, AgRP and POMC were determined by RT quantitative PCR using the DNA Engine Monitor 2 (BioRad 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 Operon Biotechnologies (Alameda, CA). The primer sequences used are described in Table 1. Primers were diluted to a working concentration of 15 µM with nuclease-free water (Sigma-Aldrich Corp., St. Louis, MO). The RT quantitative 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 curve
prepared from purified NPY, AgRP and POMC cDNA PCR product was used to calculate the steady-state concentration of NPY, AgRP and POMC mRNA in triplicate wells for each sample. The PCR amplification products were electrophoretically separated 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 the QIAquick Gel Extraction Kit (Qiagen Sciences) 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.

The data were analyzed as an incomplete block design using MIXED model procedures of SAS (Version 9.1, SAS Institute, Cary, NC), testing the random effects of lamb and block, and the fixed effects of treatment. Because block effect was not significant ($P > 0.10$) for any of the three variables, the block effect was removed from the model. Because the objective of the experiment was to evaluate the effect of the metabolites and hormones on mRNA concentration, a Fisher's protected LSD test was used to compare the control treatment (low glucose) versus the rest of the treatments.

Results and Discussion

This is the first study that compared the in vitro effect of glucose, propionate, insulin, CCK, GLP-1, and PYY on sheep neuropeptide concentrations. Hypothalamic concentration of NPY and AgRP mRNA did not change due to any of the incubation treatments ($P = 0.18$ and $P > 0.20$; Figure 3 and 4, respectively). However, the
combination of insulin and glucose increased the relative concentration of POMC mRNA ($P < 0.01$; Figure 5).

Lee et al. (2005) showed in mice hypothalamus incubated with different concentrations of glucose an increase in NPY and AgRP mRNA concentration but not changes in POMC concentration. Those results in mice agree with the glucostatic theory of regulation of feed intake proposed by Mayer (1953). The glucostatic theory of regulation on feed intake has been discredited for ruminants (Manning et al., 1959). Therefore, the lack of an effect of high glucose concentration in the media on the sheep hypothalamic neuropeptides in the present study was expected.

A decreased in DMI was observed after ruminal infusion of propionate in ruminants (Oba and Allen, 2003). The decrease in DMI was associated with an increase in plasma concentration of glucose, insulin and propionate. The decrease in DMI due to propionate infusion may be due to an increase in propionate oxidation in the liver (Anil and Forbes, 1988). However, the effect plasma propionate concentration on neuropeptides that regulate feed intake had not been tested. In the present study, no changes in mRNA for hypothalamic neuropeptides NPY, AgRP or POMC were observed with propionate incubation. These neuropeptides are known to regulate feed intake in non-ruminants. Because propionate incubation did not affect mRNA concentration in our sheep hypothalamus samples, it seems likely that this is not the main mechanism by which propionate regulates DMI in ruminants.

In the present study, significant changes in any hypothalamic neuropeptide due to insulin when combined with a low concentration of glucose in the media were not observed; however, POMC mRNA concentration increased when insulin was added in a high
glucose concentration media. Sato et al. (2005) reported no changes in NPY mRNA on in vitro slices of rat hypothalamus incubated with increased concentrations of insulin. However, a decrease in mRNA NPY concentration was observed in fasted rats that received intracerebroventricular (ICV) infusions of insulin compared with fasted rats which received saline solution infusion. Similar to the results reported in the present study, Benoit et al. (2002) report an increased in POMC mRNA concentration in fasted rats after ICV insulin infusion every 12 h. Benoit et al. (2002) also report a decrease in feed intake when insulin was infused ICV 1 h before feeding in rats. The reason for the lack of response in insulin with low glucose in the present study but not when insulin was incubated with higher glucose concentrations could be explained by the interaction of insulin and glucose, or other metabolites or hormones. This interaction has been observed previously in vivo and was defined as a dose response (Grosvum 1995; Figure 1), in which insulin has an effect of decreasing DMI only when plasma glucose concentration is above a basal concentration. Adam et al. (2002) reported a decrease in NPY and AgRP, and no changes in POMC mRNA concentration in sheep fed ad libitum compared with restricted feeding of sheep. The differences in results from this in vitro study compared with the in vivo study from Adam et al. (2002) may be due to multiple changes in plasma concentration of hormones and metabolites, such as leptin and NEFA observed by Adam et al. (2002). Even though an increase in POMC mRNA concentration was observed in the present study, it cannot confirm that the in vitro increase in POMC observed in this study was responsible for the decrease in DMI observed in vivo when plasma glucose and insulin concentration increased.
Studies where hypothalamic tissue was incubated in media containing gut peptides have not been reported. However, there are some in vivo studies where gut peptides were ICV infused. Intracerebroventricular infusion of CCK decreases DMI and meal size in sheep (Della-Fera and Baile, 1980). Similar results have been observed in non-ruminants (Bi et al. 2004). However, Bi et al. (2004) showed that the changes in NPY expression due to CCK were species specific. Rats lacking the receptor 1 for CCK (CCK 1R) had an increase in hypothalamic mRNA concentration for NPY, which did not happened in mice without the CCK 1R. In the present study there were not changes of NPY, AgRP or POMC when the hypothalamus was incubated in a media that contained CCK.

In vitro hypothalamic incubation in media containing GLP-1 also did not change mRNA concentration for NPY, AgRP or POMC. This result is similar to previous in vivo results in rats (Turton et al., 1996), where ICV infusion of GLP-1 did not change mRNA concentration for NPY compared with saline infused rats. However, Seo et al. (2008) showed that ICV infusion of GLP-1 decreased NPY and AgRP and increased POMC mRNA concentration in the hypothalamus of fasted rats. The change in mRNA concentration for the different neuropeptides was associated with changes in feed intake. From the results in the current study, and the discrepancy in non-ruminant data, the role of GLP-1 on the regulation of neuropeptides that regulate intake cannot be confirmed. Peptide YY decreases DMI in non-ruminants (Batterham et al. 2002). In rats the central effect of PYY is by binding to a Y 2 receptor, and decreasing NPY gene expression (Batterham et al., 2002; Challis et al., 2003). This was demonstrated in both in vitro and in vivo hypothalamic samples and by increasing mice hypothalamic POMC mRNA (Challis et al., 2003) in vivo. In the present study, no changes were observed in mRNA
concentration for NPY, AgRP and POMC when bovine PYY was added to the media. However, the role of PYY has not been reported for ruminants. Onaga et al. (2000) reported in sheep that plasma PYY concentration did not change over a two-day period, even after the ingestion of diets based on either forage or concentrate. Therefore, it is possible that PYY does not play a role in the regulation of DMI in ruminants. However; it is also possible that the functional structure of bovine PYY is not similar to ovine PYY, which has not been reported yet. A different structure of the peptide could change the three dimensional conformation of the peptide, which would affect the binding of the peptide to the receptor (Keire et al., 2000).

In conclusion the incubation of sheep hypothalamus in media containing metabolites or hormones by themselves did not change mRNA concentration for the neuropeptides NPY, AgRP or POMC. However, the combination of insulin and glucose increased POMC mRNA concentration. This interaction suggests both hormones and metabolites are needed to elicit a change in one of the major neuropeptides known to regulate DMI. This may imply that the central mechanism of DMI in ruminants is regulated by the interaction of changes in concentration of different hormones and metabolites.
References


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\(^1\) NPY = Neuropeptide Y; AgRP = Aguti-related peptide; POMC = Proopiomelanocortin.

Table 1. Primer sequences used for the reverse transcriptase quantitative PCR.
Figure 3. In vitro hypothalamic sheep neuropeptide Y (NPY) concentration (normalized with cyclophilin B; CYC) after 2 h incubation in DMEM containing the treatments: low glucose (control, C; 1mM of glucose), high glucose (HG; 10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1mM glucose), glucose-insulin (G x I; 1nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulfide and 1 mM glucose), glucagon-like peptide-1(GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY 3-36 and 1 mM of glucose).
Figure 4. In vitro hypothalamic sheep aguti-related peptide (AgRP) concentration (normalized with cyclophilin B; CYC) after 2 hours incubation in DMEM containing the treatments: low glucose (control, C; 1 mM of glucose), high glucose (HG; 10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1 mM glucose), glucose-insulin (G x I; 1 nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulfide and 1 mM glucose), glucagon-like peptide-1 (GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY 3-36 and 1 mM of glucose).
Figure 5. In vitro hypothalamic sheep proopiomelanocortin (POMC) concentration (normalized with cyclophilin B; CYC) after 2 hours incubation in DMEM containing the treatments: low glucose (control, C; 1 mM of glucose), high glucose (HG; 10 mM of glucose), propionate (P; 1 mM of Na propionate and 1 mM of glucose), insulin (I; 1 nM of bovine insulin and 1 mM glucose), glucose-insulin (G x I; 1 nM of insulin and 10 mM of glucose), cholecystokinin (CCK; 80 pM of CCK-8 sulfide and 1 mM glucose), glucagon-like peptide-1(GLP-1; 120 pM of GLP-1 (7-36) amide and 1 mM of glucose) and peptide YY (PYY; 100 pM of bovine PYY 3-36 and 1 mM of glucose). * P < 0.01 for G x I compared with C.
CHAPTER 4

EFFECT OF RESTRICTION FEEDING AND SUPPLEMENTAL DIETARY FAT ON GUT PEPTIDE AND HYPOTHALAMIC NEUROPEPTIDE CONCENTRATIONS IN GROWING WETHERS

Abstract

Our objectives were to evaluate the effect of supplemental fat and amount of metabolizable energy intake (MEI) on plasma concentration of the gut peptides: glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), ghrelin and oxyntomodulin (OXM). A further objective was to determine the association of these peptides with DMI and the hypothalamic concentration of mRNA for the neuro-peptides neuropeptide Y (NPY), agouti-related peptide (AgRP) and proopiomelanocortin (POMC). In a complete randomize block design with a 2 x 2 factorial arrangement of treatments, 32 pens with two wethers each were restricted-fed (2.45 Mcal/lamb/d) or ad libitum-fed (Intake main factor, n=16) and the diet with or without 6% supplemental fat (Fat main factor, n=16) for a period of 30 d. Dry matter intake was measured daily. On d 8, 15, 22, and 29 body weight was measured before feeding; and 6 h after feeding, blood samples were collected for plasma insulin,
GLP-1, CCK, ghrelin, GIP, OXM, glucose and NEFA analysis. On d 29, blood was collected 30 min before feeding for the same hormone and metabolite analyses. On d 30, the wethers were slaughtered and the hypothalamus was collected to measure messenger (m) RNA concentration of NPY, AgRP and POMC. Feeding ad libitum (resulting in increased MEI), increased plasma insulin, GIP and NEFA concentration ($P < 0.02$, 0.01 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 concentration ($P < 0.05$ and 0.01 respectively) and increased or tended to increased plasma GIP ($P < 0.01$) and hypothalamic mRNA concentration for NPY and AgRP ($P < 0.07$ and 0.11, respectively). Plasma GLP-1 and CCK concentration increased for wethers fed ad libitum compared with those restricted-fed, but within those fed ad libitum, the response was higher when they had supplemental fat in the diet (Fat x Intake interaction, $P < 0.04$). Pre-feeding plasma ghrelin concentration was higher in the restricted compared with the ad libitum-fed wethers, but the concentration was similar after feeding (Intake x Time interaction, $P < 0.01$). Supplemental dietary fat did not affect ($P > 0.10$) plasma ghrelin concentration. We conclude that the hormones insulin, ghrelin, CCK and GLP-1 may regulate DMI by regulating the hypothalamic gene expression of NPY, AgRP and POMC.
Introduction

Dry matter intake (DMI) regulation is important in ruminants as DMI is a major determinant of milk and meat production. 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 non-ruminants (Dakley et al., 2004; Gale et al., 2004). Previous studies in ruminants have shown that supplemental dietary fat (Relling and Reynolds, 2007b; Bradford et al., 2008) increases plasma concentrations of GLP-1 and CCK and decreases the pre-prandial surge of ghrelin; these changes are associated with a decrease in DMI in lactating dairy cows. In contrast, despite the increased metabolizable energy intake (MEI), the increased plasma CCK and GLP-1 concentration in early lactation was a response to the physiological stage and not to DMI or MEI (Relling and Reynolds, 2007a). Plasma GIP concentration increased due to supplemental dietary fat (Relling and Reynolds, 2007b) but not when fat was postruminally infused (Relling and Reynolds, 2008), and increased due to an increase in MEI when starch and casein were postruminally infused. In all these studies the effect of supplemental dietary fat on gut peptides is confounded with the amount of DMI, because all animals were fed ad libitum. Therefore, it is not known if the increases in plasma gut peptides concentration respond as a mediator of DMI or just to the presence of extra dietary fat. An increase in hypothalamic mRNA concentration of neuropeptide Y (NPY) and agouti-related peptide (AgRP) was observed in fasting sheep (Adam et al., 2002). These neuropeptides are potent stimulators of DMI (Valassi et al. 2008). However, there was no effect of fasting on the amount of hypothalamic mRNA for pro-opiomelanocortin (POMC), a peptide that decreases DMI. The effect of amount of feed intake and its interaction with
supplemental dietary fat I on GLP-1, CCK, OXM, ghrelin and GIP has not been studied in growing sheep. In addition, the association of plasma concentration of these gut hormones with the concentration of hypothalamic neuropeptides that regulate DMI has not been reported for sheep. The objectives of the present study were to determine in growing sheep the effect of supplemental fat and amount of DMI and MEI on plasma concentration of the gut peptides GLP-1, CCK, GIP, ghrelin and OXM, and the associated effect on the hypothalamic concentration of the neuro-peptides NPY, AgRP and POMC.

Materials and Methods

Animals and diets

Sixty four Targhee x Hampshire wethers were used in a complete randomize block design with a 2x2 factorial arrangement of treatments. Animal care followed guidelines recommended in Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1998). The wethers were blocked by weight and divided into two groups to be used in two consecutive periods. In each period, they were housed in 16 pens with 2 wethers per pen. Each period was blocked again by body weight into a heavy and a light group. Wethers were fed a pelleted mixed diet containing 40% forage and 60% concentrate (Table 2). The diet was formulated to meet nutrient requirements for finishing wethers according to the National Research Council (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; NRC 2001) and DMI.
Treatments

The experimental design was a complete randomized block design, in a 2 x 2 factorial arrangement of treatments. The treatments were the amount of feed offered (intake effect; restricted versus ad libitum DMI) and supplemental dietary fat (fat effect; control (CON) versus 6% of Ca salts of palm oil on DM basis (FAT)) as main effects during 30-d treatment periods. The amount of feed provided for the restricted-fed/CON wethers was 2.5% of the pen average body weight from the day before the experiment started, and wethers were offered that amount of feed daily until the end of the experiment. For the wethers fed the restricted/FAT treatment, the amount fed was isocaloric on an estimated ME basis with the restricted/CON fed wethers; therefore, they received 2.265% of body weight. For the ad libitum treatment, the diet was provided at 110% of the anticipated ad libitum intake. The amount of fat fed in the fat 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 concentration of gut peptides in dairy cattle (Relling and Reynolds, 2007b). The control diet, without the addition of fat, was fed for ad libitum intake for 15 d prior to the start 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 ration DM fed for the first day of the treatment period, 4% on the second day and the total 6% on the fourth day. On sampling days, feed delivery was staggered at 3-min intervals per pen to allow blood sampling to be at the same relative time post-feeding.
Sampling

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 FA (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 amount of DMI from feeding to when blood was collected.

Blood samples were collected from the jugular vein on d 8, 15, 22 and 29, starting 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 HCl (1.6 and 4.7 mg/mL of blood, respectively) and placed on ice. After centrifugation for 25 min (1,800 x g and 4°C) blood was aliquoted into individual polypropylene tubes for each hormone analysis to be performed, flash-frozen using liquid N\textsubscript{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 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 on d 30 starting at 0600 h. The hypothalamus was removed after slaughter as in Chapter 3 (Glass et al., 1984). In brief, the frontal landmark for the hypothalamus was the optic chiasm. Caudal of the optic chiasm is the 3\textsuperscript{rd} 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 3rd 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 by 1.5 by 0.6 cm. The hypothalamus samples were flash-frozen in liquid N\textsubscript{2} within 10 min after slaughter.

Hormone and metabolite analysis.

Concentrations of insulin, GLP-1, and CCK-8 were measured using RIA as described previously (Benson and Reynolds, 2001), and 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 (2007a) using a different primary antibody (G-27-07, Phoenix Pharmaceuticals, Inc.) and 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 on a pooled sample of plasma from both wethers in each pen taken 6 h after feeding on d 29. The assay used was a commercial kit (Oxyntomodulin RIA Kit # RKU-028-22, Phoenix Pharmaceuticals, Inc., Belmont, CA), validated as described in Appendix 2. In brief, the displacement of OXM binding due to incremental additions of sheep plasma was compared with an OXM standard curve. Plasma ghrelin concentration was measured using the 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 due to 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 phenyl-methylsulfonyl fluoride (10 mg/ml) as recommended in the kit protocol, to decrease the breakdown of active ghrelin. The intra-assay CV averaged less than 7.9, 13.4, 11.0, 10.4, 10.3 and 7.2% for insulin, GLP-1, OXM, GIP, CCK-8, and ghrelin assays respectively. Minimum sensitivities (90% of zero standard binding) of the insulin, GLP-1, OXM, GIP, CCK-8, 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 two-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). The relative mRNA concentration of NPY, AgRP and POMC were determined by RT quantitative PCR using the DNA Engine Monitor 2 (BioRad 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 Operon Biotechnologies (Alameda, CA). The primer sequences used are described in Table 1. Primers were diluted to a working concentration of 15 µM with nuclease-free water (Sigma-Aldrich Corp., St. Louis, MO). The RT quantitative 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 was used to calculate the steady-state concentration of NPY, AgRP and POMC mRNA in triplicate wells for each sample. The PCR amplification products were electrophoretically separated 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 the QIAquick Gel Extraction Kit (Qiagen Sciences) 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 post-feeding plasma insulin, GLP-1, CCK, GIP and metabolite concentrations, BW on d 8, 15, 22 and 29, and daily DMI and MEI; pen averages of were statistically analyzed as repeated measures using MIXED models procedures of SAS (Version 9.1, SAS Institute, Cary, NC), testing the random effects of pen and block, and fixed effects of fat, intake, time (day of sampling), the fat by intake interaction and the fat by intake by time interaction. The restricted treatments were not included in the analysis for DMI and MEI as 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 non-homogeneous variance due to the restricted intake for all the variables, intake was included in the model as a group effect, and the SEM are presented independently in the tables. 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 day 29 of sampling, the concentrations at 0730 h (pre-feeding) were compared only with the concentrations 6 h after feeding (post-feeding) sampling of that day, using the same model as for the previous analysis but with the pre- and post-feeding concentration as repeated measurements. In all analyses, if the three way interaction, fat x intake x 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 (Version 9.1, SAS Institute, Cary, NC) using the average of each pen for d 29 for both variables. Using the same procedure, the correlation of plasma GLP-1 and GIP concentration with plasma NEFA concentration were measured using the individual animal concentrations for d 8, 15, 22 and 29 (pre- and post-feeding samples).
Results

Body weight, DMI and MEI

Body weight (Fat x Intake x 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 (Figure 6). Supplemental dietary fat did not decrease or increase BW (\( P > 0.60 \); Figure 6). Feeding Ca salts of palm oil numerically decreased daily DMI and daily MEI by ad libitum-fed wethers but these differences were not significant (\( P = 0.12 \) and 0.20, respectively; Table 3). Restricting intake changes the pattern of feed consumption. At 6 h post-feeding (at the time of bleeding), DMI was actually greater for the restricted-fed wethers than for those fed ad libitum; within the restricted-fed group, DMI was greater for those fed the CON diet than for those fed the FAT diet (Fat x Intake \( P < 0.02 \), Table 3). Metabolizable energy intake was also higher at 6 h after feeding for the restricted-fed wethers compared with those fed ad libitum. Within the ad libitum group, wethers fed the CON diet had a lower MEI than those fed the FAT diet (Fat x 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 x intake interaction (\( P < 0.05 \)) was detected for plasma GLP-1 and CCK concentration (Table 4). Ad libitum-fed wethers had a higher plasma GLP-1 and CCK concentration than the restricted-fed wethers. Feeding fat increased plasma GLP-1 and CCK concentration in the ad libitum-fed wethers but not in the restricted-fed wethers. Plasma OXM did not show significant
changes ($P > 0.30$) due to the intake regime or the addition to dietary fat (Table 4). Plasma GIP concentration was higher in the wethers fed ad libitum and in those fed fat for the first 3 wk of the experiment, however plasma concentration of GIP was not affected by intake or fat supplementation on d 29 of the experiment (Fat x Intake x Time interaction $P < 0.10$; Table 4 and Figure 7). Plasma glucose concentration was higher in wethers fed the CON diet ($P < 0.01$) than those fed the FAT diet (Table 4). The amount of feed offered did not change plasma glucose concentration ($P > 0.20$). Plasma NEFA concentration (Table 4) increased due to addition of dietary fat ($P < 0.01$) and due to the increase in feed offered ($P < 0.02$).

Plasma concentration of hormones and metabolites pre- and post-feeding

A three way interaction ($P < 0.03$) between intake level, fat supplementation, and time relative to feeding was detected for plasma insulin concentration (Table 5). For the restricted-fed wethers, plasma insulin concentration was higher 6 h after feeding than pre-feeding. Wethers fed ad libitum had higher plasma insulin concentration than those whose intake was restricted. Also, plasma insulin concentration did not change after feeding in the wethers fed the CON diet, but was higher post-feeding for those fed the FAT diet. Plasma GLP-1 concentration was higher for wethers pre-feeding than post feeding for both FAT and CON diets when fed ad libitum and for the FAT diet when intake was restricted, but not for the CON diet when intake was restricted (Fat x Intake and Time; $P < 0.07$). Plasma GLP-1 concentration increased in ad libitum-fed wethers compared with those fed at restricted intakes (Table 5). Plasma CCK concentration was higher pre-feeding than post feeding for wethers fed FAT and CON diets ad libitum and
for those restricted-fed the FAT diet, but not for those restricted-fed the CON diet (Fat x Intake, and Time; \(P < 0.08\) and 0.07 respectively; Table 5). Plasma CCK concentration was higher in ad libitum-fed than in restricted-fed wethers; this response was higher when fat was added in the diet (Table 5). Plasma GIP concentration (Time \(P < 0.01\)) was higher 6 h after feeding than in the pre-feeding 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 higher pre-feeding only in the restricted-fed wethers (Time x Intake; \(P < 0.01\)). The addition of fat did not change plasma ghrelin concentration (\(P > 0.20\)). Plasma glucose concentration was higher in the ad libitum-fed than the restricted-fed wethers. The after feeding plasma glucose concentration was higher than the pre-feeding concentration for wethers fed CON and FAT diets at restricted intake and for those fed the CON diet ad libitum, but not when the FAT diet was offered ad libitum (Time x Intake, and Time x Fat; \(P < 0.01\); Table 5). Plasma NEFA concentration was higher in the pre-feeding samples only in the restricted wethers (Time x Intake; \(P < 0.01\)).

Hypothalamic mRNA concentrations of NPY, AgRP and POMC.

Hypothalamic mRNA concentration for NPY and AgRP were higher (\(P < 0.07\) and 0.02, respectively) for restricted-fed than for ad libitum-fed wethers (Table 6). Supplemental dietary fat trended to increase NPY and AgRP mRNA concentration (\(P < 0.07\) and 0.11 respectively) compared to CON. The relative concentration of POMC in the hypothalamus was numerically, but not significantly (\(P = 0.15\)), greater for wethers fed
ad libitum 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

Body weight, DMI and MEI

By design, the restricted wethers consumed less feed than those fed ad libitum. This resulted in a decrease in BW during the first week due to the decrease in feed provided and potential effects of less gut fill. All lambs gained weight after d 8 of the experiment, but the rate of gain was higher for the wethers fed ad libitum, 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 significant. The addition of a similar concentration of dietary fat decreased DMI in previous experiments on lactating sheep (Reynolds et al. 2006). Based on visual observation, the restricted-fed lambs finished consuming their ration within approximately the first hour of the feeding period. As a result of this eating behavior, at the time of bleeding the DMI and MEI was actually higher for the restricted-fed wethers compared with those fed ad libitum. This pattern of meal consumption could influence plasma concentrations of hormones and metabolites at 6 h post feeding.
Overall changes in plasma concentration of hormones and metabolites.

This study is the first that I am aware of to report effects of intake level and fat supplementation on plasma concentrations of GLP-1, GIP, CCK, OXM and ghrelin in finishing sheep.

Similar results on plasma insulin concentration have been observed previously (Sano et al., 1999). In restricted-fed sheep, plasma insulin concentration decreased compared with 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, 2007b). The decrease in DMI would decrease the absorption of insulin secretagogues such as propionate.

The 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, 2007b). In those studies, the increase in plasma gut peptide concentrations was associated with a decrease in DMI. In the present study, there was a numerical decrease in ad libitum DMI for the wethers consuming the supplemental fat diet, which could have been caused by the increase in GLP-1 and CCK observed. Gut peptides have been reported to be associated with regulation of feed intake in non-ruminants (Gale et al., 2004). To our knowledge this is the first study to measured plasma concentration of gut peptides in sheep when intake was controlled. In the present study, plasma GLP-1 and CCK concentration was not changed by the addition of supplemental fat in the diet when wethers were fed at a restricted intake. The interaction between fat and intake could imply that CCK and GLP-1 regulate DMI in ruminants to maintain a similar MEI when they are ad libitum-fed. In restricted-fed animals the addition of fat in the diet did not
change the plasma concentration of CCK and GLP-1, eventhough they consumed almost double the amount of fatty acids (31.1 g/d in the CON versus 61.7g/d in the FAT). However, when the lambs had free access to a higher energy density diet, the plasma gut peptide concentration increased to regulate MEI to a constant amount. Therefore, the presence of fat in the diet per se does not increase plasma CCK and GLP-1 concentration. This could be seen when we compare the fatty acid intake from the restricted-FAT (61.7 g/d) with the fatty acid intake of the ad libitum-CONT (65.2 g/d), in which an increase in fatty acids of approximately 5 % produced an approximate 20 and 50 % greater response on plasma GLP-1 and CCK concentration, respectively.

Oxyntomodulin is a peptide derived from the proglucagon gene from the L cells in the small intestine (Holst, 1997). The amino acid (AA) sequence of OXM contains the sequence of glucagon plus eight AA more on the C-end of the molecule. The OXM molecule also is included in a bigger protein called glicentin. Glicentin and OXM were called “gut glucagon” (GUT) due to the cross-reactivity that they have with the pancreatic glucagon molecule in some assays (Manns, 1971). With the development of antibodies specific for pancreatic glucagon, GUT was measured as the difference between the total glucagon, which was measure with the assays that cross-react with pancreatic and the GUT assay, and the one specific for pancreatic glucagon (Manns, 1971; Ghatel et al., 1983). All the molecules of the progulcagon gene, such as GLP-1, GLP-2 and OXM or glicentin, are co-secreted by the L cells (Holst, 2007). In non-ruminants, it has been reported that OXM reduces food intake (Dakin et al., 2001; Cohen et al., 2003) by binding the GLP-1 receptor (Small and Bloom, 2004). In the present study with lambs, we did not observed significant changes in plasma OXM
concentrations due to amount of feed offered or supplemental dietary fat. However, there was a trend for plasma GLP-1 and OXM concentration to be correlated ($r = 0.28; P = 0.11$). It is not known if the L cells in ruminants secrete OXM, glicentin or other peptides which include glicentin. The metabolic pathway of the peptide in ruminants has not been reported. In a study with multi-catheterized lactating dairy cows, Benson and Reynolds (2001) showed GUT 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 increased due to an increase in MEI and by supplemental dietary fat. This difference was diminished during the 4 wk of sampling. An increase in plasma GIP concentration was observed when fat was fed in lactating dairy cows (Relling and Reynolds, 2007b) and in pre-ruminant 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 due to an increase in energy intake from abomasal infusion of casein or starch. In the present study, plasma GIP concentration in restricted/FAT-fed wethers was higher than for the ad libitum/CON-fed wethers, even though the average total fat consumption in the latter group was higher (61.7 g vs. 65.2 g, respectively). The reason for this could be due to a possible role for GIP in regulating energy redistribution, not only influenced by the total MEI, but also dependent on the source of that energy. This assumption has not been proved 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 respiratory quotient (Relling et al. 2009), indicating that GIP could play a regulatory role in energy utilization in ruminants. Plasma glucose concentration decreased due to the infusion of fat but did not differ due to the amount of DMI or MEI. A similar effect was observed previously when fat was fed ad libitum in ruminants (Relling and Reynolds, 2007b). The lack of difference in plasma glucose due to intake is likely the result of insulin regulation of glucose. In this study, there was not an association between plasma glucose concentration and plasma GIP and GLP-1 concentration. In non-ruminants, GLP-1 and GIP have been shown to decrease plasma glucose concentration (Knapper et al., 1996). However, it has been proposed for non-ruminants (Holst, 1997) and for ruminants (Martin et al, 1993a; Martin and Faulkner, 1993) that for these hormones to have role in modulating glucose, the minimum plasma glucose concentration should be 4.5 mM. This value is higher than the average plasma concentration for the wethers in the current study.

The increase in NEFA concentration due to supplemental fat and intake in the 6 h post-feeding was observed previously (Gagliostro and Chilliard, 1991; Relling and Reynolds, 2007b). The increase in 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, due to a higher plasma concentration of lipoproteins (Gagliostro and Chilliard, 1991). The increase in plasma insulin concentration for the CON animals compared with those fed the FAT 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 vs. NEFA (r
-0.20; $P < 0.02$), but not for GLP-1 and NEFA ($P > 0.30$). The negative correlation of GIP and NEFA concentration is possibly due to the role of GIP in decreasing lipolysis in ovine adipose tissue (Martin et al., 1993b); however, there are other factors that may be influencing plasma NEFA concentration. As mentioned earlier, 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 post-feeding

Plasma insulin concentration was higher in the post-feeding sampling for all treatments except for lambs fed the CON diet ad libitum. The post-prandial insulin response was observed previously in sheep (Matsunaga et al., 1999). It may be due to an increase in the absorption of insulin secretagogues such as propionate. A physiological explanation is unclear for the similar plasma insulin concentration in the CON ad libitum before and after feeding.

For plasma GLP-1 and CCK concentration, based on previous studies (Relling and Reynolds, 2007b), we were expecting higher concentrations 6 hours after feeding than before feeding. The increase in plasma concentration of GLP-1 and CCK pre-feeding compared with post feeding in the restricted-fed wethers could be due to the feeding behavior and rate of feed consumption. The restricted-fed wethers consumed all their daily diet within one hour, which could affect the continuous flow of digesta to the small intestine. It is possible that the daily pattern of secretion for GLP-1 and CCK are different in cattle than in sheep. This could explain why there was a higher plasma concentration of GLP-1 and CCK pre-feeding than after feeding. However, in two consecutive
samplings, taken at 5 and 7 h after feed in sheep (Chapter 5), there was not a significant time effect. This is similar to what was observed in dairy cattle (Relling and Reynolds; 2007b). However, it is worth noting that plasma GLP-1 and CCK concentrations have a similar trend for fat and intake effect comparing pre- and post-feeding concentrations.

As we had expected, plasma GIP concentration was higher after feeding than before feeding. This increase in plasma GIP concentration was observed previously due to an increase in nutrient absorption in sheep (McCarthy et al., 1992) and in lactating dairy cows (Relling and Reynolds, 2007b). The reason for this increase may be due to a possible role of GIP in nutrient partitioning in ruminants (Faulkner and Martin 1991). However, the design of this experiment could not confirm this assumption.

The intake x time interaction observed in this study for plasma ghrelin concentration was due to an increase only in the pre-prandial ghrelin plasma concentration for the restricted-fed wethers. Plasma ghrelin concentration was similar pre- and post-prandial in the ad libitum-fed and post-prandial for the restricted-fed wethers. This results was similar to the one observed by Wertz-Lutz et al. (2008), in which they observed an increase in plasma ghrelin concentration in beef cattle during a nutrient restriction. In the restricted-fed steers in the study by Wertz-Lutz et al. (2008), the plasma ghrelin concentration was always higher than in the non-restricted fed steers, but in the present study post prandial concentration was similar in the restricted and ad libitum-fed wethers. A possible explanation for this difference is that in the Wertz-Lutz et al. (2008) study, the animals were consuming only 80 % of their nutrient requirements and the animals were losing weight. In the present study, the restriction was smaller and lambs were gaining weight. This was also observed in ad libitum-fed dairy cows that are not in a negative energy
balance, in which there were not changes in pre- versus post-prandial plasma ghrelin concentration (Bradford and Allen, 2008). The pre-prandial increase in plasma ghrelin concentration could be due to the lower MEI imposed for the restricted-fed wethers. Pre-prandial plasma ghrelin may be stimulating appetite and contributing to the aggressive eating behavior by the restricted-fed wethers. In the present study the wethers finished their ration within one hour of being fed. In the present study, we did not observed a fat effect on plasma ghrelin concentration. However, in dairy cows studies (Bradford et al., 2008; Chapter 7) an increase in fat intake produced a decrease in plasma ghrelin concentration compared with control cows in samples taken immediately before feeding. In the present study, we did not observed a pre-prandial increase in plasma ghrelin concentration in the wethers fed the CON diet as was observed in previous experiments (Bradford et al., 2008; Wertz-Lutz et al., 2008). This does not mean that the increase did not occur in the present study, but probably the sampling schedule in the present study did not allowed us to observe that difference.

Pre-prandial plasma glucose concentration was lower than post-prandial concentration in the restricted wethers. This occurred despite higher plasma insulin post-feeding vs. pre-feeding 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 post-feeding for lambs fed the CON diet ad libitum. Unlike the restricted-fed lambs, this occurred when plasma insulin was lower. Lambs fed the FAT diet ad libitum had lower plasma glucose post-feeding than pre-feeding. These lambs also had the highest plasma insulin concentration.
The increase in pre-prandial NEFA concentration in the restricted-fed lambs may be due to an increase in fat mobilization due to a lower plasma insulin concentration. This increase pre-feeding was not observed in lambs fed ad libitum. The pre-prandial concentration of NEFA was inversely correlated with the plasma concentration of GIP/GLP-1. This inverse association could be 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 did 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, we assume that these mechanisms are interacting to drive the animal to eat.

Hypothalamic mRNA concentrations of NPY, AgRP and POMC.

In the present study, we observed a decrease in the neuropeptides that stimulate DMI (orexigenic neuropeptides NPY and AgRP), when lambs were fed ad libitum than when intake was restricted. Hypothalamus POMC mRNA concentration did not change due to 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 due to feeding fat. Results in rat models showed conflicting data of the effect of fat on neuropeptides (Wang et al., 2002; Dziedzic et al., 2007). In the study presented by Wang et al. (2002), mRNA NPY
concentration decreased for rats consuming saturated fats, but increases 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 due to high fat diets rich in saturated fat, but there were no changes for high fats rich in n-3 and n-6 fatty acids diets 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 concentration for NPY and AgRP was associated with lower DMI but similar MEI for the wethers fed FAT than for the wethers fed CON. However, the changes in hypothalamic concentration for the neuropeptides could be associated with changes in hormones and metabolites that occur in these wethers. The lower mRNA concentration in the ad libitum-fed lambs is associated with a greater degree of satiety for the ad libitum-fed lambs. The decrease in these neuropeptide concentrations for the restricted intake and the control-fed diets is probably due to the interaction of many peripheral signals that are processed in the hypothalamus. The signals that may influence the decreased in mRNA concentrations for NPY and AgRP for the ad libitum-fed lambs are: the increased plasma concentration of insulin and CCK, or decreased NEFA and pre-feeding 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, we have an inverse association between plasma insulin concentration and NPY mRNA concentration, not only due to differences in DMI, but also due to the additional dietary fat. Also in rats, it has been found 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 concentration. 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 following insulin action. 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 intra ventricular infusion of GLP-1 (Turton et al., 1996). Therefore, it is possible that GLP-1 regulates the decrease in DMI observed in the FAT ad libitum-fed wethers compared with the CONT ad libitum-fed wethers 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 concentration (Dimaraki and Jaffe, 2006) in non-ruminants. Ours is the first study that I am aware of to show the association between plasma ghrelin concentration and the hypothalamic mRNA concentration of NPY, AgRP and POMC in growing sheep. In our study, there was a positive association between pre-feeding plasma ghrelin concentration and the mRNA concentration of the orexigenic peptides. From this, we can assume that the increase in ghrelin concentration observed pre-feeding in this and other studies (Wertz-Lutz et al., 2008; Bradford and Allen, 2008) are in part responsible for an increase in appetite. It has been reported that fatty acids inhibit the expression of NPY and AgRP in non-ruminants (Lam et al., 2005). In this study, we also found a positive association between NEFA and NPY and AgRP mRNA concentration due to the additional dietary fat, but a negative association due to amount of DMI. The increase 6 h after feeding in plasma NEFA concentration due to ad libitum feeding could be another
factor that contributes to the decrease in NPY and AgRP concentration. 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 of 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 they may reflect the interaction of those changes.

In conclusion, the decrease in DMI observed when supplemental fat is provided to sheep offered feed ad libitum is associated with a down regulation of expression of the orexigenic neuropeptides. The data in this study support the assumption that the mechanisms of how the 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. However, more studies need to be conducted to be able to determine causative relationships and the separate effects of each hormone in the regulation of DMI.
References


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 (accepted to the joint national meeting of ADSA and ASAS)


<table>
<thead>
<tr>
<th>Item</th>
<th>CONT</th>
<th>FAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong></td>
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<td></td>
</tr>
<tr>
<td>Alfalfa meal, 17% CP</td>
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<td>20.00</td>
</tr>
<tr>
<td>Soy hulls</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Ground corn</td>
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<td>43.90</td>
</tr>
<tr>
<td>Megalac$^1$</td>
<td>-</td>
<td>6.00</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
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<td>8.09</td>
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<tr>
<td>Limestone</td>
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<td>0.05</td>
</tr>
<tr>
<td>Trace mineral salt</td>
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</tr>
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<td>Vitamin A, 30 kIU/g</td>
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<tr>
<td>Vitamin D, 3 kIU/g</td>
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</tr>
<tr>
<td>Vitamin E, 44 IU/g</td>
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<td>0.05</td>
</tr>
<tr>
<td>Selenium, 200mg/g</td>
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<td>Animal-vegetable fat</td>
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<td>Ammonium chloride</td>
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<tr>
<td>CP</td>
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<tr>
<td>NDF</td>
<td>28.01</td>
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</tr>
<tr>
<td>Ash</td>
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</tr>
<tr>
<td><strong>Total fatty acids</strong></td>
<td>3.31</td>
<td>7.26</td>
</tr>
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</table>

Table 2. Formulation and chemical composition (% of DM basis) of the control diet and diet supplemented with 6% Ca-salts of palm oil$^1$

$^1$ Ca-salts of palm oil (Megalac®, Church and Dwight Co., Inc., Princeton, NJ).

$^2$ CONT= control diet; FAT= diet with the addition of 6% of Ca-salts of palm oil.
<table>
<thead>
<tr>
<th>Item</th>
<th>Restricted CON&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Restricted FAT&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ad libitum CON&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ad libitum FAT&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SEM rest&lt;sup&gt;3&lt;/sup&gt;</th>
<th>SEM adlib&lt;sup&gt;3&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;4&lt;/sup&gt; F</th>
<th>P-value&lt;sup&gt;4&lt;/sup&gt; I</th>
<th>P-value&lt;sup&gt;4&lt;/sup&gt; FxI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n wethers (pens)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Daily DMI, kg</td>
<td>0.94</td>
<td>0.85</td>
<td>1.97</td>
<td>1.74</td>
<td>-</td>
<td>0.08</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Daily MEI, Mcal</td>
<td>2.46</td>
<td>2.46</td>
<td>5.20</td>
<td>5.01</td>
<td>-</td>
<td>0.23</td>
<td>0.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DMI at bleeding&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.94</td>
<td>0.85</td>
<td>0.64</td>
<td>0.66</td>
<td>0.01</td>
<td>0.025</td>
<td>0.26</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>MEI at bleeding&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.46</td>
<td>2.46</td>
<td>1.67</td>
<td>1.91</td>
<td>0.03</td>
<td>0.179</td>
<td>0.02</td>
<td>0.01</td>
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</table>

Table 3. Mean DMI and MEI of growing wethers, ad libitum-fed or restricted-fed diets with or without the addition of 6 % Ca salts of palm oil<sup>1</sup>.

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 CONT= control diet; FAT= diet with the addition of 6 % of Ca-salts of palm oil.

3 rest= restricted-fed wethers; adlib= ad libitum-fed wethers.

4 F= main effect of addition of fat in the diet; I= main effect of the difference in intake; FxI= interaction of fat and intake main effects.

5 Pen was used as the experimental unit.

6 DMI (kg) and MEI (Mcal) at bleeding is the amount that the wethers had consumed after 6 h the feed was offered.
<table>
<thead>
<tr>
<th>Item</th>
<th>Restricted CON²</th>
<th>Restricted FAT²</th>
<th>Ad libitum CON²</th>
<th>Ad libitum FAT²</th>
<th>SEM rest³</th>
<th>SEM adlib³</th>
<th>P-value ⁴</th>
<th>F</th>
<th>I</th>
<th>FxI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, pM</td>
<td>287</td>
<td>211</td>
<td>308</td>
<td>295</td>
<td>21</td>
<td>21</td>
<td>0.04</td>
<td>0.02</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>GLP-1a, pM</td>
<td>21</td>
<td>24</td>
<td>29</td>
<td>38</td>
<td>1</td>
<td>2</td>
<td>0.02</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>OXM³, pM</td>
<td>177</td>
<td>188</td>
<td>197</td>
<td>186</td>
<td>9</td>
<td>12</td>
<td>0.98</td>
<td>0.44</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>CCK⁵, pM</td>
<td>4.94</td>
<td>6.07</td>
<td>9.14</td>
<td>17.38</td>
<td>0.47</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>GIPb, pM</td>
<td>79</td>
<td>103</td>
<td>93</td>
<td>122</td>
<td>5</td>
<td>6</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>Glucose, mM</td>
<td>4.33</td>
<td>4.06</td>
<td>4.35</td>
<td>4.17</td>
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<td>0.06</td>
<td>0.01</td>
<td>0.218</td>
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</tr>
<tr>
<td>NEFAa, mM</td>
<td>60</td>
<td>74</td>
<td>65</td>
<td>86</td>
<td>3</td>
<td>4</td>
<td>0.01</td>
<td>0.01</td>
<td>0.126</td>
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</tbody>
</table>

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 Each mean represents blood samples taken on d 8, 15, 22 and 29 of the experiment.

2 CONT= control diet; FAT= diet with the addition of 6 % of Ca-salts of palm oil.

3 rest= restricted-fed wethers; adlib= ad libitum-fed wethers.

4 F= main effect of addition of fat in the diet; I= main effect of the difference in intake; FxI= interaction of fat and intake main effects.

5 GLP-1= glucagon-like peptide-1; OXM= oxyntomodulin; CCK= cholecystokinin; GIP= glucose-dependent insulinotropic polypeptide.

a Time effect P < 0.05.

b Intake x Fat x Time P <0.10.
<table>
<thead>
<tr>
<th>Item</th>
<th>Restricted</th>
<th>Ad libitum</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON^1 FAT^1</td>
<td>CON^1 FAT^1</td>
<td>SEM rest^2 adlib^2</td>
<td>SEM fat intake F x I time F x I x T</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre feeding</td>
<td>137 121</td>
<td>284 300</td>
<td>22 27</td>
<td>0.70 0.01 0.06 0.01 0.03</td>
</tr>
<tr>
<td>Post feeding</td>
<td>245 190</td>
<td>268 359</td>
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<td></td>
</tr>
<tr>
<td>GLP-1^4, pM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre feeding</td>
<td>23 31</td>
<td>35 44</td>
<td>3 3</td>
<td>0.01 0.01 0.37 0.05 0.07</td>
</tr>
<tr>
<td>Post feeding</td>
<td>22 26</td>
<td>30 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCK^4, pM</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pre feeding</td>
<td>5.75 9.27</td>
<td>12.71 20.45</td>
<td>1.43 3.08</td>
<td>0.03 0.01 0.08 0.07 0.20</td>
</tr>
<tr>
<td>Post feeding</td>
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<tr>
<td>GIP^4, pM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pre feeding</td>
<td>44 47</td>
<td>68 87</td>
<td>6 8</td>
<td>0.01 0.16 0.01 0.01 0.61</td>
</tr>
<tr>
<td>Post feeding</td>
<td>82 79</td>
<td>81 100</td>
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<tr>
<td>Ghrelin^a, pM</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre feeding</td>
<td>49 52</td>
<td>18 23</td>
<td>4 1</td>
<td>0.22 0.01 0.80 0.01 0.91</td>
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<tr>
<td>Post feeding</td>
<td>17 23</td>
<td>15 24</td>
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Table 5. Plasma hormone and metabolite concentrations in growing wethers 30 min pre-feeding and 6 h post-feeding after 29 d of feeding ad libitum or at restricted intake, diets with or without the addition of 6 % Ca salts of palm oil.
Table 5 continued.

<table>
<thead>
<tr>
<th></th>
<th>Glucose&lt;sup&gt;a,b&lt;/sup&gt;, mM</th>
<th>NEFA&lt;sup&gt;a&lt;/sup&gt;, mM</th>
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<tbody>
<tr>
<td></td>
<td>Pre feeding</td>
<td>Post feeding</td>
</tr>
<tr>
<td></td>
<td>3.73 3.68 4.14 4.31</td>
<td>4.24 3.88 4.37 4.20</td>
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<tr>
<td></td>
<td>0.06 0.07 0.07 0.01</td>
<td>0.01 0.01</td>
</tr>
<tr>
<td></td>
<td>0.01 0.87</td>
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</table>

CONT = control diet; FAT = diet with the addition of 6% of Ca-salts of palm oil.

rest = restricted-fed wethers; adlib = ad libitum-fed wethers.

fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F x I = interaction of fat and intake main effects; time = Main effect of pre and post feeding; F x I x T = interaction of fat, intake and time main effects.

GLP-1 = glucagon-like peptide-1; CCK = cholecystokinin; GIP = glucose-dependent insulinotropic polypeptide.

<sup>a</sup> Time x intake P < 0.01.

<sup>b</sup> Time x fat P < 0.01.
Table 6. Relative mRNA concentration for hypothalamic neuropeptides that regulate DMI after 30 d of ad libitum or restricted feeding diets with or without the addition of 6% Ca salts of palm oil.

1 CONT = control diet; FAT = diet with the addition of 6% of Ca-salts of palm oil.

2 fat = main effect of addition of fat in the diet; intake = main effect of the difference in intake; F x I = interaction of fat and intake main effects.

3 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.
Figure 6. Average BW (kg) of growing lambs restricted-fed with (FAT, □) or without (CONT, ○) the addition of 6 % Ca salts of Palm oil, and ad libitum-fed with (FAT, ■) or without (CONT, ●) the addition of 6 % Ca salts of Palm oil after a 29 d feeding period. $P < 0.01$ for intake x fat x time interaction.
Figure 7. Plasma GIP concentration (pmol/ml) of growing lambs restricted-fed with (FAT, □) or without (CONT, ○) the addition of 6 % Ca salts of Palm oil, and ad libitum fed with (FAT, ■) or without (CONT, ●) the addition of 6 % Ca salts of Palm oil after a 29 d feeding period. * indicates $P < 0.05$ for fat x intake x time means separated using the slices option of SAS.
CHAPTER 5

EFFECT OF PHYSIOLOGICAL INTRA-JUGULAR INFUSION OF GLUCAGON-LIKE PEPTIDE-1 AND CHOLECYSTOKININ ON DRY MATTER INTAKE, DIGESTIBILITY, AND CROMIUM OXIDE RATE OF PASSAGE IN GROWING WETHERS

Abstract

A decrease in DMI when fat is added to ruminant diets has been associated with increased plasma concentrations of some gastrointestinal hormones, such as glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK). However, a cause and effect relationship between these hormones and regulation of feed intake has not been established and the effect of infusing physiological amounts of these hormones on DMI has not been investigated in ruminants. The objectives of this experiment were to achieve an increase in plasma GLP-1 and CCK concentration via jugular infusion in sheep similar to the increase due to feeding fat; and to determine the effect of GLP-1 or CCK infusion on daily and post-feeding DMI, DM and nutrient digestibility, and rate of passage of orally dosed Cr in growing wethers. Eighteen cross-bred growing lambs were fed a pelleted control diet containing (DM basis) 60% concentrate and 40% forage in a 9 x 2
replicated incomplete Latin square with two week periods. The treatments were a control (0% added fat), addition of two amounts of Ca salts of palm oil fatty acids (4 or 6% of ration DM), and 3 jugular vein infusion doses of GLP-1 (0.052, 0.103, or 0.155 µg/kg BW/d) or CCK (0.069, 0.138, or 0.207 µg/kg BW/d). Amounts infused were based on first order kinetics, hormone half-life, and estimated plasma volume. Each infusion period was six days. Hormone concentrations were measured in jugular vein plasma from samples taken on d 1, 4 and 6 of each period. Increases in GLP-1 and CCK concentrations during hormone infusions were comparable to increases observed when increasing amounts of fat were fed. Feeding fat tended to decrease DMI (linear, P < 0.13). The infusion of 0.052 µg/kg BW/d GLP-1, but not the greater doses, tended to decrease DMI (cubic, P < 0.09). Infusion of CCK did not affect DMI. Feeding fat and GLP-1 infusion (linear, P < 0.09 and 0.12, respectively) tended to decrease DMI during the first hour after feeding. For CCK infusion, DMI during the first hour after feeding increased for the lowest and highest doses of CCK but decreased for the middle dose (quadratic, P < 0.01) during the first hour after feeding. Retention time of Cr in the rumen and total GIT decreased (linear, P < 0.01) when fat was fed, but was not affected by GLP-1 or CCK infusion. In conclusion, jugular vein infusion rates based on first-order kinetics produced similar plasma CCK and GLP-1 concentrations as observed when fat was fed. Infusion of GLP-1 had effects on DMI and initial meal size similar to effects of feeding fat. However, it is likely that effects of feeding fat on DMI are due to additive effects of multiple hormones and metabolic signals, not solely to changes in plasma GLP-1 or CCK concentration.
Introduction

The addition of dietary fat decreases DMI in sheep (Reynolds et al., 2006) and cattle (Relling and Reynolds, 2007b). The decrease in DMI due to feeding fat has been associated with an increase in plasma concentration of the gut hormones glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) (Chapter 4, Relling and Reynolds, 2007b). In non-ruminants, the effects of GLP-1 and CCK on feed intake have been widely studied. Glucagon-like peptide-1 decreased feed intake when infused intravenously in humans (Holst, 2004) or infused intracerebrally in rats (Turton et al., 1996). Also, GLP-1 has been reported to decrease small intestine motility (Spiller et al., 1984, Holst, 1997). Cholecystokinin decreases feed intake by reducing meal size in non-ruminants (Moran, 2004). Also, CCK increases exocrine pancreas secretion and reduces gut motility (Walsh, 1994).

In ruminants, there are no data on the effects of GLP-1 infusion on DMI or gut motility. In sheep, pharmacological doses of CCK resulted in decreased DMI when CCK was infused into blood circulation (Grovum, 1981) or into the central system (Della Ferra and Baile, 1979). Infusion of CCK also decreased gut motility (Grovum, 1981; Romański, 2004) and increased exocrine pancreas secretion (Tachibana et al., 1995). However, the effect of a physiological CCK infusion dose that mimics the increase in CCK concentration produced by feeding fat, has not been studied previously.

Based on the literature, the hypotheses tested in the current study were: 1) the addition of dietary fat increases plasma concentration of GLP-1 and CCK, decreases DMI, decreases meal size and reduces the rumen and total rate of digesta passage through the gut, and does not change MEI; 2) GLP-1 or CCK can be infused at rates based on a first order
kinetics model to increase plasma concentration of these hormones to concentrations similar to those observed when feeding fat; 3) the infusion of GLP-1 decreases DMI and rate of digesta passage through the gut, does not affect meal size, and increases DM digestibility; 4) the infusion of CCK does not change DMI, decreases rumen, and total rate of digesta passage, increases DM and CP digestibility, and decreases meal size.

The objective of the experiment was to determine the effect of physiological concentrations of GLP-1 and CCK on daily and post feeding DMI, rate of passage of orally dosed Cr$_2$O$_3$, and DM and nutrient digestibility in growing lambs. To accomplish this, a first order kinetic model of hormone degradation was used to estimate the amount of hormone needed to infuse to be able to achieve similar concentrations of GLP-1 and CCK via infusion of the peptides to that observed when fat was fed.

Materials and methods

Animals and diets

All animal procedures were approved by the Agricultural Animal Care and Use Committee of The Ohio State University, following guidelines recommended in Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1998). For one month before the start of the experiment, twenty-four Targhee x Hampshire wethers (36.5 ± 2.5 kg BW) were fed a pelleted control diet (Table 7) formulated to meet nutrient requirements of growing lambs according to the National Research Council (1985). The lambs were grouped by weight and housed in two pens with 12 wethers each. Daily rations were provided at 0800 h, and lambs were fed for ad libitum intake of DM (10% refusal).
Treatments and experimental design.

Treatments were six days of: 1) control diet (without supplemental fat); 2) supplemental dietary fat at 4% of ration DM (control diet plus the addition of Ca-salts of palm oil - Table 7); 3) supplemental dietary fat at 6% of ration DM; 4 to 6) incremental intravenous (jugular vein) infusion of CCK-8 sulfide (C2175 Sigma-Aldrich Inc, St Louis, MO) at 0.069, 0.138, or 0.207 µg/kg BW/d, respectively; 7 to 9) or incremental intravenous infusion of GLP-1 (H6795 GLP-1(7-36) amide, Bachem California Inc, CA) at 0.052, 0.103, or 0.155 µg/kg BW/d, respectively. The hormone solutions were prepared using one L of sterile saline solution (9 g/L of NaCl). The animals fed the control and the fat supplemented diets were intravenously infused with one L of sterile saline solution (9 g/L of NaCl). The targeted doses of the hormones infused were calculated using a single compartment, first-order kinetic hormone degradation, based on the equation:

\[ \text{Increase in hormone concentration} \times 0.693/\text{half life} \]

The half life used for this equation was 5 min for GLP-1 (Perfetti and Merkel; 2000) and 1.5 min for CCK (Hoffmann et al. 1993). The target increase for the 0.138 and 0.103 µg/kg BW/d doses for CCK and GLP-1 respectively were based on a previous study in which fat was fed to lactating dairy cows and CCK-8 and GLP-1 were measured (Relling and Reynolds, 2007a). These were designated as 100% doses; the remaining doses were 50 or 150% of the 100% doses. The liter of saline solution was infused at a rate of 0.725 ml/min during 23 h.

The experimental design was a 9 x 2 incomplete duplicated Latin square. Each of the two groups of lambs was considered as a square, and they were used in two periods, with an interval of two weeks between each period. The treatments were randomly distributed,
with the restriction that in the second period the wethers were not allowed to repeat their period 1 treatment.

Two weeks before the experiment began the wethers were housed in individual pens. Wethers fed supplemented fat were adapted to the diet with incremental increases in fat concentration in the diet; 2 % of ration DM fed for the first day of the adaptation period, 4 % on the second day, and the total 6 %, for those specific treatment animals, on d 4. One week before the experiment started, the lambs were moved into metabolic crates described previously (Murphy, et al. 1994). During the week prior to the experiment, the animals were adapted to all the procedures that were conducted during the sampling week, including fecal sampling and periodically removing and weighing the feed from the bunks to measure pattern of DMI within the day. Forty-five hours before the experiment started, catheters were established in the jugular vein of nine wethers as described previously (Huntington et al. 1989), with a slight adjustment in the length of the catheter (36 cm total length, of which 20 cm were inside the jugular vein). Selection of the nine wethers from the group of twelve for catheterization was based on previous consistency of DMI. The infusions started at 1000 h. The bottles with sterile saline solution and the ones with hormones in solution were kept refrigerated during the infusion. The infusion line from the bottle to the animal was sterilized using an Anprolene ethylene oxide (EtO) gas sterilizer system (EW-100778-00, Cole-Parmer, Vernon Hills, IL). The connection between the bottle with the infusion and the infusion line had a sterile 25-mm syringe filter of 0.45 µm (05-713-387 Whatman International Ltd, Florham Park, NJ). Between the end of each day’s infusion and the start of the
following day, the infusion lines were flushed with sterile saline solution (9 g/L of NaCl) and the filters were changed.

Sampling and analyses.
Feed was offered daily at 1300 h and the refusals were removed and weighed 23 h later at 1200 h. The DM content of feed and refusal was measured by drying at 100 °C for 24 h. These measures were used to determine DMI. Feed samples were taken on each day and were pooled for analysis of crude protein (CP), fatty acids (FA), neutral detergent fiber (NDF) and ash concentration as described previously (Beckman and Weiss, 2005). Five minutes before feeding on the third day of the experimental period, 2 g of Cr₂O₃ was given in an intraruminal bolus delivered in a gelatin capsule via the esophagus with the aid of a balling gun. Fecal samples were collected via the rectum at 6, 11, 18, 24, 30, 35, 42, 48, 54, 59, 66, 72, 78, 83, 90 and 96 h after the Cr₂O₃ dosing to determine fecal Cr concentrations over time. The estimate of Cr rate of passage was calculated from the natural logarithms of the fecal Cr concentrations over time using the line plus exponential curve fitting function of Genstat 10.2 (VSN International Ltd., Hemel Hempstead, UK). Parameters to describe Cr rate of passage were calculated using a multicompartamental model (Dhanoa et al., 1985). The model describes Cr flow through an infinite number of gastrointestinal pools that are associated with fractional outflow rate. The reciprocal of the fractional outflow rate is the mean retention time (h) of Cr in that compartment. In the model, the two smallest fractional outflow rates, k₁ and k₂, represent the two pools within the gastrointestinal tract with the longest retention times, whereas transit time (TT; h) describes passage through the remaining minor pools. Total mean retention time
(TMRT; h), representing the mean retention time of Cr in the entire digestive tract and was calculated as:

\[ \text{TMRT} = \frac{1}{k_1} + \frac{1}{k_2} + TT. \]

Assuming \( k_2 \), the ascending slope, describes the rate at which chromium particles pass through the hindgut, and \( k_1 \), the descending slope, represents outflow rate of chromium from the rumen (Gasa et al., 1991; Grovum and Williams, 1973), mean rumen retention time (RMRT; h) was calculated as:

\[ \text{RMRT} = \frac{1}{k_1} + \frac{1}{k_2}. \]

Consequently, TT describes the passage time of Cr through the section of the digestive tract posterior to the rumen.

To measure digestibility, total fecal collection was performed daily during the last 5 d of each experimental period. Five percent of the total daily feces was collected and pooled for analysis of DM, NDF, CP, FA, and ash concentration. The total intake was compared with the fecal output to calculate digestibility (Murphy, et al. 1994). Blood samples (10 ml) were taken 6 and 8 h after feed was offered on d 1, 4 and 6 of each experimental period. Blood samples were immediately transferred into polypropylene tubes containing solutions of disodium EDTA and benzamidine HCl (1.6 mg and 4.7 mg/ml blood, respectively) and placed on ice. After centrifugation for 25 min at 1800 X g and 4°C, plasma was partitioned into individual polypropylene tubes for each analysis to be performed, flash frozen using liquid N\textsubscript{2} within 40 min of sample collection, and stored at -80°C until analyzed. For CCK analysis, equal volumes of plasma from both sequential samples were composited in a tube containing 500 Kallikrein inhibitor units (KIU) of dried aprotinin (Trasylol, Bayer, Germany) per ml of plasma added (Benson and
Reynolds, 2001), flash frozen after each addition and then mixed when thawed for analysis.

Hormone and metabolite analysis.
Concentrations of insulin, GLP-1, and CCK-8 were measured using RIA as described previously (Benson and Reynolds, 2001), and validated based on parallel displacement of labeled hormone binding by serial addition of ovine plasma samples. The intra-assay CV averaged less than 12.5 %, 10 %, and 10.3 % for insulin, GLP-1 and CCK-8 assays, respectively. Minimum sensitivities (90% of zero standard binding) of the insulin, GLP-1, and CCK-8 assays were 0.0027, 0.001 and 0.0006 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 two-reaction, enzyme-based assay (Wako Chemicals USA, Richmond, VA) as described by Johnson and Peters (1993).

Statistical analysis
The data were analyzed as 9 x 2 incomplete replicated Latin square with repeated measurements in time using the MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC) with a model testing the random effects of wether, period and square, and the fixed effect of treatment and time and their interaction. For digestibility, a similar statistical model was used without the effect of time. The two daily plasma samples taken at 6 and 8 h post-feeding on the three days of sampling in each experimental period were analyzed in the lab individually but the average hormone and metabolite concentrations
of both were used in the statistical analysis as one daily value. Orthogonal polynomial contrasts were used to partition total treatment sums of squares into the following comparisons: linear and quadratic effect of fat, and linear quadratic and cubic effect of GLP-1 or CCK-8. Because the unequal spacing of the fats treatments (0, 4 and 6%) Proc IML of SAS was used to estimate the appropriate coefficients.

Results

Plasma concentration of hormones and metabolites.

Increasing inclusion of fat in the diet did not \( (P > 0.15) \) increase plasma GLP-1 concentration but caused a linear \( (P < 0.02) \) and quadratic \( (P < 0.03) \) increase in plasma CCK concentration (Table 8). There were no effects \( (P > 0.2) \) of supplemental fat on plasma insulin, glucose or NEFA concentration.

Plasma GLP-1 and CCK concentrations during the infusion of these hormones were similar to the plasma concentrations obtained when fat was fed, i.e., fat 4% vs. CCK 0.138 µg/kg BW/d and GLP-1 0.103 µg/kg/d; and fat 6% vs. CCK 0.207 µg/kg BW/d and GLP-1 0.155 µg/kg/d. However, the increases of plasma CCK and GLP-1 were not statistically significant. Incremental infusion of GLP-1 did not affect insulin, GLP-1, CCK, or glucose plasma concentration \( (P > 0.20; \text{Table 9}) \), but tended to increase plasma NEFA concentrations \( (P < 0.08; \text{Table 9}) \). The incremental infusion of CCK increased plasma CCK concentration (Linear \( P < 0.07 \); cubic \( P < 0.02 \)), but did not affect insulin, GLP-1, glucose or NEFA plasma concentrations \( (P > 0.15; \text{Table 10}) \).
DMI, MEI and digestibility

Supplemental dietary fat tended to decrease DMI (Linear $P < 0.12$), and decreased ash digestibility (Linear $P < 0.05$; Quadratic $P < 0.01$). It also tended to increase CP digestibility (Linear $P < 0.11$), but did not affect ($P > 0.15$) MEI, or the digestibility of DM, NDF, FA, and OM (Table 11). The infusion of GLP-1 decreased DMI (Cubic $P < 0.09$) and MEI (Cubic $P < 0.09$), but did not affect ($P > 0.15$) the digestibility of DM, NDF, CP, FA, OM, or ash (Table 12). Incremental infusion of CCK did not affect ($P > 0.15$) DMI, MEI, DM, NDF, FA, OM, or ash digestibility (Table 13); however, the dose of 0.207 µg/kg BW/d of CCK decreased (Quadratic $P < 0.09$) CP digestibility (Table 13).

Pattern of intake

Feeding increasing amounts of fat decreased DMI during the first h after feeding (Linear $P < 0.09$), and from 8 to 16 h after feeding (Linear $P < 0.12$; Table 14). There was a quadratic ($P < 0.01$) effect of supplemental fat on DMI for 2 to 4 h after feeding, with the lowest DMI for wethers fed the diet containing 6% supplemental fat. The cumulative effect of DMI over time when fat was fed followed the same trend that was observed for the first hour after feeding. A linear decrease in DMI with increasing dietary fat was observed for cumulative intakes at 2, 4, 8, and 16 h after feeding ($P < 0.12$, 0.08, 0.04, and 0.02; respectively). Incremental infusion of GLP-1 tended to decrease meal size during the first h after feeding (Linear $P < 0.12$; Table 15) and during the period of 8 to 16 h (Linear $P < 0.08$) after feeding. There was a cubic ($P < 0.04$) effect during the period between 4 to 8 h after feeding when GLP-1 was infused, due to an increase in DMI on the 0.103 µg/kg BW/d dose of GLP-1. As was observed for increasing dietary
fat, the incremental increase in infusion of GLP-1 tended to linearly decreased cumulative DMI at 2 and 4 h after feeding ($P < 0.14, 0.10$; respectively), there was an equilibration at 8 h after feeding ($P > 0.20$) and a trend for decreased intake at 16 h after feeding ($P < 0.12$). Incremental infusion of CCK affected DMI on the first h after feeding (Cubic $P < 0.01$) with an increased intake for the 0.069 $\mu$g/kg BW/d treatment, a decrease for 0.138 $\mu$g/kg BW/d and an increase for the 0.207 $\mu$g/kg BW/d infusion (Table 16). During the period from 1 to 2 h after feeding CCK infusion increased DMI for the 0.069 $\mu$g/kg BW/d and 0.138 $\mu$g/kg BW/d doses, compared to the Control and the 0.207 $\mu$g/kg BW/d infusion treatment (Quadratic $P < 0.04$). There was an effect on DMI during the period of 4 to 8 h after feeding (Cubic $P < 0.04$), due to a decrease in DMI for the 0.069 $\mu$g/kg BW/d treatment. From 16 to 23 h after feeding, increasing infusion of CCK resulted in increased DMI (Linear, Quadratic, and Cubic $P < 0.01$). The increase in DMI with incremental CCK infusion was observed also for the cumulative DMI at 4 and 8 h after feeding (Linear, Quadratic, and Cubic $P < 0.01$), but at 2 and 16 h after feeding the cumulative DMI was not significant different among CCK infusions ($P > 0.30$).

Chromium rate of passage

Feeding incremental amounts of dietary fat decreased rumen (Linear $P < 0.01$; Cubic $P < 0.04$) and total mean retention time for Cr(Linear $P < 0.01$; Cubic $P < 0.12$; Table 17) and resulted in a slight increase in ruminal passage rate (Linear $P < 0.02$). Incremental increases in infusion of GLP-1 or CCK did not affect ruminal or total tract Crretention time ($P > 0.17$; Table 18 and Table 19). Increasing CCK infusion dose increased ruminal Crpassage rate (Linear $P < 0.01$; Quadratic $P < 0.01$).
Discussion

Plasma concentration of hormones and metabolites.

In the current study, increasing amounts of dietary fat increased plasma GLP-1 and CCK concentration. Compared to the Control diet, 6% supplemental fat resulted in a 20 and 70% increase in plasma GLP-1 and CCK, respectively. The numerical increase in GLP-1 concentration lacked significance, perhaps due to variation or insufficient replication. Perhaps the lack of a significant effect of fat supplementation for GLP-1 in this study compared with the response observed in lambs fed ad libitum in Chapter 4 could be associated with the fact that the wethers in crates in the present study did not reach the same DMI or ADG as the wethers from Chapter 4 that were housed in pens. Additionally, there was greater replication in Chapter 4.

Using a single compartment, first-order kinetic hormone degradation equation to calculate infusion doses, the plasma concentration of GLP-1 and CCK were similar to the plasma GLP-1 and CCK concentration reached when fat was fed. Infusion of GLP-1 numerically increased plasma GLP-1 concentration, but the increase was not statistically significant. The plasma GLP-1 for the 0.103 µg/kg BW/d dose of GLP-1 did not fit the pattern of response observed for the 0.052 and 0.155 µg/kg BW/d doses. The unexplainably low concentration for the 0.103 µg/kg BW/d dose does not fit a biological response and the value may be spurious. The infusion of CCK produced an increase in plasma CCK concentration as would be expected. It is noteworthy that infusion of increasing doses of GLP-1 did not affect plasma CCK and infusion of CCK did not affect plasma GLP-1.
The infusion of GLP-1 produced a linear increase in plasma NEFA concentration, which was not expected, based on previous results that showed GLP-1 decreases lipolysis in the adipose tissue (Martin et al., 1993). However, this increase in NEFA concentration does not confirm that the change was due to changes in lipolysis within the adipose tissue. More research is needed to further understand the role of GLP-1 in adipose tissue metabolism. Increasing fat supplementation and infusion of GLP-1 and CCK did not affect plasma concentration of glucose or insulin.

DMI, MEI and DM and chemical digestibility

In previous studies where supplemental fat was fed to ruminants, a decrease in DMI was observed (Reynolds et al., 2006, Relling and Reynolds, 2007b). In the current study, there was an 8-15% decrease in DMI, but the difference was not statistically significant. As explained for the plasma hormone data, the lack of significance may be due to insufficient replication or lambs housed in the metabolism crates did not reach their maximum growth capacity or intake.

The digestibility of DM, CP, FA and OM was not affected by the amount and source of supplemental dietary fat used in this experiment. Similar trends were observed in lactating dairy cows for DM, OM, NDF, CP, and FA digestibility (Palmquist, 1991; Harvatine and Allen, 2006). It is worthy of mention that the values for apparent fatty acid digestibility reported here are similar to those reported by Palmquist (1991). Based on Palmquist (1991), the true digestibility of fatty acids would be expected to decrease with the addition of dietary fat. The quadratic response for ash digestibility in the present
study was not expected; however, the decrease observed in the wethers fed 6% of Ca salts of palm oil fatty acids may be due to an increase of Ca lost in the feces.

The infusion of GLP-1 decreased DMI compared to the Control for the 0.052 µg/kg BW/d dose, but this decrease was not as dramatic for the 0.103 and 0.155 µg/kg BW/d doses. As discussed above, the plasma GLP-1 concentration for the 0.103 µg/kg BW/d dose was lower than would be expected. It is not known if this affected the total DMI responses observed. The effects of GLP-1 infusion on details of meal consumption patterns will be discussed later. Because the diet in all the treatments of GLP-1 infusion had the same ME, MEI had the same trend as observed for DMI. This is the first study that I am aware of in ruminants where GLP-1 was infused in physiological doses, and DMI was reported. In non-ruminants, GLP-1 decreases feed intake (Holst, 2006), via a hypothalamic mechanism (Turton et al., 1996). However, when sheep hypothalamus was cultivated in vitro in media containing GLP-1, the mRNA concentration for neuropeptides that regulate DMI did not change (Chapter 3). Because DMI is regulated by many factors (Forbes, 1985), the physiological increase of GLP-1 by itself may not be enough to depress total daily DMI. In the present study, the infusion of 0.052 µg/kg BW/d of GLP-1 resulted in a greater plasma concentration than the 0.103 µg/kg BW/d dose and DMI followed a similar pattern. From these data, we cannot confirm that the increase within physiological ranges in plasma GLP-1 concentration is what causes decreased total daily DMI, but because of the inverse association between plasma GLP-1 and DMI, it is possible that GLP-1 contributes to the regulation of DMI. This regulation may involve changes in feeding behavior and meal consumption patterns as discussed later.
In the present study there was no effect of CCK infusion on DMI, MEI, or DM, NDF, FA, OM, and ash digestibility. In previous studies in sheep, the infusion of CCK decreased DMI when it was infused in the jugular vein (Grovum, 1981) or intracerebrally infused (Della Ferra and Baile, 1979). Grovum (1981) observed a decrease in DMI when CCK was infused at an approximate dose of 5 µg/kg BW/d. This was an acute response (10 min) after infusing CCK in fasting sheep. In that study, no differences in intake were observed for smaller doses. Using the single compartment, first-order kinetic hormone degradation equation described for the present study, the doses used by Grovum would be considered to be pharmacological in nature. Additionally, Grovum (1981) did not describe the active form of CCK used. It is known that feeding fat increases plasma CCK concentration (Relling and Reynolds, 2007b). That increase was measured using a kit that binds to CCK-8 sulphate and also cross-reacts with CCK-33 sulphate. Despite the ability to measure such an increase, it does not mean that there are not other CCK forms secreted in ruminants, as occurs in humans (where the predominant CCK circulating form is CCK-33; Walsh, 1994) or in dogs (where CCK-58 is a bioactive form; Reeve et al., 2003). It has been observed in rats that bioactive CCK-8 is synthesized in the hypothalamus (De Fanti et al., 1998). The reason for the decrease in intake observed when CCK-8 was centrally (CSV) infused (Della Ferra and Baile, 1979) with smaller doses than were used in the present study may be due to paracrine functions of CCK in the hypothalamus as a neuropeptide. Although the dose used by Della Ferra and Baile (1979) was smaller than the one used in the present experiment, the final concentration in the ventricle was likely greater.
Because CCK is known to increase gallbladder contraction (Walsh, 1994; Romański, 2004), and increase pancreatic secretion (Walsh, 1994; Tachibana, 1995) an increase in DM, CP and FA digestibility was hypothesized for infusion of CCK in the present study. However, no changes in DM and FA digestibility were observed. There was a quadratic effect of CCK infusion on CP digestibility, with a slight increase for the 0.069 µg/kg BW/d dose, and a decrease for 0.207 µg/kg BW/d dose. In the present study we did not measure pancreatic exocrine secretion or gallbladder contraction, but it can be assumed that the effect of CCK for the present study was not similar to what was observed by Tachibana et al. (1995) for pancreas secretion, or by Romański (2004) for gallbladder contraction. This assumption is based in the pharmacological doses used in both of those experiments. The dose used by Tachibana et al. (1995) was a bolus dose of 0.121 µg/kg BW, which was the same amount used in the middle dose; however, the dose in the current experiment was infused in 24 h, not given as a bolus. On the other hand Romański (2004) used bolus doses that went from 0.02 to 2 µg/kg BW.

Pattern of intake

As hypothesized, feeding increasing amounts of dietary fat reduced DMI during the first h after feeding. The assumption, based on non-ruminant literature (Moran, 2004), was that the decrease in DMI was produced by the resultant increase in plasma CCK concentration; however, the plasma CCK concentration for the wethers fed 4 % fat was greater than for the wethers fed 6 % supplemental dietary fat. It is possible that the decrease in DMI observed due to increased fat consumption was regulated by GLP-1 (D’Alessio, 2008). In the time period between 2 and 4 h after feeding, the wethers fed 4
% additional dietary fat had an increase in DMI resulting in greater DMI than for lambs fed the control diet during that period. This was likely a compensatory response affected by the reduced intake during the earlier periods. However, the cumulative DMI follows the same pattern throughout the day. Cumulative intakes throughout the day showed a consistent linear decrease in response to supplemental dietary fat. During the first 4 hours post-feeding, the lambs fed 6% supplemental fat had a 45% reduction in DMI compared with the Control lambs. This pattern persisted up to 16 h post-feeding, although the effect of fat was not as dramatic for the later times. These data indicate that the decrease in DMI intake caused by feeding fat is influenced by size of individual meals and pattern of intake as well as by a daily regulation of DMI to maintain caloric intake.

When GLP-1 was infused at increasing doses, DMI during the first hour after feeding showed a similar trend as when fat was fed. The cumulative DMI for most of the day showed a linear decrease in cumulative DMI with increasing GLP-1 infusion. This relationship was even more pronounced if the actual plasma GLP-1 concentrations are considered. The unexplainably low plasma GLP-1 concentration for the 0.103 µg/kg BW/d GLP-1 infusion coincided with a higher cumulative intake pattern than was observed for the lower and higher doses. These responses to GLP-1 infusion and plasma concentration were nearly identical to the responses in plasma GLP-1 and decreased cumulative intake during the day observed for increased supplemental dietary fat. The highest dose of GLP-1 infused resulted in nearly identical plasma GLP-1 as observed for wethers fed 6% supplemental fat. The decrease in cumulative DMI for the highest GPL-1 infusion and 6% supplemental fat was very similar at each time point post-feeding. These data provide compelling evidence for GLP-1’s role in regulating DMI when fat is fed.
When CCK was infused the response pattern for cumulative DMI showed a different trend than the effect of fat, not only during specific time periods after feeding, but also for the cumulative DMI data. These data suggest that the increase in plasma CCK when fat was fed was coincidental and not the causative factor in decreasing DMI. From the data in the current study it can be concluded that GLP-1 may play a more important role in regulating DMI in response to dietary fat than CCK.

Chromium rate of passage

The main assumption for this part of the study was that a decrease in rumen and small intestine motility would reduce the rate of passage of Cr. In the present study, the increase in dietary fat increased the rate of passage of Cr through the gastrointestinal tract, mainly due to an increase in rate of passage from the rumen. Based on ruminant and non-ruminant data, the opposite result was expected. Feeding fat increased plasma GLP-1 and CCK concentration. In non-ruminants, GLP-1 reduced gastrointestinal motility (Holst, 1997), which would decrease the rate of passage. In ruminants, CCK infusion decreased reticulo-rumen (Grovum, 1981) and small intestine (Romański, 2004) motility. The infusion of GLP-1 in wethers used in the present study did not change the Cr rate of passage from the rumen or the digestive tract part posterior to the rumen. I am not aware of any previous reports in the literature on the effect of GLP-1 on gut motility in ruminants. The lack of response to GLP-1 in the present study could be because in ruminants GLP-1 does not have that function. However, it is possible that the absence of a response in gut motility due to GLP-1 was because the animals were not at maximum growth potential. The increasing infusion of CCK created a trend to increase Cr retention.
time through the digestive tract posterior to the rumen, but the difference was not statistically significant. However, that trend agrees with Romański (2004) who reported CCK infusion decreased small intestine motility in sheep. It is possible that the response in this study was not as marked as the results observed previously (Romański, 2004) because the doses used in this experiment were designed to maintain physiological plasma concentrations of the hormone, and the ones used by Romański (2004) were pharmacological.

In conclusion, despite similar increases in plasma concentration of GLP-1 and CCK when fat was fed as when the peptides were infused, the increase in plasma concentration of each peptide in particular did not reproduce the effect of feeding fat. However, there were some trends due to infusion similar to feeding fat such as the decrease in DMI daily, the first hour after feeding, as well as the cumulative DMI at 2, 4 and 16 h after feeding when GLP-1 was infused. These results suggest that GLP-1 plays a role in the regulation of DMI when fat is fed, but it is not the only factor involved. More research has to be conducted to understand the interactive roles the gut peptides have in regulating in ruminant metabolism in general, and DMI in particular.
References


<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>4 % fat</th>
<th>6 % fat</th>
</tr>
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<tr>
<td><strong>Ingredients</strong></td>
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</tr>
<tr>
<td>Alfalfa meal, 17% CP</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
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<tr>
<td>Soy hulls</td>
<td>20.00</td>
<td>20.00</td>
<td>20.00</td>
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<tr>
<td>Ground corn</td>
<td>48.59</td>
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<td>43.90</td>
</tr>
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<td>4.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
<td>8.00</td>
<td>8.03</td>
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</tr>
<tr>
<td>Urea</td>
<td>0.50</td>
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<tr>
<td>Limestone</td>
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<td>Monosodium phosphate</td>
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<td>0.50</td>
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<td>0.01</td>
<td>0.01</td>
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<tr>
<td>Vitamin D, 3 kIU/g</td>
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<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin E, 44 IU/g</td>
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<td>0.05</td>
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<td>Selenium, 200mg/g</td>
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<tr>
<td><strong>Chemical composition</strong></td>
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<tr>
<td>NDF</td>
<td>28.01</td>
<td>26.22</td>
<td>24.68</td>
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<tr>
<td>CP</td>
<td>14.96</td>
<td>15.13</td>
<td>15.75</td>
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<td>Ash</td>
<td>5.92</td>
<td>6.02</td>
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<tr>
<td>Total fatty acids</td>
<td>2.88</td>
<td>5.57</td>
<td>7.25</td>
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</tbody>
</table>

Table 7. Formulation and chemical composition (% of DM basis) of the control diet and diet supplemented with 4 and 6 % Ca-salts of palm oil<sup>1</sup>

<sup>1</sup> Ca-salts of palm oil (Megalac®, Church and Dwight Co., Inc., Princeton, NJ).

<sup>2</sup> CONT= control diet; FAT= diet with the addition of 6 % of Ca-salts of palm oil.
<table>
<thead>
<tr>
<th>Item</th>
<th>Fat treatments(^1)</th>
<th></th>
<th></th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
</tr>
</thead>
<tbody>
<tr>
<td>n wethers</td>
<td>CONT= control diet</td>
<td>4%</td>
<td>6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>298</td>
<td>321</td>
<td>255</td>
<td>42.1</td>
<td>0.61</td>
<td>0.27</td>
</tr>
<tr>
<td>GLP-1(^3), pM</td>
<td>24.0</td>
<td>26.8</td>
<td>28.9</td>
<td>3.8</td>
<td>0.17</td>
<td>0.87</td>
</tr>
<tr>
<td>CCK(^3), pM</td>
<td>6.53</td>
<td>13.96</td>
<td>11.11</td>
<td>1.34</td>
<td>0.02</td>
<td>0.03</td>
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<tr>
<td>Glucose, mM</td>
<td>4.17</td>
<td>4.23</td>
<td>4.27</td>
<td>0.87</td>
<td>0.43</td>
<td>0.95</td>
</tr>
<tr>
<td>NEFA(^3), mM</td>
<td>53.4</td>
<td>87.8</td>
<td>70.7</td>
<td>11.7</td>
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<td>0.11</td>
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</tbody>
</table>

Table 8. Mean plasma hormone and metabolite concentrations in growing wethers, fed a control diet or a control diet plus addition of 4 or 6 % Ca salts of palm oil.

\(^1\) CONT= control diet; 4 % = diet with the addition of 4 % of Ca-salts of palm oil; 6 % = diet with the addition of 6 % of Ca-salts of palm oil.

\(^2\) L= linear contrast; Q= quadratic contrast.

\(^3\) GLP-1= glucagon-like peptide-1; CCK= cholecystokinin; GIP= glucose-dependent insulinotropic polypeptide; NEFA=non esterified fatty acid.
Table 9. Mean plasma hormone and metabolite concentrations in growing wethers fed a control diet and infused with 0, 0.052, 0.103, and 0.155 µg/kg BW/d of GLP-1.

1 CONT= control diet infused with saline solution; 0.052 = control diet infused with 0.052 µg/kg BW/d of GLP-1; 0.103 = control diet infused with 0.103 µg/kg BW/d of GLP-1; 0.155= control diet infused with 0.155 µg/kg BW/d of GLP-1.

2 L= linear contrast; Q= quadratic contrast; C=Cubic contrast.

3 GLP-1= glucagon-like peptide-1; CCK= cholecystokinin; GIP= glucose-dependent insulinotropic polypeptide; NEFA=non esterified fatty acid.
Table 10. Mean plasma hormone and metabolite concentrations in growing wethers fed a control diet and infused with 0, 0.069, 0.138, and 0.207 µg/kg BW/d of CCK.

1 CONT= control diet infused with saline solution; 0.069 = control diet infused with 0.069 µg/kg BW/d of CCK; 0.138 = control diet infused with 0.138 µg/kg BW/d of CCK; 0.207 = control diet infused with 0.207 µg/kg BW/d of CCK.

2 L= linear contrast; Q= quadratic contrast; C=Cubic contrast.

3 GLP-1= glucagon-like peptide-1; CCK= cholecystokinin; GIP= glucose-dependent insulinotropic polypeptide; NEFA=non esterified fatty acid.
<table>
<thead>
<tr>
<th>Item</th>
<th>Fat treatments&lt;sup&gt;1&lt;/sup&gt;</th>
<th>$P$-value &lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONT</td>
<td>4% fat</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>1.83</td>
<td>1.55</td>
</tr>
<tr>
<td>MEI (Mcal/d)</td>
<td>4.82</td>
<td>4.31</td>
</tr>
<tr>
<td>DM Digestibility&lt;sup&gt;3&lt;/sup&gt;</td>
<td>66.9</td>
<td>67.4</td>
</tr>
<tr>
<td>NDF digestibility&lt;sup&gt;3&lt;/sup&gt;</td>
<td>40.0</td>
<td>38.7</td>
</tr>
<tr>
<td>CP digestibility&lt;sup&gt;3&lt;/sup&gt;</td>
<td>63.8</td>
<td>63.3</td>
</tr>
<tr>
<td>FA digestibility&lt;sup&gt;3&lt;/sup&gt;</td>
<td>68.2</td>
<td>70.9</td>
</tr>
<tr>
<td>OM Digestibility&lt;sup&gt;3&lt;/sup&gt;</td>
<td>69.4</td>
<td>68.7</td>
</tr>
<tr>
<td>Ash Digestibility</td>
<td>43.9</td>
<td>47.3</td>
</tr>
</tbody>
</table>

Table 11. Daily DMI MEI, and digestibility (%) in growing wethers, fed a control diet or a control diet plus addition of 4 or 6 % Ca salts of palm oil.

<sup>1</sup> CONT= control diet; 4 % = diet with the addition of 4 % of Ca-salts of palm oil; 6 % = diet with the addition of 6 % of Ca-salts of palm oil.

<sup>2</sup> L= linear contrast; Q= quadratic contrast.

<sup>3</sup> DM = dry matter; NDF = neutral detergent fiber; CP= crude protein; FA= fatty acid; OM = organic matter.
<table>
<thead>
<tr>
<th>Item</th>
<th>CONT</th>
<th>0.052</th>
<th>0.103</th>
<th>0.155</th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI (kg/d)</td>
<td>1.95</td>
<td>1.52</td>
<td>1.84</td>
<td>1.80</td>
<td>0.87</td>
<td>0.87</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>MEI (Mcal/d)</td>
<td>5.14</td>
<td>3.99</td>
<td>4.81</td>
<td>4.79</td>
<td>0.25</td>
<td>0.88</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>DM Digestibility^3</td>
<td>67.9</td>
<td>65.7</td>
<td>66.0</td>
<td>66.9</td>
<td>1.3</td>
<td>0.64</td>
<td>0.26</td>
<td>0.99</td>
</tr>
<tr>
<td>NDF digestibility^3</td>
<td>40.0</td>
<td>33.1</td>
<td>34.6</td>
<td>37.1</td>
<td>3.2</td>
<td>0.63</td>
<td>0.19</td>
<td>0.91</td>
</tr>
<tr>
<td>CP digestibility^3</td>
<td>63.8</td>
<td>63.0</td>
<td>62.2</td>
<td>62.9</td>
<td>1.2</td>
<td>0.55</td>
<td>0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>FA digestibility^3</td>
<td>68.2</td>
<td>65.5</td>
<td>72.4</td>
<td>73.3</td>
<td>6.8</td>
<td>0.49</td>
<td>0.80</td>
<td>0.42</td>
</tr>
<tr>
<td>OM Digestibility^3</td>
<td>69.4</td>
<td>67.1</td>
<td>67.3</td>
<td>68.2</td>
<td>1.3</td>
<td>0.58</td>
<td>0.25</td>
<td>0.92</td>
</tr>
<tr>
<td>Ash Digestibility</td>
<td>43.9</td>
<td>43.4</td>
<td>45.6</td>
<td>45.3</td>
<td>2.0</td>
<td>0.49</td>
<td>0.98</td>
<td>0.39</td>
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</table>

Table 12. Daily DMI, MEI, and digestibility (%) in growing wethers fed a control diet and infused with 0, 0.052, 0.103, and 0.155 µg/kg BW/d of GLP-1.

1 CONT= control diet infused with saline solution; 0.052 = control diet infused with 0.052 µg/kg BW/d of GLP-1; 0.103 = control diet infused with 0.103 µg/kg BW/d of GLP-1; 0.155= control diet infused with 0.155 µg/kg BW/d of GLP-1.

2 L= linear contrast; Q= quadratic contrast; C=Cubic contrast.

3 DM = dry matter; NDF = neutral detergent fiber; CP= crude protein; FA= fatty acid; OM = organic matter.
<table>
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<th>Item</th>
<th>CCK treatments(^1)</th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI (kg/d)</td>
<td>CONT 1.81 1.75 1.83 1.95</td>
<td>0.10</td>
<td>0.21</td>
<td>0.39</td>
<td>0.45</td>
</tr>
<tr>
<td>MEI (Mcal/d)</td>
<td>4.65 4.81 4.82 5.01</td>
<td>0.25</td>
<td>0.29</td>
<td>0.95</td>
<td>0.77</td>
</tr>
<tr>
<td>DM Digestibility</td>
<td>67.9 67.4 67.4 64.8</td>
<td>1.6</td>
<td>0.17</td>
<td>0.46</td>
<td>0.65</td>
</tr>
<tr>
<td>NDF digestibility</td>
<td>40.0 36.2 37.9 34.2</td>
<td>3.5</td>
<td>0.35</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>CP digestibility</td>
<td>63.8 65.1 65.0 59.1</td>
<td>1.9</td>
<td>0.13</td>
<td>0.09</td>
<td>0.56</td>
</tr>
<tr>
<td>FA digestibility</td>
<td>68.2 78.3 68.9 69.0</td>
<td>4.9</td>
<td>0.76</td>
<td>0.34</td>
<td>0.25</td>
</tr>
<tr>
<td>OM Digestibility</td>
<td>69.4 68.7 68.9 66.2</td>
<td>1.4</td>
<td>0.16</td>
<td>0.49</td>
<td>0.68</td>
</tr>
<tr>
<td>Ash Digestibility</td>
<td>43.9 46.1 44.4 42.3</td>
<td>2.2</td>
<td>0.52</td>
<td>0.35</td>
<td>0.51</td>
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Table 13. Daily DMI, MEI, and digestibility (%) in growing wethers fed a control diet and infused with 0, 0.069, 0.138, and 0.207 µg/kg BW/d of CCK.

\(^1\) CONT = control diet infused with saline solution; 0.069 = control diet infused with 0.069 µg/kg BW/d of CCK; 0.138 = control diet infused with 0.138 µg/kg BW/d of CCK; 0.207 = control diet infused with 0.207 µg/kg BW/d of CCK.

\(^2\) L = linear contrast; Q = quadratic contrast; C = cubic contrast.

\(^3\) DM = dry matter; NDF = neutral detergent fiber; CP = crude protein; FA = fatty acid; OM = organic matter.
<table>
<thead>
<tr>
<th>Item</th>
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<th>6 %fat</th>
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<th>L</th>
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<tr>
<td>Period, h post-feeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>452</td>
<td>332</td>
<td>238</td>
<td>73</td>
<td>0.09</td>
<td>0.82</td>
</tr>
<tr>
<td>1 to 2 h</td>
<td>18</td>
<td>34</td>
<td>49</td>
<td>5</td>
<td>0.27</td>
<td>0.83</td>
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<tr>
<td>2 to 4 h</td>
<td>141</td>
<td>223</td>
<td>90</td>
<td>16</td>
<td>0.30</td>
<td>0.01</td>
</tr>
<tr>
<td>4 to 8 h</td>
<td>327</td>
<td>299</td>
<td>257</td>
<td>58</td>
<td>0.45</td>
<td>0.81</td>
</tr>
<tr>
<td>8 to 16 h</td>
<td>616</td>
<td>501</td>
<td>501</td>
<td>48</td>
<td>0.12</td>
<td>0.56</td>
</tr>
<tr>
<td>16 to 23 h</td>
<td>413</td>
<td>301</td>
<td>439</td>
<td>51</td>
<td>0.99</td>
<td>0.04</td>
</tr>
<tr>
<td>Cumulative, h post-feeding</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>452</td>
<td>332</td>
<td>238</td>
<td>73</td>
<td>0.09</td>
<td>0.82</td>
</tr>
<tr>
<td>2h</td>
<td>470</td>
<td>366</td>
<td>287</td>
<td>70</td>
<td>0.12</td>
<td>0.86</td>
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<tr>
<td>4h</td>
<td>611</td>
<td>480</td>
<td>337</td>
<td>78</td>
<td>0.08</td>
<td>0.81</td>
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<tr>
<td>8h</td>
<td>938</td>
<td>779</td>
<td>634</td>
<td>79</td>
<td>0.04</td>
<td>0.68</td>
</tr>
<tr>
<td>16h</td>
<td>1554</td>
<td>1280</td>
<td>1135</td>
<td>96</td>
<td>0.02</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 14. Dry matter intake (g) at different times after feeding in growing wethers fed a control diet or a control diet plus addition of 4 or 6 % Ca salts of palm oil.

1 CONT= control diet; 4 % = diet with the addition of 4 % of Ca-salts of palm oil; 6 % = diet with the addition of 6 % of Ca-salts of palm oil.

2 L= linear contrast; Q= quadratic contrast.
<table>
<thead>
<tr>
<th>Item</th>
<th>GLP-1 treatment</th>
<th>P-value ≤²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONT, 0.052, 0.103, 0.155</td>
<td>SEM</td>
</tr>
<tr>
<td>Period, h post-feeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>452 360 350 262</td>
<td>66</td>
</tr>
<tr>
<td>1 to 2 h</td>
<td>18.69 37 77 53</td>
<td>24</td>
</tr>
<tr>
<td>2 to 4 h</td>
<td>141 144 161 132</td>
<td>34</td>
</tr>
<tr>
<td>4 to 8 h</td>
<td>327 317 405 319</td>
<td>24</td>
</tr>
<tr>
<td>8 to 16 h</td>
<td>61 575 533 485</td>
<td>47</td>
</tr>
<tr>
<td>16 to 23 h</td>
<td>407 364 465 498</td>
<td>71</td>
</tr>
<tr>
<td>Cumulative, h post-feeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>452 358 350 262</td>
<td>66</td>
</tr>
<tr>
<td>2h</td>
<td>470 379 428 304</td>
<td>62</td>
</tr>
<tr>
<td>4h</td>
<td>611 523 589 436</td>
<td>54</td>
</tr>
<tr>
<td>8h</td>
<td>938 830 995 763</td>
<td>59</td>
</tr>
<tr>
<td>16h</td>
<td>1554 1456 1528 1257</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 15. Dry matter intake (g) at different times after feeding in growing wethers fed a control diet and infused with 0, 0.052, 0.103, and 0.155 µg/kg BW/d of GLP-1.

¹ CONT = control diet infused with saline solution; 0.052 = control diet infused with 0.052 µg/kg BW/d of GLP-1; 0.103 = control diet infused with 0.103 µg/kg BW/d of GLP-1; 0.155 = control diet infused with 0.155 µg/kg BW/d of GLP-1.

² L = linear contrast; Q = quadratic contrast; C = cubic contrast.
<table>
<thead>
<tr>
<th>Item</th>
<th>CONT</th>
<th>0.069</th>
<th>0.138</th>
<th>0.207</th>
<th>SEM</th>
<th>L</th>
<th>Q</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Period, h post-feeding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 h</td>
<td>381</td>
<td>526.16</td>
<td>244</td>
<td>426</td>
<td>61</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>1 to 2 h</td>
<td>19</td>
<td>109</td>
<td>79</td>
<td>37</td>
<td>25</td>
<td>0.84</td>
<td>0.04</td>
<td>0.54</td>
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<tr>
<td>2 to 4 h</td>
<td>141</td>
<td>93</td>
<td>111</td>
<td>146</td>
<td>27</td>
<td>0.79</td>
<td>0.16</td>
<td>0.64</td>
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<td>4 to 8 h</td>
<td>322</td>
<td>308</td>
<td>374</td>
<td>365</td>
<td>23</td>
<td>0.11</td>
<td>0.92</td>
<td>0.04</td>
</tr>
<tr>
<td>8 to 16 h</td>
<td>614</td>
<td>575</td>
<td>510</td>
<td>537</td>
<td>60</td>
<td>0.26</td>
<td>0.51</td>
<td>0.30</td>
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<td>16 to 23 h</td>
<td>351</td>
<td>257</td>
<td>562</td>
<td>489</td>
<td>51</td>
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<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Cumulative, h post-feeding</strong></td>
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<td></td>
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<td></td>
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<td>381</td>
<td>526</td>
<td>244</td>
<td>426</td>
<td>61</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
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<td>2h</td>
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<td>538</td>
<td>426</td>
<td>446</td>
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<td>0.01</td>
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<td>843</td>
<td>905</td>
<td>57</td>
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<td>16h</td>
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<td>1459</td>
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<td>109</td>
<td>0.79</td>
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<td>0.73</td>
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</table>

Table 16. Dry matter intake (g) at different times after feeding in growing wethers fed a control diet and infused with 0, 0.069, 0.138, and 0.207 µg/kg BW/d of CCK.

1 CONT = control diet infused with saline solution; 0.069 = control diet infused with 0.069 µg/kg BW/d of CCK; 0.138 = control diet infused with 0.138 µg/kg BW/d of CCK; 0.207 = control diet infused with 0.207 µg/kg BW/d of CCK.

2 L = linear contrast; Q = quadratic contrast; C = Cubic contrast.
<table>
<thead>
<tr>
<th>Item</th>
<th>Fat treatment&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P-value &lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
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<td>CONT</td>
<td>4 %</td>
</tr>
<tr>
<td>&lt;sub&gt;1&lt;/sub&gt;&lt;sub&gt;k&lt;/sub&gt;&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.0745</td>
<td>0.0837</td>
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<td>0.419</td>
<td>0.472</td>
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<tr>
<td>RMRT&lt;sup&gt;3&lt;/sup&gt;</td>
<td>16.62</td>
<td>14.07</td>
</tr>
<tr>
<td>TT&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11.52</td>
<td>10.05</td>
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<tr>
<td>TMRT&lt;sup&gt;3&lt;/sup&gt;</td>
<td>28.09</td>
<td>24.18</td>
</tr>
</tbody>
</table>

Table 17. Mean chromium kinetics through the gastrointestinal tract in growing wethers fed a control diet or a control diet plus addition of 4 or 6 % Ca salts of palm oil.

<sup>1</sup> CONT= control diet; 4 % = diet with the addition of 4 % of Ca-salts of palm oil; 6 % = diet with the addition of 6 % of Ca-salts of palm oil.

<sup>2</sup> L= linear contrast; Q= quadratic contrast.

<sup>3</sup> <sub>k</sub> = rate (proportion per hour) at which chromium particles pass out of the rumen; RMRT = mean retention time of chromium within the rumen; TT= time taken for chromium to pass through the section of the digestive tract posterior to the rumen; TMRT = total mean retention time of chromium in the entire digestive tract.
### Table 18. Mean chromium kinetics through the gastrointestinal tract in growing fed a control diet and infused with 0, 0.052, 0.103, and 0.155 µg/kg BW/d of GLP-1.

1 CONT= control diet infused with saline solution; 0.052 = control diet infused with 0.052 µg/kg BW/d of GLP-1; 0.103 = control diet infused with 0.103 µg/kg BW/d of GLP-1; 0.155= control diet infused with 0.155 µg/kg BW/d of GLP-1.

2 L= linear contrast; Q= quadratic contrast; C=Cubic contrast.

3 $k_1$ = rate (proportion per hour) at which chromium particles pass out of the rumen; $k_2$ = rate (proportion per hour) at which chromium particles pass out of the hindgut; RMRT = mean retention time of chromium within the rumen TT= time taken for chromium to pass through the section of the digestive tract posterior to the rumen; TMRT = total mean retention time of chromium in the entire digestive tract.

<table>
<thead>
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<tr>
<td>$k_1^3$</td>
<td>0.0745</td>
<td>0.0821</td>
</tr>
<tr>
<td>$k_2^3$</td>
<td>0.411</td>
<td>0.471</td>
</tr>
<tr>
<td>RMRT</td>
<td>16.65</td>
<td>15.02</td>
</tr>
<tr>
<td>TT</td>
<td>11.60</td>
<td>11.67</td>
</tr>
<tr>
<td>TMRT</td>
<td>28.25</td>
<td>26.46</td>
</tr>
<tr>
<td>Item</td>
<td>CCK treatment</td>
<td>SEM</td>
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<tr>
<td>------</td>
<td>---------------</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.069</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.0821</td>
<td>0.0840</td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.411</td>
<td>0.400</td>
</tr>
<tr>
<td>RMRT</td>
<td>16.65</td>
<td>14.77</td>
</tr>
<tr>
<td>TT</td>
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<td>12.88</td>
</tr>
<tr>
<td>TMRT</td>
<td>28.25</td>
<td>27.65</td>
</tr>
</tbody>
</table>

Table 19. Mean chromium kinetics through the gastrointestinal tract in growing wethers fed a control diet and infused with 0, 0.069, 0.138, and 0.207 µg/kg BW/d of CCK.

1 CONT = control diet infused with saline solution; 0.069 = control diet infused with 0.069 µg/kg BW/d of CCK; 0.138 = control diet infused with 0.138 µg/kg BW/d of CCK; 0.207 = control diet infused with 0.207 µg/kg BW/d of CCK.

2 L = linear contrast; Q = quadratic contrast; C = Cubic contrast.

3 $k_1$ = rate (proportion per hour) at which chromium particles pass out of the rumen; $k_2$ = rate (proportion per hour) at which chromium particles pass out of the hindgut; RMRT = mean retention time of chromium within the rumen; TT = time taken for chromium to pass through the section of the digestive tract posterior to the rumen; TMRT = total mean retention time of chromium in the entire digestive tract.
CHAPTER 6

EFFECT OF INTRAVENOUS GLUCAGON-LIKE PEPTIDE-1 INFUSION ON DRY MATTER INTAKE AND mRNA EXPRESSION OF NEUROPEPTIDE Y, AGOUTI RELATED PEPTIDE AND PROOPIO MELANOCORTIN IN GROWING WETHERS

Abstract

The effect of glucagon-like peptide-1 (GLP-1) on dry matter intake (DMI) in ruminants is not clear. The objectives of the present study were to evaluate the effect of intra-jugular vein infusions of GLP-1 at physiological doses on DMI, nutrient digestibility and on hypothalamic mRNA concentration of neuropeptides that regulate appetite, i.e., neuropeptide Y (NPY), agouti related peptide (AgRP), and proopi melanocortin (POMC). In experiment 1, thirty six cross bred wethers were fed a pelleted control diet containing (DM basis) 60% concentrate and 40% forage in a complete randomize block design. The treatments were dietary addition of 6 % Ca salts of palm oil fatty acids, and 6-d jugular vein infusions of 0.155 µg/kg BW/d of GLP-1. Amounts infused were based on single compartment, first-order kinetics, hormone half-life, and estimated plasma volume. Hormone concentrations were measured in jugular vein plasma from samples taken on d 1, 4 and 6 of each period. On d 7, the wethers were slaughtered for hypothalamus collection to measure mRNA concentration of NPY, AgRP and POMC.
The addition of 6 % of Ca salts of palm oil increased plasma GLP-1 concentration \((P < 0.01)\) and decreased DMI on d 1, but by d 6 DMI of lambs fed supplemental fat was similar to those fed the control diet (time x treatment interaction \(P < 0.05\)). The infusion of GLP-1 did not change DMI \((P > 0.20)\) and increased neutral detergent fiber digestibility \((P < 0.01)\). There were no differences in mRNA concentration for NPY, AgRP or POMC due to GLP-1 infusion or feeding fat. In experiment 2, three lambs fed a control diet (60% concentrate and 40% forage) in a completely randomized design were incrementally (step-up) infused with 0.155, 0.310, 0.620 and 1.240 µg/kg BW/d of GLP-1 for 2 d at each dose. Daily measurements for DMI and plasma GLP-1 concentration were taken. Increasing the dose of GLP-1 infusion did not decrease DMI \((P > 0.15)\), but increased plasma GLP-1 concentration (Linear, \(P < 0.01\)). In conclusion, physiological plasma GLP-1 concentration did not decreased DMI. Therefore, it is likely that effects of feeding fat on DMI are due to additive effects of multiple hormones and metabolic signals, not solely changes in plasma GLP-1 concentration.

Introduction

Using non-ruminants models, glucagon-like peptide-1(7, 36) amide (GLP-1) has been associated with decreases in feed intake (Turton et al., 2002). In ruminants, an increase in plasma GLP-1 concentration has been associated with a decrease in dry matter intake (DMI) when fat was added to the diet (Relling and Reynolds, 2007; Bradford et al., 2008, Chapter 4). However, the jugular vein infusion of physiological doses of GLP-1 did not have the same effect on daily DMI as feeding fat, despite the similar increase in plasma
GLP-1 concentration observed when fat is fed (Chapter 5). Also, the central mechanism of how GLP-1 regulates feed intake is controversial. Results from Turton et al. (1996) in rats, where GLP-1 was intracerebroventricular (ICV) infused, did not change mRNA concentration for neuropeptide Y (NPY) compared with saline infused rats. However, Seo et al. (2008) showed that ICV infusion of GLP-1 decreased NPY and agouti-related peptide (AgRP) and increased proopiomelanocortin (POMC) mRNA concentration in the hypothalamus of fasted rats. In ruminants, in vitro culture of sheep hypothalamus in media containing GLP-1 did not change the relative concentration of NPY, AgRP or POMC mRNA, neuropeptides that regulate feed intake in non-ruminants (Valassi et al. 2008), compared with similar media without GLP-1 (Chapter 3). However, an increase in NPY and AgRP was associated with an increase in plasma GLP-1 concentration and a decreased in DMI when fat was fed (Chapter 4) to growing lambs. Therefore the objectives of this study were to determine the effect of jugular vein infusion of GLP-1 on DMI and mRNA concentration of the neuropeptides NPY, AgRP and POMC in growing wethers.

Materials and methods

Animal care followed guidelines recommended in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1998).

Experiment 1

Three weeks before the start of the experiment, thirty six Targhee x Hampshire wethers (40.7 ± 3.3 kg BW) were fed a pelleted control diet (Table 6) formulated to meet nutrient
requirements of growing lambs according to the National Research Council (1985). The wethers were grouped by weight and housed in three pens with 12 wethers each. Daily rations were provided at 0800 h, and wethers were fed for ad libitum intake of DM (10% refusal).

Treatments were 7 d of: 1) control diet (CONT; without supplemental fat; table 6); 2) supplemental dietary fat at 6% of ration DM (FAT; control diet plus the addition of 6% of Ca-salts of palm oil; Table 6); 3) intravenous GLP-1 (GLP-1; 0.155 µg/kg BW/d of GLP-1(7-36) amide H6795, Bachem California Inc, CA and fed CONT). The wethers on the CONT and FAT treatments received an infusion of 1 L of sterile saline solution per day. The hormone solutions and the saline solutions were made and infused as described in Chapter 5. The concentration of hormone used in the infusion followed the same rationale as used for Chapter 5.

The experiment was conducted as a completely randomized block design. Each of the three groups of wethers was considered as a block. Within each block, the twelve wethers were allocated randomly to the three treatments. Two weeks before the experiment began, the wethers were housed in individual pens. Wethers fed supplemented fat were adapted to the diet with incremental increases in fat concentration in the diet; with an amount equal to 2% of ration DM fed for the first day of the adaptation period, 4% on the second and third days, and the total 6% on d 4. One week before the experiment started, the lambs were moved into metabolic crates described previously (Murphy, et al. 1994). During the week prior to the experiment, the animals were adapted to all of the procedures that were to be conducted during the sampling week. Forty five hours before the experiment started, jugular catheters were established in the jugular vein as described
previously (Chapter 5). Two animals experienced a drop in DMI to less than 50% of the previous day’s intake when lambs were moved into the metabolic crates. Therefore, before the infusion started, one wether on the control treatment in block one and a wether on the GLP-1 treatment in block three were removed from the experiment. The infusions started at 1000 h.

Feed was offered daily at 1300 h and the refusals were removed and weighed 23 h later at 1200 h. For digestibility and plasma samples, samples were collected and processed as described in Chapter 5. Briefly, to measure digestibility, total fecal collection was performed daily during the last 5 d of each experimental period. Five percent of the total daily feces was collected and pooled for analysis of DM, NDF, CP, FA, and ash concentration. Blood samples (10 ml) were taken 6 and 8 h after feed was offered on d 1, 4, and 6 of each experimental period. Blood samples were immediately transferred into polypropylene tubes containing solutions of disodium EDTA and benzamidine HCl (1.6 mg and 4.7 mg/ml blood, respectively) and placed on ice. After centrifugation for 25 min at 1800 x g and 4°C, plasma was partitioned into individual polypropylene tubes for each analysis to be performed, flash frozen using liquid N₂ within 40 min of sample collection, and stored at -80°C until analyzed.

Concentrations of insulin and GLP-1 were measured using RIA as described previously (Chapter 5). The intraassay CV averaged less than 12.5% for insulin and less than 11% for GLP-1. Minimum sensitivities (90% of zero standard binding) of the insulin and GLP-1 assays were 0.0027 and 0.001, 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 two-reaction, enzyme-based assay (Wako Chemicals USA, Richmond, VA) as described by Johnson and Peters (1993).

The morning of the seventh day, the lambs were transported 165 km (transport time was 100 min) to the abattoir in the Animal Science Building (OSU, Columbus, OH) for hypothalamus collection. The hypothalamus was collected as described in Chapter 3. During hypothalamus collection, one sample from block three on the GLP-1 treatment was lost due to damage of the brain caused by the captive bolt.

To measure mRNA concentration for NPY, AgRP and POMC, the protocol and primers used were the same as the ones described in Chapter 3.

The data were analyzed as a complete randomized block design with repeated measurements in time using the MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC) with a model testing the random effects of wether and block, and the fixed effect of treatment and time. For digestibility and mRNA concentration data, a similar statistical model was used without the effect of time. The two daily plasma samples for hormones and metabolites from the three days of sampling in each experimental period were analyzed in the lab individually but the average of both were used in the statistical analysis as one daily value. Fisher's protected LSD test was done comparing treatments at a $P$ value of 0.10.

Experiment 2

Three wethers were housed in metabolic crates and fed a pelleted diet without the addition of supplemental fat (Control diet, Table 6). The wethers were adapted to the diet for at least a month before the experiment started. The pelleted diet was provided once a day at
1100 h for ad libitum intake (10% refusal). On the same day that the wethers were moved into the crates, a jugular vein catheter was placed as described previously (Chapter 5). The first 3 d were for adaptation to the crates and the catheters. On d 4 and 5, DMI was measured (based on DM of the feed consumed and the refusals) and used as the basal DMI value. The GLP-1 infusion treatments started 30 min before feeding on d 6 the wethers were in the crates. The infusions were a step-up infusion of 0.155, 0.310, 0.620 and 1.240 µg/kg BW/d of GLP-1 (GLP-1 (7-36) amide H6795, Bachem California Inc, CA). Each dose was infused for two consecutive days. The preparation of the hormone solutions and the protocol for the infusion were described previously (Chapter 5).

Samples of feed and refusals were collected daily for DMI measurements, and analyzed as in Experiment 1. Blood was collected daily as described previously (Chapter 5), 6 h after feeding, and was analyzed as described for Experiment 1.

The data obtained in this experiment has a confounding factor of day by dose of GLP-1 because the same animal was used as its own control when GLP-1 infusion was increased. Therefore, the response variable in this experiment was time by dose. The data were analyzed as a completely randomized design with repeated measurements of time x dose, using the MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC) with a model testing the random effects of wether and the fixed effect of dose by time. The basal DMI was used as a covariate. Orthogonal polynomial contrast was used to partition total treatment sums of squares into the following comparisons: linear, quadratic, and cubic effects for the 0.155, 0.310, 0.620 and 1.240 µg/kg BW/d of GLP-1 doses.
Results

Experiment 1

There was a time by treatment interaction for DMI ($P < 0.05$; data not shown), due to a greater DMI for GLP-1 and control-fed wethers compared with the fat-fed wethers on d 1, but no difference in DMI by d 6. Metabolizable energy intake and digestibility of DM, CP, FA and OM was not different among the treatments ($P > 0.15$; Table 20). The addition of dietary fat decreased and there was a GLP-1 infusion trend to increase ($P < 0.10$) NDF digestibility compared with control fed wethers.

Feeding fat or GLP-1 infusion did not change plasma concentrations of insulin and glucose ($P > 0.30$; Table 21) compared with the control wethers. Compared with control wethers, plasma GLP-1 and NEFA concentrations increased due to additional dietary fat ($P < 0.05$), but did not due to GLP-1 infusions.

Hypothalamic mRNA concentrations of NPY, AgRP and POMC were similar ($P > 0.25$) among wethers and those fed fat or infused with GLP-1 (Table 22).

Experiment 2

There was a numerical decrease in DMI due to increased GLP-1 infusion, but the changes were not significant (Linear, $P > 0.18$; Table 23). Plasma GLP-1 concentration showed a linear increase ($P < 0.01$) due to the increasing rate of GLP-1 infusion. Plasma insulin, glucose and NEFA concentrations did not change due to increased doses of GLP-1 over time ($P > 0.30$).
Discussion

Experiment 1

There was a time by treatment interaction for DMI due to a greater DMI for GLP-1 and control wethers compared with those fed supplemental fat on the first day, but that difference decayed over time such that by d 6, there was no treatment effect on DMI. The wethers in metabolism crates may not have been eating and growing to their maximum potential which could have attenuated their response to the treatments imposed. Similar amounts of fat or GLP-1 infusion tended to decreased DMI in sheep (Reynolds et al. 2006, Chapter 4 and 5). Previously (Chapter 5), the inclusion of 6 % fat in the diet numerically decreased NDF digestibility; in the present study, similar amounts of fat also decreased NDF digestibility. Harvatine and Allen (2006) shown that the inclusion of fat in the diet decreased ruminal digestibility, but not total tract digestibility of NDF. A possible reason for the decrease in NDF digestibility observed in the present experiment could be due to an increased rate of passage, as observed in Chapter 5, but rate of passage was not measured in the current experiment. The infusion of GLP-1 tended to increase NDF digestibility compared with control fed wethers. Our hypothesis was that an increase in NDF digestibility would be due to a decrease in gut motility resulting in increased retention time of nutrients in the small intestine. However, previously (Chapter 5), GLP-1 infusion did not decrease rate of passage nor increase NDF digestibility. The reasons for the increase in NDF digestibility are not clear.

Feeding fat increased plasma GLP-1 concentration, but the infusion of GLP-1 did not change plasma GLP-1 concentration compared with control-fed wethers. This lack of response is puzzling and may have affected animal responses to this treatment. In Chapter
we observed similar plasma GLP-1 concentration for wethers fed 6 % fat as for those infused with 0.155 µg/kg BW/d of GLP-1. However, these responses were not significantly different from those of the control wethers. The lack of increase in plasma GLP-1 concentration observed in the present study due to GLP-1 infusion could be the reason why there was no decrease in DMI due to infusion. This does not explain the observed increase in NDF digestibility. As has been observed previously, the increase in dietary fat increased plasma NEFA concentration (Gagliostro and Chilliard, 1991; Relling and Reynolds, 2007) and the reason for that may be due to a higher plasma concentration of lipoproteins (Gagliostro and Chilliard, 1991).

In the present study, there were no differences in mRNA concentrations for the neuropeptides NPY, AgRP and POMC due to feeding fat or GLP-1 infusion. It is been observed that feeding fat increases NPY and AgRP (Chapter 4) in growing wethers. The reason for the lack of response for neuropeptides concentration in the present experiment is unknown. In the case of the GLP-1 infusion treatment, it may have been due to the inability to achieve increased plasma concentrations with the dose infused.

Experiment 2

The linear increase in plasma GLP-1 concentration with increased GLP-1 dose resulted in a trend to decrease DMI. In this experiment, there was almost a 3-fold increase in plasma GLP-1 concentration at the higher infusion dose, and yet, there was not a drastic decrease in DMI. It is possible that the decrease in DMI associated with increased plasma GLP-1 concentration observed previously (Chapter 4) when fat was fed, was not due to a the sole
action of GLP-1, but more due to the combination of endocrine changes that occur (Chapter 3; Chapter 4; Chapter 5) when fat is fed.

The incretin effect of GLP-1 for increased glucose-stimulated insulin secretion and decreased in lipogenesis was not observed in the present study. For insulin to respond to GLP-1, the plasma glucose concentration has to be greater than 4.5 mM (Holst, 1997; Faulkner and Martin, 1999), and in the current experiment the average plasma glucose concentration was 3.7 mM. Glucagon like peptide-1 decreases lipolysis in sheep (Martin et al., 1993), and therefore would be assumed to decrease plasma NEFA concentration. However, in the current study, there were no changes in plasma NEFA concentration due to GLP-1 infusion. However, plasma NEFA concentrations would depend also on MEI and plasma insulin concentration, which did not change in the current experiment.

In conclusion, a physiological increase in plasma GLP-1 by itself was not enough to drive changes in DMI or metabolism in growing lambs in metabolic crates over the course of the infusion period. Therefore, more studies need to be conducted to understand the role of GLP-1 in growing lambs.
Bibliography


Table 20. Dry matter intake, metabolizable energy intake and digestibility (%) in growing wethers fed a control diet, 6 % Ca salts of palm oil, or infused with 0.155 µg/kg BW/day of GLP-1.

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<th>GLP-1</th>
<th>SEM</th>
<th>P-value</th>
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<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI², kg/d</td>
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<td>1.29</td>
<td>1.35</td>
<td>0.07</td>
<td>0.83</td>
</tr>
<tr>
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<td>50.79</td>
<td>49.74</td>
<td>1.45</td>
<td>0.22</td>
</tr>
</tbody>
</table>

¹ CONT= control diet; 6 % fat = diet with the addition of 6 % of Ca-salts of palm oil; GLP-1= control diet infused with 0.155 µg/kg BW/d of GLP-1.

² Treatment x time P < 0.05.

³ DM = dry matter; NDF = neutral detergent fiber; CP= crude protein; FA= fatty acid; OM = organic matter.

abc values with different superscript P < 0.10.
<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>6% fat</th>
<th>GLP-1</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, pM</td>
<td>312</td>
<td>270</td>
<td>270</td>
<td>23</td>
<td>0.34</td>
</tr>
<tr>
<td>GLP-1&lt;sup&gt;2&lt;/sup&gt;, pM</td>
<td>23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>3.63</td>
<td>3.55</td>
<td>3.57</td>
<td>0.09</td>
<td>0.73</td>
</tr>
<tr>
<td>NEFA&lt;sup&gt;2&lt;/sup&gt;, mM</td>
<td>49.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.81</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 21. Plasma hormone and metabolite concentration in growing wethers fed a control diet, 6 % Ca salts of palm oil, or infused with 0.155 µg/kg BW/day of GLP-1.

<sup>1</sup> CONT= control diet; 6 % fat = diet with the addition of 6 % of Ca-salts of palm oil; GLP-1= control diet infused with 0.155 µg/kg BW/d of GLP-1.

<sup>2</sup> GLP-1= glucagon-like peptide-1; NEFA= non esterified fatty acid.

<sup>ab</sup> values with different superscript P < 0.05
<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>6% fat</th>
<th>GLP-1</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY/CYC(^2)</td>
<td>0.786</td>
<td>0.216</td>
<td>0.137</td>
<td>0.33</td>
<td>0.37</td>
</tr>
<tr>
<td>AgRP/CYC(^2)</td>
<td>0.200</td>
<td>0.0314</td>
<td>0.0458</td>
<td>0.09</td>
<td>0.40</td>
</tr>
<tr>
<td>POMC/CYC(^2)</td>
<td>0.311</td>
<td>0.168</td>
<td>0.0843</td>
<td>0.09</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 22. Hypothalamic concentration of mRNA in growing wethers fed a control diet, 6% Ca salts of palm oil, or infused with 0.155 µg/kg BW/day of GLP-1.

\(^1\) CONT = control diet; 6% fat = diet with the addition of 6% of Ca-salts of palm oil; GLP-1 = control diet infused with 0.155 µg/kg BW/d of GLP-1.

\(^2\) 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.
Table 23. Dry matter intake and hormone and metabolites concentrations in growing wethers infused with increasing doses of GLP-1

1 Two days infusion of: 0.155 = 0.155 µg/kg/day of GLP-1; 0.310 = 0.310 µg/kg/day of GLP-1 after two days of 0.155 µg/kg/day of GLP-1; 0.620 = 0.620 µg/kg/day of GLP-1 after two days of 0.310 µg/kg/day of GLP-1; 1.240 = 1.240 µg/kg/day of GLP-1 after two days of 0.620 µg/kg/day of GLP-1.

2 GLP-1= glucagon-like peptide-1; NEFA= non esterified fatty acid.
CHAPTER 7

ABOMASAL SOYBEAN OIL OR CASEIN INFUSION DECREASE PRE-PRANDIAL PLASMA GHRELIN CONCENTRATION, BUT DO NOT ALTER PLASMA OXYNTOMODULIN CONCENTRATION IN LACTATING DAIRY COWS.

Abstract

The effects of increased postruminal supply of specific nutrients on plasma concentrations of the appetite regulating gut peptide hormones ghrelin and oxyntomodulin (OXM) were investigated. Our objectives were to determine acute (hours) and chronic (1 week) effects of abomasal supply of protein, carbohydrate, or fat to the small intestine on plasma concentrations of ghrelin and OXM. Four mid-lactation Holstein cows were used in a 4×4 Latin square design experiment. Treatments were 7-day abomasal infusions of water, soybean oil (500 g/d), corn starch (1100 g/d), or casein (800 g/d). Jugular vein plasma was obtained every 30 min for 7 hours on d 1 and 7. Oil and casein infusion decreased pre-prandial plasma ghrelin concentration on both days; however, DMI was only depressed after 7 d of oil infusion. The infusion of oil, starch or casein did not change plasma OXM concentration. The present data indicate that plasma ghrelin concentration is depressed by increased postruminal lipid and protein supply
immediately before feeding, but not during the postprandial period. Plasma ghrelin concentration was not altered by increased postruminal supply of carbohydrate. In addition, plasma OXM concentration did not respond to postruminal nutrient infusion in a manner similar to GLP-1, in spite of the fact that they originate from the same precursor molecule.

Introduction

The plasma concentrations of different gut peptide hormones, and their changes due to nutrition, have been studied recently in lactating dairy cows, in part due to their importance in regulation of DMI (Bradford et al. 2008; Relling and Reynolds 2008). In lactating dairy cows feeding or infusing lipids increased glucagon like peptide 1 (GLP-1) (Benson and Reynolds 2001; Relling and Reynolds, 2007). Feeding lipids also increased plasma cholecystokinin (CCK) and decreased plasma ghrelin concentration in lactating dairy cows (Bradford et al. 2008). However, the infusion of lipids does not change plasma CCK concentration (Benson and Reynolds, 2001, Relling and Reynolds 2008). Ghrelin is a gut peptide discovered in 1999 by Kojima et al. (1999) as the natural ligand for growth hormone secretagogue receptor. In non-ruminants, plasma ghrelin concentration is higher before a meal (Perez-Tilve et al., 2006). This association was also observed in beef cows (Hayashida et al., 2001) and sheep (Sugino et al., 2004). In non-ruminants ghrelin simulates appetite (Kojima and Kangawa, 2005) and in lactating dairy cows, a low pre-feeding plasma ghrelin concentration was associated with a decrease in DMI when different supplemental fats were fed (Bradford et al. 2006). However, the
effect of abomasal infusion of casein, starch, and oil on plasma ghrelin concentration has not been reported in lactating dairy cows.

Oxyntomodulin (OXM) is a peptide derived from the proglucagon peptide secreted from the L cells in the small intestine (Holst, 1997). In non-ruminants OXM has been reported to reduce food intake (Dakin et al., 2001; Cohen et al., 2003) by binding the GLP-1 receptor (Small and Bloom, 2004). To my knowledge, plasma OXM concentration has not been reported previously for cattle.

The objectives of the present study were to evaluate the short- and long-term effects of abomasal infusions of isoenergetic amounts of different macronutrients (protein, starch or fat) on plasma concentrations of ghrelin and OXM in lactating dairy cows.

Materials and Methods

All animal procedures were reviewed and approved by the Agricultural Animal Care and Use Committee at The Ohio State University. Animals, diets, treatments and experimental design have been previously described (Relling and Reynolds, 2008). Briefly, four multiparous, rumen cannulated, mid-lactation Holstein cows (714±16 kg body wt) were used in a 4 x 4 Latin Square design experiment for which cows were abomasally infused during the second week of each 2-wk period with 12 kg/d water (control), 1100 g/d of raw corn starch (A.E Staley MFC, Decatur, IL), 500 g/d of soybean oil (restaurant grade refined salad oil, Eli Lilly Co., Lafayette, IN), or 800 g/d of casein protein (Erie Casein Co., Inc., Erie, IL). The amount of oil infused was based in part on previous studies in lactating dairy cows (Benson and Reynolds, 2001; Relling and Reynolds 2007) in which abomasal oil infusion decreased DMI and increased plasma...
GLP-1 concentration, with the target rate of oil infused estimated to be approximately 3% of expected DMI. This is an amount of supplemental fat that is typically added to diets fed to lactating dairy cows in practice. Starch and casein were infused to provide gross energy equal to the oil infused. Casein and starch solutions were suspended in 12 kg of water, while during oil infusions 12 kg/d of water was infused simultaneously to balance the water volume supplied with other treatments. Cows were fed once daily at 0900 h for ad libitum DMI and orts were removed daily at 0830 h.

Blood samples (10 ml) for hormone analysis were taken every 30 min, starting at 0800 h and finishing at 1500 h during the first and seventh day of each infusion week. Blood samples were immediately transferred into polypropylene tubes containing solutions of disodium EDTA and benzamidine HCl (1.6 and 4.7 mg/ml blood, respectively) and placed on ice. After centrifugation for 30 min at 1800 × g and 4 °C, equal volumes of plasma for three sequential samples were composited in a tube containing 500 Kallikrein inhibitor units (KIU) of dried aprotinin (Trasylol, Bayer, Germany) per ml of plasma added and flash frozen after each addition. Active ghrelin was measured in the first three sampling pools which includes the samplings from 0800 h to 1200 h, using the octanoylated ghrelin kit (Active Ghrelin Kit #GHRA-88HK, Linco Research, St. Charles, MO) which was validated for bovine plasma previously (Wertz el al., 2003). Before thawing, plasma samples (500 µL) used for ghrelin analysis were acidified with 25 µL of 1 M HCl, and 5 µL of phenyl-methylsulfonyl fluoride (10 mg/ml) was added to limit the degradation of active ghrelin.

Plasma OXM concentration was measured using a commercial kit (Oxyntomodulin RIA Kit # RKU-028-22, Phoenix Pharmaceuticals, Inc., Belmont, CA). Binding of labeled
human OXM by the anti-OXM antibody with the addition of increasing amounts of unlabelled human OXM and bovine plasma showed a 77% displacement by immunoreactive peptide in bovine plasma (Figure 1). The average recovery of human OXM in bovine plasma was 104.3 ± 6.4%. Because GLP-1 and OXM are co-secreted (Holst, 1997), and there was no time effect on plasma GLP-1 concentration in these cows (Relling and Reynolds, 2008), a single pool of plasma was analyzed for OXM concentration for each day of sampling. The intra assay CV averaged less than 13 and 11% for the ghrelin and OXM assays respectively. Minimum sensitivities (90% of zero standard binding) of the ghrelin and OXM assays were 0.00022 and 0.00451 pmol/tube respectively.

Ghrelin data were analyzed as repeated measures over sampling times within each day (1 or 7) using the Mixed Procedure of SAS (Version 9.1, SAS Institute, Cary, NC) and the model testing for random effects of cow and period and the fixed effects of treatment infusion, day, sampling time, and their interaction. Because day and its interactions were not significant (P > 0.2) the interactions were removed from the model, and the data presented represent the average of d 1 and 7 of the experimental period. The slice option of the Mixed procedure was used to test for treatment by sampling time effects. 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). Oxyntomodulin data were analyzed using the same model but without the time
within day effect. Because day effect was not significant the data presented represent the average of d 1 and 7 of the experimental period.

Results and Discussion

In the present study, there was a time by treatment interaction (P < 0.10) for plasma ghrelin concentration (Figure 9) due to lower pre-prandial plasma ghrelin concentration on the soy oil and casein infusion treatments compared with the control animals. However, abomasal infusion of starch did not decreased pre-prandial plasma ghrelin concentration compared with control infused cows. The post-prandial decrease in plasma ghrelin concentration for the control and starch infused cows was similar to post-prandial decreases in plasma ghrelin concentration observed previously in lactating dairy cows (Roche et al., 2007). Also, a similar effect of lipids on pre-prandial ghrelin concentration in lactating dairy cows has been reported previously when dairy cows were fed ruminal inert fats (Bradford et al., 2008); however, to our knowledge the effect of increased postruminal supply of starch or protein on plasma ghrelin concentration has not been reported previously for lactating dairy cows. Likewise, I am not aware of any previous report comparing the direct effect of different macronutrients on plasma ghrelin concentration. In humans, it is been observed that high protein diets (Smeets et al., 2008) or protein preloads (Bowen et al., 2006) decreased plasma ghrelin concentration. As observed in this study, feeding proteins produced a pre-prandial decreased in plasma ghrelin concentration compared with carbohydrates (Bowen et al., 2006). Pre-prandial plasma ghrelin concentration was not different between control cows and those infused with starch, and plasma ghrelin concentration decreased after feeding in the starch
infused and in the control cows. A similar post-prandial decrease in plasma ghrelin concentration was observed previously in horses (Gordon and McKeever, 2006) and humans (Shiiya, 2002). In the present study (Relling and Reynolds, 2008), DMI decreased on day 7 of oil infusion but not on day 1 compared with control cows; however, concentration of ghrelin, a hormone that stimulates DMI in other species, was lower than control when oil was infused. As explained previously for GLP-1 (Relling and Reynolds, 2008), this could be because on day 1, DMI measurements began before abomasal infusions began (Relling and Reynolds, 2008). It is also possible that changes in plasma hormone concentration on day 1 are an adaptation process of the animal, which occurs prior to the change in DMI. In previous studies in cattle (Wertz-Lutz et al., 2006; Wertz-Lutz et al., 2008), plasma ghrelin concentration was higher in animals with lower MEI. In the present study we observed a decrease in plasma ghrelin concentration associated with an increase in MEI when casein was infused (Relling and Reynolds, 2008), but a decrease in plasma ghrelin concentration when oil was infused and MEI was similar to the control treatment. The increase in MEI on day 7 of starch infusion was not associated with changes in plasma ghrelin concentration compared with the control treatment. A possible reason for this difference is that in the present study the animals were fed ad-libitum and received supplemental nutrients via abomasal infusion, in contrast to a restricted feeding regime reported in previous studies (Wertz-Lutz et al., 2006; Wertz-Lutz et al., 2008).

Plasma concentration of immunoreactive OXM did not change due to treatments, day, or their interaction (P > 0.2; Figure 10) in the present study. Plasma OXM concentration and its effect on DMI have not been reported previously in dairy cows. The amino acid (AA)
sequence of OXM contains the sequence of glucagon plus eight additional AA on the carboxy end of the molecule. The OXM molecule is included in a bigger protein called glicentin. Glicentin and OXM were called “gut glucagon” (GUT) due to the cross-reactivity that they have with pancreatic glucagon antibodies in some assays, and the fact that they contain the pancreatic glucagon peptide. With the development of antibodies specific for pancreatic glucagon, GUT was measured as the difference between the total glucagon concentration, which was measured with the antibodies that cross-react with pancreatic and GUT peptides, and pancreatic glucagon concentration (Manns, 1971; Ghatei et al., 1983). In a previous study in lactating dairy cows (Benson and Reynolds, 2001), abomasal oil infusion increased plasma GUT and GLP-1 concentration; however, we can not confirm if that increase was due to an increase in OXM, glicentin or another protein from the metabolism of GUT that cross-reacted with that particular antibody. In non-ruminants, OXM decreases food intake (Small and Bloom, 2004) and is co-secreted with GLP-1 (Holst, 1997). However, in the present study we did not find any correlation (P > 0.2) between plasma OXM and GLP-1 or DMI (data not shown).

In conclusion, abomasal oil and casein, but not starch infusions decreased pre-prandial plasma ghrelin concentration compared with control cows. The decrease in plasma ghrelin concentration when fat was infused was associated with a decrease in DMI (Relling and Reynolds, 2008), but not when casein was infused. Abomasal macronutrient infusions did not increase plasma OXM concentration, despite an effect of oil infusion on plasma GLP-1 concentration.
References


Figure 8. Binding displacement of OXM on bovine plasma serial dilution (□) compared with OXM standard curve (♦). Logit = %B0/(1-%B0). The slopes were -2.50 and -1.92 for OXM standard curve and OXM for plasma bovine serial dilution. The ratio of the slopes was 0.7703.
Figure 9. Average plasma ghrelin concentrations of three 30-minutes consecutive samplings during days 1 and 7 of abomasal infusions of water (control ■), casein (■), starch (■) or oil (□) in lactating dairy cows. Cows where fed immediately after sampling at 0900 h.
Figure 10. Daily average plasma OXM concentration during day 1 and 7 of abomasal infusions of water (control), casein, starch or oil in lactating dairy cows.
CHAPTER 8

CONCLUSIONS

A series of six experiments was conducted to gain a better understanding of mechanisms involved in feed intake regulation by ruminants. Specifically, this dissertation investigated the effect of changes in concentrations of metabolites (i.e., glucose and non-esterified fatty acids (NEFA)), and hormones (i.e., insulin, glucagon like peptide-1 (GLP-1), glucose-dependent insulino tropic polypeptide (GIP), ghrelin, cholecystokinin (CCK) and oxyntomodulin (OXM)), on dry matter intake (DMI) and hypothalamic mRNA concentration of neuropeptide Y (NPY), agouti-related peptide (AgRP) and proopiomelanocortin (POMC) in ruminants.

The role of specific nutrients and hormones on in vitro mRNA expression of neuropeptides that regulate DMI in sheep has not been studied previously. Therefore, in the experiment reported in Chapter 3, the objective was to determine the effects of increased concentrations of glucose, propionate, or hormones (insulin, CCK, GLP-1, PYY) on mRNA abundance for the orexigenic neuropeptides NPY and AgRP, and the anorexic peptide POMC in sheep hypothalamus tissue cultured ex-vivo. These data differ from non-ruminant data where the presence of only one metabolite or hormone is enough to produce changes in mRNA abundance of one of these neuropeptides in the hypothalamus. However, in Chapter 3 I report that insulin and glucose in combination did increase POMC mRNA concentration. This study is the first to show an in vitro response
of POMC mRNA in sheep hypothalamus due to an interaction of insulin and glucose in the media. This interaction suggests hormones (in this case insulin) and metabolites are both needed in sheep to elicit a change in one of the major neuropeptides known to regulate DMI. Also, this could explain why the cerebral infusion of glucose does not change DMI in ruminants, except when plasma glucose and insulin are elevated above physiological concentrations.

Feeding fat to ruminants has been associated with a decrease in DMI, an increase in plasma GLP-1 and CCK concentration, and a decrease in plasma ghrelin concentration. Because most of the studies conducted where fat was supplemented were in ad libitum-fed animals, it is not known if the change in gut peptides is produced just because of the presence of fat in the diet or if is the peptides represent a mechanism for sensing the amount of MEI consumed by the animal. These gut peptides may play a major role in the regulation of DMI. In addition, I am not aware of studies in ruminants where the plasma concentration of gut peptides were associated with changes in mRNA expression for NPY, AgRP, and POMC. Therefore, for the experiment reported in Chapter 4, the objectives were to determine the effect of supplemental fat and amount of DMI and MEI on plasma concentrations of the gut peptides GLP-1, CCK, GIP, ghrelin and OXM, and the association of these peptides with the hypothalamic concentration of the neuropeptides NPY, AgRP and POMC. As expected, a numerical decrease in DMI was observed when supplemental fat was provided to sheep offered feed ad libitum, which was associated with an increase in plasma GLP-1 and CCK concentration. However, this is the first study in ruminants to report no changes in plasma GLP-1 and CCK concentration in sheep fed diets supplemented with fat if these diets are fed at a restricted
intake. These results imply that the plasma concentration of GLP-1 and CCK do not change solely in response to the presence of fat in the diet, but may play a role in the regulation of DMI in ad libitum-fed ruminants. Also as hypothesized, ad libitum-fed wethers had a smaller abundance of the orexigenic neuropeptides than restricted-fed wethers. This indicates that the hypothalamic regulation of DMI may not be fully responsible for changes in NPY, AgRP or POMC mRNA concentration, but additional regulatory mechanisms may exist. These mechanisms could include a direct effect of the hormones on the second order neurons that regulate DMI, or the secretion of the neuropeptides. Therefore, it is possible that the decrease in DMI is due to the effect of the gut peptides GLP-1 and CCK via a different mechanism. Plasma ghrelin concentration did not respond to supplemental fat. This may imply that plasma ghrelin concentration in growing wethers is more a reflection of energy status, and under the conditions of the current study, ghrelin was not a mechanism that stimulated the animals to eat. The time by fat treatment response for plasma GIP concentration in growing sheep has not been reported previously. The increase in plasma GIP concentration at the beginning of the study (during the first two weeks of the experiment) when fat was added to the diet, but not at the end of the feeding period may imply a mechanism of adaptation to energy redistribution when fat is fed. Plasma OXM did not increase due to treatments; however, its concentration was correlated with plasma GLP-1 concentration. It is possible that the L-cells in the small intestine in ruminants produce a different peptide from the N terminal part of the proglucagon gene, in addition to OXM. This has been reported in porcine, where glicentin is produced to a greater extent than OXM.
The data presented in Chapter 4 support the assumption that the mechanisms by which the animal senses an increase in energy density in the diet and “tells” the hypothalamus to decrease DMI are probably via endocrine signals such as changes in insulin or gut peptide concentration. Despite progress made in our understanding, more studies need to be conducted to be able to determine causative relationships and the separate and combined effects of each hormone and metabolite on the regulation of DMI.

As mentioned previously, an association of increased plasma CCK and GLP-1 concentrations and a reduction in DMI had been reported previously, but the long term effect of physiological increases in plasma CCK and GLP-1 concentrations due to intravenous infusion have not been reported in ruminants. Thus, for the experiment reported in Chapter 5, a single compartment, first-order kinetic model of hormone degradation was used to determine amount of hormone infusion needed to achieve similar concentrations of GLP-1 and CCK via infusion of the peptides, as those observed when fat was fed and increased GLP-1 and CCK concentrations were observed. The objectives were to determine the effect of physiological concentrations of GLP-1 and CCK (achieved via continuous intravenous infusion) on meal pattern and DMI, rate of passage of orally dosed Cr, and DM and nutrient digestibility in growing lambs. This is the first study in ruminants where the effect of plasma concentration of GLP-1 and CCK has been studied independent of the effects of feeding fat. This model was designed to allow determination of a causative role in DMI regulation, rather than just a coincidental association. Plasma concentration of GLP-1 and CCK were similar when fat was fed as when the peptides were infused. Thus, the single compartment, first-order kinetic model was successful in determining infusion doses resulting in concentrations that mimicked
those observed when fat was supplemented. This study showed some trends due to peptide infusion that were similar to feeding fat, such as a decrease in DMI (daily, the first hour after feeding, and the cumulative 23 h DMI) when GLP-1 was infused. These responses in DMI were not observed when CCK was infused. Therefore, GLP-1, but not CCK, may be a key endocrine factor that regulates the decrease in DMI when fat is fed. However, the mechanism for this function is not clear and more studies need to be conducted to understand the role of GLP-1 in DMI regulation in ruminants.

The mechanisms of how GLP-1 regulates DMI are not completely known. In non-ruminants, some studies have shown intracerebral infusions of GLP-1 changed mRNA expression of neuropeptides that regulate appetite; however, those results were inconsistent. In the experiment reported in Chapter 6, the objective was to determine the effect of jugular infusion of GLP-1 on DMI and mRNA concentration of the neuropeptides NPY, AgRP and POMC in growing wethers. With physiological doses of GLP-1 infusion used in the present study, plasma GLP-1 concentration did not increase to the same concentrations that occurred when fat was fed. Using higher doses, an increase in plasma GLP-1 concentration was achieved, but in contrast to the results of the experiment reported in Chapter 5, long term infusion of GLP-1 or feeding fat did not decrease DMI compared with the control animals. The lack of response in DMI may be the reason for the lack of difference in mRNA concentration for NPY, AgRP, and POMC. While a decrease in DMI is usually observed when fat is supplemented, this is not always the case and may be related to diet and/or the stage of production of the animals. Part of the lack of response due to feeding fat or infusing GLP-1 could be that the wethers in the present study were not able to reach their maximum productivity.
potential because they were confined in metabolism crates. However, it is also possible that the homeostatic mechanisms that regulate DMI and plasma hormone concentration were not changed enough by the treatments imposed to produce significant changes in DMI or hormone and neuropeptide concentration. In the second part of this study, an increasing amount of infusion of GLP-1 over time increased plasma GLP-1 concentration. The increase in plasma GLP-1 concentration produced a numerical decrease in DMI. However, this decrease in DMI was not statistically significant, due to the variability of the response. The totality of data collected in these experiments suggests that GLP-1 is largely responsible for the decrease in DMI when fat is fed.

The effect of abomasal infusion of macronutrients on plasma concentration of ghrelin and OXM had not been reported in lactating dairy cows. The objectives of the experiment reported in Chapter 7 were to determine the short- and long-term effects of abomasal infusions of macronutrients (protein, starch, or fat) on plasma concentrations of ghrelin and OXM in lactating dairy cows. Abomasal macronutrient infusions did change plasma OXM concentration compared with control cows. It has been proposed for non-ruminants that all the products of the proglucagon peptide, GLP-1 GLP-2 and OXM/glicentin, are co-secreted. The changes on plasma OXM and GLP-1 concentration reported in Chapter 4 and 7 were not similar, which may imply that the metabolism (secretion and degradation of the hormone) of OXM does not follow similar patterns as observed for GLP-1. However, plasma GLP-1 and OXM concentrations were correlated. It is also possible that the assay utilized in these experiments was not sensitive enough to detect similar changes. Abomasal oil and casein, but not starch, infusions decreased pre-feeding plasma ghrelin concentration compared with control cows. The decrease in
plasma ghrelin concentration when fat was infused was associated with a decrease in DMI, but this did not occur when casein was infused. From these results, it can be conclude that ghrelin by itself does not regulate DMI.

The overall conclusion for this dissertation is that the regulation of DMI depends on the interaction of many hormones and metabolites, and is not due to changes in solely one factor. However, it does appear that the gut peptide GLP-1 plays a more important role than CCK in the regulation of daily DMI in sheep. Also, the decrease in DMI observed when fat is fed is not regulated only by changes in gene expression of NPY, AgRP or POMC, but by other mechanisms. This could be a direct effect of GLP-1 in the second order neurons, as has been reported in non-ruminants. In all the studies where GLP-1, NEFA and glucose were measured; there was not an association between plasma GLP-1 concentration and the plasma concentrations of these metabolites. Although the primary focus of this dissertation was not to investigate the effect of insulin on DMI, the results in Chapter 3 and 4 show that insulin is important in the regulation of DMI. Mainly, insulin regulates DMI by regulating the gene expression of orexigenic neuropeptides. However, for insulin to have a regulatory effect on DMI, it needs to be associated with other metabolic changes, such as changes in plasma glucose or NEFA concentrations.

Another interesting finding of this dissertation was the lack of response in plasma OXM concentration to supplemental fat. Despite the expectation that plasma OXM concentration would respond in the same manner as plasma concentration of GLP-1, i.e., plasma OXM concentration would increase when fat is fed or abomosally infused, plasma OXM concentration did not change in either experiment (Chapter 4 and Chapter 7). Thus, it is possible that OXM is not the main product from the N terminal part of the
proglucagon gene in the L-cells in the small intestine. However, the lack of response may be due to the assay used, and more studies are needed to understand the role of OXM, glicentin and GUT in ruminants.

In summary, this dissertation shows that plasma GLP-1 concentration may be a key hormone responsible of the decrease in DMI observed when fat is fed. Such a decrease in DMI is caused partially by changes in gene expression of hypothalamic neuropeptides; however, it is possible that there are mechanisms, independent of the neuropeptides, by which feeding fat regulates DMI via the gut peptides.
Due to the lower concentration of GIP observed for the samples reported in Chapter 4, Relling et al. (2009), and in Larsen et al. (2009), compared with values observed previously (Relling 2006), a RIA was run to compare different antibodies and standard sources. For Chapter 4, Relling et al. (2009) and in Larsen et al. (2009), the antibody G027 (Phoenix Pharmaceutical, Inc) was used. In the assays described in Relling (2006) the antibody used was RAB-027-02 (Phoenix Pharmaceutical, Inc), but that particular antibody is no longer available commercially. This antibody was replaced with the G027-02 antibody (Phoenix Pharmaceutical, Inc). This new antibody is from the same animals but has been purified. Also in Relling (2006), the standard used was Porcine GIP (G5512, Sigma), which is also no longer available. For this reason, Human GIP standard (G2269, Sigma) was used. The results obtained using the G027 antibody were a tenth of those previously reported. For that reason, a way to compare both assays was explored.

Phoenix pharmaceutical has a GIP kit (RK 027-02) which has an antibody similar to RAB-027-02; they also have porcine GIP (027-11). Therefore, the two different antibodies and the two sources of GIP peptides were compared. Also, a displacement and a recovery of the four variables were performed with bovine and ovine plasma. The nomenclature used to represent the results was first the species of the GIP peptide used in the standard curve (P vs. H) and then the source of antibody (G027 vs. kit), i.e. a PG027 represent the standard curve run using porcine GIP and the antibody G027-02. Also, two bovine samples were run using the G027-02 and the concentration was compared with the four different standard curves.

In Figure 11 a difference in the slopes and in the intercept can be observed (Table 24). The difference in the intercept is probably due to a difference in B0 (percentage of the
radioactive hormone that remains in the tube due to binding to the antibody) of both antibodies (42 % for G027 vs. 57 % for the kit). The difference in slope is mainly due to a difference in displacement of the different standards with each antibody, in which the displacement of the radioactive $^{125}$I GIP, also known as trace, due to the porcine GIP standard is less than the displacement due to human GIP.

The displacement of bovine and ovine plasma compared with the different standard curves using both antibodies (Figures 12 and 13; Tables 25 and 26) showed that the type of antibody and standard that is recommended to use depends on the species where the plasma comes from. For example, for bovine plasma, a porcine standard is recommended for both antibodies (the use of the kit or for G027-20). However when sheep plasma is used, the use of the kit is not recommended. Also for sheep plasma porcine or human standard can be used.

To determine the recovery of the peptides, three different amounts of porcine and human GIP (with both Ig G sources in bovine and ovine plasma), were compared. The results of that recovery are in Table 27.

To confirm if there was any difference in the measurement of GIP using the different standards and antibodies, GIP concentration in two random samples using the different assays was measured. This revealed that the plasma concentration of GIP quantified (Table 28) in bovine plasma differed according the combination of sources of the standard and antibody. This could explain the differences observed previously (Relling, 2006) with the ones reported in Larsen et al. (2009), Relling et al. (2009), and in Chapter 4.
References:


Larsen, M., A. E. Relling, C. K. Reynolds, and N. B. Kristensen. Plasma concentrations of incretins (GIP and GLP-1) did not increase in periparturient cows abomasally infused with glucose. Submitted to the XIth International Symposium on Ruminant Physiology.
### Table 24

<table>
<thead>
<tr>
<th>Standard</th>
<th>Antibody</th>
<th>Intersection</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>porcine</td>
<td>Kit</td>
<td>4.306</td>
<td>-1.321</td>
</tr>
<tr>
<td>porcine</td>
<td>G027</td>
<td>4.204</td>
<td>-1.770</td>
</tr>
<tr>
<td>human</td>
<td>Kit</td>
<td>5.925</td>
<td>-2.101</td>
</tr>
<tr>
<td>human</td>
<td>G027</td>
<td>6.516</td>
<td>-3.118</td>
</tr>
</tbody>
</table>

Table 24. Intersections and slopes of the comparison of the standard curves using porcine and human GIP peptides and two different Ig G against human GIP (G027-02 and RK 027-02).

### Table 25

<table>
<thead>
<tr>
<th>Item</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine Std. curve</td>
<td>-1.321</td>
</tr>
<tr>
<td>Human Std curve</td>
<td>-2.101</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>-1.321</td>
</tr>
<tr>
<td>Ovine plasma</td>
<td>-0.366</td>
</tr>
</tbody>
</table>

Table 25. Slopes of serial dilutions of bovine and ovine plasma compared with human and porcine GIP standard curves using the RK-027-02 Ig G.

### Table 26

<table>
<thead>
<tr>
<th>Item</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine std. curve</td>
<td>-1.770</td>
</tr>
<tr>
<td>Human Std curve</td>
<td>-3.118</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>-2.059</td>
</tr>
<tr>
<td>Ovine plasma</td>
<td>-2.397</td>
</tr>
</tbody>
</table>

Table 26. Slopes of serial dilutions of bovine and ovine plasma compared with human and porcine GIP standard curves using G027-02 Ig G.
<table>
<thead>
<tr>
<th>Plasma source</th>
<th>Ig G Antibody</th>
<th>Recovery of GIP (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human</td>
<td>Porcine</td>
</tr>
<tr>
<td>Bovine</td>
<td>Kit</td>
<td>N/A</td>
<td>124.26</td>
</tr>
<tr>
<td>Ovine</td>
<td>Kit</td>
<td>86.29</td>
<td>92.33</td>
</tr>
<tr>
<td>Bovine</td>
<td>G027-02</td>
<td>124.26</td>
<td>95.29</td>
</tr>
<tr>
<td>Ovine</td>
<td>G027-02</td>
<td>126.22</td>
<td>121.75</td>
</tr>
</tbody>
</table>

Table 27. Recoveries of human and porcine GIP in bovine and ovine plasma.

<table>
<thead>
<tr>
<th>Plasma source</th>
<th>Ig G Antibody</th>
<th>GIP source for std curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>Sample 1</td>
<td>Kit</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>G027-02</td>
<td>0.074</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Kit</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>G027-02</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Table 28. Plasma GIP concentration (nM) on two random bovine plasma samples using the combination of porcine or human standard, and G-027-02 or RK-027-02.
Figure 11. Standard curves of porcine and human GIP using two different antibodies against human GIP (G027-20 and RK 027-02).
Figure 12. Comparison of serial dilution of bovine and ovine plasma with porcine and human standard curves using the antibody from RK 027-02.
Figure 13. Comparison of serial dilution of bovine and ovine plasma with porcine and human standard curves using G027-20 Ig G.
APPENDIX B

OXYNTOMODULIN ASSAY VALIDATION
Oxyntomodulin (OXM) is a peptide produced by the L cells, from the proglucagon gene (Figure 2). It is possible that in bovine and ovine, OXM is a peptide that has been measured and reported as gut glucagon (GUT). The measurement of (GUT) in dairy cows was measured by differential assays (Manns, 1972; Benson and Reynolds, 2001), as the difference between total and pancreatic glucagon. Total glucagon is measured using an antibody that binds to the glucagon and to gut glucagon. However, it is not clear which peptides are included as GUT; it could be OXM or glicentin. The antibody used in Benson and Reynolds (2001) was reported to have 40% binding to OXM, but it was not reported if it cross-reacted with any of the other gut glucagon products. For that reason, a series of standard curves was run to compare the binding of the total glucagon antibody (#4660-0204, Biogenesis, Poole, UK) to some of the products from the proglucagon gene, such as GLP-1 (1-37), GLP-1 (7-36), GLP-2, glicentin related polypeptide (GRPP), glucagon and OXM. As can be seen in Figure 14, the antibody binds to glucagon and OXM, but not to the other gut peptides. The intersection and slopes are in Table 29.

To be able to compare the increase in GUT, the sensitivity for the assays for total and pancreatic glucagon have to be similar. However, when the standard curves were compared using total and pancreatic (#1032, Linco, St. Louis, MO) glucagon, the sensitivity of the antibodies were not similar (Figure 15). Therefore the approach used by Benson and Reynolds (2001) would not be appropriate to use to measured “gut glucagon” or OXM.

That led the need to validate/develop a more proper assay to measured OXM. The ultrasensitive OXM RIA kit from Phoenix Pharmaceutical (RKU-028-22) was used in bovine and ovine plasma. The displacement was not parallel (Figure 16), but the ratio of
the slopes (Table 30) were 53.59 % and 59.12 % for bovine and ovine serial plasma dilution, respectively. The logarithm of the concentration and the Logit, which is the natural logarithm of the percentage of B0 divided one minus the percentage of B0 (Logit = Ln(%B0/(1 − %B0)), were plotted to obtain the slopes.

The recoveries of OXM in bovine and ovine plasma are reported in Table 31.

The protocol that was used was based in the one from Phoenix Pharmaceutical with some modifications. Some of the changes that were made were mainly to use half of the primary antibody, and the trace. It is appropriate to check the B0 in the validation; the reason for this change is that Phoenix Pharmaceutical reported a B0 of 38 %; however, the B0 in the present experiment was 26.6%.

References


<table>
<thead>
<tr>
<th>Standard</th>
<th>Intersection</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxyntomodulin</td>
<td>-0.2195</td>
<td>-0.265\ln</td>
</tr>
<tr>
<td>Glucagon</td>
<td>-0.4391</td>
<td>-0.188\ln</td>
</tr>
</tbody>
</table>

Table 29. Intersections and slopes of the comparison of Oxyntomodulin and glucagon standards curves using total glucagon antibody (4660-0204, Biogenesis, Poole, UK)

<table>
<thead>
<tr>
<th>Item</th>
<th>Slope</th>
<th>Displacement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human OXM std. curve</td>
<td>-3.1477</td>
<td>-</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>-1.6869</td>
<td>53.59</td>
</tr>
<tr>
<td>Ovine plasma</td>
<td>-1.861</td>
<td>59.12</td>
</tr>
</tbody>
</table>

Table 30. Slopes and displacements of serial dilutions of bovine and ovine plasma compared with human oxyntomodulin.

<table>
<thead>
<tr>
<th>Item</th>
<th>Recovery of human OXM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>99.47</td>
</tr>
<tr>
<td>Ovine plasma</td>
<td>97.03</td>
</tr>
</tbody>
</table>

Table 31. Recovery of 10, 20 and 40 pg/tube of OXM in 50 µl of bovine and ovine plasma
Figure 14. Percentage of B0 binding for the different proglucagon peptides
Figure 15. Logarithmic of the percentage of B0 binding for glucagon using antibodies for total and pancreatic glucagon.
Figure 16. Displacement of serial dilution of bovine and ovine plasma using percentage of B0 binding for OXM compared with OXM standard curve.


Chan, J. L., V. Stoyneva, T. Kelesidis, P. Raciti, and C. S. Mantzoros. 2006. Peptide YY levels are decreased by fasting and elevated following caloric intake but are not regulated by leptin. Diabetologia. 49:169-173.


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