Doctoral Thesis

Studies on the Interaction of Nutrients and Ghrelin in Lactating Dairy Cows

Rika Fukumori

Graduate School of Biosphere Science

Hiroshima University

March 2013

Doctoral Thesis

Studies on the Interaction of Nutrients and Ghrelin in Lactating Dairy Cows

Rika Fukumori

Department of Bioresource Science

Graduate School of Biosphere Science

Hiroshima University

March 2013

Contents

Chapter 1

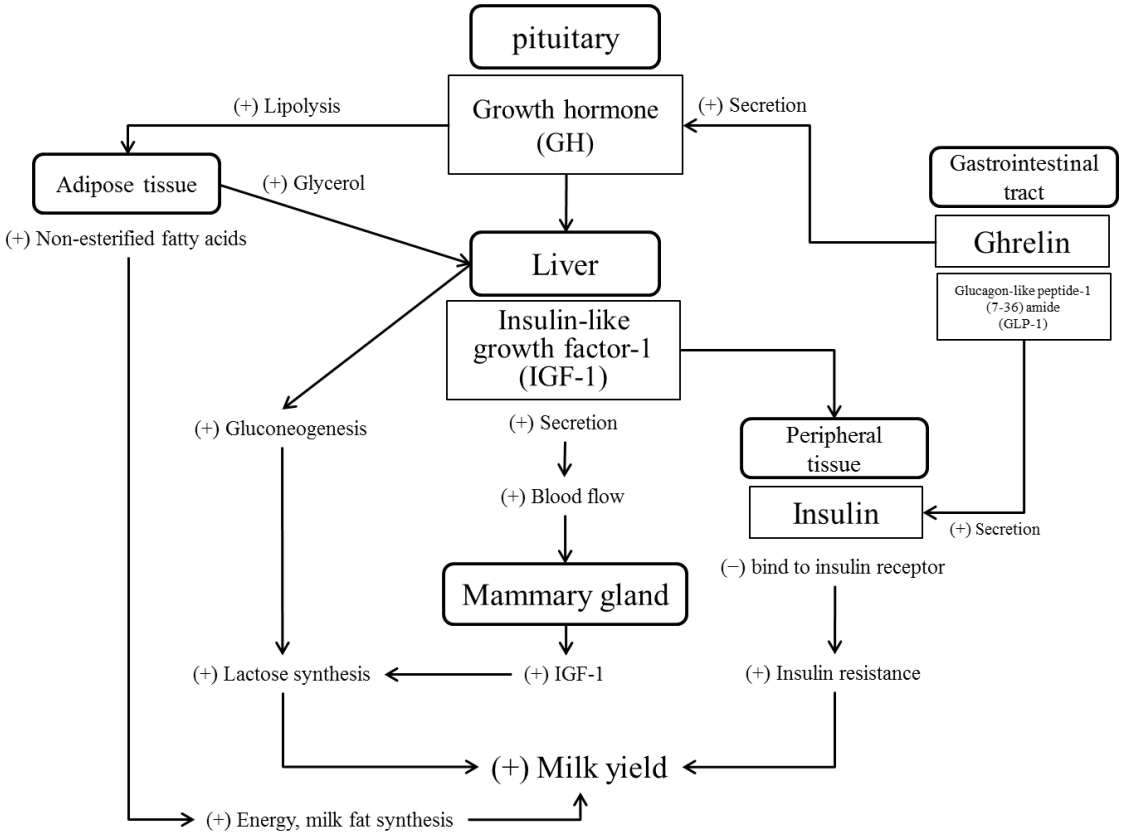
General Introduction

1.1. The endocrine control systems for lactation of dairy cows

Dairy cows produce milk through the use of nutrients derived from ingested feeds and mobilized body tissues when their energy intake is insufficient. The metabolic processes partitioning nutrients to milk are important factors to determine milk yield for lactating dairy cows, and are under the endocrine control of several metabolic hormones. In particular, growth hormone (GH) plays a primary role in increasing milk production for lactating dairy cows (Peel et al., 1982; Burton et al., 1994 [Figure 1-1]). GH stimulates gluconeogenesis in the liver and lipolysis in the adipose tissue, to provide energy and a substrate of milk fat synthesis (Bauman and Currie, 1980). Simultaneously, GH increases plasma insulin-like growth factor-1 (IGF-1) concentration (Davis et al., 1987), which enhances mammary gland blood flow in lactating ruminants (Prosser et al., 1990, 1994) and causes insulin resistance in the adipose and muscular tissues (Burton et al., 1994). Numerous trials have confirmed the galactopoietic effects of recombinant bovine GH (rbGH), and 6-41% increases in milk yield were observed (Burton and McBride, 1989; Bauman and Vernon, 1993; Bauman et al., 1985). In the United States, the usage of rbGH are permitted for increasing milk production, whereas it has not been allowed on the market in Japan, Canada, Australia, New Zealand, Israel and all European Union countries with consumer's concern over safety and security of food. Therefore, it is necessary to

develop the management systems for improving the production efficiency without relying on exogenous administration of rbGH.

Figure 1-1



The galactopoietic effects of GH and association with ghrelin.

1.2. Ghrelin: a novel GH-releasing peptide derived from the gastrointestinal tract

GH release is stimulated by hypothalamic GH-releasing hormone (GHRH) and inhibited by somatostatin (Anderson et al., 2004). Recently, however, the third independent pathway regulating GH release has been identified from the studies on GH

secretagogues (GHSs). Using a reverse pharmacology paradigm with a stable cell line expressing GHS receptor (GHS-R), Kojima et al. (1999) found a novel endogenous ligand for GHS-R from rat stomach, and named it ghrelin. Ghrelin can potently stimulate GH secretion from the pituitary gland (Kojima et al., 1999; Takaya et al., 2000). In addition, ghrelin has been found in the hypothalamic arcuate nucleus, an important region for controlling appetite (Kojima et al., 1999). This suggests that ghrelin play a role in controlling food intake. In fact, injection of ghrelin into the cerebral ventricles of rats potently stimulates food intake (Nakazato et al., 2001). Therefore, ghrelin may have some preferable effects for lactating cows.

1.3. Ghrelin secretion and function in ruminants

Ghrelin is mainly secreted by the abomasum in ruminants (Hayashida et al., 2001) and can stimulate GH secretion (Itoh et al., 2005; Hosoda and Kangawa, 2008; Fukumori et al., in press-b). Plasma ghrelin concentration is related to the feeding and nutritional status of ruminants (Sugino et al., 2004). In lactating cows, negative energy balance increases periprandial ghrelin concentrations (Bradford and Allen, 2008). Sugino et al. (2006) showed that plasma ghrelin concentration increased at postpartum and a variation of plasma GH concentration was related to the change of ghrelin in dairy cows. In addition, the ghrelin-induced GH secretion might be augmented during early lactating period (Itoh et al., 2005). Additionally, Itoh et al. (2006) reported that ghrelin increased plasma pancreatic hormones and glucose levels during only lactating period. Roche et al. (2008) also reported that long-term infusion of ghrelin increased plasma non-esterified fatty acids (NEFA) concentration in lactating cows. Thus, ghrelin could

play an important role to regulate the secretion and action of metabolic hormones, changing the nutrient metabolism for milk production in lactating cows.

1.4. Effects of nutrients on plasma ghrelin concentration in ruminants

Plasma ghrelin concentrations are related to feeding; A preprandial rise and a postprandial fall in circulating ghrelin concentrations have been observed in humans (Cummings et al., 2002) and sheep (Sugino et al., 2002). Some reports propose that circulating metabolites affect the depression of circulating ghrelin concentrations. In non-ruminants, both oral and intravenous doses of glucose depressed plasma ghrelin concentrations (Nakagawa et al., 2002, Shiiya et al., 2002). In ruminants, however, the major energy substrate is volatile fatty acids (VFA). Therefore, it is likely that the inhibiting factor for ghrelin secretion may differ between non-ruminants and ruminants. In fact, VFA (acetate, propionate and butyrate) injections depressed plasma ghrelin concentrations in wethers and calves (Fukumori et al., 2011; Fukumori et al., in press-a), while glucose injection did not depress in sheep and calves (Sugino et al., 2010; Fukumori et al., in press-a). In addition, a high-protein diet consumed sheep showed higher plasma ghrelin concentration compared with a low-protein diet in sheep (Takahashi et al., 2009), and the intravenous infusion of amino acids increased plasma ghrelin concentration in sheep (Sugino et al., 2010). These results suggest that available amino acids can increase plasma ghrelin concentration. Furthermore, plasma ghrelin concentration was raised by feeding medium chain fatty acids in rat (Nishi et al., 2005). Additionally, the previous study using lactating ewes showed feeding long-chain fatty acids with methionine increased milk production with an increase in

DMI (Goulas et al., 2003). Thus, it was hypothesized that medium-chain and long-chain fatty acids with methionine would be effective to increase plasma ghrelin concentration, although the effects of these nutrients on plasma ghrelin concentration had not been proved in ruminants. The nutritional strategies modifying the supply of amino acids and fatty acids to control plasma ghrelin concentration may result in the preferable milk production via alterations of plasma GH and pancreatic hormone concentrations. Therefore, the objectives of this study were to determine the effect of nutrients on secretion and action of ghrelin, and relationship with milk production in lactating dairy cows including three sub-objectives:

Sub-objective 1:

To determine the effects of amino acids on plasma ghrelin concentration, and to elucidate the ghrelin action inducing GH, glucagon and insulin secretion in lactating cows.

Sub-objective 2:

To determine the effects of medium-chain fatty acids on plasma concentration and action of ghrelin, and to investigate the interaction of metabolic hormones, metabolites and milk production in lactating cows.

Sub-objective 3:

To determine the effects of long-chain fatty acids plus methionine on plasma ghrelin concentration, and to investigate the interaction of metabolic hormones, metabolites and milk production in lactating cows.

The nutritional strategy that focused on regulating endogenous hormone secretion to get efficient production is an unique attempt. These new findings would contribute to understand ghrelin secretion and its role in regulating lactation performance of dairy cows.

Chapter 2

Effects of Intravenous Infusion of Amino Acids on Plasma Ghrelin Concentration, and GH, Insulin and Glucagon Secretion Induced by Ghrelin Injection in Lactating Dairy Cows

2.1. Introduction

The relationship between available nutrients and ghrelin secretion was documented in several published papers. A high protein diet increased ghrelin plasma levels in rats (Vallejo-Cremades et al., 2004), and oral low-dose of an essential amino acid mixture increased plasma ghrelin concentration in fasting humans (Knerr et al., 2003). In sheep, the intravenous infusion of an amino acid mixture also increased plasma ghrelin concentration in sheep (Sugino et al., 2010). These previous results suggest that amino acids possibly increase plasma ghrelin concentration in lactating cows. Therefore, the objective of the present study was to investigate the effect of amino acids infusion into the jugular vein on plasma ghrelin concentration in lactating dairy cows. Additionally, nutrient availability may affect ghrelin action. Itoh et al. (2006) demonstrated that ghrelin injection increased plasma GH, glucagon, insulin and glucose concentrations during the lactating period of dairy cows. Thus, the second aim of this study was to determine the effect of amino acids on ghrelin action in regulating GH secretion, pancreatic hormone secretion and nutrients (glucose and NEFA) metabolism in lactating dairy cows.

2.2. Materials and Method

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock and Grassland Science Animal Care Committee, and which were formulated to comply with Japanese regulations.

2.2.1. Animals

Six lactating Holstein cows [calving number: 3.3 ± 0.8, initial days in milk (DIM): 57.2 ± 2.1, initial body weight (BW): 687 ± 18 kg] were fed a diet according to the Japanese Feeding Standard for Dairy Cattle (Agriculture, Forestry, and Fisheries Research Council Secretariat, 2006), twice daily (0900 h and 1800 h). The cows were allowed free access to the diet and water. Refusals were weighed daily before the morning feeding. The crude protein (CP) and total digestible nutrient (TDN) contents in the diet and the level of TDN offered were 13.0% and 69.8% on the dry matter (DM) basis, respectively. Cows were milked twice daily before each feeding. The daily DM intake, milk yield, TDN and CP sufficiency through the experiment were 23.1 ± 1.0 kg, 32.5 ± 0.4 kg, 105.7 ± 3.5% and 97.0 ± 4.3%, respectively. The cows were inserted chronic catheters (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) into both sides of the jugular vein at least 1 day before the first treatment. These catheters were used for both reagent infusion and blood sampling.

2.2.3. Treatments and blood sampling

Cows were randomly given two infusion treatments with mixture solution of

amino acids [AMI: Aminic (Ajinomoto Pharmaceuticals Co., Ltd., Tokyo, Japan) plus methionine dissolved into saline, total amino acids: 91.1 mg/mL, infusion rate: 4.0 mg/kg of metabolic body weight ($BW^{0.75}$)/min] or saline (CON) in a cross-over design. Each treatment was carried out at least 1 week interval to avoid measurement values being affected by the previous treatment. The amino acids composition of AMI is presented in Table 2-1. The time schedule for the infusion and injection treatment, and the blood sampling is showed in Figure 2-1. The infusion amount of AMI was equal to about 6% of the requirement of digestible crude protein (CP) and compensated for the lack of CP intake. At 4 h after morning feeding (1300 h), a mixture solution of amino acids or saline was continuously infused into the jugular vein catheter on the left side. The solutions were infused with a peristaltic pump (Model 312/MP-4; Gilson, Inc., Middleton, France) for 4 h. At two hours after the initiation of AMI and CON infusion, synthetic bovine ghrelin (1 μg/kg BW; KNC Laboratories Co., Ltd., Kobe, Japan) was single dosed into the right side jugular vein through the catheter. This injection dose of ghrelin was chosen based on Itoh et al. (2006). Their report demonstrated that a dose of ghrelin similar to that used in this study had clear effects on insulin and glucagon secretion in lactating cows. After ghrelin injection, 10 mL of saline was injected for flushing the inner of the catheter. Blood samples were taken at −130, −120, −10, 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 150 and 180 min relative to the ghrelin injection from the catheter of the right side jugular vein and collected into tubes containing heparin (10 U/mL of blood; Wako Pure Chemical Industries Ltd., Osaka, Japan) and aprotinine [500 kilo inhibitor unit (KIU)/mL of blood; Trasyrol, Bayer, Leverkusen, Germany]. The obtained blood samples were centrifuged at 1,500 × *g* for 20 min at 4°C. Harvested plasma was stored at −80°C prior to assay.

2.2.4. Sample analysis

Plasma ghrelin, GH and insulin levels were measured with time-resolved fluoro-immunoassay (TR-FIA). Assay for bioactive ghrelin and GH was conducted as described previously (Sugino et al., 2004). The ghrelin concentration was measured by competitive solid-phase immunoassay using europium (Eu)-labeled synthetic rat ghrelin (Peptide Institute, Inc., Osaka, Japan) and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin (Kitasato University, Aomori, Japan) and anti-rat ghrelin serum (National Cardiovascular Center Research Institute, Osaka, Japan). The ghrelin antibody is particular with acylated ghrelin (positive form of ghrelin). Intra- and inter-assay coefficients of variation were 1.3 and 1.5%, respectively. Least detectable dose in this assay system was 0.025 ng/mL. The GH concentration was measured by competitive solid-phase immunoassay using Eu-labeled synthetic ovine GH (NIDDK, Bethesda, USA) and polystyrene microtiter strips coated with anti-rabbit γ-globulin and anti-ovine GH serum (NIDDK, Bethesda, USA). Intra- and inter-assay coefficients of variation were 2.6 and 3.6%, respectively. Least detectable dose in this assay system was 0.158 ng/mL. Insulin assay was conducted as described previously (Takahashi et al., 2006). The insulin concentration was measured by competitive solid-phase immunoassay using Eu-labeled synthetic bovine insulin (Sigma-Aldrich Inc., Tokyo, Japan) and polystyrene microtiter strips coated with anti-guinea pig γ-globulin (JAPAN LAMB CO., LTD., Hiroshima, Japan) and anti-human insulin serum (Yanaihara Institute Inc., Shizuoka, Japan). Intra- and inter-assay coefficients of variation were 2.2 and 1.8%, respectively. Least detectable dose in this assay system was 0.016 ng/mL.

Plasma glucagon concentration was measured using commercially available radioimmunoassay kit (glucagon assay kit; Daiichi Radioisotope Laboratories Ltd., Tokyo, Japan). Glucagon concentration was measured in the same assay, and the intra-assay coefficient of variation was 3.4%. Least detectable dose was 1.5×10^{-5} ng/mL.

Plasma glucose concentrations were determined using a glucose analyzer (GA-1151; Arkray, Inc., Kyoto, Japan). Plasma alpha amino nitrogen (AAN) concentration was analyzed following the procedures of Goodwin (1968). Plasma NEFA concentrations were determined with the commercially available kit (NEFA C test Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.2.5. Calculations and statistics

The values of plasma hormones and metabolites were expressed as means of six cows with standard error (SE). The area under the curve (AUC) was calculated for hormones and metabolites after ghrelin injection over 0-60 min, 60-120 min and 120-180 min terms. The values were expressed as means of six cows with SE. Data were analyzed as a crossover design with a mixed linear model using restricted maximum likelihood by the JMP program package (version 5.01 for Windows computer system, SAS Institute, Inc., Cary, NC, USA). Dependent variables were summarized as least squares means and least squares SE. The model included the treatment, time and the interaction between treatment and time as the fixed effects, and cow and period as the random effects. The temporal changes of the concentrations for each infusion treatment were evaluated by multiple comparisons with Tukey's test. The differences in plasma concentrations at each time and AUC at each term between

CON and AMI were evaluated by Student's t-test. $P < 0.05$ was considered significant.

2.3. Results and Discussion

In rats, ghrelin expression and secretion were enhanced by feeding of a high protein diet (Vallejo-Cremades et al., 2004; 2005). Knerr et al. (2003) reported that oral low-dose of essential amino acid mixture bolus elicited hyper-aminoacidemia and an increased ghrelin secretion in fasting humans. In sheep, plasma ghrelin concentrations were increased by abomasal infusion of casein plus starch (Sugino et al., 2010). Thus, the absorption of amino acids may be one of the stimulating factors of ghrelin secretion in both ruminant and non-ruminant animals. In addition, a recent study demonstrated that the intravenous infusion of amino acids increased plasma ghrelin concentrations in sheep (Sugino et al., 2010). These previous reports suggest that the increased circulating levels of amino acids may play a key role in enhanced ghrelin secretion. In this study, the amino acid infusion increased plasma AAN concentration (pre-infusion: 0.328 mg N/dL, post-infusion: 0.462 mg N/dL, $P < 0.05$, Figure 2-2A) and AAN AUC was greater in AMI compared with CON (0-60 min; CON: 20.9 mg N·min/dL, AMI: 27.2 mg N·min/dL and 60-120 min; CON: 19.2 mg N·min/dL, AMI: 27.2 mg N·min/dL, $P < 0.05$, Figure 2-4A), but did not affect the plasma ghrelin concentration (Figures 2-3A, 2-5A). Sugino et al. (2010) demonstrated that more than 66% rise of plasma AAN concentration stimulated ghrelin secretion, but not in 27% rise of plasma AAN concentration. In the present study,

41% rise of plasma AAN levels by amino acids infusion has not changed plasma ghrelin levels in lactating cows. The present study examined the effect of the amino acid infusion under the deficient of CP intake (97%) to make the infusion effective. The infusion amount of amino acids was the extent to compensate for the shortage of the feed. On the other hand, previous reports tested more extreme differences between control and treatment (Sugino et al., 2010; Takahashi et al., 2009). Takahashi et al. (2009) examined high-CP diet (40%) versus low-CP diet (10%) in wethers, resulting high-CP diet tended to increase plasma ghrelin concentration. The infusion amount of amino acid by Sugino et al. (2010) was equal to about 60% of the requirement of digestible CP. Thus, the infusion amount of amino acids in the present study would be too low to increase plasma ghrelin concentration. The types of amino acids may be also an important factor for ghrelin secretion. Kuhara et al. (1991), who infused intravenously 17 kinds of amino acids at the each dose of 3.0 mmol/kgBW over 30 min, observed that acidic amino acids stimulated GH secretion, neutral amino acids stimulated insulin secretion, and neutral straight-chain amino acids stimulated glucagon secretion in sheep. The total amount of the mixture of amino acids was nearly equal level (2.68 mmol/kgBW over 120 min) with the study by Kuhara et al (1991), but the infusion level of each amino acid was lower, which might be the reason of no response of GH, insulin and glucagon to AMI observed in the present experiment. There may be a threshold level of plasma AAN and the kind of amino acid stimulating ghrelin and metabolic hormone secretion.

Plasma ghrelin concentration increased after ghrelin injection ($P < 0.05$, Figure 2-3A) with no difference between CON and AMI (Figures 2-3A, 2-5A). Plasma GH concentrations were increased by ghrelin injection in both treatments ($P < 0.05$, Figure

2-3B). Plasma GH concentration peaked at 5 min after ghrelin injection, and then decreased to the basal level within 50 min in both treatments. The GH secretory response to ghrelin was not different between both treatments (Figures 2-3B, 2-5B). Takahashi et al. (2009) reported that ghrelin stimulated GH secretion more in the satiety state than in the fasting state in sheep. Thus, the GH secretory response to ghrelin might be affected by feeding states or energy balance. The GH secretory response to ghrelin injection in this study was investigated under the same feeding state. Thus, GH secretion by ghrelin injection likely did not be affected by the difference in the level of the circulating amino acids.

Plasma glucagon concentration increased after ghrelin injection in both treatments ($P < 0.05$, Figure 2-3D). Itoh et al. (2005) have reported that ghrelin enhanced glucagon secretion only in lactating cows and that ghrelin induced pancreatic glucagon secretion in diabetic rats, but not in normal rats (Adeghate and Parvez, 2002). This ghrelin action in stimulating glucagon secretion could be expressed when insulin resistance is enhanced under the conditions such as lactating or diabetes. A significant increase of plasma glucagon concentration was observed at 5 min after ghrelin injection in both treatments, and thereafter, plasma glucagon concentrations rapidly declined in CON, but not in AMI (25 min; CON: 0.0896 ng/mL, AMI: 0.112 ng/mL, $P < 0.05$, Figure 2-3D). Consequently, glucagon AUC from 0 to 60 min in AMI was greater compared with CON (0-60 min; CON: 5.10 ng･min/mL, AMI: 6.13 ng･min/mL, $P < 0.05$, Figure 2-5D). In the current study, there was no effect of the amino acid infusion on plasma glucagon concentration before ghrelin injection. However, the rise of plasma glucagon concentration by ghrelin injection was augmented in AMI compared with CON. Thus, it may be possible that the infused

amino acids enhance glucagon secretion through a ghrelin-dependent process. In this study, however, such mechanism was unclear and further investigations are suggested for more clarification.

Plasma glucose and NEFA concentrations increased after ghrelin injection in both treatments ($P < 0.05$, Figures 2-2B, 2-2C). These responses were preceded by increased plasma GH and glucagon concentrations by ghrelin injection. It is well known that GH and glucagon enhance glucose and NEFA release into the blood circulation through their gluconeogenic, glycogenolytic and lipolytic actions. Because ghrelin inhibits insulin signaling in skeletal muscle, liver, and fat tissue (Murata et al., 2002; Barazzoni et al., 2008), ghrelin-induced hyperglycemia may also be related to the limited peripheral glucose uptake and/or to the enhanced gluconeogenesis. Although there has been no information about an acute response to ghrelin on plasma NEFA concentration, long-term infusion of ghrelin in lactating cows increased plasma NEFA concentration with no changes in plasma GH concentration (Roche et al., 2008). In the present study, therefore, ghrelin injection possibly increased plasma glucose and NEFA concentrations via GH and glucagon.

Plasma insulin concentration increased after ghrelin injection with observed higher peak at 10 min in AMI compared with CON (CON: 2.967 ng/mL, AMI: 3.875 ng/mL, $P < 0.05$, Figure 2-3C), although insulin AUC was not significantly different between two infusion treatments (Figure 2-4C). Although the increased plasma glucose and NEFA concentrations after ghrelin injection were not different in the peak values between CON and AMI (Figures 2-2B, 2-2C), the decline in plasma glucose concentration from 60 to 120 min was greater in AMI compared with CON ($P < 0.05$, Figures 2-2B, 2-3B). The reason might be related to a greater insulin peak level in

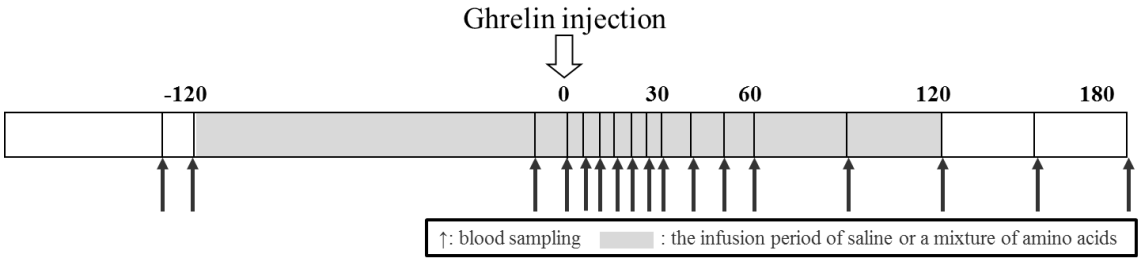
AMI. The insulin response followed the increases in plasma glucagon and glucose concentrations. Glucagon stimulates insulin secretion directly by paracrine manner in the pancreas or via hyperglycemia by enhancing glucose release from the liver (Johnson et al., 1972). Takahashi et al. (2006, 2007) demonstrated that intrajugular ghrelin administration stimulated glucose-induced insulin secretion in meal-fed sheep. In the present study, ghrelin might increase plasma glucagon and glucose concentrations, thereby enhancing insulin secretion. Sugino et al. (2010) revealed that hyper-aminoacidemia and hyper-ghrelinemia enhanced glucose induced insulin secretion in sheep. Thus, the high values of plasma insulin concentration in AMI might be resulted because of the high values of plasma AAN and ghrelin concentrations compared with CON.

In conclusion, the increase in plasma AAN concentration may enhance ghrelin action to increase insulin and glucagon secretions into blood circulation without affecting plasma ghrelin concentration during lactating period in cows.

Table 2-1. Composition of amino acids mixture used in this study

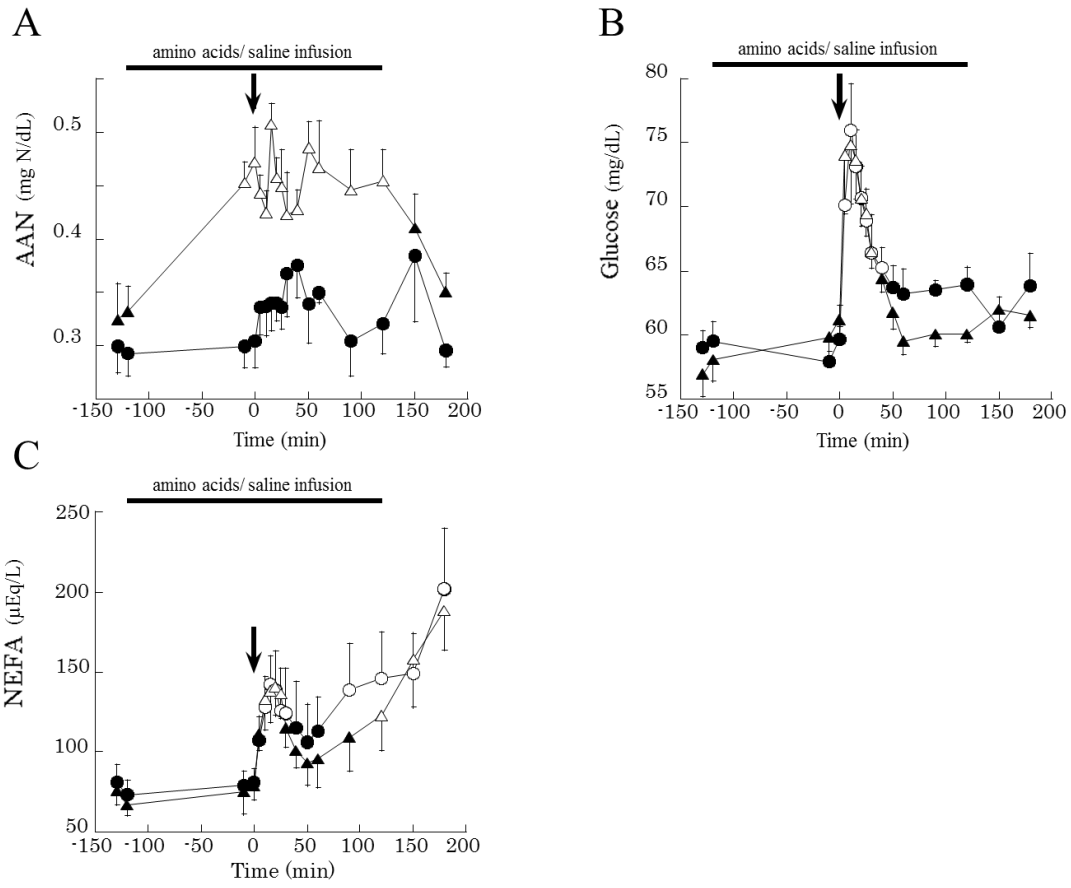
Item	w/v (%)
L-valine	1.17
L-leucine	1.08
L-lysine acetate	0.833
L-isoleucine	0.758
L-arginine	0.750
L-threonine	0.625
L-alanine	0.592
Glycine	0.583
L-phenylalanine	0.583
L-proline	0.417
L-histidine	0.417
L-methionine	0.867
L-serine	0.142
L-tryptophan	0.108
L-aspartate	0.0833
L-glutamate	0.0417
L-tyrosine	0.0333
L-cystine	0.0292
Total amino acids	9.11
Total N (mg/mL)	12.7

Figure 2-1



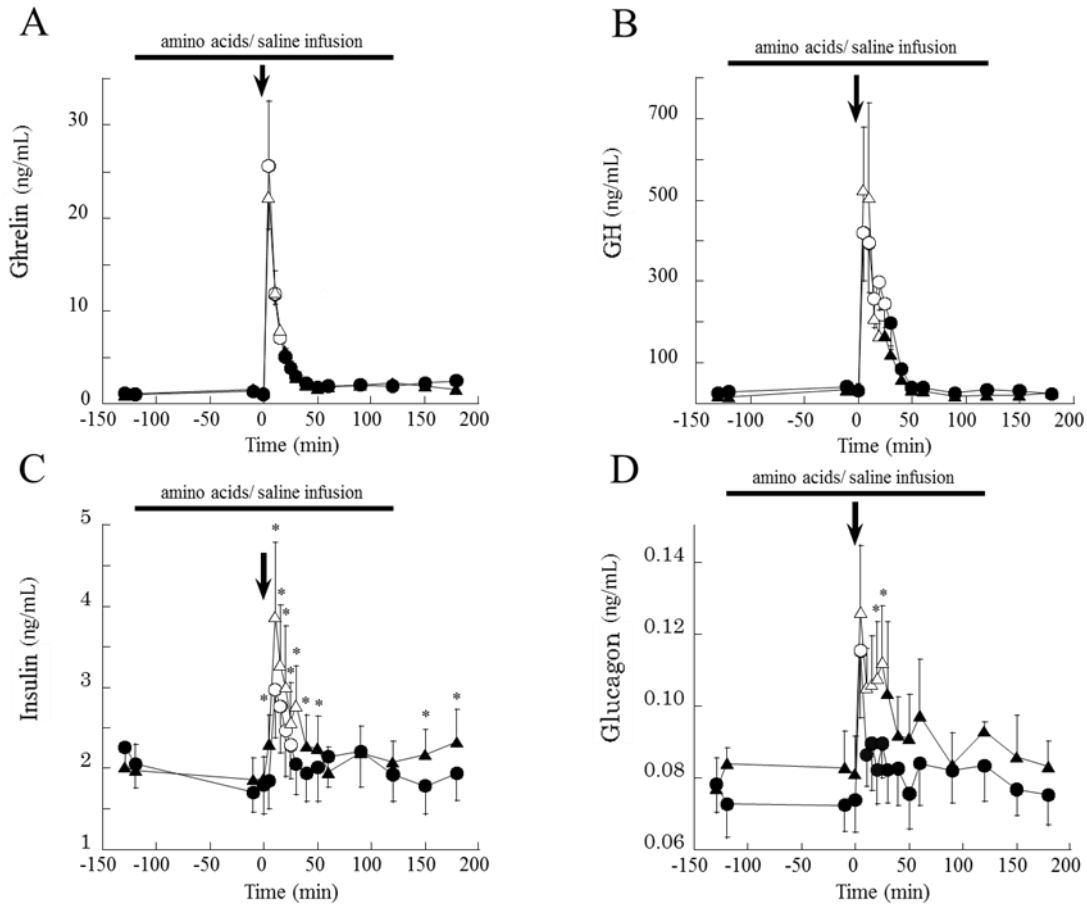
The time schedule of saline or amino acids infusion, ghrelin injection and blood sampling.

Figure 2-2



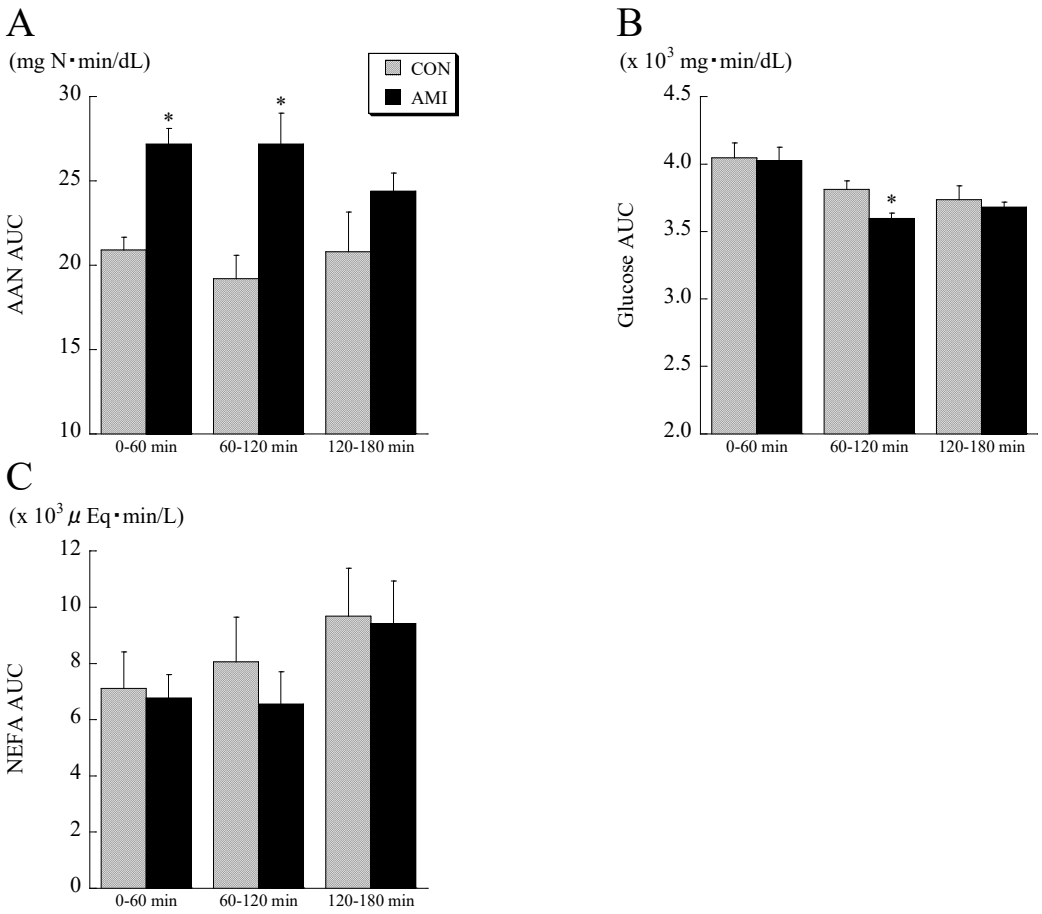
Plasma AAN (A), glucose (B) and NEFA (C) concentrations in amino acids (AMI) and saline (CON) treatments before and after ghrelin injection. Values are expressed as mean ± SE (vertical bar, n = 6). Circles and triangles show the CON and AMI treatments, respectively. *: $P < 0.05$ compared between CON and AMI at each time. Open plot: $P < 0.05$, difference between pre amino acids or saline infusion (mean values from −130 and −120 min). The horizonal bar and arrow show the period of the amino acids or saline infusion period and ghrelin injection time, respectively.

Figure 2-3



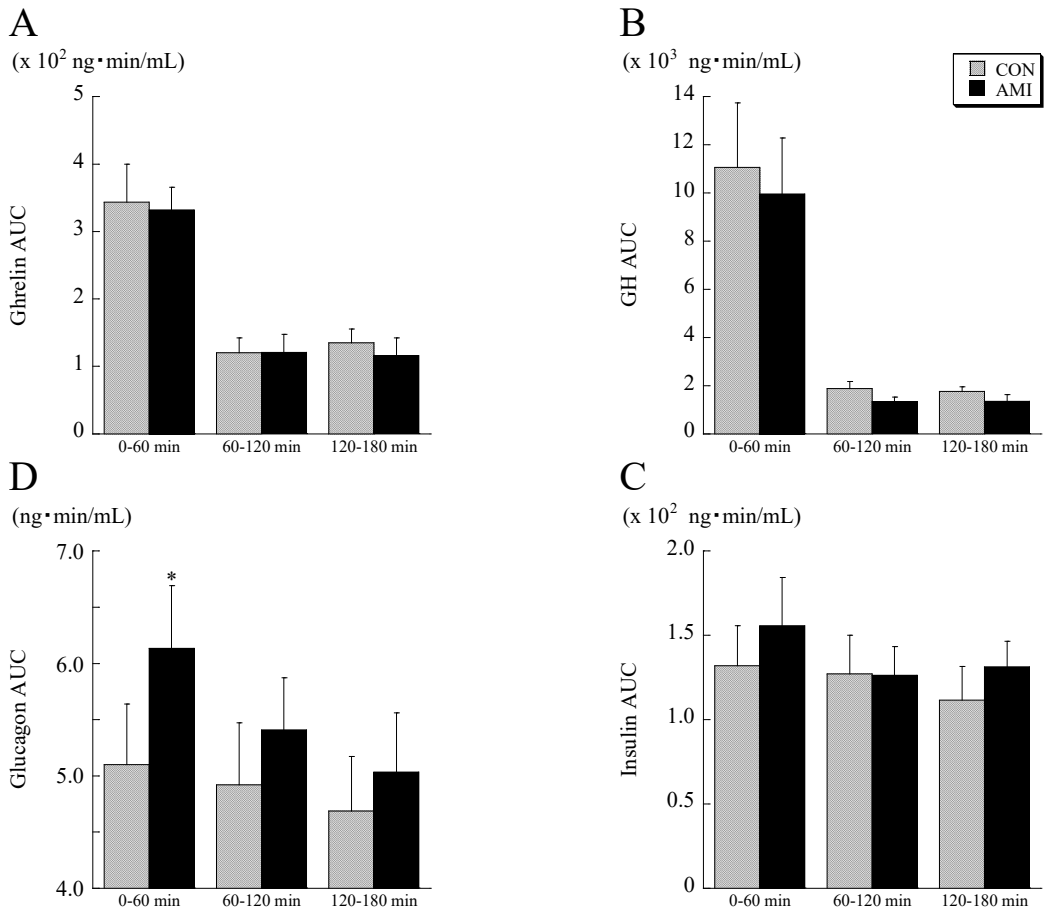
Plasma ghrelin (A), GH (B), insulin (C) and glucagon (D) concentrations in amino acids (AMI) and saline (CON) treatments before and after ghrelin injection. Values are expressed as mean ± SE (vertical bar, n = 6). Circles and triangles show the CON and AMI treatments, respectively. *: $P < 0.05$ compared between CON and AMI at each time. Open plot: $P < 0.05$, difference between pre amino acids or saline infusion (mean values from −130 and −120 min). The horizonal bar and arrow show the period of the amino acids or saline infusion period and ghrelin injection time, respectively.

Figure 2-4



The area under the curves (AUCs) of plasma AAN (A), glucose (B) and NEFA (C) concentrations over 0-60 min, 60-120 min and 120-180 min following ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, n = 6). *: $P < 0.05$ compared between CON and AMI at each period.

Figure 2-5



The area under the curves (AUCs) of plasma ghrelin (A), GH (B), insulin (C) and glucagon (D) concentrations over 0-60 min, 60-120 min and 120-180 min following ghrelin injection in amino acids (AMI) and saline (CON) treatments. Values are expressed as mean ± SE (vertical bar, n = 6).
*: $P < 0.05$ compared between CON and AMI at each period.

Chapter 3

Effects of Feeding Medium-Chain Fatty Acids on Plasma Concentration and Action of Ghrelin and Milk Production in Lactating Dairy Cows

3.1. Introduction

Ghrelin has a unique characteristic that it requires acylation with octanoate (C8:0) at its Ser 3 residue in order to become a proper ligand for its only known receptor, the growth hormone secretagogue receptor 1a (GHS-R1a) (Nakazato et al., 2001). In rats, medium-chain fatty acids (MCFA, C6:0–C12:0) ingestion increased stomach octanoyl ghrelin, whereas the amount of total ghrelin remained unchanged in rats (Nishi et al., 2005). Kirchner et al. (2009) also showed an increase of more than double the concentration of octanoyl ghrelin in mice fed a medium-chain triglyceride-enriched diet. These studies suggested that dietary MCFA are used directly in ghrelin acylation. Therefore, MCFA ingestion can increase plasma octanoyl ghrelin concentration in lactating cows. However, the effect of MCFA on plasma ghrelin concentration has not been examined in ruminants. Furthermore, MCFA may be an energy supplement for lactating cows because they are rapidly metabolized in the body. However, several reports showed that MCFA had adverse effects on ruminal fermentation (Dohme et al., 1999, 2001) and including MCFA in the diets as a triglyceride decreased dry matter intake (DMI) in lactating cows (Grummer and Socha, 1989; Reveneau et al., 2012). MCFA, especially lauric acid (C12:0),

exhibit potent antiprotozoal properties and reduce ruminal methane production (Dohme et al., 1999, 2001). Calcium salts of fatty acids are products aimed to depress the adverse effects on ruminal fermentation (Schneider et al., 1988) and several reports showed that calcium salts of long-chain fatty acids were used for lactating cows succeeded to avoid the adverse effect on ruminal fermentation (Schauff and Clark, 1989; Schneider et al., 1988). Therefore, the aim of this study was to investigate the effect of calcium salts of MCFA (MCFA-Ca) on plasma ghrelin concentration. Simultaneously, the present study evaluated the other metabolic and gastrointestinal hormones. In this study, plasma GH, IGF-1, glucagon-like peptide-1 (7-36) amide (GLP-1), glucagon and insulin concentrations were investigated as evaluating metabolic hormonal changes. Additionally, MCFA ingestion may change ghrelin action as described in Chapter 2, and they may have some preferred effects on milk production. GLP-1, secreted by the small intestine, stimulates insulin secretion (Kreymann et al., 1990; Fukumori et al., 2012-b; Hare et al., 2010). Glucagon and insulin are closely related to nutrient metabolism and may contribute to milk production.

In this study, the effects of the ingested MCFA-Ca on plasma concentration and action of ghrelin, and their relationship with milk production were evaluated in lactating cows.

3.2. Materials and Methods

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock

and Grassland Science Animal Care Committee, and which were formulated to comply with Japanese regulations.

3.2.1. Animals and Management

Five multiparous Holstein cows (initial DIM: 73.6 ± 2.2, calving number: 2.6 ± 0.4, initial BW: 572.6 ± 31.1 kg) were fed one of the two diets formulated to meet the nutrient requirements according to the Japanese Feeding Standard for Dairy Cattle (2006). The ingredients and composition of the diets are presented in Table 3-1. The cows were managed in individual tie stalls, allowed free access to water, and provided experimental diets twice daily at 0900 and 1800 h. They were milked twice daily before each feeding (0840 and 1740 h) and weighed every week.

3.2.2. Treatments and Experimental Design

The cows were randomly assigned to one of the two dietary treatments in a crossover design with 2-week periods. One group was fed a diet supplemented with MCFA-Ca (Star mate; Yuka Sangyo Co., Ltd., Tokyo, Japan), and the other was fed the same diet without the supplement (control). The MCFA-Ca containing 75% FAs (FA composition: C8:0, 25%; C10:0, 20%; and C12:0, 55%) was added to 1.5% of the dietary DM, following the protocol of a previous study (Fukumori et al., 2012-b). Cows were offered the food ad libitum, allowing for 10% refusal for the first 10 d for each treatment. For the last 4 d of each period, the cows were fed 95% of ad libitum intake to maintain a constant metabolizable energy intake (MEI).

3.2.3. Sampling

Samples of the diets and refusals were collected and pooled for the last 4 d of each period. Milk samples were collected for the last 4 d of each period. Sodium azide was added as a preservative to these samples, and the samples were stored at 4°C until analyses of fat, protein, lactose, total solids (TS) and solid not fat (SNF). Blood samples were taken on d 13 or d 14 from the jugular vein catheter (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) inserted on day 13 of each period. The blood samples (8 mL) were taken at 10-min intervals from 0800 to 1100 h. To evaluate ghrelin action, synthetic bovine ghrelin (0.5 μg/kgBW) was single dosed into the jugular vein through the catheter. After ghrelin injection, 10 mL of saline was injected for flushing the inner of the catheter. Blood samples were taken at −10, 0, 1.5, 3, 4.5, 6, 9, 12, 15, 20, 25, 30, 40, 50, 60, 90 and 120 min relative to the ghrelin injection from the catheter. The incremental area was calculated for hormones (GH, insulin and glucagon) and metabolites (glucose and NEFA) after ghrelin injection over 0-60 min and 60-120 min terms. Collected blood samples were immediately placed in tubes containing heparin (10 U/mL of blood; Wako Pure Chemical Industries Ltd., Osaka, Japan) with aprotinin (500 KIU/mL of blood; Sigma-Aldrich Inc., Tokyo, Japan), and centrifuged at 1,500 ×*g* for 20 min at 4°C. Harvested plasma samples were stored at −80°C until assay.

3.2.4. Sample Analysis

The diet samples and refusals were analysed for DM, CP, neutral detergent fiber (NDF), ether extract (EE) and crude ash contents according to the procedures of AOAC (1990). Metabolizable energy (ME) and net energy (NE) contents in the

treatment diets were estimated by NRC (2001). Retained energy was calculated with the following formula: Retained energy (Mcal/d) = NE_{intake} – ($NE_{maintainance}$ + $NE_{lactation}$).

Milk samples were measured for fat, protein, lactose, TS and SNF by infrared analysis (Milko-Scan 1344 A/BN; Foss Electric Company Inc., Hillerød, Denmark).

Plasma ghrelin, GH and insulin concentrations were measured as described at Chapter 2.2.4. Plasma IGF-1 concentration was measured by TR-FIA using Eu-labeled human IGF-1 (GroPep bioreagents Pty Ltd., Adelaide, Australia) and polystyrene microtiter strips coated with anti-rabbit γ-globulin and anti-human IGF-1 (GroPep bioreagents Pty Ltd., Adelaide, Australia). Plasma GLP-1 concentration was measured by TR-FIA using Eu-labeled rat GLP-1 (Yanaihara Institute Inc., Shizuoka, Japan) and polystyrene microtiter strips coated with anti-rabbit γ-globulin and anti-human GLP-1 (Peptide Institute, Inc., Osaka, Japan). Plasma glucagon concentration was measured by TR-FIA using Eu-labeled porcine glucagon (Sigma-Aldrich, Inc., Tokyo, Japan) and polystyrene microtiter strips coated with anti-guinea pig γ-globulin and anti-bovine glucagon (Acris Antibodies GmbH, Herford, Germany). Intra- and inter-assay of coefficients of variation were 2.8% and 2.1%, respectively, and, least detectable dose was 0.019 ng/mL.

Plasma glucose concentration was determined using a glucose analyzer (GA-1151; Arkray Co, Ltd., Kyoto, Japan). Plasma beta-hydroxy butyrate (BHBA), NEFA, triglyceride (TG), total-cholesterol (T-CHO) and urea nitrogen (UN) concentrations were determined using an automated biochemical analyser (Beckman Coulter, Inc., CA, USA).

3.2.5. Statistics

Data for feed intake, milk yield and milk composition were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). The mixed model included treatment as a fixed effect, and cow and period as random effects. For the statistical analysis of plasma hormone and metabolite concentrations, sampling time and sampling time × treatment were added to the model. Results are expressed as least squares means and standard error of means. Significant differences were set at $P < 0.05$ and trends were set at $P < 0.15$.

3.3. Results and Discussion

3.3.1. Feed Intake, Milk Yield, Milk Composition and BW Change

DMI and MEI were lower in the MCFA-Ca diet-fed cows than in the control diet-fed cows ($P = 0.006$ and $P = 0.013$, respectively, Table 3-2). Grummer and Socha (1989) reported that medium-chain triglyceride (C8:0 and C10:0) supplementation reduced DMI in lactating cows. Dohme et al. (2004) reported that supplementation with lauric acid (C12:0) also reduced DMI in lactating cows. Dietary medium-chain fatty acids or triglyceride supplements often reduce DMI likely because of the modification of ruminal digestion (Hristov et al., 2009; Reveneau et al., 2012). Although rumen-protected fat was used in this study, it might affect ruminal fermentation and fiber digestion to reduce DMI. Kawamura et al. (1990) reported that calcium salts of capric acid and lauric acid decreased protozoa concentration in the rumen fluid in lactating dairy cows, suggesting the MCFA-Ca might affect ruminal fermentation. In addition, fat supplements can reduce DMI even when they are fed in

the rumen-protected form or by abomasal infusion (Sutton et al., 1983; Relling and Reynolds, 2008). Therefore, reduction in DMI may have resulted partly from factors other than the adverse effects of fat on ruminal digestion.

Milk yield tended to be decreased by the MCFA-Ca diet relative to the control diet (P = 0.077, Table 3-2). Milk protein content and yield were decreased by the MCFA-Ca diet (P = 0.021 and P = 0.008, respectively, Table 3-2). Milk lactose content and yield were also decreased by the MCFA-Ca diet (P = 0.020 and P = 0.021, respectively, Table 3-2). However, weekly BW changes tended to be higher in MCFA-Ca diet-fed cows (P = 0.119, Table 3-2). In beef heifers, feeding refined coconut oil (rich in MCFA) increased average daily gain and gain efficiency, although DMI and gross energy intake were unaffected (Jordan et al., 2006). On the other hand, retained energy tended to be decreased by the MCFA-Ca diet (P = 0.080, Table 3-2). This result probably reflected that 4% FCM did not change in spite of the reduction of energy intake in the MCFA-Ca diet-fed cows. It is controversial whether MCFA-Ca supplementation preferentially distributes the ingested energy to body accumulation or milk production. MCFA, especially lauric acid, exhibit potent antiprotozoal properties and reduce ruminal methane production (Dohme et al., 1999, 2001), suggesting MCFA can avoid an energy loss from the methane emission. Therefore, actual MEI and retained energy may be underestimated because using calcium salts of long-chain fatty acids as substitute for MCFA-Ca. A proper examination that determines MEI or NE_{intake} in MCFA-Ca diet-fed cows should be done.

3.3.2. Responses of Hormones and Metabolites to Diets

Plasma ghrelin, GH, GLP-1, insulin, glucose, BHBA, NEFA, TG, T-CHO and UN concentrations varied over time ($P < 0.05$, Figure 3-1, 3-2). No significant interactions of time and treatment were observed. Plasma ghrelin, glucose, GH and NEFA concentrations decreased postprandially, whereas plasma GLP-1, insulin, BHBA, TG, T-CHO and UN concentrations increased after feeding.

3.3.3. Plasma Hormone concentrations

The MCFA-Ca diet resulted in an increase in plasma ghrelin concentrations relative to the control diet ($P = 0.021$, Table 3-3). Ghrelin is produced by gastrointestinal ghrelin cells. Its unique characteristic is that it requires acylation with n-octanoate (C8:0) at its Ser3 residue in order to become a proper ligand for its only known receptor, that is the growth hormone secretagogue receptor 1a (GHS-R1a) (Nakazato et al., 2001). In rats, MCFA ingestion increased stomach octanoyl ghrelin, whereas the amount of total ghrelin was unchanged in rats (Nishi et al., 2005). Kirchner et al. (2009) also showed an increase of more than double the concentration of octanoyl ghrelin in mice fed a medium-chain triglyceride-enriched diet. These studies suggested that dietary MCFA are used directly in ghrelin acylation, thereby affecting circulating ghrelin concentration. The acylated active form of ghrelin was determined in this assay (Sugino et al., 2004); therefore, the increase in plasma acylated ghrelin concentrations by MCFA-Ca supplementation in our study may have been caused by an increase in MCFA supply.

Plasma ghrelin concentration was higher in the MCFA-Ca diet-fed cows, but plasma GH concentration was not different between the treatments (Table 3-3). In

addition, MCFA-Ca diet induced a decrease in plasma IGF-1 concentration ($P < 0.001$, Table 3-3). High-protein diets or higher energy intake increases plasma IGF-1 concentration (Fontana et al., 2008). Therefore, in this study, the decrease in plasma IGF-1 of MCFA-Ca diet-fed cows might be caused by lower energy and CP intake. In this study, milk yield decreased in MCFA-Ca diet fed cows might be contributed by the lower IGF-1 concentration.

GLP-1 was produced by L cells mainly in the small intestine and secreted in response to carbohydrates and lipids. Several studies reported that dietary LCFAs increased plasma GLP-1 concentrations in cows (Litherland et al., 2005; Relling and Reynolds, 2007, 2008). However, in this study, no change in plasma GLP-1 concentrations was noted in the MCFA-Ca diet-fed cows compared with the control diet-fed cows (Table 3-3). Although the mechanisms regulating fat-stimulated GLP-1 release have not been clarified in detail, Beglinger et al. (2010) reported that intraduodenal infusion of sodium caprylate did not stimulate GLP-1 release but that sodium oleate was effective in humans. On the other hand, Hirasawa et al. (2005) demonstrated that the stimulation of G protein-coupled receptor 120 (GPR120), a receptor for unsaturated LCFA, promoted GLP-1 secretion in vitro and in vivo in mice. Therefore, MCFA might not directly induce GLP-1 release because they are not the ligands of GPR120.

Ghrelin and GLP-1 are known to have a function in food intake regulation. Ghrelin enhances food intake and GLP-1 decreases food intake in non-ruminants and ruminants (Gutzwiller et al., 1999; Nakazato et al., 2001; Harrison et al., 2008; Relling et al., 2011; Wertz-Lutz et al., 2006). As a result, no adverse effect of gut hormones on reduction in DMI was found in the MCFA-Ca diet-fed cows in this study. Thus,

reduction in DMI due to MCFA may have been caused by factors other than ghrelin and GLP-1. Higher concentrations of MCFA, bound to albumin, trnsfered to the liver through the portal blood and are rapidly oxidized by the liver compared with LCFAs because MCFA do not require transformation by carnitine palmitoyl transferase I to penetrate the mitochondria (Bach and Babayan, 1982). Reduction in DMI by MCFA may be attributable to increased oxidation of MCFA in the liver (Allen et al., 2009).

Plasma insulin concentration was lower ($P < 0.001$, Table 3-3) in the MCFA-Ca diet-fed cows than in the control diet-fed cows, whereas no difference in plasma glucagon concentrations was observed (Table 3-3). Although lower levels of insulinemia due to MCFA supplementation were shown in the report by Piot et al. (1999), other studies in rats (Turner et al., 2009; Wein et al., 2009) and calves (Mills et al., 2010) observed no change in plasma insulin concentrations. Thus, MCFA by itself may not have affected insulin release. Dohme et al. (1999) reported that coconut oil adversely affected fiber degradation in the rumen. The result suggests that VFA absorption might be lower in the MCFA-Ca. Although rumen or plasma VFA concentration was not determined in this study, the decreases in plasma GLP-1 and insulin concentrations might been caused by lower VFA absorption in the MCFA-Ca diet-fed cows.

3.3.4. Plasma metabolite concentrations

In this study, various changes in plasma metabolites were observed with MCFA-Ca supplementation. MCFA-Ca diet induced increases in plasma BHBA, NEFA, T-CHO, and UN concentrations ($P \leq 0.001$, Table 3-3). Plasma glucose concentration was lower in the MCFA-Ca diet-fed cows than in the control diet-fed

cows (P = 0.011, Table 3-3). The increase in plasma NEFA concentration induced by MCFA was consistent with previous findings (Keller et al., 2002; Mills et al., 2010). The increase in plasma BHBA concentrations may have occurred because of rapid MCFA oxidation as demonstrated in previous studies (Dias et al., 1990; Shinohara et al., 2005). The higher plasma T-CHO concentration may be caused by the higher β-oxidation of MCFA-Ca because cholesterol is made from acetyl-CoA. The previous study observed a decrease in DMI-enhanced urea production (Obitsu et al., 2011). In addition, MCFA reduced protozoa to decrease ruminal ammonia (Dohme et al., 1999, 2001), suggesting that a paucity of ammonia is necessary for urea production in order to enhance Gln catabolism (Nieto et al., 2002). This hypothesis accords with the higher plasma UN concentrations in the MCFA diet-fed cows in this study. A correlation between the rate of β-oxidation of MCFA-Ca in the liver and muscle, and increased glucose sensitivity has been demonstrated by insulin challenge (Han et al., 2003; Perdomo et al., 2004). This insulin-stimulated decrease in plasma glucose concentration was greater in the MCFA diet-fed calves (Mills et al., 2010). Although the insulin response was not directly measured in this study, the decrease in plasma glucose concentration and milk lactose content and yield may have been partly due to enhanced insulin action. Alternatively, ketone bodies could decrease lactate removal and glucose production in hepatocytes (Kummel, 1984), which may be responsible for the lower plasma glucose concentrations and milk lactose yield in this study.

3.3.5. The effect of MCFA on ghrelin-induced increases of plasma hormone and metabolite concentrations

Because MCFA-Ca diet affected basal values of these plasma concentrations,

incremental areas, expressed by deducting from AUC for the pre-injection areas, were used for the comparisons. Incremental areas of plasma hormone (GH, glucagon, and insulin) and metabolite (glucose and NEFA) concentrations are presented in Table 3-4. As previous reports described (Itoh et al., 2006; Roche et al., 2008), ghrelin injection also increases plasma pancreatic hormone, glucose and NEFA concentrations during lactating period. Furthermore, in Chapter 2, amino acid infusion enhanced ghrelin-induced insulin and glucagon secretion and glucose uptake. In the current study, there were no differences between treatments in incremental areas of plasma GH, glucagon, insulin, and glucose concentrations. The incremental area of NEFA from 60 to 120 min in MCFA-Ca diet-fed cows tended to be greater compared with control diet-fed cows (P = 0.091, Table 3-4). However, overall, there were little effects of MCFA on ghrelin action which increases plasma hormone and metabolite concentrations.

3.3.6. Conclusion

In the present experiment, the effect of MCFA on lactating dairy cows was examined with a focus on changes in plasma ghrelin, GH, the other hormone and metabolite concentrations, ghrelin action, and their relationship with milk production. The observations suggested that MCFA-Ca supplementation might increase plasma ghrelin concentration although it might not contribute to higher plasma GH and IGF-1 concentrations. MCFA-Ca supplementation indicated the possibility to increase production efficiency, but milk yield was not increased probably because of the decrease in DMI. A reduction in DMI due to MCFA-Ca supplementation remains an unsolved problem. Therefore, improving the adverse effects of MCFA on feed intake

is an important subject. In addition, many researchers have pointed out that MCFA supplementation induces liver lipidosis in rodents (Turner et al., 2009; Wein et al., 2009) and calves (Piot et al., 1999; Graulet et al., 2000; Mills et al., 2010). The use of MCFA-Ca for dietary supplementation in lactating dairy cows requires further research.

Table 3-1. Ingredient and chemical composition of the diets

Item	Treatment	
	Control	MCFA-Ca[1]
Ingredient (% DM basis)		
Corn silage	40.0	39.4
Alfalfa hay cubes	8.3	8.1
Sudan grass hay	5.1	5.0
Mixed concentrate[2]	32.7	32.3
Soybean meal	5.6	5.5
Beet pulp pellet	8.3	8.2
Calcium salts of fatty acids[3]	0.0	1.5
Chemical composition (DM basis)		
OM[4] (%)	93.1	92.9
CP[5] (%)	16.1	16.0
NDF[6] (%)	39.5	39.0
EE[7] (%)	2.7	3.9
ME[8] (Mcal/kg)	2.6	2.7

[1]MCFA-Ca = calcium salts of medium-chain fatty acids.

[2]Mixed concentrate contained 55% corn grain, 20% corn gluten feed, 10% soybean meal, 7% canola meal, 8% others (wheat middlings, wheat bran, molasses, calcium carbonate, salt, and vitamin premix).

[3]Star mate: Declared fatty acids contained 25% caprylic acid, 20% capric acid, 55% lauric acid.

[4]OM = organic matter.

[5]CP = crude protein.

[6]NDF = neutral detergent fiber.

[7]EE = ether extract.

[8]ME = Metabolizable energy: Estimated value from NRC (2001).

Table 3-2. Feed intake, milk production and composition during experimental period in lactating cows

Item	Treatment		SEM	*P*-value
	Control	MCFA-Ca[1]		
DMI[2] (kg/d)	22.2	19.5	0.733	0.006
MEI[3] (Mcal/day)	57.7	52.0	1.94	0.013
Milk yield (kg/d)	28.4	27.5	2.50	0.077
4% FCM (kg/d)[4]	28.0	26.2	0.149	0.149
Milk composition (%)				
Fat	3.93	3.75	0.213	0.357
Protein	3.27	3.15	0.145	0.021
Lactose	4.34	4.23	0.057	0.020
TS[5]	12.5	12.2	0.305	0.131
SNF[6]	8.59	8.38	0.120	0.004
Milk composition yield (kg/d)				
Fat	1.10	1.00	0.094	0.246
Protein	0.915	0.854	0.056	0.008
Lactose	1.24	1.16	0.115	0.021
Body weight changes (kg/week)	-0.695	8.79	6.76	0.119
Retained energy (Mcal/d)	6.19	3.78	1.26	0.080

Data are shown by least squares means (LSM) and standard errors of the LSM (SEM).

[1]MCFA-Ca = calcium salts of medium-chain fatty acids.

[2]DMI = dry matter intake.

[3]MEI = metabolizable energy intake.

[4]4% FCM (kg/d) = 4% fat correct milk = 0.4 × Milk yield (kg/d) + 15 ×Milk yield (kg/d) × Milk fat (%).

[5]TS = total solid.

[6]SNF = solid not fat.

Table 3-3. Plasma hormone and metabolite concentrations during feeding period in lactating cows

Item	Treatment		SEM	*P*-value		
	Control	MCFA-Ca[1]		Time	Treatment	Treatment × Time
Hormones (ng/mL)						
Ghrelin	0.129	0.185	0.045	<0.001	0.021	0.903
GH[2]	33.1	40.1	12.9	<0.001	0.216	0.986
IGF-1[3]	70.2	62.7	13.5	0.947	<0.001	0.940
GLP-1[4]	1.00	1.01	0.342	0.012	0.174	0.309
Glucagon	0.353	0.332	0.058	0.640	0.118	0.245
Insulin	5.06	3.76	0.82	<0.001	<0.001	0.131
Metabolites						
Glucose (mg/dL)	68.9	67.5	2.01	<0.001	0.011	0.203
BHBA[5] (μmol/L)	407	507	50.5	<0.001	<0.001	0.888
NEFA[6] (μEq/L)	85.1	106	6.87	<0.001	<0.001	0.597
TG[7] (mg/dL)	7.25	7.26	0.394	<0.001	0.509	0.758
T-CHO[8] (mg/dL)	167	190	15.1	<0.001	0.001	0.889
UN[9] (mgN/dL)	15.7	16.7	0.949	<0.001	<0.001	0.999

Data are shown by least squares means (LSM) and standard errors of the LSM (SEM).

[1]MCFA-Ca = calcium salts of medium-chain fatty acids.

[2]GH = growth hormone.

[3]IGF-1 = insulin-like growth factor-1.

[4]GLP-1 = glucagon-like peptide-1 (7-36) amide.

[5]BHBA = beta-hydroxy butyrate.

[6]NEFA = non-esterified fatty acids.

[7]TG = triglyceride.

[8]T-CHO = total-cholesterol.

[9]UN = urea nitrogen.

Table 3-4. Incremental areas of plasma hormone and metabolite concentrations by intravenous ghrelin injection

Item	Treatment		SEM	*P* value
	Control	MCFA-Ca[1]		
GH[2] (ng/mL·min)				
0 - 60	43.4	52.2	24.66	0.278
60 - 120	2.14	4.22	2.75	0.629
Glucagon (pg/mL·min)				
0 - 60	0.0583	0.0270	0.0144	0.132
60 - 120	0.0754	0.0135	0.0274	0.197
Insulin (ng/mL·min)				
0 - 60	1.76	2.95	1.03	0.146
60 - 120	1.66	0.698	0.730	0.336
Glucose (mg/dL·min)				
0 - 60	5.84	3.88	1.32	0.251
60 - 120	12.1	2.41	4.73	0.207
NEFA[3] (μEq/L·min)				
0 - 60	22.8	36.6	7.57	0.198
60 - 120	27.6	8.67	8.57	0.091

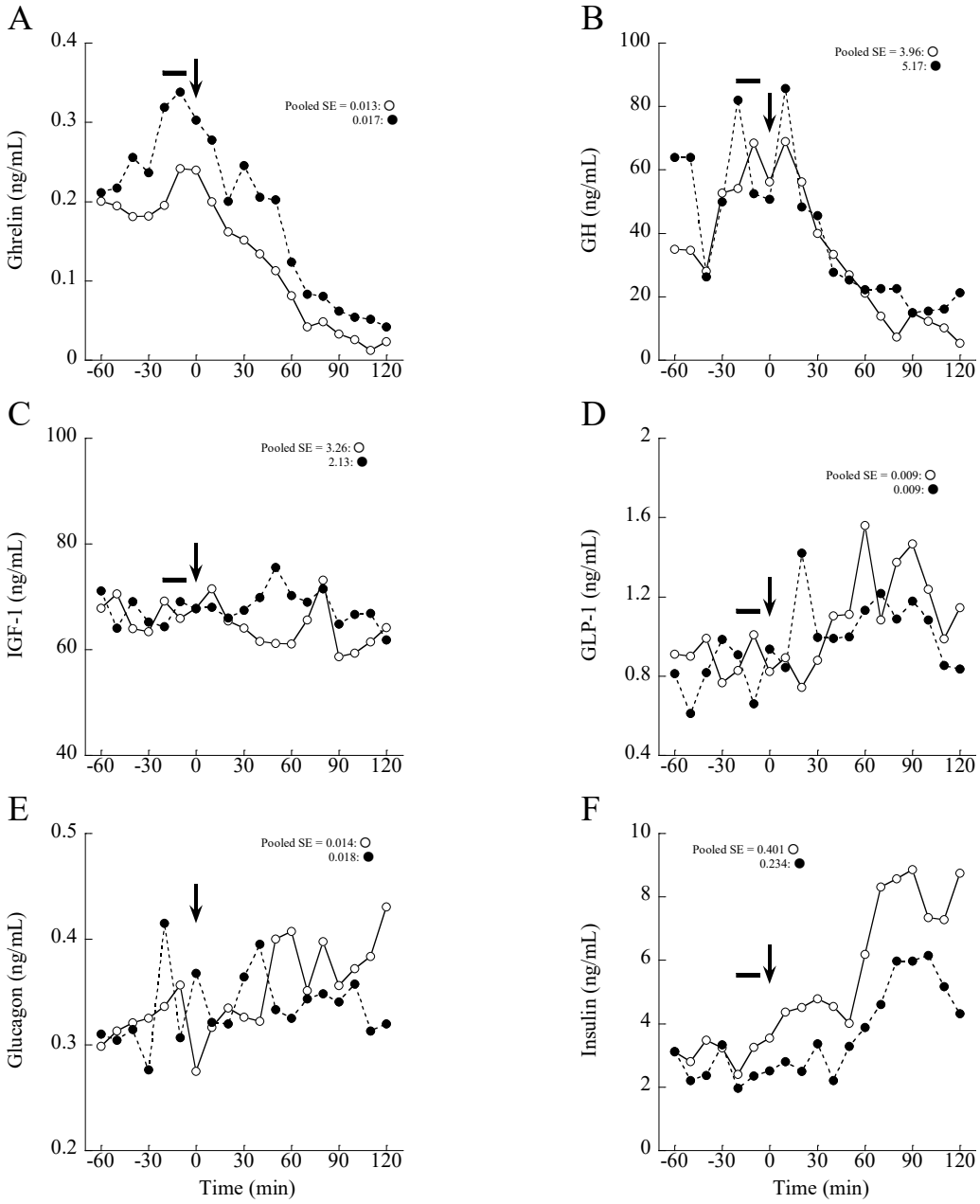
Data are shown by least squares means (LSM) and standard errors of the LSM (SEM).

[1]MCFA-Ca = calcium salts of medium-chain fatty acids.

[2]GH = growth hormone.

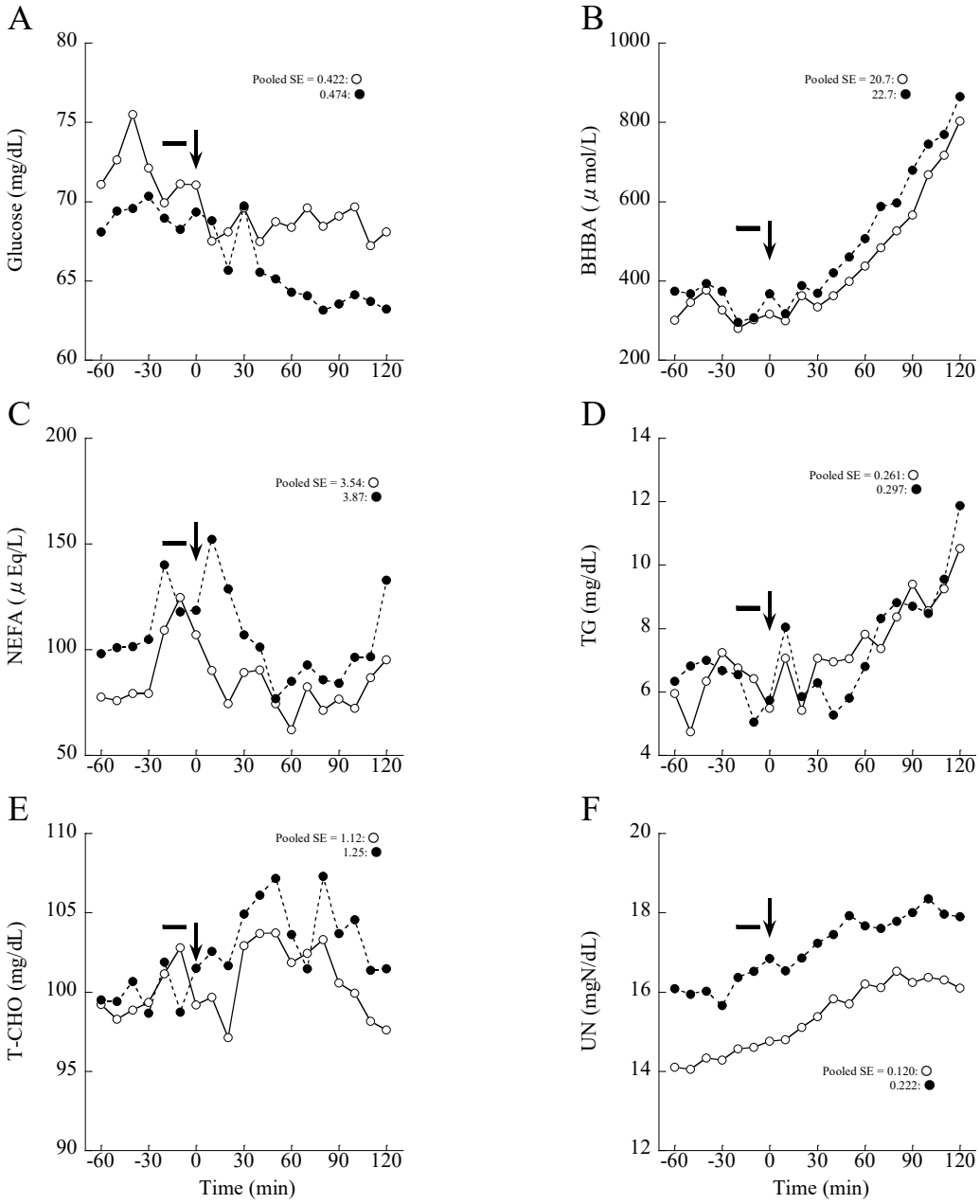
[3]NEFA = non-esterified fatty acids.

Figure 3-1



Plasma hormone (ghrelin: A, GH: B, IGF-1: C, GLP-1: D, Insulin: E and Glucagon: F) concentrations in cows fed control diet (○with a solid line) and MCFA-Ca (● with a doted line). Values are expressed as least square of means (n = 5). The horizontal bar and arrow show the milking period and feeding time, respectively.

Figure 3-2



Plasma metabolite (glucose: A, BHBA: B, NEFA: C, TG: D, T-CHO: E and UN: F) concentrations in cows fed control diet (○with a solid line) and MCFA-Ca (● with a doted line). Values are expressed as least square of means (n = 5). The horizontal bar and arrow show the milking period and feeding time, respectively.

Chapter 4

Effects of Feeding Long-Chain Fatty Acids and Rumen-Protected Methionine on Plasma Ghrelin Concentration and Milk Production in Lactating Dairy Cows

4.1. Introduction

In Chapter 3, MCFA diet could increase plasma ghrelin concentration. However, higher plasma ghrelin concentration did not contribute to higher GH concentration and milk production. Fat supplementation derived from long-chain fatty acids is valid to increase dietary energy density (NRC, 2001). However, increasing the dietary fat content often depresses DMI of lactating cows (Allen, 2000). The mechanism of hypophagia induced by fat supplementation is incompletely understood and assumed to be affected by various factors involving the absorption process and metabolism of fatty acids. Methionine (Met) improves lipid metabolism (Yagasaki et al., 1989) partially by altering the activities of hormone-sensitive lipase and lipoprotein lipase (Kawasaki et al., 2010). In dairy cows, Met is the first limiting amino acid for milk production (Schwab et al., 1976). Supplementation of Met hydroxyl analog increases milk fat synthesis with hypertriglycemia (Huber et al., 1984). Conversely, an insufficiency of Met during the periparturient period results in the development of hepatic lipidosis (Shibano and Kawamura, 2005). These reports suggest that Met may affect lipid metabolism in dairy cows. In lactating ewes, supplementation of rumen-protected Met (RPM) combined with fat increased DMI,

milk yield and milk fat secretion compared with the supplementation of fat only (Goulas et al., 2003), but the effects of Met on secretions of ghrelin and other metabolic hormones were unknown. Therefore, the effects of adding calcium salts of long-chain fatty acids (LCFA-Ca) and RPM on plasma ghrelin, GH and other hormone concentrations and milk production in lactating cows were investigated in this study.

4.2. Materials and Methods

The procedures used in the present study were carried out in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock and Grassland Science Animal Care Committee, and which were formulated to comply with Japanese regulations.

4.2.1. Animals and Management

Four lactating Holstein cows [calving number: 1.75 ± 0.25 (primiparous: n = 1, multiparous: n = 3), initial DIM: 143.5 ± 3.9, initial BW: 547.3 ± 14.7 kg] were fed four diets formulated to meet the nutrient requirements according to the Japanese Feeding Standard for Dairy Cattle (2006). The ingredients and composition of the diets are presented in Table 4-1. The cows were managed in individual tie stalls, allowed free access to water, and provided experimental diets twice daily at 0900 and 1800 h. They were milked twice daily before each feeding (0840 and 1740 h), and weighed every week.

4.2.2. Treatments and Experimental Design

The cows were used in a 4 × 4 Latin square design in each 2 week period. Four treatments consist of basal diet only (without supplement), supplemented with LCFA-Ca made from palm and soybean oil [Megalac R (Church & Dwight Co., Inc., Princeton, NJ, USA)], with RPM [Lactet SP (Nippon Soda Co., Ltd., Tokyo, Japan)], and with LCFA-Ca plus RPM. The LCFA-Ca containing 85% fatty acids was added to 1.5% of the diet DM, following a previous study (Bilby et al., 2006). The RPM containing 67% of DL-Met was supplemented at 20 g/d for RPM or LCFA-Ca plus RPM treatment. Cows were offered each diet ad libitum, allowing for 15% refusal for the first 10 d for each treatment. For the last 4 d of each period, the cows were fed 95% of ad libitum intake. Refusals were weighed daily before each morning feeding (0800 h).

4.2.3. Sampling

Samples of the diets and the refusals were collected and pooled for the last 4 d of each period. Milk samples were collected for the last 4 d of each period, added with sodium azide as preservative, and stored at 4°C until analysis. Blood samples were taken at 12 d from the jugular vein catheter inserted on 10 d of each period. Blood samples (8 mL) were taken at 10 min intervals from 0800 to 1100 h. Blood samples were collected into heparinized tubes with aprotinin (500 KIU/mL of blood) and centrifuged at 1,500 × *g* for 20 min at 4°C. Harvested plasma samples were stored at -80°C until assay.

4.2.4. Sample Analysis

The diet samples, refusals and milk samples were analysed as described at Chapter 3.2.4. Plasma ghrelin, GH, IGF-1, GLP-1, insulin and glucose concentrations were determined every 10 min and plasma glucagon, BHBA, NEFA, TG, T-CHO and UN concentrations were determined every 20 min. Plasma amino acid concentrations were measured at 0900h. Assays of plasma ghrelin, GH, glucagon and insulin concentrations were conducted as described at Chapter 2.2.4. Plasma IGF-1, GLP-1, glucose, BHBA, NEFA, TG, T-CHO and UN concentrations were determined as described at Chapter 3.2.4. Plasma amino acid concentrations were determined by ion-exchange chromatography using an automatic amino acid analyzer (JLC-500; JEOL Ltd., Tokyo, Japan) after deproteinization of plasma with an equal volume of sulfosalicylic acid (3% wt/vol).

4.2.5. Statistics

Data for feed intake, milk yield and milk composition were analyzed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The mixed model included treatment as a fixed effect, and cow and period as random effects. For the statistical analysis of plasma hormone and metabolite concentration, sampling time and sampling time × treatment were added to the model. A 2 × 2 factorial design was used to test the main effects of LCFA-CA supplementation (LCFA-Ca vs. non-LCFA-Ca), RPM supplementation (RPM vs. non-RPM), and their interaction. Results are reported as least squares means and SEM. Significant differences were set at $P < 0.05$ and trends were set at $P < 0.15$.

4.3. Results and Discussion

4.3.1. Feed Intake, Milk Yield and Milk Composition

DMI in cows fed LCFA-Ca diets compared with cows fed non-LCFA-Ca diets was lower ($P = 0.025$, Table 4-2), whereas MEI was not affected. Generally, high fat inclusion in diets reduces fiber digestion, increases fatty acid absorption, inhibits abomasal motility (Nicholson and Omer, 1983) and increases gut hormone secretion (Choi et al., 2000; Relling and Reynolds, 2007). All of these factors would reduce DMI. Supplementation of LCFA-Ca had been expected to alleviate such negative effects on ruminal fermentation and fiber digestibility in lactating cows (Schauff and Clark, 1989; Schneider et al., 1988). However, DMI was decreased by LCFA-Ca in this study consistent with other reports (Choi and Palmquist, 1996; Schauff and Clark, 1992). RPM did not improve suppression of DMI by LCFA-Ca contrary to results found by Goulas et al. (2003) who observed feeding fat with RPM increased DMI in lactating ewes. Additionally, Chillard and Doreau (1997) observed no improvement effect of RPM on DMI of cows fed a fish oil supplemented diet during mid-lactation, although such non-protected fat including high polyunsaturated fatty acids could largely inhibit dietary fiber digestion and depress DMI.

Milk yield and composition, except for total solid contents, were unaffected by LCFA-Ca and RPM. Milk total solid contents tended to be lower in cows fed LCFA-Ca diets compared with cows fed non-LCFA-Ca diets ($P = 0.107$). In lactating ewes, supplementation of RPM combined with fat increased milk yield and milk fat secretion compared with the supplementation of fat only (Goulas et al., 2003). In their report, such an increase in milk production was accompanied with higher feed intake. In the present study, DMI or MEI did not increase in combination of

LCFA-Ca and RPM.

4.3.2. Responses of Hormones and Metabolites to Diets

Plasma ghrelin, insulin, glucose, BHBA, NEFA, TG and UN (except for non-LCFA-Ca + RPM) concentrations varied largely through time ($P < 0.002$, Figures 4-1, 4-2), and no significance of the interaction time by treatment was observed ($P > 0.192$, Figures 4-1, 4-2). The time effect for plasma ghrelin, glucose and NEFA concentrations was due to a postprandial decrease. Plasma insulin, BHBA, TG and UN concentrations increased after feeding.

4.3.3. Plasma Hormone Concentrations

Plasma ghrelin was higher ($P < 0.001$, Table 4-3) and plasma GH concentration tended to higher ($P = 0.054$, Table 4-3) in cows fed LCFA-Ca compared with cows not fed LCFA-Ca. Compared with cows fed non-LCFA-Ca, plasma IGF-1, glucagon and insulin concentrations in cows fed LCFA-Ca were lower ($P < 0.001$, $P = 0.002$, and $P = 0.012$, respectively, Table 4-3), and plasma GLP-1 concentration tended to be lower ($P = 0.061$, Table 4-3). Plasma IGF-1 concentration was lower ($P = 0.017$, Table 4-3) and plasma insulin concentration tended to be higher ($P = 0.092$, Table 4-3) in cows supplemented RPM than those without RPM. There were interactions between LCFA-Ca and RPM for ghrelin and glucagon ($P = 0.002$ and $P = 0.041$, respectively, Table 4-3): LCFA-Ca plus RPM increased plasma ghrelin, glucagon, but RPM alone did not show such effects. There tended to be an interaction between LCFA-Ca and RPM for GH ($P = 0.130$, Table 4-3): Plasma GH concentration showed the highest level in LCFA-Ca plus RPM.

The increase in plasma ghrelin concentration of cows fed LCFA-Ca is consistent with that of rats that ingested some fatty acids (Nishi et al., 2005). In addition, RPM decreased plasma ghrelin concentration in cows not fed LCFA-Ca, whereas LCFA-Ca plus RPM compared with LCFA-Ca alone increased plasma ghrelin concentration. Thus, only a simultaneous inclusion of LCFA-Ca and RPM may enhance ghrelin secretion via the changes in lipid metabolism. Ghrelin enhances feed intake in both nonruminants (Nakazato et al., 2001) and ruminants (Harrison et al., 2008; Wertz-Lutz et al., 2006). As a result, we could not find the relationship between plasma ghrelin concentration and feed intake in this study. Since reports on ghrelin responses to lipid metabolism in ruminants are limited, further research is desirable.

Many researchers have reported that high fat supplementation increased GLP-1 concentration (Benson and Reynolds, 2001; Bradford et al., 2008; Litherland et al., 2005; Relling and Reynolds, 2007). Litherland et al. (2005) demonstrated that abomasal infusion of fat increased plasma GLP-1 concentration dependent on dosage. Compared with others, the decrease of plasma GLP-1 concentration with LCFA-Ca in this study could be attributed to a fewer fat ingestion level of 280 g/d. GLP-1 plays a role in food intake (Turton et al., 1995) and Relling and Reynolds (2007) have reported that dietary fats increased GLP-1 in dairy cows. Additionally, Relling et al. (2011) observed that intrajugular infusion of GLP-1 tended to decrease DMI in growing wethers. In this study, however, plasma GLP-1 concentration was decreased by LCFA-Ca, suggesting that GLP-1 did not mediate DMI.

Plasma insulin concentration decreased in cows fed LCFA-Ca with decreasing DMI consistent with previous reports (Choi and Palmquist, 1996; Cummins and Sartin, 1987). The depression of plasma insulin concentration might be due to a lower VFA

production caused by decreased DMI. Lower plasma BHBA concentration in cows fed LCFA-Ca was possibly caused by less butyrate absorption by depressed ruminal fermentation. And, plasma GLP-1 concentration in cows fed LCFA-Ca tended to decrease at the same time, suggesting decreased GLP-1 may be related to insulin depression.

LCFA-Ca also decreased plasma glucagon concentration. The result is consistent with that of Cummins and Sartin (1987), but inconsistent with that of Khorasani and Kennelly (1998). In several species, elevated NEFA decreases plasma glucagon concentration (Gerich et al., 1976; Lavau et al., 1979). However, the present results showed that the decrease of plasma glucagon with LCFA-Ca was attenuated by RPM. In Chapter 2, glucagon secretion was more strongly enhanced when amino acids and ghrelin were simultaneously administered in comparison with the administration of ghrelin alone in lactating cows. Therefore, the combination of increase in available Met and higher plasma ghrelin concentration likely increased plasma glucagon concentration in LCFA-Ca fed cows.

4.3.4. Plasma Metabolite Concentrations

Compared to the diets without LCFA-Ca, plasma NEFA, TG, T-CHO and UN concentrations in cows fed LCFA-Ca were higher ($P < 0.001$, $P = 0.029$, $P < 0.001$, and $P < 0.001$, respectively, Table 4-3), but with lower plasma BHBA concentration ($P = 0.006$, Table 4-3). In cows fed RPM diets, plasma glucose concentration was higher ($P = 0.001$, Table 4-3), plasma T-CHO concentration was lower ($P = 0.009$, Table 4-3), and plasma NEFA and UN concentration tended to be lower ($P = 0.15$ and $P = 0.121$, respectively, Table 4-3). Interactions between LCFA-Ca and RPM were

observed for TG and T-CHO (P = 0.049 and P = 0.003, respectively, Table 4-3): RPM decreased plasma TG and T-CHO concentrations in cows fed LCFA-Ca, but did not affect the cows not fed with LCFA-Ca. In cows fed RPM diets, plasma Met concentration tended to be higher (P = 0.101, Table 4-4). There was an interaction between LCFA-Ca and RPM for Met (P = 0.096, Table 4-4): RPM alone increased plasma Met concentration, but LCFA-Ca plus RPM decreased.

The increase in plasma glucose concentration of cows fed RPM was consistent with Berthiaume et al. (2001), while inconsistent with Bertics and Grummer (1999). Such discrepancies might be explained by the differences in the physiological state of cows: the present study and the study by Berthiaume et al. (2001) used mid-lactating cows, but the study by Bartics and Grummer used early-lactating cows.

Plasma NEFA, T-CHO and TG concentrations increased by LCFA-Ca in the current study has been previously observed in cows (Choi et al., 2000; Gobert et al., 2009). On the other hand, RPM with LCFA-Ca decreased plasma T-CHO and TG concentrations, and tended to decrease plasma NEFA concentration (Table 4-3). Met is a methyl group donor for phosphatidylcholine in dairy cows (Pinotti et al., 2002) to enhance plasma lipoprotein. In laboratory animals, casein (a Met-rich protein) or L-Met induced hypercholesterolemia in rabbits and rats (Khosla et al., 1989; Oda et al., 1991). In calves, however, a high fat diet supplemented with L-Met did not increase plasma VLDL concentration (Auboiron et al., 1993), and Met hydroxyl analog did not affect hepatic TG accumulation in cows (Bertics and Grummer, 1999). Whether Met enhanced VLDL that was not measured in this study is unknown. On the other hand, because Met is converted to taurine in dairy cows, plasma taurine concentration increased linearly with an increase of postruminal Met infusion (Pisulewski et al.,

1996). Taurine conjugates with bile acids to become taurocholate in the liver, and promotes lipid absorption and cholesterol consumption (Yokogoshi and Oda, 2002). Yagasaki et al. (1989) showed that dietary Met and glycine reduced serum T-CHO and increased fecal sterol excretion in rats. Furthermore, Met as a sulfur amino acid suppressed serum NEFA and TG concentration through the control of hormone-sensitive lipase activity and the restoration of lipoprotein lipase activity in peripheral tissues of hepatoma-bearing rats (Kawasaki et al., 2010). In this study, therefore, suppression of plasma T-CHO, NEFA and TG with LCFA-Ca plus Met might be induced by enhanced uptake and use of lipids by various tissues.

Berthiaume et al (2001, 2006) reported that feeding RPM clearly increased arterial Met concentration. However, in this study, plasma Met concentration did not clearly increased because of the peripheral venous value. Ingested RPM was likely taken up by the peripheral tissues.

4.3.5. Conclusion

The present study did not show any favorable effects of RPM on feed intake and milk production in cows fed with LCFA-Ca, although plasma ghrelin concentration increased. Further studies are desirable to elucidate the effects of lactating stage and Met supplemental level with LCFA-Ca on DMI and milk production.

In conclusion, the responses to LCFA-Ca are associated with the increases in plasma concentration of ghrelin and the decreases of insulin, glucagon and GLP-1. RPM plus LCFA-Ca modulated plasma lipid concentrations and concomitantly elevated plasma ghrelin concentration. These metabolic and endocrine changes may be induced by absorbed LCFA and Met, but the increase in plasma ghrelin

concentration did not involve with MEI or an increase in milk production.

Table 4-1. Ingredient and chemical composition of the diets

Item	non-LCFA-Ca	LCFA-Ca[1]
Ingredient (% DM basis)		
Corn silage	42.2	41.6
Alfalfa hay cubes	7.2	7.1
Sudan grass hay	5.7	5.6
Mixed concentrate[2]	37.8	37.2
Beet pulp pellets	7.1	7.0
Calcium salts of fatty acids[3]	0.0	1.5
Chemical composition (DM basis)		
OM[4] (%)	93.0	92.7
CP[5] (%)	14.1	13.9
NDF[6] (%)	32.2	31.7
EE[7] (%)	3.2	4.5
ME[8] (Mcal/kg)	2.4	2.5

[1]LCFA-Ca = calcium salts of long-chain fatty acids.

[2]Mixed concentrate contained 55% corn grain, 20% corn gluten feed, 10% soybean meal, 7% canola meal and 8% others (wheat middlings, wheat bran, molasses, calcium carbonate, salt and vitamin premix).

[3]Megalac R: Declared fatty acids contained 26% palmitic acid, 4% stearic acid, 33% oleic acid, 32% linoleic acid and 5% linolenic acid.

[4]OM = organic matter.

[5]CP = crude protein.

[6]NDF = neutral detergent fiber.

[7]EE = ether extract.

[8]ME = Metabolizable energy: Estimated value from NRC (2001).

Table 4-2. Feed intake, milk production and composition during experimental period in lactating cows

	Treatment							
	non-LCFA-Ca		LCFA-Ca [1]			Contrast[3] (*P* -value)		
Item	non-RPM	RPM	non-RPM	RPM[2]	SEM	LCFA-Ca	RPM	LCFA × RPM
DMI[4] (kg/d)	22.4	22.2	21.3	21.7	0.675	0.025	0.803	0.322
MEI[5] (Mcal/d)	56.7	56.2	55.4	56.4	1.72	0.463	0.794	0.325
Milk yield (kg/d)	27.2	27.1	27.3	27.5	1.83	0.508	0.982	0.655
4% FCM[6] (kg/d)	29.9	28.8	30.0	30.3	1.63	0.505	0.766	0.574
Milk composition (%)								
Fat	4.46	4.35	4.48	4.42	0.44	0.825	0.685	0.909
Protein	3.51	3.56	3.47	3.45	0.132	0.177	0.729	0.508
Lactose	4.62	4.59	4.61	4.61	0.059	0.736	0.743	0.662
TS[7]	13.6	13.5	12.7	12.5	1.01	0.107	0.759	0.966
SNF[8]	9.13	9.11	9.08	9.11	0.124	0.736	0.973	0.710
Milk composition yield (kg/d)								
Fat	1.19	1.16	1.20	1.21	0.062	0.772	0.369	0.558
Protein	0.950	0.957	0.942	0.947	0.037	0.517	0.302	0.954
Lactate	1.26	1.24	1.26	1.27	0.089	0.870	0.316	0.139
Body weight changes (kg/week)	14.3	18.5	13.5	12.7	4.60	0.359	0.612	0.474
Retained energy (Mcal/d)	3.98	3.22	3.44	3.22	0.986	0.437	0.990	0.440

Data are shown by least squares means (LSM) and SEM.

[1]LCFA-Ca = calcium salts of long-chain fatty acids.

[2]RPM = rumen-protected methionine.

[3]*P* -value for factorial contrasts: LCFA-Ca, RPM and the interaction between LCFA-Ca and RPM.

[4]DMI = dry matter intake

[5]MEI = metabolizable energy intake.

[6]4% FCM = 4% fat corrected milk (kg/d) = 0.4 × Milk yield (kg/d) + 15 × Milk yield (kg/d) × Milk fat (%).

[7]TS = sotal solid.

[8]SNF = solid not fat.

Table 4-3. Plasma hormone and metabolite concentrations during feeding period in lactating cows

	Treatment							
	non-LCFA-Ca		LCFA-Ca[1]			Contrast[3] (*P* -value)		
Item	non-RPM	RPM	non-RPM	RPM[2]	SEM	LCFA-Ca	RPM	LCFA × RPM
Hormones (ng/mL)								
Ghrelin	0.106	0.087	0.116	0.126	0.027	<0.001	0.301	0.002
GH[4]	14.1	11.2	14.9	17.8	3.55	0.054	0.993	0.130
IGF-1[5]	107	102	95.5	86.9	31.8	<0.001	0.017	0.649
GLP-1[6]	0.519	0.464	0.452	0.448	0.128	0.061	0.185	0.258
Glucagon	0.991	0.936	0.603	0.855	0.462	0.002	0.187	0.041
Insulin	2.51	2.86	2.36	2.41	0.128	0.012	0.092	0.202
Metabolites								
Glucose (mg/dL)	62.9	63.4	62.1	63.6	1.91	0.872	0.001	0.344
BHBA[7] (μmol/L)	595	579	514	517	43.4	0.006	0.796	0.729
NEFA[8] (μEq/L)	95.4	90.2	116	110	7.13	<0.001	0.150	0.842
TG[9] (mg/dL)	7.57	8.29	9.86	8.41	0.82	0.029	0.501	0.049
T-CHO[10] (mg/dL)	197	200	247	211	14.9	<0.001	0.009	0.003
UN[11] (mgN/dL)	8.79	8.13	9.26	9.23	0.70	<0.001	0.121	0.152

Data are shown by least squares means (LSM) and SEM.

[1]LCFA-Ca = calcium salts of long-chain fatty acids.

[2]RPM = rumen-protected methionine.

[3]*P* -value for factorial contrasts: LCFA-Ca, RPM and the interaction between LCFA-Ca and RPM.

[4]GH = growth hormone.

[5]IGF-1 = insulin-like growth factor-1.

[6]GLP-1 = glucagon-like peptide-1 (7-36) amide.

[7]NEFA = non-esterified fatty acids.

[8]BHBA = beta-hydroxy butyrate.

[9]TG = triglyceride.

[10]T-CHO = total-cholesterol.

[11]UN = urea nitrogen.

Table 4-4. Plasma amino acid concentrations during experimental period in lactating cows

	Treatment							
	non-LCFA-Ca		LCFA-Ca[1]			Contrast[3] (*P* -value)		
Item	non-RPM	RPM	non-RPM	RPM[2]	SEM	LCFA-Ca	RPM	LCFA × RPM
EAA[4] (nmol/mL)								
Arginine	60.4	91.7	102	92.5	12.6	0.322	0.081	0.092
Histidine	30.6	39.5	33.1	27.6	6.51	0.671	0.261	0.105
Isoleucine	110	134	134	131	15.2	0.487	0.462	0.371
Leucine	125	145	158	147	20.8	0.710	0.223	0.271
Lystine	66.4	92.3	95.9	90.7	13.0	0.401	0.270	0.226
Methionine	25.2	36.1	38.3	36.0	4.03	0.243	0.101	0.096
Phenylalanine	37.6	43.8	47.8	44.9	6.40	0.713	0.232	0.329
Threonine	95.5	115	134	109	16.3	0.864	0.320	0.195
Tryptophan	39.9	41.8	37.3	34.3	3.14	0.851	0.122	0.426
Valine	229	259	267	252	24.4	0.739	0.506	0.325
NEAA[5] (nmol/mL)								
Alanine	296	332	348	324	31.1	0.799	0.356	0.234
Asparagine	55.5	69.8	78.0	70.7	8.57	0.686	0.202	0.239
Aspartate	6.11	7.19	8.13	7.14	0.714	0.952	0.216	0.200
Citrulline	89.0	94.2	99.1	92.1	10.4	0.839	0.403	0.220
Cystine	1.85	2.31	0.697	1.12	1.01	0.541	0.138	0.979
Glutamine	273	282	307	267	20.7	0.402	0.603	0.202
Glutamate	56.9	62.7	61.4	53.2	3.15	0.583	0.273	0.014
Glycine	449	460	476	449	58.7	0.982	0.563	0.706
1-methyl histidine	8.72	9.97	8.99	9.04	0.679	0.156	0.441	0.184
3-methyl histidine	8.01	8.31	7.78	7.34	1.60	0.954	0.618	0.756
Ornithine	40.5	54.7	54.5	54.6	6.36	0.306	0.316	0.309
Proline	72.8	104	98.0	100	12.1	0.139	0.314	0.194
Serine	116	78.8	132	54.8	13.5	0.400	0.747	0.159
Taurine	33.6	43.9	42.6	42.6	4.99	0.236	0.359	0.232
Tyrosine	41.5	51.6	59.8	54.4	8.17	0.724	0.149	0.268

Data are shown by least squares means (LSM) and standard errors of the LSM (SEM)

[1]LCFA-Ca = calcium salts of long-chain fatty acids.

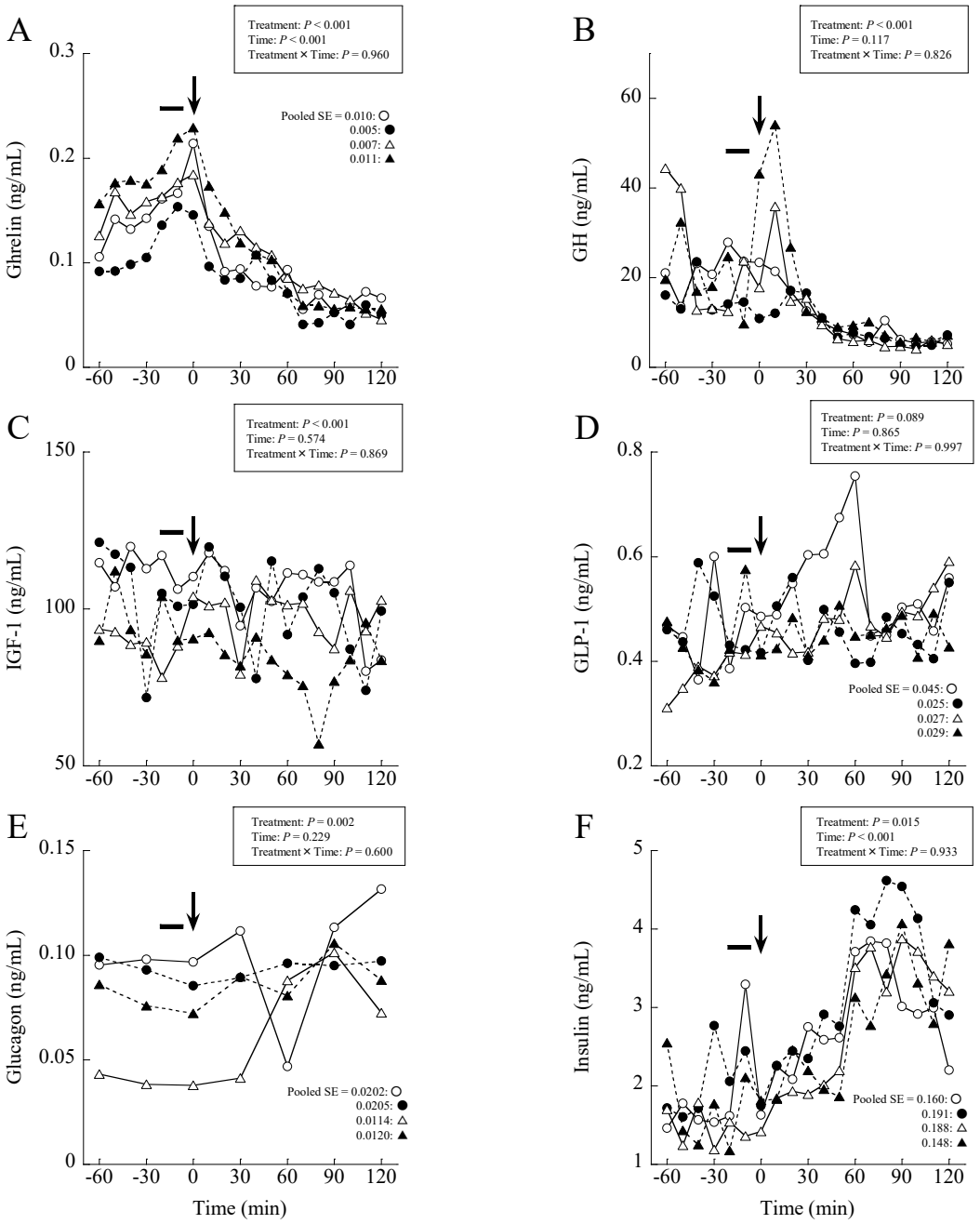
[2]RPM = rumen-protected methionine.

[3]*P* -value for factorial contrasts: LCFA-Ca, RPM and the interaction between LCFA-Ca and RPM.

[4]EAA: Essential amino acid.

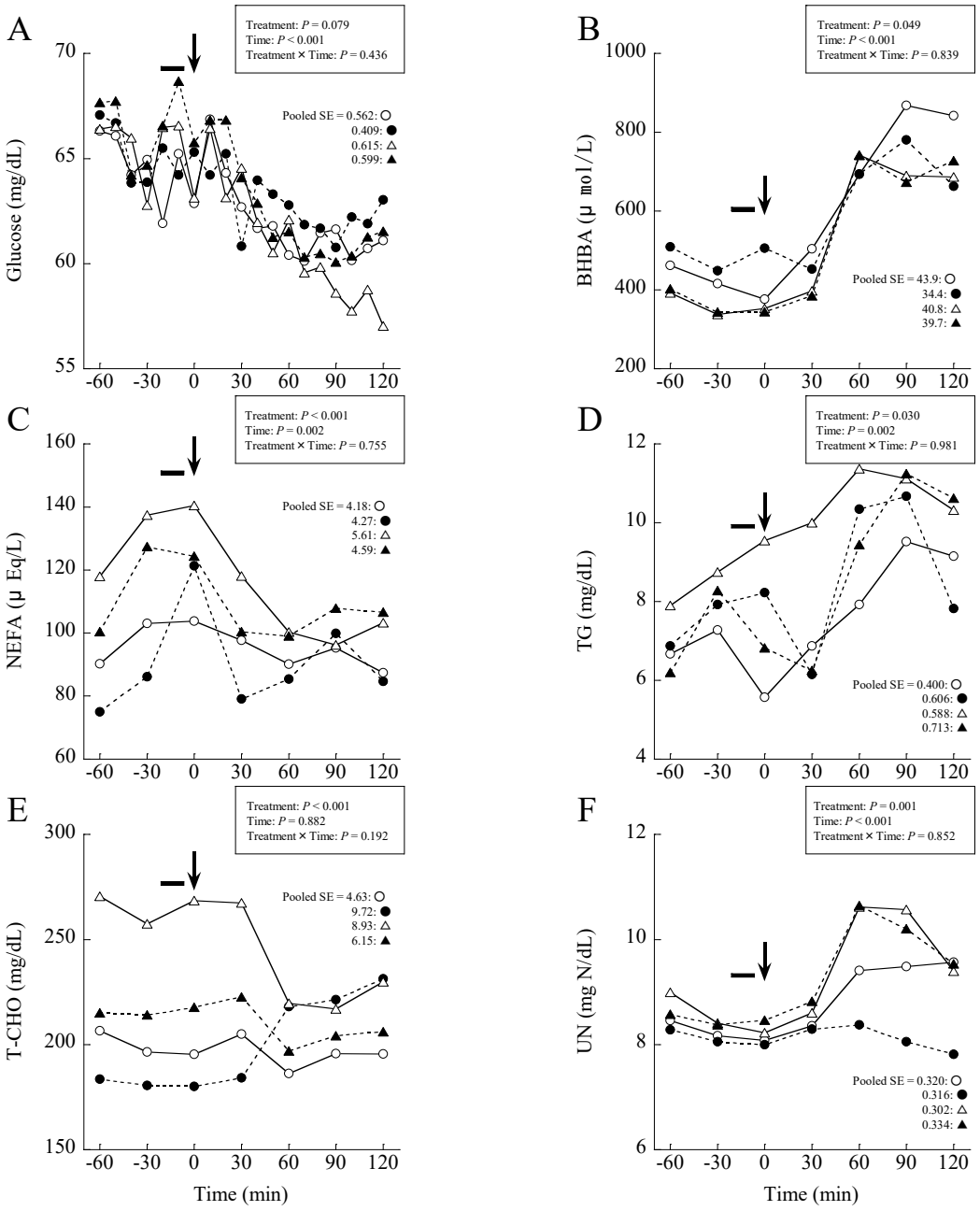
[5]NEAA: non-essential amino acid.

Figure 4-1



Plasma hormone (ghrelin: A, GH: B, IGF-1: C, GLP-1: D, glucagon: E and insulin: F) concentrations in cows fed each treatment diet: non-LCFA-Ca + non-RPM (○ with a solid line), non-LCFA-Ca + RPM (● with a dotted line), LCFA-Ca + non-RPM (△ with a solid line) and LCFA-Ca + RPM (▲ with a dotted line). Values are expressed as least square of means (n = 4). P-value for effects of treatment, time and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding time, respectively.

Figure 4-2



Plasma metabolite (glucose: A, BHBA: B, NEFA: C, TG: D, T-CHO: E and UN: F) concentrations in cows fed each treatment diet: non-LCFA-Ca + non-RPM (○ with a solid line), non-LCFA-Ca + RPM (● with a dotted line), LCFA-Ca + non-RPM (△ with a solid line) and LCFA-Ca + RPM (▲ with a dotted line). Values are expressed as least square of means (n = 4). P-value for effects of treatment, time and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding time, respectively.

Chapter 5

General Discussion

In recent dairy industry, cows are made to undergo a coordinated series of physiological adaptations to support milk synthesis. This is most noticeable during the early lactating period, when the energy demand for milk yield output is greater than the ingested energy via feeds. The energy deficit places cows in a state of negative energy balance, resulting in the mobilization of body reserves. Although GH plays a central role in the regulation of nutrient partitioning and key processes for milk production, the artificial control of GH secretion and action is very difficult. Ghrelin, one of gastrointestinal hormones, stimulates GH secretion. In addition, ghrelin is related to secretions of other major metabolic hormones (insulin and glucagon), which regulate nutrient metabolism in the whole body and involve in milk production. Secretions of gastrointestinal hormones are strongly related to ingested nutrients (digestion and absorption). Manipulated ghrelin secretion via feeding management may make it possible to modify milk production via the secretions of metabolic hormones and metabolic changes. The present study conducted three experiments whith different nutrients (amino acids and fatty acids): the primary aim of this study was determine whether available nutrients modify plasma ghrelin concentration. Second aim was to determine whether the modification of plasma ghrelin concentration by the ingested nutrients affects plasma metabolic hormone concentrations and nutrient metabolism, and milk production. The general summary

of these examinations is shown in Table 5-1.

5.1. The effects of nutrients on plasma ghrelin concentration

The previous studies have reported the effects of some nutrients on plasma ghrelin concentration using sheep and cattle (Sugino et al., 2010; Fukumori et al., 2011; Fukumori et al., in press-a). Among these studies, post-ruminally available protein or circulating amino acids may be important factors to regulate ghrelin secretion during feeding management (Sugino et al., 2010). Therefore, the amino acid infusion intravenously to lactating cows was conducted in Chapter 2. As a result, the amino acid infusion did not affect plasma ghrelin concentration. One of the possible reasons are those, the infused level of amino acids would be a limiting factor because the infusion level was less than the previous studies in sheep (Sugino et al., 2010; Takahashi et al., 2009), and lactating cows demands more amino acids due to lactation. The previous study by Sugino et al. (2010) and Takahashi et al. (2009) loaded numerous amino acids in relative to the individual's requirement. Their doses of amino acids would be excess as an actual utilization. Therefore, developing more effective way is necessary to increase plasma ghrelin concentration by amino acids. The individual amino acid may differentially affect plasma ghrelin concentration. Kuhara et al. (1991) demonstrated the varied responses of GH, glucagon and insulin to 17 kinds of amino acids. In their report, aspartic acid, glutamic acid, and arginine enhanced GH secretion, which may also be consistent with ghrelin secretion. Therefore, it is necessary to determine which amino acid possesses a stimulating effect on ghrelin secretion.

The effect of MCFA on plasma ghrelin concentration was investigated in Chapter 3. As a result, feeding MCFA could increase plasma ghrelin concentration as same as the previous report by non-ruminants (Nishi et al., 2005). The ingested MCFA are supposed to be directly used for synthesis of the active-form ghrelin. LCFA also increased plasma ghrelin concentration in chapter 4. LCFA are not used for acylation of ghrelin, and therefore, the mechanism how LCFA increases plasma ghrelin concentration is unclear. As one of the possible reasons, changes in lipid metabolism might increase plasma ghrelin concentration. In support to this, the accelerated lipid metabolism by LCFA combined with Met augmented ghrelin secretion. It was supposed that Met itself did not enhance ghrelin secretion because cows fed Met alone did not increase plasma ghrelin concentration.

Energy intake level would be one of factors determining plasma ghrelin concentration because negative energy balance and feed deprivation increase plasma ghrelin concentration (Bradford and Allen, 2008; Wertz-Lutz et al., 2006). Therefore, the possibility that lower MEI in cows by MCFA affected the increases in plasma ghrelin concentration cannot be ruled out. However, our previous study showed that the difference in MEI between 100% and 120% of requirement did not affect plasma ghrelin concentration (The graduation thesis by Ishizu, 2010), suggesting that the variety of energy intake in the positive energy balance may not affect plasma ghrelin concentration. Because all cows used in Chapters 3 and 4 were in the positive energy status (Tables 3-2, 4-2), higher plasma ghrelin concentration by MCFA and LCFA plus Met treatments was likely due to a difference in available nutrients.

Overall, obtained results indicate that nutritional treatment may alter plasma ghrelin concentration in lactating cows. Although amino acids did not affect plasma

ghrelin concentration, MCFA, LCFA and LCFA plus Met treatments might be effective for increase plasma ghrelin concentration. Plasma ghrelin concentration when cows were fed LCFA and LCFA plus Met showed 9% and 19% rises relative to the concentration when they were fed control diet, while MCFA diet showed 43% rises in plasma concentration relative to the control diet. In addition, the previous studies determined that VFA (short-chain fatty acids) decrease plasma ghrelin concentration (Fukumori et al., 2011; Fukumori et al., in press-a). These results suggest that the differences in carbon chain-length of fatty acids may affect plasma ghrelin concentration, and MCFA can largely augment plasma ghrelin concentration.

5.2. The effects of nutrients on the responses of metabolic hormones to ghrelin injection

The past studies demonstrated that ghrelin has characteristic actions in the lactating period: the impact of ghrelin on GH secretion was greater in the early lactating period compared with other physiological states (Itoh et al., 2005) and the ghrelin-induced increases in plasma glucagon, glucose and insulin concentrations were observed in the lactating period, but not in other physiological states (Itoh et al., 2006). The ghrelin-induced glucagon and glucose releases were shown also in type 2 diabetic rats (Adeghate and Parvez, 2002). Although lactating cows are not in a pathological condition, they are thought to be in a state resembling the diabetic state with respect to glucose metabolism. Thus, these results indicate that ghrelin may augment the catabolic condition associated with insulin resistance.

This study determined for the first time the effects of available nutrients on the

ghrelin actions. In Chapter 2, the effects of intravenous amino acids infusion on ghrelin induced GH, insulin and glucagon secretions were determined. As a result, the amino acid infusion did not affect the GH response to ghrelin injection, but augmented glucagon and insulin releases after ghrelin injection. Subsequently, the amino acid infusion stimulated the recovery of plasma glucose concentration from its peak level via augmented insulin release. This result suggests that amino acids may counteract the ghrelin induced catabolic action. In Chapter 3, the effects of feeding MCFA on ghrelin induced GH, insulin and glucagon secretions were determined. In the experiment, although feeding MCFA tended to inhibit the increase in plasma NEFA concentration after ghrelin injection, there was not much difference with the control diet. Thus, the amino acid infusion modified the ghrelin action in lactating cows, which may contribute to an improvement of insulin sensitivity.

5.3. The relationship between ghrelin and metabolic hormones

Ghrelin is a GH releasing factor and it is sometimes observed that there is a link between plasma ghrelin and GH concentration in the short-term period (pre- and post-prandial changes) (Sugino et al., 2002) and the long-term observation (from prepartum to lactating period) (Sugino et al., 2006; Bradford and Allen, 2008). Therefore, an alteration of plasma ghrelin concentration by nutrients may affect plasma GH concentration. In Chapters 3 and 4, feeding MCFA and LCFA increased plasma ghrelin concentration. The higher plasma ghrelin concentration induced by feeding LCFA and LCFA plus Met tended to be accompanied by plasma GH concentration. On the other hand, higher ghrelin concentration induced by feeding MCFA did not

affect plasma GH concentration although the rise in plasma ghrelin concentration was greater in MCFA treatment than LCFA and LCFA plus Met treatments. Date et al. (2000) demonstrated that the continuous administration of ghrelin increased plasma GH concentration on d 6 after the continuous administration, but the high plasma GH concentration did not last on d 12, suggesting the continuous ghrelin administration induced homologous down-regulation of GHS-R. Therefore, the long-term sustaining of high plasma ghrelin concentration may not be effective to increase plasma GH concentration. In the present study, plasma GH concentration was not increased by 41% rises in plasma ghrelin concentration, but 9 to 19% rises in plasma ghrelin concentration induced high plasma GH concentration after 14 d treatment. The moderate continuous increase of plasma ghrelin concentration may be effective in case requiring a persistent increase in plasma GH concentration. Increased plasma ghrelin concentration did not contribute to the increase in plasma IGF-1 concentration in both Chapters 3 and 4. Twenty-four hour continuous infusion of ghrelin increased plasma IGF-1 concentration with high plasma GH concentration in human (Veldhuis et al., 2008). However, in the long-term period, plasma IGF-1 concentration is positively correlated with protein intake (Law et al., 2009) and energy balance (Pedernera et al., 2008). Although GH stimulates IGF-1 synthesis in the liver, the GH receptor abundance is regulated by the energy intake level and a limiting factor for plasma IGF-1 concentration (Rhoads et al., 2007). Actually, plasma IGF-1 concentration is not consistent with plasma GH concentration in lactating period. Butler et al. (2003) demonstrated hyperinsulinemic euglycemic clamp for 96 h increased plasma IGF-1 concentration with high expression of GH receptor mRNA. In the present study, plasma insulin concentration decreased with MCFA and LCFA, suggesting the low

plasma IGF-1 concentration related to plasma insulin concentration rather than plasma ghrelin and GH concentrations.

Plasma glucagon and insulin concentrations were also modified by feeding MCFA, LCFA and LCFA plus Met. Unlike obtained results in Chapter 2, high plasma ghrelin concentration by feeding MCFA and LCFA did not increase plasma glucagon and insulin concentrations. The decrease in plasma insulin concentration in MCFA and LCFA treatments are possibly associated with lower plasma GLP-1 concentration and DMI, and are altered by rumen fermentation rather than by plasma ghrelin concentration. However, plasma glucagon and insulin concentrations were increased when cows were fed LCFA plus Met than when cows were fed LCFA only. The result resembles the result in Chapter 2 where amino acids enhanced glucagon and insulin secretion induced by ghrelin injection.

5.4. A role of ghrelin in regulating lactation.

Ghrelin has catabolic function (stimulating GH secretion) and anabolic function (enhancing appetite and fat deposition) in laboratory animals. It has been unclear how ghrelin acts in lactating cows. In Chaper 2, amino acids did not affect plasma ghrelin concentration, but altered ghrelin action. In the study, amino acids stimulated insulin secretion and glucose removal after ghrelin injection. Although milk production was not observed in this experiment due to a short-term infusion, providing more amino acids to lactating cows in the state of negative energy balance may lead to restore insulin resistance. In Chapters 3 and 4, feeding MCFA and LCFA plus Met did not increase milk production despite of the increase in endogenous plasma ghrelin

concentration. However, MCFA could increase milk production with high plasma ghrelin concentration if MEI were equivalent to the control diet. In Chapter 4, using mid-lactating cows, plasma ghrelin and GH concentrations were increased by feeding LCFA and LCFA plus Met without the increase in milk production. As same as in Chapter 3, feeding LCFA and LCFA plus Met may also bring some effects to milk production in early-lactating period.

In conclusion, the nutritional treatment in this study succeeded in increasing plasma ghrelin concentration. However, the endogenous increase in plasma ghrelin concentration did not always result in an increase in plasma GH concentration. Additionally, the nutritional strategy for stimulating ghrelin secretion did not contribute to enhancing milk production, but some possibilities to modify milk production could be found out because of manipulating endogenous ghrelin secretion by nutritional treatments was accompanied with several alterations of metabolic hormones and nutrient metabolism. The feed supplements such as MCFA or LCFA plus Met may lead to higher milk production when the supplements are fed in the other lactating stage or for a prolonged period.

Table 5-1. The summary of the results of 3 studies

Item	Amino acid mixture*	MCFA-Ca[1]*	LCFA-Ca[2]*	LCFA-Ca+Met[3]†
DMI[4]	—[5]	↓	↓	↗
Milk yield	—	↘	→	→
Plasma concentrations of hormones and metabolites				
Ghrelin	NS	↑ ↑	↑	↑
GH[6]	NS	→	↗	↗
IGF-1[7]	—	↓	↓	↓
GLP-1[8]	—	→	↘	→
Glucagon	NS	→	↓	↗
Insulin	NS	↓	↓	↗
Glucose	NS	↓	→	↑
BHBA[9]	—	↑	↓	→
NEFA[10]	NS	↑	↑	↓
TG[11]	—	—	↑	↓
T-CHO[12]	—	↑	↑	↓
UN[13]	—	↑	↑	→
Ghrelin action[14]				
GH	→	→	—	—
Insulin	↑	→	—	—
Glucagon	↑	→	—	—
Glucose	↓	→	—	—
NEFA	→	↘	—	—

[1]MCFA = medium-chain fatty acids.

[2]LCFA = long-chain fatty acids.

[3]Met = methionine.

[4]DMI = dry matter intake.

[5]—: Not tested in the study.

[6]GH = growth hormone.

[7]IGF-1 = insulin-like growth factor-1

[8]GLP-1= glucagon-like peptide-1 (7-36) amide.

[9]BHBA = beta-hydroxy butyrate.

[10]NEFA = non-esterified fatty acids.

[11]TG = triglyceride.

[12]T-CHO = total-cholesterol.

[13]UN = urea nitrogen.

[14]The area under curve or incremental area of each hormone and metabolites after ghrelin injection.

*Compared with the control treatment.

†Compared with LCFA-Ca treatment.

Chapter 6

General Summary

Feed intake and metabolic use of nutrients are important factors to maximize milk production of lactating dairy cows, and they are partly regulated by metabolic hormones. Growth hormone (GH) is well documented for the increase in milk production through some metabolic regulation to promote lipolysis in the adipose tissue and to enhance gluconeogenesis and biosynthesize of insulin-like growth factor-1 (IGF-1) in the liver. IGF-1 has a galactopoietic effect with increasing blood flow into the mammary gland and with causing insulin resistance. Thus, GH plays a central role in regulating milk production in lactating cows, and hence, the approach to enhance the endogenous GH secretion potentially leads to the increase in milk production. However, such approach has been not established because GH secretion is strongly regulated by central nervous systems. Ghrelin, secreted from the gastrointestinal tract, was identified as a potent GH secretagogue in recent years. In addition, ghrelin is related to appetite regulation; ghrelin injection increases the food intake in laboratory animals. Because circulating ghrelin is sensitive to ingested nutrients, hence the nutritional management or feeding strategy to control ghrelin secretion may be able to adjust the GH secretion. The previous study demonstrated that plasma ghrelin concentration in ruminants was decreased by volatile fatty acids, and was not affected by glucose as a secretory inhibitor in non-ruminants. Another previous study revealed that plasma ghrelin concentration was increased by amino

acids in sheep. However, there has been still little information of which nutrients affect plasma ghrelin concentration in ruminants. The objectives of this study were to determine the effects of some nutrients on plasma ghrelin concentration and ghrelin-induced metabolic changes, and to consider ghrelin's role in controlling milk production of lactating cows.

In the 1st trial, the effects of amino acids on plasma ghrelin concentration and ghrelin action were investigated in lactating cows. A mixture solution of amino acids (AMI) or saline (CON) were intravenously infused to determine plasma ghrelin concentration, and after then, during the infusion of AMI or CON, ghrelin was intravenously injected to determine the ghrelin action. In this experiment, six lactating Holstein cows were randomly assigned to two infusion treatments (AMI or CON) in a cross-over design. There was no difference in plasma ghrelin concentration between AMI and CON before the ghrelin injection. After the ghrelin injection, plasma GH, glucose, and non-esterified fatty acids (NEFA) concentrations in comparison with before injection immediately increased ($P < 0.05$) with no difference between two infusion treatments. Plasma insulin and glucagon concentrations were also increased by ghrelin injection in two infusion treatments ($P < 0.05$). The peak value of plasma insulin concentration was greater in AMI compared with CON ($P < 0.05$). Plasma glucagon concentration showed no difference at the peak value reached at 5 min in both treatments, and after then, showed sustained higher values in AMI compared with CON ($P < 0.05$). After plasma glucose concentration reached the peak, the degree of decline was greater in AMI compared with CON ($P < 0.05$). These results indicated that the plasma amino acids directly do not affect plasma ghrelin concentration, but may modify the ghrelin actions; amino acids which stimulate

glucagon, insulin, and glucose release into the blood circulation.

In the 2nd trial, the effects of medium-chain fatty acids (MCFA) on plasma ghrelin, GH, other metabolic hormone, metabolite concentrations, and milk production were investigated. Five early-lactating Holstein cows were randomly assigned to two dietary treatments in a cross-over design for 2-week periods. The diets were with a supplement of MCFA (MCFA-Ca, 1.5% added on dry matter basis) and without MCFA-Ca (CON). Plasma concentrations of hormones and metabolites in jugular vein were measured around the morning feeding on day 14 of each period. Dry matter intake (DMI) and metabolizable energy intake (MEI) were decreased, and milk yield tended to be decreased in the MCFA-Ca compared with CON diet ($P < 0.05$). Milk protein and lactose contents were decreased in the MCFA-Ca diet ($P < 0.05$). The MCFA-Ca diet increased plasma ghrelin concentration ($P < 0.05$), but did not affect plasma GH concentration, and decreased plasma IGF-1 concentration ($P < 0.05$). The MCFA-Ca diet did not affect plasma glucagon-like peptide-1 (7-36) amide (GLP-1) concentration. Plasma insulin concentration was decreased ($P < 0.05$), and plasma glucagon concentration was not changed with the MCFA-Ca diet. Plasma NEFA, total cholesterol (T-CHO) and beta-hydroxy butyrate (BHBA) concentrations were increased in the MCFA-Ca diet ($P < 0.05$). In conclusion, although plasma GH concentration did not link plasma ghrelin concentration, the MCFA diet increased plasma ghrelin concentration in lactating cows.

In the 3rd trial, the effects of long-chain fatty acids (LCFA) and methionine (Met) on plasma ghrelin, GH, other metabolic hormone, metabolite concentrations, and milk production were investigated. Four lactation Holstein cows were used in a 4 x 4 Latin square experiment for each 2-week period. Cows were fed diets with

supplements of calcium salts of LCFA (LCFA-Ca, 1.5% added on dry matter basis), rumen-protected Met (RPM, 20 g/d), LCFA-Ca plus RPM and without supplements (CON). Jugular blood samples were taken from 1 h before to 2 h after morning feeding at 10 min intervals on day 12 of each period. The LCFA-Ca decreased DMI ($P < 0.05$), but RPM did not affect DMI. Both supplements of LCFA-Ca and RPM did not affect MEI and milk yield and composition. Plasma concentrations of NEFA, triglyceride (TG), and T-CHO were increased with LCFA-Ca alone ($P < 0.05$), but the degrees of increases in plasma TG and T-CHO concentrations were moderated by LCFA-Ca plus RPM ($P < 0.05$). The LCFA-Ca increased plasma ghrelin concentration ($P < 0.05$) and the ghrelin concentration with LCFA-Ca plus RPM was the highest among the treatments. Similarly, plasma GH concentration tended to be increased by LCFA-Ca ($P = 0.056$) and showed the highest level in LCFA-Ca plus RPM. On the other hand, plasma IGF-1 concentration was decreased by LCFA-Ca and RPM ($P < 0.05$). Plasma GLP-1, glucagon, and insulin concentrations were decreased with LCFA-Ca ($P < 0.05$), whereas there was the interaction between LCFA-Ca and RPM in plasma glucagon concentration ($P < 0.05$); the LCFA-Ca plus RPM mitigated the decrease of plasma glucagon concentration by LCFA-Ca. These results showed that the supplementation of RPM together with LCFA-Ca increases plasma ghrelin and glucagon concentrations. Although the GH concentration was linked with plasma ghrelin concentration, plasma IGF-1 concentration was not associated with plasma GH and ghrelin concentrations.

In lactating cows, the exogenous ghrelin injection shows certain catabolic effects such as stimulating GH and glucagon secretions, associated with NEFA and glucose releases into the circulation in lactating cows. This study showed that amino acids

did not affect plasma ghrelin concentration, but modified these ghrelin actions; ghrelin-induced glucagon and insulin secretions were augmented, and simultaneous glucose removal from the circulation was enhanced by amino acids. This suggests amino acids may counteract the catabolic action of ghrelin. The present study demonstrated that plasma ghrelin concentration varies according to the kinds of available nutrient. MCFA increased plasma ghrelin concentration because they are directly used for acylation of ghrelin. LCFA also increased plasma ghrelin concentration. When Met was fed with LCFA, plasma ghrelin concentration was further increased. This might be caused not by a direct action of Met, but by the indirect action of Met because Met modulates lipid metabolism. The incremental degree of plasma ghrelin concentration was greater in cows fed MCFA (43%) compared with cows fed LCFA plus Met (19%). Nonetheless, the endogenous increase in plasma ghrelin concentration did not increase plasma GH concentration in cows fed MCFA, but increased in cows fed LCFA plus Met. The present study showed that continuous hyper plasma ghrelin concentration did not increase GH secretion always, suggesting that keeping of plasma ghrelin concentration above threshold level is not effective for the stimulation of GH secretion. The increased plasma ghrelin concentration by LCFA plus Met might be also related to some metabolic changes such as the higher plasma glucagon and glucose concentrations.

In conclusion, the kind of available nutrients affect plasma concentration and action of ghrelin in lactating cows. Milk production is not enhanced by the feed supplements as MCFA and LCFA because of their adverse effect on feed intake. However, the changes in plasma ghrelin concentration induced by these feed supplements could mediate major metabolic hormones to control nutrients use in the

whole body of lactating cows.

Acknowledgments

I would like to acknowledge with gratitude, the guidance, and supervision of Dr. TANIGUCHI Kohzo during the course of this study and the author is grateful for his constructive criticism and encouragement during the preparation of this thesis.

I would like to express my sincere thanks to my graduate committee members: Dr. YOSHIMURA Yukinori, Dr. KUSHIBIKI Shiro, Dr. BUNGO Takashi, Dr. OBITSU Taketo, and Dr. SUGINO Toshihisa, for their kindly reviewing my doctoral thesis with invaluable comments and detailed suggestions.

I owe a special debt of thanks to Dr. SUGINO Toshihisa for his endoless help with hormone analysis and valuable advices during the course of this study. Thanks are also given to Dr. OBITSU Taketo for his help with laboratory analysis and statistical analysis.

The author would like to thank Dr. LIAO M Lawrence, Graduate School of Biosphere Science, Hiroshima University (Higashi-Hiroshima, Japan), for critical reading of the manuscripts.

I would like to thank Dr. KUSHIBIKI Shiro, Dr. SHINGU Hiroyuki, Dr. MORIYA Naoko, and Dr. KOBAYASHI Hisami, and Dr. YAMAJI Kayoko, National Institute of Livestock and Grassland Science (Tsukuba, Japan) for helping animal and laboratory works and giving valuable advices. I would like to thank Dr. ITOH Fumiaki and Dr. ASAKUMA Sadaki, Hokkaido region agricultural research center (Sapporo, Japan) for helping animal and laboratory works and giving valuable advices. I would like to thank all farm staffs in National Institute of Livestock and Grassland Science (Tsukuba, Japan) for animal care and technical helping for the experiments.

I would like to acknowledge all students of our laboratory (Animal Nutrition and Feeding) for their assistance and for their technical advices. The author would like to thank all farm staffs in Terrestrical Field Science (Saijo Station), Hiroshima University.

I would like to thank Yuka Sangyo Co., Ltd. (Tokyo, Japan) for donating calcium salts of medium-chain fatty acids in Chapter 3. Thanks are also given to Nippon Soda Co., Ltd. (Tokyo, Japan) and Nisso Shoji Co., Ltd. (Tokyo, Japan) for donating rumen-protected methionine and calcium salts of long-chain fatty acids, respectively in Chapter 4.

The research described in this paper was funded by a Grant-in-Aid for Scientific Research No. 18780202 and Grant-in-Aid for JSPS Fellows from the Japan Society for the Promotion of Science.

References

Adeghate E, Parvez H. Mechanism of ghrelin-evoked glucagon secretion from the pancreas of diabetic rats. Neuroendocrinology Letter. 2002. 23:432–436.

Agriculture, Forestry, and Fisheries Research Council Secretariat. Japanese Feeding Standards for Dairy Cattle, Tokyo, Japan, 2006.

Allen MS. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. Journal of Dairy Science. 2000. 83:1598-1624.

Allen MS, Bradford BJ, Oba M. The hepatic oxidation theory of the control of feed intake and its application to ruminants. Journal of Animal Science. 2009. 87:3317-3334.

Anderson LL, Jeftinija S, Scanes CG. Growth hormone secretion: molecular and cellular mechanisms and in vivo approaches. Experimental Biology and Medicince. 2004. 229:291-302.

AOAC. Official methods of analysis. Vol. 1. 15th ed. Association of Official Analytical Chemists, Arlington, VA. 1990.

Auboiron S, Durand D, Bauchart D, Robert J, Chapman MJ. Lipoprotein metabolism in the preruminant calf: Effect of a high fat diet supplemented with L-methionine. Journal of Dairy Science. 1993. 77:1870-1881.

Bach AC, Babayan VK. Medium-chain triglycerides: an update. The American Journal of Clinical Nutrition. 1982. 36:950-962.

Barazzoni R, Zanneti M, Cattin MR, Visintin L, Vinci P, Cattin L, Stebel M, Guarnieri G. Ghrelin enhances in vivo skeletal muscle but not liver AKT signaling in rats. Obesity. 2008. 15:2614-2623.

Bauman DE, Currie WB. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. Journal of Dairy Science. 1980. 63:1514–1529.

Bauman DE, Eppard PJ, DeGeeter MJ, Lanza GM. Responses of high-producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. Journal of Dairy Science. 1985. 68:1352-1362.

Bauman DE, Vernon RG. Effects of exogenous somatotropin on lactation. Annual Review of Nutrition. 1993. 13:437-461.

Beglinger S, Drewe J, Schirra J, Goke B, D'Amato M, Beglinger C. Role of fat hydrolysis in regulating glucagon-like peptide-1 secretion. Journal of Clinical. Endocrinology and Metabolism. 2010. 95:879-886.

Benson JA, Reynolds CK. Effects of abomasal infusion of long-chain fatty acids on splanchnic metabolism of pancreatic and gut hormones in lactating dairy cows. Journal of Dairy Science. 2001. 84:1488-1500.

Bertics SJ, Grummer RR. Effects of fat and methionine hydroxyl analog prevention or alleviation of fatty liver induced by feed restriction. Journal of Dairy Science. 1999. 82:2731-2736.

Berthiaume R, Dubreuil P, Stevenson M, McBride BW, Lapierre H. Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. Journal of Dairy Science. 2001. 84:194-203.

Berthiaume R, Thiverge MC, Patton RA, Dubreuil P, Stevenson M, McBride BW, Lapierre H. Effect of ruminally protected methionine on splanchnic metabolism of amino acids in lactating dairy cows. Journal of Dairy Science. 2006.

89:1621-1634.

Bilby TR, Block J, do Amaral BC, Sa Filho O, Silvestre FT, Hansen PJ, Staples CR, Thatcher WW. Effects of dietary fatty acids on oocyte quality and follicular development in lactating dairy cow in summer. Journal of Dairy Science. 2006. 89:3891-3903.

Bradford BJ, Allen MS. Negative energy balance increases periprandial ghrelin and growth hormone concentrations in lactating dairy cows. Domestic Animal Endocrinology. 2008. 34:196-203.

Bradford BJ, Harvatine KJ, Allen MS. Dietary unsaturated fatty acids increase plasma glucagon-like peptide-1 and cholecystokinin and may decrease premeal ghrelin in lactating dairy cows. Journal of Dairy Science. 2008. 91:1443-1450.

Burton JL, McBride BW. Recombinant bovine somatotropin (rbST): is there a limit for biotechnology in applied animal agriculture? Journal of Agriculture Ethics. 1989. 2:129-159.

Burton JL, McBride BW, Block E, Glimm DR, Kennelly JJ. A review of bovine growth hormone. Canadian Journal of Animal Science. 1994. 74:167-201.

Butler ST, Marr AL, Pelton SH, Radcliff RP, Lucy MC, Butler WR. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. Journal of Endocrinology. 2003. 176: 205-217.

Chilliard Y, Doreau M. Influence of supplementary fish oil and rumen-protected methionine on milk yield and composition in dairy cows. Journal of Dairy Research. 1997. 64:173-179.

Choi BR, Palmquist DL. High fat diets increase plasma cholecystokinin and pancreatic

polypeptide, and decrease plasma insulin and feed intake in lactating cows. Journal of Nutrition. 1996. 126:2913-2919.

Choi BR, Palmquist DL, Allen MS. Cholecystokinin mediates depression of feed intake in dairy cattle fed high fat diet. Domestic Animal Endocrinology. 2000. 19:159-175.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 2002. 50:1714-1719.

Cummins KA, Sartin JL. Response of insulin, glucagon, and growth hormone to intravenous glucose challenge in cows fed high fat diets. Journal of Dairy Science. 1987. 70:277-283.

Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, Nakazato M. Central effects of a novel acylated peptide, ghrelin on growth hormone release in rats. Biochemical and Biophysical Research Communications. 2000. 275:477-480.

Davis SR, Gluckman PD, Hart IC, Henderson HV. Effects of injecting growth hormone or thyroxine on milk production and blood plasma concentrations of insulin-like growth factors I and II in dairy cows. Journal of Endocrinology. 1987. 114:17-24.

Dias VC, Fung E, Snyder FF, Carter RJ, Parsons HG. Effects of medium-chain triglyceride feeding on energy balance in adult humans. Metabolism. 1990. 39:887-891.

Dohme F, Machmuller F, Estermann BL, Pfister P, Wasserfallen A, Kreuzer M. The role of the rumen cilate protozoa for methane suppression caused by coconut oil. Letter in Applied Microbiology. 1999. 29:187-192.

Dohme F, Machmuller A, Wasserfallen A, Kreuzer M. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Letter in Applied Microbiology. 2001. 32:47-51.

Dohme F, Machmuller A, Sutter F, Kreuzer M. Digestive and metabolic utilization of lauric, myristic and stearic acid in cows, and associated effects on milk fat quality. Archives of Animal Nutrition. 2004. 58:99-116.

Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging Cell. 2008. 7:681-687.

Fukumori R, Sugino T, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Taniguchi K. Plasma ghrelin concentration is decreased by short chain fatty acids in wethers. Domestic Animal Endocrinology. 2011. 41:50-55.

Fukumori R, Yokotani A, Sugino T, Itoh F, Kushibiki S, Shingu H, Moriya N, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Taniguchi K. Effects of amino acids infused into the vein on ghrelin induced GH, insulin and glucagon secretion in lactating cows. Animal Science Journal. 2011. 82:267-273.

Fukumori R, Sugino T, Shingu H, Moriya N, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Kushibiki S, Taniguchi K. Effects of calcium salts of long-chain fatty acids and rumen-protected methionine on plasma concentrations of ghrelin, glucagon-like peptide-1 (7 to 36) amide and pancreatic hormones in lactating cows. Domestic Animal Endocrinology. 2012-a. 42:74-82.

Fukumori R, Mita T, Sugino T, Obitsu T, Taniguchi K. Plasma concentrations and effects of glucagon-like peptide-1 (7-36) amide in calves before and after weaning. Domestic Animal Endocrinology. 2012. 43:299-306.

Fukumori R, Mita T, Sugino T, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Taniguchi K. Effects of glucose and volatile fatty acids on blood ghrelin concentrations in calves before and after weaning. Journal of Animal Science (in press-a).

Fukumori R, Mita T, Sugino T, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Taniguchi K. Effects of intravenous ghrelin injection on plasma growth hormone, insulin, and glucose concentrations in calves at weaning. Animal Science Journal (in press-b).

Gobert M, Martin B, Ferlay A, Chilliard Y, Graulet B, Pradel P, Bauchart D, Durand D. Plant polyphenols associated with vitamin E can reduce plasma lipoperoxidation in dairy cows given n-3 polyunsatuated fatty acids. Journal of Dairy Science. 2009. 92:6095-6104.

Gerich JE, Charles MA, Grodsky GM. Regulation of pancreatic insulin and glucagon secretion. Annual Review of Physiology. 1976. 38:353-388.

Goodwin JF. On the measurement of urinary nitrogen with 1-fluoro-2, 4-dinitrobenzene. Clinica Chemica Acta. 1968. 21:231-240.

Goulas C, Zervas G, Papadopoulos G. Effect of dietary animal fat and methionine on dairy ewes milk yield and milk composition. Animal Feed Science and Technology. 2003. 105:43-54.

Graulet B, Gruffat-Mouty D, Durand D, Bauchart D. Effects of milk diets containing beef tallow or coconut oil on the fatty acid metabolism of liver slices from preruminant calves. British Journal of Nutrition. 2000. 84:309-318.

Grummer RR, Socha MT. Milk fatty acid composition and plasma energy metabolite concentrations in lactating cows fed medium-chain triglycerides. Journal of Dairy

Science. 1989. 72:1996-2001.

Gutzwiller, JP., B. Göke, J. Drewe, P. Hildebrand, S. Ketterer, D. Handschin, R. Winterhalder, D. Conen and C. Beglinger. Glucagon-like peptide-1: a potent regulator of food intake in humans. Gut. 1999. 44:81-86.

Han J, Hamilton JA, Kirkland JL, Corkey BE, Guo W. Medium-chain oil reduces fat mass and down-regulates expression of adipogenic genes in rats. Obesity Research. 2003. 11:734-744.

Hare KJ, Vilsbøll T, Asmar M, Deacon CF, Knop FK, Holst JJ. The glucagonostatic and insulinotropic effects of glucagon-like peptide-1 contribute equally to its glucose-lowering action. Diabetes. 2010. 59:1765-1770.

Harrison JL, Miller DW, Findlay PA, Adam CL. Photoperiod influences the central effects of ghrelin on food intake, GH and LH secretion in sheep. Neuroendocrinology. 2008. 87:182-192.

Hayashida T, Murakami K, Mogi K, Nishihara M, Nakazato M, Mondal MS, Horii Y, Kojima M, Kangawa K, Murakami N. Ghrelin in domestic animals: distribution in stomach and its possible role. Domestic Animal Endocrinology. 2001. 21:17-24.

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto M, Miyazaki S, Tsujimoto G. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nature Medicine. 2005. 11:90-94.

Hosoda H, Kangawa K. The autonomic nervous system regulates gastric ghrelin secretion in rats. Regulatory Peptides. 2008. 146:12-18.

Hristov AN, Vander Pol M, Agle M, Zaman S, Schneider C, Ndegwa P, Vaddella VK, Johnson K, Shingfield KJ, Karnati KR. Effect of lauric acid and coconut oil on

ruminal fermentation, digestion, ammonia losses from manure, and milk fatty acid composition in lactating cows. Journal of Dairy Science. 2009. 92:5561-5582.

Huber JT, Enery RS, Bergen WG, Liesman JS, Kung L Jr., King KJ, Gardner RW, Checketts M. Influences of methionine hydroxyl analog on milk and milk fat production, blood serum lipids, and plasma amino acids. Journal of Dairy Science. 1984. 67:2525-2531.

Itoh F, Komatsu T, Yonai M, Sugino T, Kojima M, Kangawa K, Hasegawa Y, Terashima Y, Hodate K. GH secretory responses to ghrelin and GHRH in growing and lactating dairy cattle. Domestic Animal Endocrinology. 2005. 28:34-45.

Itoh F, Komatsu T, Kushibiki S, Hodate K. Effects of ghrelin injection on plasma concentrations of glucose, pancreatic hormones and cortisol in Holstein dairy cattle. Comparative Biochemistry and Physiology. 2006.143:97-102.

Johnson ME, Das NM, Butcher FR, Fain JN. The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl adenosine monophosphate, and fatty acids. The Journal of Biological Chemistry. 1972. 247:3229-3235.

Jordan E, Lovett DK, Monahan FJ, Callan J, Flynn B, O'Mara FP. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. Journal of Animal Science. 2006. 84:162-170.

Kawamura N, Kato Y, Takahashi A, Sugiura S. Effects of a series of saturated fatty acids with differerent length of alkyl redicle in the form of calcium soaps on the milk composition and milk fatty acids in cows. Research Bulletin of the Aichi-ken Agricultural Research Center (in Japanese). 1990. 22:329-337.

Kawasaki M, Miura Y, Funabiki R, Yagasaki M. Comparison of the effects on lipid

metabolism of dietary methionine and cystine between hepatoma-bearing and normal rats. Bioscience, Biotechnology, and Biochemistry. 2010. 74:158-167.

Keller U, Turkalj I, Laager R, Bloesch D, Bilz S. Effects of medium- and long- chain fatty acids on whole body leucine and glucose kinetics in man. Metabolism. 2002. 51:754-760.

Khorasani G., Kennelly JJ. Effects of added fat on performance, rumen characteristics, and plasma metabolites of midlactation cows. Journal of Dairy Science. 1988. 81:2459-2468.

Khosla P, Samman S, Carroll KK, Huff MW. Turnover of 125I-VLDL and 131I-LDL apolipoprotein B in rabbits fed diets containing casein or soy protein. Biochimica et Biophysica Acta. 1989. 1002:157–163.

Kirchner H, Gutierrez JA, Solenberg PJ, Pfluger PT, Czyzyk TA, Willency JA, Schürmann A, Joost HG, Jandacek RJ, Hale JE, Heiman ML, Tschöp MH. GOAT links dietary lipids with the endocrine control of energy balance. Nature Medicine. 2009. 15:741-745.

Knerr I, Gröschl M, Rascher W, Rauh M. Endocrine effects of food intake: insulin, ghrelin, and leptin responses to a single bolus of essential amino acids in humans. Annals of Nutrition and Metabolism. 2003. 47:312-318.

Kojima M, Hosoda M, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999. 402:656-660.

Kuhara T, Ikeda S, Ohneda A, Sasaki Y. Effects of intravenous infusion of 17 amino acids on the secretion of GH, glucagon and insulin in sheep. American Journal of Physiology-Endocrinology and Metabolism. 1991. 260:E21-E26.

Kummel L. Evidence for possible interrelationship between gluconeogenesis and ketogenesis? Biochemistry International. 1984. 8:393-400.

Kreymann B, Williams G, Ghatei MA, Bloom SR. Glucagon like peptide-1 7-36: a physiological incretin in man. Journal of Endocrinology. 1990. 126:169-173.

Lavau M, Fried SK, Susini C, Freychet P. Mechanism of insulin resistance in adipocytes of rats fed a high-fat diet. The Journal of Lipid Research. 1979. 20:8-16.

Law RA, Young FJ, Patterson DC, Kilpatrick DJ, Whylie ARG, Mayne CS. Erratum to "Effect of dietary protein content on animal production and blood metabolites of dairy cows during lactation". 2009:1001-1012.

Litherland NB, Thire S, Beaulieu AD, ReynoldsCK, Benson JK, Drackley JK. Dry matter intake is decreased more by abomasal infusion of unsaturated free fatty acids than by unsaturated triglycerides. Journal of Dairy Science. 2005. 88:632-643.

Mills JK, Ross DA, Van Amburgh ME. The effects of feeding medium-chain fatty triglycerides on the growth, insulin responsiveness, and body composition of Holstein calves from birth to 85 kg of body weight. Journal Dairy Science. 2010. 93:4262-4273.

Murata M, Okimura Y, Iida K, Matsumoto M, Sowa H, Kaji H, Kojima M, Kangawa K, Chihara K. Ghrelin modulates the downstream molecules of insulin signaling in hepatoma cells. The Journal of Biological Chemistry. 2002. 277:5667-5674.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. Nature. 2001. 409:194-198.

Nakagawa E, Nagaya N, Okumura H, Enomoto M, Oya H, Ono F, Hosoda H, Kojima M, Kangawa K. Hyperglycaemia suppresses the secretion of ghrelin, a novel

growth-hormone-releasing peptide: responses to the intravenous and oral administration of glucose. Clinical Science (London, England). 2002.103:325-328.

National Research Council. Nutrient Requirements of Dairy Cattle. 7th Rev. ed. Washington, DC: Natl. Acad. Press; 2001.

Nicholson T, Omer SA. The inhibitory effect of intestinal infusions of unsaturated long-chain fatty acids on forestomach motility of sheep. British Journal of Nutrition. 1983. 50:141-149.

Nieto R, Obitsu T, Fernández-Quintela A, Bremner D, Milne E, Calder AG, Lobley GE. Glutamine metabolism in ovine splanchnic tissues: effects of infusion of ammonium bicarbonate or amino acids into the abomasum. British Journal of Nutrition. 2002. 87:357-366.

Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, Yanase T, Nawata H, Kangawa K, Kojima M. Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. Endocrinology. 2005. 146:2255-2264.

Obitsu T, Kamiya M, Kamiya Y, Tanaka M, Sugino T, Taniguchi K. Effects of high ambient temperature on urea-nitrogen recycling in lactating dairy cows. Animal Science Journal. 2011. 82:531-536.

Oda H, Fukui H, Hitomi Y, Yoshida A. Alteration of serum lipoprotein metabolism by polychlorinated biphenyl and methionine in rats fed a soybean protein diet. Journal of Nutrition. 1991. 121:925-933.

Peel CJ, Fronk TJ, Bauman DE, Gorewit RC. Effect of exogenous growth hormone in early and late lactation on lactational performance of dairy cows. Journal of Dairy Science. 1983. 66:776-782.

Pedernera M, Garcia SC, Horagadoga A, Barchia I, Fulkerson WJ. Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. 2008. 91:3896-6907.

Perdomo G, Commerford SR, Richard AM, Adams SH, Corkey BE, O'Doherty RM, Brown NF. Increased beta-oxdation in muscle cells enhances insulin-stimulated glucose metabolism and protects against fatty acid-induced insulin resistance despite intramyocellular lipid accumulation. The Journal of Biological Chemistry. 2004. 279:27177-27186.

Pinotti L, Baldi A, Dll'Orto V. Comparative mammalian choline metabolism with emphasis on high-yielding dairy cows. Nutriton Research Reviews. 2002. 15:315-331.

Piot C, Hocquette JF, Veerkamp JH, Durand D, Bauchart D. Effects of dietary coconut oil on fatty acid oxidation capacity and of the liver, the heart and skeletal muscles in the preruminant calf. British Journal of Nutrition. 1999. 82:299-308.

Pisulewski H, Rulquin H, Peyraud JL, Verite R. Lactational and systemic responses of dairy cows to postruminal infusions of increasing amounts of methionine. Journal of Dairy Science. 1996. 79:1781-1791.

Prosser CG, Fleet IR, Corps AN, Froesch ER, Heap RB. Increase in milk secretion and mammary blood flow by intra-arterial infusion of insulin-like growth factor-I into the mammary gland of the goat. Journal of Endocrinology. 1990. 126:437-443.

Prosser CG, Davis SR, Farr VC, Moore LG, Gluckman PD. Effects of close-arterial (external pudic) infusion of insulin-like growth factor-ii on milk-yield and mammary blood-flow in lactating goats. Journal of Endocrinology. 1994. 142:93-99.

Relling AE, Reynolds CK. Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating cows. Journal of Dairy Science. 2007. 90:1506-1515.

Relling AE, Reynolds CK. Abomasal infusion of casein, starch and soybean oil differentially affect plasma concentrations of gut peptides and feed intake in lactating cows. Domestic Animal Endocrinology. 2008. 35:35-45.

Relling AE, Reynolds CK, Loerch SC. Effects of feeding fat or intrajugular infusion of glucagon-like peptide-1 and cholecystokinin on dry matter intake, digestibility, and digesta rate of passage in growing wethers. Journal of Animal. Science. 2011. 89:168-178.

Reveneau C, Karnati SKR, Oelker ER, Firkins JL. Interaction of unsaturated fat or coconut oil with monensin in lactating dairy cows fed 12 times daily. I. Protozoal abundance, nutrient digestibility, and microbial protein flow to the omasum. Journal of Dairy Science. 2012. 95:2046-2060.

Rhoads RP, Kim JW, Van Amburgh ME, Ehrhardt RA, Frank SJ, Boisclair YR. Effect of nutrition on the GH responsiveness of liver and adipose tissue in dairy cows. Journal of Endcrinology. 2007. 195:49-58.

Roche JR, Sheahan AJ, Chagas LM, Blache DP, Berry DP, Kay JK. Long-term infusion of ghrelin and obestatin in early lactation dairy cows. Journal of Dairy Science. 2008. 91:4728-4740.

Schauff DL, Clark JH. Effects of prilled fatty acids and calcium salts of fatty acids and calcium salts of fatty acids on rumen fermentation, nutrient digestibilities, milk production, and milk composition. Journal Dairy Science. 1989. 72:917-927.

Schauff DJ, Clark JH. Effects of feeding diets containing calcium salts of long-chain

fatty acids to lactating dairy cows. Journal of Dairy Science. 1992. 75:2990-3002.

Schneider PL, Sklan D, Chalupa W, Kronfeld DS. Feeding calcium salts of fatty acids to lactating cows. Journal of Dairy Science. 1988. 71:2143-2150.

Schwab CG, Satter LD, Clay AB. Response of lactating cows to abomasal infusion of amino acids. Journal of Dairy Science. 1976. 59:1254-1270.

Shibano K, Kawamura S. Serum free amino acid concentration in hepatic lipidosis of dairy cows in the periparturient period. Journal of Veterinary Medical Science. 2005. 68:393-396.

Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukawa S. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. The Journal of Clinical Endocrinology and Metabolism. 2002. 87:240-244.

Shinohara H, Ogawa A, Kasai M, Aoyama T. Effect of randomly interesterified triacylglycerols containing medium- and long-chain fatty acids on energy expenditure and hepatic fatty acid metabolism in rats. Bioscience, Biotechnology, and Biochemistry. 2005. 69:1811-1818.

Sutton JD, Knight R, McAllan AB, Smith RH. Digestion and synthesis in the rumen of sheep given diets supplemented with free and protected oils. British Journal of Nutrition. 1983. 49:419-432.

Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K, Terashima Y. A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. Biochemical Biophysical Research Communications. 2002. 295:255-260.

Sugino T, Nakamura K, Ieki H, Shinde S, Isobe N, Hasegawa Y, Kojima M, Kangawa K,

Obitsu T, Taniguchi K. Plasma levels of ghrelin and glucagon-like peptide-1 in periparturient Holstein dairy cows. 2006. 7th AAAP Animal Science Congress. 517 (proceeding)

Sugino T, Hasegawa Y, Kurose Y, Kojima M, Kangawa K, Terashima Y. 2004. Effects of ghrelin on food intake and neuroendocrine function in sheep. Animal Reproduction Science. 2004. 82-83:183-194.

Sugino T, Kawakita Y, Fukumori R, Hasegawa Y, Kojima M, Kangawa K, Obitsu T, Taniguchi K. Effects of glucose and amino acids on ghrelin secretion in sheep. Animal Science Journal. 2010. 81:199-204.

Takahashi H, Kurose Y, Kobayashi S, Sugino T, Kojima M, Kangawa K, Hasegawa Y, Terashima Y. Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep. Journal of Endocrinology. 2006. 189:67-75.

Takahashi H, Kurose Y, Sakaida M, Suzuki Y, Kobayashi S, Sugino T, Kojima M, Kangawa K, Hasegawa Y, Terashima Y. Ghrelin differentially modulates glucose-induced insulin secretion according to feeding status in sheep. Journal of Endocrinology. 2007. 194:621-625.

Takahashi H, Kurose Y, Suzuki Y, Kojima M, Yamaguchi T, Yoshida Y, Ogino M, Hodate K, Azuma Y, Sugino T, Kojima M, Kangawa Y, Kobayashi S. Ghrelin differentially modulates the GH secretory response to GHRH between the fed and fasted states in sheep. Domestic Animal Endocrinology. 2009. 37:55-60.

Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. Journal of Clinical Endocrinology and Metabolism. 2000. 85:4908-4911.

Turner N, Hariharan K, TidAng J, Frangioudakis G, Beale SM, Wright LE, Zeng XY, Leslie SJ, Li JY, Kraegen EW, Cooney GJ, Ye JM. Enhancement of muscle mitochondrial oxdative capacity and alterations in insulin action are lipid species dependent: potent tissue-specific effects of medium-chain fatty acids. Diabetes. 2009. 58:2547-2554.

Turton MD, Shea D, Gunn I, Beak SA, Edwards CMB, Wilding KJPH, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature. 1995. 379:69-72.

Vallejo-Cremades MT, Gomez-Garcia L, Chacatas-Cortesao M, Moreno C, Sanchez M, De Miguel E, Gomez De Segura IA. Enriched protein diet-modified ghrelin expression and secretion in rats. Regulatory Peptides. 2004. 121:113-119.

Vallejo-Crenades MT, Gomez de Segura IA, Gomez-Garcia L, Perez-Vicente J, De Miguel E. A high-protein dietary treatment to intestinally hypotrophic rats induces ghrelin mRNA content and serum peptide level changes. Clinical Nutrition. 2005. 24:904-912.

Veldhuis JD, Reynolds GA, Iranmanesh A, Bowers CY. Twenty-four hour continuous ghrelin infusion augments physiologically pulsatile, nycthemeral, and entropic (feedback-regulated) modes of growth hormone secretion. The Journal of Clinical Endocrinology and Metabolism. 2008. 93:3597-3603.

Wein S, Wolffram S, Schrezenmeir J, Gasperiková D, Klimes I, Seböková. IE. Medium-chain fatty acids ameliorate insulin resistance caused by high-fat diets in rats. Diabetes/Metabolism Research and Reviews. 2009. 25:185-194.

Weltz-Lutz AE, Knight TJ, Pritchard RH, Daniel JA, Clapper JA, Smart AJ, Trenkle A, Beitz DC. Circulating ghrelin concentrations fluctuate relative to nutritional status

and influence feeding behavior in cattle. Journal of Animal Science. 2006. 84:3285-3300.

Yagasaki M, Machida M, Funabiki R. Effects of dietary methionine and glycine on serum lipoprotein profiles and fecal sterol excretion in normal and hepatoma-bearing rats. Journal of Nutritional Scienence and Vitaminology. 1989. 36:45-54.

Yokogoshi H, Oda H. Dietary taurine enhances cholesterol degradation and reduce serum and liver cholesterol concentrations in rats fed a high-cholesterol diet. Amino Acids. 2002. 23:433-439.

Abbreviations

AAN: alpha-amino nitrogen

AUC: area under the curve

BW: body weight

CP: crude protein

d: day

DIM: days in milk

DM: dry matter

DMI: dry matter intake

EE: ether extract

Eu: Europium

FA: fatty acids

FCM: fat correct milk

g: gravity

GH: growth hormone

GHRH: growth hormone releasing hormone

GHS: growth hormone secretagogue

GHS-R: gwowth hormone secretagogue receptor

GLP-1: glucagon-like peptide-1 (7-36) amide

GPR-120: G-protein coupled receptor-120

h: hour

IGF-1: insulin-like growth factor-1

KIU: kilo inhibitor unit

LCFA: long-chain fatty acids

LCFA-Ca: calcium salts of long-chain fatty acids

MCFA: medium-chain fatty acids

MCFA-Ca: calcium salts of medium-chain fatty acids

ME: metabolizable energy

MEI: metabolizable energy intake

Met: methionine

min: minute

N: nitrogen

NDF: neutral detergent fiber

NEFA: non-esterified fatty acids

OM: organic matter

rbGH: recombinant bovine GH

RPM: rumen protected methionine

SNF: solid non fat

T-CHO: total-cholesterol

TG: triglyceride

TR-FIA: time-resolved fluoroimmunoassay

TS: total solid

UN: urea nitrogen