Studies on the Effects of Dietary Urea and Negative Energy Balance on Metabolisms of Glucose and Urea in Sheep

A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

Paramintra VINITCHAIKUL

Under the supervision of

Professor Dr. Hiroaki SANO

The United Graduate School of Agricultural Sciences

Science of Bioproduction

Animal Nutrition and Physiology

Iwate University

Contents

Chapter-4

Dedication

It is my gratefulness and warmest regard that I dedicated this dissertation to my parents.

A special feeling of gratitude to my wife

who has always loves and encourages through my time in Japan.

I also would like dedicate this dissertation to all my teachers

who have instruct and inspire through my life.

Acknowledgements

I would like to express the deepest appreciation from my heartiest to my supervisor, Professor Dr. Sano Hiroaki, who had the affectionate supervision, inspiring attitude, and exceeding genius. I am sincerely indebted for his kindly that offer my chance to study in Japan. Without his precious guidance and compassionate facilitation this dissertation would not have been possible.

I owe my deepest gratitude to my co-supervisors, Professor Dr. Kita Kazumi, Department of Animal Science, Faculty of Agriculture, Iwate University and Professor Dr. Hidaka Satoshi, Department of Life Science and Agriculture, Research Faculty, Obihiro University of Agriculture and Veterinary Medicine for their useful suggestion and kind facilitation in the completion of work.

I would like to grateful acknowledge to Chiang Mai University, Thailand for financial support to my study at Iwate University, Japan. Finally, I share the credit of my work with our members of Laboratory of Ruminant Nutrition and Physiology for their warmest friendship, grateful collaboration and kind help during my study.

Abbreviations

AA	Amino acid
ADG	Average daily gain
ALB	Albumin
BHBA	β-hydroxybutyric acid
BUN	Blood urea nitrogen
BW	Body weight
GLB	Globulin
HE	High energy
HUHE	High urea high energy
HULE	High urea low energy
HUME	High urea middle energy
LE	Low energy
LUHE	Low urea high energy
LULE	Low urea low energy
LUME	Low urea middle energy
ME	Middle energy
MNS	Microbial nitrogen supply
N	Nitrogen
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NS	Not significant difference
NUHE	Non-urea high energy
NULE	Non-urea low energy

PD	Purine derivatives
TP	Total protein
TG	Triglyceride
UHE	Urea high energy
ULE	Urea low energy
VFA	Volatile fatty acid

Chapter-1

General introduction

Present problem base situation of this study

Almost dairy farms in Thailand are currently small holder dairy farms with average herd size less than 30 heads per farm (Information and communication technology center, Department of Livestock Development, 2014). Most common important problems in dairy farm were mastitis, reproduction and lameness, respectively (Simasatitkul et al., 1999). However, these problems were only front problems but the core of the problem was deeply hid in the background. The nutritional management would be a core of the problem, especially roughage quality. Markvichitr et al. (1995) were revealed that the low quality roughage was found to be the main cause of inferior production and reproduction problems in Thailand.

In Thailand, the roughage availability is depended on the harvest season and irregular quality. Otherwise, farmers usually use agriculture by-product from food industrial factory as a roughage sources. Therefore, almost roughage diets are uncontrollable and low quality such as low protein and energy contents, lack of fiber quality and dry matter contents. Inadequate feed supplies and high production costs are among the most serious problem facing on small holder farms in Thailand. Low availability of roughage leads to the wide use of commercial concentrates which account for 60% of the on-farm variable costs of production (Kehren and Tisdell, 1996).

The farmers need to rise up the essential nutrient requirement of their animals by used commercial concentrate diet. The recent study in Thailand, commercial concentrate diets in market were usually used urea as protein source in the ration (Promma and Suriyasathaporn, 2005). In practical, the farmers often suddenly increase a concentrate diet quantity during peak of milk production after calving period. Unfortunately, the blood urea nitrogen (BUN) is quantities with protein intake. The dairy cows will be inescapable suffer from high BUN situation. Moreover, concomitance during peak of milk production is same period as negative energy balance situation which always happen at 2-3 weeks after parturition. Both of high BUN and negative energy balance are largely affected on reproduction and production cycle in dairy industry.

Effects of urea in ruminants

Urea is a nonprotein nitrogen with simple nitrogenous compound. It can be used for plant fertilizer or protein substitution in a ruminant diet. Ruminal enzymes called ureases readily hydrolyze or split the urea compound molecule into ammonia (NH_3) and carbon dioxide (CO_2) in the rumen (Johnson et al., 1979). When there is an excess of ammonia relative to energy in the rumen, ruminal ammonia concentration will be increased. Excess of unused ruminal ammonia is enters into the blood circulation pass through the rumen wall and is transported to the liver where it is converted into urea nitrogen (Hammond, 1997). This urea becomes part of the BUN pool in the body. The BUN pool has three ultimate fates: recycling, secretion in milk, or excretion in urine. Recycling of urea via saliva and across the rumen wall, can provide nitrogen for rumen

microbial protein synthesis. Abundant urea is filtered from the blood by the kidney and excreted in the urine. (Swensen and Reece, 1993).

BUN is an indicator of diet adequacy and nitrogen utilization efficiency (Kohn, 2007). Balanced diets for lactating dairy cows were associated with average BUN concentration of 5.35 mmol/L (Roseler et al., 1993). However, many studies showed negative effects of high BUN level in last decade. Especially, negative effects on the fertility problems both confinement and grazing herds (Butler, 1998, Laven and Drew, 1999, Westwood et al., 1998, Wittwer et al., 1999). Pregnancy rates were decreased by about 20% when BUN was higher than 6.78 mmol/L (Butler et al., 1996). High dietary protein (nitrogen) intake resulting in BUN higher than 7.14 mmol/L. This high BUN level was associated with uterine environment and decreased fertility (reduced conception rate, decreased pregnancy rate) in both of lactating dairy cows and heifers (Butler et al., 1996, Elrod and Butler, 1993, Elrod et al., 1993, Ferguson et al., 1993).

Several mechanisms of high urea nitrogen on reproductive performance were elucidated. High urea nitrogen was toxic to bovine gametes and/or embryos (Visek, 1984, Ferguson et al., 1988, Staples et al., 1993), directly affected on oocytes (Gath et al., 1999) and alters uterine pH (Elrod and Butler, 1993, Elrod et al., 1993, Rhoads et al., 2004).

Most recently in large field study found that even lower urea nitrogen levels at 5.5 mmol/L, pregnancy rate was reduced (Rajala-Schultz et al., 2001). Cows exposed with urea nitrogen level >5.7 mmol/L at 30 day before first service during summer were high risk of not pregnancy (Melendez et al., 2000). Therefore, Ferguson (2007) who recommended a mean of urea nitrogen from 3.2 to 5 mmol/L was sufficient for adequate milk production and was ensured for no negative effects on reproduction.

Effects of negative energy balance in ruminants

The transition period is the period between late gestation and early lactation, also known as the periparturient period, the three weeks pre- and post-parturition in the dairy cow (Goff and Horst, 1997). This time is characterized by extreme physiological, metabolic, and nutritional changes in the body. The dairy cows are characterized by a physical transition from gestation to parturition and lactation. They are challenged to adjusting to these dynamic physiological, metabolic, and nutritional shifts (Bauman and Currie, 1980). Cows which have difficulty in making a successful transition are subject to experiencing negative energy balance (NEB).

NEB in a ewe is differs from a dairy cow. NEB in pregnant ewe with twin or triplet is an inadequate nutrition situation during the late stages of gestation. The primary predisposing cause is inadequate nutrition during late gestation, usually due to insufficient energy density of the ration and decreased rumen size as a result of fetal growth. Especially, in the last 4 weeks of gestation, metabolizable energy requirements rise dramatically due to rapid fetal growth rate (Cynthia, 2010).

Consequent of NEB and reduced dry matter intake are serious. Most of the metabolic diseases occur during the first few weeks of lactation before peak milk yield (Goff and Horst, 1997; Ingvartsen, 2006), because NEB reaches its in maximum (Butler and Smith, 1989), The cows will risk to exposed the metabolic disorders such as fatty liver and ketosis (Doherty, 2002; Bertics et al., 1992), displaced abomasum (Duffield et al. 2009; LeBlanc et al., 2005), retained placenta (Cameron et al., 1998), lameness (Lean, 2011) and immunosuppression (Goff, 2008). The combined effects of all these challenges are reduced fertility and milk production resulting in diminishing profits (Esposito et al., 2014).

Metabolic diseases can interrelated with one disease increases risk for other diseases. The energy-associated diseases include ketosis, displaced abomasum, fatty liver, retained placenta, metritis and possibly mastitis (William, 2005). The root cause of these conditions is an NEB situation, where the animal responds inappropriately to the NEB during peak of the production. NEB of early lactation will be emphasized by milk production rises in dairy cow and of late gestation will be enhanced by twin or more fetuses in ewe as well.

Responding of negative energy balance in ruminants

Negative energy balance will be occurred when the metabolic energy demand (for tissue maintenances, daily activity and production) exceed than energy intake from their diet. This cause body reserves mobilized to compensate for the energy deficit between dry matter intake and the energy demand (Bauman and Currie, 1980). Body energy reserves are mainly adipose tissues which is a source of lipolytic nutrients. The animal will adapt to satisfy their energy demand by mobilize triglyceride in adipose tissues into non-esterified fatty acid (NEFA) (McNamara, 1991) which results in elevated blood NEFA concentration. This mobilization increase in gluconeogenesis from glycerol precursor and provides the necessary energy from free fatty acid to support the energy demand.

These NEFAs are used to produce energy by body tissues. However, available data suggest that the liver takes up NEFAs in proportion to their supply (Emery et al., 1992). Unfortunately, the liver typically does not have sufficient capacity to completely dispose of NEFAs through export into the blood or catabolism for energy. Thus, the animal predisposed to accumulate triglycerides in the liver tissue by re-esterifies process. This situation would be impaired a general health of an animal and induced other metabolic diseases.

Metabolic interrelation between urea and glucose kinetics

Mostly during early postpartum period in dairy cow or late gestation period in ewe is a peak of highest energy demand in biological production cycle. The important key of dietary management during this period is carbohydrate and protein managements due to for maximized production level. Glucose net availability is mainly dependent on gluconeogenesis and the main glucogenic nutrients are propionic acid, glucogenic amino acids, and lactate (Danfaer, 1994). The liver is a major role for maintenance of blood glucose homeostasis by controlling the balance between gluconeogenic and glycogenolytic pathways under an increasing of glucose demands.

When animal receive high protein in their diet, the animal would need more carbohydrate supply as an energy source in diet ration. Because of ruminal microbial organisms would be necessary use energy for converse rumen ammonia into microbial protein. Unfortunately, the animal normally reduces dietary intake or inappetite during NEB, opposite direction with high energy demand. Excess protein or poorly balanced protein relative to carbohydrate supply might be happened.

High blood ammonia concentration might be occurred especially when urea is used instead of rumen degradable protein. Because the rate of ammonia release from urea is faster (Cherdthong and Wanapat 2010). Sharp rise in rumen ammonia concentration resulted in elevated concentrations of ammonia and urea in blood (Bartley et al. 1981). High ammonia concentration reduces the capacity of liver to synthesize glucose. Overton et al. (1999) showed ammonia decreased the ability of hepatocytes to synthesize glucose from propionate. Furthermore, ammonia loading by either infusion into the mesenteric vein or supplemental urea to feed (Wilton et al. 1988; Maltby et al. 1991) caused a decrease in the net hepatic production of glucose.

Concurrently, NEB condition is associated with depressed capacity of hepatic gluconeogenesis due to lower hepatic enzymes activities (Rukkwamsuk et al., 1999). Moreover, the animal with triglyceride accumulation in the liver during NEB appears to impair liver function. Zhu et al. (2000) who suggested accumulation of triglycerides in the liver of cows as occurs during early lactation may result in higher ammonia concentrations, because ureagenesis is inhibit. Overton (2001) mentioned on the inhibition of glucose synthesis may occur in vivo when triglycerides accumulate in liver during NEB. He concluded again including with decreased capacity for gluconeogenesis and ureagenesis (Overton, 2003).

Because, glucose absorbed from the gastrointestinal tract of ruminants is very small, most of the glucose is supplied from gluconeogenesis (Widiawati et al., 2014). Moreover, almost all of urea in ration is rapid hydrolyzed to ammonia in the rumen (Cherdthong and Wanapat, 2010) and urea by itself very slow transport across the rumen wall for direct absorption (MooNey and O'Donovan, 1970). Therefore, the glucose and urea absorption are limited in ruminants.

The rates of glucose and urea turnover could be used to assess gluconeogenesis and ureagenesis in the current study. At steady state condition with continuous infusion of tracers, the isotope tracers were reached to their plateau of the specific radioactivity or enrichment. The net rate of production equals the net rate of utilization with limited absorption rate, in this condition the turnover rate reflects as the endogenous production rate.

Hypothesis of the study

Use of urea during NEB may be argued as its effects on the hepatic gluconeogenesis and ureagenesis under practical feeding condition. However, to the author's knowledge there are no reports regarding for the use of dietary urea during NEB on hepatic gluconeogenesis and ureagenesis. This prompted to determine the effects of urea usage on glucose and urea kinetics during NEB by deficiency of energy supply.

Therefore, it was hypothesized that the urea usage under NEB condition in practical feeding would be more depress rate of gluconeogenesis and ureagenesis. Interaction between urea and NEB on gluconeogenesis and ureagenesis was within concurrent study.

Objectives of the study

To prove the hypothesis that urea usage under NEB condition in practical feeding would be more depress rate of gluconeogenesis and ureagenesis. The study was divided into two experiments.

The objective of each study was to determine

1. Effects of isonitrogenous replacing soybean meal by urea and NEB, high energy diet (669 kJ/ ($kg^{0.75}$·day)) following with low energy (222 kJ/ ($kg^{0.75}$·day)), on plasma glucose and urea kinetics in sheep

2. Effects of urea level (0.5 and 1.1 g/ ($kg^{0.75}$·day)) and negative energy level (167, 335 and 502 kJ/ ($kg^{0.75}$·day)) on microbial protein supply, plasma glucose and urea kinetics in sheep

Moreover, interaction of urea x energy were within concurrent examination in both experiments. In addition, nitrogen balance study, ruminal fermentation characteristics and plasma free amino acids concentration were also determined in both studies.

Using sheep for experimentation

In this study, the author intends to study by *In vivo* experiment. Sheep was represented as an experimental animal model of the ruminants. The primary reason cause by the current facilities of the laboratory organizations have been provided for sheep research. The infra-structure required for work on a large ruminant such as dairy cow, holding facilities and appropriate equipments are inconvenient.

However, sheep have been studied extensively in their own right, with much knowledge accruing in regard to ruminant nutrition and animal production (Adams and McKinley, 2009). Therefore, sheep have been used as suitable experimental subjects in such diverse fields of study including nutrition and gastrointestinal physiology as well.

There are several reasons for using sheep to make excellent experimental subjects in physiological studies. The size of the sheep represents one of its key strengths as an experimental model. The size of the sheep enables ease introduction of catheter or cannulation into various blood vessels and the rumen. The purposes for obtain samples of blood and rumen content for chemical analysis. Besides that there are major occupational healths and safety issues recommended relate to laboratory animal care and handing in sheep. Moreover, sheep are friendly and unharmed with human.

Chapter-2

Effects of replacing soybean meal with urea and negative energy balance on ruminal fermentation characteristics, plasma glucose and urea kinetics in sheep

Introduction

Urea could be substituted for rumen degradable protein in ruminant diet because ammonia from urea can be utilized by rumen microbes to form microbial protein. When urea is used instead of rumen degradable protein, the rate of ammonia release from urea is faster (Cherdthong and Wanapat 2010). Sharp rise in rumen ammonia concentration resulted in elevated concentrations of ammonia and urea in blood (Bartley et al. 1981).

Following an introduction previously, we already knew the negative effect of high ammonia concentration on hepatic gluconeogenesis (Aiello and Armentano 1987; Wilton et al. 1988; Demigne´ et al. 1991; Maltby et al. 1991 and Overton et al. 1999). Moreover, we also knew negative effect of NEB on hepatic gluconeogenesis (Rukkwamsuk et al. 1999) and ureagenesis (Strang et al. 1998; Zhu et al. 2000; Overton 2003).

The objective of this study was to determine the isonitrogenous replacement of soybean meal by urea during NEB condition would be more depress rate of gluconeogenesis and ureagenesis, which can be illustrated by decrease both of glucose and urea turnover rate. Moreover, nitrogen balance, ruminal fermentation characteristics, plasma free amino acid concentrations and interaction of urea x energy were within concurrent examination.

Materials and Methods

Animals, experimental design and diets

The experiment was carried without any noticeable stress to the animals under approval of the Laboratory Animal Care and Use Committee of Iwate University (approval no: A201255). All sheep had been keeping inside the free stall farm in Iwate University before the study was started. Four rams and two wethers healthy crossbred (Corriedale x Suffolk) with average age of 3.5 – 4 years and initial body weight (BW) of 50.7 $\pm$ 2.5 kg were used. The trial was carried out in crossover design with two isonitrogenous diets of either soybean meal or urea replaced diet. Each experimental period lasted for 24 days. The sheep were randomly assigned to each sequence of trial.

The experimental design is shown in Diagram 1 and the diet composition is stated in Table 1. The basal diet consisted of mixed hay (orchardgrass and reed canarygrass), cornstarch and soybean meal. The trial was preceded by 669 kJ/ ($kg^{0.75}$·day) of high energy diet and crude protein 5 g/ ($kg^{0.75}$·day) contained either soybean meal or replaced with 0.8 g/ ($kg^{0.75}$·day) urea (Wako, Japan) with calculated in isonitrogenous on day1-14 of adaptation period. Further, continued 5 days with same high energy diet, then NEB was induced by suddenly dropped to 222 kJ/ ($kg^{0.75}$·day) of low energy diet for 5 days later. The nitrogen source was still unchanged and crude protein level was balanced with the basal diets in each sequence of trial. Cornstarch, soybean meal and urea were weighed according with the ration treatments, then homogenous mixed and mashed together with mixed hay prior feeding time. Diets were offered in equal proportion twice a day at 08.30 and 20.30 hours and had free access to drinking water during the whole experimental period.

The animals were housed in individual pens during the adaptation period. The sheep were moved to individual metabolic cages in a controlled environmental room with 23 ± 1°C, 70% relative humidity and 08.00 – 22.00 hours of lighting during last 10 days in each treatment period.

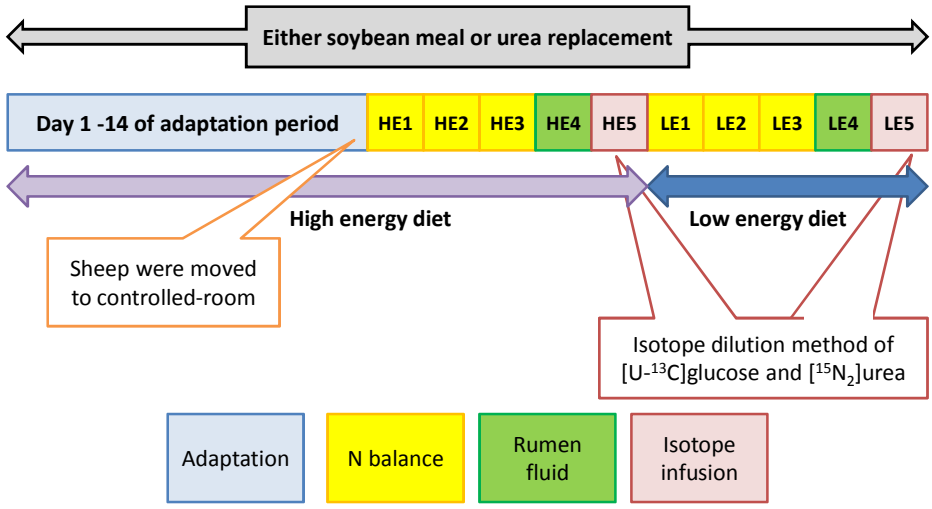

Diagram 1 Flow chart showed the experimental design in this study. Collecting samples day divided into high energy (HE1 – HE5) and low energy (LE1 – LE5) periods. Dietary treatments composed of Non-urea High Energy (NUHE), Non-Urea Low Energy (NULE), Urea High Energy (UHE) and Urea Low Energy (ULE), respectively.

Table 1 Dietary treatment formulation and chemical composition of diets

g (dry matter)/ ($kg^{0.75}$·day)	Non-urea		Urea	
	High energy	Low energy	High energy	Low energy
Dietary treatment formulation				
Mixed hay	21.5	7.2	21.8	7.6
Cornstarch	31.1	2.8	36.3	7.8
Soybean meal	5.2	8.9	0.1	3.9
Urea	0.0	0.0	0.8	0.8
Chemical composition				
Dry matter intake	57.8	18.9	59.0	20.1
Crude protein	5.3	5.0	5.3	5.1
Metabolizable energy[A] (kJ/ ($kg^{0.75}$·day))	671	221	674	223

[A] estimated from AFRC (1993)

Samples collection

The sheep were weighed weekly and feed were adjusted accordingly. On day 1-3 of each high and low energy diet, urine and feces were collected for 3 days from each sheep for nitrogen (N) balance study. The urine bucket contained 50 mL of 6 N H_2SO_4. The 24 hours urine samples were collected and a subsample of 50 mL was stored at -30°C until analysis. Feed and each 24 hours of feces samples were collected, dried at 60°C for 48 hours, ground and stored in a plastic box at room temperature until analysis.

Daily serum sample was collected at last 2 day of high energy diet and day 1-4 of low energy diet. Before the morning meal, blood was sampled from jugular vein puncture into serum separating tube (Vacutainer® SST™, BD, USA). Serum was separated by centrifuging at 3,500*g* for 15 min at 4°C (H-3R, Kokusan, Japan) and subsequently analyzed the serum triglyceride (TG), non-esterifies fatty acid (NEFA) and β-hydroxybutyric acid (BHBA) for NEB monitoring on the same day.

On day 4 of each high and low energy diet, rumen liquid samples (approximately 50 mL) were taken at pre-feeding (0 h) and at 1.5, 3 and 6 hours post-feeding. Rumen fluid pH was immediately measured (F-51, Horiba Ltd., Japan) and the rumen liquid samples were centrifuged at 8,000*g* for 10 min at 4°C (RS-18IV, Tomy, Japan). The mixture of 1 mL of the supernatant from the rumen liquid samples and 1 mL of 0.1 mol/L HCl was used for ruminal ammonia analysis. A 5 mL of supernatant of rumen liquid samples were used for ruminal volatile fatty acid (VFA) analysis. All of the rumen samples were stored at -30°C.

Isotope infusion procedure and collection

On day 5 of each high and low energy diet, the primed continuous infusion of [U-^{13}C]glucose and [$^{15}N_2$]urea was applied to determine plasma glucose and urea kineticss simultaneously. Polyvinyl catheters were inserted in both of left and right jugular veins at pre-feeding. A mixed saline solution containing 2.9 μmol/kg$^{0.75}$ of [U-^{13}C]glucose (D-glucose-$^{13}C_6$, 99 atom% excess; Cambridge Isotope Laboratories, USA) and 10.2 μmol/kg$^{0.75}$ of [$^{15}N_2$]urea ($^{15}N_2$urea, 98 atom% excess; Cambridge Isotope Laboratories, USA) was injected into the right jugular catheter as a priming dose at 12.00 hour. Same mixed saline solution was continuously

infused for 4 hours through the same catheter by using a multichannel peristaltic pump (AC-2120, Atto, Japan) at rate of 2.9 and 10.2 µmol/ ($kg^{0.75}$·h).

For stable infusion attainment, the infusion rate of tracer solution was monitored every 30 min throughout the infusion period. A 10 mL of blood sample was taken from the left jugular catheter into heparinized tubes at before priming dose infusion (0 hour) and 6 mL at 2.5, 3, 3.5 and 4 hours during infusion. The blood samples were centrifuged at 10,000*g* for 10 min at 4°C and plasma was collected and stored at -30°C until further analysis.

Chemical analysis

Nitrogen contents of diets, feces and urine were determined by the Kjeldahl method (AOAC 1990, Tecator 2520 and Kjeltec 2300, FOSS, Sweden). A factor of 6.25 was used to convert N into crude protein. N absorption, N retention and N digestibility were calculated by (N intake – fecal N), (N intake – (fecal N + urinal N)) and (N absorption/ N intake) x 100, respectively. Rumen ammonia concentration was determined with a colorimetric method (Weatherburn 1967) by using a spectrophotometer (V-630, JASCO, Japan).

The VFA concentration was measured by using gas chromatography (HP-5890, Hewlett Packard, USA) after stream distillation as described by Sano et al. (2009). Briefly, rumen fluid sample was centrifuged (0ºC, 8000 rpm, 10 min) and 5 mL of supernatant was taken in VFA glass tube, added with 1 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 1 mL of 20% H_2SO_4. Then, the tube was set with steam distillation set. The liquid of steam distillation was collected into a glass beaker and titrated with 0.1 N NaOH. The titrated distillate volume was reduced first with open flame and then dried at 60ºC in the oven. Finally, molar percentage

of individual VFA was determined using the gas chromatography acidifying with 1 mL of 5 N H_3PO_4 solution.

Serum TG, NEFA and BHBA were analyzed by using automatic chemistry analyzer (Toshiba TBA 40FR Accute, Diamond Diagnostics, USA) based on enzymatic method (determiner-L TG II), ACS-ACOD method (NEFA-HAII) and cyclic enzymatic method (autokit 3-HB), respectively. The plasma glucose concentration was determined by the glucose oxidase method (Huggett and Nixon 1957).

The plasma free amino acids and ammonia concentrations at pre-infusion period were determined by using an automated amino acid analyzer (JLC-500/V, JEOL, Japan). Briefly, 1 mL of blood plasma was mixed with 1 mL of 3% sulfosalicylic acid (SSA) and kept into refrigerator for overnight. Then, it was centrifuged (4ºC, 3,000 rpm, 15 min) twice and supernatant was transferred into new another tube. Finally, supernatant was filter through cellulose acetate 0.45 μm hydrophilic filter (Toyo Roshi Kaisha Ltd., Japan) and transferred to vials for determining plasma free amino acids and ammonia concentration.

The plasma [$^{15}N_2$]urea enrichment was analyzed according to Wood et al. (2006) by using gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan). Briefly, 0.5 mL of plasma sample was added with 0.5 mL of 4% SSA and 50 μL of internal standard. Then, the mixer was well mixed and kept into refrigerator for 30 min. The plasma samples were centrifuged at 0ºC, 12,000 rpm for 10 min twice. The supernatant was passed through cation exchange resin (Dowex 50 X8 (H^+ form, 200-400 mesh, 0.5 mL) and washed with 0.5 mL distilled water. The distilled water after passed cation exchange resin was kept for plasma glucose enrichment determination. Again, washed the sample with 0.5 mL distilled water and discarded. The 1 mL of 8 N ammonia was added into the sample and washed twice with 1 mL

distilled water. Then, 0.02 mL of supernatant transferred into glass screw capped tube and placed in desiccation chamber until drying. After drying, 25 µL of MTBSTFA+1% TBDMS-Cl and 25 µL of acetonitrile was added, mixed, capped and incubated at 80ºC for 60 min. Finally, the sample was taken into chromatographic vials for analysis of urea enrichment. The derivatives were analyzed with ionized monitoring as m/z 231 and m/z 233 for plasma [$^{15}N_2$]urea. The plasma isotope enrichments were obtained by comparing their peak areas and peak area abundances with standard curve.

The plasma urea concentrations were calculated from the ratio of unlabeled urea and [^{13}C,$^{15}N_2$]urea as internal standard (^{13}C, 99%,·$^{15}N_2$, 98% atom excess; Cambridge Isotope Laboratories, USA) with m/z 231 and m/z 234.

The plasma [U-^{13}C]glucose enrichment was determined according to the method of Tserng and Kalhan (1983) with modified by Fujita et al. (2006) by using gas chromatography mass spectrometry. Briefly, the distilled water after passed cation exchange resin (Dowex 50 X8 (H^+ form, 200-400 mesh, 0.5 mL) from previous plasma urea enrichment analysis was again passed through anion exchange resin (Dowex 1 X8 (CH_3COO^- form, 200-400 mesh, 1 mL). Then, 0.1 mL of supernatant transferred into glass screw capped tube and placed in desiccation chamber until drying. After drying, 0.5 mL of HCl hydroxylaminephyridine was added, capped and incubated at 90ºC for 30 min. The sample was added with 0.5 mL of acetic anhydride, again capped and incubated at 90ºC for 1 hour. Then, the sample kept into chilled ice for 30 min, mixed with 2 mL of distilled water and 1 mL of chloroform, shacked vigorously for 1 min and removed upper layer. Thereafter, it was mixed with 2 mL of distilled water, shacked vigorously for 1 min and upper part removed and centrifuged at 0ºC, 3,000 rpm for 5 min again and removed upper part. Finally, lower organic portion was transferred into small glass tube,

dried at 30ºC by N_2 gas and kept into desiccation chamber. After drying, mixed with 100 μL of chloroform and taken into chromatographic vials for analysis of glucose enrichment. The ions determined are m/z 314 and m/z 319. The plasma isotope enrichments were obtained by comparing their peak areas and peak area abundances with standard curve.

Calculations and statistical analysis

The glucose and urea turnover rates were calculated according to the equation described by Wolfe (1984), as follows:

$$\text{Turnover rate (mmol/ (kg}^{0.75}\cdot\text{h))} = I \text{ x } (1 / \text{E-1})$$

Where *I* represents the infusion rate of [U-^{13}C]glucose or [$^{15}N_2$]urea. E represents the isotope enrichment of the plasma [U-^{13}C]glucose or [$^{15}N_2$]urea at the steady state condition, respectively.

The mean values and pooled s.e.m. were generated for all data. All data were analyzed by using MIXED procedure in SAS (1996). The model composed of the fixed effects as periods (first and second), urea (soybean meal and urea), energy (high and low energy) and the interaction of urea x energy. Sheep were randomized as random effect. Ruminal fermentation characteristics were subjected to repeated measurement analysis. Tukey-Kramer adjustment was used to identify diets with different effects on the variable involved. The level of significance was set at $P<0.05$ throughout the analysis.

Results

Negative energy balance induction

Effects of replacing soybean meal with urea and NEB on average daily gain (ADG) and serum metabolites are presented in Table 2. No significant difference (P>0.05) of ADG with urea replacement was observed. However, ADG was lower (P<0.01) in low energy when compared to high energy as illustrated in Fig 1. Interaction effect of urea x energy was not found (P>0.05). Serum TG and BHBA concentrations had no difference (P>0.05) either in urea replacement or low energy. Serum NEFA concentration was not affected (P>0.05) by urea replacement but higher (P<0.01) during low energy. Interaction effect of urea x energy was not found (P>0.05).

Time course change on serum TG, NEFA and BHBA concentrations, from before negative energy induction until finished five day of induction, are illustrated in Fig 2. Serum NEFA concentration did not differ (P>0.05) between urea replacement and non-urea diet. However, serum NEFA concentration was higher in day by day from first day of negative energy induction until end of induction. Interaction effect of urea x time was not found (P>0.05). Serum TG and BHBA concentrations did not differ (P>0.05) between urea replacement and non-urea diet. Serum TG and BHBA concentrations were stable and no significant difference (P>0.05) in time and interaction of urea x time.

Table 2 Effects of replacing soybean meal with urea and negative energy balance on average daily gain and serum metabolite concentrations in sheep

	Non-urea		**Urea**		**Pooled**	***P* value**		
	High energy	Low energy	High energy	Low energy	**s.e.m.**	Urea	Energy	Urea x Energy
ADG (kg/day)	-0.05	-0.40	0.05	-0.60	0.06	0.63	<0.01	0.13
Serum metabolites (mmol/L)								
TG	0.07	0.09	0.07	0.08	<0.01	0.42	0.35	0.49
NEFA	0.14	0.46	0.08	0.52	0.05	0.93	<0.01	0.30
BHBA	0.27	0.30	0.44	0.40	0.03	0.06	0.90	0.57

ADG - Average daily gain, TG - Triglyceride, NEFA - Non-esterified fatty acid, BHBA - β-hydroxybutyric acid

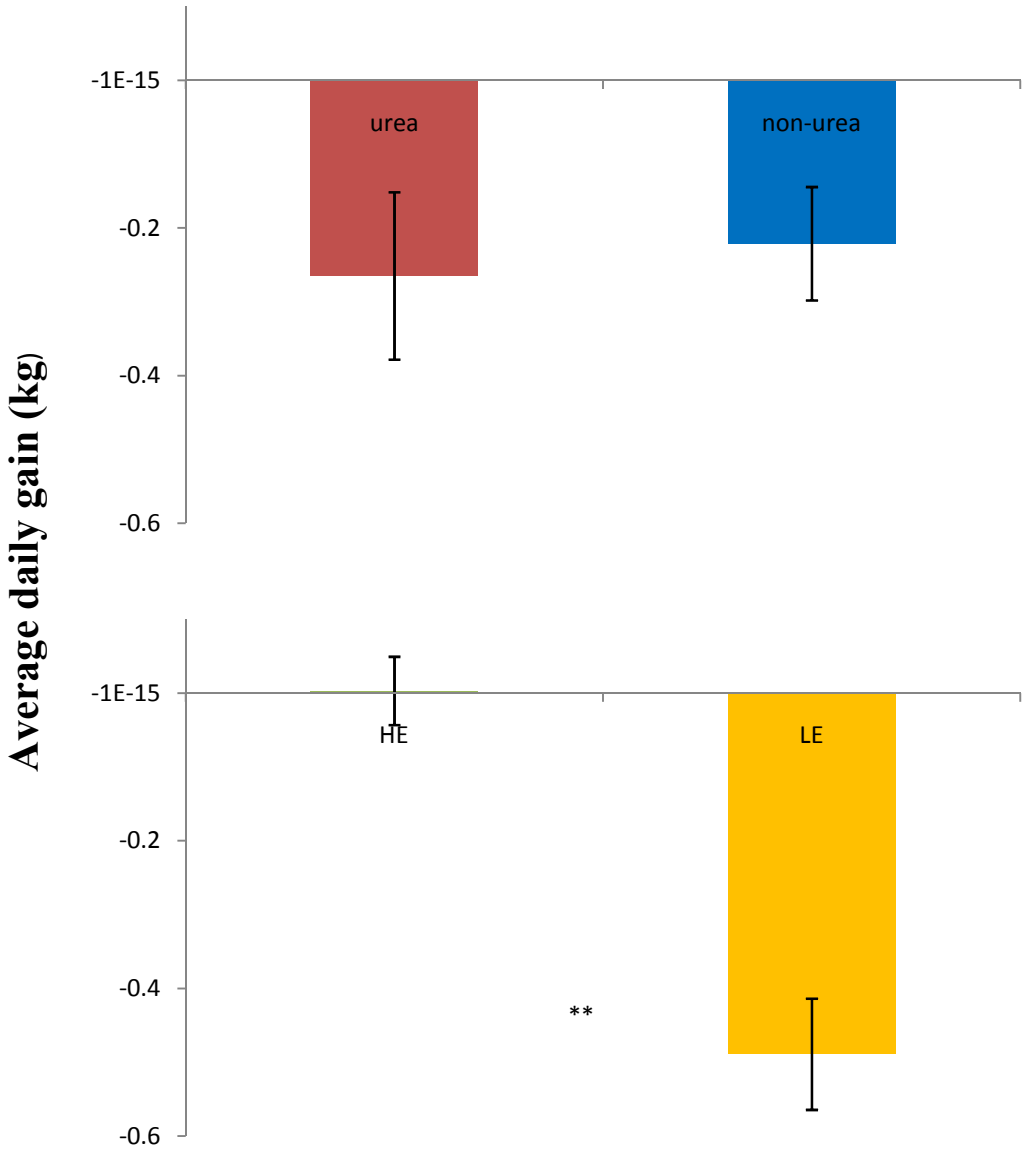

Fig 1 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on average daily gain (kg) in sheep. ** differ significantly ($P<0.01$)

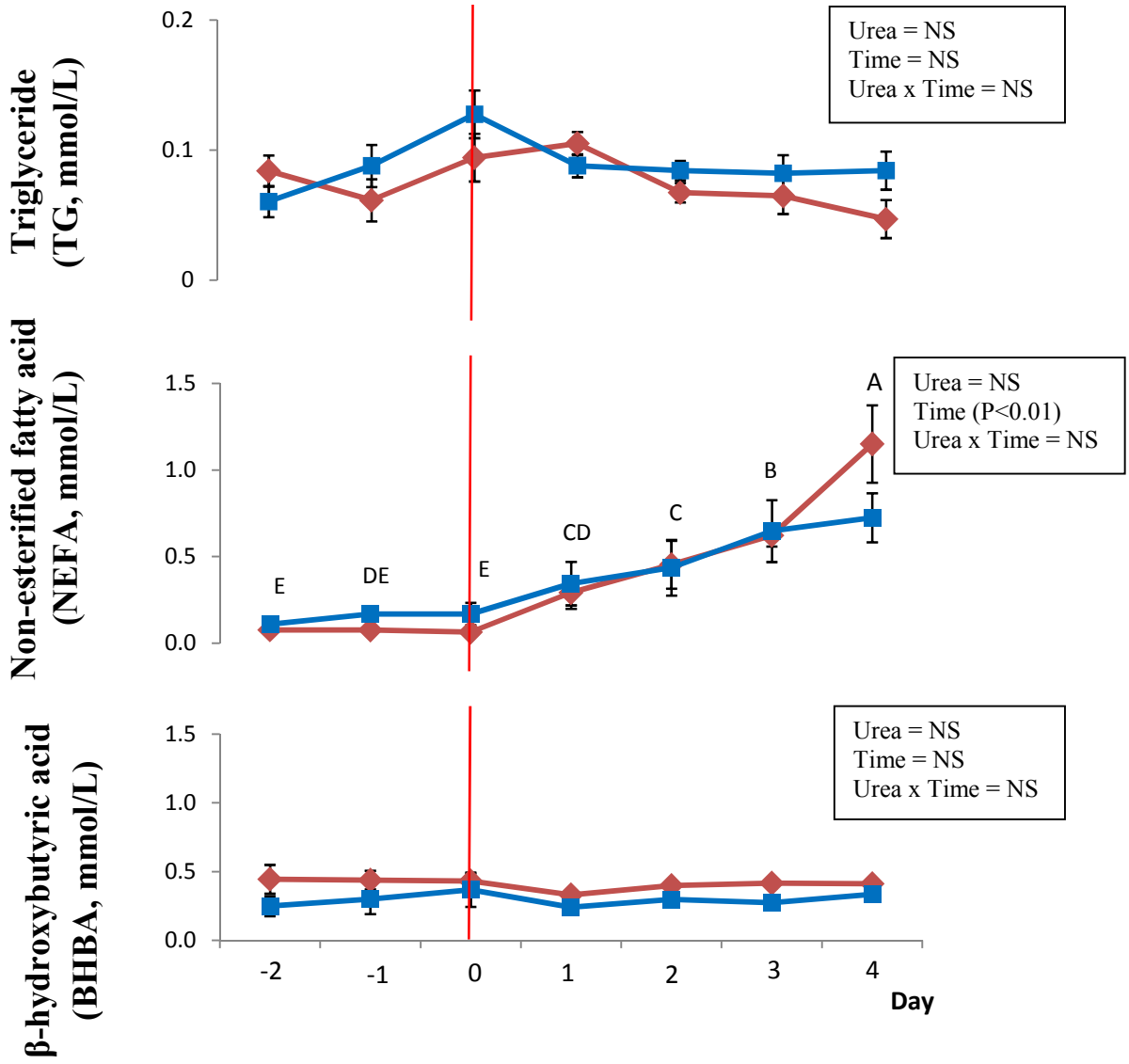

Fig 2 Effects of replacing soybean meal (NON-UREA) with urea (UREA) during negative energy induction on serum triglyceride (TG), non-esterified fatty acid (NEFA) and β-hydroxybutyric acid in sheep. NS = not significant difference (P>0.05);
A, B, C, D and E times differ significantly (P<0.05); —— negative energy induction

Nitrogen balance

Effects of replacing soybean meal with urea and NEB on N balances are presented in Table 3. N intake was influenced ($P<0.01$) by urea replacement as well as energy. Fecal N, N absorption and digestibility were also affected ($P<0.05$) by urea replacement and energy. Urea replacement did not affect ($P>0.05$) on urinary N excretion but was higher ($P<0.01$) in low energy. Consequently, the lower ($P<0.01$) in N retention during low energy was accompanied by an increase in urinary N excretion. However, urea replacement did not affect ($P>0.05$) on N retention. N digestibility in low energy diet was higher ($P<0.01$) than high energy diet. Urea and energy interaction effect was not seen in any of the N balance parameters.

Effects of replacing soybean meal with urea and NEB on N balances are illustrated in Fig 3 - 4. N balances study did not differ ($P>0.05$) between urea replacement and non-urea diet. However, N intake, fecal N and N retention were higher ($P<0.01$) in high energy diet while urinal N and N absorption were higher ($P<0.01$) in low energy diet. N digestibility did not differ ($P>0.05$) between urea replacement and non-urea diet but higher in low energy than high energy diet.

Table 3 Effects of replacing soybean meal with urea and negative energy balance on nitrogen (N) balance in sheep

N balance	**Non-urea**		**Urea**		**Pooled**	***P* value**		
g/ ($kg^{0.75}$·day)	High energy	Low energy	High energy	Low energy	**s.e.m.**	Urea	Energy	Urea *x* Energy
N intake	0.84	0.80	0.85	0.80	<0.01	<0.01	<0.01	0.36
Fecal N	0.46	0.20	0.36	0.14	0.02	0.02	<0.01	0.46
Urinary N	0.23	0.64	0.30	0.65	0.03	0.23	<0.01	0.29
N absorption	0.39	0.60	0.50	0.66	0.02	0.01	<0.01	0.43
N retention	0.16	-0.04	0.20	0.02	0.03	0.31	<0.01	0.88
N digestibility (%)	46	75	58	82	2.50	0.02	<0.01	0.45

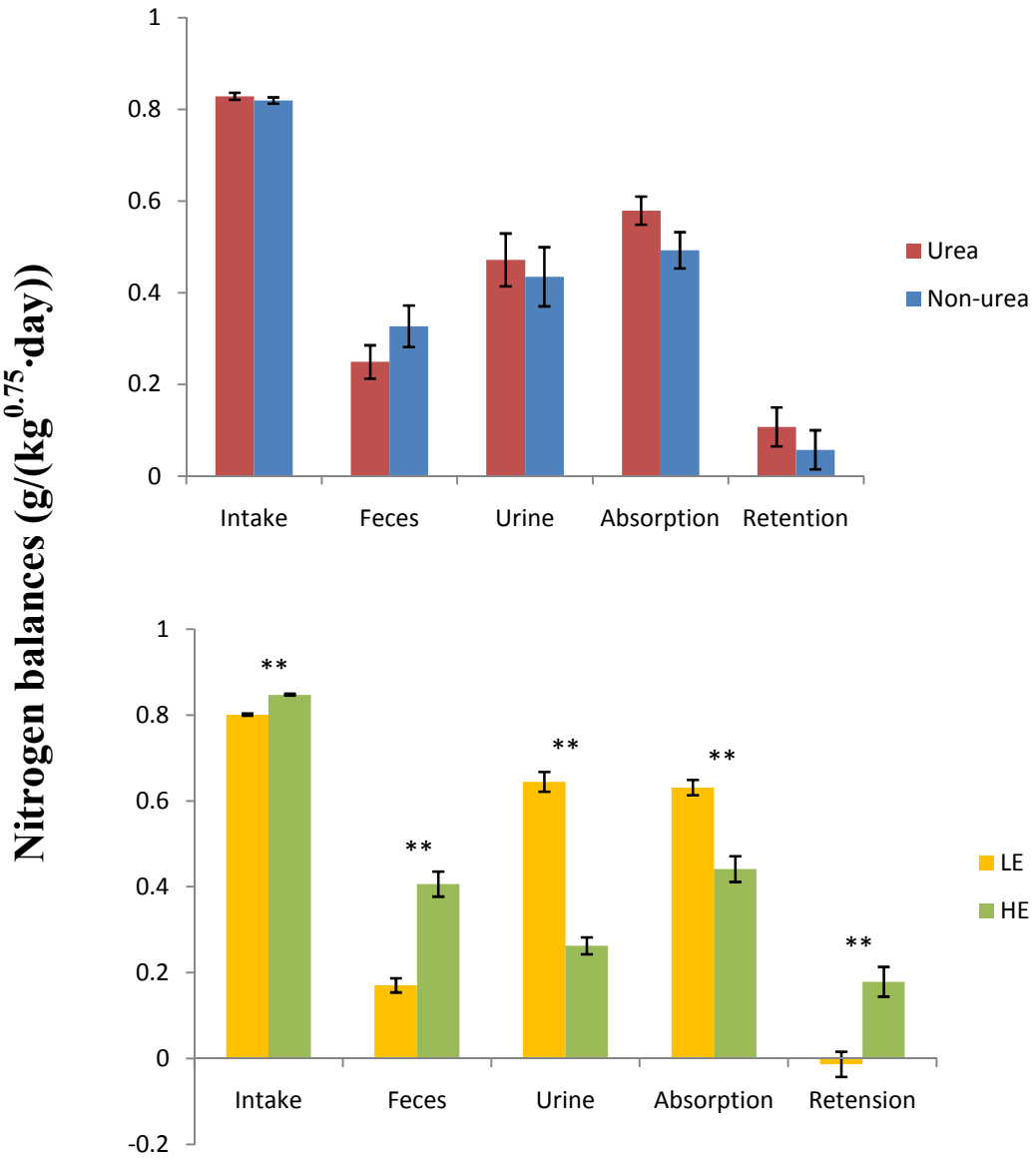

Fig 3 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on nitrogen balances (g/(kg$^{0.75}$·day)) in sheep. ** differ significantly (P<0.01)

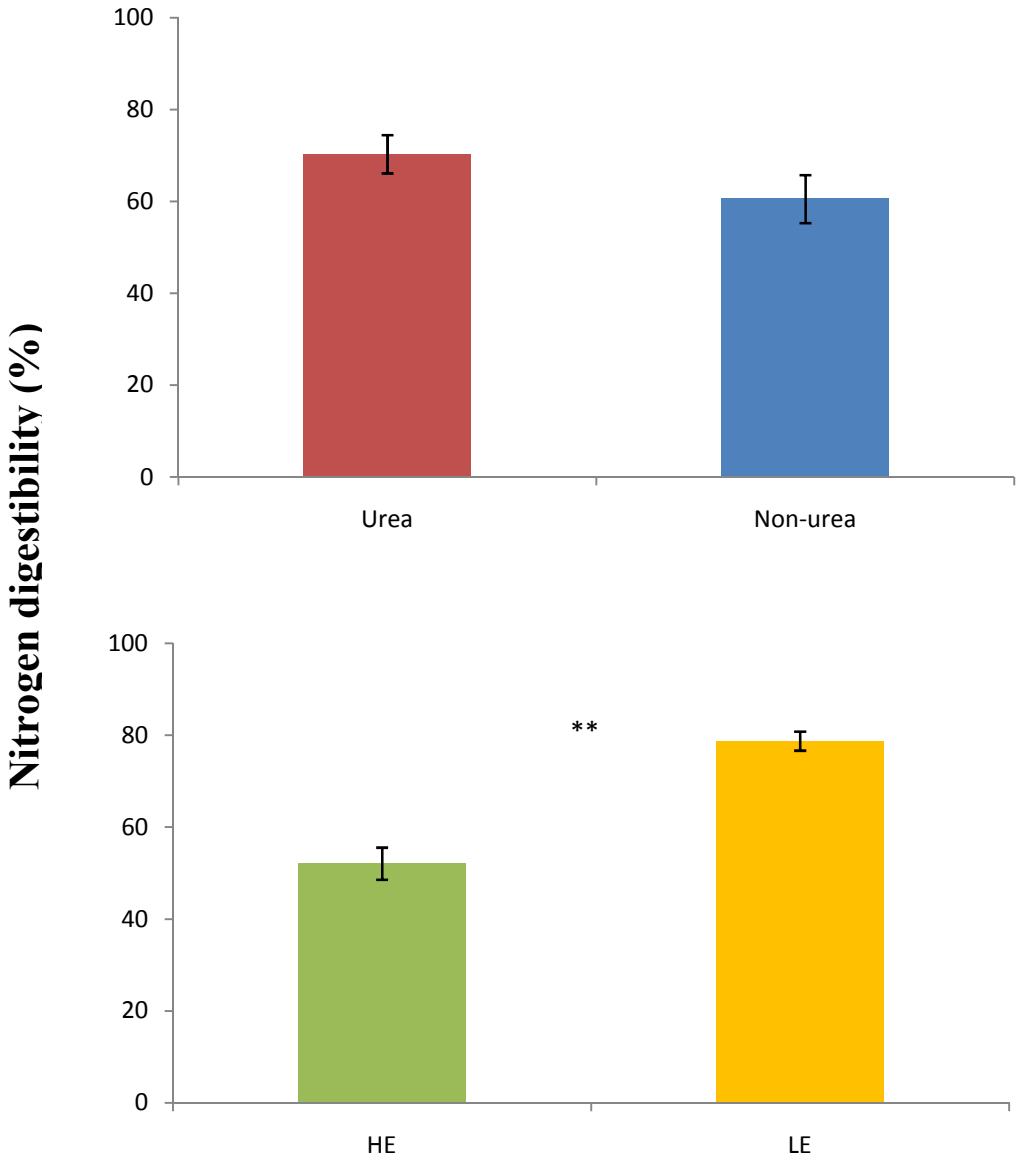

Fig 4 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on nitrogen digestibility (%) in sheep. ** differ significantly ($P<0.01$)

Ruminal fermentation characteristics

Average (0-6 hours) of rumen fermentation characteristics are presented in Table 4. Rumen pH and ammonia concentration were higher with urea replacement (P=0.04) and low energy (P<0.01). Total VFA, acetate, propionate and butyrate concentrations were not affected (P>0.05) by urea replacement but were higher (P<0.01) in high energy. Interaction effect of urea x energy was found in acetate, butyrate concentrations (P<0.05) and acetate: propionate ratio (P<0.01). Acetate concentration was highest (P<0.05) in high energy while butyrate concentration was lowest (P<0.05) in non-urea low energy diet.

Time course change on rumen pH, ammonia concentration, total rumen VFA concentration, individual VFAs concentration and acetate: propionate ratio at pre-feeding until 6 hours post-feeding are illustrated in Fig 5 - 8. Rumen pH did not differ (P=0.05) between urea replacement and non-urea diet. Interaction of urea x time was not found (P>0.05). Rumen pH was tended to decrease concentration after feeding until 3 hours post-feeding and was stabled until 6 hours post-feeding. Rumen pH in low energy was higher (P<0.01) than high energy with interaction of energy x time (P<0.01). Rumen pH in low energy seemed to be stable while high energy decreased concentration after feeding until 3 hours post-feeding and was stabled until 6 hours post-feeding.

Rumen ammonia concentration in urea replacement diet was higher (P<0.01) than non-urea diet with interaction of urea x time (P<0.01). Rumen ammonia concentration in urea replacement diet was peaked at 1.5 hour post-feeding while non-urea diet decreased concentration after feeding until 3 hours post-feeding and was stabled until 6 hours post-feeding. Rumen ammonia concentration in low energy diet was higher (P<0.01) than high

energy diet without interaction of urea x time (P>0.05) and was peaked at 1.5 hour post-feeding.

Total rumen VFA concentration in urea replacement diet was higher (P<0.01) than non-urea diet without interaction of urea x time (P>0.05). Total rumen VFA concentration was peaked at 3 hours post-feeding and was stabled until 6 hours post-feeding. Total rumen VFA concentration in high energy diet was higher (P<0.01) than low energy diet with interaction of energy x time (P<0.01). Total rumen VFA concentration in high energy diet was peaked at 3 hours post-feeding and was stabled until 6 hours post-feeding while low energy diet was stabled along the time. Individual rumen VFAs concentration was similar trend with total rumen VFA concentration.

Table 4 Effects of replacing soybean meal with urea and negative energy balance on average (0-6 hours) of ruminal fermentation characteristics in sheep

Rumen fermentations (mmol/L)	**Non-urea**		**Urea**		**Pooled s.e.m.**	***P* value**		
	High energy	Low energy	High energy	Low energy		Urea	Energy	Urea x Energy
pH	6.35	6.99	6.72	7.01	0.1	0.04	<0.01	0.06
Ammonia	2.54	8.66	3.76	9.88	0.7	0.04	<0.01	0.99
Total VFA	64.4	35.4	60.2	38.2	3.1	0.59	<0.01	0.14
Acetate	40.7[a]	21.2[b]	36.1[a]	24.6[b]	1.9	0.72	<0.01	0.03
Propionate	11.9	6.7	14.6	5.3	1.0	0.68	<0.01	0.17
Butyrate	8.6[a]	4.4[c]	8.2[a]	6.5[b]	0.4	0.12	<0.01	0.02
Isobutyrate	1.0	1.2	0.3	0.7	0.1	0.01	0.15	0.83
Valerate	0.6	0.6	0.5	0.4	<0.1	0.08	0.33	0.18
Isovalerate	0.9	1.3	0.4	0.8	0.1	<0.01	0.01	0.71
A/P ratio	4.0[a]	3.6[ab]	2.6[b]	4.7[a]	0.2	0.73	0.04	<0.01

a, b, and c – means with different letters in a row differ significantly (P<0.05)
A/P - Acetate: Propionate ratio

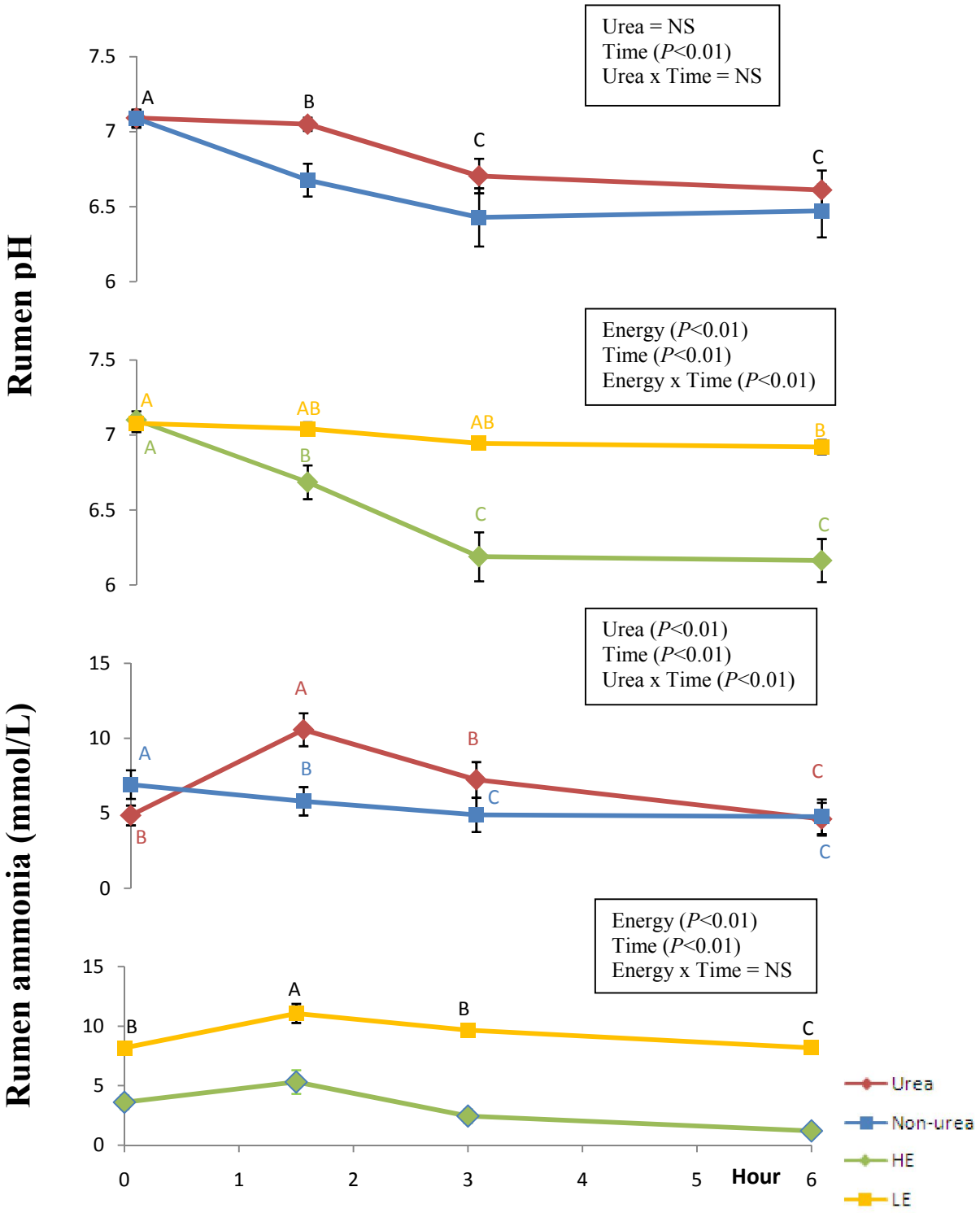

Fig 5 Effects of replacing soybean meal with urea (urea and non-urea) and negative energy balance, low energy (LE) and high energy (HE), on rumen pH and ammonia concentration (mmol/L) in sheep. NS = not significant difference ($P>0.05$); A, B and C times differ significantly ($P<0.05$)

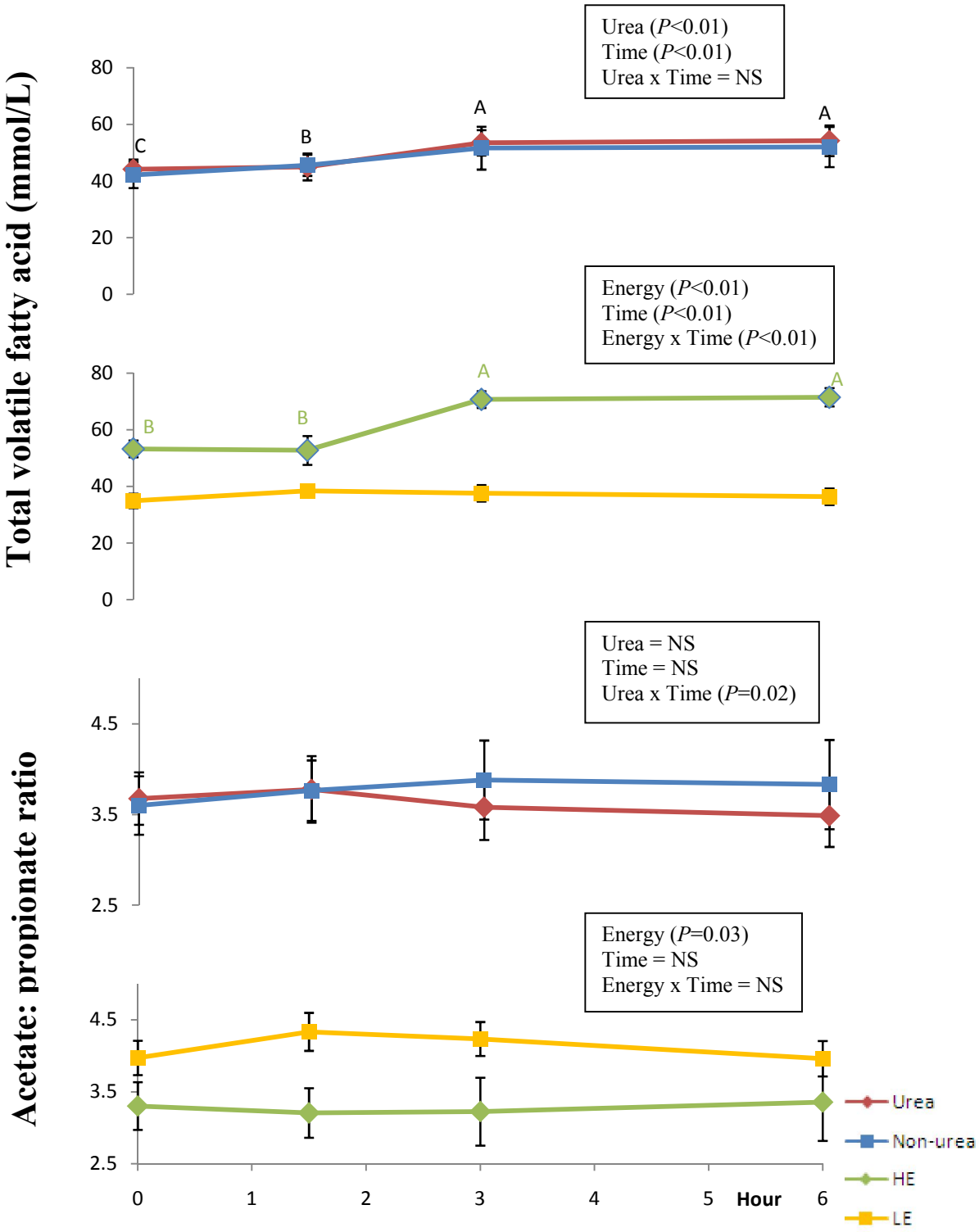

Fig 6 Effects of replacing soybean meal with urea (urea and non-urea) and negative energy balance, low energy (LE) and high energy (HE), on rumen total volatile fatty acid concentration (mmol/L) and acetate: propionate ratio in sheep. NS = not significant difference ($P>0.05$); A, B and C times differ significantly ($P<0.05$)

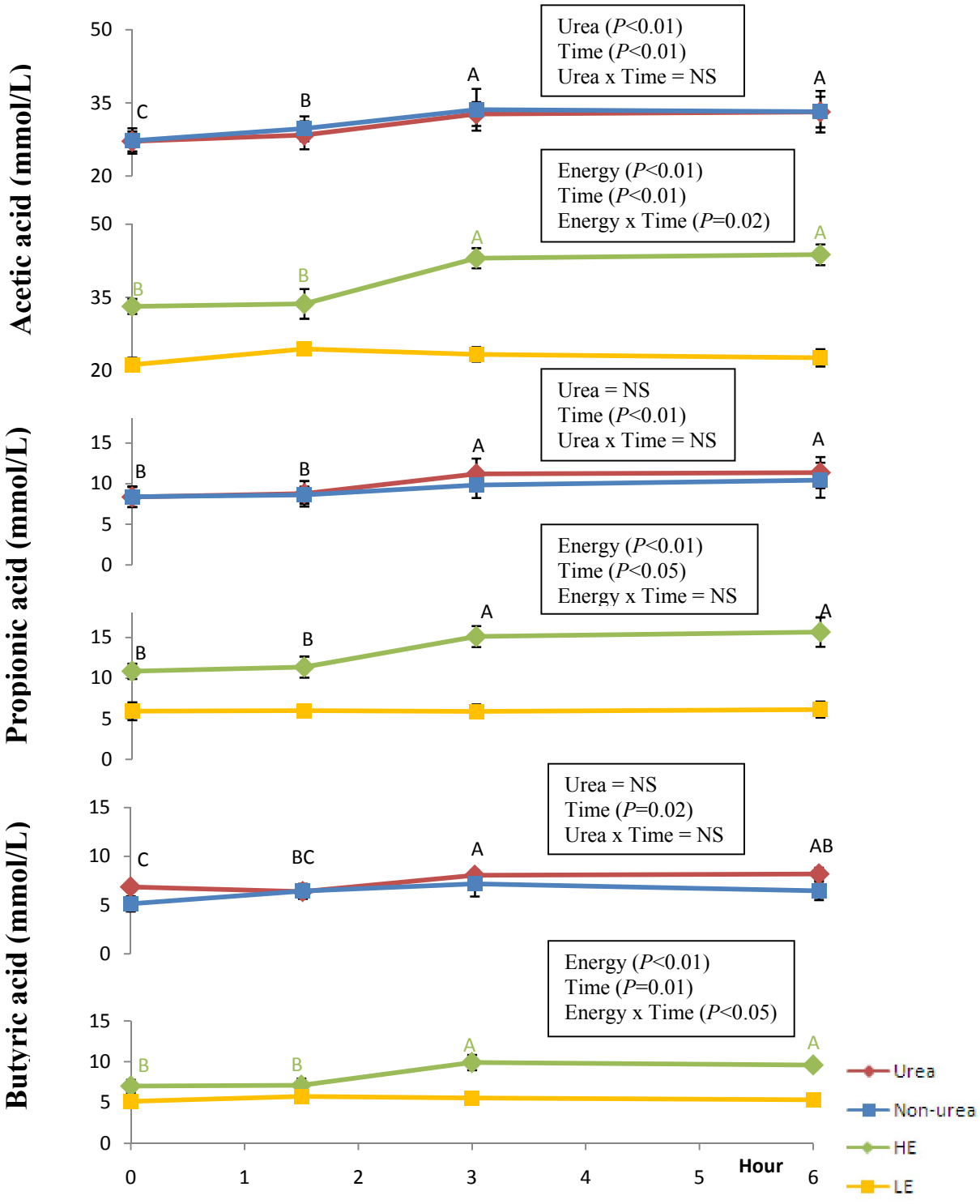

Fig 7 Effects of replacing soybean meal with urea (urea and non-urea) and negative energy balance, low energy (LE) and high energy (HE), on rumen acetic, propionic and butyric acid concentration (mmol/L) in sheep. NS = not significant difference ($P>0.05$); A, B and C times differ significantly ($P<0.05$)

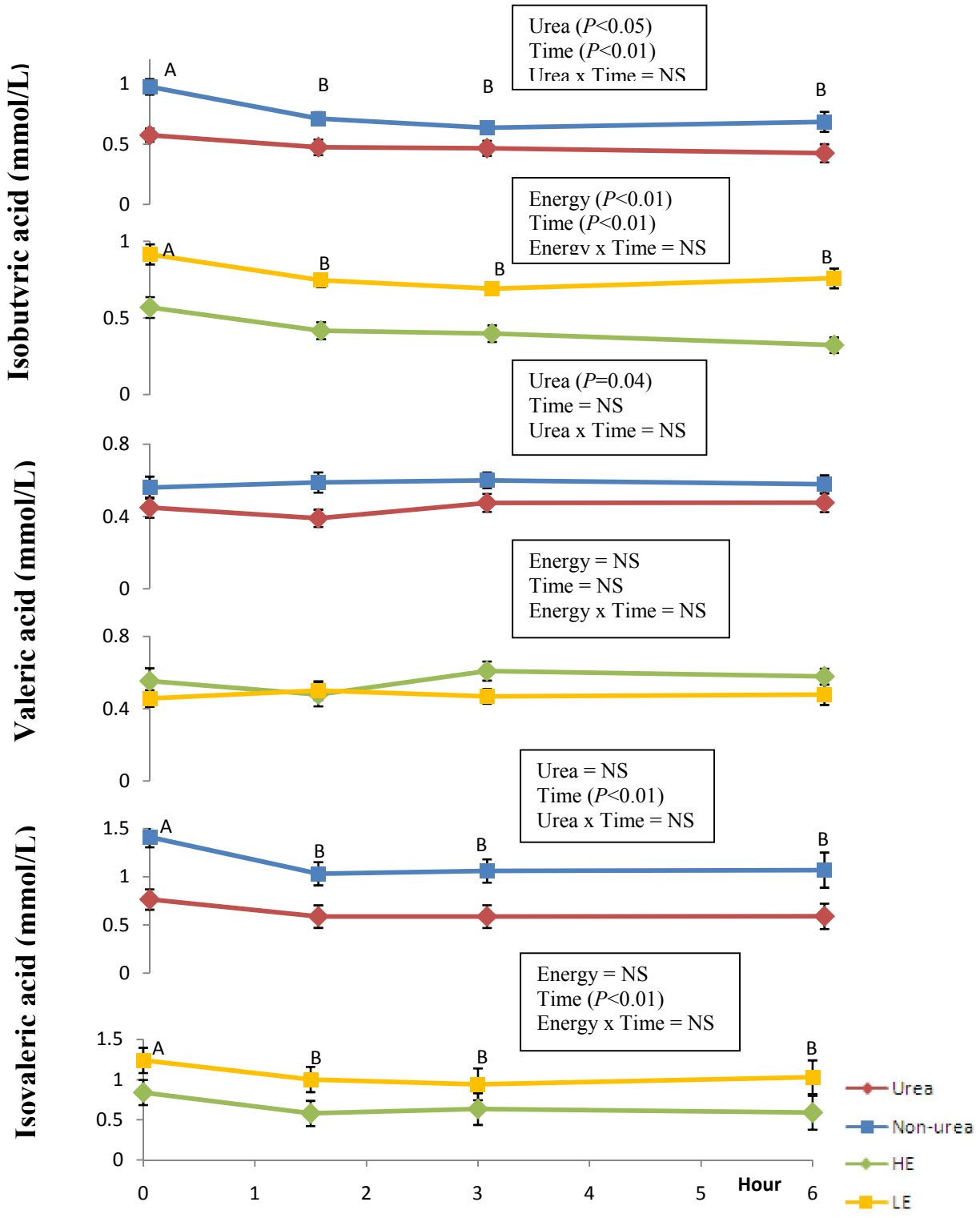

Fig 8 Effects of replacing soybean meal with urea (urea and non-urea) and negative energy balance, low energy (LE) and high energy (HE), on rumen isobutyric, valeric and isovaleric acid concentration (mmol/L) in sheep. NS = not significant difference ($P>0.05$); A and B times differ significantly ($P<0.05$)

Plasma free amino acids, glucose, ammonia and urea concentration

Plasma free amino acids, glucose, ammonia and urea concentrations at pre-isotope infusion are presented in Table 5 and Fig 9 - 10. Plasma free amino acid concentrations were not influenced (P>0.05) by urea replacement, except for glutamine. Plasma threonine, valine, methionine, isoleucine, leucine, phenylalanine, serine, asparagines, glutamic acid, glutamine, glycine, alanine, tyrosine and proline concentrations were higher (P<0.05) in high energy. Interaction effects of urea x energy were not found (P>0.05).

Plasma glucose and ammonia concentrations did not differ (P>0.05) at pre-infusion levels in all dietary conditions. Plasma urea concentration was not affected (P>0.05) by urea replacement while it was higher (P<0.01) in low energy. Urea and energy interactions were not seen (P>0.05).

Table 5 Effects of replacing soybean meal with urea and negative energy balance on plasma free amino acids, glucose, ammonia and urea concentrations at pre-isotope infusion in sheep

Plasma concentrations	**Non-urea**		**Urea**		**Pooled s.e.m.**	***P* value**		
	High energy	Low energy	High energy	Low energy		Urea	Energy	Urea x Energy
Amino acids (μmol/L)								
Threonine	161	79	197	76	13	0.32	<0.01	0.24
Valine	195	135	208	111	11	0.73	<0.01	0.24
Methionine	17	12	19	11	1	0.87	<0.01	0.53
Isoleucine	80	62	83	58	3	0.89	<0.01	0.54
Leucine	108	84	102	71	5	0.27	<0.01	0.68
Phenylalanine	46	28	48	28	3	0.86	<0.01	0.75
Histidine	42	40	48	42	2	0.18	0.20	0.51
Lysine	107	88	127	113	6	0.06	0.15	0.79
Aspartic acid	5.3	N/D	6.9	3.7	1	N/D	N/D	N/D
Serine	140	79	225	105	17	0.05	<0.01	0.30
Asparagine	84	48	92	48	6	0.78	<0.01	0.79
Glutamic acid	75	55	73	47	6	0.51	0.02	0.14
Glutamine	248	171	335	259	20	<0.01	0.04	0.76
Glycine	772	416	1157	508	76	0.26	<0.01	0.66
Alanine	236	141	251	169	24	0.15	<0.01	0.42
Tyrosine	60	40	80	37	8	0.13	<0.01	0.09
Arginine	123	124	123	124	5	0.41	0.77	0.40
Proline	95	53	112	54	6	0.28	<0.01	0.36
Glucose (mmol/L)	4.5	4.6	4.3	4.7	0.2	0.92	0.57	0.77
Ammonia (mmol/L)	0.14	0.14	0.12	0.14	<0.01	0.96	0.75	0.80
Urea (mmol/L)	3.4	5.7	3.7	5.5	0.3	0.66	<0.01	0.25

N/D - not detected

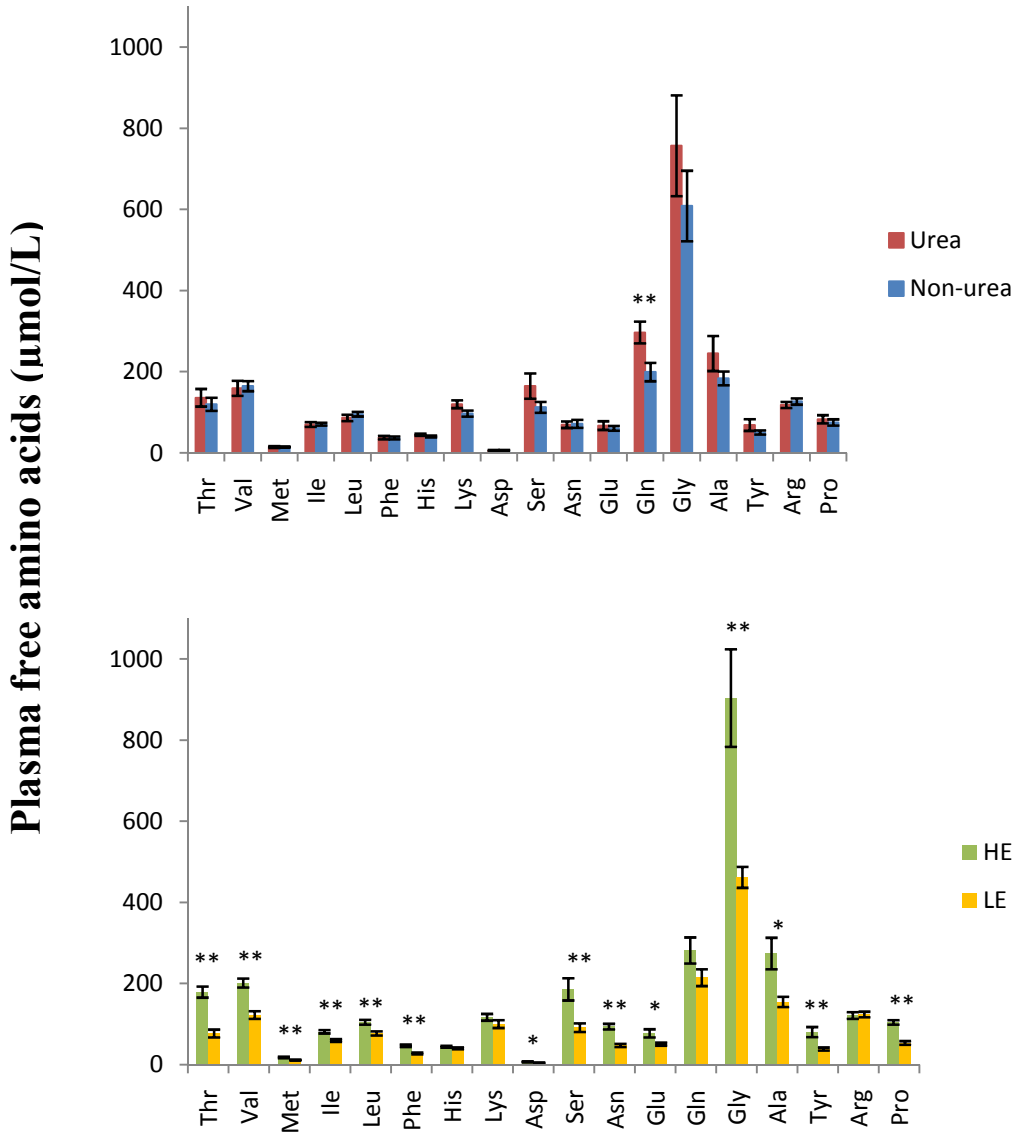

Fig 9 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on plasma amino acids concentration (μmol/L) at pre-isotope infusion in sheep. * differ significantly (P<0.05) ** differ significantly (P<0.01)

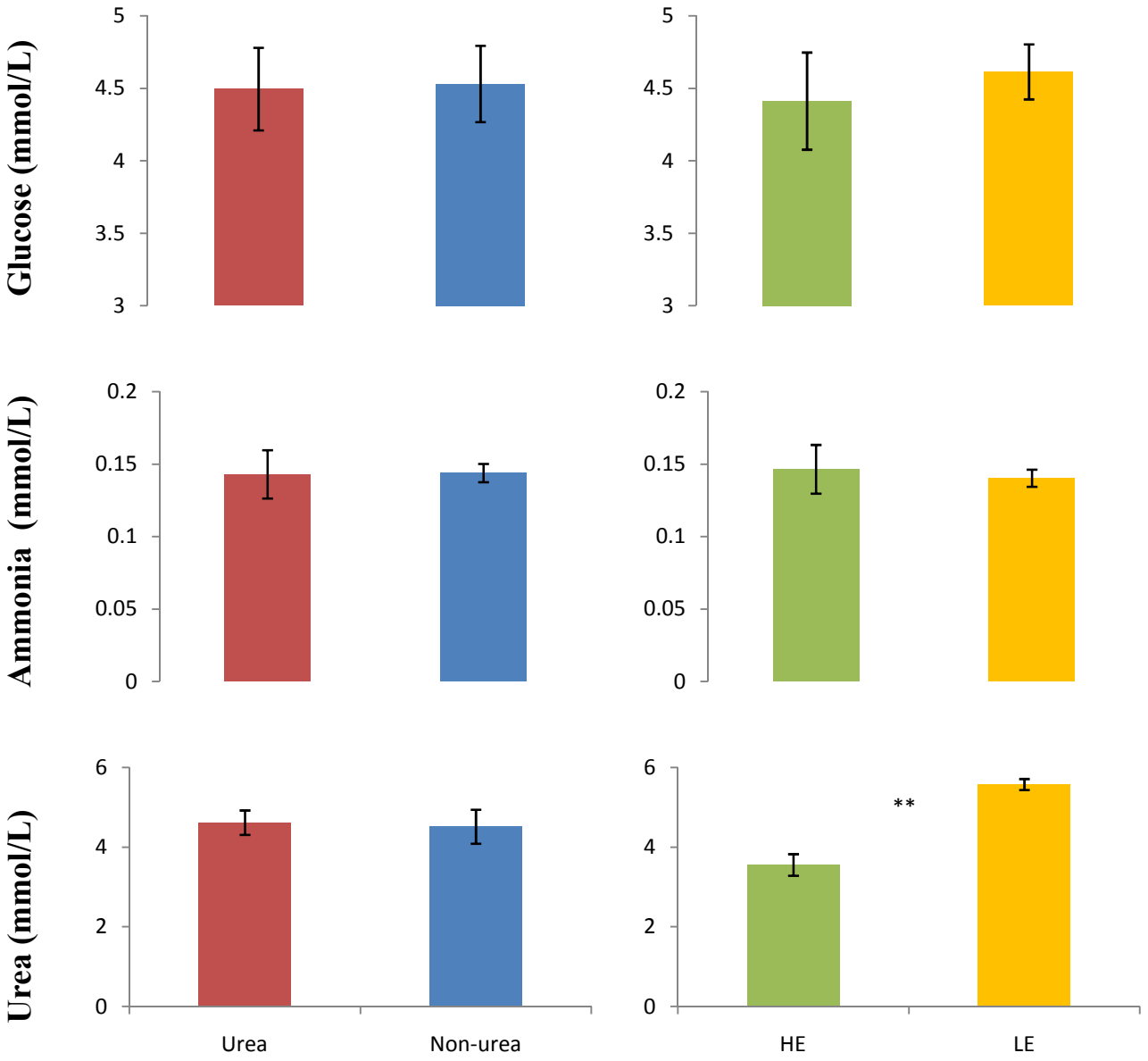

Fig 10 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on plasma glucose, ammonia, urea concentration (mmol/L) at pre-isotope infusion in sheep. ** differ significantly (P<0.01)

Plasma glucose and urea kinetics

Plasma [U-^{13}C]glucose enrichment and glucose concentration almost reached in a steady state during 2.5 - 4 hours of isotope infusion in all dietary treatments as illustrated in Fig 11. Plasma glucose turnover rate is presented in Fig 13. Plasma glucose kinetics during isotope infusion is presented in Table 6. During primed continuous infusion period, plasma glucose concentration did not change (P>0.05) and interaction effect of urea x energy was not found (P>0.05) among dietary treatments. Urea replacement did not influence (P>0.05) on plasma glucose turnover rate. However, lower (P<0.01) plasma glucose turnover rate was found in low energy but no interaction effect (P>0.05) of urea x energy was observed.

Plasma [$^{15}N_2$]urea enrichment and urea concentration almost reached in a steady state during 2.5 - 4 hours of isotope infusion in all dietary treatments as illustrated in Fig 12. Plasma urea turnover rate is presented in Fig 13. Plasma urea kinetics during isotope infusion is presented in Table 6. Plasma urea concentration and turnover rate were not affected (P>0.05) by urea replacement. Nevertheless, higher (P<0.01) plasma urea concentration and turnover rate were found in low energy but no interaction effect (P>0.05) of urea x energy was observed.

Table 6 Effects of replacing soybean meal with urea and negative energy balance on plasma glucose and urea kineticss during 2.5-4 h of primed continuous infusion in sheep

Plasma kineticss	**Non-urea**		**Urea**		**Pooled**	***P* value**		
	High energy	Low energy	High energy	Low energy	**s.e.m.**	Urea	Energy	Urea x Energy
Glucose concentration (mmol/L)	4.4	3.8	4.8	4.5	0.2	0.08	0.09	0.60
Glucose turnover rate (mmol/ ($kg^{0.75}$·h))	1.8	1.2	1.6	1.0	0.1	0.13	<0.01	0.83
Urea concentration (mmol/L)	2.9	5.4	3.0	5.2	0.3	0.64	<0.01	0.57
Urea turnover rate (mmol/ ($kg^{0.75}$·h))	1.9	5.4	1.5	3.3	0.4	0.06	<0.01	0.15

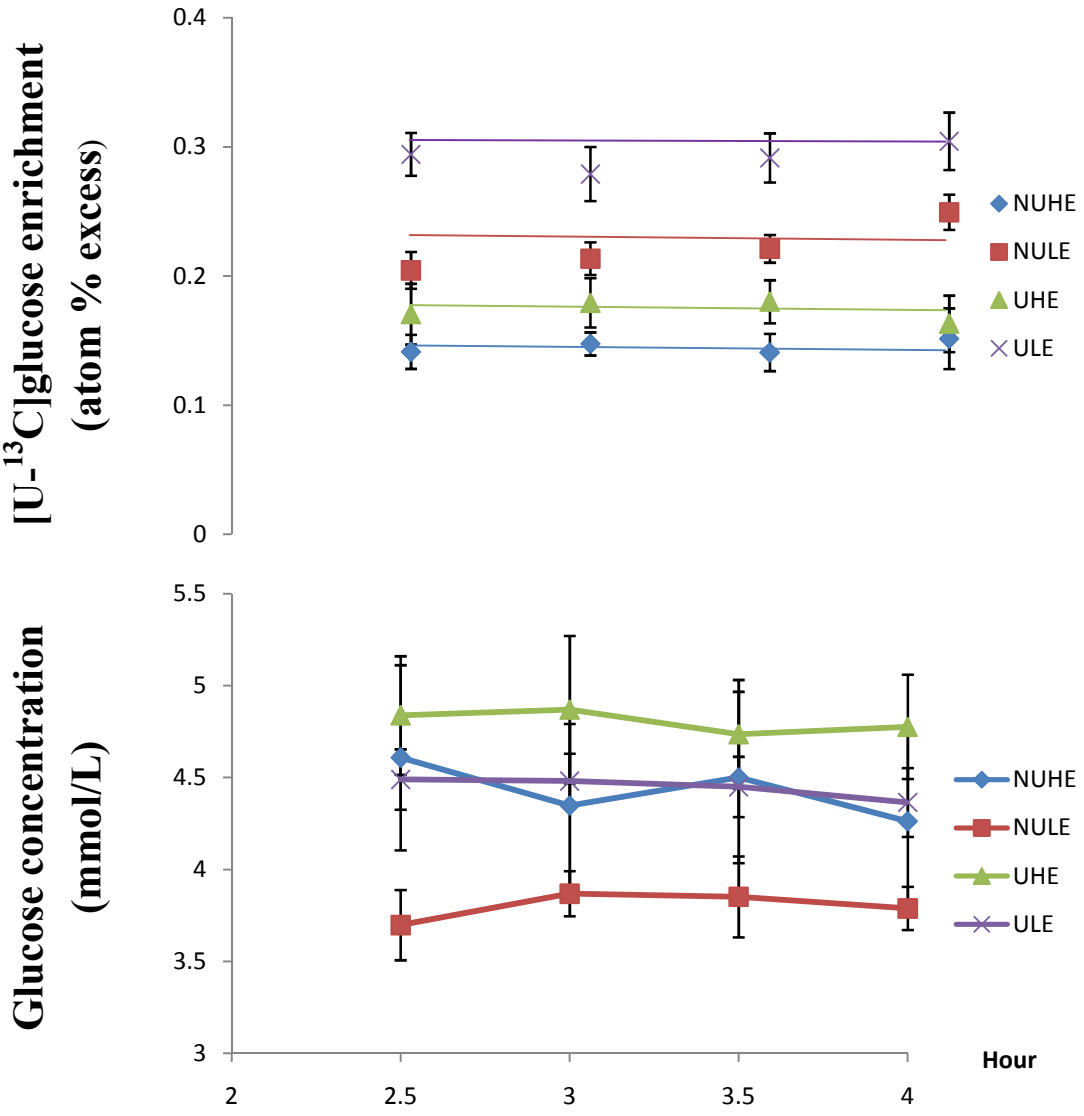

Fig 11 Time course change on plasma [U-^{13}C]glucose enrichment (atom % excess) and glucose concentration (mmol/L) during 2.5 – 4 hours of primed continuous infusion with four dietary treatments; Non Urea High Energy (NUHE), Non Urea Low Energy (NULE), Urea High Energy (UHE) and Urea Low Energy (ULE) in sheep.

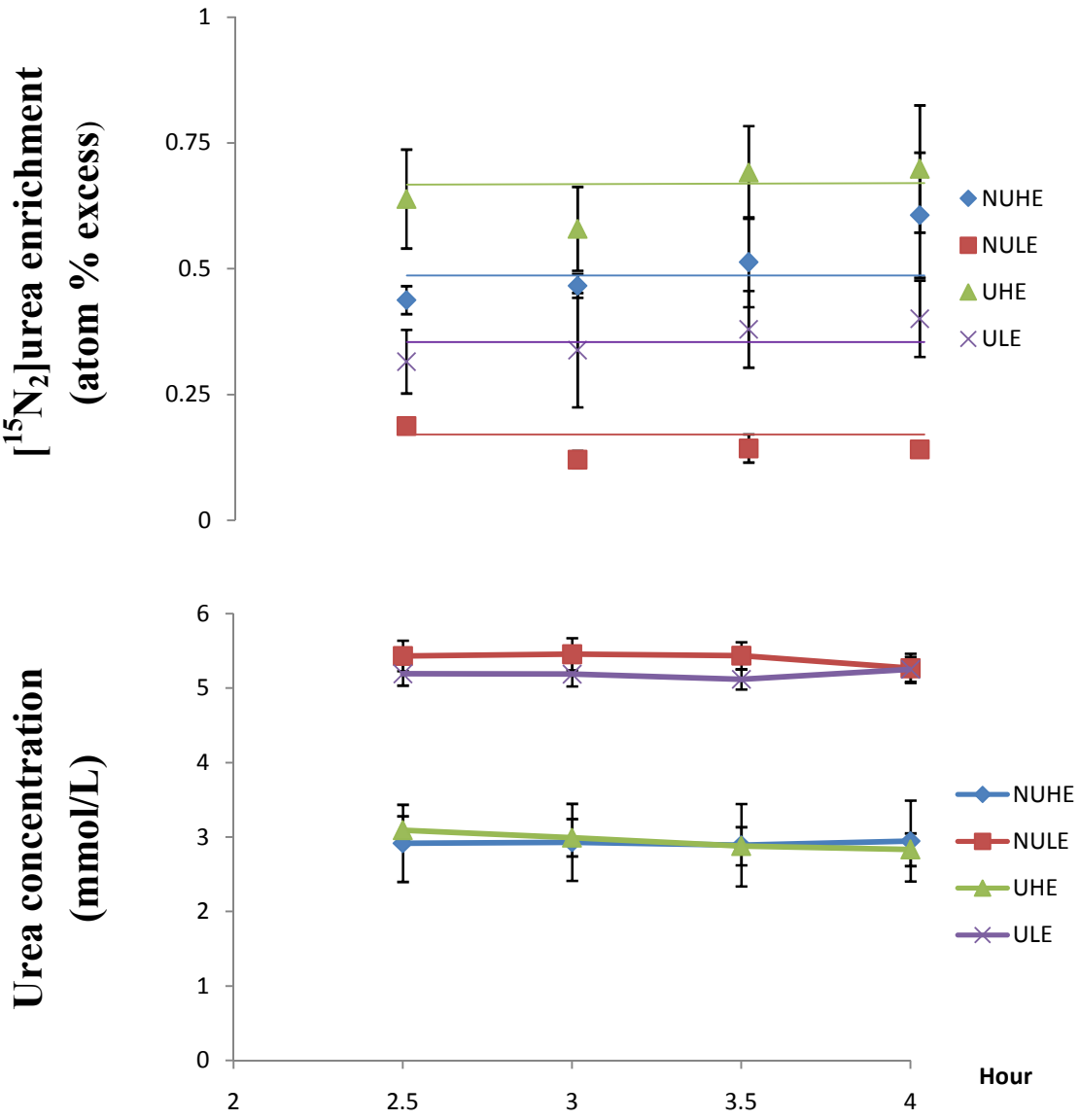

Fig 12 Time course change on plasma [$^{15}N_2$]urea enrichment (atom % excess) and urea concentration (mmol/L) during 2.5 – 4 hours of primed continuous infusion with four dietary treatments; Non Urea High Energy (NUHE), Non Urea Low Energy (NULE), Urea High Energy (UHE) and Urea Low Energy (ULE) in sheep.

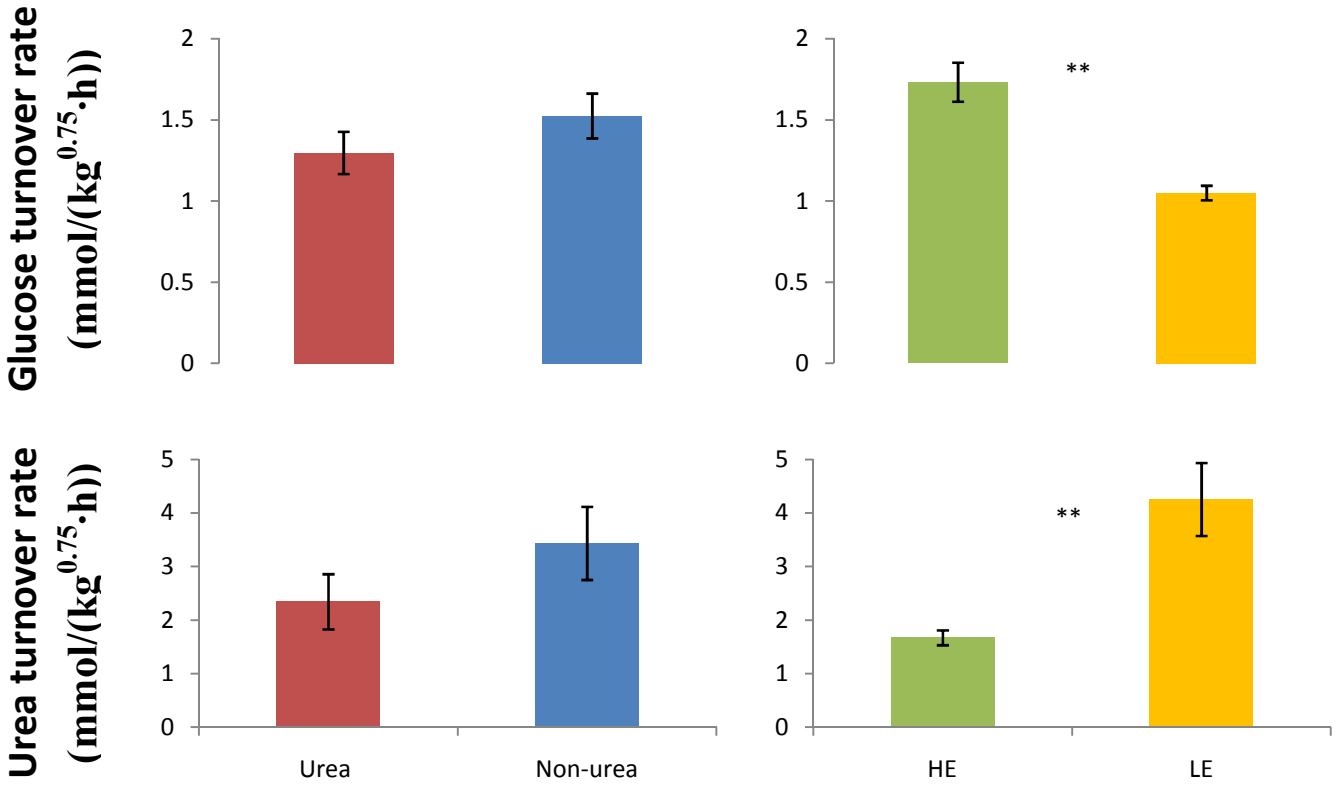

Fig 13 Effects of replacing soybean meal with urea (urea ■ and non-urea ■) and negative energy balance, low energy (LE ■) and high energy (HE ■), on plasma glucose and urea turnover rate (mmol/(kg$^{0.75}$·h)) during 2.5 – 4 hours of primed continuous infusion in sheep.
** differ significantly (P<0.01)

Discussion

Effects of urea and NEB on glucose kinetics

Both urea replacement and low energy treatments had no effect on basal glucose concentration at pre-isotope infusion. The basal glucose concentration (4.3-4.7 mmol/L) was slightly above than standard serum glucose concentration of 1.7 – 3.6 mmol/L in sheep (Jackson and Cockcroft, 2002). This might be because of blood samples were collected 3 hours post-feeding and rumen VFA concentrations peaked at that time. However, blood glucose concentration is subjected by hormonal regulation (Weekes, 1991).

In our study, urea replacement had no effect on plasma glucose turnover rate. This result did not accord with the hypothesis that isonitrogenous replacement of soybean meal by urea could impair glucose turnover rate. Low plasma ammonia concentration might be responsible for that. Even rumen ammonia concentration was higher with urea replacement diet and also higher during NEB. Our results showed lower plasma ammonia concentration (<0.15 mmol/L) than previous studies that successfully decrease glucose production (Weekes et al., 1978; Demigné et al., 1991). Because the amount of urea is rapidly hydrolyzed to ammonia in the rumen (Golombeski et al., 2006; Highstreet et al., 2010) and ammonia utilization was intrinsically related to energy supply from rumen carbohydrate availability (Delgado-Elorduy et al., 2002; Reynolds, 2006).

Plasma glucose turnover rate was lower during NEB. The result stated that glucose production rate was lower with low availability of energy according to our expectation that NEB condition would depress rate of glucose turnover. In contrast, glucose turnover rate is higher with high energy intake and gluconeogenic supply to the liver (Brockman, 1993;

Danfaer et al., 1995). Ortgues-Marty et al. (2003) who reviewed that the effect of intake level are unanimously considered as being highly determinant for glucose turnover.

Total VFA concentration was higher during high energy diet, because high dietary intake increases VFA production (Doreaua et al., 2003). In addition, the rumen pH was lower in high energy diet because of higher VFA production. High energy diet improved N retention under isonitrogenous intake, with decreases in digestible N. These changes in N metabolism might be associated with increased ammonia utilization as well as might be improved microbial N supply. Superior microbial N supply with high energy level could be due to more efficient synchronizing between dietary energy and N supply (Sinclair et al., 1995).

Besides that, fecal N excretion was higher in high energy diet. Cornstarch was a major composition in high energy diet. Even cornstarch was highly fermented in the rumen but a part of unfermented could escape from ruminal fermentation (Ørskov 1986). The escape starch was fermented in the large intestine and increased microbial protein production. Therefore, fecal N excretion loss would be expected (Owens and Soderlund 2006).

Effects of urea and NEB on urea kinetics

Plasma ammonia concentration at pre-isotope infusion did not differ among treatments. However, plasma urea concentration at pre-isotope infusion was higher in low energy. Plasma urea concentration was 1.5 times higher in low energy (5.5-5.7 mmol/L) than high energy (3.4-3.7 mmol/L) but both remained in normal serum concentration (3–10 mmol/L) in sheep (Jackson and Cockcroft, 2002). Plasma urea turnover rate was higher in low energy with no effects by urea replacement and interaction. Similar results were observed by Cocimano and Leng (1967) who found a linear relationship between plasma urea concentration and urea turnover rate in sheep. The increase of plasma urea turnover rate was a contrary result to our hypothesis that the NEB condition would be associated with a depressed capacity of hepatic ureagenesis. The possible reasons might be due to lack of severe NEB induction in our study and also lack of high lipid accumulation in liver cells to impair liver function (Zhu et al., 2000; Overton, 2003).

Usually the NEFA concentration above than 0.4 mmol/L affects hepatic function in sheep (Cynthia, 2010). The slightly higher NEFA concentration (0.46-0.52 mmol/L) in our study might not be enough to affect liver function during NEB. Moreover, serum BHBA concentration remained at the normal range (<0.8 mmol/L) in healthy sheep (Cynthia, 2010). ADG during NEB decreased than before induction as well. Taken together, it reveals that the animal lost their body weight during NEB which might be indicated the fat mobilization from adipose tissues. This phenomenon may have satisfied energy balance while ketogenesis might not be activated.

As N retention is largely depended on amount of fermentable carbohydrate in the diet (Sarwar and Ajmal Khan, 2003). Low N retention was found in low energy diet in the present study. The result agrees with the observation of EL-Sabagh et al. (2009) who reported that N balance was negative at the low energy intake level.

Plasma urea turnover rate was more pronounced during NEB condition. Though propionate is a major gluconeogenic substrate, amino acids may also act as gluconeogenic substrates during increased metabolic demand of glucose, such as during NEB (Bergman and Heitmann, 1978; Overton et al., 1999). When liver increases hepatic catabolism of amino acids, ammonia production will be increased (Overton et al., 1999). The liver would compensate this increased amount of ammonia by detoxifying ammonia into urea and accordingly resulted in increased urea turnover rate (Nolan and Leng, 1970).

Moreover, almost all plasma free amino acid concentrations were lower during NEB. Nozière et al. (2000) showed similar findings of decreased concentration of almost all essential and non-essential amino acids, when dietary intake was reduced. However, the potential of amino acids as gluconeogenic substrate during NEB in our experiment were unclear due to study design. In addition, only plasma glutamine concentration was higher in urea replacement diet. Glutamine is a major amino acid to carry ammonia from peripheral tissues to corporate into urea at the liver or excrete in the urine at the kidney. The urea usage in the diets for ruminants enhanced synthesis of glutamine, which inhibited the synthesis of nitric oxide from arginine in urea cycle (Wu G, 2013).

Urinary N was also higher during NEB. This data showed inefficient ruminal utilization of ammonia that was reflected by the urinary nitrogen excretion (Henning et al., 1993). Similarly Lindberg and Jacobsson (1990) reported that the proportion of urea N excretion increased in low energy diet and decreased in high energy diet.

Finally, from our result high plasma urea turnover was simultaneous with low plasma glucose turnover during NEB. This might be resulted from the competition for cytoplasmic oxaloacetate between ureagenesis and gluconeogenesis pathways (Krebs et al., 1979). Moreover, after ammonia addition in the incubated liver cells, Meijer et al. (1978) found decreased gluconeogenesis because of decreased flux through phosphoenolpyruvate carboxykinase (PEPCK) and a fall of malate concentration which in turn cause a fall of oxaloacetate concentration. However, our finding in this experiment is unclear for this mechanism and may need further study for more understanding.

Chapter-3

Effects of urea and energy levels on microbial protein supply, ruminal fermentation characteristics, plasma glucose and urea kinetics in sheep

Introduction

From the first experiment, it was hypothesized that the isonitrogenous replacement of soybean meal by urea during NEB condition would be depressed rate of gluconeogenesis and ureagenesis. However, the results were at all contrasted from the hypothesis. Urea replacement was not affected on gluconeogenesis. Even urea replacement raised up a rumen ammonia concentration higher than non-replacement diet but not elevated plasma ammonia concentration. Because low plasma ammonia concentration (<0.15 mmol/L) when compared with other studies that successfully decrease glucose production (Weekes et al. 1978; Demigné et al. 1991), the gluconeogenesis in the liver was not interfered. Moreover, ureagenesis was higher during NEB and was also contrasted from the hypothesis. The reason might be due to not severe NEB induction in our study and also may be due to lack of high lipid accumulation in liver cells to impair liver function (Zhu et al. 2000; Overton 2003).

Because isonitrogenous replacement of urea in previous study did not affect on plasma ammonia concentration, the dietary urea treatment was designed into higher level than pervious study for achieved the hypothesis. Moreover, NEB induction was induced more negative energy than previous study. In addition, the synergism between urea and energy supplementations are essential significant on the maximum production rate of microbial protein. The rumen microorganisms might be interrupted due to asynchronous between urea and energy supply when

using urea during NEB. The rumen microorganisms may be an important regulator for ammonia level in the rumen. Therefore, microbial nitrogen supply (MNS) was within our focus in this study.

It was hypothesized in this study that the high level of dietary urea supply during NEB would be depressed rate of gluconeogenesis and ureagenesis which can be determined by decrease glucose and urea turnover rates. Moreover, nitrogen balance, ruminal fermentation characteristics, plasma free amino acid concentrations, microbial protein supply and interaction of urea x energy were within concurrent examination.

Materials and Methods

Animals, experimental design and diets

The experiment was carried without any noticeable stress to the animals under the approval of Laboratory Animal Care and Use Committee of Iwate University (approval no: A201425). Six adult crossbred (Corriedale x Suffolk) with an initial BW of 43.1 $\pm$ 1.8 kg were used. The experiment was carried out by 6 x 5 incomplete Latin square designs with five dietary treatments in each sheep. Each experimental period lasted for 19 days. The sheep were randomly assigned to each sequence of trial.

The experimental design is shown in Diagram 1 and the diet composition is stated in Table 1. The basal diet consisted of timothy hay, crush corn, soybean meal and urea. Adaptation dietary was maintained at crude protein 5 g/ ($kg^{0.75}$·day) and energy 502 kJ/ ($kg^{0.75}$·day) on day 1-14 of adaptation period. The animals were housed in individual pens during the adaptation period.

Dietary treatments composed of two levels of urea, 0.5 and 1.1 g/ ($kg^{0.75}$·day), and would be kept total protein content balance into 5 and 7 g/ ($kg^{0.75}$·day) in the ration. Three levels of energy; 167, 335 and 502 kJ/ ($kg^{0.75}$·day), were combined with two levels of urea. Therefore, six dietary treatments were created as Low Urea Low Energy (LULE), Low Urea Middle Energy (LUME), Low Urea High Energy (LUHE), High Urea Low Energy (HULE), High Urea Middle Energy (HUME) and High Urea High Energy (HUHE), respectively. Dietary treatments were introduced on day 15-19 for five consecutive day period.

The sheep were moved to individual metabolic cages in a controlled environmental room with 23 $\pm$ 1°C, 70% relative humidity and 08.00 – 22.00 hours of lighting during last 6 days in each treatment period. Diets were offered in equal proportion twice a day at 08.30 and 20.30 hours and had free access to drinking water during the whole experimental period.

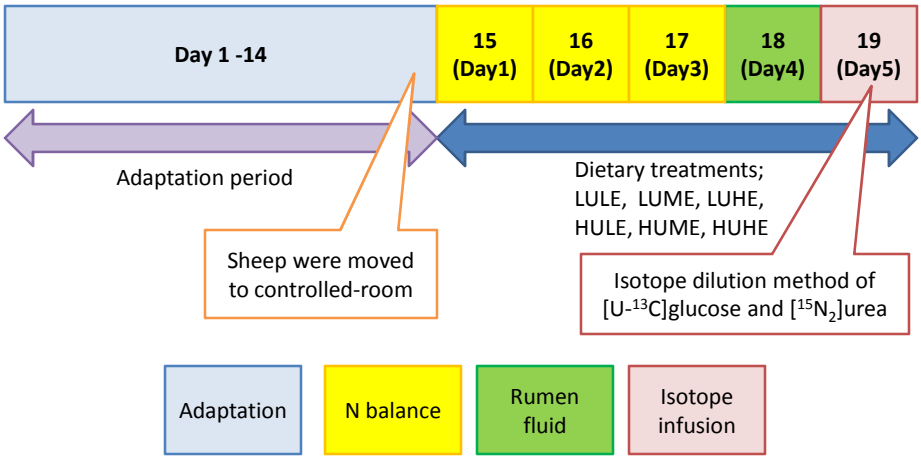

Diagram 1 Flow chart showed the experimental design in this study. Collecting samples period composed of five days period (Day1-5). Dietary treatments composed of Low Urea Low Energy (LULE), Low Urea Middle Energy (LUME), Low Urea High Energy (LUHE), High Urea Low Energy (HULE), High Urea Middle Energy (HUME) and High Urea High Energy (HUHE), respectively.

Table 1 Dietary treatment formulation and chemical composition of diets

g (dry matter)/ ($kg^{0.75}$·day)	Adaptation	Low Urea			High Urea		
		Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy
Dietary treatment formulation							
Timothy hay	28.7	10.2	19.7	29.5	10.1	20.4	29.3
Crush corn	19.1	0.0	8.7	17.5	0.0	7.3	16.5
Soybean meal	0.0	6.0	3.7	1.3	7.0	4.8	2.4
Urea	0.8	0.5	0.5	0.5	1.1	1.1	1.1
Chemical composition							
Dry matter intake	48.6	16.8	32.6	48.8	18.2	33.6	49.2
Crude protein	5.2	5.0	5.0	5.0	7.0	7.0	7.0
Metabolizable energy[A] (kJ/ ($kg^{0.75}$·day))	502	166	334	504	178	335	503

[A]NRC, 6th revised edition (1985)

Samples collection

The sheep were weighed weekly and feed were adjusted accordingly. On day 15-17 of each dietary treatment, urine and feces were collected for 3 days from each sheep for nitrogen (N) balance study. The sampling method was same as previous study.

Blood was sampled before NEB induction (day 14) and daily from day 15-18 in each dietary treatment. Blood collection protocol was same as early experiment. Daily serum sample was analyzed for the serum total protein (TP), albumin (ALB), blood urea nitrogen (BUN), NEFA and BHBA for NEB monitoring on the same day.

On day 18, rumen liquid samples were taken at pre-feeding (0 hour) and at 1.5, 3 and 6 hours post-feeding by using previous technique and protocol. Rumen fluid sample was used for pH, ammonia concentration and VFAs analysis.

On day 19, the primed continuous infusion of [U-^{13}C]glucose and [$^{15}N_2$]urea was applied to determine plasma glucose and urea kineticss. Isotope infusion procedure and collection were mentioned in early study.

Chemical analysis

Serum TP, ALB, BUN, NEFA and BHBA concentrations were analyzed by using automatic chemistry analyzer (Toshiba TBA-40 FR Acute, Diamond Diagnostics, USA) based on biuret method, bromcresol green (BCG) method, urease-GLDH method, ACS-ACOD method (NEFA-HAII) and cyclic enzymatic method (autokit 3-HB), respectively. Serum globulin (GLB) concentration was calculated by serum TP concentration minus with serum ALB concentration.

The plasma free amino acid and ammonia concentration protocol were slightly improved. After mixed plasma sample with 3% sulfosalicylic acid (SSA) and kept into refrigerator for overnight. The centrifuge speed was changed from 3,000 into 10,000 rpm for more agglutinated and purified the samples.

The purine derivatives (PD) excretion, namely allantoin, uric acid and xanthine plus hypoxanthine from diluted urine samples were determined by the procedure described by Chen and Gomes (1992) using the spectrophotometer (U-1000, HITACHI, Japan). The analysis protocols explain in brief as follow;

For allantoin determination, 1 mL of urine and standard samples were taken into 15 mL glass tube, mixed with 5 mL of distillated water and 1 mL of NaOH (0.5 mol/L). Then, the tubes were placed in the boiling water bath for 7 min. The tubes were cooled in ice tray, then added with 1 mL of HCl (0.5 mol/L) and 1 mL of phenylhydrazine (0.023 mol/L), and boiled again for another 7 min. After boiling, the tubes were cooled again in ice tray for several min and added with 3 mL of concentrated HCI (11.4 N) and 1 mL of potassium ferricyanide (0.05 mol/L). After 20 min, the absorbance was read at 522 nm. The allantoin concentration was calculated (mg/L) based on the linear equation.

For uric acid, 2.5 mL of urine and standard samples were taken into 10 mL glass tube and mixed with 1 mL of KH_2PO_4 phosphate buffer (0.67 mol/L). The sample and standard were duplicated. In each set, 150 µL of phosphate buffer and in other set 150 µL of uricase solution were added, mixed by vortexing and incubated in the 37ºC water bath for 90 min. Then, the absorbance was read at 293 nm and uric acid concentration was calculated based on the standard equation.

For xanthine and hypoxanthine, 1.0 mL of urine and standard samples were taken into 10 mL glass tubes and added with 2.5 mL of KH_2PO_4 phosphate buffer (0.2 mol/L)and 0.35 mL of L-histidine (4.3 mmol/L). The sample and standard were duplicated. In each set, 150 µL of phosphate buffer and in other set 150 µL of xanthine oxidase solution were added, mixed by

vortexing and incubated in the 37ºC water bath for 60 min. Then, the absorbance was read at 293 nm and xanthine and hypoxanthine concentration was calculated based on the standard equation.

Nitrogen contents, rumen ammonia concentration, rumen VFAs concentration, plasma glucose concentration, plasma glucose enrichment, plasma glucose turnover rate, plasma urea concentration, plasma urea enrichment and plasma urea turnover rate were analyzed as same as previous method.

Calculations and statistical analysis

The glucose and urea turnover rates were calculated according as previous equation. The mean values and pooled s.e.m. were generated for all data. All data were analyzed by using MIXED procedure in SAS (1996). The model composed of the fixed effects as periods (first and second), urea (low and high), energy (low, middle and high) and the interaction of urea x energy. Sheep were randomized as random effect. Ruminal fermentation characteristics were subjected to repeated measurement analysis. Tukey-Kramer adjustment was used to identify diets with different effects on the variable involved. The level of significance was set at $P<0.05$ throughout the analysis.

Estimation of MNS

The amount of microbial purines absorbed (X mmol/d) corresponding to the PD excreted (Y mmol/d) was calculated based on the relationship described by Chen et al. (1990) as follows:

$$Y = 0.84X + (0.150BW^{0.75} e^{-0.25X})$$

Where, 0.84 is the recovery of absorbed purines as PD in urine and $0.150BW^{0.75}$ represents the endogenous contribution of purine excretion. The calculation of X from Y based on the equation was made by means of the Newton's iteration process.

Finally MNS was calculated (Chen and Gomes, 1992) as follows:

MNS (g/d) = 70 x X(mmol/d) / 0.83x0.116x1000

Where, 0.83 = digestibility of microbial purines, 70 = N content of purines (mg/mmol) and 0.116 = ratio of purine N to total N in mixed microbial biomass (Chen, 1989).

Results

Negative energy balance induction

Effects of urea and energy on ADG and serum metabolites are presented in Table 2. No significant difference (P>0.05) of ADG with urea level was observed. However, ADG was affected (P=0.01) by low energy treatments. Serum TP, ALB and GLB concentrations had no difference (P>0.05) on both urea and energy treatments. Serum BUN concentration was higher (P<0.01) in high urea level and was affected (P<0.01) by low energy treatments. Serum NEFA and BHBA concentration were significantly differed (P<0.01 and P=0.02) in low energy treatments. Interaction effects of urea x energy were not found (P>0.05) in all of serum metabolites.

Time course changes of BW and serum metabolites are illustrated in Fig 1 - 4.

BW highly increased during adaptation period. Then, BW sharply decreased during low energy treatment until finished negative energy induction. Serum TP, ALB and GLB concentrations were not affected (P>0.05) by urea and energy treatments while were unstable (P<0.05) and varied on induction day.

Interaction response of urea x time and energy x time were identified (P<0.01) in serum BUN concentration. Serum BUN concentration was higher (P<0.01) in high urea than low urea level and inverse variation (P<0.01) with energy level. However, serum BUN concentration increased from first induction day and kept in stable 2 day after induction.

Interaction effect of energy x time was found (P<0.01) in serum NEFA concentration. Serum NEFA concentration had differed response from energy treatments with highest in low energy than middle and high energy. Moreover, trend to positive increased day by day without (P>0.05) urea treatment effect.

Interaction effect of energy x time was also found (P<0.01) in serum BHBA concentration. Serum BHBA concentration had differed response from energy treatments with lowest in low energy than middle and high energy. However, general trend was unstable (P<0.01) during negative energy induction without (P>0.05) urea treatment effect.

Table 2 Effect of urea and energy levels on average daily gain and serum metabolite concentrations in sheep

	Low Urea			High Urea			Pooled	P value		
	Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy	s.e.m.	Urea	Energy	Urea x energy
ADG (kg/d)	-0.40	-0.43	-0.08	-0.50	-0.21	-0.06	0.05	0.52	0.01	0.37
Serum metabolites										
TP (g/dL)	6.17	6.18	6.01	6.17	6.02	6.06	0.02	0.86	0.62	0.99
ALB (g/dL)	3.47	3.44	3.46	3.50	3.43	3.38	0.01	0.69	0.44	0.87
GLB (g/dL)	2.70	2.74	2.55	2.67	2.58	2.68	0.02	0.99	0.78	0.98
BUN (mmol/L)	8.28	6.58	4.94	10.03	8.14	6.29	0.20	<0.01	<0.01	0.81
NEFA (mmol/L)	0.60	0.23	0.14	0.65	0.27	0.11	0.03	0.88	<0.01	0.91
BHBA (mmol/L)	0.30	0.35	0.36	0.30	0.35	0.40	<0.01	0.22	0.02	0.98

ADG - Average daily gain, TP – Total protein, ALB – Albumin, GLB – Globulin,
BUN – Blood urea nitrogen, NEFA - Non-esterified fatty acid, BHBA - β-hydroxybutyric acid

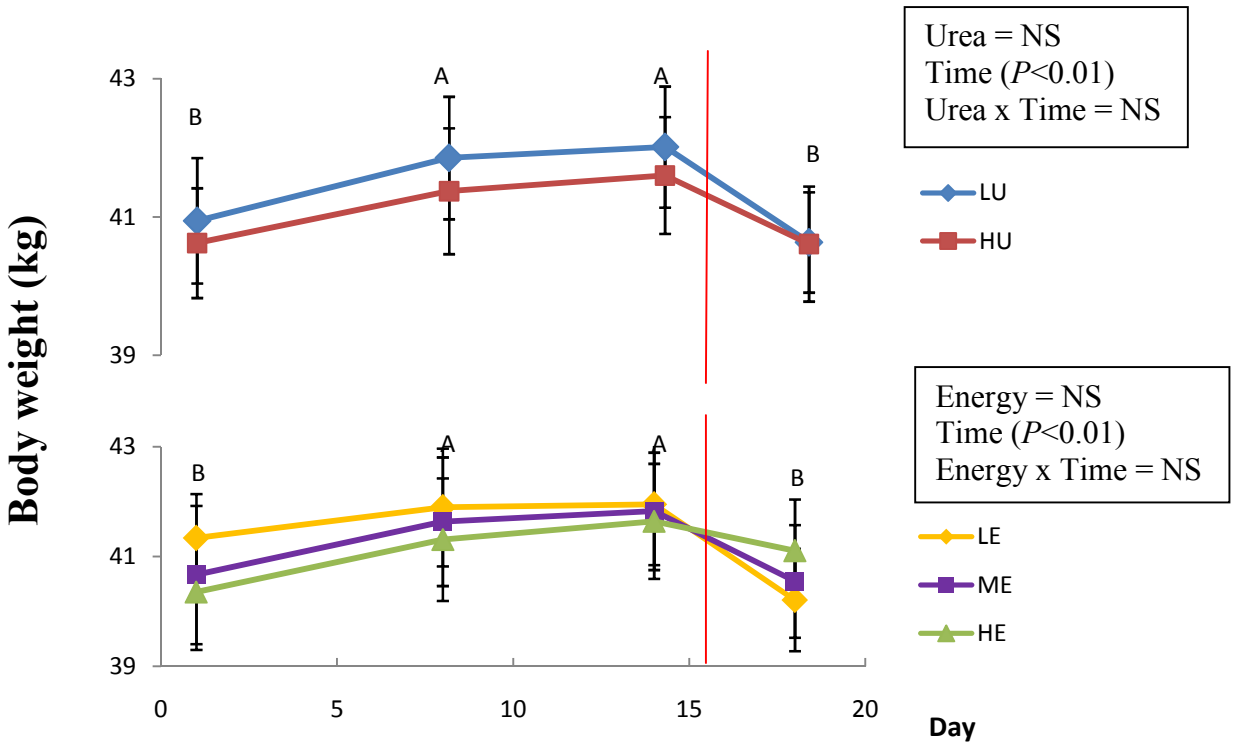

Fig 1 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on body weight (kg) in sheep. NS = not significant difference ($P>0.05$); A and B times differ significantly ($P<0.05$); — negative energy induction

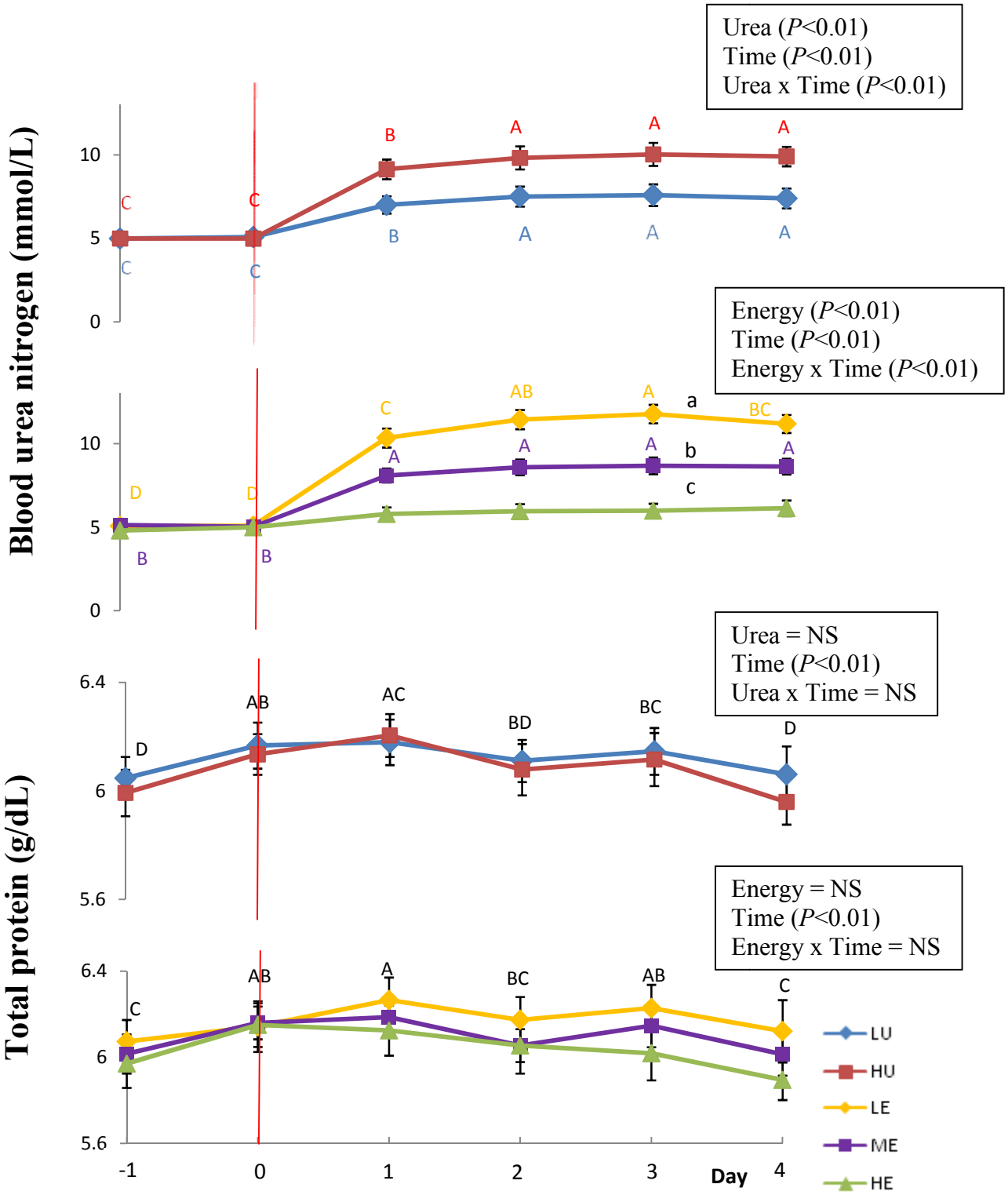

Fig 2 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on serum blood urea nitrogen (mmol/L) and total protein (g/dL) in sheep. NS = not significant difference ($P>0.05$); A, B, C and D times differ significantly ($P<0.05$); a, b and c energy differ significantly($P<0.05$); —— negative energy induction

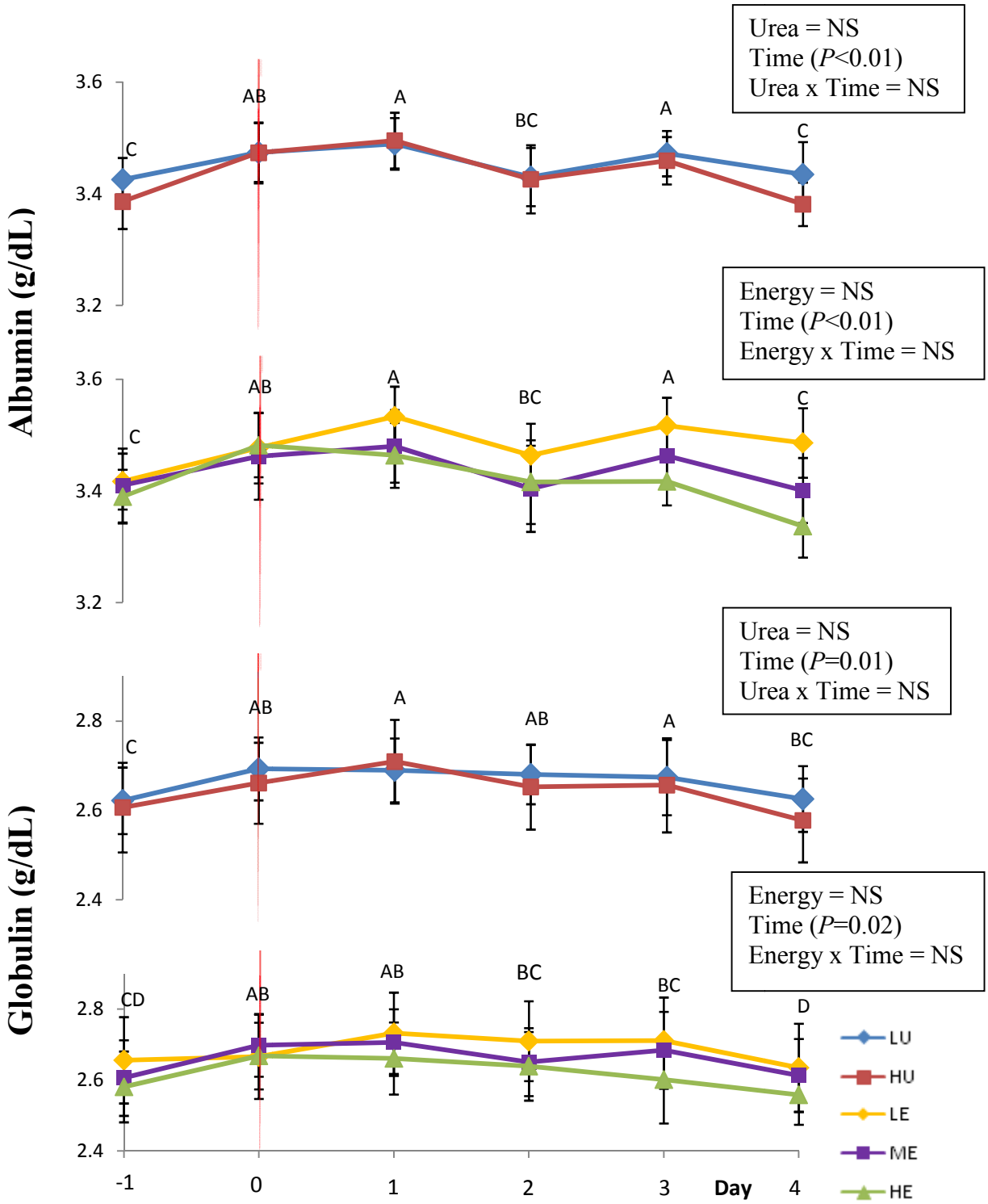

Fig 3 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on serum albumin and globulin concentration (g/dL) in sheep. NS = not significant difference (P>0.05); A, B, C and D times differ significantly (P<0.05); —— negative energy induction

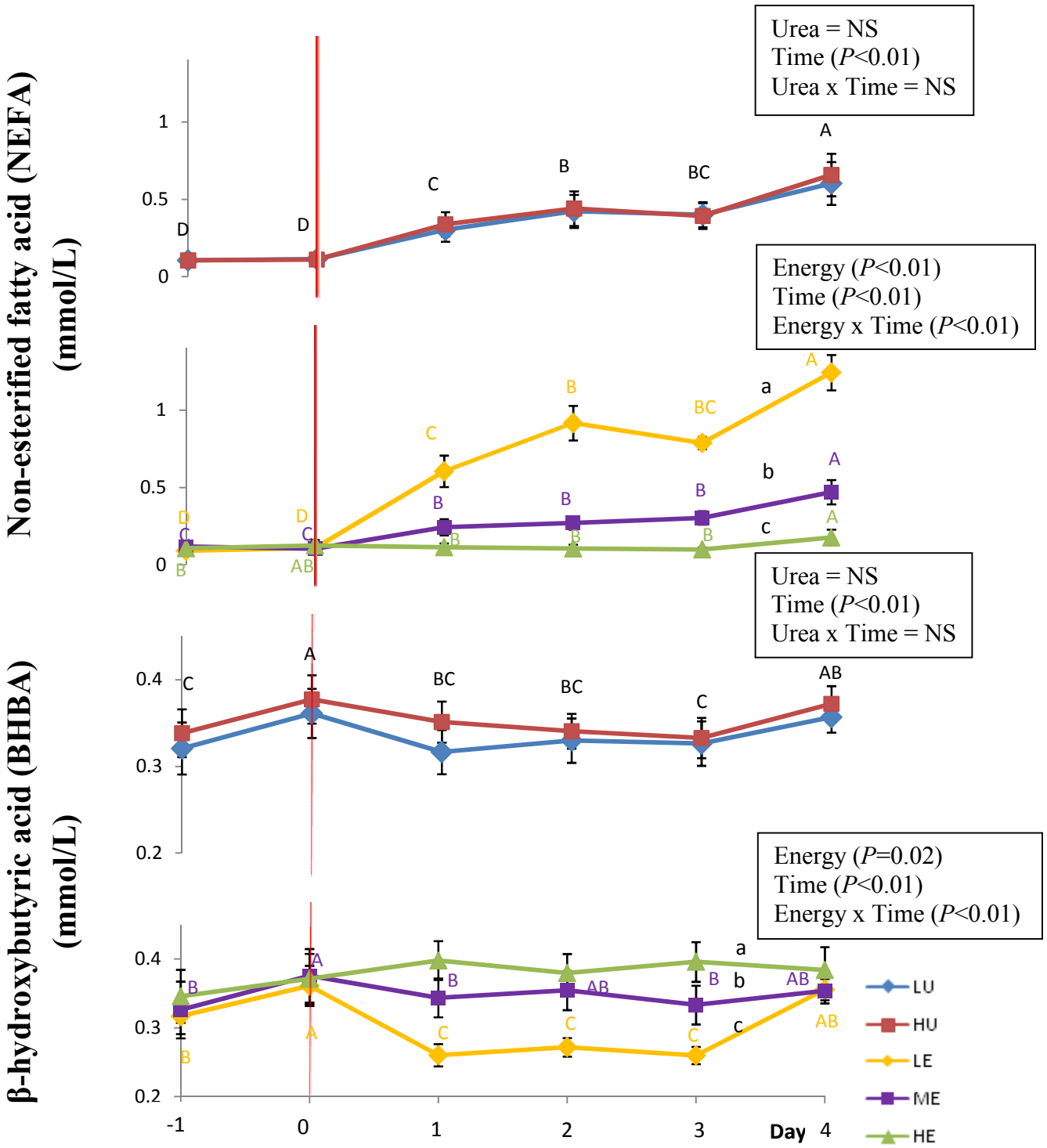

Fig 4 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on serum non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHBA) concentration (mmol/L) in sheep. NS = not significant difference (P>0.05); A, B, C and D times differ significantly (P<0.05); a, b, and c energy differ significantly (P<0.05); —— negative energy induction

Nitrogen balance

Effects of urea and energy levels on N balance are presented in Table 3. N intake was influenced ($P<0.01$) by urea level as well as energy level. Fecal N was affected ($P<0.01$) by low energy treatments. Urinary N was higher ($P=0.01$) in high urea level than low urea level and was affected ($P<0.01$) by low energy supply. N absorption, retention and digestibility were higher ($P<0.01$) in high urea level. Low energy treatments influenced on N retention ($P=0.01$) and N digestibility ($P<0.01$). Urea and energy interaction effect was not seen in the N balances study.

Figure of N balance and N digestibility are show in Fig 5 and 6. N intake, urinary N, N absorption, N retention and N digestibility were higher ($P<0.05$) in high urea than low urea levels except fecal N did not differ ($P>0.05$). On the other hand, fecal N was highest ($P<0.05$) according with energy level (LE<ME<HE) while urinary N was highest ($P<0.05$) in low energy than middle and high energy (LE>ME~HE). N digestibility was lowest ($P<0.05$) in high energy than middle and low energy (LE~ME>HE).

Table 3 Effect of urea and energy levels on nitrogen (N) balance in sheep

N	**Low Urea**			**High Urea**			**Pooled**	**P value**		
Balance	Low	Middle	High	Low	Middle	High	**s.e.m.**	**Urea**	**Energy**	**Urea x**
g/(kg$^{0.75}$·day)	Energy	Energy	Energy	Energy	Energy	Energy				**energy**
N intake	0.83	0.86	0.89	1.17	1.24	1.29	0.04	<0.01	<0.01	0.11
Feces N	0.13	0.17	0.24	0.12	0.17	0.24	<0.01	0.13	<0.01	0.66
Urinary N	0.70	0.53	0.50	0.90	0.69	0.55	0.03	0.01	<0.01	0.73
N absorption	0.70	0.69	0.65	1.05	1.08	1.06	0.04	<0.01	0.13	0.26
N retention	<0.01	0.16	0.15	0.22	0.39	0.51	0.04	<0.01	0.01	0.77
N digestibility (%)	84	80	73	90	87	82	1.06	<0.01	<0.01	0.17

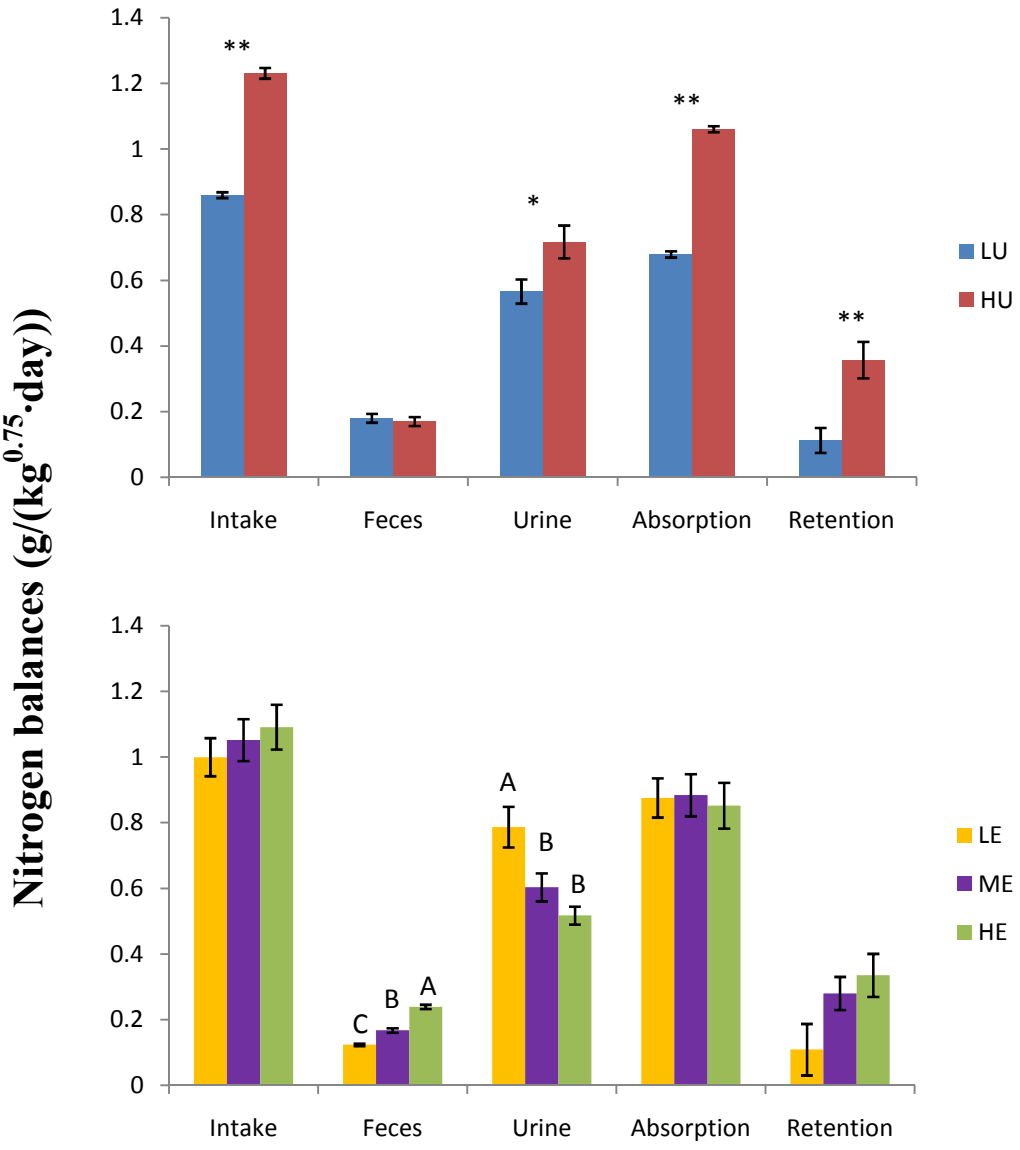

Fig 5 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on nitrogen (N) balances (g/(kg$^{0.75}$·day)) in sheep. ** differ significantly (P<0.01); A, B, C and * differ significantly (P<0.05)

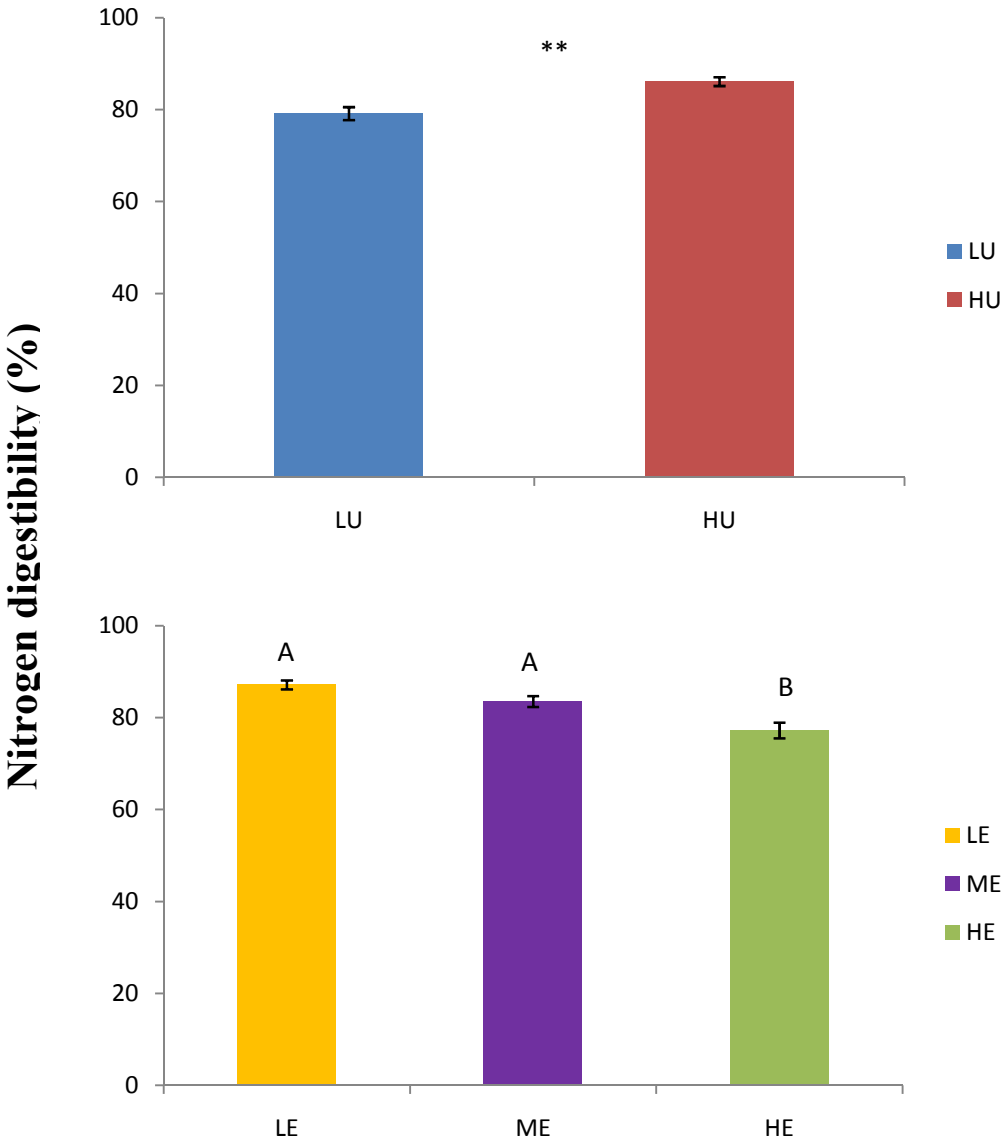

Fig 6 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on nitrogen digestibility (%) in sheep. ** differ significantly (P<0.01); A and B differ significantly (P<0.05)

Ruminal fermentation characteristics

Effects of urea and energy levels on average (0-6 hours) of rumen pH, ammonia, total VFA, individual VFA concentrations and acetate: propionate ratio are presented in Table 4. The interaction effect of urea x energy with different responding of dietary treatments in rumen pH was identified (P=0.02). The rumen pH was highest *(*P<0.05) in HULE and was lowest (P<0.05) in LUHE. Rumen ammonia concentration was higher (P<0.01) in high urea level than low urea level and was affected (P<0.01) by low energy treatments. Total VFA and acetic acid concentrations were influenced (P<0.01) by both urea level and low energy treatments. Propionic acid concentration was affected (P<0.01) by low energy treatments. Interaction effect of urea x energy was found (P<0.01) in butyric acid concentration. Butyric acid concentration was highest (P<0.05) in LUHE and was lowest (P<0.05) in LULE. Acetate: propionate ratio was not affected (P>0.05) by both urea and energy treatments.

Time course changes of rumen pH, ammonia, total VFA, individual VFA concentrations and acetate: propionate ratio are illustrated in Fig. 7 - 10. Rumen pH was higher (P<0.01) in high urea level than low urea level and was stabled (P>0.05) through 6 hours post-feeding. Moreover, rumen pH was also highest (P<0.05) in low energy than middle and high energy (LE>ME>HE). Rumen pH decreased after feeding until 1.5 hour post-feeding and then stable with energy treatments. Interaction between urea and time with different responded in rumen ammonia concentration was found (P=0.03). Rumen ammonia concentration was higher (P<0.01) in high urea level than low urea level and was highest (P<0.05) in low energy than middle and high energy (LE>ME>HE). In general trend, there was peaked at 1.5 hour post-feeding and then descended until 6 hours post-feeding.

The results of time course analysis in VFAs data were showed that total VFA, acetic acid and propionic acid concentrations were higher (P=0.02, P=0.03 and P=0.01) in low urea level than high urea level and was highest (P<0.05) in high energy than middle and low energy (LE<ME<HE). The greatest of total VFA concentration was observed at 1.5 hour post-feeding, then maintained their concentration and finally decreased at 6 hours post-feeding. Acetic concentration was maintained (P>0.05) in stable concentration from pre-feeding until 6 hours post-feeding. The peak of propionic acid concentration was observed at 1.5 hour post-feeding and then descended. Butyric acid concentration was highest (P<0.05) in high energy than middle and low energy (LE<ME<HE) with no affected (P>0.05) by urea treatment. Butyric acid concentration was kept (P>0.05) in stable concentration from pre-feeding through 6 hour post-feeding.

Table 4 Effect of urea and energy levels on rumen fermentation characteristics in sheep

Rumen fermentations (mmol/L)	Low Urea			High Urea			Pooled s.e.m.	P value		
	Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy		Urea	Energy	Urea x energy
pH	6.92[b]	6.59[d]	6.36[e]	7.00[a]	6.80[c]	6.61[d]	0.1	<0.01	<0.01	0.02
Ammonia	5.70	3.79	2.59	7.23	5.58	3.86	0.2	<0.01	<0.01	0.70
Total VFA	29.7	45.9	58.9	32.3	40.2	45.1	1.3	<0.01	<0.01	0.06
Acetate	20.2	30.5	39.3	20.9	26.3	28.9	1.5	<0.01	<0.01	0.11
Propionate	5.4	8.6	11.8	6.1	7.8	9.8	0.5	0.12	<0.01	0.42
Butyrate	2.2[e]	4.9[b]	6.3[a]	2.8[d]	4.2[c]	5.1[b]	0.3	0.03	<0.01	<0.01
Isobutyrate	0.6	0.5	0.4	0.9	0.6	0.4	0.1	0.07	<0.01	0.07
Valerate	0.4	0.5	0.5	0.5	0.5	0.5	0.1	0.86	0.09	0.44
Isovalerate	0.9	0.9	0.6	1.1	0.8	0.6	0.1	0.33	<0.01	0.10
A/P ratio	3.8	3.7	3.5	3.5	3.5	3.3	0.1	0.27	0.34	0.89

a, b, c, d and e – different letters in a row among overall dietary treatments differ significantly (P<0.05), A/P - Acetate: Propionate ratio

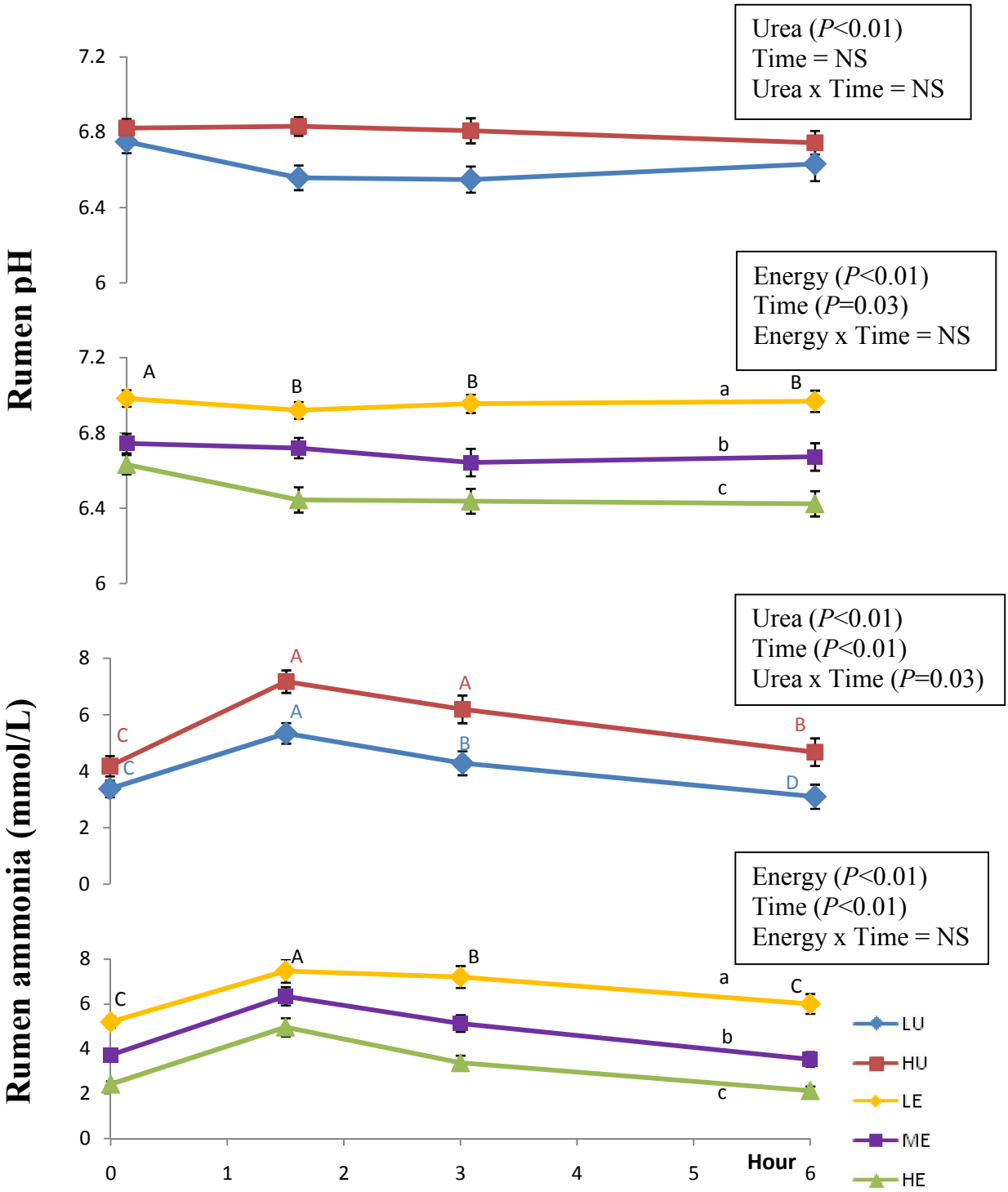

Fig 7 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on rumen pH and ammonia concentration (mmol/L) in sheep. NS = not significant difference ($P>0.05$);
A, B, C and D times differ significantly ($P<0.05$); a, b and c energy differ significantly ($P<0.05$)

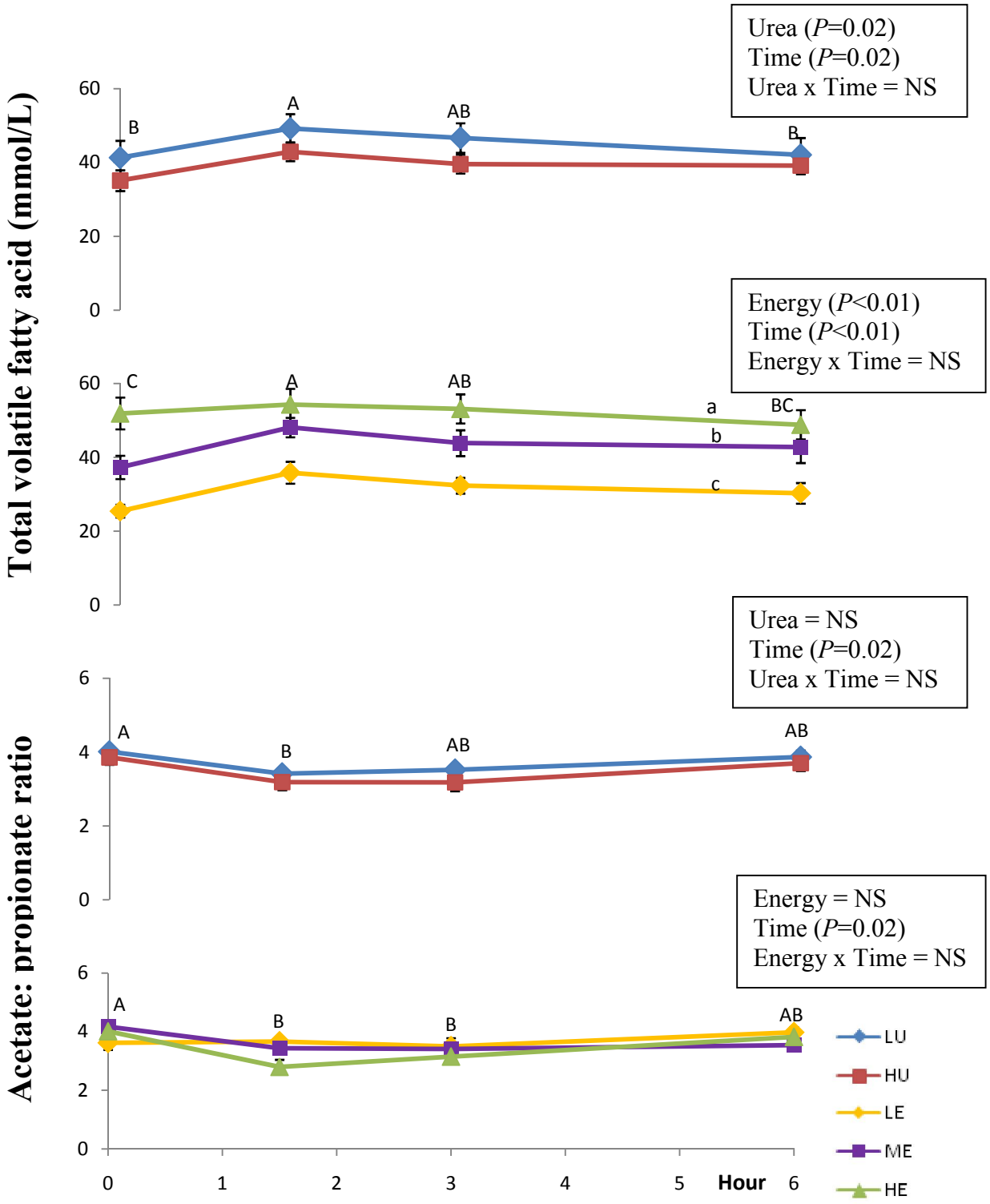

Fig 8 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on rumen total volatile fatty acid concentration (mmol/L) and acetate: propionate ratio in sheep. NS = not significant difference ($P>0.05$); A, B and C times differ significantly ($P<0.05$); a, b and c energy differ significantly ($P<0.05$)

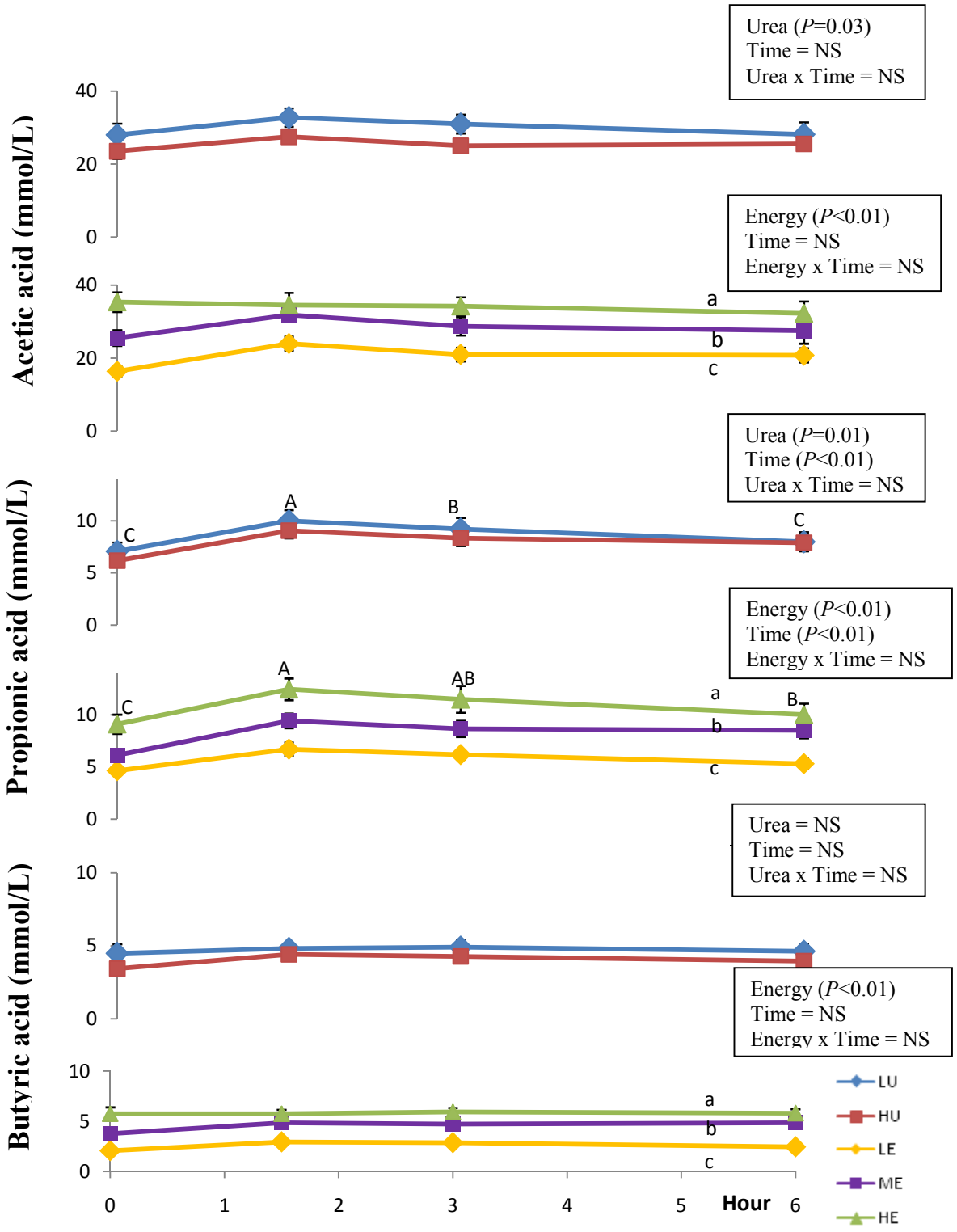

Fig 9 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on rumen acetic, propionic and butyric acid concentration (mmol/L) in sheep. NS = not significant difference (P>0.05); A, B and C times differ significantly (P<0.05); a, b and c energy differ significantly (P<0.05)

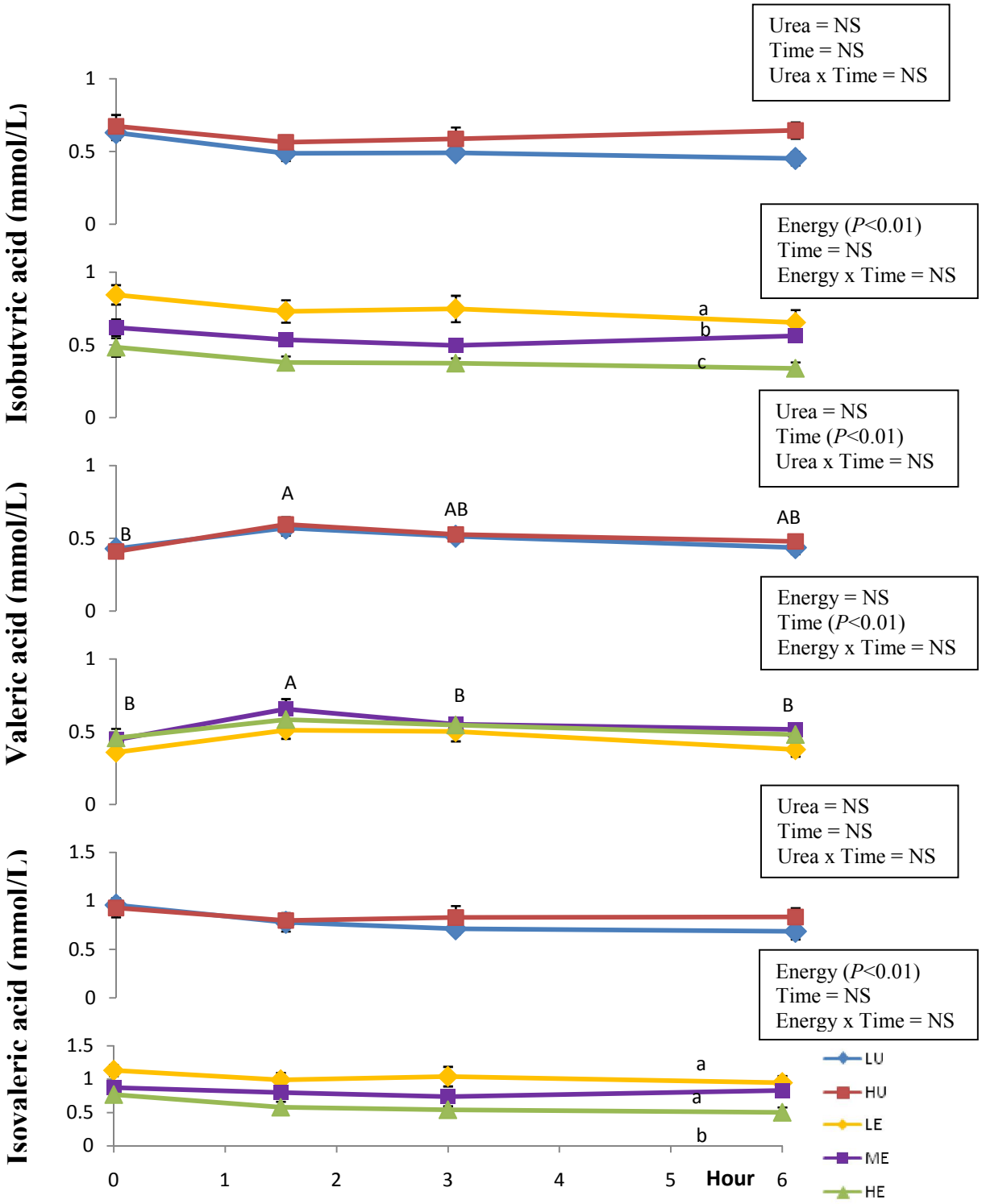

Fig 10 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE, middle energy ME and high energy HE) on rumen isobutyric, valeric and isovaleric acid concentration (mmol/L) in sheep. NS = not significant difference (P>0.05); A and B times differ significantly (P<0.05); a, b and c energy differ significantly (P<0.05).

Plasma free amino acids, glucose, ammonia and urea concentration

Plasma free amino acids, glucose, ammonia and urea concentrations at pre-isotope infusion are presented in Table 5. Plasma threonine, valine, methionine, isoleucine, leucine, phenylalanine, serine, asparagine, glutamine, glycine, alanine, arginine and proline concentrations were affected ($P<0.05$) by urea level. Plasma phenylalanine, asparagine, glutamic acid, tyrosine, arginine and proline concentrations were affected ($P<0.05$) by energy levels. Interaction effect of urea x energy was found ($P=0.04$) in asparagine and glutamine concentrations. Plasma asparagine concentration was highest ($P<0.05$) in LUHE and lowest ($P<0.05$) in HULE. Plasma glutamine concentration was highest ($P<0.05$) in HUME and lowest ($P<0.05$) in LULE.

Effects of urea and energy levels on plasma free amino acids, glucose, ammonia and urea concentrations at pre-isotope infusion are illustrate in Fig 11 - 12. Plasma threonine, valine, methionine, isoleucine, leucine, phenylalanine, serine, glycine and alanine, concentrations were higher ($P<0.05$) in low urea level than high urea level, except for glutamine concentration was lower ($P<0.01$).

Plasma phenylalanine and glutamic acid concentrations were affected ($P<0.05$) by energy as LE≤ME≤HE, respectively. Plasma asparagine, tyrosine and proline concentrations were lowest ($P<0.05$) in low energy than middle and high energy (LE<ME~HE), except for arginine concentration was lowest ($P<0.05$) in high energy than middle and high energy (LE~ME>HE).

At pre-infusion level, plasma glucose concentration did not differ ($P>0.05$) among dietary treatments. Plasma ammonia and urea concentration were higher ($P<0.01$) in high urea level than low urea level. Plasma urea concentration was lowest ($P<0.05$) in high energy than middle and low energy (LE~ME>HE). Urea and energy interactions were not found ($P>0.05$).

Table 5 Effect of urea and energy levels on plasma free amino acids, glucose, ammonia and urea concentrations at pre-isotope infusion in sheep

Plasma concentrations	Low urea			High urea			Pooled s.e.m.	P value		
	Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy		Urea	Energy	Urea x Energy
Amino acids (μmol/L)										
Threonine	141	120	152	119	142	122	8	0.04	0.27	0.34
Valine	133	127	149	107	120	133	5	0.02	0.24	0.63
Methionine	13	13	15	10	12	11	1	<0.01	0.36	0.53
Isoleucine	64	66	75	53	57	51	3	<0.01	0.62	0.48
Leucine	85	83	101	65	71	72	3	<0.01	0.22	0.58
Phenylalanine	34	35	43	31	34	36	1	0.03	<0.01	0.74
Histidine	51	50	55	54	53	43	1	0.41	0.67	0.06
Lysine	102	86	91	91	90	76	3	0.09	0.20	0.32
Tryptophan	28	30	28	25	29	31	1	0.72	0.33	0.21
Aspartic acid	3.4	4.7	5.2	5.5	7.0	6.4	1	0.36	0.31	0.30
Serine	106	98	120	93	108	97	5	0.01	0.24	0.06
Asparagine	60^{b}	70^{b}	100^{a}	48^{c}	78^{b}	72^{b}	4	0.03	<0.01	0.04
Glutamic acid	53	66	72	57	67	74	3	0.73	0.03	0.94
Glutamine	324^{c}	344^{b}	397^{b}	399^{b}	452^{a}	377^{b}	14	<0.01	0.05	0.04
Glycine	452	431	552	368	409	384	21	<0.01	0.12	0.09
Alanine	169	181	183	137	151	135	10	<0.01	0.18	0.27
Tyrosine	38	48	63	37	50	50	2	0.10	<0.01	0.23
Arginine	177	155	139	202	183	142	6	0.03	<0.01	0.77
Proline	58	77	104	58	78	79	4	0.03	<0.01	0.13
Glucose (mmol/L)	3.4	3.3	3.5	3.4	3.4	3.5	0.1	0.87	0.45	0.86
Ammonia (mmol/L)	0.15	0.16	0.16	0.19	0.19	0.18	<0.01	<0.01	0.79	0.50
Urea (mmol/L)	5.7	5.0	3.9	6.4	5.9	5.2	0.2	<0.01	<0.01	0.38

a, b, c – different letters in a row among overall dietary treatments differ significantly (P<0.05)

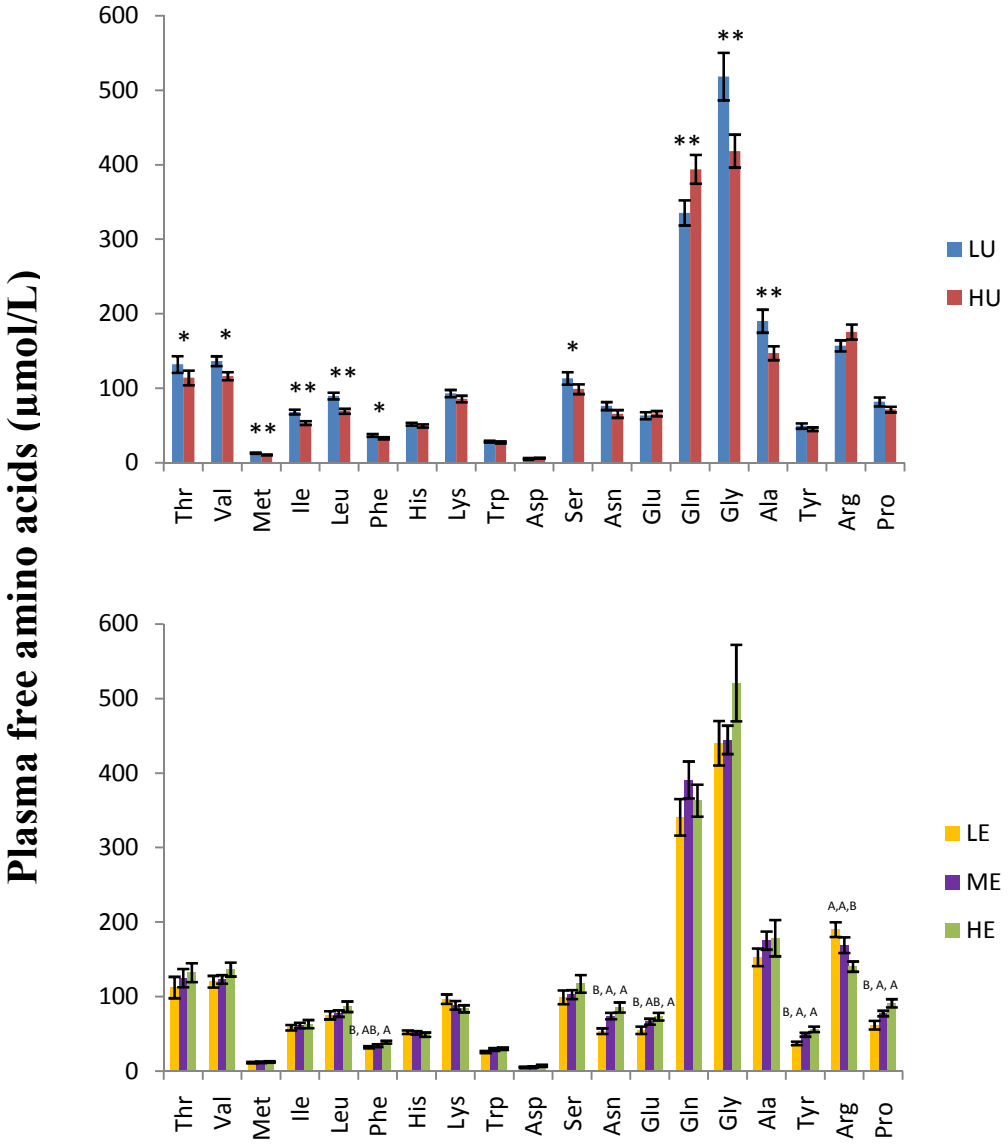

Fig 11 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on plasma amino acids concentration (μmol/L) at pre-isotope infusion in sheep. * differ significantly (P<0.05); ** differ significantly (P<0.01); A and B differ significantly (P<0.05)

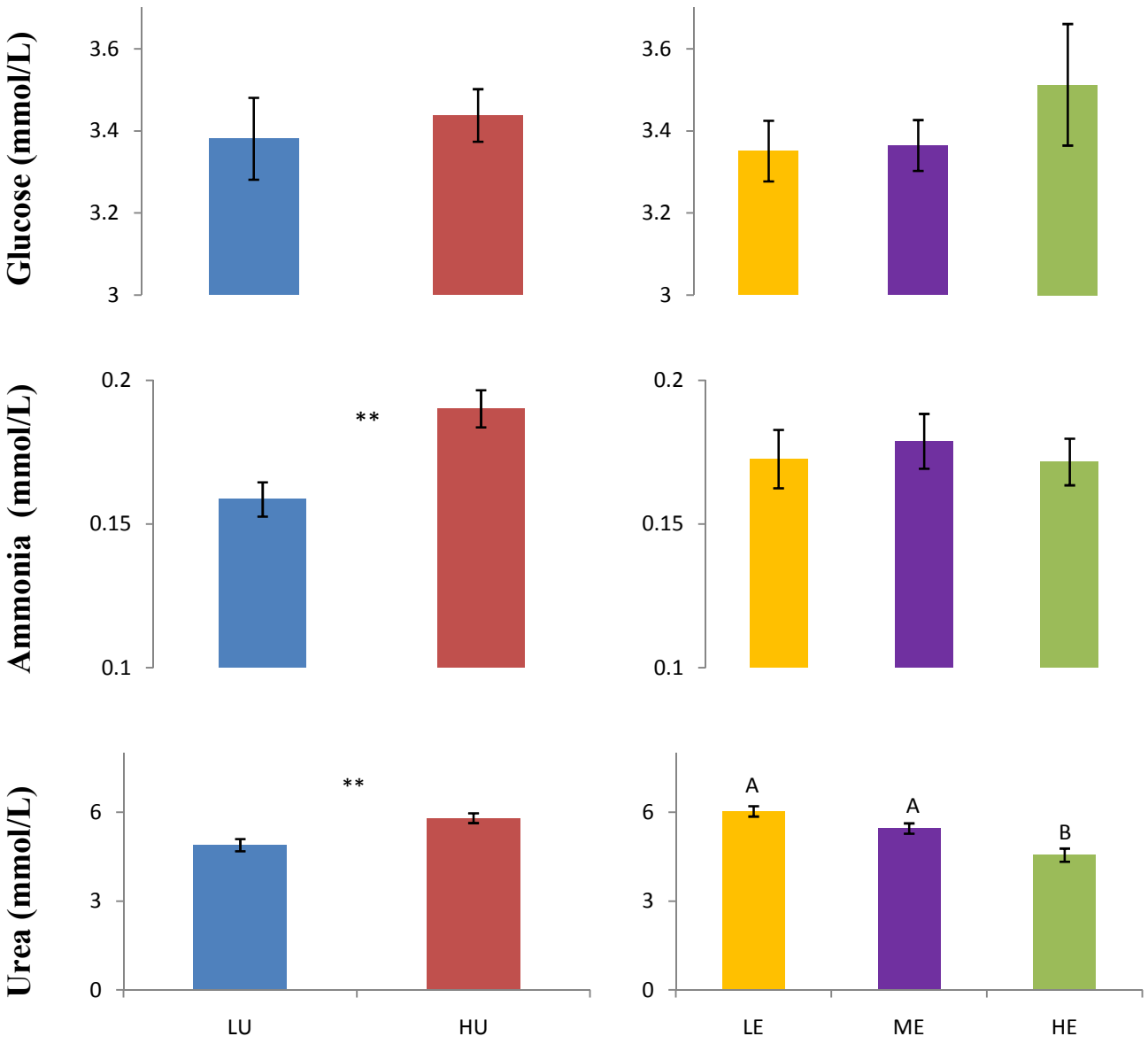

Fig 12 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on plasma glucose, ammonia and urea concentration (mmol/L) at pre-isotope infusion in sheep. ** differ significantly (P<0.01); A and B differ significantly (P<0.05)

Plasma glucose and urea kinetics

Plasma glucose concentration, [U-^{13}C]glucose enrichment, urea concentration and [$^{15}N_2$]urea enrichment almost reached in a steady state during 2.5 - 4 hours of isotope infusion in all dietary treatments as illustrated in Fig 13 and 14. Plasma glucose and urea kinetics during isotope infusion are presented in Table 6. Plasma glucose and urea turnover rates are illustrated in Fig 15.

Plasma glucose concentration was not influenced (P>0.05) by urea and energy treatments and interaction effect of urea x energy was not found (P>0.05). Urea treatment did not influence (P>0.05) on plasma glucose turnover rate. However, plasma glucose turnover was positive influenced (P<0.05) according with energy levels (LE≤ME≤HE) without (P>0.05) interaction effect of urea x energy. Plasma urea concentration and the turnover rate were higher (P>0.05) in high urea level than low urea level. Plasma urea turnover rate was highest (P<0.05) in low energy than middle and high energy (LE>ME~HE), while no interaction effect (P>0.05) of urea x energy was observed.

Table 6 Effect of urea and energy levels on plasma glucose and urea kinetics during 2.5-4 hours of primed continuous infusion in sheep

Plasma kineticss	**Low Urea**			**High Urea**			**Pooled**	**P value**		
	Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy	**s.e.m.**	Urea	Energy	Urea x energy
Glucose concentration (mmol/L)	3.4	3.4	3.4	3.4	3.5	3.6	0.1	0.22	0.78	0.50
Glucose turnover rate (mmol/(kg$^{0.75}$·h))	1.0	1.2	1.4	1.0	1.3	1.7	0.1	0.44	0.03	0.75
Urea concentration (mmol/L)	5.4	4.9	3.5	6.2	5.5	5.0	0.2	<0.01	<0.01	0.06
Urea turnover rate (mmol/(kg$^{0.75}$·h))	5.0	3.5	2.6	7.5	5.4	4.8	0.3	<0.01	<0.01	0.76

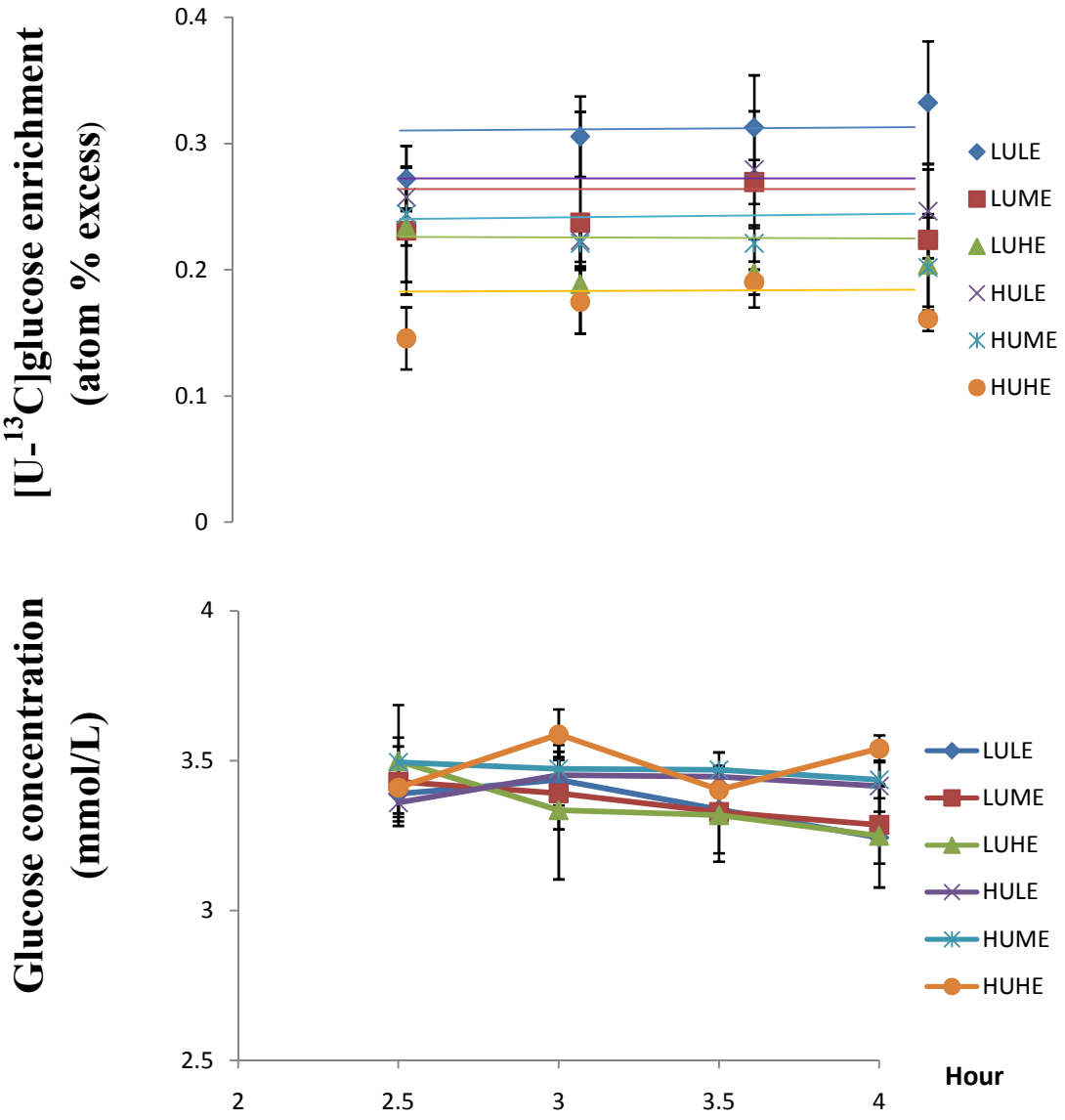

Fig 13 Time course change on plasma [U-^{13}C]glucose enrichment (atom % excess) and glucose concentration (mmol/L) during 2.5 – 4 hours of primed continuous infusion with six dietary treatments; Low Urea Low Energy (LULE), Low Urea Mid Energy(LUME), Low Urea High Energy (LUHE), High Urea Low Energy (HULE), High Urea Mid Energy (HUME) and High Urea High Energy (HUHE), respectively in sheep.

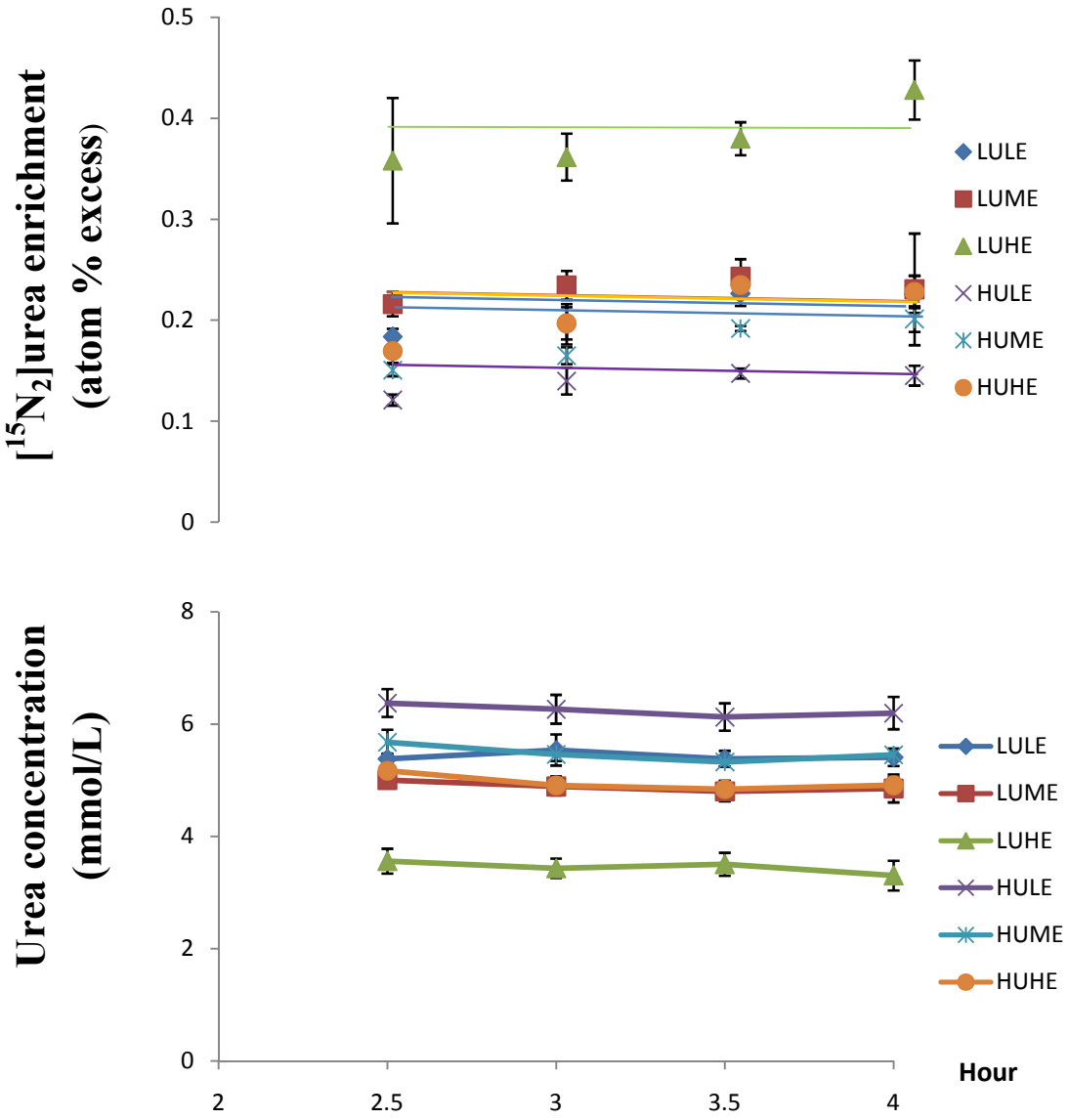

Fig 14 Time course change on plasma [$^{15}N_2$]urea enrichment (atom % excess) and urea concentration (mmol/L) during 2.5 – 4 hours of primed continuous infusion with six dietary treatments; Low Urea Low Energy (LULE), Low Urea Mid Energy(LUME), Low Urea High Energy (LUHE), High Urea Low Energy (HULE), High Urea Mid Energy (HUME) and High Urea High Energy (HUHE), respectively in sheep.

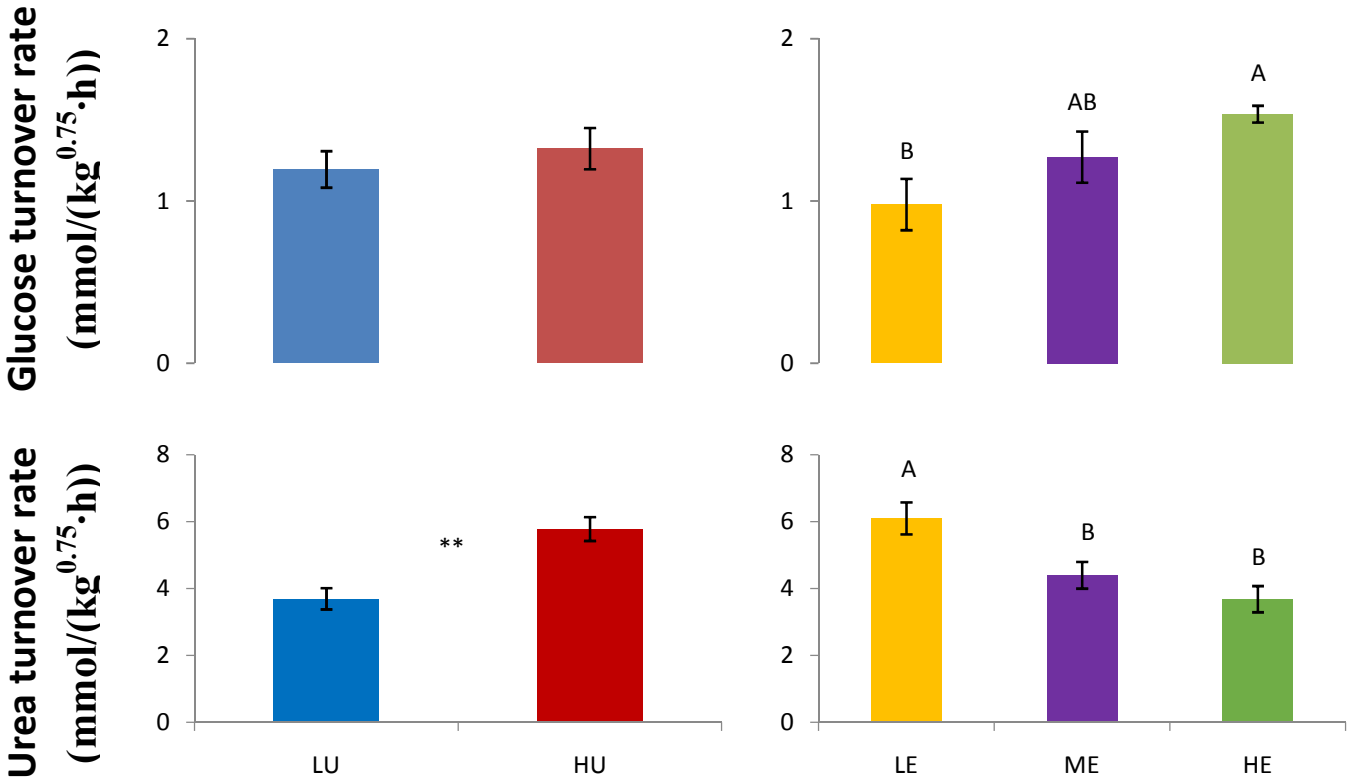

Fig 15 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on plasma glucose and urea turnover rate (mmol/(kg$^{0.75}$·h)) during 2.5 – 4 hours of primed continuous infusion in sheep.
** differ significantly (P<0.01); A and B differ significantly (P<0.05)

Microbial protein supply

Effects of urea and energy levels on PD excretion and MNS in sheep are illustrated in Table 7 and Fig 16 - 17. Urinary allantoin excretion had no difference (P>0.05) either in high or low urea level, but was higher (P<0.05) according with energy level (LE<ME<HE). Uric acid and xanthine plus hypoxanthine did not differ (P>0.05) among the dietary treatments. Total PD excretion had no difference (P>0.05) between high and low urea levels. However, total PD excretion was affected (P<0.05) by energy as LE≤ME≤HE and resulting to total MNS was also responded (P<0.05) by energy in the same way. Urea and energy interactions were not found (P>0.05) in all of MNS parameters.

Table 7 Effect of urea and energy levels on purine derivatives (PD) excretion and microbial nitrogen supply (MNS) in sheep

mmol/($kg^{0.75}$·day)	**Low Urea**			**High Urea**			**Pooled**	**P value**		
	Low Energy	Middle Energy	High Energy	Low Energy	Middle Energy	High Energy	**s.e.m.**	Urea	Energy	Urea x energy
Allantoin	0.24	0.34	0.45	0.26	0.33	0.41	0.02	0.64	<0.01	0.70
Uric acid	0.17	0.14	0.16	0.18	0.19	0.17	0.01	0.56	0.98	0.79
Xanthine + hypoxanthine	0.04	0.03	0.04	0.03	0.03	0.01	<0.01	0.05	0.44	0.26
Total PD	0.45	0.51	0.65	0.47	0.55	0.60	0.02	0.89	<0.01	0.58
Total MNS (g/($kg^{0.75}$·day))	0.35	0.43	0.56	0.39	0.46	0.51	0.02	0.94	<0.01	0.60

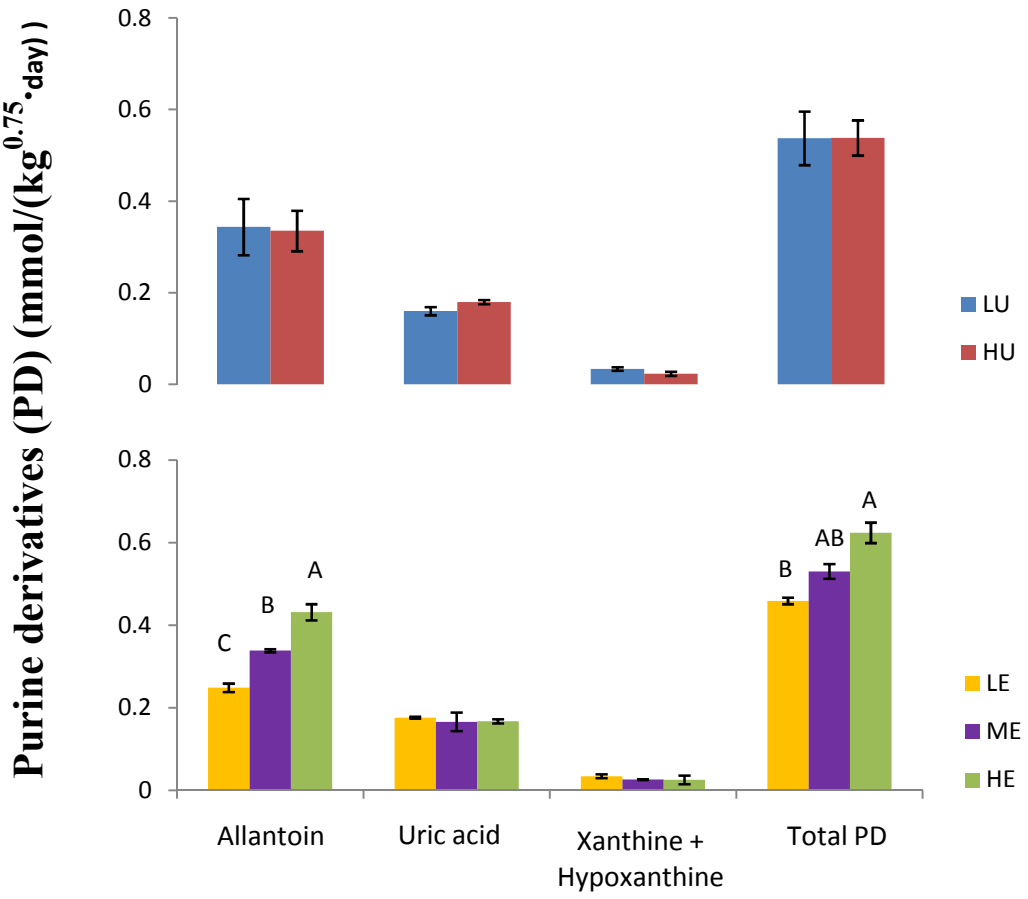

Fig 16 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on purine derivatives (PD) (mmol/(kg$^{0.75}$·day)) in sheep. A, B and C differ significantly (P<0.05)

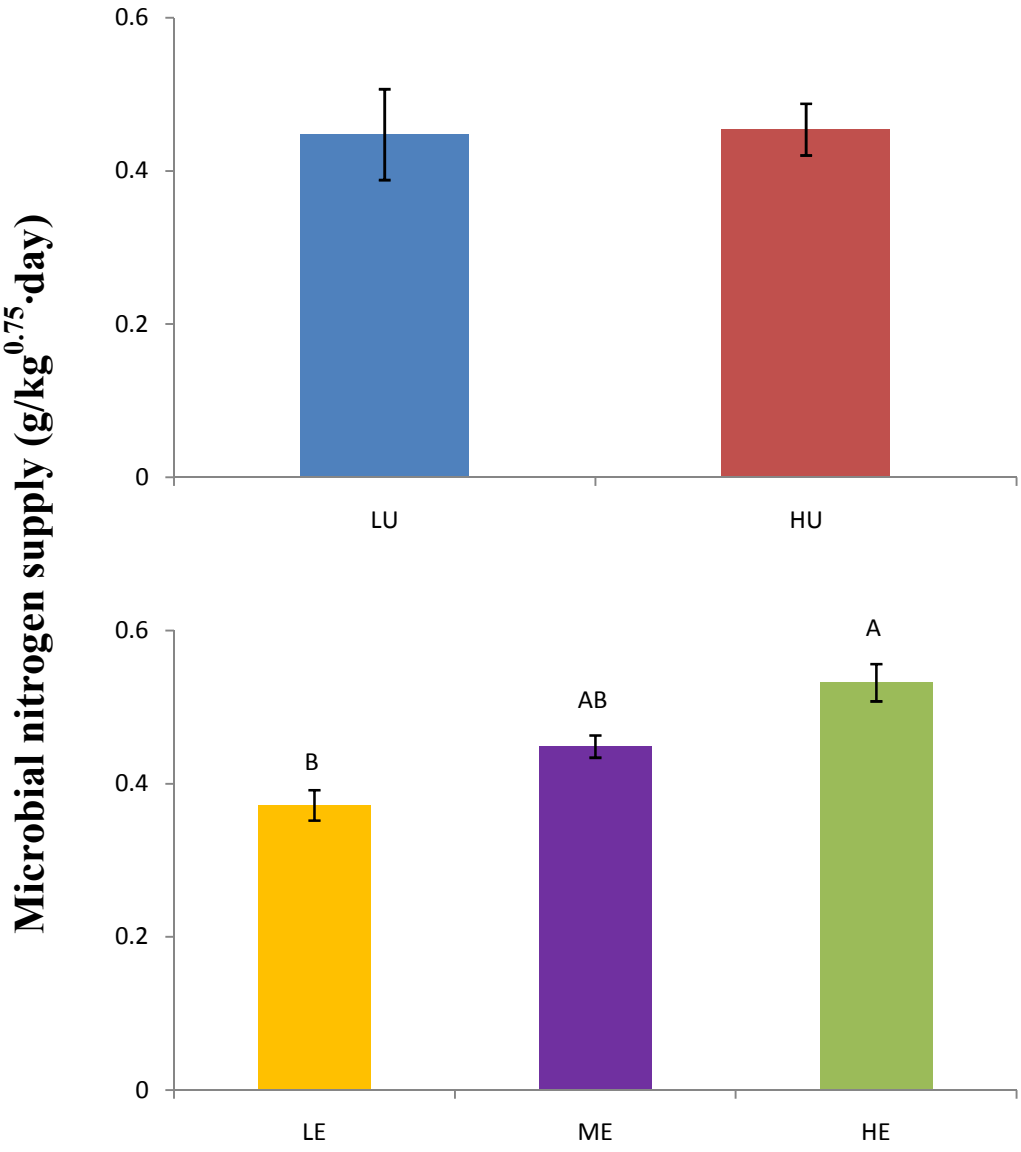

Fig 17 Effects of urea levels (low urea LU and high urea HU) and energy levels (low energy LE , middle energy ME and high energy HE) on microbial nitrogen supply (g/(kg$^{0.75}$·day)) in sheep. A and B differ significantly (P<0.05)

Discussion

Effect of urea and NEB on glucose kinetics

Most of glucose concentrations were within glucose standard concentration of 1.7 – 3.6 mmol/L in sheep (Jackson and Cockcroft. 2002). Because blood glucose concentration is especially modulated by hormone regulation (Bergman et al. 1966), both urea and energy had no effects on basal glucose concentration at pre-isotope infusion.

In the first experiment, the result does not accord with hypothesis that the isonitrogenous replacement of soybean meal by urea could effect on plasma glucose turnover rate. The reason may be caused by low plasma ammonia concentration. In this second experiment, urea treatment increased from 0.8 g/ ($kg^{0.75}$·day) up to 1.1 g/ ($kg^{0.75}$·day) in high urea dietary treatment. However, the result was still same as previous experiment. High urea level treatment had no effects on plasma glucose turnover rate. Even plasma ammonia concentration was higher (0.15 – 0.19 mmol/L) than first experiment (0.12 – 0.14 mmol/L). The plasma ammonia concentration was still lower than 1 mmol/L that other studies successfully decrease the glucose production (Weekes et al. 1978; Demigné et al. 1991). Because rumen ammonia was utilized by rumen microbes and intrinsically related to energy supply from carbohydrate availability in rumen (Delgado-Elorduy et al. 2002; Reynolds 2006), rumen ammonia concentration was inversely accorded with energy levels.

Plasma glucose turnover rate was highest in high energy while lowest during low energy treatment. The result insisted that glucose production rate depends on energy availability as same as previous experiment. This result confirmed the hypothesis that NEB condition depress rate of glucose production and consistent with other studies (Steel and Leng, 1973; Brockman 1993; Danfaer et al. 1995).

Because VFA production depends on dietary intake level (Doreaua et al. 2003), total rumen VFA concentration was also positive accorded with energy levels. In the current study, interaction of urea x energy was identified (P=0.02) in rumen pH. The highest of rumen pH (7.00) was found in HULE and the lowest (6.36) was found in LUHE. This result might be due to urea hydrolysis elevates rumen pH (Kertz, 2010) in high urea treatment. Total ruminal VFA of high energy diet was build up and can reduce the rumen pH (Dijkstra et al., 2012) as a concomitant in the rumen.

Crush corn was a major component in high energy treatment in this study which is approximately 60% of rumen undegradable protein (Lardy, 2013). Because fecal N excretion increased linearly in response to increased rumen undegradable protein supplementation (Wright et al. 1998), fecal N excretion was greater in high energy than low energy. Moreover, high energy treatment was higher dietary intake which increases digesta and microbial flow as a result in enhanced total MNS production (Chen et al., 1992). In addition, superior microbial N supply in high energy level could be due to more efficient synchronizing between dietary energy and N supply (Sinclair et al., 1995).

Furthermore, high urea level in dietary ration also enhanced N absorption, N retention and N digestibility. These changes in N metabolism were probably associated with increased ammonia utilization. Greater N retention may indicate better N supply in high urea level treatment. This is in accordance with Habib et al. (2008). Addition of urea 10 g per day was significantly increased the capacity of sheep to retain more N in the body.

Effect of urea and NEB on urea kinetics

Rumen ammonia concentration was higher in high urea level treatment than low urea level treatment. This increasing of rumen ammonia concentration leads to elevate concentrations of ammonia and urea in blood (Lewis, 1957; Bartley et al., 1981). This phenomenon accorded with our results that plasma ammonia and urea concentrations at pre-isotope infusion were also higher in high urea than low urea level treatment. However, plasma urea concentration remained in normal blood concentration (3 – 10 mmol/L) in sheep (Jackson and Cockcroft 2002).

Following the hypothesis in the study, NEB condition was associated with depressed capacity of hepatic ureagenesis or decreased plasma urea turnover rate. A contrary result was showed that plasma urea turnover rate was higher during NEB induction. The primary possible might be due to lack of severe NEB induction and lack of high lipid accumulation in liver cells to impair liver functions (Zhu *et al*. 2000; Overton 2003). Even NEB induction in this experiment (167 kJ/ ($kg^{0.75}$·day)) was lower than previous experiment (222 kJ/ ($kg^{0.75}$·day)), the result still corroborated with early experiment. Plasma urea turnover rate was higher in lower energy than high energy treatments. Moreover, plasma urea turnover rate was also higher in high urea level than low urea level, because urea pool size increased and positively responded with increasing of dietary protein content in the ration (Cocimano and Leng, 1967; Zhou et al., 2015).

Serum NEFA concentration was highest (1.24 mmol/L) in low energy treatment and was lowest in high energy treatment (0.18 mmol/L) in day 5 of NEB induction. This value was higher than previous study (1.15 mmol/L) in the same period of NEB induction and higher than normal level (<0.4 mmol/L) in sheep as well. This result presented the mobilization of NEFA from adipose tissue, especially in low energy treatment. Serum BHBA concentration increased (approximate from 0.3 to 0.4 mmol/L) according with energy level. Nevertheless, serum BHBA

concentration remained in the normal range (<0.8 mmol/L) in sheep (Cynthia, 2010). It reveals that NEFA mobilization may have satisfied the energy balance without activation of ketogenesis. Lowest N retention was also found in low energy treatment cause by NEB as same as previous experiment.

Changes in the amounts of plasma proteins may result from alteration in synthesis, catabolism or from protein losses. Albumin level reflects the balance between hepatic synthesis from dietary nitrogenous intake and endogenous demands/losses. Moreover, the serum albumin level was a negative correlation between the severities of fatty accumulation in the liver (Sevinc and Aslan, 1998; Sevinc et al., 2001). However, serum TP and ALB concentrations had no influence by all dietary treatments and were kept in normal level (5.9 – 7.8 and 2.7 – 3.7 g/dL) in sheep (Cynthia, 2010). It might be declare that the liver functions were not severely impaired by dietary treatments.

Although, serum globulin concentration was not affected by all dietary treatments, the concentration was lower (2.55 -2.74 g/dL) than the standard level (3.2 – 5 g/dL) in sheep (Cynthia, 2010). Low serum globulin level typically indicates liver disease, inability of digest or absorbs dietary protein and inflammatory bowel diseases. Another possibility might be related with lower immunoglobulins (Kaneko et al. 1997). However, the reasons were still unclear in this study.

Plasma free amino acid concentrations in this study were various responded with urea and energy treatments. Almost of free amino acid concentrations were lower in high urea level than low urea level treatment. A similar trend was found in sheep fed urea as source of N in rations had lowered levels of branched-chain amino acids (valine, isoleucine, and leucine), lysine and phenylalanine (Bergen et al., 1973; Young et al., 1973). Chalupa et al. (1970) reasoned that

feeding urea as the sole N source caused decreases in activities of carbamyl phosphate synthetase, ornithine transcarbamylase and arginase. Decreases in these enzyme systems were concluded to be the result of ammonia causing derangements in cellular energy metabolism or suboptimum amino acids.

Almost of free amino acid concentrations were also lower during NEB due to act as gluconeogenic substrate (Bergman and Heitmann 1978; Overton et al. 1999), except arginine concentration was higher. Arginine plays an important role in removing ammonia in urea cycle which is catalyzed from arginosuccinate by using arginosuccinate lyase. An increase in plasma arginine concentration may be associated with the change in plasma urea N concentration, because plasma arginine was synthesized in urea cycle (Fujita et al., 2006).

Urinay N excretion was higher during low energy treatment and also higher during treated with high urea level diet. This result elucidated inefficiency of ammonia utilization in the rumen, because insufficient energy in the ration (Lindberg and Jacobsson, 1990; Henning et al., 1993). These mentions are proved by the result of lower total MNS in lower energy levels. Moreover, the liver increased hepatic catabolism of amino acids in responding with glucose demand during NEB. This mechanism will be increased ammonia production and urea excretion in the urine (Overton et al., 1999). However, in this study cannot distinguish apart of the urea excretion caused by between unutilized or catabolic ammonias due to study design.

Chapter-4

Summary and conclusions

For economical reason, urea could be substituted for rumen degradable protein in ruminant diet because ammonia from urea can be utilized by rumen microbes to form microbial protein. Rumen ammonia, blood ammonia and blood urea concentrations were elevated when urea was hydrolyzed by rumen microbes. High blood ammonia concentration was negative relation with hepatic gluconeogensis. Moreover, negative energy balance (NEB) situation in ruminant production cycle, early lactation period in dairy cow or late gestation in ewe, was associated with depressed gluconeogenesis and also decreased capacity of ureagenesis. Therefore, urea usage during NEB may be argued as it compromises the hepatic gluconeogenesis and ureagenesis under practical feeding condition. It was hypothesized that the urea usage during NEB condition in practical feeding would be more depress rate of gluconeogenesis and ureagenesis.

The first experiment, the objective was to determine effects of isonitrogenous replacing soybean meal by urea and NEB on nitrogen balance, ruminal fermentations, plasma glucose and urea kinetics in sheep. A crossover design with two different types of isonitrogenous source, either soybean meal or replaced with urea, was assigned to six sheep. High energy diet following with low energy diet treatment was nested in each isonitrogenous source for NEB induction. Plasma glucose and urea turnover rates were determined by the primed continuous infusion. NEB induction was modest damage with indication by increased (P<0.01) of serum non-esterified fatty acid (NEFA) concentration, while triglyceride and β-hydroxybutyric acid (BHBA) concentrations did not change. Nitrogen retention was lower (P<0.01) in low energy.

Rumen pH and ammonia concentration were higher with urea replacement (P=0.04) and low energy (P<0.01). Almost all rumen volatile fatty acid (VFA) concentrations and plasma free amino acid concentrations were lower (P<0.01 and P<0.05) in low energy. Plasma glucose and ammonia concentrations were not influenced by urea replacement and low energy. The glucose turnover rate was lower (P<0.01) in low energy. However, plasma urea concentration and turnover rate were higher (P<0.01) in low energy while had no (P>0.05) effects of urea replacement. These findings suggest that NEB has strong influence on both of glucose and urea kinetics without urea replacement effect and interaction of urea x energy.

The result from first experiment does not accord with hypothesis that the isonitrogenous replacement of soybean meal by urea could effect on plasma glucose turnover rate. The reason may be caused by lower plasma ammonia concentration when compared with other studies that successfully decrease the glucose production. For achieved the hypothesis, urea treatment was increased in second experiment. Moreover, a contrary result from first experiment was showed that plasma urea turnover rate was higher during NEB induction. The primary possible might be due to lack of severe NEB induction and lack of high lipid accumulation in liver cells to impair liver functions. Therefore, NEB induction in second experiment was intended to lower than first experiment.

The second experiment, the objective was to clarify effects of high urea supplementation and negative energy level on nitrogen balance, ruminal fermentations, microbial protein supply, plasma glucose and urea kinetics in sheep. The experiment was carried out by using 6 x 5 incomplete Latin square designs. The six dietary treatments composed of two levels of urea (low and high) and three levels of energy (low, middle and high) were randomly introduced to six sheep. Plasma glucose and urea turnover rates were determined by using as same as previous

method. NEB induction was mild damage with indicate by increase (P<0.01) of serum NEFA concentration according with lower energy levels, while serum BHBA concentration kept in normal range in sheep. Serum total protein, albumin and globulin concentrations did not change. Nitrogen retention was higher (P=0.01) in high energy than lower energy treatment and was also higher (P<0.01) in high urea level than low urea level. The interaction effect of urea x energy with different responding of dietary treatments in rumen pH was identified (P=0.02). Rumen ammonia concentration was higher (P<0.01) in low energy than high energy treatment and was higher (P<0.01) in high urea level than low urea level as well. Total rumen VFA, acetate and propionate concentrations were higher (P<0.01) according with energy levels. Almost of free amino acid concentrations were lower (P<0.05) in high urea level than low urea level treatment and were also lower (P<0.05) during low energy, except arginine concentration was higher (P<0.01). Plasma glucose concentration was not influenced by any dietary treatments. Plasma ammonia and urea concentrations were higher (P<0.01) in high urea level than low urea level while plasma urea concentration was lower (P<0.01) in high energy than lower energy levels. Total microbial nitrogen supply increased (P<0.01) related with increasing of energy levels. The plasma glucose turnover rate was positive related (P=0.03) according with energy levels, but the plasma urea turnover rate was negative related (P<0.01) with energy levels and was higher (P<0.01) with high urea level treatment. These findings conclude that NEB crucially influences on both glucose and urea kinetics independently without interactions of urea x energy. Urea level supplementation was positive related with ureagenesis.

Finally, the results of this study not accorded with the hypothesis. The major reason is insufficient NEB due to physiological status of the experimental animals. Even NEB was induced by feeding half or one-third of maintenance energy level, the animals can recover in

energy homeostasis. However, the natural NEB by increase energy demands, lactating in dairy or high fetal growth rate in ewe, may influence on glucose and urea metabolisms following in this hypothesis.

General conclusion

Soybean meal replaces with urea or high level of urea supplementation in the ration did not affect on glucose turnover rate. However, low energy treatment seriously decreases glucose turnover while increases urea turnover rate independently without interaction effects of urea and energy. The implication of this study raveled that urea replacement or low urea level supplementation was suggested in dietary ration due to limited effects on glucose and urea metabolisms. Unfortunately, NEB is the major inescapable situation with influences on both glucose and urea metabolisms in sheep. Further investigations need to find on the compromising ways between glucose and urea metabolisms during NEB.

References

Adams, D., McKinle, M., 2009. The sheep. ANZCCART Fact Sheet, Faculty of Veterinary Science, University of Sydney. pp. 1-13.

AFRC, 1993. Energy and Protein Requirements of Ruminants. CAB International, Wallingford, Oxfordshire.

Aiello, R.J., Armentano, L.E., 1987. Gluconeogenesis in goat hepatocytes is affected by calcium, ammonia and other key metabolites but not primarily through cytosolic redox state. Comp. Biochem. Physiol. 88, 193.

AOAC, 1990. Official Methods of Analysis of International Association of Official Analytical Chemists. 16th ed., AOAC International, Arlington.

Barley, E.E., Avery, T.B., Nagaraja, T.G., Watt, B.R., Davidovich, A., Galitzer, S., Lassman, B., 1981. Ammonia toxicity in cattle. V. Ammonia concentration of lymph and portal, carotid and jugular blood after the ingestion of urea. J. Anim. Sci. 53, 494-498.

Bauman, D.E., Currie, W.B., 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63, 1514–1529.

Bergen, W.G., Henneman, H.A., Magee, W.T., 1973. Effect of dietary protein level and protein source on plasma and tissue free amino acids in growing sheep. J. Nutr. 103, 575-585.

Bergman, E.N., Heitmann, R.N., 1978. Metabolism of amino acids by the gut, liver, kidneys and peripheral tissues. Fed. Proc. 37, 1228-1232.

Bergman, E.N., Roe, E.R., Kon, K., 1966. Quantitative aspects of propionate metabolism and gluconeogenesis in sheep. Am. J. Physiol. 211, 793-799.

Bertics, S.J., Grummer, R.R., Cadorniga-Valino, C., Stoddard, E.E., 1992. Effect of prepartum dry matter intake on liver triglyceride concentration and early lactation. J. Dairy Sci. 75, 1914–1922.

Brockman, R.P., 1993. Glucose and short-chain fatty acid metabolism. In: Quantitative aspects of ruminant digestion and metabolism, CAB International. pp. 249–265.

Butler, W.R., 1998. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J. Dairy Sci. 81, 2533-2539.

Butler, W.R., Calaman, J.J., Beam, S.W., 1996. Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle. J. Anim. Sci. 74, 858-865.

Butler, W.R., Smith, R.D., 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J. Dairy Sci. 72, 767–783.

Cameron, R.E., Dyk, P.B., Herdt, T.H., Kaneene, J.B., Miller, R., Bucholtz, H.F., Liesman, J.S., Vandehaar, M.J., Emery, R.S., 1998. Dry cow diet, management, and energy balance as risk factors for displaced abomasum in high producing dairy herds. J. Dairy Sci. 81, 132-139

Chalupa, W., Clark, J., Opliger, P., Lavker, R., 1970. II Detoxication of ammonia in sheep fed soy protein or urea. J. Nutr. 100, 170-176.

Chen, X.B., 1989. Excretion of purine derivatives by cattle and sheep and its use for the estimation of absorbed protein. Ph.D Dissertation. Aberdeen Univ.

Chen, X.B., Gomes, M.J., 1992. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives - An overview of the technical details. International Food Resources Unit, Rowett Research Institute, Bucksburn Aberdeen AB2 9SB.

Chen, X.B., Hovell, F.D.D., Ørskov, E.R., Brown, D.S., 1990. Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivatives excretion by cattle. Br. J. Nutr. 63, 131.

Cherdthong, A., Wanapat, M., 2010. Development of urea products as rumen slow-release feed for ruminant production: A review. Aust. J. Basic Appl. Sci. 4, 2232-2241.

Cocimano, M.R., Leng, R.A., 1967. Metabolism of urea in sheep. Brit. J. Nutr. 21, 353-371.

Cynthia, M.K., 2010. The Merk Veterinary Manual. 10th ed., MERK & CO. INC., Whitehouse station.

Danfaer, A., 1994. Nutrient metabolism and utilization in the liver. Livest. Prod. Sci. 34, 115-127.

Danfaer, A., Tetens, V., Agergaard, N., 1995. Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. Comp. Biochem. Physiol. B111, 201–210.

Delgado-Elorduy, A., Theurer, C.B., Huber, J.T., Alio, A., Lozano, O., Sadik, M., Cuneo, P., De Young, H.D., Simas, I.J., Santos, J.E.P., Nussio, L., Nussio, C., Webb, Jr.K.E., Tagari, H., 2002. Splanchnic and mammary nitrogen metabolism by dairy cows fed steam-rolled or steam-flaked corn. J. Dairy Sci. 85, 148–159.

Demigne´, C., Yacoub, C., Morand, C., Re ´me´sy, C., 1991. Interactions between propionate and amino acid metabolism in isolated sheep hepatocytes. Br. J. Nutr. 65, 301.

Dijkstra, J., Ellis, J.L., Kebreab, E., Strathe, A.B., López, S., France, J., Bannink, A., 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Tech. 172, 22-33.

Doherty, M.L., 2002. Fatty liver syndrome in cattle (lipidosis). In: Animal health and production compendium. Wallingford, CAB International.

Doreaua, M., Michalet-Doreau, B., Grimaud, P., Atti, N., Nozière, P., 2003. Review: Consequences of underfeeding on digestion and absorption in sheep. Small Ruminant Res. 49, 289-301.

Duffield, T.F., Lissemore, K.D., McBride, B.W., Leslie, K.E., 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. J. Dairy Sci. 92, 571–580.

EL-Sabagh, M., Sugino, T., Obitsu, T., Taniguchi, K., 2009, Effect of energy intake on splanchnic net flux and whole body balance of nitrogen in mature sheep fed lucerne hay cubes. In: Ruminant physiology: digestion, metabolism and effects of nutrition on reproduction and welfare. Wageningen academic publisher. pp. 170-171.

Elrod, C.C., Butler, W.R., 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. J. Anim. Sci. 71, 694-701.

Elrod, C.C., Van Amburgh, M., Butler, W.R., 1993. Alterations of pH in response to increased dietary protein in cattle are unique to the uterus. J. Anim. Sci. 71, 702-706.

Emery, R.S., Liesman, J.S., Herdt, T.H., 1992. Metabolism of long chain fatty acids by ruminant liver. J. Nutr. 122, 832–837.

Esposito, G., Irons, P.C., Webb, E.C., Chapwanya, A., 2014.Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim. Reprod. Sci. 144, 60-71.

Ferguson, J.D., 2007. Protein and fertility. In: Proc. The 5th mild-Atlantic nutrition conference. Maryland Feed Industry Council, Timonium. pp. 109-114.

Ferguson, J.D., Blanchard, T., Galligan, D.T., Hoshall, D.C., Chalupa, W., 1988. Infertility in dairy cattle fed a high percentage of protein degradable in the rumen. J. Am. Vet. Med. Assoc. 192, 659-662.

Ferguson, J.D., Galligan, D.T., Blanchard, T., Reeves, M., 1993. Serum urea nitrogen and conception rate: the usefulness of test information. J. Dairy Sci. 76, 3742-3746.

Fujita, T., Kajita, M., Sano, H., 2006. Response of whole body protein synthesis, nitrogen retention and glucose kinetics to supplemental starch in goats. Comp. Biochem. Physiol. B144, 180-187.

Gath, V.P., Lonergan, P., Boland, M.P., O'Callaghan, D., 1999. Effect of diet type on establishment of pregnancy and embryo development in heifers. Theriogenology 51, 224.

Goff, J.P., 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176, 50–57.

Goff, J.P., Horst, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80, 1260–1268.

Golombeski, G.L., Kalscheur, K.F., Hippen, A.R., Schingoethe, D.J., 2006. Slow-release urea and highly fermentable sugars in diets fed to lactating dairy cows. J. Dairy Sci. 9, 4395–4403.

Habib, G., Raza, M., Saleem, M., 2008. Effect of tree leaves with or without urea as a feed supplement on nutrient digestion and nitrogen balance in sheep. Anim. feed sci. tech. 144, 335-343.

Hammond, A.C., 1997. Update on BUN and MUN as a guide for protein supplementation in cattle. In: Proc. Florida Ruminant Nutr Symp. Univ Florida, Gainesville. pp. 43-52.

Henning, P.H., Steyn, D.G., Meissner, H.H., 1993. Effect of synchronization of energy and nitrogen supply on ruminal characteristics and microbial growth. J. Anim. Sci. 71, 2516- 2528.

Highstreet, A., Robinson, P.H., Robison, J., Garrett, J.G., 2010. Response of Holstein cows to replacing urea with a slowly rumen released urea in a diet high in soluble crude protein. Livest. Sci. 129, 179–185.

Huggett, A.G., Nixon, D.A., 1957. Enzymatic determination of blood glucose. Biochem. J. 66, 368-370.

Information and communication technology center, 2014. Dairy farmer data 2014. Department of Livestock Development, Thailand. http://ict.dld.go.th/th2/index.php/th/report/276-report-thailand-livestock/reportsurvey56/485-report56-milk/. Accessed March, 2015. (in Thai).

Ingvartsen, K.L., 2006. Feeding and management-related diseases in the transition cow. Anim. Feed Sci.Tech. 126, 175–213.

Jackson, G.G.P., Cockcroft, D.P., 2002. Clinical Examination of Farm Animals. Blackwell science.

Johnson, L.J., Dinusson, W.E., Erickson, D.O., 1979. Nitrogen in animal production. Farm Res. 37, 30-36.

Kaneko, J.J., Harvey, J.W., Bruss, M.L., 1997. Clinical Biochemistry of Domestic Animals. 5th ed., Academic Press, San Diego, California, pp. 932.

Kehren, T., Tisdell, C.A., 1996. Women and common property resources in the management and health of livestock in Thai villages. Research Papers and Reports in Animal Health Economics, The University of Queensland, Brisbane 24.

Kertz, A.F., 2010. Review: Urea feeding to dairy cattle: a historical perspective and review. Prof. Anim. Sci. 26, 257-272.

Kohn, R., 2007. Use of milk and blood urea nitrogen to identify feed management inefficiencies amd estimate nitrogen excretion by dairy cattle and other animals. In: Florida ruminant nutrition symposium. Best western gateway grand, Gainesville.

Krebs, H.A., Lund, P., Stubbs, M., 1979. Interactions between gluconeogenesis and urea synthesis. In: Gluconeogenesis: Its regulation in mammalian species. John Wiley & Sons.

Lardy, G., 2013. Feeding corn to beef cattle. NDSU extension service, North Dakota State University, Fargo, North Dakota.

Laven, R.A., Drew S.B., 1999. Dietary protein and the reproductive performance of cows. Vet. Rec. 145, 687-695.

Lean, I.J., 2011. Non-Infectious Diseases: Ketosis. Encyclopedia of Dairy Sciences. 2nd ed., Vol. 2, Camden, Elsevier. pp, 815–823.

LeBlanc, S.J., Leslie, K.E., Duffield, T.F., 2005. Metabolic predictors of displaced abomasum in dairy cattle. J. Dairy Sci. 88, 159–170.

Lewis, D., 1957. Blood-urea concentration in relation to protein utilization in the ruminant. J. Agri. Sci. 48, 438-446.

Lindberg, J.E., Jacobsson, K.G., 1990. Nitrogen and purine metabolism at varying energy and protein supplies in sheep sustained on intragastric infusion. Brit. J. Nutr. 64, 359-370.

Maltby, S.A., Lomax, M.A., Beever, D.E., Pippard, C.J., 1991. Effect of increased ammonia and amino acid supply on post-prandial portal drained viscera and hepatic metabolism

in growing steers fed maize silages. In: Energy metabolism of farm animals. ETH-Zurich, pp, 23.

Markvichitr, K., Tumwasorn, S., Thanindratarn, B., 1995. Losses due to health and fertility problems in purebred Holstein Friesian cows under Thai farmer condition. Thai J. Agric. Sci. 28, 201-207.

McNamara, J.P., 1991. Regulation of adipose tissue metabolism in support of lactation. J. Dairy Sci. 74, 706–719.

Meijer, A.J., Gimpel, J.A., Deleeuw, G., Tischler, M.E., Tager, J.M., Williamson, J.R., 1978. Interrelationships between gluconeogenesis and ureogenesis in isolated hepatocytes. J. Biol. Chem. 253, 2308-2320.

Melendez, P., Donovan, A., Hernandez, J., 2000. Milk urea nitrogen and infertility in Florida Holstein cows. J. Dairy Sci. 83, 459-463.

MooNey, P., O'Donovan, D.J., 1970. The permeability of the rumen to simple nitrogenous compounds. Biochem. J. 119, 18-19.

National Research Council, 1985. Nutrient requirement of sheep. 6th ed., National Academy Press, Washington, DC.

Nolan, J.V., Leng, R.A., 1970. Metabolism of urea in late pregnancy and the possible contribution of amino acid carbon to glucose synthesis in sheep. Brit. J. Nutr. 24, 905−915.

Nozière, P., Rémond, D., Bernard, L., Doreau, M., 2000. Effect of underfeeding on metabolism of portal-drained viscera in ewes. Brit. J. Nutr. 84, 821–828.

Ortigues-Marty, I., Vernet, J., Majdoub, L., 2003. Whole body glucose turnover in growing and non-productive adult ruminant: meta-analysis and review. Reprod. Nutr. Dev. 43, 371-383.

Overton, T.R., 2001. Healthy livers make for healthy cow. Adv. Dairy Tech. 13, 169-180.

Overton, T.R., 2003. Managing the metabolism of transition cows. In: Proc. The 6th westhern dairy management conference. Reno, Nevada. pp. 7-16.

Overton, T.R., Drackley, J.K., Ottemann-Abbamonte, C.J., Beaulieu, A.D., Emmert, L.S., Clark, J.H., 1999. Substrate utilization for hepatic gluconeogenesis is altered by increased glucose demand in ruminants. J. Anim. Sci. 77, 1940-1951.

Owens, F.N., Soderlund, S., 2006. Ruminal and postruminal starch digestion by cattle. In: Cattle grain processing symposium, Oklahoma State University. pp. 116-128.

Ørskov, E.R., 1986. Starch digestion and utilization in ruminants. J. Anim. Sci. 63, 1624- 1633.

Promma, A., Suriyasathaporn, S., 2005. Factors associated with milk urea nitrogen in lactating cows from dairy farm in Chiang Mai. D.V.M. thesis, Chiang Mai University, Thailand. (in Thai).

Rajala-Schultz, P.J., Saville, W.J.A., Frazer, G.S., Wittum, T.E., 2001. Association between milk urea nitrogen and fertility in Ohio dairy cows. J. Dairy Sci. 84, 482-489.

Reynolds, C.K., 2006. Production and metabolic effects of site of starch digestion in lactating dairy cattle. Anim. Feed Sci. Tech.130, 78–94.

Rhoads, M.L., Gilbert, R.O., Lucy, M.C., Butler, W.R., 2004. Effects of urea infusion on the uterine luminal environment of dairy cows. J. Dairy Sci. 87, 2896-2901.

Roselere, D.K., Ferguson, J.D., Sniffen, C.J., Herrema, J., 1993. Dietary protein degradability effects on plasma and milk urea nitrogen and milk nonprotein nitrogen in Holstein cows. J. Dairy Sci. 76, 525-534.

Rukkwamsuk, T., Wensing, T., Geelen, M.J., 1999. Effect of fatty liver on hepatic gluconeogenesis in periparturient dairy cows. J. Dairy Sci. 82, 500-505.

Sano, H., Shibasaki, S., Sawada, H., 2009. The effect of the source of nitrogen supplementation on nitrogen balance, rate of plasma leucine turnover, protein synthesis and degradation in sheep. Ach. Anim. Nutr. 63, 401-412.

Sarwar, M., Ajmal Khan, M., 2003. Nitrogen retention and chemical composition of urea treated wheat straw ensiled with organic acids or fermentable carbohydrates. Asian Australas J. Anim. Sci. 16, 1583-1592.

SAS, 1996. SAS/STAT® Software: Changes and Enhancement through Release 6.11. SAS Inst. Inc. Cary, NC.

Sevinc, M., Aslan, V., 1998. The relationship between parturient paresis and fat cow syndrome in dairy cows. Tr. J. of Vet. Anim. Sci. 22, 23-28.

Sevinc, M., Basoglu, A., Birdane, F.M., Boydak, M., 2001. Liver function in dairy cows with fatty liver. Revue de Medecine Veterinaire. (In France).

Simasatitkul, N., Phongphaew, A., Jierwiriyapant, P., ter Meulen, U., 1999. Disease prevention and control in dairy cattle of smallholders in Chiang Mai, Thailand. Deutcher Tropentag-Berlin.

Sinclair, L.A., Garnsworthy, P.C., Newbold, J.R., Buttery, P.J., 1995. Effects of synchronizing the rate of dietary energy and nitrogen release in diets with a similar carbohydrate composition on rumen fermentation and microbial protein synthesis in sheep. J. Agr. Sci. 124, 463-472.

Staples, C.R., Garcia-Bojalil, C., Oldick, B.S., Thatcher, W.W., 1993. Protein intake and reproductive performance of dairy cows: a review, a suggested mechanism, and blood and milk urea measurements. In: Proc.The fourth Florida Ruminant Nutri. Symp., Gainesville. pp. 37-52.

Steel, J.W., Leng, R.A., 1973. Effects of plane of nutrition and pregnancy on gluconeogenesis in sheep. Br. J. Nutr. 30, 451-473.

Strang, B.D., Bertics, S.J., Grummer, R.R., Armentano, L.E., 1998. Effect of long-chain fatty acids on triglyceride accumulation, gluconeogenesis, and ureagenesis in bovine hepatocytes. J. Dairy Sci. 81, 728-739.

Swenson, M.J., Reece, W.O., 1993. Water balance and excretion. In: Physiology of domestic animals, 11th ed., Cornell Univ. Press. pp. 573–604.

Tserng, K.Y., Kalhan, S.C., 1983. Estimation of glucose carbon recycling and glucose turnover with [U-^{13}C]glucose. Am. J. Physiol. 245, 476-482.

Visek, W.J., 1984. Ammonia: its effects on biological systems, metabolic hormones, and reproduction. J. Dairy Sci. 67, 481-498.

Weatherburn, M.W., 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971-974.

Weekes, T.E.C., 1991. Hormonal control of glucose metabolism. In: Physiological aspects of digestion and metabolism in ruminants. Academic Press. pp. 183-200.

Westwood, C.T., Lean, I.J., Kellaway, R.C., 1998. Indications and implications for testing of milk urea in dairy cattle: a quantitative review. Part 2. Effect of dietary protein on reproductive performance. N. Z. Vet. J. 46, 123-130.

Weekes, T.E.C., Richardson, R.I., Geddes, N., 1978. The effect of ammonia on gluconeogenesis by isolated sheep liver cells. Proc. Nutr. Soc. 38, 3.

Widiawati, Y., Teleni, E., Suharyono, S., 2014. Glucose metabolism in sheep fed grass supplemented with *Gliricidia Sepium*. Atom Indonesia 40, 121-127.

William, B., 2005. Risk factors for metabolic disease. In: Tri-State Dairy Nutrition Conference Proceedings Department of Veterinary Preventive Medicine, Ohio State University.

Wilton, J.C., Gill, M., Lomax, M.A., 1988. Uptake of ammonia across the liver of forage-fed cattle. Proc. Nutr. Soc. 47, 153.

Wittwer, F.G., Gallardo, P., Reyes, J., Optiz, H., 1999. Bulk milk urea concentrations and their relationship with cow fertility in grazing dairy herds in southern Chile. Prev. Vet. Med. 38, 159-166.

Wolfe, R.R., 1984. Tracers in Metabolic Research: Radioisotope and Stable Isotope/ Mass Spectrometry Methods. Liss, Inc.

Wood, P.L., Khan, M.A., Moskal, J.R., 2006. Neurochemical analysis of amino acids, polyamines and carboxylic acids: GC–MS quantitation of tBDMS derivatives using ammonia positive chemical ionization. J. Chromatogr. B831, 313-319.

Wright, T.C., Moscardini, S., Luimes, P.H., Susmel, P.. McBride, B.W., 1998. Effect of rumen undegradable protein and feed intake on nitrogen balance and milk protein production in dairy cows. J. Dairy Sci. 81, 784-793.

Wu, G., 2013. Amino Acids: Biochemistry and Nutrition, Taylor & Francis Group.

Young, A.W., Boling, J.A., Bradley, N.W., 1973. Performance and plasma amino acids of steers fed soybean meal, urea or no supplemental nitrogen in finishing rations. J. Anim. Sci. 36, 803-808.

Zhou, J.W., Guo, X.S., Degen, A.A., Zhang, Y., Liu, H., Mi, J.D., Long, R.J., 2015. Urea kinetics and nitrogen balance and requirements for maintenance in Tibetan sheep when fed oat hay. Small Ruminant Res. (In press).

Zhu, L.H., Armentano, L.E., Bremmer, D.R., Grummer, R.R., Bertics, S.J., 2000. Plasma concentration of urea, ammonia, glutamine around calving, and the relation of hepatic triglyceride, to plasma ammonia removal and blood acid-base balance. J. Dairy Sci. 83, 734-740.