CHAPTER 3

Materials and methods

3.1 Materials

3.1.1 Instruments and equipments

	Instrument and equipment	Company	Country
1	Asbestos	-	-
2	Balances	Mettler-Tooledo	Switzerland
3	Beaker 50, 100, 250, 500 ml	Pyrex	USA
4	Boiling sterilizer	Applied Mpedic	Thailand
5	Controlled intravaginal drug release (CIDR) devices	Pfizer Animal Health	New Zealand
6	Crucible Haldenwanger	-	-
7	Crucible fiber apparatus	Gerhardt	Germany
8	Desiccator	Glaswerk	Germany
9	Digestion apparatus	Buchi	Thailand
10	Dispensing tube	Buchi	Thailand
11	Distillation apparatus	Gerhardt	Germany
12	Drying oven	Memmert	Germany
13	Erlenmeyer Flask 50, 250, 500 ml	Schortt	Germany
14	Frizzier -20^{o}C	-	-
15	Flask heater	Gerhardt	Germany
16	Glass crucible	-	-
17	Hammer mill	Thomass-willey	-
18	Heat sealer	Audion Elektro	Netherlands
19	Hot plate	Northem chemical	Thailand
20	Incubator	Memmert	Germany
21	Insert device	-	Switzerland

22	Magnetic Stirrer	Slangor	Malays
23	Microscope Ultrasound	Honda Electronic	Japan
24	Muffle furnace 550^{o}C	Heraeus Hauau	-
25	Needle	-	-
26	Refrigerator	-	-
27	Round bottom 100, 500, 2000 ml	Schott	Germany
28	Soxhlet apparatus	-	-
29	Suction pump	W. Kranich	Germany
30	Syring	-	-
31	Titration apparatus	Brand	-
32	Volumetic flask 100, 5000 ml	Schott	Germany
33	Vortex mixer	Scientific industries	USA
34	Water bath	W. Krannich	Germany

3.1.2 Chemicals

Chemical		**Company**	**Country**
1	Acetone	Merck	Germany
2	NaOH	Univar	Australia
3	Boric acid	Merck	Germany
4	Calcium Oxide	-	-
5	Cety trimethlylamonium bromide	Merck	Germany
6	Chromic sulphuric	Merck	Germany
7	Cupper sulphate	Merck	Germany
8	Dichloromethane	-	-
9	Disodium hydrogen phosphate	Merck	Germany
10	Disodium ethylene diaminetetra	Kerck	Germany
11	Acetate (EDTA)	-	-
12	Ditilled water	-	-
13	2- ethoyethano	Merck	Germany
14	Human chorionic gonadotropin hormone (hCG)	Intervet International B.V	New Zealand
15	HCL	Merck	Germany
16	H_2SO_4	Merck	Germany

17	Selenium mixture	-	-
18	Sodium borate decahydrate	-	-
19	Sodium laury sulphate	Merck	Germany

3.2 Methods

3.2.1 Paper mulberry leaves collection

The fresh paper mulberry leaves were collected every day around the Northern Agriculture and Forestry College and nearby the villages, Luang prabang district and province, Laos. (Figure 3.1; Figure 3.2).

Figure 3.1 Paper mulberry leaves collection **Figure 3.2** Paper mulberry field

3.2.2 Concentrate preparation

The concentrate was formulated for approximately 15 % crude protein including corn 20 %, rice bran 30 %, soy bean meal 15 %, broken rice 32 %, premix 2 % and salt 1 % (Figure 3.3; Figure 3.4)

Figure 3.3 Concentrate preparation **Figure 3.4** Concentrate after mixed

3.2.3 Chemical analysis

The chemical composition (Dry mater, Organic matter, Crude fiber, Ether extract, Crude protein, Nitrogen free extract) of paper mulberry leaves and concentrate were analysed by proximate analysis (AOAC, 1990). Fiber content (Neutral detergent fiber, Aciad detergent fiber, Acid detergent lignin) of paper mulberry leaves and concentrate were analysed by detergent methods (Van Soest *et al.*, 1991).

Experiment I: Study on the effects of concentrate supplementation levels on growth performance of local female goats in Laos

3.2.4 Animals and housing

Twenty- four local female goats with average 10 months of age, and 22.5 ± 3.3 kg of live weight were used. All goats were houses in individual pen as in the Figure 3.5. The pen was made by wooden and roofed by tile with dimension of 1.0 m in width, 1.5 m in length, and 1.5 m in height (Figure 3.6).

Figure 3.5 Experimental housing

Figure 3.6 The local female goats in separated pens

3.2.5 Experimental design

The randomise completely block designed (RCBD) was applied in the present study. The animals were blocked according to live weight. The goats were divided into 4 treatments and 6 goats per treatment, by following of concentrate supplementation levels as follows:

T1 : Control, paper mulberry leaves (PML) *ad libutum*

T2 : PML *ad libutum* + Concentrate 200 g/head/day

T3 : PML *ad libutum* + Concentrate 300 g/head/day

T4 : PML *ad libutum* + Concentrate 400 g/head/day

3.2.6 Feeding

Animal were fed two times per day at 8:30 am and 16:00 pm. Each animal was provided with fresh water freely all time. The basal diet was paper mulberry leave (Figure 3.7) and supplementation of various level of concentrate (Figure 3.8) were used as in the treatment.

Figure 3.7 Weighed the paper mulberry leaves

Figure 3.8 Concentrate feeding

3.2.7 Data collection

1. Feed intake and average daily weight gain

The quantities of offered feeds and refusals were measured daily for 92 days. Goats were individually weighted for every week before feeding and watering in the morning to determine average daily gain (ADG) and to evaluated body weight changes.

2. Digestibility trial

After feeding trial, feces from each goat were weighted and recorded every day. The feces sample were collected for 7 days, then pooled and thoroughly mixed together. After that, 10 % of samples was taken and stored at -20^{o}C for further analysis (Figure 3.9). During feces collection, all feed offer and refusal were also collected to evaluate the percentage of digestibility. Sample of fed diets and feces were

used for the analysis to chemical composition by method of AOAC (1990). Neutral detergent fiber, and acid detergent fiber were determined by detergent method of (Van Soest *et al.*, 1991). The apparent digestibility was evaluated by nutrient in feed and nutrient in feces as the formula follow:

$$\text{Apparent digestibility (\%)} = \frac{\text{(Nutrient in feed - Nutrient in feces) x 100}}{\text{Nutrient in feed}}$$

The total digestible nutrient (TDN) was used the formula as follow:

TDN (%) = DCP + DCF + DNFE + (DEE x 2.25)

DCP = Digestible Crude Protein

DCF = Digestible Crude fiber

DNFE = Digestible Nitrogen Free Extract

DEE = Digestible Ether Extract

The digestible energy (DE) was used the formula as follow (NRC, 2001):

DE (Mcal/kg) = 0.04409 x TDN(%)

Metabolizable energy (ME_1) was used the formula as follow (NRC, 2001):

ME_1 (Mcal/kg) = -0.45 + 0.4453 TDN*

Metabolizable energy (ME_2) was used the formula as follow Shiemann *et al.* (1971) and Lee *et al.* (2000):

ME_2 (MJ/kg) = 15.2DCP + 34.2DEE + 12.8DCF + 15.9DNFE

Net energy for lactation (NE_L) was used the formula as follow (NRC, 2001):

NE_L (Mcal/kg) = 0.0245TDN - 0.12

Figure 3.9 Technique for collection of the feces

3.2.8 Statistical Analysis

The data were presented as mean ± SEM. The different between group of body weight (BW), average daily gain (ADG), and dry matter intake (DMI) were evaluated by ANOVA using the PROC MIXED. The significance of different between treatments mean were tested by using the Duncan's New Multiple Range Test (Steel *et al.*, 1997). All statements of significances were based on probability less than $P<0.05$.

Experiment II: Study on effect of concentrate supplementation levels on response to estrous synchronization of local female goats in Laos

3.2.9 Animal

Before starting the experiment, local female goats (13 months of age and 25.4 ± 0.7 kg of BW) were observed for one complete cycle (spontaneous estrous cycle) then were selected for study. All goats were determined to ensure an absence of reproductive problems and all goats remained healthy throughout the study. The estrous cycles of female goats were synchronized by CIDR devices (Eazi-Breed CIDR, 0.3 g P_4; Pfizer Animal Health, New Zealand) for 14 days. The estrous behavior was monitored in the presence of two males at 06:00 h and 18:00 h for 5 days, following the removal of CIDR devices. Commencement of estrus was defined as the time when the female goats first stood to be mounted by the male goats (Ozyurtlu *et al.,* 2010). The first day of estrus was designated as day 0 of the nutritional period. Consequently, 24 local female goats that exhibited estrus were selected for the dietary treatments.

3.2.10 Experimental design, feeding, and housing

Nutritional treatment was conducted for a period of 61 days, with 42 days for pre-synchronization (covering of two estrous cycles), 14 days for estrous synchronization, and 5 days for post-synchronization periods. Twenty-four local female goats were randomly assigned to either of the two dietary treatments as follows. Group 1 (PML; n=12): female goats which received paper mulberry leaves as the basal diet. Group 2 (PML + CONc; n=12): female goats which received paper mulberry leaves as the basal diet and 400 g/head/day of the concentrate, which contained 15 % of CP (were mixed by 30% rice bran; 20% corn; 15% soy bean meal; 32% broken rice; 2% premix; 1% salt). The experimental diets were fed twice daily at 08:30 h and 16:00 h. The feed refusals were removed and weighed each time. The female goats in the two groups were given *ad libitum* access to fresh clean water. The animals were kept in individual pens made of wood, with roofs of tiles, with the pens having the following dimension: 1.0 m in width, 1.5 m in length, and 1.5 m in height.

3.2.11 Estrous synchronization protocol

On day 42 of the nutritional treatments, all the female goats in the both groups received CIDR insert for estrus of 14 days, and animals were treated with an intramuscular injection (i.m.) of 300 international unit (IU) of human chorionic gonadotropin (hCG; Chorulon®, Intervet International B.V., New Zealand) at the removal of CIDR (day 56) (Navanukraw *et al.*, 2014) (Figure 3.10). After the withdrawal of CIDR devices, the overt signs of estrus were detected twice daily for a 5-day period (at 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, 96 h, 108 h, and 120 h after CIDR removal; Figure 3.11) with the aid of two male goats.

Figure 3.10 Technique for estrous synchronization in female goats

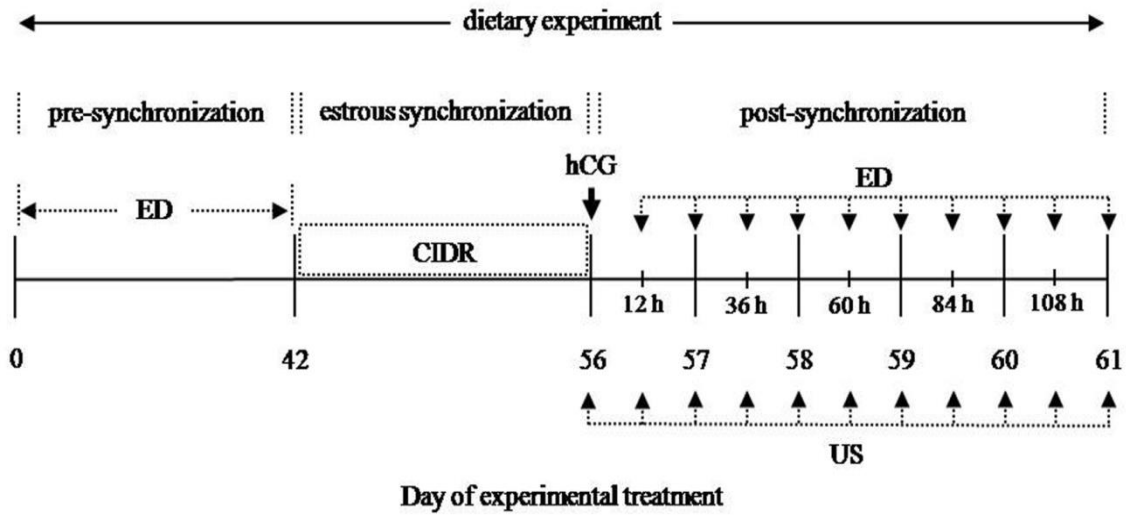

Figure 3.11 A schematic representation of the experimental design. CIDR = controlled intravaginal drug release devices; hCG= human chorionic gonadotropin; ED= estrous detection; US= ultrasonography.

3.2.12 Measurement of growth performance

During the nutritional treatments, all the animals were weighed individually each week before feeding and giving water in the morning. To calculate ADG, female goats were weighed at day 0 and day 61. The feed offer and the residual feed were collected individually every day in the morning, and the individual feed intake was used to calculate the individual DMI.

3.2.13 Metabolizable energy calculation

Animal was examined for the metabolizable energy (ME) intake of the feed that the goats had consumed. Feces of goats were collected individually from day 42 to day 61, which was in the periods of estrous synchronization and post-synchronization. The ME was calculated from the digestion coefficient, which was derived from the *invivo* digestion trial using the equation of Shiemann *et al.* (1971) and Lee *et al.* (2000), as follows:

$$ME\ (MJ/kg) = 15.2DCP + 34.2DEE + 12.8DCF + 15.9DNFE.$$

3.2.14 Estrous response

All the female goats in the two groups were monitored for estrus twice daily for a minimum of 30 min per detection with two teaser male goats within 12 h to 120 h after the removal of CIDR . The onset of estrus was defined as the time when the female goat first stood to be mounted by the male goats (Figure 3.12). The duration of estrus was defined as the interval between the onset of estrus and the end of estrus. The end of estrus was considered to be the time when the female goats did not accept the male goats. On the basis of response measurements, the estrous rate (number of female goats exhibiting estrus/total number of synchronized goats × 100; %), the interval to the onset of estrus (h), and the duration of estrus (h) were calculated for each of the treatment groups.

3.2.15 Ovarian ultrasonography

Ovarian follicular determination was carried out according to the procedures described by Moonmanee and Yammuen-art (2015) using transrectal ultrasonography (HS-1600V, Honda Electronics, Japan) . Briefly, the ovaries were scanned using transrectal ultrasonography with a 7.5 MHz rectal transducer on the day of CIDR withdrawal (day 56) to evaluate the diameter of the largest preovulatory follicle as shown in the Figure 3.12. Then, the size of the largest preovulatory follicle was measured and recorded on follicular maps, which allowed the identification for subsequent analyses. The ovaries were scanned by the same operator on the day of CIDR removal and every 12 h after CIDR withdrawal (up to 120 h if incidence of ovulation had not occurred) to evaluate the ovulation time.

Ovulation of the largest preovulatory follicle was considered to occur when only one follicle, greater than 5.0 mm in diameter and observed in the previous scanning, had disappeared (Menchaca *et al.*, 2007). On the basis of ovulatory measurements, the ovulation rate (number of female goats exhibiting ovulation/total number of synchronized goats × 100; %), the diameter of the preovulatory follicle (mm), the interval from the CIDR removal to ovulation (h), and the interval from estrus to ovulation (h) were calculated for each of the treatment groups.

Figure 3.12 Observation female goats after CIDR removal and ovaries scanning

3.2.16 Measurement of reproductive performance

After the end of the synchronization period, female goats exhibiting estrous behavior were taken to a separate pen where they were mated by natural service using fertile male goats. On the basis of reproductive measurements, the fecundity (number of pregnant female goats/number of female goats mating × 100; %), the infertility (number of non-pregnant female goats/number of female goats mating × 100; %), the fertility (number of female goats kidding/number of female goats mating × 100; %), the single kidding rate (number of female goats with single kid/number of female goats kidding × 100; %), the twin kidding rate (number of female goats with twin kids/number of female goats kidding × 100; %), the productivity (number of kids born alive/total number of female goats in each treatment group), the kid yield (number of kids born alive/total number of female goats mating), and the prolificacy (number of

kids born alive/total number of female goats kidding) were also calculated for each treatment groups (Haldar *et al.*, 2014; Gallego-Calvo *et al.*, 2015). Additionally, the average number of kids varies according to birth type and gender was also determined for each treatment groups.

Kid born After kidding kid cleaning

Figure 3.13 Kidding of female goat after supplementation with dietary treatment

3.2.17 Statistical analyses

The data are presented as mean ± SEM. The BW, BWC, ADG, FCR, the diameter of the largest preovulatory follicle, the time of ovulation, the interval to the onset of estrus, the duration of estrus, the productivity, the kid yield as well as the prolificacy were analyzed. Differences between the means were used for the student's *t*-test. The estrous rate, the ovulation rate, the fecundity, the infertility, the fertility**,** the kidding rate, and the proportion of kids varies according to gender were analyzed by chi-square analysis (Steel *et al.*, 1997). Differences with $P \leq 0.05$ were considered significant, and those with $0.05 < P < 0.10$ were considered a tendency (Lima *et al.*, 2009).

3.2.18 Experimental place and duration of the experiment

The study was conducted at the goat production farm of the Northern Agriculture and Forestry College (NAFC), at 20 km from Luang prabang district and Luang prabang province, Laos. Duration of the experiment was last for 7 months, started from March to October 2015.