CHAPTER V
EXPERIMENT 3
EFFECT OF STAIR-STEP FEEDING REGIMEN DURING ESTROUS CYCLE ON FERTILITY OF THAI-NATIVE GOATS

1 Introduction

Animals in the wild, particularly ruminants but also other herbivore, experience periods of alternating food abundance and poverty. Even under domestication the derivatives of these animals and others which humans have chosen to meet their needs do not always have sufficient food available at particular times to allow a full expression of their genetic potential for growth. In such cases a smooth progression along the sigmoid-shaped growth curve, predetermined for the individual by its genetic template, is disrupted. When this occurs and growth falls below genetic potential, it has been shown in many experiments that when food supplies again become abundant, growth rates accelerate and exceed those achieved by comparable animals fed well and continuously. This phenomenon is known as compensatory growth and is a term which may be regarded as synonymous with the often-used alternatives of catch-up growth, rebound growth and rehabilitative growth (Lawrence and Fowler, 2002).

Throughout the world, productivity of ruminants and profitability of their rearing depends in large part on climate and the resultant nutritional plane. Weather impacts both the quantity and quality of feedstuffs consumed by ruminants. An obvious practice to avert decreased productivity in dry periods is supplementary feeding. Hence, livestock producers must compare cost of supplementation with projected effects of not supplementing on current and future productivity. In addition, the maximum length of time with low nutrient intake that can be allowed without impairing future production potential is a consideration. In general, ruminants can partially or completely compensate for an earlier period of slow or no growth or BW loss with a low nutritional plane through increased feed intake and/or more efficient feed utilization (Hornick et al., 2000). The magnitude and nature of compensatory

growth is influenced by factors such as the severity of feed restriction, level of realimentation, characteristics of diets fed during and after nutrient restriction (e.g., dietary protein and energy concentrations), lengths of nutrient restriction and realimentation periods and breed and age of the animal (Horton and Holmes, 1978; Coleman and Evans, 1993; Drouillard et al., 1991; Hays et al., 1995; Hornick et al., 2000).

Recently, researchers have developed a nutrition regimen to a so called "stair-step feeding regimen" that is a combination of alternating dietary energy restriction and realimentation phases (Ford and Park, 2001; Park, 2005). The basic concept of this model is to exploit the biological nature of both dietary energy restriction and the compensatory growth phenomenon in concert with one or more hormone dependent allometric phases of body composition development (i.e., prepuberty, puberty, and late gestation). Energy restriction (i.e., providing all known essential nutrients but reducing caloric intake) has a profound influence on the biology and health of animals including the retardation of aging and the reduction of cancer incidence and other late-life diseases (Hursting et al., 2003). Through modulation of endocrine and enzymatic status, energy restriction shifts the physiological focus to energy-conserving activities, mainly maintenance and repair functions, and decreases certain energy-wasteful metabolic pathways (e.g., substrate cycles) that are not essential for growth. Realimentation (refeeding) after energy restriction induces compensatory growth, which is characterized by an accelerated anabolism, a reduced maintenance requirement, an activated endocrine status, and an altered tissue composition (Wilson and Osbourn 1960; Ashworth and Millward, 1986). Compensatory growth enhances the efficiency of general body development and induces hyperplasia and hypertrophy of tissues and organs, including the mammary gland (Park, 2002; 2005).

Therefore, the research objectives were to focus and investigate of compensatory growth application (using stair-step feeding regimen) on reproductive management can be effective protocol in goats for Thai-native goat production expecting that these appropriate technologies and management regimens could be used to enhance the efficiency of goat production.

2. Materials and Methods

2.1 Animal Ethics

The experiment was approved by the Animal Ethics Committee of Khon Kaen University (No. AEKKU 35/2551; November 20th, 2008; Attached in the appendices). The study was conducted during the winter and dry season (October, 2009 - March, 2010). The University is located at 102 degrees east longitude and 16 degrees north latitude with a tropical climate. The experiment was carried out at the experimental farm, the small ruminant unit, Department of Animal Science, Faculty of Agriculture, Khon Kaen University.

2.2 Animals and Treatments

Sixteen Thai-native non pregnant goats were used with the average age and body weight of 8 months and 17±0.6 kg (Mean±SE), respectively. The does exhibited at least normal 2 estrous cycles and were housed in 1 m^2 steel cages. Heat detection was recorded using a vasectomized buck on daily, at 6.00 A.M. and 5.00 P.M. according to the method of Gordon (1997) and Cognie (1999). The estrus was detected and designed as Day 0. The female goats were randomly assigned into two groups: Control (n= 8) and Stair-step feeding group (n=8). In the control group, goats were fed 100% of nutrients requirement for growth in puberty periods (NRC, 1981).

Table 5.1 Nutrient requirement for goat (Maintenance)

Body Weights (kg)	TDN (g)	ME (Mcal)	NE (Mcal)	TP (g)	Ca (g)	P (g)	Vitamin A (1000 IU)	Vitamin D (IU)
10	153	0.57	0.32	22	1	0.7	0.4	84
20	267	0.96	0.54	38	1	0.7	0.7	144
30	363	1.30	0.73	51	2	1.4	0.9	195
40	448	1.61	0.91	63	2	1.4	1.2	243
50	530	1.91	1.08	75	3	2.1	1.4	285

Source: NRC (1981)

Does were offered Ruzi grass hay, TDN=51%, CP=8.5%, DM=500 g/head/d and concentrate supplemented at 1% of body weight (CP=16%), or 170 g/head/d. Total feed intake: DM=670 g/head/d, ME=1.25 Mcal/kg/d, CP=55 g/head/d during Day 1-42. In the Stair-step feeding group, treatment divided two phases: feed restriction and realimentation. In the first phase, goats were fed roughage and concentrate 70% of the average consumption of control group during day 1-21 (the 1st estrous cycle). In the second phase, goats were offered 130% of the average consumption of control group during at day 22-42 (the 2nd estrous cycle). Goats were scheduled to determine follicular growth and number on day 41 by surgical laparotomy. The follicular aspiration for assessment of oocyte quality on day 45 (2nd laparotomy), after ovariectomy, ovarian tissues were analyzed for proliferative cell nuclear antigen (PCNA) by Immunohistochemistry and mRNA Bcl-2 quantitation.

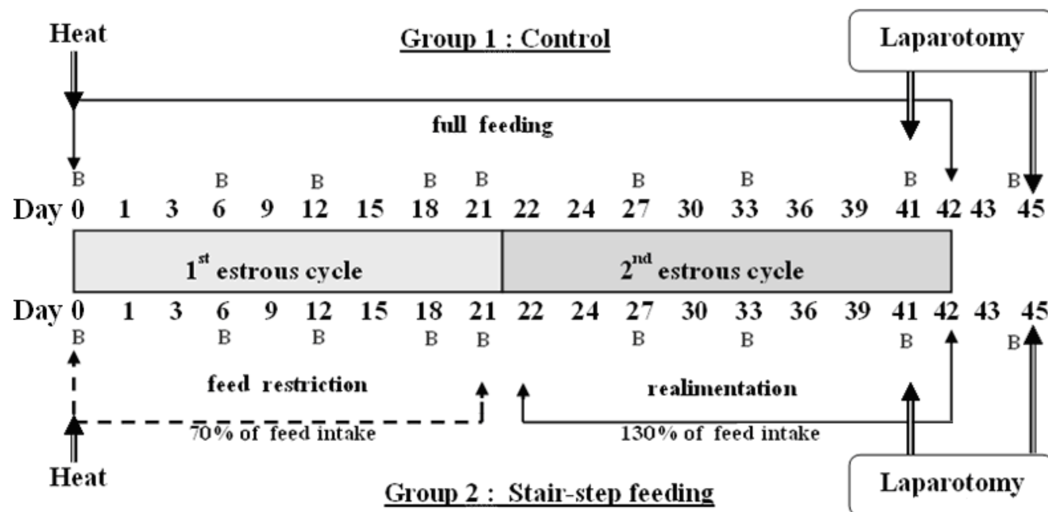

Figure 5.1 Description of Stair-step feeding regimen, Day 0 = heat, B = blood collection

2.3 Feeding management

The goats were provided with ruzi grass hay and concentrate supplemented at 1% of body weight twice daily, clean water and mineral block for ad libitum consumption and housed in 1 m^2 steel cages. The concentrate feed consisted of 50% cassava chip, 30% sun flower seed meal, 17% fine rice bran, 1% urea, 1% salt, di-calcium phosphate 1% and 1% premix (Table 5.2). Before 30 days of the experiment

started, animals were adapted to the diet. The animals were weighed when the experiment started and then every day at 6:00 h in the morning before feeding. For all animals, roughage and concentrates were provided two time every morning (7.00 AM) and evening (5.00 PM). In the control group, goats were fed 100% of nutrients requirement for growth in puberty periods (NRC, 1981). Does was offered a diet according of body weight on daily (Table 5.2).

Table 5.2 The scheduled of concentrate and roughage offered to does on daily (adjusted to the body weight)

	Control group		**Stair-step group**			
			Restrict phase		**Realiment phase**	
Body weight Kg	**concentrate g**	**hay g**	**concentrate g**	**hay g**	**concentrate g**	**hay g**
14	140	410	98	287	182	533
14.5	145	425	101.5	297.5	188.5	552.5
15	150	440	105	308	195	572
15.5	155	455	108.5	318.5	201.5	591.5
16	160	470	112	329	208	611
16.5	165	485	115.5	339.5	214.5	630.5
17	170	500	119	350	221	650
17.5	175	515	122.5	360.5	227.5	669.5
18	180	530	126	371	234	689
18.5	185	545	129.5	381.5	240.5	708.5
19	190	560	133	392	247	728
19.5	195	575	136.5	490	253.5	747.5
20	200	590	140	413	260	767
20.5	205	605	143.5	423.5	266.5	786.5
21	210	620	147	434	273	806
21.5	215	635	150.5	444.5	279.5	825.5
22	220	650	154	455	286	845
22.5	225	665	157.5	465.5	292.5	864.5
23	230	680	161	476	299	884

The samples were analyzed for DM, CP, neutral detergent fiber (NDF), acid detergent fiber (ADF) and ash. DM, CP, ADF and ash were analyzed according to the standard methods of AOAC (1990). NDF was determined by the method of Van Soest et al. (1991) using sodium sulphite and amylase, and was expressed with residual ash. Animals were vaccinated according to the requirement of the

Department of Livestock Development and the University farm for foot and mouth disease (FMD), hemorrhagic septicemia (HS) and brucellosis.

Table 5.3 Composition of concentrate (% air dried basis)

Item	(%) Proportion
Fine rice bran	17
Cassava chip	50
Sun flower seed meal	28
Urea	1
Di-calcium phosphate	1
Salt	1
Sulfur	1
Premix	1
Total	100

Table 5.4 Chemical composition of concentrate and roughage used in the experiment (% DM basis)

Ingredient	Composition (% DM)						
	DM (%)	CP	Ash	ME	NDF	ADF	Ca
Concentrate	90.78	16.00	4.87	3.00	18.61	13.78	0.42
Roughage							
Ruzi grass hay	89.6	7.3	6.9	2.3	66.2	35.1	0.35

DM = dry matter, CP = crude protein, Ash = total ash, ME = metabolizable energy, NDF = neutral detergent fiber, ADF = acid detergent fiber, Ca = calcium

2.4 Data Collection

2.4.1 Body weight and body condition scoring

Goats were initially weighed and then every day interval throughout the experimental period. The animals were weighed at 06:00 hours prior to feeding. At the end of the experimental period, the average weight of individual animal was also

recorded. Body condition scores (BCS) were assessed by the herd manner at before experiment started to determine the effect of BCS on response variables. BSC was assigned to each using a quarter-point scale from 1 to 5, where 1=emaciated and 5=obese. Goats were categorized into 2 groups according to BCS at before into experiment: those with less than average BCS and those with greater than or equal to average BCS.

2.4.2 Feed intake

Dry matter (DM) and nutrients intake by goats were estimated by measuring the refusals of feed. In every morning, feed refusals were collected and then weighed to determine the daily feed intake of each animal, i.e., Feed intake (FI) = Feed consumption/ interval in days. Average daily gain was calculated as the difference in weight between the final and initial weights divided by the interval in days from the dates the initial and final weights were taken, i.e., ADG = Weight gain/ interval in days. Feed conversion ratio (FCR) was calculated as the amount of feed consumed between the dates the initial weights divided by the difference in weight between the final and initial weights were taken, i.e., FCR = Feed consumed/weight gain (Dzakuma et al., 2004).

2.4.3 Blood sampling and collection

An amount of 5 ml blood samples were taken by jugular vein puncture for progesterone determinations on days 0 (standing heat of doe), 6, 12, 18, 21(1st estrous cycle), 27, 33, 41(1st laparotomy) and day 45 (2nd laparotomy). Samples were allowed to clot at room temperature and were centrifuged at 3,000 g for 10 min within 1 h after collection. The serum was stored at -20◦C until assayed for progesterone. Serum progesterone concentrations were determined by competitive ELISA (Crane et al., 2006). Goat anti-mouse IgG (H+L) was made in mouse by using a P4-horse radish peroxidase conjugate. Intraassay coefficients of variation were 2.65%, and assay sensitivity was 0.025 ng/ml.

Blood samples collected from goats by jugular vein puncture 5 ml for glucose, insulin, blood urea nitrogen (BUN) and non esterified fatty acid (NEFA) determinations prior feeding (0 hour) and after feeding (4 hour) on days 12 and 33. Samples were allowed to clot at room temperature and were centrifuged at 3,000 g for 10 min within 1 h after collection and then transported to the laboratory. Serum

glucose, NEFA, and blood urea nitrogen (BUN) concentrations were analyzed according to the methods of Kannan et al. (2007). Plasma insulin concentration was analyzed according to the method of Tanaka et al. (2004).

2.4.4 The counting follicular number and oocyte quality

Follicular growths determine by number and size of follicle (preovulatory follicle). This experiment divided follicle in 3 groups by size, such as 1-3, 4-6, and >7 mm according with Gonzalez-Bulnes et al. (2003). Follicular aspiration from beside any ovary (follicular size >2mm) for assessment of oocyte quality. Oocytes were classified into 2 groups: good and poor quality groups (Islam et al., 2007).

2.4.5 Ovary collection and preparation

Reproductive tracts with ovaries were collected by the 2nd laparotomy on day 45. Ovariectomy of another ovary was placed on ice, and immediately transported to the laboratory. Then it was cut into 2 sections; one section collected follicular tissues (theca and granulosa layer, keep in TCM-199 at -80◦C until Bcl-2 gene expression determine). Another used immunohistochemical detection of proliferative cell nuclear antigen for performed to determine the rate of cell proliferation of follicles, and expression of labeling index. Ovary was cut cross sectionally into several pieces (1-2 mm/piece) and was fixed in Carnoy's solution (ethanol:chloroform:acetic acid, 6:3:1) for 2 h at room temperature. Ovarian pieces were then stored in 70% ethanol until further processing. Fixed ovarian pieces were dehydrated by using a graded series of ethanol, cleared with a histological clearing agent (Histo-Clear®, National Diagnostics, Atlanta, GA), paraffin embedded, sectioned at 6 μm, and mounted onto glass slides as previously described (Grazul-Bilska et al., 2007).

2.4.6 Tissue collection

Thecal and granulosa tissues were obtained from follicles of ovary. The ovary was placed into a 60 mm Petri dish containing tissue culture media 199 (TCM-199) on ice. Granulosa cells were removed from follicular layers and rinsed with the TCM-199 into follicular cavity using siliconized Pasteur pipette, and then transferred to micro tube containing with media. Thecal tissue peeled from each follicle using a forceps, and then transferred to micro tube. Thecal and granulosa tissues each goat

were to snap frozen for evaluation of Bcl-2 genes expression using a real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

2.4.7 Proliferating cell nuclear antigen (PCNA)

Immunohistochemical detection of proliferative cell nuclear antigen was performed to determine the rate of cell proliferation of healthy and unhealthy follicles, expressed as a labeling index. Additional paraffin-embedded sections (6 µm) form each ovary was used for detection of PCNA using a specific monoclonal antibody as described previously (Zheng et al., 1994; Navanukraw, 2003; Grazul-Bilska et al., 2007). Twenty nine sections were chosen by size from each ovary and stained for PCNA immunoperoxidase. The remaining follicles less than 3 mm were not used for evaluation. Sections were deparafinized with 3% xylene for 3 min, rehydrated in ethanol, and quenched in 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity. After washes with water and PBS, normal horse serum was added for 20 min at 4°C to prevent nonspecific binding. PCNA, monoclonal mouse antibody (1:100 dilution, MAB no. 2586, Cell Signaling Technology, CA) was added as primary antibody, and incubated 24 h at 4°C as previously described (Grazul-Bilska et al., 2007). Primary antibody was detected by a biotinylated second antibody (antimouse antibody-IgG; 1:100 dilution, Vector Labolatories, Burligame, CA). Then, specimens were incubated for 60 min at at 4°C. For color development, diaminobenzadine (DAB) substrate was used for 5 min. After immunostaining the sections were counterstained with hematoxylin. For PCNA control, the PCNA antibody was replaced with PBS (200 µl/section).

2.4.8 Labeling index and image analysis

Tissue sections were positioned under the 40x objective lens so that either the granulosa or theca layer was brought into view. Then, images were taken from granulosa and theca layer areas of mapped healthy and unhealthy follicles. To determine changes in labeling index (LI), the percentage of the total area that exhibited positive staining for the number of PCNA-positive (proliferating; brown color) cells, and the total number of cells per area were determined quantitatively by using an image analysis system (Motic Images Plus 2.0, Olympus, JA) as described

previously (Guntaprom, 2010). For each follicle, 8 randomly chosen fields (0.05 mm^2 per field) were evaluated separately for granulosa or theca layers. The data were expressed as the mean percentage±SEM of the total area that exhibited positive staining within each tissue area. The LI was calculated as a percentage (%) of proliferating cells out of the total number of cells within each tissue area. The LI for theca cells included all labeled cells, and no attempt was made to distinguish between theca cell types within the theca layer.

2.4.9 Quantitative RT- PCR

Fluorescent, real-time quantitative RT-PCR was used to determine differences in mRNA expression of Bcl-2. Total RNA was extracted from granulosa and theca cells by the High Pure RNA Tissue Kit® (Roche Applied Science, Mannheim, Germany). Optical densities at 260 and 280nm were measured to determine the quantity and purity of RNA samples. An amount of 2 mg total RNA was subjected to reverse transcription with Transcriptor High Fidelity cDNA Synthesis Kit® (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol, using 2 µl random hexamers. Primers (Table 5.5) for quantitative RT-PCR were made with Primer Quest software (Integrated DNA Technologies, Coralville, IA, USA) according to the manufacturer's restrictions. Each PCR reaction (total volume of 50µl) consisted of 5µl cDNA template, 25 µl Faststart SYBR Green Master, 0.5 µl forward primer, 0.5 µl reverse primer, and 19 µl water (PCR grade). Thermal cycling conditions were as follows: 10 min at 95 ^oC for pre-incubation, 50 cycles of 30 s at 95^oC for denaturing, 20 s at 55 ^oC for annealing, and 45 s at 72 ^oC for extension, followed by a ramp from 65 ^oC to 95 ^oC over 1 min to determine the melting curve. Quantification of Bcl-2 gene, primer and condition, was previously published by Kelli et al. (2005).

Table 5.5 Quantitative RT-PCR primers

Gene	Nucleotide sequence	Accession number
Bcl-2		
Forward primer	5' - CTGCACCTGACGCCCTTCAC- 3'	XM_586976
Reverse primer	5' - GCGTCCCAGCCTCCGTTGT- 3'	XM_586976

2.4.10 Statistical Analysis

Continuous data such as FI, ADG, FCR, BUN, NEFA, Insulin, glucose, and P4 were analyzed using procedure GLM of SAS. The BCS was analyzed using Chi-square analysis (Cochran-Mantel-Haenszel) of SAS. Plasma P4 concentrations were analyzed with a nested analysis of variance with treatment, animal (treatment), and day included in the model, and differences between specific means were evaluated by using the student *t*-tests (SAS, 2000).

3. Results and Discussion

3.1 Effect of stair-step feeding regimen on growth performance

Characteristics of BCS (body condition score) in goats were not different between control and stair-step feeding groups in this study (2.76±0.07 vs 2.98±0.06 respectively, P>0.05). The result showed that average final weight, weight gain and average daily gain of control group compared with stair-step group were not different (P>0.05). However, ADG of stair-step feeding group was tended (P=0.09) greater than that of control group (Table 5.6).

Table 5.6 Effect of stair-step feeding regimen on growth performance

Items	Control	Stair-step	P-value
No. of goats treated	8	8	
BCS	2.76±0.07	2.98±0.06	0.53
Initial body weight (kg)	16.50±0.22	16.37±0.32	0.54
Final body weight (kg)	18.35±0.28	18.48±0.33	0.25
Weight gain (kg)	1.85±0.12	2.11±0.13	0.21
ADG (g/d)	46.25±3.21	52.81±2.69	0.09

Total DM intake included (roughage and concentrate) and FCR did not differ between stair-step and control groups. Percentage of DM intake was 3.63±0.03 in control and 3.56±0.02 in stair-step group (Table 5.7).

Table 5.7 Effect of stair-step feeding regimen on feed intake

Items	Control	Stair-step	P-value
Dry matter intake			
Total feed intake (g/d)	638.71±5.67	636.97±12.41	0.90
Concentrate (g/d)	171.63±1.60	172.57±4.33	0.80
Roughage (g/d)	467.08±4.82	464.41±8.22	0.79
% BW	3.63±0.03	3.56±0.02	0.72
g/kgBW075	74.96±0.66	n73.30±0.37	0.90
Nutrient intake (roughage + concentrate)			
CP (g/d)	61.55±0.51	61.51±1.07	0.97
ME (Mcal/d)	1.59±0.01	1.58±0.02	0.92
NDF (g/d)	341.14±3.32	339.55±5.85	0.83
ADF (g/d)	187.59±1.79	186.78±3.21	0.80
FCR	14.36±0.90	12.18±0.91	0.10

3.2 Effect of stair-step feeding on blood metabolize and hormonal change

Serum glucose concentration of stair-step feeding group during 2nd estrous cycle at 4 h was higher than control group (69.7±2.2 vs 55.3±2.8 mg/dl; P<0.05). Nevertheless, blood urea nitrogen (BUN) during estrous cycle at 0 and 4 h was not different between groups (P>0.05). Serum glucose and BUN concentration in this study were in the normal level according to Kanyinji et al. (2009). The normal level of serum glucose and BUN concentration were 40-85 mg/dl and 10-20 mg/dl, respectively. However, the serum glucose and BUN concentrations changed from 0-4 h were higher (P<0.05) during estrous cycle in stair-step feeding group compared to the control (Table 5.8).

Table 5.8 Effect of stair-step feeding on serum glucose and BUN concentration in goat

	Glucose (mg/dl)						BUN (mg/dl)					
	1st estrous cycle		Means ±SE	2nd estrous cycle		Means ± SE	1st estrous cycle		Means ± SE	2nd estrous cycle		Means ± SE
	0 h	4 h		0 h	4 h		0 h	4 h		0 h	4 h	
Control	51.0±4.7	56.0±1.7	53.5±3.0	52.0±2.5	55.3±2.8[a]	55.1±2.4	13.2±2.0	15.5±3.5	14.3±2.6	14.7±1.8	18.3±2.4	16.5±2.0
Stair-step	47.6±2.0	55.0±1.2	53.5±1.2	52.5±3.1	69.7±2.2[b]	59.6±1.9	12.0±1.1	15.7±1.2	13.4±0.5	15.8±1.1	20.0±1.0	17.7±0.9

[a,b] Means ± SE differ within column, $P<0.05$

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Blood glucose concentrations in ruminants are considerably lower than those of nonruminants, and ruminants are relatively insensitive to insulin. These major differences in carbohydrate metabolism have led to considerable speculation on the role of glucose in ruminant metabolism (Udum et al., 2008). Glucose metabolism is expected to increase with feeding, because propionate, the major precursor for gluconeogenesis, is produced in the rumen and absorbed after feeding (Sano et al., 1999). Moreover, insulin secretion is accelerated by feeding (Sano et al 1999).

Udum et al. (2008) found plasma NEFA levels to be inversely related to long-term food intake in sheep. They suggest that the different NEFA values apparently were not simply the result of the level of food intake by the sheep but might be involved in regulating food intake. Various physiological models, including chronic food-restriction or photoperiodically driven changes in voluntary food intake.

The levels of serum NEFA concentrations during the 1st estrous cycle (feed restriction phase) of stair-step feeding group compared with control group were not different (0.29±0.03 vs 0.23±0.02 mmol/L, respectively; $P>0.05$). However, in the 2nd estrous cycle (realiment phase) when add 130% feed intake, found that serum NEFA concentration of stair-step feeding group was less than control group (0.16±0.02 vs 0.26±0.03 mmol/L, respectively; $P<0.05$). Moreover, blood plasma metabolites were consistent with normal levels in goat (BUN, 10 to 20mg/dl; NEFA, < 0.45 mmol/L; albumin, 2.7 to 3.9 g/dl; glucose, 40 to 85 mg/dl; total cholesterol, 70 to 130mg/dl) reported by Kaneko (1989). Since concentrations of blood plasma metabolites have been commonly used to assess the plane of nutrition in ruminants (Russell and Wright, 1983)

The serum insulin concentrations during the 1st estrous cycle of stair-step feeding group compared with control group were not different (6.68±1.4 vs 8.0±1.1 (μU/ml, respectively; $P>0.05$). Despite, in the 2nd estrous cycle (realiment phase), found that serum insulin concentration of stair-step feeding group was higher than control group (15.57±1.57 vs 8.73±1.21 μU/ml, respectively; $P<0.05$). Tanaka et al. (2004) reported normal level of serum insulin concentration in goat was 5-25 μU/ml.

Russell and Wright (1983) reported that both glucose and insulin influence the release of NEFA. The higher insulin concentrations in 2nd estrous cycle

(realimentation phase) inhibits adipose-tissue lipolysis and decrease NEFA concentration in present study.

Insulin has been implicated in processes related to reproduction besides normal serum glucose regulatory action and has an important role in follicular development and function. The ovary has been shown to be the site of action for insulin and insulin growth factor-1 (IGF-1) in several species (Hammond et al., 1991; Gong et al., 1993). Growth factors like IGF, EGF and its families directly influence follicular growth rate by enhancing granulosa cell proliferation.

Table 5.9 Means ± SEM of serum NEFA and Insulin concentrations in goat

	NEFA (mmol/L)			Insulin (μU/ml)		
	1st estrous cycle	2 nd estrous cycle	Means ± SE	1st estrous cycle	2nd estrous cycle	Means ± SE
Control	0.22±0.02	0.26±0.03 [a]	0.24±0.02	8.00±1.10	8.73±1.21 [a]	8.37±0.83
Stair-step	0.28±0.03	0.16±0.02 [b]	0.22±0.01	6.68±1.47	15.57±1.57 [b]	10.64±1.18

[a,b] Means ± SE differ within column, P<0.05

Strubbe et al (1977) showed that insulin is low up to 5 min before a meal but increases upon the ingestion of food. Insulin is a critical regulator of energy metabolism, and evidence suggests a close relationship between circulating glucose levels and insulin secretion. Glucose, insulin and NEFA data are consistent with those reported in the literature for cattle in the fed or fasted state (Wretz-Lutz et al 2006). However, Chelikani et al (2004) demonstrated that responsiveness of plasma glucose, insulin and NEFA concentrations during fasting was diffent depending on physiological state of the animal. Blood glucose and insulin concentrations fluctuate reciprocally before and after feeding (Cummings et al 2001).

Lofgren and Warner (1972) reported a sustained increase in plasma insulin concentrations which peaked at 30 minutes in sheep fed with a high concentrate diet. All the findings presented here might possibly be explained as being a consequence of insulin secretion or inhibition in response to feeding and fasting.

Progesterone concentrations of the female goats followed a definite pattern consistent with follicular and luteal phases and with ovarian in activity postpartum. It

is recommended that progesterone levels should be monitored in the practical application of clinical endocrinology in caprine reproduction (Perkins and Fitzgerald, 1994). Serum progesterone concentration of goat during 1st estrous cycle on days 0, 6, 12, 18 and 21 were not different between stair-step feeding and control groups (P>0.05). The serum P4 concentration all goats were low (P4 < 1 ng/ml; Munro and Stabenfeldt, 1984) on day 0 or standing heat (control = 0.45±0.12; stair-step feeding = 0.47±0.08 ng/ml) and then increased on day 6 (2.82±0.13 vs 2.27±0.49 ng/ml), and very high on day 12 (6.3±0.61 vs 6.51±0.36 ng/ml) and decreased on day 18 (3.3±0.31 vs 4.17±0.63 ng/ml) and was low again (less than 1 ng/ml) on day 21 (0.9±0.16 vs 0.58±0.11 ng/ml).

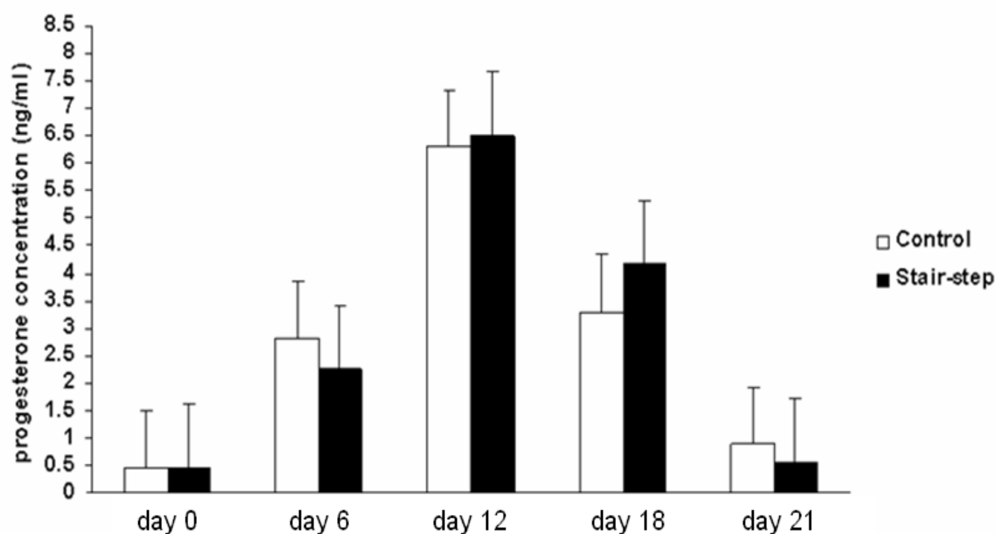


Figure 5.2 Means (± SEM) serum P4 concentrations (ng/ml) of goats on days 0, 6, 12, 18, and day 21 during 1stestrous cycle

The serum progesterone profile during the reproductive cycle of the Thai-native goat in present study (Figure 5.2) was similar to the West African Dwarf and indigenous Damascus does (Zarkawi and Soukouti, 2001), and Khanum et al. (2008) reported that progesterone profiles during estrous in Dwarf goat (*Capra hircus*).

However, in the 2nd estrous cycle (realiment phase) when add 130% feed intake, found that serum P4 concentration on day 12 of stair-step feeding group was greater than control group (6.68±0.95 vs 9.76±0.3 ng/ml, respectively; P<0.05) (Figure 5.3).

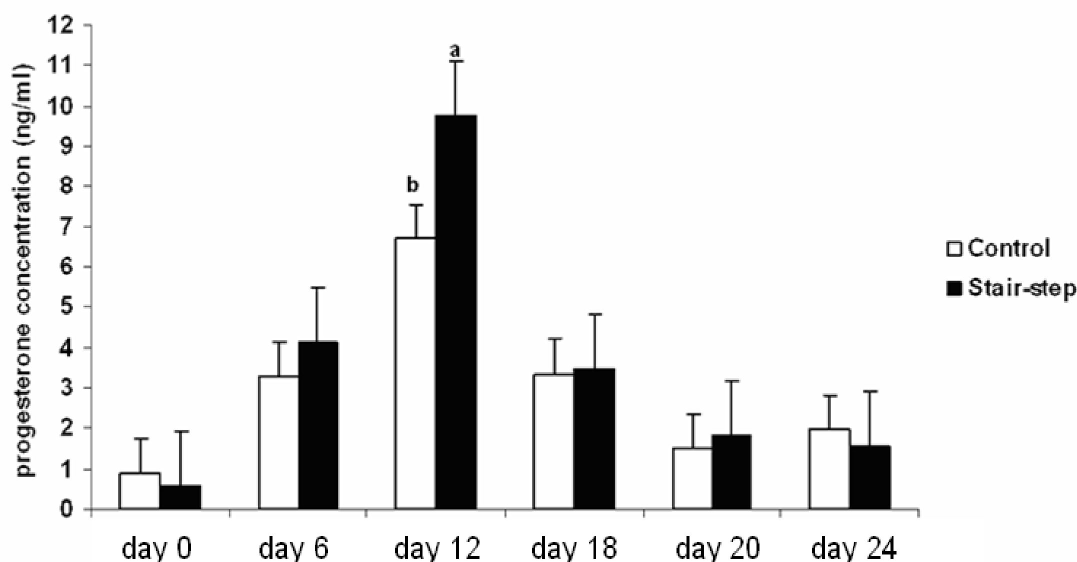


Figure 5.3 Means (± SEM) serum P4 concentrations (ng/ml) of goats on days 0, 6, 12, 18, 20, and day 24 (2nd laparotomy) during the 2nd estrous cycle
[a, b] Proportion differ (P <0.05)

3.3 Effect of stair-step feeding regimen on follicular development and oocytes quality

Follicular growths determined by number and size of follicle (preovulatory follicle) on day 41 (1st laparotomy). The result found that, total numbers of follicle in stair-step feeding group was greater than the control group (16.37±2.08 vs 10.83±0.79 follicles, respectively; P< 0.01). However, the number of follicular size 1-3 and ≥7 mm were not differed between control and stair-step feeding groups in present study (Table 5.10).

Table 5.10 Effect of stair-step feeding regimen on numbers of preovulatory follicle (1st laparotomy)

Items	Control	Stair-step	P-value
Follicular size 1-3 mm	6.62±0.59	8.00±0.75	0.17
Follicular size 4-6 mm	2.87±0.22 [a]	6.62±1.14 [b]	0.01
Follicular size ≥7 mm	1.37±0.18	1.87±0.29	0.17
Total	10.87±0.16 [a]	16.50±1.75 [b]	0.01

[a,b] Means ± SEM differ within row, P<0.01

In this study, the oocytes were classified into 2 groups: good and poor quality groups according to Islam et al. (2007). Numbers of good quality from all follicular size 1-3 and 4-6 mm were significantly different between stair-step feeding and control group (P<0.01). However, numbers of good quality from follicular size ≥7 mm was not different between control and stair-step feeding groups (Table 5.11). The results indicate that stair-step feeding regimen affects oocyte quality and hence fertility improvement in Thai-native goat. Amal et al. (2008) evaluated the effect of diet restriction and re-feeding on some reproductive performance in Egyptian-native goats, and found that it affected ovulation rate by 40% increase.

Table 5.11 Effect of stair-step feeding regimen on percentage of good oocytes quality (2nd laparotomy)

Items	Control	Stair-step	P-value
% of good quality (follicular size 1-3 mm)	5/13 (38.5%) [a]	10/11 (91%) [b]	0.01
% of good quality (follicular size 4-6 mm)	3/8 (37.5%) [a]	9/11 (82%) [b]	0.01
% of good quality (follicular size ≥7 mm)	2/3 (67.0%)	4/5 (80%)	0.67

[a,b] Percentages of oocyte quality differ within row, P<0.01 (Chi-square test)

Immunohistochemical detection of proliferative cell nuclear antigen was performed to determine the rate of cell proliferation of follicular size 1-3, 4-6 and ≥7 mm, expressed as a labeling index.

The expression of PCNA, an essential regulator of the cell cycle, appears to mark the initiation of follicle growth, coinciding with and in some cases preceding the first sign of granulosa cell enlargement and preceding theca cell enlargement. An examination of its regulation and the basis for the induction of its expression in the granulosa and theca cells at the onset of follicular growth may increase understanding of the mechanisms underlying this process. The results found that the labeling index in granulosa and theca layer of all follicular size 1-3, 4-6 and ≥7 mm were statistically different between stair-step feeding and control group (P<0.05).

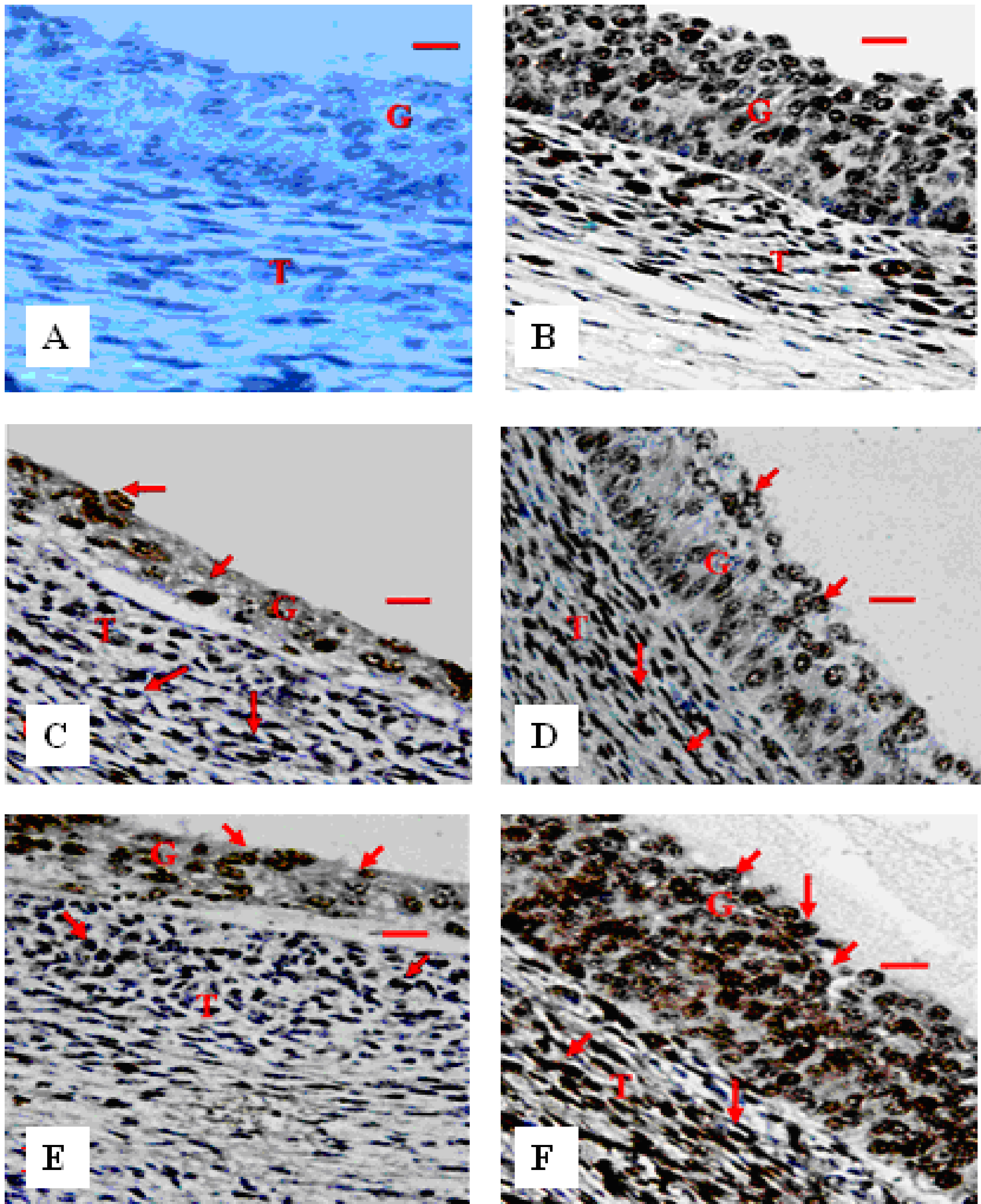
Table 5.12 Percentage of proliferative cell nuclear antigen (PCNA) of follicular size 1-3, 4-6, and ≥7 mm

Items	Follicular size 1-3 mm		Follicular size 4-6 mm		Follicular size ≥ 7 mm	
	Grnulosa cell labeling index + (%)	Thecal labeling index + (%)	Grnulosa cell labeling index + (%)	Thecal labeling index + (%)	Grnulosa cell labeling index + (%)	Thecal Labeling index + (%)
Control	27.13±1.28 [a] (n=5)	24.54±1.92 [c] (n=5)	26.50±1.45 [c] (n=4)	25.15±1.51 [c] (n=4)	24.24±1.48 [a] (n=3)	23.76±1.81 [c] (n=3)
Stair-step	38.27±1.88 [b] (n=6)	33.29±2.22 [d] (n=6)	34.57±1.64 [d] (n=6)	36.76±2.06 [d] (n=6)	33.63±2.27 [b] (n=4)	30.32±1.79 [d] (n=4)

+ Labeling index = number of proliferating cells expressed as a proportion of the total number of cells.

[ab] Within a column, means with different superscripts differ ($P < 0.01$)

[cd] Within a column, means with different superscripts differ ($P < 0.05$)



Control group **Stair-step feeding group**

Figure 5.4 Immunolocalization of PCNA; (A), in a section of the negative staining of follicle; (B), in a section of the positive staining of follicle; (C) and (D), in a section of follicular size 1-3 mm; (E) and (F), in a section of follicular size 4-6 mm; G = granulosa layer; T = Theca layer; Size bar indicates 100 µm; arrows indicate stained PCNA

Many studies have investigated cell proliferation in follicle during the estrous cycle period (Winston et al., 2004), but there is a lack of information about mitotic activity or PCNA of follicle in goat. Thus, we used the PCNA expression in situ as measures of cell proliferation. The frequencies of PCNA positive cells of granulosa and theca cells in follicle of stair-step feeding group was greater than the control group during the 2nd estrous cycle of goat in present study. This finding was in agreement with those reported in dairy cows (Isobe and Yoshimura, 2007; Guntaprome, 2010), goat (Martins et al., 2005), pig (Tomanek and Chronowska, 2006; Fricke et al., 1996) and rats (Gaytan et al., 1996). PCNA is required for DNA synthesis and appears to be involved in follicular growth. Expression of PCNA in granulosa cells begins upon the formation of a primary follicle, and its level of expression appears to increase during the gonadotropin-dependent stages of preovulatory follicular development (Oktay et al., 1995)

According to Tomanek and Chronowska (2006), the most rapid granulosa cell proliferation occurs in large preantral follicles just before the antrum formation. At that stage of development, follicles start to enlarge rapidly and extensive PCNA immunoreactivity reflects the high proliferation rates in granulosa and theca cells of healthy antral follicles. Parallelly, as a result of response to gonadotrophin stimulation, granulosa and theca cells develop steroidogenic functions and increasing amounts of steroid hormones are produced. The high rate of steroidogenesis in large follicles is accompanied by decrease in cell proliferation rate. During the follicular development, more than 99% of follicles undergo the proccess of atresia while only a few reach the final size and ovulate.

Using the PCNA as a marker, Feranil et al. (2005) found in buffalo and cattle ovary significantly higher frequency of PCNA labeled cells in healthy follicles than in the early and advanced atretic follicles. Reduced number of PCNA immunoreactive cells during atresia was also found in theca.

3.4 Expression of Bcl-2 gene in follicles

Real-time RT-PCR techniques has allowed us to quantify expression of mRNA for Bcl-2. The accuracy of each real-time PCR was validated by quantifying known numbers of target cDNA templates. Then, those templates were used for generated standard curve. In this study, those standard curves showed a highly linear relationship between ct value and known number template dilution by given highly R^2 value of standard curve are shown in Figure 5.5.

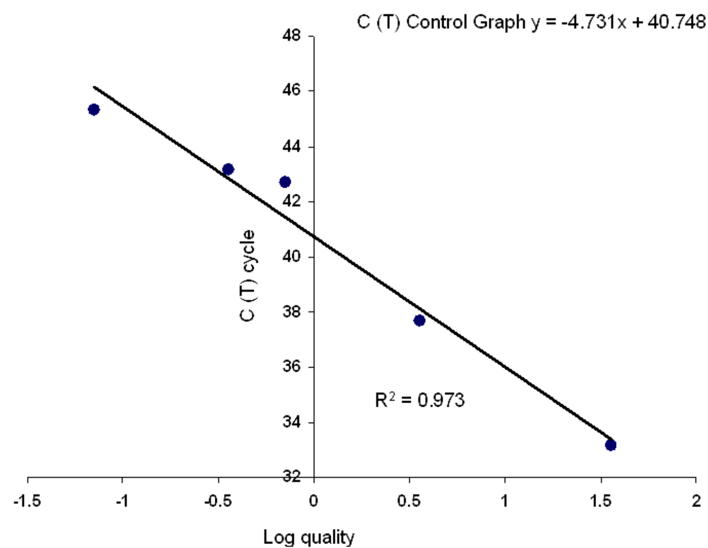


Figure 5.5 The standard curves obtained by plotting the logarithm of mRNA concentration for Bcl-2 versus threshold cycle (Ct) for population quantification by using real time PCR

The mRNA levels of Bcl-2 in thecal tissue of follicular size 1-6 mm during the 2nd estrous cycle of stair-step feeding group was greater than the control group (3.85±0.24 vs 2.95±0.38, respectively; P<0.05). However, thecal Bcl-2 mRNA levels of each follicular size (1-3 and 4-6 mm) were not differed between control and stair-step feeding groups in present study (Figure 5.7 and 5.8).

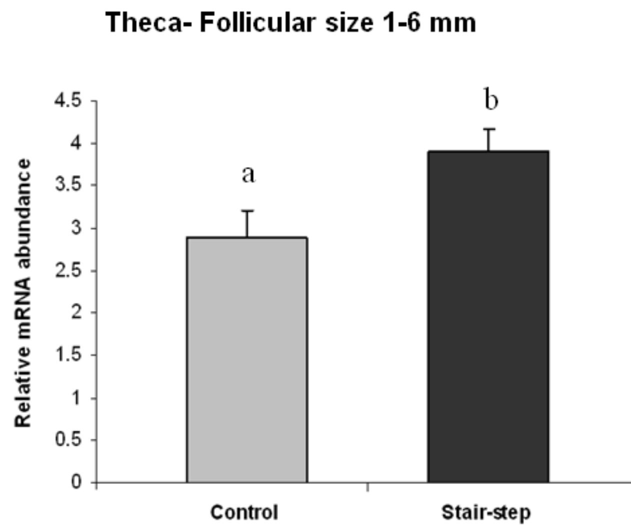


Figure 5.6 The expression of Bcl-2 mRNA in thecal tissue of follicular size 1-6 mm during the 2nd estrous cycle. Different superscripts denote statistically different values (P<0.05)

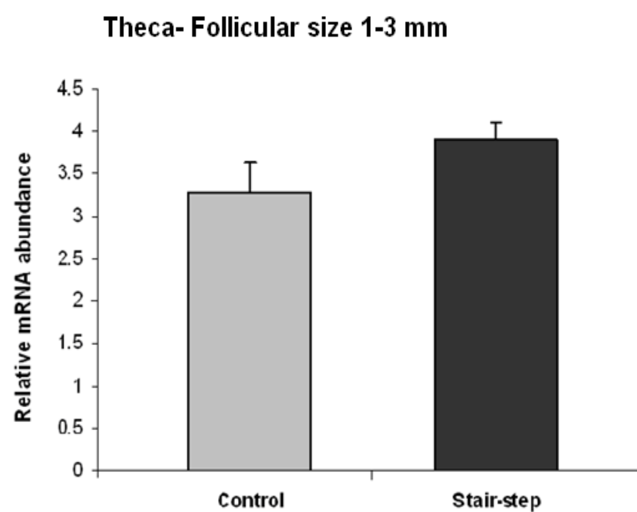


Figure 5.7 The expression of Bcl-2 mRNA in thecal tissue of follicular size 1-3 mm during the 2nd estrous cycle

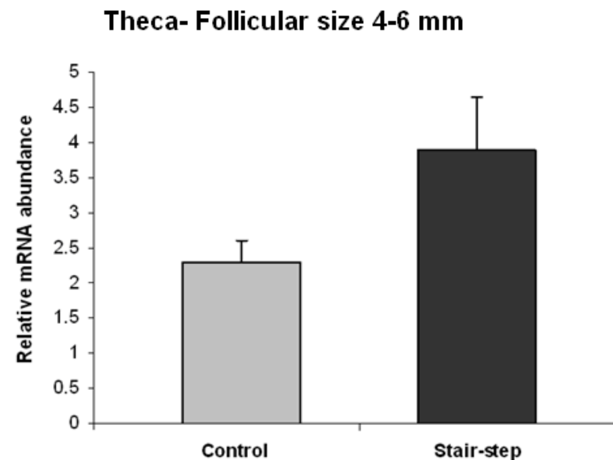


Figure 5.8 The expression of Bcl-2 mRNA in thecal tissue of follicular size 4-6 mm during the 2nd estrous cycle

The mRNA levels of Bcl-2 in granulosa tissue of follicular size 1-6 mm during the 2nd estrous cycle of stair-step feeding group was greater than the control group (1.11±0.12 vs 0.56±0.06, respectively; P<0.01). Moreover, granulosa Bcl-2 mRNA levels of each follicular size (1-3 mm) of stair-step feeding group was greater than the control group (1.26±0.15 vs 0.55±0.08, respectively; P<0.01). Nevertheless, graulosa Bcl-2 mRNA levels of follicular size 4-6 mm was not different between control and stair-step feeding groups (Figure 5.11).

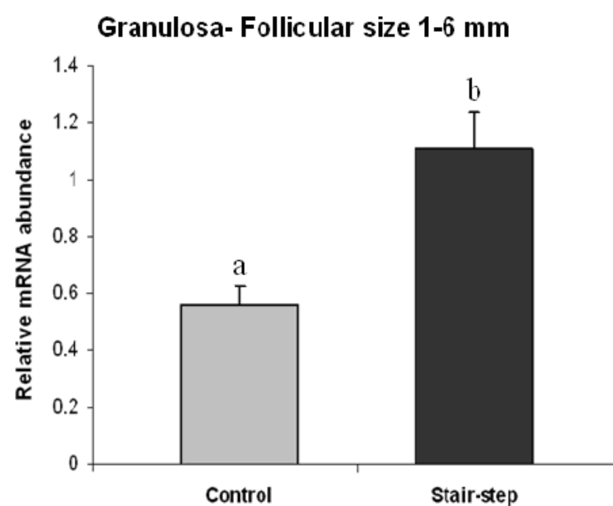


Figure 5.9 The expression of Bcl-2 mRNA in granulosa tissue of follicular size 1-6 mm during the 2nd estrous cycle. Different superscripts denote statistically different values (P<0.01)

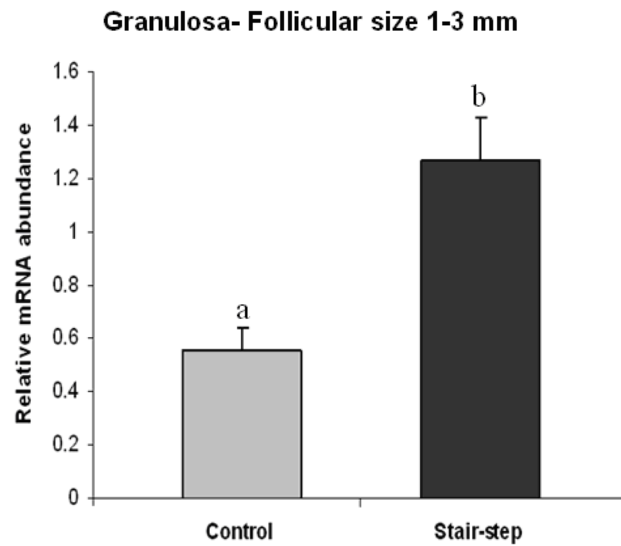


Figure 5.10 The expression of Bcl-2 mRNA in granulosa tissue of follicular size 1-3 mm during the 2nd estrous cycle. Different superscripts denote statistically different values (P<0.01)

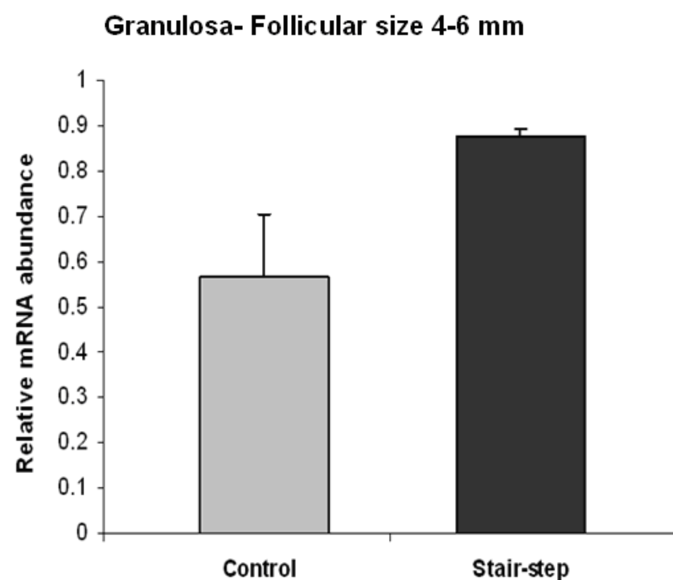


Figure 5.11 The expression of Bcl-2 mRNA in granulosa tissue of follicular size 4-6 mm during the 2nd estrous cycle

The Bcl-2 family of proteins includes members which either promote (Bax) or inhibit (Bcl-2, Bcl-xL) apoptosis. Overexpression of Bax accelerates apoptotic death response to death signals (Oltvai et al. 1993).

Bcl-2 is a survival molecule that resides in the nuclear envelope and mitochondria. Its expression is found in the granulosa cells of both fetal and adult ovaries (Hussein, 2005). Salvetti et al., (2009) showed a correlation between decreases in the level of Bcl-2 mRNA with the incidence of apoptosis in isolated granulosa cells which were cultured under different hormonal treatment conditions. This expression is related to gonadotrophin levels where higher levels of gonadotropins increase the expression of Bcl-2 and decrease the expression of Bax (Sugino et al., 2000).

In the current study, the levels of mRNA encoding Bcl-2 in granulosa and theca cell of follicular size 1-6 mm in the stair-step feeding group (during realiment phase) was greater than the control group. It is also possible that other hormonal signalling such as ovarian steroids or locally produced growth factors that can influence granulosa cell fate and serve as the primary regulators of Bcl-2 gene expression (Tilly et al., 1991; Johnson, 2003). This finding was in agreement with those reported in bovine (Kelli et al., 2005; Li et al., 2009) and rats (Salvetti et al., 2009).

4. Conclusions

In conclusion, the application of compensatory growth using stair-step feeding regimen during estrous cycle. The results found that average total feed intake, average daily gain and feed conversion ratio were not significantly different between stair-step and control group. However, the stair-step feeding regimen in order to induce compensatory growth on oocytes quality, follicular development and the labeling index in granulosa and theca layer of follicle greater than control group. Likewise, the ratio of relative levels of mRNA encoding Bcl-2 of granulosa and theca cell of follicle in the stair-step group was greater than the control. Thus, the results indicate that reproductive management in pubertal and pre-gestation periods using stair-step feeding regimen affects fertility improvement in Thai-native goat.