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Follicular Dynamics, Steroid Hormones and Blood Metabolites Concentrations During Long Term Protein Flushing in Subtropical Ewes

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ABSTRACT

The aim of this study was to investigate the influence of long-term high-protein flushing on ovarian function and metabolic status in Ossimi ewes. Fourteen cycling Ossimi ewes were divided into high protein (210 % of maintenance crude protein) or treatment group (TG; n = 7) and control group (CG; n = 7). Oestrous was synchronized by PGF2α, monitored ultrasonographically daily for two oestrous cycles. The CG fed a maintenance diet (9.5 % CP) through-out the experiment. The mean number of small (2–2.9 mm), medium (3–5 mm) ovarian follicles, and follicular waves were higher (P<0.01) in ewes of TG compared to that of CG in the first and second oestrous. The size of large, subordinate and ovulatory follicles were greater (p <0.05) in ewes of TG compared to those of CG. Ewes of the TG recorded a higher (p <0.05) ovulation rate in the first cycle while, ovulation rate did not show any significant change in second oestrous. Long term flushing of high-protein ration significantly influenced the concentration of glucose and some metabolic profiles. Concentration of serum total proteins was greater at 0, 1 and 13 days, while serum total cholesterol was greater at day 0 and 1 after first oestrous (ovulation is day 0) in ewes of TG as compared to ewes of CG. Serum glucose was increased significantly (p<0.05) at day 3 and 13 after first oestrous and at day 0 and 7 after second ovulation in TG when comparing to CG. There was significant (p < 0.05) increase in serum concentrations of progesterone at day 13 after first oestrous in TG and at day 3 after second oestrous. The level of serum estradiol 17-β increased significantly at day 0, 1, 3, 7 and 13 in the first oestrous and at day 0 on second oestrous in TG as compared to CG. In conclusion, the long term protein flushing increased the number of small and medium follicles and follicular waves, the size of ovulatory follicles in the first and second oestrous, the reproductive efficiency in subtropical ewes. Long term protein flushing increase ovulation rate in the first but had no effect on the second oestrous.

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INTRODUCTION

Nutrition can affect reproductive features by two possible pathways (Scaramuzzi *et al*., 2006). The first way is through acting on the endocrine system (GnRH, FSH-LH, and estradiol). The second way was considered by Scaramuzzi *et al*. (2006), where the nutritional inputs would affect reproduction by directly acting on the ovary and the ovarian follicles through changes in the metabolic modulatory systems (insulin–glucose, leptin and growth hormone and growth factors). The stimulation of these intra-follicular systems leads to a suppression in follicular oestradiol production. The consequence of these direct actions on the follicle is a reduced negative feedback to the hypothalamic-pituitary system and increased FSH secretion that leads to stimulation of folliculogenesis. In sheep, static body condition affects follicular dynamics and ovulation rate patterns through changes in FSH secretion (Vinoles *et al*., 2002). Bartlewski *et al*., (2011) reviewed that the growth of antral follicles reaching ostensibly ovulatory sizes occurs in a wave-like pattern throughout the breeding season in both prolific and non-prolific breeds of sheep. There are typically 3 or 4 waves of follicle development during the interovulatory interval. Follicular wave emergence is primarily controlled by changes in circulating concentrations of follicle-stimulating hormone (FSH). The largest ovarian follicles acquire the ability to secrete oestradiol from the day of emergence with peak oestradiol secretion occurring about the time they reach maximum diameter. The high ovulation rate may be achieved by the ovulation of follicles from the last two

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waves of the interovulatory interval. The endocrine influences on ovarian function have brought into existence of strong follicular dominance.

Energy or protein supplementation has been found, to promote the development of more antral follicles by maintaining adequate levels of both FSH and LH in sows (Quesnel *et al*., 1998). On the other hand, inadequate dietary protein results in a reduced number of antral follicles and a decreased ovulation rate for rats and goats (Cognie *et al*., 2003, I'Anson *et al*., 2003). This effect may be related to the fact that nutritional deficiencies reduce synthesis and secretion of FSH and LH, preventing final maturation of the ovulatory follicle (Muller *et al*., 1998).

The entry of follicles into the terminal-growth stage (secondary follicular waves) is hierarchical, independent of gonadotrophins, and seems to be related to changes in the expression of the growth factor complex (McNatty *et al*., 1999; Webb *et al*., 1999). Nutritionally influenced mediators of energy balance and reproductive function, such as insulin, the IGF system and leptin (Sansinanea *et al*., 2001) may be modified by protein supplementation (Noguchi, 2000). The hypothalamo-hypophyseal- ovarian axis appears to play a critical role in the integration of nutritional status and reproduction (Wiltbank *et al*., 2002).

In sheep, follicle populations are very sensitive to the nutritional input and folliculogenesis and ovulation rate can be readily increased by nutritional manipulation. The manipulation of reproduction using nutrition is an inexpensive management tool to control ovulation rate and litter size particularly in low cost, extensive production systems in marginal environments such as the semi arid, Mediterranean and hill farming regions of the world (Martin *et al*., 2004).

We hypothesized that high protein supplementation (dynamic effect) of non-pregnant ewes would alter the peripheral steroid hormones, follicular dynamics and blood metabolites. Therefore, the aim of the present study was to evaluate the effects of high protein supplementation on plasma concentration of steroid hormones and metabolites, and on follicular dynamics in Subtropical ewes.

MATERIALS AND METHODS

Animals and experimental design:

The experiment was carried out on the experimental farm of Faculty of Agriculture, Assiut University, Assiut (27◦N, 31◦E), and Egypt. Fourteen multiparous clinically healthy Ossimi ewes 4–5 years old and body weight = 40-50 kg raised in semi-open pens were used for this experiment. The experiment was carried out during the early summer breeding season for these animals from June to July

The oestrous cycles of ewes were synchronized by intramuscular double injection of 250 μg/mL of PGF2α analog cloprostenol (1 ml Juramate, Jurox Pty. Ltd., Australia), 10 days apart and ovulation was confirmed by ultrasonography. Animals were randomly divided into two equal groups, one group was fed on a maintenance protein level (9.5% crude protein (CP) i.e control group (CG), while the second group was fed high protein level (20 % CP, 210 % of maintenance CP level) i.e treatment group (TG). Estrus was detected using a teaser ram and ultrasonography was carried out daily to monitor the ovulation. The ingredients and chemical composition of experimental diets are shown in Table 1. Diets were mixed daily and fed twice a day. All nutrients met the requirements of 50 kg maintenance ewe sheep (NRC, 1985). Feed intake was recorded daily and their representative samples were taken for chemical analysis. The average daily feed intake was 380.6 g wheat straw and 888 g concentrate in a total feed intake of 1268.6± 22.31 g/head/day for CG and 246.4 g wheat straw 985.7 g concentrate in a total of 1232.14 ± 9.2 g/head/day. The CP intake was 120.5 g/day and 246.43 g/day for control and treated groups, respectively. The high protein diet started from the day of the first injection of PGF2α until the 13th day after second ovulation.

Ultrasonographic examination and blood sampling:

Ovarian structures of all animals were monitored ultrasonographically using a real-time, B-mode, diagnostic scanner equipped with a transrectal 5/7.5 MHz linear array transducer (Hitachi, EUB-405B, Japan). Ultrasound examinations were performed daily starting 4 days before second prostaglandin administration till 13^{th} day after the second estrous. All follicles ≥ 2 mm and CL were measured, and mapped individually for each ewe.

Follicular waves in ewes consist of 1–4 follicles attaining a similar final stage of development, and antral follicles. The largest follicles that grow to an ostensibly ovulatory diameter of ≥ 5mm before regression or ovulation emerge in an orderly succession throughout the 17-day interovulatory interval, giving typically 3 or 4 follicular waves (Bartlewski *et al*., (2011). The mean days of wave emergence (defined as the day that follicles growing to ≥5 mm in diameter were first detected at 3 mm in diameter (Bartlewski *et al*., 1999a)

Ovulation was considered to have occurred when a large growing antral follicle that had been identified and followed for several days was no longer observed. A follicle wave is considered to be the initial synchronous growth of a cohort of follicles (emergence), followed by one or more that continues growing (the dominant follicle) while others regress (subordinate follicles) (Evans, 2004). Each wave consists of the contemporaneous

recruitment of three to six follicles to grow larger than 4–5 mm in diameter. Within several days of wave initiation one follicle is selected as a dominant follicle. The dominant follicle continues to grow and differentiate, whereas its sister subordinate follicles (next largest follicle) plateau in growth and then regress. The dominant follicle of the first wave in two-wave cycles and of the first and second waves in three-wave cycles regresses. All follicles that have been recruited in the same follicular wave but weren't selected to be dominant follicles are subordinate (next largest follicle). The number of days until the appearance of the first follicular waves were calculated and recorded for each animal. The CL was examined and an image of the maximum diameter from the largest cross-sectional area was estimated. The following ovarian characteristics were determined and compared between groups: (1) ovulation rates; (2) diameter of the ovulatory follicles; (3) interval from treatment to emergence of a new follicular wave; (4) number and diameter of the CL. Estrus was detected by checking the behavior (refusal or standing) after introducing a ram to females thrice daily. The mean number of small (2–2.9 mm in diameter), medium (3–5 mm in diameter), and large follicles (>5 mm in diameter) were recorded. Blood samples were collected by jugular venipuncture daily until 13th day after second ovulation. Blood samples were centrifuged at 1.300 × *g* for 20 min at 4°C within 24 h and serum was harvested and stored at −20 ◦C until assayed for total proteins, total cholesterol, globulin, glucose, urea, albumin, estrogen and progesterone.

Analysis of blood metabolites and hormones:

Blood metabolites were analyzed by spectrophotometer (Unico, USA) using commercial test kits (Spinreact, Spain): glucose – enzymatic colorimetric method, total protein – biuret reagent, total cholesterol, and urea (Young, 2001). Estradiol 17-β and progesterone concentrations were determined using direct ELISA technique. Kits were provided by Diagnostic System Laboratory Co. (DSL, Catalogue No. 3900, USA). The coefficient of variance of intra-and interassay were 4.8 and 9.2%, for estrogen and 3.6 and 12.43% for progesterone, respectively. The sensitivity of the assay was 2 pg for estradiol and 0.12 ng for progesterone.

Statistical analysis*:*

Data were statistically analyzed using General linear model (GLM) procedure of SAS. (1996). Differences between means were tested using Duncan's multiple range test (Duncan, 1955). Probability values of less than 0.05 ($P < 0.05$) were considered significant. Results are expressed as means ± SEM.

Results:

Follicular dynamics:

In the first oestrous there was a significant ($P < 0.01$) increase in the number of small (2-2.9 mm) and medium (3–5 mm) follicles, and number of follicular waves ($p<0.05$) on ovaries of ewes in TG compared to CG (table 2). At the same time, the size of ovulatory follicles (mm) was larger ($P < 0.05$) in ewes of TG compared with those of CG. In addition, ovulation rate was higher in the TG ($p <0.05$) than that of the CG. In the second oestrous, there was a significant ($P < 0.05$) increase in the number of small (2-2.9 cm) and medium (3–5 cm) follicles and number of follicular waves in TG in comparison with CG (Table 2). No significant difference was observed between TG and CG regarding ovulation rate and corpus luteum size. Moreover, the size of subordinate (next largest follicle) and ovulatory follicles were larger ($p <0.001$) in TG compared to CG. However there was no significant difference between CG and TG in the wave length in days.

The overall means of all data of the two ovulations indicated that there were significant ($p <0.05$) increases in the size of subordinate and ovulatory follicle, mean number and total number of waves in ewes of TG compared to CG (Table 3).

Blood metabolites:

Changes in serum concentrations of blood metabolites are shown in Fig 1. The mean concentrations of serum total protein increased significantly ($p<0.05$) in ewes of TG compared to CG during first oestrous cycle at day 0, 1 and 13 (9.45± 0.59 vs 8.62± 0.57, 9.87± 0.32 vs 9.21± 0.12, 10.01± 0.91 vs 8.6± 0.36), respectively. Blood glucose increased significantly ($p<0.05$) in TG than that of CG at day 3 (62.63±4.46 mg/dl vs 41.96±3.35 mg/dl; $P < 0.05$), day 13 (65.05±1.32 mg/dl vs 53.33±1.76 mg/dl) during first oestrous cycle while, it was increased at day 0 (78.8±3.62 mg/dl vs 48.68±3.17 mg/dl) and 7 (62.63±4.46mg/dl vs 41.96±3.35 mg/dl) during second oestrous cycle. Moreover, serum total cholesterol increased significantly ($p<0.05$) at day 0 (78.28±7.22 vs 55.33±3.18 mg/dl) and day 1 (70.38±2.18vs 48.01±4.91 mg/dl) during first oestrous cycle and at day 1 (85.12±4.04 vs 58.57±7.67 mg/dl) during second oestrous cycle. Serum urea concentration increased significantly at day 7 during second oestrous cycle (71.83±4.93 vs 50.25±5.29 mg/dl).

Serum estradiol 17-β and progesterone concentrations:

There was a significant ($p<0.05$) increase in mean serum progesterone concentrations in TG on days 0 and 13 of the first oestrous cycle (0.33±0.0.02, and 4.67 ±0.15) ng/ml compared to CG (0.19±0.05 and 3.87 ±0.03)

ng/ml, respectively (Fig. 2). In addition, in the second oestrous cycle serum progesterone increased significantly ($p<0.05$) on day 3 in TG (2.00±0.15) compared to CG (0.93±0.14). At the same time serum estradiol concentration increased significantly ($p<0.05$) on day 0, 1, 3, 7 and 13 after first ovulation in TG (44.9±1.7, 31.00±1.8, 25.00±1.5, 37.50±0.88 and 29.43± 0.38) pg/ml in comparison with CG (37.36±1.59, 22.93±1.16, 18.33±1.45, 30.2±1.49 and 26.2±1.11) pg/ml. During the second oestrous cycle there was a significant ($p<0.05$) increase in serum estradiol on day 0 in TG compared to CG (44.00±0.88 vs 29.43±0.99) pg/ml. There were no significant differences between serum oestradiol and progesterone in all studied days during the two cycle comparison (Fig 3 and 4) except serum oestradiol in TG at day 13 which was significantly higher in the first oestrous than second oestrous cycle.

Discussion:

The current study demonstrated that long term protein flushing successfully increased the number of dominant follicles prior to ovulation, size of the ovulatory follicle and the ovulation rate. In the present study the significant increase in the number of small (2-2.9 mm), medium (3–5 mm) follicles and number of follicular waves on ovaries of ewes in TG was in agreement with previous studies (Davis *et al*. 1981, Fletcher 1981, Smith 1988, and Waghorn *et al*., 1990; Rhind 1993; I'Anson *et al*., 2003; Cognie *et al*., 2003; Meza-Herrera *et al*., 2008) which revealed a positive effect of protein flushing and fertility. In this concern previous studies proved that providing excess dietary CP during 5-8 days before anticipated estrus (i.e. beginning of the mid-luteal phase) increased the ovulation rate (Smith & Stewart, 1990; Smith, 1998). Increased levels of protein in the diet, in conjunction with increasing ovulation rates, increased the circulating levels of FSH during the latter half of the oestrus cycle (Knight *et al*., 1975; Davis *et al*., 1981). On the Contrary to our finding, Garnsworthy *et al*. (2008) observed no effects of dietary protein level (range 15 to 20% CP) on development of small, medium, and dominant follicles, or on ovulation timing in dairy cows. Haresign (1981) suggested that flushing for one cycle may extend its effects by preventing the late atresia of follicles. Also, Lishman *et al*. (1974) reported a greater ovarian follicular response to gonadotrophin in ewes that were on a high plane of nutrition. Our finding were consistent with the finding of previous studies which had reported the positive effect of protein-rich supplements on the ovulation rate of ewes (Smith, 1988) and Rhind (1993).The improvement in the follicular number, size and follicular waves in concomitant with the increase in ovulation rate (OR), this could be due to the change in concentrations of amino acids in the plasma particularly the branched long chain amino acids (BCAA) from the high percentage of cotton seed cake and soybean in TG diet. However, the infusion of BCAA had increased the ovulation rate in ewes in previous studies (Downing *et al*., (1995), Waghorn *et al*., 1990). Other possible explanation could be the direct action of branched chain amino acids on the ovary, since excess protein allowance enhances the circulating levels of these acids concomitantly to increase in ovulation rate in ewes (Downing *et al*., 1995). Since flushing with soybean meal results in excess protein allowance (NRC, 1985), part of the extra amino acids may be converted to glucose by gluconeogenesis. Several lines of evidence suggest that increases in the blood glucose and insulin levels regulate glucose availability at the follicular level and folliculogenesis in ewes (Munoz-Gutiérrez *et al*., 2002; Scaramuzzi *et al*., 2006, Letelier *et al*., 2008). Similarly, Hoon *et al*. (2000) and (Daghigh Kia *et al*., 2011) reported a positive effect of dietary protein on fertility rates and suggested that high protein intake increases FSH pulses, oestrogen concentrations and improves fertility rate in sheep. Scaramuzzi *et al*., (2006) presented a good explanation about the effect of nutritional flushing which alter blood concentrations of some reproductive hormones as transient increase in FSH and decrease in estradiol concentrations in the blood. The consequence of these direct actions on the follicle is a reduced negative feedback to the hypothalamic-pituitary system and increased FSH secretion that leads to a stimulation of follicle maturation and transition to a larger follicular category. The FSH is known to stimulate the final maturation of the dominant follicle together with LH.

The increased levels of blood metabolites like total protein and cholesterol in the ewe serum during oestrus may result from increased metabolic activity while the ewe is under the influence of higher levels of oestrogen. Estrogens and growth hormones cause an increase in total plasma proteins owing to their anabolic effects (Kaneko, 1989). Larson & Kendall (1957) reported an increased level of albumin and total protein in the blood of cows at the oestrous time. It is known that blood cholesterol concentrations and steroid hormones synthesis are positively related to energy intake and health of animals (Velhankar, 1973), while lower cholesterol and glucose concentrations after calving have been associated with an increased number of days from calving to conception (Kappel *et al*., 1984). Rabiee and Lean (2000) suggested that glucose might promote the cholesterol uptake into the ovarian cells and vice versa. Glucose may be a regulator of GnRH release (Randel, 1990; Short *et al*., 1990). Concentrations of plasma cholesterol were positively associated with expression of oestrus at first ovulation, interval from calving to conception, and likelihood of conception and pregnancy (Westwood *et al*., 2002). The level of blood urea is a useful tool for estimating the protein nutritional status in ruminants, as it is readily affected by the dietary intake of protein and energy (Ide *et al*., 1966), in particular by the ratio between protein and energy (Carlsson and Bergström, 1994). Postpartum urea levels in relation to nutrition vary according to protein content, protein degradability, non-protein nitrogen and energy of the diet (Park *et al*.,

2002). The high urea level on day 7 in second ovulation may have a negative effect on ovulation rate in treated ewes due to the negative effect of ammonia on reproductive tract. Excess dietary protein intake in early lactation dairy cows has been associated with decreased reproductive efficiency. Elevated plasma urea nitrogen and milk urea nitrogen concentrations have been associated with decreased conception rates in dairy cows (Roseler *et al*., 1993).

The significant increase in serum oestradiol and progesterone explained by (Bartlewski *et al*., 1999a) who reported that, during the oestrous cycle in the ewe, there are 3–4 major increases in circulating concentrations of oestradiol. The first increase occurs the day after the onset of luteolysis; this increase is concomitant with the increase in LH pulse frequency. The continued increase in oestradiol secretion during the follicular phase of the oestrous cycle reflects increased an increase in LH receptor density in preovulatory follicles. Subsequent increments in oestradiol secretion occur throughout the luteal phase, at 3- to 4-day intervals (Bartlewski *et al*., 1999a). In non-prolific breeds of sheep, the amplitude of oestradiol fluctuations associated with all non-ovulatory waves emerging during dioestrus does not differ from that during the preovulatory rise in oestradiol secretion (Bartlewski *et al*., 1999a). The largest ovarian follicles acquire the ability to secrete oestradiol from the day of emergence with peak oestradiol secretion occurring about the time they reach maximum diameter (Bartlewski *et al*., 2011).

Very low concentrations of progesterone immediately after ovulation (day 0) and during the period of CL formation, are followed by increasing concentrations between days 3 and 7, which then plateau up until ~day 12, and subsequently show rapid decline reaching a nadir prior to the next oestrus and ovulation (Bartlewski *et al*., 1999b).

In conclusion, the long term protein supplement increased the diameter of ovulatory follicles, ovulation rate, and increased the reproductive efficiency in subtropical ewes in the first and second oestrous. In addition, long term protein flushing may had no positive effect on ovulation rate in the second oestrous under present experiment conditions.

Conflict of interest:

The authors declare that they have no conflict of interest.

Table 1: Ingredients and calculated composition of experimental diets (as fed).

Ingredient, %	CG	TG
Yellow corn, ground	32.00	5.00
Cotton seed meal, undecorticated	6.00	45.20
Wheat bran	28.20	18.00
Soybean meal	2.00	10.00
Premix*	0.30	0.30
Ground limestone	1.00	1.00
Salt	0.50	0.50
Wheat straw	30.00	20.00
Total	100	100
Calculated nutrient, (%)		
ME Mcal/kg	2.04	2.04
ME MJ/kg	8.54	8.54
CP	9.5	20.00
CF	16.48	19.79
EE	2.52	3.35
NFE	66.41	51.40
Ash	5.02	5.42
Ca	0.42	0.51
P	0.54	0.74

CG: control group (9.5 % CP), TG: treated group (20 % CP, 2.1 fold higher than maintenance ration)
* Premix each 3 kg contain:
1250000 IU, Vit. A; 2500000 IU, Vit. D3; 1000 mg Vit E; 80000 mg Mn; 60000 mg Zn; 50000 mg iron, 20000 mg copper, 5000 mg iodine, 250 mg selenium, 1000 mg cobalt, completed till 3 kg by $Caco_3$.

Table 2: Effect of high protein supplementation (Mean ± SE) on follicular dynamics and corpus luteum (CL) during two consecutive oestrous cycles.

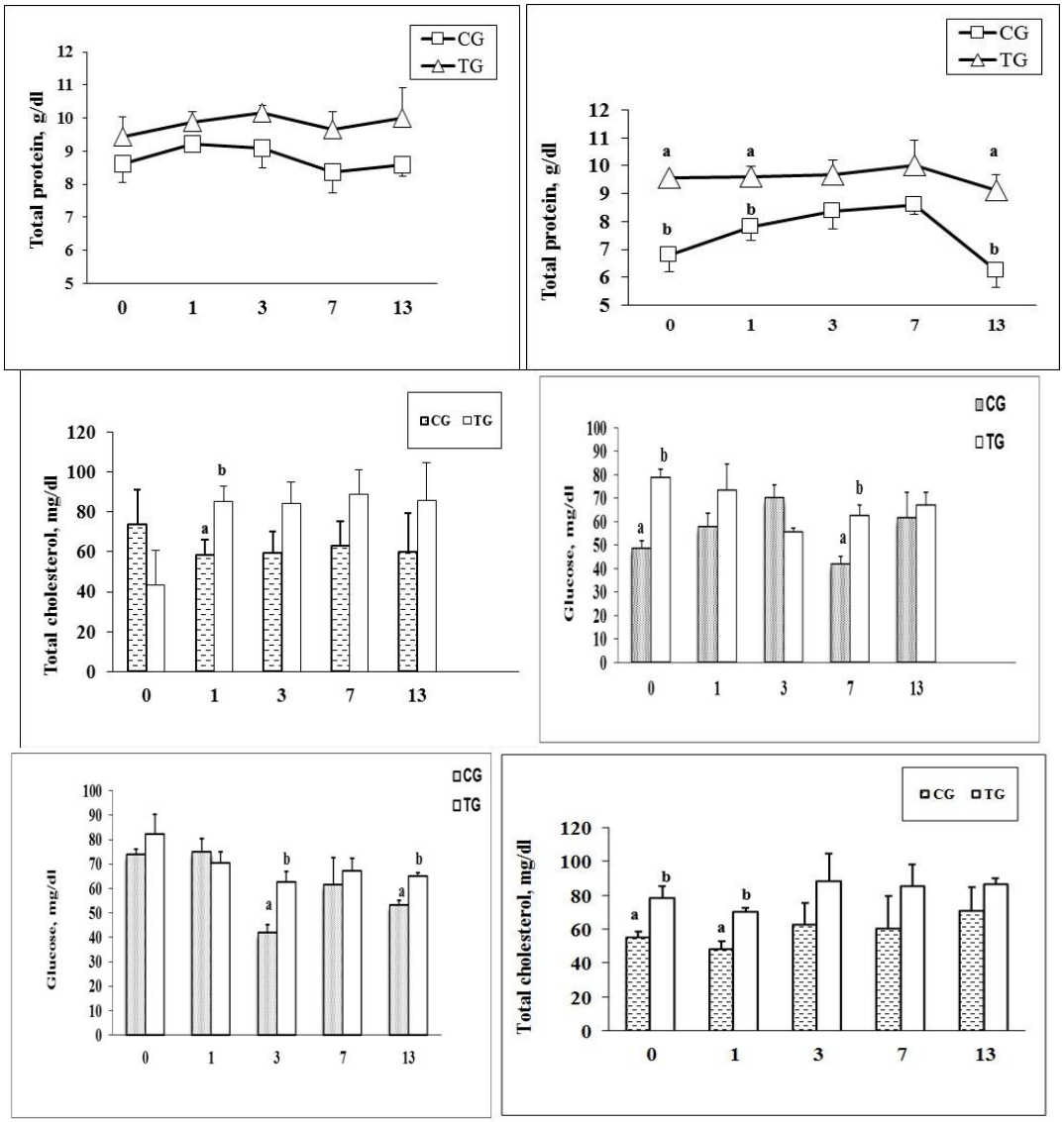
Item	Follicles	CG	TG	P
First oestrous	Small (2-2.9 mm*)	3.62± 0.15[b]	4.46±0.19 [a]	0.001
	Medium (3-5 mm*)	2.13±0.09 [b]	3.08± 0.12[a]	0.0001
	Largest Subordinate follicles (mm)	3.8 ± 0.2	4.2 ± 0.2	0.11
	Ovultory follicle (mm)	5.4± 0.1[b]	5.8±0.01[a]	0.0001
	Number of follicular waves	1.29 ± 0.18 [b]	2.14 ± 0.26[a]	0.02
	Waves length (day)	9.83 ± 0.83[b]	5.89 ± 1.05[a]	0.02
	First waves after first ovulation (day)	17.50 ± 6.56	11.33 ± 6.77	0.14
	Ovulation rate	1.0± 0.00b[b]	1.43±0.20 [a]	0.05
	CL diameter (cm)	1.04±0.01	1.04± 0.01	0.82
Second oestrous	Small (2-2.9 mm*)	3.43±0.14[b]	3.96± 0.14[a]	0.01

	Medium (3-5 mm*)	2.78±0.11[b]	4.52±0.20[a]	0.0001
	Largest Subordinate follicles (mm)	3.6 ± 0.01[b]	4.2 ± 0.1[a]	0.000
	Ovultory follicle (mm)	5.4±0.1[b]	5.8±0.1[a]	0.0001
	Number of follicular waves	2.17 ± 0.31[b]	3.00 ± 0.001[a]	0.02
	Waves length (day)	10.46 ± 2.21	7.50 ± 1.19[a]	0.2
	First waves after second ovulation (day)	7.00 ± 1.5	5.00 ± 0.8	0.38
	Ovulation rate	1.0± 0.00	1.0± 0.001	0.9
	CL diameter (cm)	0.88±0.01	0.91±0.02	0.1

* = number of follicles
Ovulation rate = determined on the basis of the number of *corpora lutea* in a given oestrous cycle/ number of animals

Table 3: Overall mean of dominant, subordinate follicles, number of follicular waves and estrous length of both first and second oestrous cycles.

Item	Control	Treatment	P
Largest Subordinate follicle (mm)	3.7 ± 0.1[b]	4.2 ± 0.1[a]	0.0001
Ovulatory follicle (mm)	5.4 ± 0.1[b]	5.8 ± 0.12[a]	0.0001
Number of follicular waves	22	33	
Average number of follicular waves	3.14 ± 0.51[b]	4.71 ± 0.52[a]	0.05
Waves length (day)	10.26 ± 1.52[b]	6.96 ± 0.87[a]	0.04
Estrous length (day)	21.57 ± 0.57	20.28 ± 0.52	0.12



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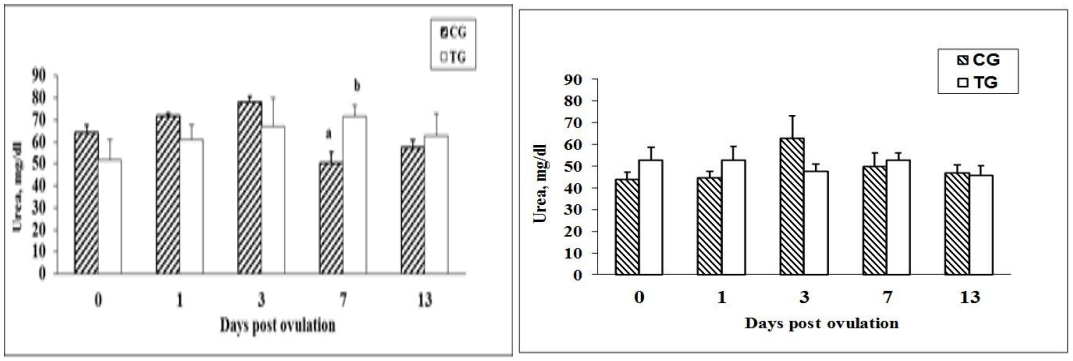
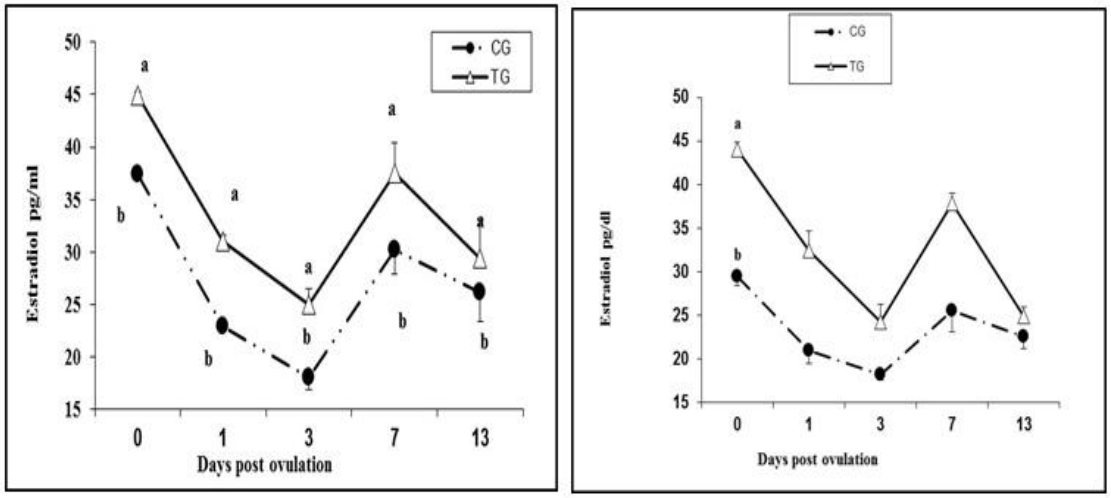


Fig. 1: Changes in serum metabolites concentrations in ewes flushed with long term protein supplementation (A) first oestrus (B) second estrus. Bras or line with different superscript indicate significant differences (B) between groups on the same day of the oestrous cycle ($p < 0.05$).
(A) First oestrus B) Second oestrus



(D) Second oestrus

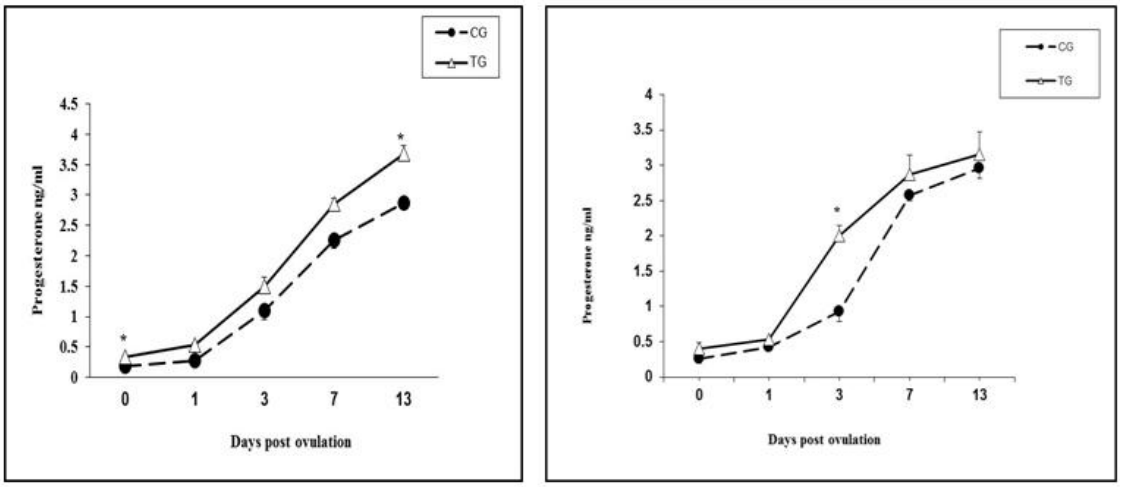


Fig. 2: Changes in serum estradiol and progesterone in ewes flushed with long term protein supplementation (C) first oestrus (D) second oestrus. Line with different superscript or * symbol superscript indicate significant differences between groups on the same day of the oestrous cycle ($p < 0.05$).

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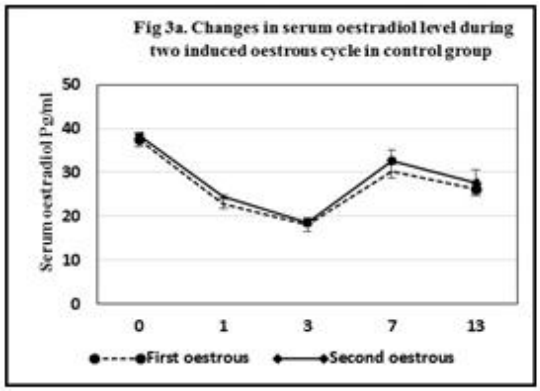
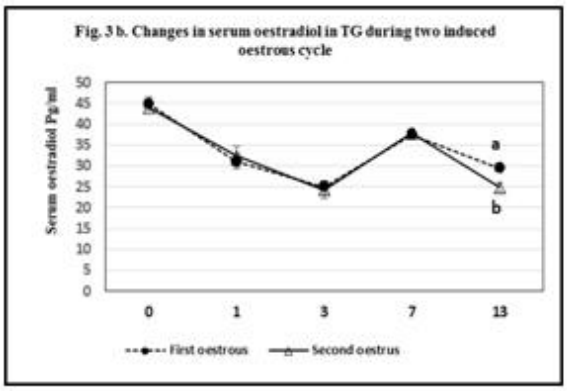
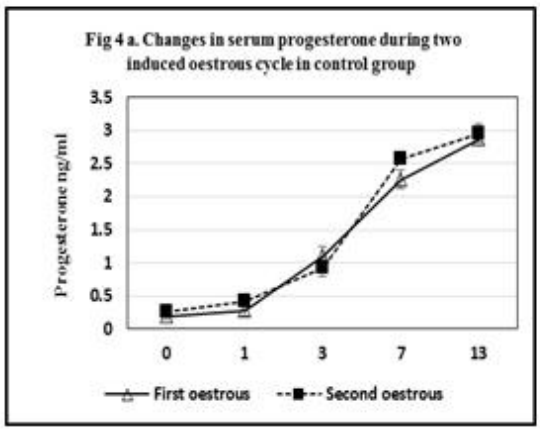
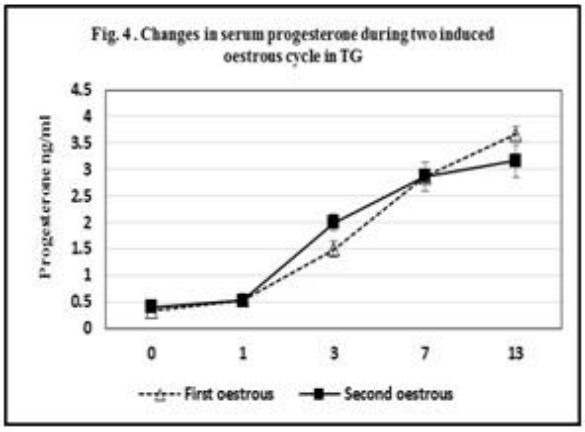
(B) First oestrus:









TG= treatment group

Fig 3 and 4: Line with different superscript indicate significant differences between first and second oestrous cycle on the same day at the same group ($p < 0.05$).

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