

## Research Article

# Effects of Different Ratios of Linoleic to Linolenic Acids in Diets During Flushing Period on Blood Metabolites, Hormone Concentrations and Reproductive Performance of Zel Ewes

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## Introduction

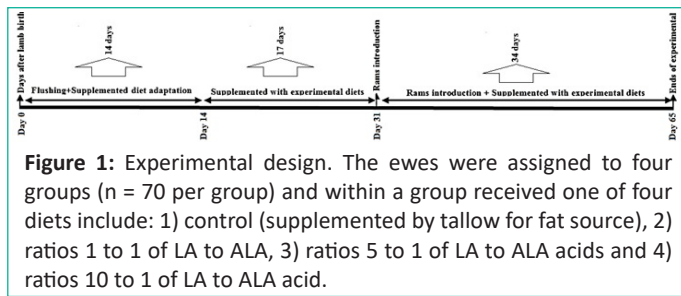
Flushing diets are commonly used in ewes with the lower body condition score to boost folliculogenesis, ovulation and increase the lambing rate [22]. It contain high energy density and fed at least two weeks before ram introduction during the breeding season. Increased level of dietary energy was shown to affect the ovarian function and follicular size [36]. Feeding energy-rich additives such as fat supplements modulates the recrudescence of hypothalamic and pituitary function and regulates the ovarian activity in females [27]. In addition, higher dietary fat ingestion have a direct impact on ovarian structures, which may lead to an increase in the size and number of the ovarian follicles [30]. The addition of fatty acids source into the diet could affect the concentrations of blood hormones [15]. The supplementation of polyunsaturated fatty acids to flushing diets had a significant impact on ewe reproductive performance through an increase in plasma concentrations of cholesterol

## Abstract

A total of 280 ewes ( $44 \pm 3$  kg and two years old) were fed to evaluate the effects of diets enriched in different ratios of omega-6 (linoleic acid; LA) and omega-3 (alpha-linolenic acid; ALA) Polyunsaturated Fatty Acids (PUFAs) on blood metabolites, some hormone concentrations and reproductive performance of Zel fresh ewes. Ewes were assigned to four experimental groups ( $n=70$  per group) and within a group received one of four diets, including control (enriched beef tallow, Group1), LA to ALA ratio of 1:1 (Group 2), LA to ALA ratio of 5:1 (Group 3), and LA to ALA ratio of 10:1 (Group 4). This trial was continued approximately for 65 days. Blood samples were collected on days 14, 31, and 45 to measure plasma concentrations of blood metabolites. A significant increase in glucose and cholesterol showed in ewes group 2 compared to the Control group ( $P<0.05$ ), but the triglyceride levels were significantly decreased ( $P<0.05$ ). The concentrations of plasma 17-beta estradiol on day 31 were markedly higher for group 2 ( $P<0.05$ ). LA to ALA ratio of 1:1 showed approximately 55 percent more plasma progesterone compared to Control ( $P<0.05$ ). Plasma insulin concentrations increased in groups 3 and 4 compared to the Control group ( $P<0.05$ ). Moreover, significant differences were observed among groups for pregnancy, lambing rates, and sex ratio of newborn lambs ( $P<0.05$ ). In conclusion, ratios of 1:1 and 5:1 (LA: ALA) of fatty acids in diets improved fertility and reproductive performance.

**Keywords:** Ewe; Fatty acids; Nutrition; Blood parameters; Reproductive performance

and progesterone [1]. For instance, supplementing flushing diets with Calcium Salts of Fatty Acids (CSFA) in Afshari ewes increased plasma glucose and Blood Urea Nitrogen (BUN), and estradiol-17  $\beta$ , which causes improvement of fertility, lambing rate, and the lambs' birth weight. Several studies have also shown that Polyunsaturated Fatty Acids (PUFAs) of the omega-3 and omega-6 family, such as ALA and LA, which are usually found in feedstuffs derived from linseed and soybean, respectively, play an essential role in animal reproductive performance [31]. Despite the presence of evidence for the beneficial effects of PUFAs on folliculogenesis and reproductive performance in different animal species, the evidence for their impact on ovarian activity in sheep during breeding season are sparse. This study aimed to evaluate the effects of flushing diets enriched with three ratios of omega-6 (linoleic acid) / omega-3 (linolenic acid) fatty acids on reproductive performance, blood metabolites and hormones in Zel ewes during breeding season period.



**Figure 1:** Experimental design. The ewes were assigned to four groups (n = 70 per group) and within a group received one of four diets include: 1) control (supplemented by tallow for fat source), 2) ratios 1 to 1 of LA to ALA, 3) ratios 5 to 1 of LA to ALA acids and 4) ratios 10 to 1 of LA to ALA acid.

**Materials and Methods**

**Animals, Nutrition Program, and Experimental Design**

A total of 280 ewes (44 ± 3 kg and two years old) were assigned to four experimental groups (n=70 per group), and received one of four diets, including the control diet (supplemented by beef tallow, Group1), LA to ALA ratio of 1:1 (Group 2), LA to ALA ratio of 5:1 (Group 3), and LA to ALA ratio of 10:1 (Group 4). The supplementation of different PUFA sources in the experimental diets was continued for 65 days (day 0= initiation of the feeding, Figure 1).

To increase the accuracy in the contribution of each LA and ALA fatty acids in ewes' diet, the profile of fatty acid in the diet was determined (Table 1). Formulation of the experimental diets are provided in Table 2. These isoenergetic and isonitrogenous experimental diets contained approximately 30-gram fatty acids compared to the level of the saturated fat present in beef tallow for the control group. The amounts of fatty acid profiles of extruded soybean and extruded linseed diets were obtained from our previous study [26]. Based on former study results, a total fatty acid in extruded soybean and linseed were 18.88 and 36.7 percent, based on dry matter, respectively. These values were calculated according to the amounts (g) of fatty acid sources added to the experimental diets. Soybean extruded

**Table 1:** Amounts of different fatty acid sources in diet (% DM).

Source of fatty acids	Fatty acid profiles (%)				
	C16:0	C18:0	C18:1	C18:2	C18:3
	Palmitic acid	Stearic acid	Oleic acid	LA	ALA
Extruded Linseed	7.64	5.22	20.05	14.81	51.28
Extruded Soybean	9.65	4.43	21.64	50.21	4.9

**Table 2:** Ingredient and chemical composition of experimental diets from week's three to six.

Ingredient (% DM)	Treatments			
	Control	Ratios of LA to ALA		
		1:01	5:01	10:01
Alfalfa Hay	27.61	26.67	25.71	25.8
Wheat – Straw	18.09	17.39	17.1	17.32
Barley Grain	15.17	13.43	14.92	14.68
Corn Dry	3.03	2.42	2.98	2.94
Soybean - Meal – 44	5.46	4.88	4.07	4
Rice Bran	27.91	27.22	25.71	25.8
Tallow	1.82	0	0	0
Soybean – Extruded	0	4.27	8.19	8.62
Linseed - Extruded	0	2.81	0.45	0
Calcium – Carbonate	0.61	0.61	0.57	0.56
Salt	0.3	0.31	0.28	0.28
Chemical components				
Crude protein (% DM)	12.3	12.3	12.3	12.3
Dietary NDF (% DM)	39.2	39.4	41.2	41.3
Dietary Fat (% DM)	5.1	5.1	5.1	5.1

supplemented in experimental diets (except the control group) was 70, 145, and 155g for ratios 1:1, 1:5, and 1:10, respectively. On the other hand, 46, 8, and 0g extruded linseed were added to experimental diets, respectively. The flushing diets were formulated using SRNS software version 1.9.5566.0 and based on the requirements of a 35±1.2 kg ewe, with a body condition score of 2.5±0.25. The amount of dry matter for each ewe was 1450 g/day. The schematic protocol for the experimental diets and the length of the feeding period of the experimental groups is shown in Figure 1. Healthy two-years-old Zel rams were used for mating in this study. One ram was used per 15 ewes. The rams spent a period of three estrus cycles of the ewes (51 days) in the herd. All rams were fed with different diets from ewes.

**Blood Metabolite and Hormone Analysis**

Blood samples were collected from the jugular vein in EDTA tubes. Four blood samples were collected from each ewe (n=7/ treatment) on days 0 (initiation of fat supplementation), 14, 31, and 45. The blood samples were centrifuged for 15 min at 2000g, and the blood plasma was stored at -20°C for future analysis. Plasma concentrations of progesterone, insulin, and estradiol were determined using ELISA (Diaplus, North York, Ontario, Canada). Intra-assay and inter-assay coefficients of variation were lower than 12%. Plasma glucose, cholesterol, and triglycerides were determined enzymatically using a spectrophotometer (Shimadzu 2100, Kyoto, Japan), according to the manufacturer's instruction.

**Reproductive Performance**

The pregnancy rate, lambing rate, and sex ratios of lambs were measured after approximately five months. The pregnancy was confirmed by measuring plasma progesterone in the mated ewes. The pregnancy rate was determined based on the number of ewes had high plasma progesterone levels and the number of ewes that had mated with the painted back. The lambing rate was calculated by the number of lambs born to the number of mated ewes. The numbers of female and male lambs were recorded at birth to measure the sex ratio.

**Statistical Analysis**

Data were tested for normal distribution of the residuals using SAS software by the PROC UNIVARIATE procedure (SAS Institute, 2006). Data on blood metabolites and hormones were analyzed as repeated measurements using PROC MIXED of SAS software the following model:

$$Y_{ijk} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + B_k + e_{ijk}$$

Where  $\mu$  is the population mean,  $\alpha_i$  is the treatment effect,  $\tau_j$  is the effect of sampling day or time,  $(\alpha\tau)_{ij}$  is the interaction effects of treatment and sampling day or time,  $B_k$  is a random effect of animal, and  $e_{ijk}$  is the residual error. Significance was declared at P<0.05. The binomially distributed reproductive data were analyzed using the logistic procedure of SAS. Significance and tendencies were declared at P<0.05, unless otherwise indicated.

**Results and Discussion**

The purpose of this study was to determine the effects of different proportions of essential fatty acids LA and ALA (linseed and soybean extruded as LA and ALA, respectively) in diets on blood metabolites, reproductive hormones, and reproductive performance. Unlike almost all previous investigations, in this study the relative amounts of ALA and LA were calculated.

Formulation of each experimental diet based on the amount of each calculated fatty acid. In each experimental diet, the ratios (1:1, 5:1, and 10:1) were formulated according to these amounts. The major aim of this procedure was to determine the probable effects the concentrations of each essential fatty acid on ewe's fertility. The percentage of omega-3 and omega-6 fatty acid sources in experimental diets had shown variable effects on ruminant reproduction in most of previous studies.

Overall, the concentration of all metabolites in the blood plasma increased from day 14 (first sampling) compared to day 45 (fourth sampling). There were significant differences in plasma glucose concentrations between the experimental groups at all sampling times ( $P < 0.05$ ) (Table 3). In the first sampling on day 14, ewes in group 4 (ratio 10 to 1 LA to ALA) had the highest (51.09 mg/dl) concentrations of plasma glucose ( $P < 0.05$ ). In the second and third sampling times on days 31 and 45, respectively, the highest levels of glucose were observed in the experimental groups 5:1 LA to ALA (54.03 mg/dl) and 1:1 LA to ALA (77.01) (Table 3). In the present study, fresh ewes who received a control diet (beef tallow as the primary fat source) had the lowest concentration of plasma glucose in comparison to other groups in all three times of blood sampling. A possible reason for the decrease in glucose concentration could be related to the decreased feed intake caused by the presence of beef tallow, and the resultant decrease in the concentrations of propionate or metabolizable proteins in the rumen fluid [1]. Unlike our results, no significant differences were observed in plasma glucose concentration of ewes received diet supplemented with the PUFAs [12]. When the plasma glucose concentration rises, the uptake of glucose and cholesterol by the ovarian cells may increase [25].

Plasma cholesterol in the second sampling time (day 14) in ewes receiving 5:1 LA to ALA ratio was significantly higher than in the other groups ( $p < 0.05$ ). There was no diet  $\times$  sample day interaction on plasma cholesterol concentrations. In agreement with the results of the present study, the concentration of plasma cholesterol in ewes fed a flushing diet supplemented with calcium salts of fatty acids was higher compared to the control group [16]. It seems that the reason for the relative increase of cholesterol concentration in the blood plasma of treatment groups (supplemented with unsaturated fatty acids) as compared to the control group, could be due to the increased level of ruminal biohydrogenation of polyunsaturated fatty acids and then absorption in the small intestine. Our result is also consistent with the findings of Marcello et al. (1971), who showed that the increasing content of fatty acids in diets increases unsaturated fatty acids C16:1 and C18:1 concentrations in animals plasma [19]. Since cholesterol is considered a major component of lipoproteins, more fatty acids are used in cholesterol formation [13]. Cholesterol is an important precursor of progesterone in the Corpus Luteum (CL), and also plays a role in the synthesis of other steroid hormones in the follicular phase [7]. Feeding diets supplemented with n-3 fatty acids compared with n-6 fatty acids resulted in higher progesterone concentrations in follicular fluid in ewes [35].

Ewes in all experimental groups showed increased plasma triglyceride concentrations from 14 to 45 days of sampling (Table 3). The highest triglyceride concentration showed on day 45 for group 1 (25.24 mg/dl). However, the plasma triglyceride concentration did not differ among experimental groups ( $P > 0.05$ ). Increasing in triglyceride concentration may be due to decreased activity of the lipogenic enzyme in the animals'

**Table 3:** Plasma concentrations of metabolites in different treatment groups.

		Treatments					
Metabolites		Control	1:01	5:01	10:01	SEM	P-Value
Glucose (mg/dl)	Day 14	48.53 <sup>b</sup>	50.02 <sup>ab</sup>	49.78 <sup>ab</sup>	51.09 <sup>a</sup>	0.794	0.044
	Day 31	50.71 <sup>b</sup>	53.27 <sup>ab</sup>	54.03 <sup>a</sup>	53.06 <sup>ab</sup>	1.509	0.023
	Day 45	67.26 <sup>b</sup>	77.01 <sup>a</sup>	70.53 <sup>ab</sup>	75.12 <sup>ab</sup>	3.321	0.041
Cholesterol (mg/dl)	Day 14	78.47	79.67	83.6	78.9	5.373	0.902
	Day 31	91.99 <sup>b</sup>	91.68 <sup>a</sup>	97.27 <sup>a</sup>	92.75 <sup>ab</sup>	1.573	0.045
	Day 45	100.83	101.55	103.53	104.68	4.2	0.91
Triglyceride (mg/dl)	Day 14	17.76	17.98	18.33	18.71	0.84	0.862
	Day 31	21.03	18.9	19.18	19.71	0.953	0.416
	Day 45	25.24	19.7	23.25	20.09	1.814	0.123

Means in the same row with different superscript are significantly different ( $P < 0.05$ ).

livers and adipose tissues [13]. Moreover, increasing the accumulation of triglycerides in the liver of ruminants may be due to increased levels of Free Fatty Acids (FFAs) in the blood.

Plasma progesterone concentrations were similar across the experimental groups at the first sampling time. On the second and third sampling days, plasma progesterone concentrations were significantly higher in the experimental groups 4 (1.36 ng/dl) and 2 (4.00 ng/dl), respectively ( $P < 0.05$ ) (Table 4). There were no ( $P > 0.05$ ) diet  $\times$  time interactions for concentrations of blood plasma progesterone. According to our results, the increasing trend of progesterone concentration in ewes' blood plasma is shown in Table 4. This result was in agreement with the observations of Wonnacott et al. (2010) who reported that higher progesterone concentration in follicular fluid in ewes fed diets supplemented with omega-3 and omega-6 fatty acids [35]. Moussavi et al. (2007) reported no significant difference in the plasma progesterone concentrations between the control group (no fat supplement in the diet) and cows fed fish meal and calcium salts of fatty acids during the luteal phase [21]. However, Walsh et al. (2011) showed that ruminants fed diets supplemented with PUFAs have a higher concentration of progesterone during the luteal phase of the estrus cycle [34]. Accordingly, Castaneda-Gutierrez et al. (2007) speculated that the increase in progesterone concentrations of plasma in cows supplemented with conjugated LA was due to an increase in IGF-I recycle [6] and the production of prostaglandin E2 from Steroidogenic Acute Regulatory (StAR) in the granulosa cells [18]. An increase in serum lipid concentrations is associated with an increase in the fat contents of small and large steroidogenic cells (estrogenic constituents) of ruminant corpus luteum. Therefore, an increase in intracellular lipids may be a reason for enhancing the level of progesterone precursors (Hawkins et al., 1995). On the other hand, the role of ALA in the prevention of PGF2 $\alpha$  synthesis is clear, which has an optimal effect on progesterone concentration [14]. Higher levels of progesterone concentrations have been shown to support embryo viability and enhance the establishment of pregnancy [30].

As shown in Table 4, the addition of different ratios (1:1, 5:1, and 10:1) of LA and ALA had no significant effect on ewes' plasma estradiol 17- $\beta$  hormone in all the experimental groups in the first and third sampling times. On the second sampling time, the highest plasma estradiol 17- $\beta$  hormone concentrations (91.56 pg/ml) were observed in experimental group 2 with a ratio of 5:1 (LA to ALA). An interestingly marked increase in the concentration of estradiol 17-beta hormone was observed only on day 31 of sampling for the experimental diet with a ratio of 1 to 1 LA to ALA. Somchit et al. (2007) reported that plasma concentration of estradiol in ewes fed lupin as a source of supplementary

**Table 4:** Plasma concentrations of hormones in different treatment groups.

Hormones	Time	Treatments				SEM	P-Value
		Control	1:01	5:01	10:01		
Insulin ( $\mu\text{m}/\text{ml}$ )	Day 14	5.88	7.15	6.17	7.46	0.737	0.386
	Day 31	8.17	8.11	7.19	9.41	0.941	1.331
	Day 45	7.87 <sup>b</sup>	8.46 <sup>ab</sup>	10.13 <sup>a</sup>	10.26 <sup>a</sup>	0.925	0.239
Progesterone (ng/dl)	Day 14	1.16	1.39	1.46	1.51	0.164	0.461
	Day 31	1.24 <sup>ab</sup>	0.97 <sup>b</sup>	1.11 <sup>ab</sup>	1.36 <sup>a</sup>	0.125	0.039
	Day 45	2.63 <sup>b</sup>	4.00 <sup>a</sup>	2.81 <sup>ab</sup>	3.71 <sup>ab</sup>	0.634	0.021
Eestradiol 17- $\beta$ (pg/ml)	Day 14	56.78	56.96	57.04	54.52	3.94	0.962
	Day 31	70.15 <sup>b</sup>	91.65 <sup>a</sup>	75.46 <sup>ab</sup>	81.87 <sup>ab</sup>	6.63	0.048
	Day 45	68.69	72.49	67.8	65.37	4.34	0.71

Means in the same row with different superscript are significantly different ( $P < 0.05$ ).

**Table 5:** Pregnancy rates, lambing rates, and sex ratio of lambs in ewe with different treatment groups.

Treatment	Pregnancy rate (%)	Lambing rate (%)	Sex ratio (% male / female)
Control	57.14 <sup>b</sup>	42.86 <sup>b</sup>	66.67/33.33 <sup>ab</sup>
1:1	85.71 <sup>a</sup>	85.71 <sup>a</sup>	50/50 <sup>b</sup>
1:5	71.43 <sup>ab</sup>	75.14 <sup>ab</sup>	75/25 <sup>a</sup>
1:10	71.43 <sup>ab</sup>	71.43 <sup>ab</sup>	71.43/28.57 <sup>a</sup>
SEM	0.175	0.184	0.262
P-Value	0.046	0.039	0.033

Means in the same columns with different superscript are significantly different ( $P < 0.05$ ).

fat in the diet was decreased, but this reduction was not significant [29]. Two papers have reported that nutritional supplementation with energetic sources decreased estradiol secretion [28,33]. In agreement with our study, serum estradiol concentration in ewes fed the fatty acid supplemented diets increased from the time of injection of PGF2 $\alpha$  (day 0) to day 3 [2]. Increasing the availability of cholesterol may result in increased steroid component synthesis and improved energy balance by supplementing fat in the diet [1]. Downing et al. (1999) reported that glucose and insulin injections into the ovarian vessels reduced estradiol secretion [9]. The reason for reduced estradiol secretion might be due to the reduced availability of substrates for the production of estradiol or the loss of capacity of granulosa cells to convert these substrates to this hormone [29].

The pregnancy rate in the control group (57.14%) was lower than in the other experimental groups (Table 5). Also, the lambing rate of the control group (42.86%) was markedly lower than the other experimental groups ( $P < 0.05$ ). No significant differences were observed in lamb birth weight between experimental groups. However, supplementing sources of fats such as oil or oilseeds resulted in an improved lambing performance in sheep [8]. Adding supplemented fat to flushing diets in ewes may have a significant impact on the animal's reproductive performance through an increase in plasma concentrations of progesterone [5].

The sex ratio was significantly affected by experimental treatments ( $P < 0.05$ ). Ewes on a diet with 5:1 of LA to ALA produced the highest number of male offspring. Published data concerning the effect of food supplements on sex ratio are controversial and primarily relate to the diet composition during the mating and fertilization period [17]. Reports by Gulliver et al. (2013) showed that further research is required to determine whether the observed increase in the proportion (58.2 vs. 43.5%) of

female-born lambs of the Merino  $\times$  Border Leicester ewes [14]. The content of MUFA, PUFA, and glucose in diet could affect the sex ratio [20]. It seems that the effect of dietary fat supplementation on sex offspring is related to female parents; feeding male rats with fat supplemented diets did not affect the sex ratio of the offspring [3].

Due to the effect of fatty acids in each stage of ovulation and pregnancy, supplementing diets with omega-3 and omega-6 fatty acids could take place in the estrous, mating, and pregnancy stages in ewe's herds. These effects might happen due to the impact of omega-3 and omega-6 fatty acids on the synthesis of steroid hormones, increased ovulation, and follicle size. There is a positive correlation between glucose and cholesterol concentrations in ovarian cells, which means that by increasing cholesterol absorption and increasing insulin concentration, glucose uptake increases [25]. The simultaneous absorption of glucose and cholesterol leads to the release of LH by influencing the gonadotrophic releasing hormones [10]. An increase in LH concentration leads to the development of larger ovarian follicles, yields higher estradiol concentration during the estrous cycle, and results in the formation of a larger corpus luteum, with aids in pregnancy establishment and continuity. Childs et al. (2008) fed beef heifers diets enriched in fish oil (omega-3 fatty acids), and even though no increase in serum progesterone was observed on day 7 of the estrous cycle [7]. They postulated that there was evidence for the more synthesis of progesterone during the whole estrous cycle due to the increase in the serum concentrations of cholesterol and the presence of a greater size corpus luteum.

## Conclusion

In conclusion, the results of this study showed that ratios of 1:1 (LA: ALA) of fatty acids in diets of experimental groups more improve fertility and reproductive performance. The levels of plasma progesterone and estrogen in ewes who received LA and ALA at a ratio of 1:1 were significantly higher than in other experimental groups. Different ratios of LA to ALA acids in the diet during the flushing period had beneficial effects on reproductive postpartum in Zel fresh ewes. According to the results of our study, we suggested further studies to be carried out on this topic accompanied by measuring different amounts of omega-3 and omega-6 fatty acids in the diets.

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