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Ovarian follicular development, hormonal and metabolic profile in prepubertal ewe lambs with moderate dietary restriction and lipid supplementation

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ABSTRACT

To evaluate the effects of moderate dietary restriction and lipid supplementation on ovarian follicular development, hormonal and metabolic profile, thirty-five prepuberal ewe lambs were blocked by body weight and randomly assigned to treatments: AL−US (control) - un-supplemented-diet *ad libitum* (3.5% ether extract, $n = 9$); R-US - intake restricted to 85% of the AL−US diet ($n = 9$); AL-LS - lipid-supplemented-diet *ad libitum* (9.8% ether extract, $n = 8$); R-LS - intake restricted to 85% of the AL−LS diet ($n = 9$), from 95 ± 8 days of age until estrus or 7 months of age. Lipid supplementation did not reduce dry matter intake. Daily weight gain was greater in lambs fed *ad libitum*. Plasma glucose was greater in the R−LS treatment group, while serum insulin was less with lipid supplementation. There was a treatment by age interaction on total cholesterol, HDL cholesterol and triglyceride serum concentrations. Estrus was detected in 43% of the animals and the overall ovulation rate was 60%. The number of follicles, diameter of the largest follicle, body weight, age and serum progesterone at puberty did not differ among treatment groups. The mean diameter of the largest follicle was greater in lambs having than in those not having ovulations and increased with age in both groups. There was an interaction between the effects of occurrence of ovulation and age on the number of follicles between 3 and 5 mm and > 5 mm. Lipid supplementation and dietary restriction altered the metabolic profile in ewe lambs with no concomitant changes in values for reproductive variables.

1. Introduction

Puberty in ewe lambs is mainly determined by body weight and photoperiod (Foster et al., 1985). In the tropics, the growth rate and body weight are more important for attainment of puberty, irrespective of the season of birth, compared with effects on puberty

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in subtropical and temperate latitudes (Mukasa-Mugerwa et al., 1991). In the Santa Inês sheep, a local breed of northeastern Brazil which do not have marked photoperiod effects on reproduction (Coelho et al., 2006; Sasa et al., 2002), nutrition is the main factor affecting age at puberty. Ewe lambs attain puberty when they 60%–75% of the body weight of an adult female, which occurs between 4 and 5 months of age and at weights between 25–35 kg (Nunes et al., 1997). Dietary restriction during the prepubertal period delays the occurrence of puberty (Day et al., 1986) by inhibiting luteinizing hormone (LH) secretion and impairing dominant follicle development (Kinder et al., 1995). Knowledge of the development of ovarian antral follicles in prepubertal ewe lambs is still limited (Bartlewski et al., 2002, 2006).

Nutrition modulates reproductive functions and physiological functions through the circulating metabolic hormones such as insulin, insulin-like growth factor I (IGF-I), growth hormone (GH) and leptin (Armstrong et al., 2003). Insulin functions centrally to stimulate LH release in ruminants (Tanaka et al., 2000) and locally at granulosa and theca cells of ovarian follicles to mediate glucose uptake, thus modulating follicular function (Williams et al., 2001). Glucose also appears to have a function in the nutritional regulation of GnRH release and, in turn, pulsatile LH secretion (Diskin et al., 2003). Feed intake is positively associated with glucose concentrations in prepubertal heifers (Bossis et al., 1999).

Lipid supplementation is a nutritional strategy that appears to be related to the improvement of reproductive function through being an energy source and by actions on reproductive processes unrelated to energy (Mattos et al., 2000). Supplementation with omega-6 (linoleic acid C18:2)- and omega-3 (linolenic acid, C18:3)- fatty acids has positive effects on ovarian follicle development in cows (Lucy et al., 1993; Thomas and Williams, 1996; Beam and Butler, 1997; Thomas et al., 1997; Staples et al., 1998; Bilby et al., 2006a, 2006b). In contrast to cattle, there are few observations on the effects of lipid supplementation on ovarian follicular numbers and diameter in goats (Mahla et al., 2017) and ewes (Zeron et al., 2002; Ghoreishi et al., 2007), and these results are somewhat inconsistent. Considering that the fatty acid profiles of dietary lipid sources may determine the effects on reproduction (Santos et al., 2008), the use of oleaginous seeds rich in polyunsaturated fatty acids (PUFA) as a source of omega-6 (n-6) and omega-3 (n-3) families is desirable. The supply of roasted soybean, a feed rich in n-6 linoleic acid, to ewe diets may, therefore, positively affect follicle development. To the best of our knowledge, however, the effects of follicular development on prepubertal ewe lambs fed a diet rich in omega-6 fatty acid have not been evaluated.

The aim of this study was, therefore, to evaluate the effects on ovarian follicular development and hormonal and metabolic profiles of lipid supplementation of diets of prepubertal ewe lambs. It is hypothesized that n-6 PUFA supplementation to *ad libitum* or restricted-fed ewe lambs improves follicular development.

2. Material and methods

The experiment was conducted at the Department of Animal Sciences of the Federal University of Lavras (UFLA). All experimental procedures were approved by the Bioethics Committee on Animal Use - UFLA (Protocol No. 085/2013).

2.1. Pre-experimental management

To obtain uniform ewes with respect to age, weight and birth period, a breeding season was conducted with 139 Santa Inês sheep between 3.0 and 4.0 (Scale 0–5, Gordon, 1997) in body condition score. During gestation and after lambing, ewes were submitted to routine management practices. After weaning at 45 days of age, the ewe lambs were maintained in pastures with Tifton 85 (*Cynodon* spp.) grasses and supplemented with concentrate prior to initiation of the experiment.

2.2. Experimental design and treatments

The experiment was arranged in a incomplete randomized block design, with four treatments. Thirty-five single-gestation ewe lambs weighing 21.5 ± 1.7 kg that were 95.0 ± 7.9 days of age (average ± SD) were assigned according to body weight into nine blocks, eight with four animals each and one with three animals, and randomly assigned to one of four treatments: AL-US (control): unsupplemented-diet *ad libitum* (n = 9); R-US: intake restricted to 85% of the AL-US treatment (n = 9); AL-LS: lipid-supplemented-diet *ad libitum* (n = 8); R-LS: intake restricted to 85% of the AL-LS treatment (n = 9).

2.3. Experimental diets and feeding regimen

Throughout the experiment a single diet was maintained for each treatment group. Lipid supplementation was in the form of roasted and cracked soybeans (90.6% dry matter - DM, 38.5% crude protein - CP, 22.3% neutral detergent fiber - NDF, 5.8% ash, 23.2% ether extract - EE, 10.2% non-fibrous carbohydrates - NFC) added to the diet. Diets were prepared according to the National Research Council (NRC, 2007) recommendations to meet the nutritional requirements of ewe lambs that weighed 20 kg and were to gain 250 g per day (Table 1).

Ewe lambs were maintained in individual stalls and orts from each pen were removed and weighed daily to record individual intake. The amount of feed offered to *ad libitum* treatment groups was adjusted daily allowing at least 10% of orts so as to not compromise growth performance. To determine the amount of feed to be offered to the restricted animals, the average intake from the previous 7 days in the respective *ad libitum* diet was used. Feed was supplied as a whole diet once a day (10 a.m.) and mixed again in the trough at 3:00 p.m. Diets were fed until the first estrus was detected or until lambs were 7 months of age. Animals had access three times a week to a dry paddock, for 40 min. Free access to water and mineral mix were provided.

Table 1
Ingredients and chemical composition of the experimental diets.

Ingredients	% Dry matter	
	Unsupplemented-diet	Lipid-supplemented-diet
Corn silage	52.12	51.74
Corn meal	16.37	10.77
Soybean meal	29.63	6.42
Roasted and cracked soybean	0.00	29.21
Limestone	0.73	0.72
White salt	0.42	0.42
Minerals[1]	0.73	0.72
Chemical composition		
Dry matter (% of as fed)	43.08	42.83
Crude protein[2]	20.57	19.35
Neutral detergent fiber[2]	34.96	37.91
Ash[2]	6.70	6.70
Ether extract[2]	3.54	9.82
Non-fibrous carbohydrate[2,3]	33.94	26.22

[1] Minerals (Vacci-phos sheep without copper®, Vaccinar, Belo Horizonte, Brazil): 155.0 g Ca; 85 g P; 5 g Mg; 15 mg S; 140 g Na; 3,500.00 mg Zn; 5,000.00 mg Mg; 42.0 mg I; 15.00 mg Se; 36.00 mg Co; 1,000.00 mg F; 1,000.00 mg Mn.
[2] % of dry matter.
[3] NFC = 100-(CP + NDF + Ash + EE).

2.4. Chemical analysis of feed and orts

Ort samples (20% of total) and corn silage samples were collected daily, whereas concentrate ingredient samples were collected once a week. Composite samples were collected every 2 weeks for orts from each pen and for corn silage and monthly for each of the concentrate ingredients and proximate analysis was conducted on these samples. Samples, except for the concentrate ingredients, were pre-dried in a convection oven for 72 h at 55 °C. All samples were ground in a 1 mm sieve mill and a sub-sample was dehydrated at 105 °C for 24 h for determination of DM content. The CP content was analyzed using a Micro-Kjeldahl steam distillation apparatus and the EE was analyzed by the Goldfisch method, as described in the AOAC (1990). Ash were determined by incinerating the sample at 600 °C for 5 h. The NDF content was determined using the filter crucible method with thermostable α-amylase (Termamyl® 2X - Novozymes Latin America Ltda. Paraná, Brazil). Non-fibrous carbohydrates (NFC) were calculated according to NRC (2001) using the formula: NFC = 100 - (CP + NDF + EE + ash).

Five 100 mg roasted soybean samples were lyophilized prior to the fatty acid profile analysis. Lipids were extracted with chloroform:methanol:water (2:1:0.8 v/v/v), according to Folch et al. (1957). The lipid extracts were saponified with 2 mL of 0.5 M NaOH in methanol (w:v) and esterified with 2.5 mL of esterifying reagent (10 g NH_4Cl, 300 mL CH_3OH, 15 mL H_2SO_4) in a boiling bath (5 min per process). After addition of a 4 mL saturated NaCl solution, methyl esters were extracted with 2.5 mL hexane and preserved in N_2 at −80 °C prior to analyses (Bligh and Dyer, 1959).

Fatty acid-methyl esters were analyzed using gas chromatography (GC 2010, Shimadzu, Italy) equipped with an automatic injector (AOC 20i Shimadzu), a flame ionization detector and a capillary column detector (SP-2560 Supelco® TM, 100 m long, 0.25 mm i.d., 0.2 mm thickness) filled with helium gas (28 cm s-1). The column temperature was programmed from 140 to 240 °C, with an increase of 4 °C/min and maintained for 30 min at that temperature and a split ratio of 1:100. Fatty acid peaks were integrated and quantified using the GC Solution software (Shimadzu, Italy). The identification was made by comparing retention times with reference to the certified internal standard (Supelco® 37 Component FAME Mix). Results (Table 2) were reported as area percentages (g/100 g).

Table 2
Fatty acid profile of the roasted soybean.

Fatty acids	% total FA[1]
Palmitic (C16:0)	10.82
Stearic (C18:0)	4.49
Oleic (C18:1n9c)	24.06
Linoleic (C18:2n6c)	52.47
Linolenic (C18:3 n3c)	6.46
Arachidic(C20:0)	0.34
Gadoleic (C20:1)	0.13
Behenic (C22:0)	0.45
Lignoceric (C24:0)	0.15
Other fats	0.63

[1] Fatty acids.

2.5. Performance measurements

Ewe lambs were weighed prior to feeding once a week throughout the experimental period for weight gain determination. Feed conversion (FC) was calculated using the formula: FC = DM intake (DMI, kg)/weight gain (kg). Subcutaneous fat depth between the 12^{th} and 13^{th} ribs was measured by ultrasonography (Aloka SSD-500, 3.5 MHz linear transducer, Tokyo, Japan) on the first day of the experimental diet supply and at 4, 5, 6 and 7 months of age. Three measurements were taken and the average was determined.

2.6. Blood sampling and assays

Blood samples were collected on the first day of the experimental period, immediately before feeding and at 3, 4, 5, 6 and 7 months of age, between 3 and 4 h after feeding for metabolites and insulin quantification. Blood sampling was performed by puncture of the jugular vein in tubes containing EDTA-K_3 solution and sodium fluoride (Anticoagulant Glucose, Labor Import, Osasco, São Paulo, Brazil) for glucose testing or without anti-coagulant (BD Vacutainer, New Jersey, USA) for triglyceride, total cholesterol, HDL cholesterol, and insulin determinations. Immediately after collection, samples were kept in coolers between 4 and 5 °C, centrifuged at 1500 x *g* for 15 min and plasma or serum stored at − 20 °C. Glucose, triglycerides, total cholesterol and HDL cholesterol analyses were performed using a microplate reader (MutiskanTH GO Microplate Spectrophotometer, ThermoScientific, Marietta, OH, USA) utilizing commercial kits of Liquiform Glucose (Labtest, Lagoa Santa, MG, Brazil), Liquiform Triglycerides (Labtest, Lagoa Santa, MG, Brazil), HDL Cholesterol (Labtest, Lagoa Santa, MG, Brazil) and Liquiform Cholesterol (Labtest, Lagoa Santa, MG, Brazil). Insulin analysis was performed using a solid-phase radioimmunoassay utilizing a commercial kit (Porcine Insulin RIA Kit, Millipore, Missouri, USA). The high intra-assay coefficient of variation (CV) was 15.37% and the low 1.51%.

Blood samples for progesterone assays were collected 4–6 h after feeding, once a week from 25 kg body weight and twice a week from the time lambs weighed 30 kg until the time of detection of estrus or until lambs were 7 months of age. Samples were collected in vacuum tubes without anticoagulant (Vacutainer®, BD, New Jersey, USA) and processed as described previously in this manuscript. Progesterone (ng/mL) was quantified using a double-antibody radioimmunoassay (Progesterone DA, MP Biomedicals Santa Ana, California, USA). High and low intra-assay coefficients of variation were 8.27% and 0.18%, respectively. High and low inter-assay coefficients of variation were 13.15% and 19.39%, respectively.

2.7. Estrous detection, ovulation and luteal phase length

Estrous detection was performed once a day after ewe lambs gained weight to the extent they weighed 25 kg with “teaser” rams. Ewe lambs were considered in estrus when allowing rams to mount consistently. Ovulation was estimated to have occurred on the sixth day preceding the time when a progesterone concentration ≥ 1 ng/mL was detected (Quircke et al., 1979; Ryan et al., 1991). The increase in serum progesterone to values ≥ 1 ng/mL was used to define whether an animal had attained puberty. The first increase in serum progesterone to values ≥ 1 ng/mL was used to compare the effect of treatments.

The occurrence of short, normal and long estrous cycles was determined using the methods previously described by Schirar et al. (1989). The weight that was recorded that was nearest to that of the date of ovulation was considered as the weight at the first ovulation, with a 4 day-deviation, because animals were weighed once a week.

2.8. Ultrasonographic examination

Ovarian functions of 34 ewe lambs was assessed by real time transrectal ultrasonography (Aloka SSD 500, 7.5 MHz linear transducer; model UST – 660, Tokyo, Japan) on alternate days for 10 days at 3, 4 and 6 months of age. A single evaluation was performed at 5 and 7 months of age. In one ewe lamb from the R−US treatment group, it was not possible to assess the ovaries properly and data from this animal were not considered in the analyses. All ultrasonic assessements were conducted by a single operator. After visualization of the bladder and the caudal portion of the uterus, the transducer was rotated 45° to 90° clockwise and counter clockwise to locate the ovaries. The number of antral follicles ≥ 3 mm and ≤ 5 mm in diameter were recorded in one category and those > 5 mm in another. The diameter of the largest follicle was also recorded. Follicle diameters were calculated as the average of the two greatest diameters in the cross section. All ultrasonic assessments were recorded for subsequent analysis.

2.9. Statistical analysis

Data were analyzed using SAS (version 9.2, SAS Institute, Inc., Cary, NC, USA). Body weight, blood metabolites (glucose, triglycerides, total cholesterol, HDL cholesterol), insulin and subcutaneous fat depth data were analyzed using a linear mixed model with random animal effect using the MIXED procedure, as defined in Verbeke and Molenberghs (2000). A mixed model was used for each variable, defined by:

$$Y_{ijk} = \beta_1 Block_k + \beta_2 T_{1i} + \beta_3 T_{2i} + \beta_4 T_{3i} + \beta_5 T_{4i} + (\beta_6 T_{1i} + \beta_7 T_{2i} + \beta_8 T_{3i} + \beta_9 T_{4i})\, t_{ij} + (\beta_{10} T_{1i} + \beta_{11} T_{2i} + \beta_{12} T_{3i} + \beta_{13} T_{4i})\, t_{ij}^2 + b_{1i} + b_{2i}\, t_{ij} + b_{3i}\, t_{ij}^2 + e_{ijk}$$

$$b_{1i} \sim N(0, \sigma_{b1}^2)$$

$$b_{2i} \sim N(0, \sigma_{b2}^2)$$

$b_{3i} \sim N(0, \sigma^2_{b3})$

where:

Y_{ijk} = animal i, at age j and block k;

$Block_k$ = effect of block k;

T_{1i}, T_{2i}, T_{3i}, T_{4i} = effects of treatments 1, 2, 3 and 4, respectively. When isolated, T refers to the constants of the regression models. When multiplying t_{ij}, refers to the first-degree effects (t_{ij}) for each treatment. When multiplying t^2_{ij}, refers to the second-degree effects (t^2_{ij}) for each treatment;

t_{ij} = first-degree effect of age;

t^2_{ij} = second-degree effect of age;

b_{1i} = random animal effect, with $b_{1i} \sim N(0, \sigma^2_{b1})$;

b_{2i} = random effect of animal, referring to the first-degree effect of age, with $b_{2i} \sim N(0, \sigma^2_{b2})$;

b_{3i} = random effect of animal, referring to the second-degree effect of age, with $b_{3i} \sim N(0, \sigma^2_{b3})$

e_{ijk} = experimental error associated with each observation, $e_{ijk} \sim N(0, \sigma^2)$.

The effect of treatments on DM and nutrient intake, average daily weight gain (ADWG), FC, days in experiment, weight, age and serum progesterone concentration at puberty was analyzed using the GLM procedure through analysis of variance and F test. The statistical model was:

$$Y_{ij} = \mu + \beta_1 X_{1ij} + \beta_2 X_{2ij} + T_i + \gamma_j + e_{ij}$$

where:

Y_{ij} = observed value referring to the animal of block j that received treatment i;

μ = a constant associated with all observations;

X_{1ij} = covariate initial weight of the animal of block j that received treatment i;

X_{2ij} = covariate days in experiment of the animal of block j that received treatment i;

T_i = effect of treatment i;

γ_j = effect of block j;

eij = random error associated with each observation Y_{ij}, $e_{ijk} \sim N(0, \sigma 2)$ with normal distribution, zero average and variance σ^2.

The percentage of ewe lambs in estrus was analyzed by generalized linear models, considering the Bernoulli distribution and logit function, using the GENMOD procedure. The considered model was:

$$log\left(\frac{k_{ij}}{1 - k_{ij}}\right) = \mu + Ti + \gamma j$$

where:

μ = constant associated with all observations;

T_i = effect of treatment i;

γ_j = effect of block j;

Follicular development was also analyzed throughout the study considering the occurrence of ovulation. The diameter of the largest follicle and the number of follicles from 3–5 mm and > 5 mm data were analyzed using generalized linear mixed models considering the Gamma (follicular diameter) or Poisson (number of follicles) distribution and logarithmic linkage function, with random animal effect using the GLIMMIX procedure. The model used to analyze the follicular diameter considered days in experiment as covariate and the fixed effects of treatment, occurrence of ovulation during the experiment (0 = not ovulating or 1 = ovulating), linear and quadratic effects of age. The model used to analyze the number of follicles from 3–5 mm and > 5 mm was similar, but there was consideration for the interactions of treatment by ovulation and age by ovulation.

$$\log(Y_{ijkl}) = b_{1ij} + \beta_1 X_{1ij} + T_i + (\beta_2 O_{0ij} + \beta_3 O_{1ij})t_l + (\beta_6 O_{0ij} + \beta_7 O_{1ij})t_l^2$$

where:

Y_{ijkl} = diameter or number of follicles of the animal from block j receiving treatment i with ovulation status k, at the age l;

b_{1ij} = constant relative to the random effect of the animal;

X_{1ij} = covariate days in experiment;

T_i = effect of the treatment i;

O_{0ij} *and* O_{1ij} = identify the regression coefficients for ovulation during the experiment (0 = did not ovulate and 1 = ovulated);

t_l *and* t_l^2 = linear and quadratic effects of age, respectively.

3. Results and discussion

Data for the effects of treatments on DM and nutrients intake, ADWG and FC are summarized in Table 3. Ewe lambs fed the soybean diet *ad libitum* (AL−LS) had a greater ($P < 0.05$) EE intake. The DM intake was, however, not reduced ($P > 0.05$) compared to those fed the control diet *ad libitum* (AL−US). Similarly, there were no differences ($P > 0.05$) in DM intake between lambs with dietary restriction (R−US and R−LS). Lipids may alter ruminal fermentation patterns and affect DM intake (Jenkins, 1993), but the

Table 3
Daily dry matter and nutrient intake and performance parameters in prepubertal ewe lambs fed unsupplemented-diet or roasted soybean supplemented-diet *ad libitum* or restricted.

	AL-US (n = 9)	R-US (n = 9)	AL-LS (n = 8)	R-LS (n = 9)
DM (g/day)	888.6 ± 36.2^{a}	745.7 ± 8.3^{b}	844.2 ± 38.4^{a}	719.1 ± 7.6^{b}
CP (g/day)	182.8 ± 9.0^{a}	148.9 ± 1.7^{bc}	162.0 ± 7.8^{b}	135.1 ± 1.3^{c}
NDF (g/day)	325.5 ± 10.6^{a}	274.7 ± 3.0^{b}	312.1 ± 13.6^{a}	283.8 ± 3.0^{b}
Ash (g/day)	62.2 ± 2.7^{a}	51.3 ± 0.6^{c}	56.6 ± 2.6^{b}	47.9 ± 0.6^{c}
EE (g/day)	31.9 ± 1.3^{c}	25.6 ± 0.3^{d}	83.9 ± 4.1^{a}	66.4 ± 0.8^{b}
NFC (g/day)	334.9 ± 14.0^{a}	270.3 ± 2.9^{b}	219.5 ± 11.3^{c}	183.5 ± 2.0^{d}
ADGW (g/day)	154.2 ± 12.7^{a}	125.0 ± 3.8^{bc}	145.8 ± 12.7^{ab}	115.1 ± 6.0^{c}
FC[1]	6.0 ± 0.4	6.2 ± 0.2	5.9 ± 0.3	6.2 ± 0.3

AL-US: unsupplemented-diet *ad libitum*; R-US: restricted to 85% of the AL-US diet; AL-LS: lipid-supplemented-diet *ad libitum*; R-LS: restricted to 85% of the AL-LS diet.
DM - Dry matter, CP - crude protein, NDF - neutral detergent fiber, EE - ether extract, NFC - non-fibrous carbohydrate, ADGW - average daily weight gain and FC - feed conversion.
[a,b,c]Means followed by different letters on the same line differ among themselves ($P < 0.05$).
Values are lsmeans ± standard error of the mean (SEM).
[1] FC = DM intake (kg)/weight gain (kg).

negative effect of lipids on diets for ruminants can be prevented when the dietary EE content is limited to 5% (NRC, 2007). Results indicate that it is possible to exceed the EE dietary content for ewes when roasted soybeans are used, because, even though there was a relatively greater lipid content (9.8% EE), there were no decreases in DM intake in the present study. The roasting process of soybeans may have contributed to the slow release of fatty acids in the rumen. Reddy et al. (1994) observed slower fatty acid release in the rumen with roasted soybean, as well as a relatively lesser ruminal hydrogenation of C18:1 and C18:2 fatty acids compared to extruded soybean or soybean oil. Diet forage ratios (51%–52% in DM) should be considered, because the fatty acid ruminal biohydrogenation is favored when feeding diets with relatively greater fiber content, reducing the negative effects of PUFA on ruminal microorganisms (Latham et al., 1972). The ADWG of ewe lambs fed *ad libitum* was greater ($P < 0.05$) than those in the respective restriction groups. The ADWG did not differ ($P > 0.05$) between ewe lambs fed *ad libitum*, as well as between those where there was dietary restriction. Furthermore, the ADWG in lambs of the AL—LS group did not differ from those of the R—US group ($P > 0.05$). The observed weight gains were less than those that were expected when diets were formulated, which can be explained by the relatively lesser genetic potential for gain of the experimental animals (Pedrosa et al., 2010) and by the fact that females have relatively lesser weight gain than males (Rodríguez et al., 2011; Silva et al., 2012). Furthermore, the nutritional recommendations used for hair sheep production systems, such as those in Northeast Brazil, are derived from studies conducted in other countries and are not always consistent with the performance observed in these Brazilian enterprises because the nutritional requirements are affected by factors such as environmental conditions, nutritional status, and genotype (Resende et al., 2008). There was no difference in FC among treatment groups ($P > 0.05$) in the present study, which was expected due to the differences observed in DM intake and weight gain.

There was an interaction between the effects of treatment and age ($P < 0.01$) on body weight in the present study, indicating that, although all the animals had similar weights at the beginning of the experimental period, at 7 months of age, the ewe lambs with dietary restriction had lesser body weights compared to ewe lambs fed *ad libitum* (Fig. 1). Subcutaneous fat depth did not differ between treatment groups (12.0 ± 0.4; 11.0 ± 0.3; 12.0 ± 0.2 and 11.0 ± 0.3 mm in AL—US, R—US, AL—LS and R—LS, respectively), and was not affected by age or by the interaction between treatment and age ($P > 0.05$).

There was no effect of the interaction of treatment x age on glucose and insulin concentrations ($P > 0.05$). Plasma glucose concentration increased linearly ($P < 0.01$, Fig. 2A) and serum insulin concentration was of a quadratic response as a function of age

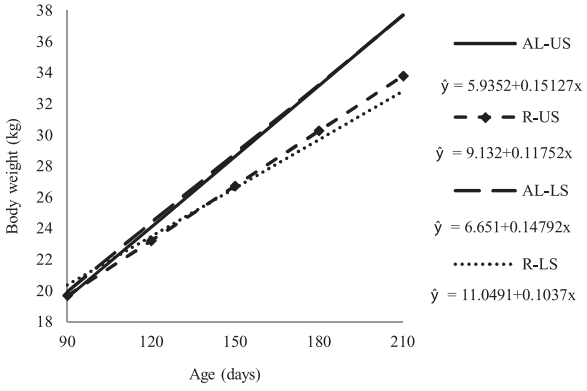

Fig. 1. Body weight of ewe lambs fed unsupplemented-diet *ad libitum* (AL-US, n = 9), unsupplemented-diet restricted to 85% of the AL-US treatment (R-US, n = 9), lipid-supplemented-diet *ad libitum* (AL-LS, n = 8) and lipid-supplemented-diet restricted to 85% of the AL-LS treatment (R-LS, n = 9).

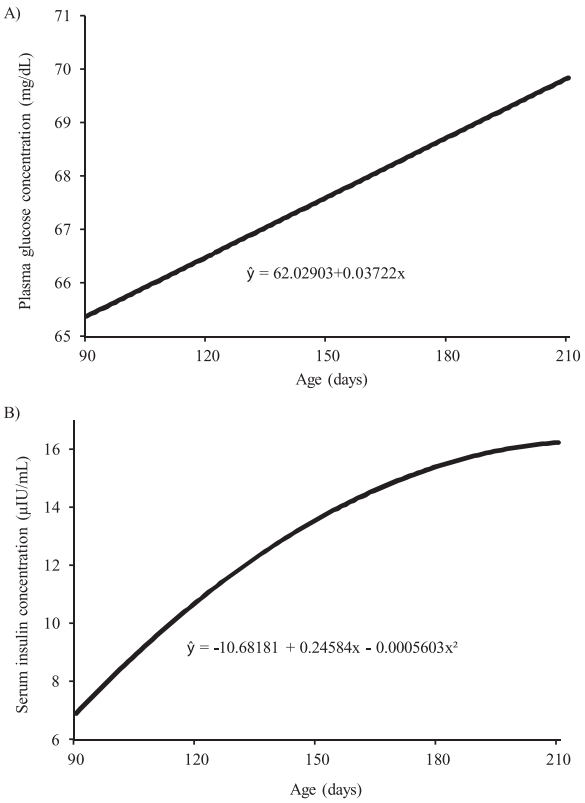

Fig. 2. Mean concentrations of (A) plasma glucose (mg/dL) and (B) serum insulin (µUI/mL) in prepubertal ewe lambs; Data were collected from 35 animals at 3, 4, 5, 6 and 7 months of age.

($P < 0.05$, Fig. 2B). Ewe lambs in the R−LS treatment group had greater plasma glucose concentrations ($P < 0.05$) than the lambs of the other treatment groups (Table 4), but plasma glucose concentration did not differ ($P > 0.05$) among ewe lambs in the AL−US, R−US, AL−LS treatment groups. Serum insulin concentration was less ($P < 0.05$) when there was lipid supplementation which is consistent with results from previous studies (Choi and Palmquist, 1996; Garnsworthy et al., 2008) where it was reported that there was a lesser insulin concentration as a result of fat inclusion in the diet. Carbohydrates are important energy sources, and in ruminants are converted to short-chain fatty acids by microbial fermentation, which stimulates insulin secretion from the pancreas (De Jong, 1982; Bergman, 1990). Insulin had a central role in metabolism by stimulating utilization of glucose in peripheral tissues such as muscle and adipose and by promoting accumulation of glycogen and lipid reserves (Garnsworthy et al., 2008). The lesser glucose concentrations in ewe lambs fed the AL−US diet are, therefore, likely induced by the greater insulin concentrations in these ewe lambs. The greater glucose concentrations in lambs fed the R−SL diet is, however, likely the result of the lesser plasma insulin concentrations in the lambs of this group.

There was a significant interaction ($P < 0.05$) between treatment by age on serum total cholesterol (Fig. 3A) in the present study. Ewe lambs from the AL−LS group had a more pronounced increase in serum total cholesterol concentration over time compared to lambs of the other treatment groups. Similarly, Balaro et al. (2012) reported that cholesterol concentrations were greater in Santa Inês sheep in which there was dietary supplementation with lipids compared with lambs fed unsupplemented diets. When there are greater concentrations of dietary EE the availability of absorbed fatty acids increases, which can be converted to acetate for

Table 4
Plasma glucose and serum insulin concentrations in prepubertal ewe lambs fed unsupplemented-diet or roasted soybean supplemented-diet *ad libitum* or restricted. Blood samples were collected between 3 and 4 h after feeding at three, four, five, six and seven months of age.

	AL-US (n = 9)	R-US (n = 9)	AL-LS (n = 8)	R-LS (n = 9)
Glucose (mg/dL)	64.35 ± 1.39[a]	65.60 ± 1.38[a]	68.19 ± 1.51[a]	73.00 ± 1.43[b]
Insulin (µUI/mL)	15.19 ± 0.98[a]	12.69 ± 1.03[ab]	11.95 ± 1.05[b]	11.25 ± 0.98[b]

AL-US: unsupplemented-diet *ad libitum*; R-US: restricted to 85% of the AL-US diet; AL-LS: lipid-supplemented-diet *ad libitum*; R-LS: restricted to 85% of the AL-LS diet.
[a,b]Means followed by different letters on the same line differ among themselves ($P < 0.05$).
Values are lsmeans ± standard error of the mean (SEM).

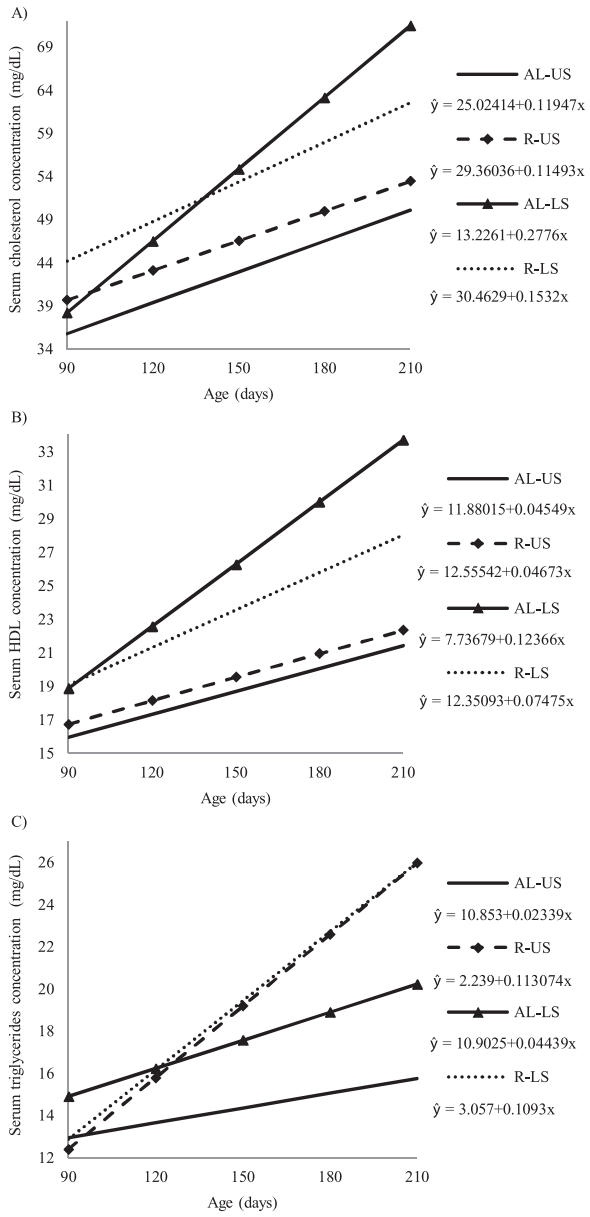

Fig. 3. Serum concentrations (mg/dL) of (A) cholesterol; (B) HDL; (C) triglycerides in ewe lambs fed unsupplemented-diet *ad libitum* (AL-US, $n = 9$), unsupplemented-diet restricted to 85% of the AL-US treatment (R-US, $n = 9$), lipid-supplemented-diet *ad libitum* (AL-LS, $n = 8$) and lipid-supplemented-diet restricted to 85% of the AL-LS treatment (R-LS, $n = 9$).

cholesterol synthesis (Nelson and Cox, 2017). The greater EE intake results in an increase in plasma cholesterol concentrations (Beynen et al., 2000).

Serum HDL cholesterol increased linearly with age, with a rapid increase in AL−LS treatment group (treatment x age interaction, $P < 0.001$, Fig. 3B), which is consistent with the increased EE intake of these animals. In goats, Beynen et al. (2000) observed that there was an increased HDL cholesterol in response to the inclusion of fat in the diet. Increased fat intake leads to an increase in fatty acids in the circulation, which results in an increase in triglycerides in circulation. Triglycerides are transported when attached to lipoproteins, resulting in increased HDL after hydrolysis (Goldberg, 1996).

There was an interaction ($P < 0.05$) between treatment and age on serum triglyceride concentrations (Fig. 3C), such that triglyceride concentrations with dietary restriction were increased markedly with advancing age with the greatest values occurring at the end of experiment, compared with ewe lambs fed *ad libitum*. Furthermore, ewe lambs from the AL−LS treatment group had greater serum triglyceride concentrations compared with ewe lambs from the AL−US treatment group. According to Kozloski (2011), in the fed state, 95% of total plasma lipids are associated with lipoproteins and only 5% are non-esterified fatty acids, which circulate bound to albumin. From the lipids associated with lipoproteins, about 90% are phospholipids and cholesterol, and only about 10%

triglycerides. Thus, increased lipid intake in AL−LS group in the present study probably resulted in a greater triglyceride concentration in circulation.

The average number of follicles ranging from 3–5 mm and > 5 mm, and the diameter of the largest follicle did not differ among treatment groups ($P > 0.05$). Similarly, in sheep supplemented with calcium salts of long-chain fatty acid the diameter of the largest follicle and the number of small (< 4 mm), medium (≥ 4 and ≤ 8 mm) and large (> 8 mm) follicles did not differ when lipid supplemented- and unsupplemented-diets were fed (Ghoreishi et al., 2007). Zeron et al. (2002) reported, however, that there was a tendency for a greater number of follicles in the ovaries of ewe lambs supplemented with calcium soaps of fish oil fatty acids. Variable results on folliculogenesis among studies could be the result of the amount of fat provided in the diet and length of supplementation, to the metabolic status of the animals (Moallem et al., 2013), as well as, be a consequence of modifications that occur to PUFA in the rumen, leading to an increase in saturated, monounsatured and polyunsatured-trans fatty acids (Jenkins, 1994). Furthermore, it was reported that the composition of fatty acids in the supplemented fat has an important function in determining the effect on reproduction (Mattos et al., 2000; Santos et al., 2008). Regarding the dietary restriction, the results of the present study are not consistent with those of Diskin et al. (2003), in which the restriction of dietary intake by 40% reduced the growth rate and diameter of the dominant follicle in cattle. Thus, it is possible that the restriction imposed in the present study (15% of the *ad libitum* treatments) was not great enough to impair follicle development.

In the analysis of follicular development considering the occurrence of ovulation, the mean diameter of the largest follicle was greater in ewe lambs that had ovulations ($P < 0.01$) than in those where there were no ovulations (4.1 compared with 3.8 mm) and there was an increase in size of the largest follicle with age in both groups ($P < 0.05$, Fig. 4A). There was interaction between the effects of ovulation and age ($P < 0.05$) on the number of follicles between 3 and 5 mm and the number of follicles > 5 mm. The number of follicles 3–5 mm in diameter increased as ages advanced in both groups, but there was a pronounced increase after ewe lambs were 120 days in those lambs which did not have ovulations during the study (Fig. 4B). The maximum number of follicles of 3–5 mm in diameter was observed at 180 days (3.7 and 2.4 follicles in animals that did and did not have ovulations, respectively) and decreased thereafter. The number of follicles > 5 mm increased as lambs aged in those lambs that did not have ovulations during the present study, reaching a maximum of 0.7 follicles at 200 days of age. There was, however, a progressive increase in the number of follicles > 5 mm from 150 days in animals that had ovulations (Fig. 4C). Reduction in the population of small follicles with advancing age, as well as, an increase in the population of large follicles, as observed in ewe lambs that had ovulations during the study, was also observed by Bartlewski et al. (2002); Mahdi and Khallili (2008) and Romero et al. (2012) in prepubertal ewe lambs and may be related to the approaching first ovulation. These observations may be due to the fact that the larger follicles secrete large amounts of estradiol and inhibin, exerting a negative feedback on the pituitary, reducing FSH concentrations (Fortune, 1994). Furthermore, increasing follicle diameters prior to the first ovulation are probably induced by an increase in the frequency of LH secretory pulses (Rawlings and Churchill, 1990).

A total of 43% (15/35) of the animals were diagnosed in estrus at 7 months of age, and there was a tendency ($P < 0.10$) for a greater percentage of ewe lambs fed *ad libitum* to attain estrus compared with those lambs with dietary restrictions (55.6%; 33.3%; 62.6% and 22.2% in the AL−US, R−US, AL−LS and R−LS, respectively). The adopted management approach in the present study of keeping the animals in individual stalls, may have contributed to the lesser than expected rate of estrous expression. It is widely accepted that social relationships that animals experience with others of the same group can affect many aspects of reproductive processes (Valasi et al., 2012). Furthermore, social cues can advance puberty onset in ewes, provided the ewe lamb has attained a critical body size (Senger, 2003).

The overall ovulation rate throughout the present study was 60% (21/35), six in AL−LS and five in the other treatment groups, even though there was a greater ADWG in ewe lambs fed *ad libitum*. The number of days in the experiment, age (days) and body weight (kg) of ewe lambs at puberty did not differ among treatment groups ($P > 0.05$, Table 5). The delay of puberty in underfed animals is attributed to a lesser frequency of GnRH pulses and accordingly of LH pulses (Foster and Olster, 1985; Foster et al., 1985; Polkowska et al., 2003) and glucose availability and circulating insulin in the developing sheep affects LH secretion (Bucholtz et al., 1996, 2000). In the present study, blood glucose and insulin concentrations were not reduced in lambs of the restricted compared with the *ad libitum* treatment groups (Table 4), as well as follicle development did not differ among these groups, which is consistent with the observation that puberty was not delayed in ewe lambs when there is moderate dietary restriction.

Similarly to results observed in the present study, Silva et al. (1988) reported that body weight in feedlot Santa Inês ewe lambs at puberty was 30.7 kg and Sousa et al. (2003) reported that Santa Inês ewe lambs attained puberty at 25–30 kg or 50%–60% of their adult body weight, which is on average 50 kg. In small ruminant breeds that do not have reproductive seasonality, the first ovulation is more closely related to body weight than to age (Foster et al., 1985) and acquisition of a minimum body weight has been a determinant in the onset of puberty (Foster and Nagatani, 1999). At 210 days of age, the body weight of the ewe lambs that did not attain puberty was (mean ± standard deviation) 33.7 ± 0.6 kg in the present study, suggesting that factors other than body weight may be involved in the attainment of puberty.

Lipid supplementation increases blood, follicular fluid and corpus luteum cholesterol (Grummer and Carroll, 1991) and as cholesterol is a precursor for the synthesis of steroid hormones (Staples et al., 1998; Mattos et al., 2000), lipid supplementation may alter ovarian steroidogenesis (Zachut et al., 2008). In the present study, even though there was a greater serum concentration of total cholesterol and HDL in the ewe lambs that were fed roasted soybeans, there was not an increase in the serum progesterone concentration ($P > 0.05$, Table 5). The absence of an effect of the lipid supplementation may be related to the large amount of variation observed in individual progesterone values. Previous studies have reported an increase (Staples et al., 1998; Ghoreishi et al., 2007; Garnsworthy et al., 2008), no effect (Bilby et al., 2006a) or even a decrease (Robinson et al., 2002) in blood progesterone concentration in cows or ewes supplemented with lipids. The inconsistent results of the lipid supplementation on reproductive tissues

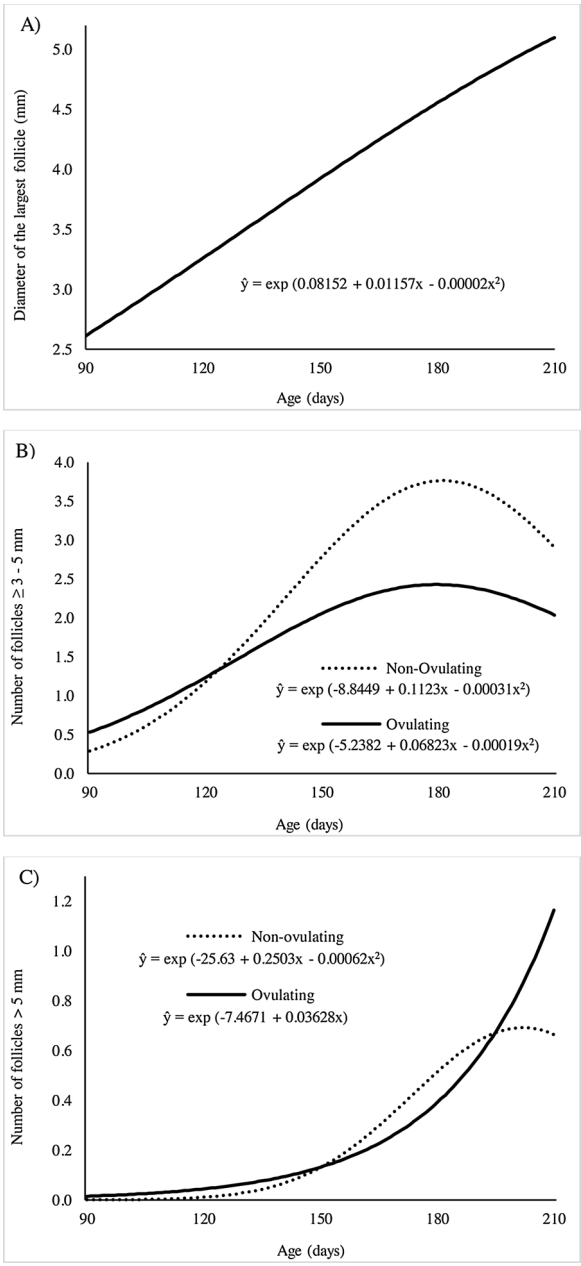

Fig. 4. Ovarian follicular development in prepubertal ewe lambs having (n = 21) or not having (n = 13) ovulations; (A) diameter of the largest follicle (mm), (B) number of follicles from 3–5 mm and (C) number of follicles greater than 5 mm; Ultrasonographic examinations were perfomed on alternate days for 10 days at 3, 4 and 6 months of age and at a single evaluation at 5 and 7 months of age.

may be related to differences in the composition of fatty acids in the supplemented fat (Mattos et al., 2000; Santos et al., 2008). It its noteworthy that the results of the present study, in which a lipid supplement rich in n-6 PUFA (52.5% linoleic acid) was provided to ewe lambs, are consistent in many ways to the results in a previous study (Wonnacott et al., 2010) in which there was increased HDL-cholesterol but decreased progesterone concentration in the follicular fluid in ewes fed a diet enriched with n-6 PUFA, in comparison to those fed a diet enriched with n-3 PUFA. There could be important differential effects of n-3 PUFA on progesterone production within theca cells, as a consequence of increased *STAR* protein (Hughes et al., 2011), that regulates cholesterol transfer within the mitochondria for ovarian steroidogenesis (Stocco and Clark, 1996).

Only eight ewes of the 21 that had ovulations (38%) had short luteal phases; three from the AL-US-, two from the R-US-, two from the AL−LS and one from the R−LS groups. Because these ewe lambs were fairly evenly distributed among treatment groups and in such small numbers, any possible effect on progesterone concentrations were not considered for analysis. Similarly, Bathaei (1996) reported that pre-pubertal ewe-lambs had irregular, short and long estrous cycles, as well as a relatively lesser intensity behavioral

Table 5
Number of days in experiment, age, weight and serum progesterone concentration at puberty in prepubertal ewe lambs fed unsupplemented-diet or roasted soybean supplemented-diet *ad libitum* or restricted.

	AL-US (n = 5)	R-US (n = 5)	AL-LS (n = 6)	R-LS (n = 5)
Days in experiment[1]	67.6 ± 13.2	79.5 ± 13.1	63.9 ± 11.7	67.0 ± 13.0
Age (days)	164.4 ± 12.7	171.5 ± 12.6	170.6 ± 11.3	171.1 ± 12.6
Body weight (kg)	30.5 ± 1.7	29.7 ± 1.7	30.4 ± 1.5	28.5 ± 1.7
Progesterone (ng/mL)[2]	2.6 ± 0.6	1.9 ± 0.6	2.1 ± 0.6	2.8 ± 0.6

AL-US: unsupplemented-diet *ad libitum*; R-US: restricted to 85% of the AL-US diet; AL-LS: lipid-supplemented-diet *ad libitum*; R-LS: restricted to 85% of the AL-LS diet.
Values are lsmeans ± standard error of the mean (SEM).
[1] Number of days in experiment until puberty. Puberty was defined as the beginning of the period when serum progesterone concentrations first exceeded 1 ng/mL.
[2] The first increase in serum progesterone to values ≥ 1 ng / mL was used to compare the effect of treatments.

estrus as compared with adult ewes; and Sasa et al. (2002) observed 22.4% irregular estrous cycles in Santa Inês ewe lambs; 11.4% short and 11.4% long. The occurrence of short estrous cycles can be explained by the short life-span of the corpus luteum, which may be related to reduced secretions of LH and FSH (Gonzalez et al., 1987). The mechanisms related to the functional failure and premature regression of the CL may involve the follicular status immediately before ovulation, which can influence the secretory capacity and the functional longevity of the CL (Camp et al., 1983). Furthermore, the insufficient gonadotropic support after ovulation, when the LH pulses are essential for the development of the CL, can be related to its premature regression, and the manifestation of short estrous cycles (Karsch et al., 1971). According to Camp et al. (1983), the progesterone production by the CL of the short luteal phase can affect the pattern of initiation of normal estrous cycles at initiation of the breeding season.

4. Conclusions

Dietary lipid supplementation in the form of roasted and cracked soybean did not affect feed intake and performance. Insulin concentrations were less with dietary lipid supplementation and there were no concomitant changes in glucose concentrations. The soybean grain diet provided a more pronounced increase in total cholesterol, HDL cholesterol and triglycerides serum concentrations as the lambs age advanced compared to the non-lipid-supplemented diet. The n-6 PUFA supplementation did not, however, result in an enhanced ovarian follicular development in prepurbertal ewe lambs fed diets *ad libitum* or in moderate restriction amounts.

Moderate dietary restriction from 3 to 7 months of age led to a reduction in weight gain and alterations in the metabolic profile, without effects on values of reproductive variables. Additionally, ewe lambs that had ovulations had a larger follicular diameter, reduced population of small follicles and increased number of large follicles around the time of puberty.

Conflicts of interest

The authors of this manuscript have no conflict of interest.

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