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Research paper

Simulated disease process during late pregnancy compromises developmental outcomes of lambs independently of the weaning method applied

Fábio Luís Henrique[a], Evaldo Antonio Lencioni Titto[a], Adoraldo José Zanella[b], Henrique Barbosa Hooper[a], Lina Fernanda Pulido-Rodriguez[a], Ana Luísa Silva Longo[a], Thays Mayra da Cunha Leme-dos-Santos[a], Alfredo Manoel Franco Pereira[c], Cristiane Gonçalves Titto[a,*]

[a] Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brazil
[b] Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, SP, Brazil
[c] Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Évora, Portugal

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ABSTRACT

Stress during pregnancy negatively affects fetal development, and artificial weaning can negatively affect animal health and welfare; however, maternal care can reverse the possible consequences of stress on the offspring. Our study aims to determine the combined effect of a prenatal disease challenge and artificial weaning on welfare and productive performance of lambs. During pregnancy, 43 ewes were distributed into three experimental groups, and at 70th and 120th days of pregnancy (Ig;n = 14; Fg = 14;), the ewes were administered with *Escherichia coli* Lipopolysaccharides (LPS). Fifteen ewes were included in the control group (Cg = 15). Cortisol and rectal temperature measurements were done subsequently to LPS or saline injection. Fourty-six lambs (21 males; 25 females) born in a six-day interval from Ig, Fg, and Cg ewes were subjected to two types of weaning, namely progressive (from 39 to 45 days) and abrupt (at 45 days of age). Lamb data, including plasma cortisol levels, rectal temperature, weaning weight, and performance in feedlot were analyzed and compared through F test and Student's *t*-test (PDIFF; P = 0.05). The injection of LPS resulted in a 619% increase in cortisol levels after two hours, and rectal temperature reached 39.48 ± 0.134 °C after four hours of LPS administration in a pregnant sheep. Both male and female lambs from the Fg group had lower birth weight ($P < 0.05$) as compared to other groups. Cortisol levels and rectal temperature decreased during progressive weaning ($P < 0.05$), in which a higher weaning weight was observed than in abrupt weaning ($P < 0.05$). On the first day at feedlot, cortisol level was reduced after 60 min upon entrance ($P < 0.05$), and higher cortisol values were observed during abrupt weaning ($P < 0.05$). Lower values of dry mater intake and average daily gain were observed for Fg males ($P < 0.05$). LPS challenge during late pregnancy compromised the lambs' indicators of productive performance. Albeit progressive weaning was less stressful during feedlot entrance and total bond separation, more days of maternal care during weaning had no relation with stress during pregnancy.

1. Introduction

Stressful situations during pregnancy, which were represented as a single challenge with lipopolysaccharide (LPS), compromise fetal development of lambs, particularly in the final third of gestation, wherein it can interfere with fetal growth and lead to life-long consequences (Iwasa et al., 2009; Hild et al., 2011; Coulon et al., 2012; Petit et al., 2015). Similarly, artificial weaning process is a well-documented source of stress in lambs even if they were not born from stressed ewes (Freitas de Melo and Ungerfeld, 2016). This phase can be firstly attributed to maternal separation and secondly to the changes in the lamb's access to food (Orgeur et al., 1998). Weaning stress causes the release of cortisol, which may decrease the level of growth hormone (GH), thus influencing the lamb's development (Kuhn et al., 1990; Orgeur et al., 1998).

On the other hand, maternal care can reverse the possible consequences of stress on offspring (Nowak, 1996; Hood et al., 2003; Hild, 2011). In this context, progressive weaning can be an alternative to modulate stress response (Napolitano et al., 2008); however, it may also

* Corresponding author.
E-mail address: crisgtitto@usp.br (C.G. Titto).

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cause an increased stress response due to repetitive separations (Alexander, 1977; Torres-Hernandez and Hohenboken, 1979; Orgeur et al., 1998). Moreover, abrupt weaning is reported to be more stressful than progressive weaning, because it disrupts the bond between an ewe and a lamb (Orihuela et al., 2004; Freitas-de-Melo and Ungerfeld, 2016).

The development outcomes of lambs can be below their genetically programmed potential due to the inhibition of cell division generated by several factors, including stress during late pregnancy (Owens et al., 1993). Moreover, after birth, lamb growth rate is influenced by several factors directly linked to stress responses. Furthermore, the activation or inhibition of endocrine responses is affected by environmental and nutritional factors (Widdowson, 1980; Gluckman, 1986).

The objective of our study is to determine the combined effects of prenatal diseases and weaning methods on the stress responses and productive performances of lambs.

2. Material and methods

2.1. Location and animals

The experiment was carried out in the campus of University of São Paulo in Pirassununga-SP under the coordinates of 21° 59′ south latitude and 47° 26′ west longitude (W.Gr), with an average altitude of 635 m. The climate was classified as Cwa under the Koppen climate classification; and in summers, it is predominantly mesothermal with rainfall and has dry winter and hot summers, with an average annual temperature of 22 °C, and an average annual rainfall that is approximately 1363 mm.

All procedures were approved by the Research Ethics Committee of Faculdade de Zootecnia e Engenharia de Alimentos of Universidade de São Paulo, protocol number 13.1.2109.74.8.

Forty-three multiparous Santa Ines ewes, which is a Brazilian native sheep breed, were inseminated with White Dorper semen on the same day, and their offsprings were used in this study (Paiva et al., 2005). Lambing was concentrated in six days in March 2014. Ewes and lambs were housed in a maternity paddock until they were seven days of age and were later moved to a rotational grazing system. All animals had access to concentrated diets, which were composed of ground corn (63.1%), soybean meal (31.1%), limestone (0.8%), and mineral supplement (5%) in creep feeding from seven days of age.

2.2. Experimental treatments

Forty-three ewes were included in the study through a 3 × 2 factorial system in which ewes were randomly grouped. Fourteen ewes were challenged with IV LPS injection during pregnancy (LPS, 0.01 g/ml, 0.8 mg/kg body weight; *Escherichia coli* 0127: B8, Sigma Aldrich, USA) at 70 days of gestation (Ig; n = 14). Fourteen ewes were injected with the same LPS doses at 120 days of gestation (Fg; n = 14). Fifteen pregnant ewes, which were kept as the control group (Cg: n = 15), received only saline IV with a volume similar as that of LPS received by other groups. During LPS challenge, blood samples were collected and rectal temperatures (RTs) were measured. For blood collection, all ewes were housed in 1.30 m long by 1.20 m wide individual boxes that allowed visual contact with other companions. The animals went through a five-day habituation period prior to LPS challenge and sampling. The blood samples were collected in vacuum tubes through jugular puncture (serum tubes and needle size: 25 × 8 mm, BD Vacutainer), starting from the basal prechallenge sample (05 h 00 min).

After LPS or saline injection, two more blood collections were carried out (06 h 00 min and 07 h 00 min). Further samplings were taken at 120-min interval until the end of 24 h (05 h 00 min). RT was concomitantly measured with blood sampling using a digital thermometer (V966F/V965F Vicks® ComfortFlex® Digital).

Sixty-one lambs were born from 43 ewes during an interval of six days: 24 from Cg (2 still birth, 1 dead after birth, 1 dead after weaning), 17 from Ig (1 stillbirth), 20 from Fg (3 still birth, 7 dead after birth). A total of 46 lambs were used in the rest of our study: 27 females, 19 males, 13 twins, and 20 single births. Birth weight was collected after the lamb suckled colostrum. Afterwards, they were identified with ear tags.

2.3. Weaning

Forty-six lambs were weaned using two management strategies during the experimental period: a) progressive weaning (Pw) and b) abrupt weaning (Aw). Definitive ewe and lamb separations were accomplished for both approaches when the lambs reached 45 ± 6 days of life. The animals were randomly distributed to either abrupt or progressive weaning, with the goal of attaining an equal number of lambs from each prenatally treated group (Cg, Ig, and Fg).

Progressive weaning was carried out for six days, and the ewes were removed daily from the paddock at 7 h 00 min; and the lambs were kept separated from their mothers until 17 h 00 min.

RT was measured ensuing blood sampling. Three blood samples were collected daily, from the 39th to 44th days of the lamb's life. The samples were collected after maternal separation: first collection was done when lambs and ewes still had auditory contact with each other (T0); second collection was done 60 min after ewe and lamb separation (T60); and the third collection was made 180 min after separation (T180). When the lambs were 45 days old, weaning was completed, and the animals were introduced into the feedlot. In the feedlot, blood samples were collected at three different periods. The first collection was made during the last contact with the mother (T0), the second at 60 min after entering the feedlot (T60), and the third at 180 min after entering the feedlot (T180). During the 46th and 47th days of life, blood samples were taken once a day for 24 and 48 h after introduction into the feedlot.

In abrupt weaning, blood samples and RT measures were obtained at the 45th, 46th, and 47th day of the lamb's life in a similar manner as in progressive weaning.

For blood and RT measures, the sampling order was randomly established on the first evaluation day. This selection method followed the same previously used order of animals in all sample collections of different weaning types (ordered samples). All measurements were done inside the corral, with minimal disturbance to minimize stress associated with restraint and sample collection.

The blood samples, 10 ml each, were collected through jugular venipuncture (serum tubes and needle size: 25 × 8 mm, BD Vacutainer) to evaluate cortisol levels. The tubes containing serum were stored at −20 °C until cortisol determination using an Electrochemiluminescence immunoassay kit (Roche Cobas Cortisol assay, Roche Diagnostics). All samples were re-assayed if the duplicates yielded a difference of more than 10%. Inter-assay coefficient of variance (CV) was 9.25%, and intra-assay CV was 12.1%. The kits were validated through the demonstration of parallel curves between standard concentrations and serially diluted samples.

The combination of stress during pregnancy and weaning resulted in six groups: CgPw (n = 9), CgAw (n = 11), IgPw (n = 8), IgAw (n = 8), FgPw (n = 5), and FgAw (n = 5).

2.4. Feedlot productive performance

When the experimental animals reached 45 days of age, they were housed in individual pens, measuring 1.20 m by 1.30 m with external view, where they stayed for 73 days preceded by seven days of adaptation. The diet was uniform for all groups, with corn silage as forage food, 30% dry matter (DM) basis proportion, and concentrate composed of ground corn (49.6%), soybean meal (17.7%), mineral salts (1.8%), and limestone (0.9%) in the proportion of 70% dry matter basis (DM). Food was given twice a day at 07 h 00 min and 16 h 00 min.

The daily amount of food supplied was the result of the consumption in the previous day. The leftovers were daily weighed, with an increase of 20% to allow better food selection. Individual drinkers were cleaned daily to maintain the availability of high quality water to all animals. Food remain samples were collected weekly and frozen for later DM determination according to procedure described by Association of Official Agricultural Chemists (1990). Lamb's performance in the feedlot was assessed according to their dry matter intake (DMI). It was calculated using the total DM amounts of concentrate to forage offered per animal per day, and lesser amount of DM remained in the feeder. The feed conversion (FC) was calculated by dividing the DMI by weight gain of the lambs throughout the whole feedlot period. Feed efficiency (FE) was calculated by dividing average daily gain (ADG) by average daily DMI. The animals were also weighed every two weeks to obtain the ADG values, which were assigned to the lambs weight on feedlot output, without taking into consideration the body weight, divided by the number of experimental days that the animals were confined.

2.5. Data analysis

The experimental design was completely randomized (CRD), with 3 × 2 factorial as the distribution pattern of treatments consisting of three groups during pregnancy combined with progressive and abrupt weaning: control, Cg; application of LPS at 70th day of gestation, Ig; and application of LPS at 120th day of gestation, Fg. The type of birth, whether single or multiple births (two lambs), was considered as a fixed effect in all ewes models. However, as it was not significant for any parameter, it was removed from the final analysis.

Analyses of cortisol data and RT during pregnancy were performed through analysis of variance (MIXED-SAS), with the division of phases of pregnancy. In the intermediate phase (70 days), the application of LPS and their interactions were included as fixed effects of sampling time (14 measures in 24 h). The means were compared through F test and Student's *t*-test (PDIFF). In late pregnancy (120 days), the analyses were similar but were rather included as random effects of cortisol peak observed in the intermediate stage of pregnancy.

For birth weight analyses, gender and its interactions, as well as random effect cortisol peak and RT peak at gestation, were added as fixed effects of stress challenge (control, intermediate and final). The means were compared using the F test and Student's *t*-test (PDIFF).

Weaning was divided into three different analyses. In the first analysis of progressive weaning, cortisol and body temperature in the first six days of lamb and mother separation were included; and the fixed effects of gender, day of separation (from 39th to 44th days of life), sample time (T0, T60, and T180), LPS stress challenge in pregnancy (control, intermediate, and final), and their interactions were analyzed as well. The second analysis of progressive and abrupt weaning was performed on the final separation day (45th day of life) and included fixed effects of gender, sample time after separation (T0, T60, and T180), stress challenge via LPS in pregnancy (control, intermediate, and final), and types of weaning, as well as their interactions. The third analysis was performed for the subsequent three days after lamb and ewe separation, with fixed effects of gender, separation day (45th, 46th, and 47th day of life), LPS stress challenge in pregnancy (control, intermediate, and final), types of weaning, and their interactions. Birth weight was used as a covariate in the model. As mentioned before, means were compared by F test and Student's *t*-test (PDIFF).

Analysis of variance was performed for the performance variables, with fixed effects of LPS stress challenge in pregnancy (control, intermediate, and final), gender, types of weaning, and their interactions. It was included as covariates effect the birth weight and weaning weight. The means were compared using F test and Student's *t*-test (PDIFF). All data were presented as mean ± SEM, and the analysis for this paper was generated using SAS software, Version 2008 of the SAS System for Windows.

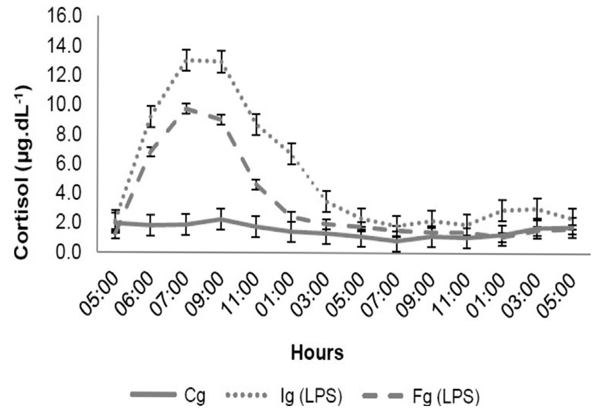

Fig. 1. Circadian rhythm of cortisol in pregnant ewes on Control treatment (Cg) and subject to the application of lipopolysaccharide (*E. coli*) in the Intermediate Phase (Ig) and in the Final phase of pregnancy (Fg).

3. Results

3.1. During pregnancy

LPS challenge increased cortisol levels in the intermediate and late phases of gestation (Fig. 1).

No differences were found in the baseline samples ($P > 0.05$) among the three treatments. However, it can be observed that the cortisol levels of Ig and Fg treatments had greater response two to four hours after the application of LPS. The baseline sampling was higher for Ig than for Fg, in which 10.9 μg. dL^{-1} and 8.50 μg. dL^{-1}, respectively ($P < 0.05$).

The peak on cortisol levels occurred between 6 h 00 min and 13 h 00 min. Furthermore, cortisol level was found to be significantly higher in Fg-treated group as compared to others treatments ($P < 0.01$). At 15 h 00 min, the blood cortisol level returned to baseline, and from this moment, no differences in the cortisol levels of the three treatments were noted ($P > 0.05$).

During LPS challenge, Ig-treated groups had a higher increase in RT than Fg-treated ewes four hours after the application of LPS ($P < 0.01$) (Fig. 2).

3.2. Birth weight

Regarding birth weight, an interaction between stress during pregnancy and sex was observed ($P < 0.05$). Male lambs from Cg and Ig groups had comparable birth weights unlike the females from the same groups ($P > 0.05$; Fig. 3). For Fg, the birth weights of both males and

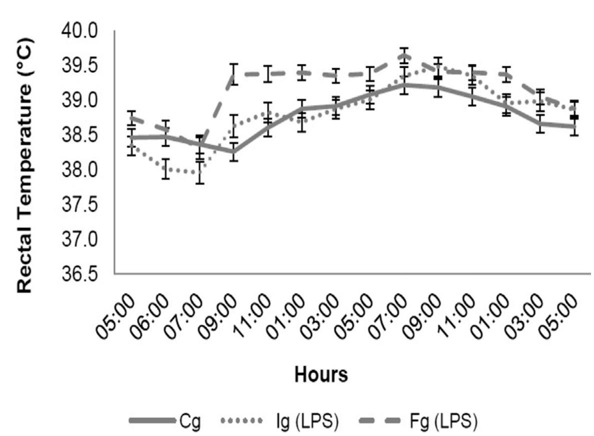

Fig. 2. Circadian rhythm of rectal temperature (°C) in pregnant ewes on Control treatment (Cg) and subject to the application of lipopolysaccharide (*E. coli*) in the Intermediate Phase (Ig) and in the Final phase of pregnancy (Fg).

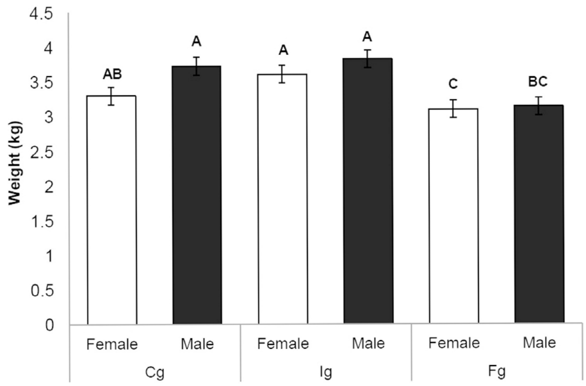

Fig. 3. Birth weight of females and males lambs from ewes on Control treatment (Cg) and subject to the application of lipopolysaccharide (*E. coli*) in the Intermediate Phase (Ig) and in the Final phase of pregnancy (Fg). Different capital letters differs between pregnancy phase x gender by F and *T* student test at 5%.

females were comparable (P > 0.05), and LPS application in the final phase of pregnancy resulted in lowest BW (P < 0.05). Lambs from Cg and Ig did not differ for BW (P > 0.05).

3.3. Weaning

No effect of LPS during pregnancy and no interaction between LPS and weaning methods were observed (P > 0.05). The cortisol levels were higher on the first day of progressive weaning as compared to the ensuing six days (P < 0.05; Fig. 4).

Lambs had the highest RT on the first day of separation as compared to other days of separation (P < 0.05). Furthermore, a lower RT was observed on the third day of separation (41st) (P < 0.05) (Fig. 4). In relation to the hours after separation, the lowest RT was measured on the first sampling time after separation. The mean was lower (P < 0.05) than the other two samples, which were taken one hour and three hours after separation.

At 45 days of age or the first day at the feedlot, the cortisol levels at T0 were similar with that taken at T60 and T180 after housing the lambs in individual pens (P > 0.05; Table 1). The lowest value of RT was observed as the lambs entered the feedlot; however, there were no significant differences in the two subsequent RTs (P < 0.05).

The type of weaning is the only factor that yielded a significant effect when comparing the mean cortisol levels on 45th, 46th and 47th days (P < 0.05). Lambs subjected to the progressive weaning had lower mean cortisol than those subjected to abrupt weaning (P < 0.05; Table 1). The highest RT values were found on progressively weaned

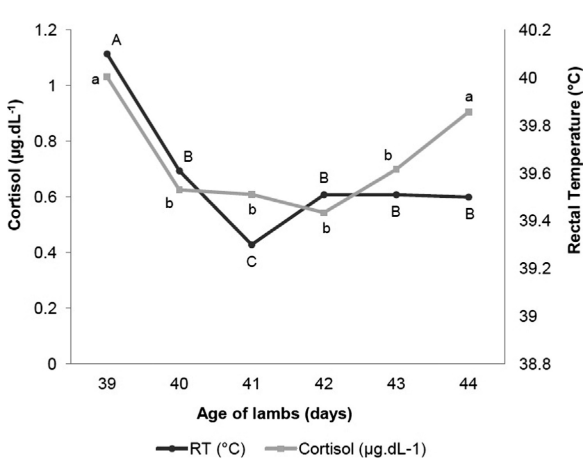

Fig. 4. Mean of cortisol levels and rectal temperature in lambs during the progressive weaning before feedlot. Different small letters (cortisol) and capital letters (rectal temperature) differs by F and *T* student test at 5%.

lambs, with 39.1 ± 0.07 °C, as compared to abruptly weaned lambs, which only had 38.9 ± 0.07 °C (P < 0.05).

Weaning weight was different between types of weaning, in which 11.8 ± 0.70 kg was measured for progressively weaned lambs and 9.9 ± 0.70 kg for abruptly weaned lambs.

The first blood sample collection was done during the last contact of the lamb with the ewe (T0); second collection was done 60 min after the lambs entered the feedlot (T60); and the third collection was done 180 min after the lambs entered the feedlot (T180). The capital letters differ between sampling times, and the small letters differ between weaning types as computed with F and Student *t*-tests at 5%.

3.4. Feedlot performance

For DMI, an interaction between LPS challenge during pregnancy and gender, but not with weaning method, was observed (Table 2). The higher DMI values were found for Cg males as compared with Fg males (P < 0.05). Moreover, no difference was found on the stress during pregnancy between Ig group and other groups (P > 0.05). For all female lambs, no differences on the effect of stress during pregnancy were observed (P > 0.05).

Stress during pregnancy and gender affected lambs' ADG, but no interaction between ADG and weaning methods was observed (Table 2). The Cg group presented a numerically higher average when the comparison was made between the male lambs; however, the average did not significantly differ from the values observed in the Ig group (P > 0.05). Moreover, both Ig and Cg groups had higher ADG values as compared with the ADG of Fg group (P < 0.05). Significant differences were also found between genders for Cg and Ig groups, wherein higher ADG values were assigned to males (P < 0.05); however, the same results were not observed in the Fg group (P > 0.05). No differences in the ADG values of the females from the three groups were observed during pregnancy (P > 0.05). Both FC and FE were not affected by stress during pregnancy, gender, nor weaning (P > 0.05).

Different small letters differ between the pregnancy groups Cg (control), Ig (stress at the intermediate stage of pregnancy), and Fg (stress in late pregnancy). Different capital letters differ between genders (P < 0.05) for each parameter.

4. Discussion

4.1. Pregnancy and birth

Previous studies reported that LPS injection during pregnancy leads to changes in the ewes' cortisol circadian rhythm and RT, hence setting a stressful situation and that in late gestation period, the ewes and lambs are more vulnerable to the effects of LPS administration (McClure et al., 2005; Iwasa et al., 2009; Hild et al., 2011). In our study, the increase in cortisol levels two hours after LPS application was higher than the observed increase in RT (Laburn et al., 1988). In other studies, different results were observed on RT, which indicates that RT peaked two hours after LPS application (*E. coli*,0.3 μg kg^{-1}) or four hours later (McClure et al., 2005; Dwyer, 2008).

Highest mortality rate was found in the offspring of ewes that were under stress during the final phase of pregnancy. Lamb mortality is usually higher in the first few days of the animal's life – having a curvilinear relationship with birth weight (Hight and Jury, 1970; Schlafer, 1994). This fact has been supported by our present data, which shows that gender had an influence on birth weight. Our results show that Fg males became more vulnerable to mortality as compared with Ig lambs or those who did not suffer from stress; and higher losses for male lambs than females were found in the same treated group (Lesage et al., 2004; Nowak and Poidron, 2006; Mairesse et al., 2007).

Tissues proliferate during embryonic development; however, specialized cells, such as skeletal muscle cells, lose their ability to replicate after birth (Owens et al., 1993). It is possible that lower birth weight in

Table 1
Mean and standard error of blood cortisol in lambs subjected to progressive weaning (Pw) or abrupt (Aw) in relation to sampling time on entrance date in the feedlot (45 days old).

	T0	T60	T180	Weaning
PW (μg.dL^{-1})	1.57 ± 0.347	0.67 ± 0.183	0.55 ± 0.282	0.93 ± 0.091B
AW (μg.dL^{-1})	2.12 ± 0.327	1.55 ± 0.231	1.55 ± 0.229	1.74 ± 0.078A
Time after entrance (μg.dL^{-1})	1.85 ± 0.426A	1.11 ± 0.420B	1.05 ± 0.415B	

The first blood sample collection was done during the last contact of the lamb with the ewe (T0); second collection was done 60 minutes after the lambs entered the feedlot (T60); and the third collection was done 180 minutes after the lambs entered the feedlot (T180). The capital letters differ between sampling times, and the small letters differ between weaning types as computed with F and Student t tests at 5%.

Table 2
Dry matter intake (DMI) and average daily gain (ADG) of male and female lambs from ewes challenged with LPS (*E. coli*) during pregnancy.

	Cg	Ig	Fg
DMI (kg.head^{-1}.day^{-1})			
Male	1.195 ± 0.086 a	1.167 ± 0.122 ab	0.855 ± 0.108 b
Female	0.970 ± 0.093	0.905 ± 0.080	1.146 ± 0.132
ADG (kg.head^{-1}.day^{-1})			
Male	0.263 ± 0.015 aA	0.259 ± 0.021 aA	0.190 ± 0.019 b
Female	0.192 ± 0.016 B	0.191 ± 0.014 B	0.220 ± 0.023

Different small letters differ between the pregnancy groups Cg (control), Ig (stress at the intermediate stage of pregnancy), and Fg (stress in late pregnancy). Different capital letters differ between genders ($P < 0.05$) for each parameter.

male lambs can be attributed to the possible effect of stress during pregnancy on the cellular development that gives origin to muscle tissues, as males present higher number of skeletal muscle cells than females (Greenwood et al., 2000).

4.2. Weaning and production performance

The reduction in cortisol levels during progressive weaning after a few days of separation demonstrated the improvement in the welfare of the lambs, as they were conditioned to a routine (Formkman et al., 2007). However, the opposite was reported by Orgeur et al. (1998). This type of weaning has positive attributes for both social interaction and productive performance of the lambs (Schichowski et al., 2008).

The higher cortisol level of the lambs on the day of their entrance in feedlot was expected in abrupt weaning it was the first separation experience. However, increase in cortisol levels was also observed in progressively weaned lambs at day one in feedlot. Albeit the lambs were used to maternal separation, they were not used to social separation, as the feedlot had individual pens (Syme and Elphick, 1983).

On the other hand, cortisol values of abruptly weaned lambs remained higher than that of progressively weaned lambs 24 and 48 h after feedlot entrance – thus showing that bond separation, together with social separation, was more stresfull (Grubb and Jewell, 1966; Orihuela et al., 2004; Freitas-de Melo and Ungerfeld, 2016).

Animals' productive performance was affected by weaning type as well. The highest weight observed for progressive weaning can be attributed to the previous solid food consumption, which contributed to the heavier weight of the animal during feedlot entrance (Napolitano et al., 1995; Orgeur et al., 1998).

Stress induced by LPS in the third trimester of pregnancy showed the poorest productive parameters for male lambs as compared to female lambs. Some studies showed the influence of stress during pregnancy on birth weight, as evidenced by the fetal gender in development (Lesage et al., 2004; Nowak and Poidron, 2006; Mairesse et al., 2007). In a normal pregnancy, the influence of gender on these assessments can be explained as normal consequences of different nutrition requirements for DMI and consequently for ADG and body size of each gender, which predicts that the gain is mainly directly related to consumption (Mertens, 1994; NRC, 1996). However, after being subjected to stress during pregnancy, the present data show that birth weight is lower in lambs born from stressed mother at the third trimester of gestation and male lambs were not able to recover from this stress. The results point out that high cortisol levels during pregnancy may modify the activity of other hormones in several species, and that high cortisol levels negatively influence the development of male lambs and can even promote changes in the reproductive organs (Ward and Weisz, 1984; Von Holst, 1998; Mack et al., 2014).

5. Conclusion

LPS challenge, as a simulated disease process during late pregnancy, compromised the lambs' indicators of productive performance. Although progressive weaning was found to be more efficient than abrupt weaning in reducing stress during feedlot entrance and total bond separation, weaning type had no relation with stress during pregnancy.

Acknowledgements

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