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The Relationship Between Litter Size and Maternal Cortisol Levels in Late Gestation of Romanov Sheep and Effect on the Survival of Lambs

The aim of this study was to determine the relationship between litter size and cortisol levels—a hormone known to be associated with stress—in late-gestation Romanov ewes capable of carrying multiple lambs, as well as the effect of maternal cortisol levels on lamb survival rates. Fifty-five healthy Romanov ewes in the final stage of pregnancy were used. Blood samples were collected from the jugular vein into biochemical test tubes on days -21, -14, -7 before parturition, and within the first 6 hours postpartum. The collected blood samples were centrifuged at 4,000 rpm for 10 minutes, and the resulting serum samples were stored at -20°C. Subsequently, maternal cortisol levels were measured using the Sheep Cortisol ELISA kit. The live weights of the lambs were determined between 0 and 4 hours postpartum, and the lambs were monitored to assess the survival rate. A significant decrease in maternal cortisol levels was found on day 0 compared to days -21, -14, and -7 and no correlation found between the lambs' live weights and survival rate and maternal cortisol levels. In conclusion, it was observed that litter size did not affect cortisol levels in Romanov sheep during late pregnancy, and there was no correlation between cortisol hormone levels and the lambs' survival rates.

Key Words: Romanov, lamb, cortisol

Romanov Koyunlarında Geç Gebelikte Yavru Sayısı ile Maternal Kortizol Düzeyleri Arasındaki İlişki ve Kuzuların Yaşama Gücüne Etkisi

Bu çalışmada, çoklu yavru taşıma özelliğine sahip geç gebelik dönemindeki Romanov koyunlarında yavru sayısı ile stres hormonu olarak bilinen kortizol seviyesi arasındaki ilişki ve maternal kortizol seviyesinin yavruların canlı kalma oranları üzerindeki etkisini belirlemek amaçlandı. Gebeliğin son döneminde bulunan 55 adet sağlıklı Romanov ırkı koyun kullanıldı. Kan örnekleri, doğum öncesi 21., 14. ve 7. günlerde ve postpartum dönemin ilk 6 saati içerisinde, juguler venden biyokimyasal analiz tüplerine alındı. Alınan kan örnekleri 4000 devirde 10 dakika santrifüje edildi ve elde edilen serum örnekleri -20°C de muhafaza edildi. Daha sonra Sheep Cortisol ELISA kit ile maternal kortizol seviyeleri ölçüldü. Doğumdan sonraki 0. ve 4. saatler arasında yavruların canlı ağırlıkları belirlendi ve yaşama oranını belirlemek için yavrular gözlemlendi. Maternal kortizol seviyesinde -21., -14. ve -7. günlere göre 0. günde anlamlı azalma bulundu. Kuzuların canlı ağırlıkları ve yaşama oranı ile maternal kortizol seviyesi arasında bir ilişki saptanmadı. Sonuç olarak, geç gebelik dönemindeki Romanov ırkı koyunlarında yavru sayısının kortizol seviyesini etkilemediği ve kortizol hormonu seviyesi ile yavruların canlı kalma oranları arasında bir ilişki olmadığı görüldü.

Anahtar Kelimeler: Romanov, kuzu, kortizol

Introduction

The period from three weeks before birth to three weeks after birth is defined as the periparturient period. During this period, significant changes occur in the animal's endocrine metabolism (1). Lambs reach approximately 70% of their total birth weight during the final 6 weeks of gestation. During this period, the presence of multiple fetuses also causes physiological stress and leads to an increase in maternal plasma cortisol levels (2).

Cortisol is a natural glucocorticoid produced by the adrenal glands that plays a key role in the maturation of fetal organs during the final stages of pregnancy (3). Cortisol secretion is regulated by the central nervous system. Stress stimulates the hypothalamus, which in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH travels through the bloodstream to the adrenal cortex, where it increases cortisol synthesis and secretion (4). Increased maternal cortisol levels have a detrimental effect on offspring (5). It has been reported that there is a positive correlation between litter size and maternal cortisol levels in Afec-Assaf sheep, and that elevated maternal cortisol levels in ewes carrying multiple lambs reduce fetal weight (6).

The Romanov sheep is classified as one of the most productive breeds in with respect to litter size (7). The number of offspring in a single litter can reach up to seven (8). However, a large litter size can increase the mortality rate of Romanov lambs. Particularly in the presence of stress, there is a negative correlation between lamb

survival and birth weight (9). The poor growth performance of lambs with low birth weight also leads to high susceptibility to disease (10).

The aim of this study is to investigate the effect of litter size on maternal cortisol levels during the prenatal period in Romanov sheep and to determine the relationship between maternal cortisol levels and the birth weight and survival of the lambs. Based on the findings of this study, it may be possible to contribute to the development of appropriate management strategies aimed at supporting the sustainability of production performance in Romanov sheep with high reproductive efficiency.

Materials and Methods

Research and Publication Ethics: This study was approved by the Local Ethics Committee for Animal Experiments at Siirt University as compliant with ethical guidelines (January 7, 2021).

Animal Material: The animal subjects used in the study consisted of sheep housed at the Siirt University Center for Small and Large Ruminant Breeding, Research, and Application. All sheep were kept under standard housing and feeding conditions throughout the study. To synchronize estrus, all ewes received intravaginal progesterone-containing sponges for 12 days, followed by equine chorionic gonadotropin (eCG) administration at sponge removal. Estrus detection was subsequently performed, and ewes were naturally mated using fertile rams.

Pregnancy examinations were performed on the 30th day post-mating using an ultrasonographic (SIUI Apogee 2100) scanning method. Routine general examinations (body temperature, pulse and respiratory rates, lymph nodes, abdominal palpation, and lung auscultation) were conducted throughout the study. A total of 55 Romanov ewes in the late stages of pregnancy, ranging in age from 2 to 6 years and with no health issues, were included in the study. The number of lambs born was recorded as follows: 5 single lambs, 18 twin lambs, 27 triplet lambs, and 5 quadruplet lambs. Since triplets and quadruplets could not be analyzed separately, Romanov sheep in the late stage of pregnancy were grouped into those with a litter size ≤ 2 (2 or fewer) and those with a litter size >2 (more than 2) to obtain statistically significant results. The live weights of the newborn lambs were determined between 0 and 4 hours after birth. The lambs were monitored for survival rates during the first 24 hours. The absence of neonatal APGAR scoring represents a limitation of the present study, and future investigations should include standardized neonatal vitality assessments to better evaluate lamb viability.

Blood samples: Blood samples were collected 21 days before birth (-21), 14 days before birth (-14), 7 days before birth (-7), and after birth (day 0—within the first 6 hours). All blood samples were collected during the late gestation period before parturition; therefore, none of the ewes had entered the lactation period at the time of

sampling. After ensuring aseptic and antiseptic conditions in the area, 5 mL of blood was collected from the jugular vein using a needle and placed into biochemical tubes. The collected blood samples were centrifuged at 4,000 rpm for 10 minutes. The resulting serum samples were transferred to eppendorf tubes and stored at -20°C until analysis. Serum cortisol concentrations were measured using a commercially available Sheep Cortisol ELISA Kit (Bioassay Technology Laboratory, Catalog No: E0003Sh), validated by the manufacturer for ovine serum samples. The intra-assay and inter-assay coefficients of variation were $<8\%$ and $<10\%$, respectively, according to the manufacturer's instructions.

Statistical Analysis: "SPSS for Windows version 22.0" was used to perform the statistical analyses (SPSS Inc., Chicago, IL, USA). All data obtained were tested for normality and homogeneity. The non-parametric "Friedman test" was used to identify differences in blood cortisol levels between days -21, -14, -7, and 0 of the perinatal period, while the "Wilcoxon signed-rank test" was used to determine which measurements showed differences between groups. To determine the presence of a positive or negative relationship (correlation) between the number of pregnancies per ewe, the ewe's age, the number of lambs born, the average live weight of lambs born, the total live weight of lambs born, the lamb survival rate, cortisol levels on day -21, cortisol levels on day -14, cortisol levels on day -7, day, and cortisol on day 0. Differences between groups at the $p<0.05$ level were considered statistically significant. Relationships among these variables were evaluated using Spearman's rank correlation analysis due to the non-normal distribution of the data.

Results

When comparing cortisol levels on days 21, 14, and 7 before birth and the postnatal period (day 0) based on the number of lambs born, a statistically significant decrease in cortisol levels was observed on day 0 ($p<0.05$) (Table 1).

When cortisol levels were examined according to the number of pregnancies in the sheep, no significant difference was found ($p>0.05$) (Table 2).

No significant difference was observed between the number of lambs born and their total average live weights based on the number of pregnancies in the ewes ($p>0.05$) (Table 3).

Table 4 presents the correlation results between pregnancy number and sheep age (0.921**); pregnancy number and lamb average weight (-0.291*); pregnancy number and lamb survival rate (-0.359**); sheep age and lamb average weight (-0.368**); sheep age and lamb survival rate (-0.392**); lamb number and lamb average weight (-0.475**); lamb number and lamb total weight (0.792**); lamb average weight and lamb survival rate (0.267*); cortisol on day -21 and cortisol on day -14 (0.752**); cortisol on day -21 and cortisol on day -7 (0.817**); cortisol on day -21 and cortisol on day 0

(0.489**); cortisol on day -14 and cortisol on day -7 (0.754**); cortisol on day -14 and cortisol on day 0 (0.305*); and cortisol on day -7 and cortisol on day 0 (0.481**) (* $p<0.05$, ** $p<0.01$).

The average live weights of singleton lambs were 3.4 kg, twin lambs 3.03 kg, triplet lambs 2.78 kg, and quadruplet lambs 2.24 kg. A total of 142 lambs were included in the survival analysis. During the first 24 hours after birth, 123 lambs survived and 19 lambs died, corresponding to a survival rate of 86.6% and a mortality rate of 13.4%.

Table 1. Pre-partum cortisol levels in ewes on different days based on the number of lambs born (ng/mL)

Days	Number of newborn lambs		<i>p</i>
	≤2 (n:23)	>2 (n:32)	
-21 day	13.72±1.20 ^{ab}	13.40±1.42 ^a	0.366
-14 day	15.23±1.44 ^a	13.49±1.20 ^a	0.125
-7 day	12.44±0.79 ^b	13.71±1.64 ^a	0.318
1st postpartum day	7.39±0.72 ^c	8.38±0.69 ^b	0.432

p: Significance level of the relationship between sheep cortisol levels and the number of lambs born, Mann-Whitney U test. abc: Values in the same column that do not share a common letter are statistically different, Friedman/Wilcoxon test ($p<0.05$).

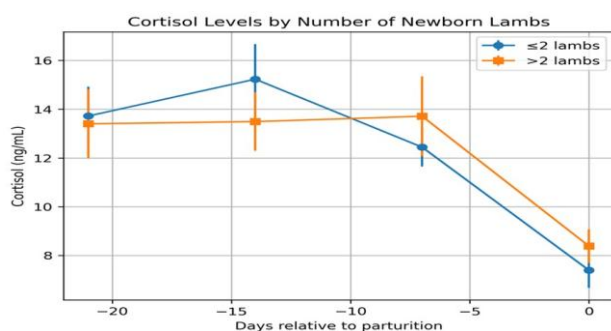


Figure 1. Temporal changes in maternal cortisol concentrations (mean ± SD) according to number of newborn lambs

Table 2. Pre-partum cortisol levels in sheep on different days, according to the number of pregnancies (ng/mL)

Days	Number of sheep pregnancies		<i>p</i>
	≤2 (n:37)	>2 (n:18)	
-21 day	14.51±1.32 ^a	11.52±0.98 ^a	0.240
-14 day	15.01±1.24 ^a	12.57±1.15 ^a	0.236
-7 day	13.92±1.45 ^a	11.65±0.64 ^a	0.733
1st postpartum day	7.86±0.60 ^b	8.18±0.94 ^b	>0.999

Within each column, values with different superscript letters differ significantly among sampling time points (Friedman test followed by Wilcoxon signed-rank test, $p<0.05$). Between-group comparisons at each sampling time point were performed using the Mann-Whitney U test.

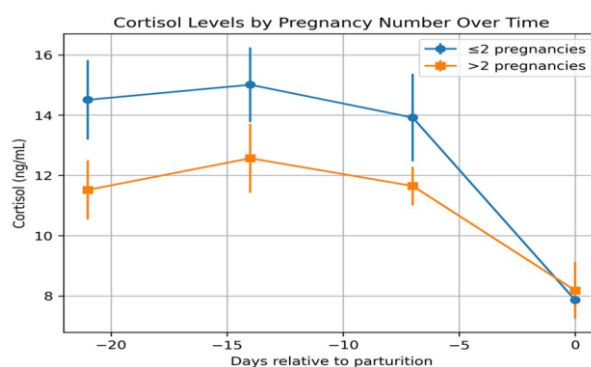


Figure 2. Temporal changes in maternal cortisol concentrations (mean ± SD)

Table 3. The average number of lambs born and their total live weight by number of pregnancies in ewes (kg)

	Number of newborn lambs	Total weight of lambs
≤2 (n:37)	2.48±0.13	7.10±0.29
>2 (n:18)	2.77±0.15	7.44±0.56
<i>p</i>	0.163	0.562

p: The significance level of the difference between the average number of lambs born and their total live weights according to the number of pregnancies in ewes; T-test for independent samples.

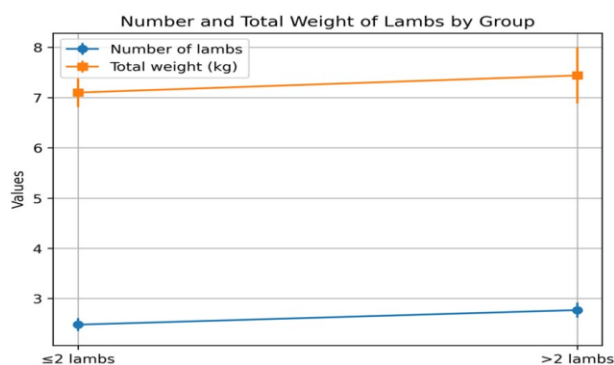


Figure 3. Comparison of number of lambs and total lamb weight (mean ± SD) between groups

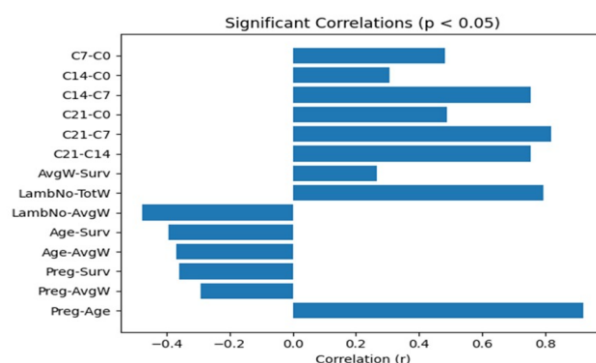


Figure 4. Significant correlations ($p<0.05$) among study variables presented as a bar chart

Table 4. Spearman correlation coefficients showing only statistically significant relationships ($p < 0.05$)

Variable 1	Variable 2	Correlation (r)
Pregnancy number	Sheep age	0.921**
Pregnancy number	Lamb average weight	-0.291*
Pregnancy number	Lamb survival rate	-0.359**
Sheep age	Lamb average weight	-0.368**
Sheep age	Lamb survival rate	-0.392**
Lamb number	Lamb average weight	-0.475**
Lamb number	Lamb total weight	0.792**
Lamb average weight	Lamb survival rate	0.267*
Cortisol -21 day	Cortisol -14 day	0.752**
Cortisol -21 day	Cortisol -7 day	0.817**
Cortisol -21 day	Cortisol 0 day	0.489**
Cortisol -14 day	Cortisol -7 day	0.754**
Cortisol -14 day	Cortisol 0 day	0.305*
Cortisol -7 day	Cortisol 0 day	0.481**

* $p < 0.05$, ** $p < 0.01$

Discussion

Glucocorticoids, such as cortisol, play a crucial role in regulating metabolism in both adult and fetal tissues. Circulating glucocorticoid concentrations typically increase during late gestation; however, exposure of the dam to stressors such as undernutrition, infection, or transportation may induce a premature elevation in cortisol levels (11). In addition, multiple fetuses may further influence the increase in maternal cortisol concentrations during late gestation (2). In a study conducted in sheep, cortisol concentrations were reported to reach peak levels approximately two weeks prior to parturition (12). Similarly, a study in sheep demonstrated that cortisol concentrations were higher in twin-bearing ewes compared with those carrying a single fetus (4). In a study conducted in Afec-Assaf ewes, maternal cortisol concentrations during late gestation were reported to differ between those carrying two or more fetuses and those bearing a single fetus (6). Tharwat et al. (13) reported that cortisol concentrations reached their peak at parturition in goats during the transition period. Based on the findings of the present study, no differences were observed in cortisol concentrations between days -21, -14, and -7 among groups stratified according to litter size and parity in Romanov ewes. However, cortisol concentrations on day 0 were found to be significantly lower than those measured at the other time points ($p < 0.05$). In the present study, the absence of marked changes in cortisol concentrations during late gestation—particularly in Romanov ewes carrying triplets and quadruplets—may indicate a greater resilience to stress in this breed.

Maternal stress is widely recognized as one of the primary contributors to elevated fetal stress hormone levels and, in addition to adversely affecting fetal development, may predispose offspring to health disorders later in life (14). Glucocorticoid administration in pregnant monkeys, sheep, and mice has been

reported to result in impaired fetal growth (15). Downs et al. (16) and Alon et al. (6) reported a negative association between maternal cortisol concentrations and lamb birth weights in studies conducted in Dall and Afec-Assaf sheep, respectively. In contrast, no negative association was observed between lamb birth weights and maternal cortisol concentrations in the present study. Further large-scale investigations are warranted to elucidate the underlying reasons for the absence of such a relationship between late-gestation cortisol levels and birth weight in Romanov ewes.

Another key determinant of fetal growth is the number of fetuses carried by the dam. In sheep, an increase in litter size is generally associated with a reduction in lamb birth weight (17). Consistent with the findings of Miller et al. (18), the present study also demonstrated a negative relationship between litter size and the mean birth weights of lambs.

It is known that there is a relationship between lamb survival and growth rates and birth weight. Generally, smaller lambs have lower survival rates and daily weight gains compared to larger lambs (19). Schreurs et al. (20) found a negative correlation between birth weight and mortality rates in twin lambs. Juengel et al. (19) reported that reduced body weight in lambs adversely affects both survival and developmental performance. Consistent with these findings, the present study demonstrated a negative association between lamb birth weight and mortality rate, which may be attributed to decreased disease resistance in lower-weight lambs. A possible explanation for the absence of an association between maternal cortisol concentrations and lamb birth weight is that cortisol concentrations measured during late gestation may not fully reflect cumulative fetal exposure to glucocorticoids throughout pregnancy. Furthermore, genetic characteristics of Romanov sheep, maternal nutritional status, placental efficiency, and other endocrine mechanisms may have exerted a stronger influence on fetal growth than maternal cortisol concentrations alone.

Aktaş et al. (21) reported that the age of the dam influences lamb survival rates. In their study on Central Anatolian Merino sheep, lambs born to ewes aged 3.5–4.5 years exhibited higher survival rates compared with those born to 1.5-year-old ewes. Sawalha et al. (22) reported that lambs born to young (1–2 years) and older (≥ 5 years) ewes exhibited lower survival rates compared with those born to middle-aged ewes (3–5 years) in Scottish Blackface sheep. Consistent with these findings, the present study demonstrated a negative association between maternal age and parity and lamb survival rates. The observed negative correlations between ewe age and lamb survival rate, as well as lamb average birth weight, may be related to differences in maternal physiological condition, body condition, and reproductive performance among ewes of different ages. These factors may influence fetal development during gestation and the lambs' subsequent survival.

In conclusion, it is noteworthy that in highly prolific Romanov ewes, maternal cortisol concentrations during

late gestation—a key stress hormone—appear to be unaffected by litter size and do not exert a significant influence on lamb survival rates. Further comprehensive and advanced studies are warranted to elucidate the underlying mechanisms of this finding. Moreover, considering these superior reproductive traits, Romanov sheep may be regarded as an economically advantageous breed for livestock producers. One limitation of the present study was the relatively small number of ewes carrying triplets and quadruplets, which prevented separate statistical evaluation of these

groups. Additional metabolic and endocrine parameters potentially affecting lamb survival were not evaluated. Future studies including larger populations of highly prolific Romanov ewes are warranted to confirm the present findings. In addition, an a priori sample size (power) calculation was not performed, and the study population was limited to the available eligible animals in the research flock. Therefore, larger prospective studies with predefined sample size calculations are recommended to validate these findings.

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