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Investigation of some acute phase protein levels of Awassi lambs in neonatal period and their relationship with lamb weight

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Acute phase proteins (APPs) have been paid attention to as sensitive and reliable biomarkers used for monitoring general health status, diagnosis, prognosis, and treatment strategies of diseases. In this study, changes in some APPs, globulin (Glb), and total protein (TP) concentrations and the correlation of these proteins with lamb weight were investigated in the neonatal period of Awassi lambs (*Ovis aries*). Repeated blood samples were taken from 59 lambs at 0, 1, 3, 7, 14, 21, and 28 days of age. There were statistical differences in average haptoglobin (Hp), serum amyloid-A (SAA), ceruloplasmin (Cp), and albumin (Alb) concentrations during the neonatal period. The lowest concentrations of both Hp and SAA were measured in precolostrum samples and increased to the highest level on the 3rd day of the neonatal period. Ceruloplasmin concentrations followed a constant course in the samples of the first 3 days and reached the highest level in the 14th day samples. The highest levels of Alb were measured in precolostrum samples, and the lowest levels were detected in the 14th day samples. Acute phase protein concentration changes did not differ significantly by gender except Cp. There was a negative correlation between the weights of lambs and Cp and Alb. It was concluded that the time-dependent change of APPs in the neonatal period could help in understanding the neonatal period of Awassi lambs and that its primary purpose is survival as a physiological part of the adaptation process to the extrauterine environment.

Keywords: Acute phase protein, lamb, neonatal period, lamb weight.

INTRODUCTION

In all mammalian species, the neonatal period, between birth and 4 weeks of age, represents a critical and complicated period in which remarkable structural and functional changes occur in the newborn for successful adaptation to the extrauterine environment (Tóthová *et al.*, 2015; Samimi, 2019; Dinler *et al.*, 2020). In this stage, the neonate is in the most vulnerable time of life. At birth, neonates of mammals suddenly move from the sterile environment in their mother's uterine into nature, where many potential pathogens can be found (McGill *et al.*, 2022). Unstable metabolic conditions caused by changing environmental conditions increase the risk of various diseases, neonatal morbidity, and mortality in lambs (Dinler *et al.*, 2020). Early-life diseases and deaths in small ruminants are among the major causes of economic losses in livestock activity (Valavi *et al.*, 2022). The struggle to reduce neonatal mortality has focused on the search for new biochemical markers that can predict the risk of diseases early and accurately observe neonates (Mussap *et al.*, 2013). Determination of acute-phase proteins, which are among these biomarkers, in neonates assists veterinarians in the context of differential diagnosis, determination of disease severity, and applying the proper therapeutic strategy (Samimi, 2019).

The neonate has an immature but functional immune system (Morein *et al.*, 2002). Difficulties such as adequate maternal antibody intake, immunological maturity, and adaptation to food may lead to impaired homeostasis and subsequent changes in the levels of some biomarkers of the acute phase response (APR) (Ramos *et al.*, 2019). Acute phase response activates a series of biochemical defense mechanisms. The change in the synthesis of a group of proteins, which can be synthesized by the liver and peripheral tissues as a part of the

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immune response and called the acute phase proteins (APPs), is one of the most important metabolic indicators of the APR (Tóthová *et al.*, 2014; Heller and Johns, 2015; Schmidt and Eckersall, 2015; Ulutas *et al.*, 2017; Ramos *et al.*, 2019).

APPs have been paid attention as sensitive and reliable biomarkers used for monitoring general health status, diagnosis, prognosis, and treatment strategies of diseases in both human and veterinary medicine (Schrödl *et al.*, 2016). The importance of APPs in diagnosis and prognosis largely depends on the animal species. In animals, some proteins have been identified as APPs and these are basically divided into positive and negative APPs. The concentrations of positive APPs increase during the APR, while those of negative APPs decrease. Positive APPs are classified as major, intermediate, and minor according to the folds of the increase in their concentrations (100-1000, 2-10, 0.5-1-fold, respectively) (Cray *et al.*, 2009; Tóthová *et al.*, 2014; Idoate *et al.*, 2015; Iliev and Georgieva, 2018; Reczyńska *et al.*, 2018). Haptoglobin and SAA are major APPs, Cp is a minor APP, and Alb is a negative APP in sheep (Eckersall and Bell, 2010; Iliev and Georgieva, 2018; Reczyńska *et al.*, 2018).

In the prevention of neonatal disease and death determining the reference intervals of specific APPs of the neonatal period, both on a species basis and depending on time, could provide a more accurate assessment of clinical pathology data and increase the diagnostic ability of neonatal disease. There are few studies describing normal APP concentrations in neonates of various small ruminant breeds. However, to the best of our knowledge, there is no study of APPs and the relationship between these proteins and lamb weights in noeonatal Awassi lambs. For this reason, it was aimed to determine the age-related changes in some APPs, Glb, and TP concentrations in neonatal Awassi lambs and the correlation of these proteins with lamb weight in the current study.

MATERIALS AND METHODS

***Animals*:** The study was carried out at the Experimental Animal Application and Research Center (HDAM) Farm Animals Unit, with the permission of Harran University Animal Experiments Local Ethics Committee (HRUHADYEK) numbered 2021/001/02.

This study was carried out in the farm animals unit of Harran University Experimental Animal Application and Research Center (HDAM) in Şanlıurfa province (37° 10' N: 38° 47' E) located in southeast Türkiye. Fifty-two pregnant healthy Awassi ewes of 2 and 3 years old were used in this study. The ewes were monitored by ultrasonography during the pregnancy period. A total of 59 healthy lambs (31 females and 28 males; 45 single and 14 twins) born between January 2021 and February 2021 were included in the study. The lambs were born without any complications from normal-length pregnancies. The Apgar system was used to evaluate neonatal vitality in the selection of lambs (Vannucchi *et al.*, 2012). In this system, a scale between 0-2 is given for each parameter at birth and the following 5th and 60th minutes, and thus Apgar scores were calculated between 0-10. In this scoring system, lambs with a score of 7 and above were included in the study. In addition, all lambs were followed up with daily physical examinations including sucking reflexes, respiratory rates, body temperatures, pulse, mucous membrane examination, and appetite assessment. Instantly after birth, all the neonates received colostrum from their dams, and afterward, they were fed with their mother's milk by suckling. The first feeding was 40±15 minutes after birth. No vaccines or drugs were applied to the ewes and neonates throughout the study. The body weights of the lambs were measured twice with digital scales immediately after birth and at 28 days of age.

***Blood sampling and laboratory analysis*:** Blood samples were taken within 45 min after birth (0^{th} day), and on days 1, 3, 7, 14, 21, and 28 after birth from the jugular vein using an evacuated serum tube without anticoagulant. The samples taken before feeding the neonate with colostrum were considered as the day zero sample. The remaining sample collections were applied at 9:00 am before the daily physical examination to avoid possible diurnal variation and the effects of stress during physical assessment. All blood samples were separated by centrifugation (3000 rpm for 10 min) and stored at -20°C until further analysis.

Serum concentrations of Hp were determined using the methods of Skinner *et al.* (1991), while serum Cp concentrations were determined spectrophotometric method, developed by Colombo and Ricterich (1964). The concentration of SAA in the lamb's serum was measured using a solid-phase sandwich commercial ELISA kit (Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) according to the manufacturer's instructions. Serum Alb and TP concentrations were measured by the colorimetric method using commercial kits (Biolabo®, France) (Epoch, BioTek, USA). The serum Glb concentration was calculated by subtracting the Alb concentration from the TP concentration.

***Statistical analysis*:** The JMP® (2019) program was used for statistical analysis, and the R program "MetaboAnalystR" (Pang *et al.*, 2020) package was used for graphics. The data were statistically analyzed using Levene's test for variance equality assumption and Shapiro Wilk test for normal distribution assumption. For this purpose, two-way analysis of variance (ANOVA) in repeated measures and Tukey HSD multiple comparison tests was used to determine the differences between the groups and analyzed to determine whether there was a difference between the groups. An independent sample t-test was used to determine the gender differences in terms of birth weights and one-month weights. Pearson correlation analysis was applied to examine the relationships between measurements. Significance was calculated at $P<0.05$ in all tests.

RESULTS

Alterations in Hp, SAA, Cp, Alb, Glb, and TP concentrations, and statistical significance of the time-related change of lambs during the first 28 days of their life are demonstrated in Table 1 and Fig. 1.

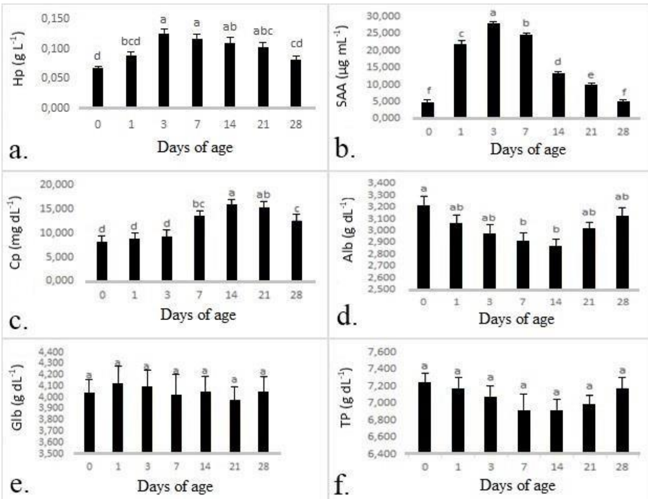

Figure 1. a-f Distribution of the concentrations of Hp (a), SAA (b), Cp (c), Alb (d), Glb (e), and TP (f) in Awassi lambs during the first 28 days of life.

There were statistical differences in average Hp, SAA, Cp, and Alb concentrations during the neonatal period while there was no statistically significant time-dependent change in Gl and TP concentrations. Hp concentrations on days 3 and 7 samples had the highest mean concentrations, while the lowest mean concentrations were measured on the day 0 samples. There was a significant difference between the mean Hp concentrations in repeated measurements except for the 3rd and 7th days. The lowest levels of SAA were measured in the 0 and 28-day samples. SAA, which started to increase in the 1st day samples, reached the highest level in the 3rd day samples. The decline, which continued until the last day of the study, continued until it reached the precolosrtum level. SAA concentrations were similar on the first and last days of the study, but statistically different in all other day samples. While the Cp concentrations followed a constant course in the first 3 days of samples, they increased from the 7th day and reached the highest level in the 14th day samples. Different from other APPs measured in the study, the highest levels of Alb were measured in precolostrum (0th day) samples and the lowest levels were detected in the 7th and 14th day samples. When the effect of gender on the time-dependent change of various proteins in the neonatal period was investigated; it was determined that there was no effect of gender on Hp, SAA, and Alb, but Cp, Glb, and Tp levels were higher in male lambs than in females ($P<0.05$).

Table 1. Changes of Hp, SAA, Cp, Alb, Glb, and TP (mean±standard deviation) in Awassi lambs from birth to 28 days of age.

	Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28	Gender	P-value
Hp (g L^{-1})									
F (n: 31)	0.07±0.02	0.09±0.04	0.12±0.04	0.13±0.07	0.12±0.07	0.10±0.05	0.08±0.05	0.10±0.05^a	N.S.
M (n: 28)	0.07±0.02	0.08±0.03	0.13±0.06	0.11±0.05	0.09±0.06	0.10±0.06	0.08±0.04	0.09±0.05^a	
T (n: 59)	0.07±0.02^d	0.09±0.04^{bcd}	0.13±0.05^a	0.12±0.06^a	0.11±0.07^{ab}	0.10±0.06^{abc}	0.08±0.05^{cd}		***
SAA(µg mL^{-1})									
F (n: 31)	4.78±2.09	23.59±8.59	28.07±5.28	24.14±3.64	13.66±2.66	9.65±2.70	4.45±1.32	14.03±3.77^a	N.S
M (n: 28)	4.81±1.69	19.54±8.15	27.59±4.57	25.14±5.24	12.80±3.38	9.37±2.53	4.31±1.01	13.07±3.56^a	
T (n: 59)	4.79±1.89^f	21.67±8.56^c	27.84±4.92^a	24.61±4.46^b	13.25±3.03^d	9.51±2.60^e	4.38±1.17^f		***
Cp (mg dL^{-1})									
F (n: 31)	7.88±2.96	8.90±3.17	8.49±3.43	12.47±3.65	15.53±4.39	15.18±2.54	12.54±5.23	11.42±3.62^a	*
M (n: 28)	8.75±3.79	8.66±3.18	10.42±4.02	14.76±4.30	16.32±3.92	15.61±3.91	12.76±4.19	12.09±3.84^b	
T (n: 59)	8.29±3.38^d	8.78±3.15^d	9.41±3.81^d	13.56±4.10^{bc}	15.91±4.16^a	15.38±3.24^{ab}	12.64±4.72^c		***
Alb (g dL^{-1})									
F (n: 31)	3.16±0.62	3.05±0.60	2.98±0.73	2.88±0.53	2.91±0.39	2.97±0.45	3.23±0.54	3.05±0.56^a	N.S.
M (n: 28)	3.27±0.59	3.06±0.63	2.96±0.50	2.94±0.59	2.82±0.47	3.05±0.46	3.02±0.51	3.03±0.53^a	
T (n:59)	3.21±0.60^a	3.05±0.61^{ab}	2.97±0.62^{ab}	2.91±0.56^b	2.87±0.43^b	3.01±0.45^{ab}	3.13±0.53^{ab}		**
Glb (g dL^{-1})									
F (n: 31)	3.90±0.89	3.90±1.03	3.93±0.10	3.82±1.45	3.95±1.15	3.97±1.03	4.04±0.96	3.95±1.01^a	
M (n: 28)	4.18±1.00	4.36±1.30	4.28±1.28	4.22±1.54	4.15±1.13	3.97±0.91	4.04±1.21	4.16±1.14^b	*
T (n: 59)	4.03±0.95^a	4.12±1.18^a	4.10±1.14^a	4.01±1.50^a	4.04±1.14^a	3.97±0.97^a	4.04±1.08^a		N.S.
TP (g dL^{-1})									
F (n: 31)	7.06±0.87	6.95±1.02	6.91±0.85	6.71±1.46	7.16±1.33	6.94±0.82	7.27±0.90	7.00±0.92^a	*
M (n: 28)	7.45±0.84	7.42±0.97	7.25±1.12	7.16±1.33	6.97±1.09	7.02±0.80	7.06±1.11	7.20±0.99^b	
T (n: 59)	7.25±0.95^a	7.18±1.02^a	7.07±0.10^a	6.92±1.41^a	6.91±1.06^a	6.98±0.80^a	7.17±1.00^a		N.S.

F: Female, M: Male, T: Total; a-f Different letters in the same row indicate significant differences (*:$P<0.05$, **:$P<0.01$, ***:$P<0.001$). N.S.: Not Significant

Body weights of female and male lambs immediately after birth and at 28 days of age are given in Table 2. It was observed that gender did not have a significant effect on birth weights, but the weight determined by 28-day weights was significantly higher in male lambs. Table 3 represents the Pearson correlations coefficient between weight and Hp, SAA, Cp, Alb, Glb, and TP concentrations of days zero and 28 samples. A strong negative correlation was detected between the body weight with Cp (R= -0.385) (P<0.01) and Alb (R= -0.265) (P<0.05) concentrations of 0. days samples. In terms of 28. days values, there was a strong negative correlation between body weight and Alb (R= -0.330) (P<0.05) concentrations.

There were correlations between APPs obtained from the sampling days of the neonatal period of Awassi lambs and Glb and TP values (Fig. 2). In the precolostrum samples, there

Table 2. Birth and 28-day weights of lambs.

	Birth weight (kg)			P-value	28 days weight (kg)			P-value
	Min	Max	X±Sx		Min	Max	X±Sx	
Female (n:31)	3.14	6.01	4.51 ± 0.84^{a}	N.S.	5.35	18.10	10.86 ± 2.53^{a}	**
Male (n: 28)	2.82	7.02	4.61 ± 0.84^{a}		8.50	18.45	12.83 ± 2.75^{b}	
Total (n:59)	2.82	7.02	4.57±0.84		5.35	18.45	11.80±2.79	

$^{a-b}$ Different letters in the same row indicate significant differences (**: P<0.01). N.S.: Not Significant

Table 3. Pearson correlations coefficient between weight and Hp, SAA, Cp, Alb, Glb, and TP concentrations of 0 and 28 days samples.

	Day 0						Day 28					
BW	Hp	SAA	Cp	Alb	Glb	TP	Hp	SAA	Cp	Alb	Glb	TP
Day 0	-0.049	-0.118	-0.385^{**}	-0.265^{*}	0.056	-0.122	-0.207	-0.006	-0.044	0.208	-0.294^{*}	-0.205
Day28	0.021	0.099	0.009	0.169	-0.053	0.059	0.011	0.011	-0.192	-0.330^{*}	0.049	-0.123

BW: Body weight, * P<0.05, ** P<0.01

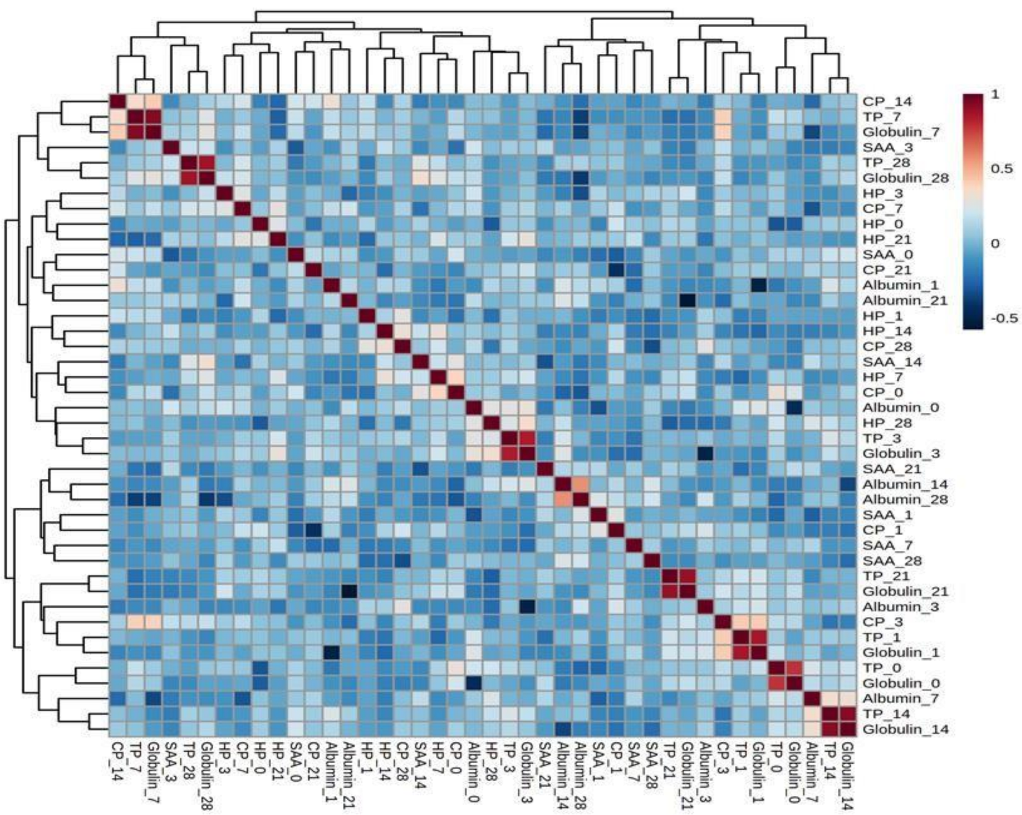

Figure 2. Hierarchical clustering heatmap of Pearson correlation coefficients between serum concentrations of Hp, SAA, Cp, Alb, Glb, and TP in Awassi lambs during the first 28 days after birth.

was a negative correlation between Hp with Glb and TP ($P<0.05$), and a positive correlation between Cp with TP ($P<0.05$). There was a positive correlation between SAA with Cp ($P<0.05$) in the 1st day samples, in the 7th day samples negative correlation between Cp with Alb ($P<0.05$), and in the 28th day samples negative correlations between SAA and Cp ($P<0.05$). There were strong correlations between Glb with Alb and TP values on all days of the study. These correlations were negative with Alb and positive with TP ($P<0.01$).

DISCUSSION

In this study, changes in the concentrations of APPs, Glb, and TP in the neonatal period of Awassi lambs and the relationship between these proteins and lamb weights were investigated. In lambs, the lowest concentrations of Hp and SAA were measured after birth, before colostrum intake. Both Hp and SAA concentrations, which are major APPs in sheep, increased to the highest level on the 3rd day of the neonatal period. The reason for the increase in Hp and SAA from the first day with colostrum intake could be explained within the scope of adaptation to extrauterine life contributed by colostrum. As a matter of fact, studies reporting that colostrum has a large amount of proinflammatory cytokine content and that these colostral cytokines transfer into the blood support the knowledge that colostrum has a beneficial role for the neonate's well-being and health (Hernández-Castellano *et al.*, 2014, 2015; Ulutas *et al.*, 2017; Peetsalu *et al.*, 2019). This increase in Hp and SAA, which starts with colostrum intake of lambs and continues in the first 7 days of life, is similar to other studies conducted with small ruminants (Ulutas *et al.*, 2017; Peetsalu *et al.*, 2019; Ramos *et al.*, 2019; Samimi, 2019; Dinler *et al.*, 2020). The decrease in Hp levels started after the first week and continued until the end of the fourth week. The decrease in SAA concentration started before the first week and it continued until the end of the fourth week. Changes in these proteins during the neonatal period may contribute to the development of defense and adaptation mechanisms or a reflection of the role of the inflammatory process in the adaptation process of the immune system. In the age-related change patterns of SAA and Hp, the more significant differences in SAA might be explained by SAA could be more sensitive than Hp in the neonatal inflammatory response in lambs.

Studies describing the pattern of Cp and its contribution to the adaptation to the extra-uterine environment of the neonatal period are scarce. Cp, whose hepatic synthesis is increased by a transcriptional mechanism by the proinflammatory agonists of the APR, acts as an antioxidant and an acute phase reactant (Kurhaluk *et al.*, 2021), was stable at the lowest concentrations in the first 3 days of the neonatal period, while the increase starting with the 7th day reached its highest level at the age of 2 weeks. This pattern was similar to studies conducted with cross-bred Kivircik lambs (Dinler *et al.*, 2020) and Saanen goat kids (Ulutas *et al.*, 2017). Cp is a copper-containing glycoprotein and it is responsible for transferring about 70 % of the circulating copper (Tóthová *et al.*, 2015). According to the results of a study conducted by Kojouri *et al.* (2020), serum copper concentration was increased significantly at the age of 14 days in lambs. Not only the increase in copper and Cp concentrations on similar days of the neonatal period suggests the relationship between serum Cp and copper concentrations, and also it might be explained by the liver's ability of neonates to synthesize Cp these days (Tóthová *et al.*, 2015; Dinler *et al.*, 2020; Kojouri *et al.*, 2020).

Albumin the main protein of mammalian serum, at the same time, is reported as the negative APP of the APR (Tóthová *et al.*, 2014). Serum Alb concentrations in neonatal lambs ranged from 2.86-3.21 g dL^{-1}, the results of this study agreed with those reported within the literature (Piccione *et al.*, 2013; Kilpi, 2015). In the current study, Alb indicated a decrease during the first 14 days of life after the highest peak on day 0 of life, and following it started to increase constantly from the beginning of the 3rd week of life until the end of the observation. It has been reported that the middle half-life of Alb ranges from 14 to 16 days in ruminants, and after this period the liver is responsible for Alb synthesis (Thrall, 2004). In other studies, with small ruminant kids, the tendency of serum Alb concentration during the neonatal period is consistent with this study (Piccione *et al.*, 2011, 2013; Kilpi, 2015). According to Fig. 1, although there was no statistically significant correlation, the visible inverse correlation between the Alb with Hp and SAA change pattern might be an indication of Hp and SAA being positive APP and Alb being negative APP. In addition, the highest serum Alb concentration in samples taken immediately after birth could be explained as the neonate is not affected by APR, infection, or stress in the intrauterine environment.

In this study, lambs were weighed as soon as they were born and at 28 days of age. While no gender difference was observed in the birth weights of the lambs, it was observed that the 28-day live weight gain in male lambs was higher than in female lambs (Table 2). There was a strong negative correlation between day 0 body weights and Cp and Alb, and a strong negative correlation between day 28 body weights and Alb (Table 3). Since lamb weight was not measured except for the 0th and 28th days of the neonatal period, a pattern and correlations of weight gain could not be determined. However, the inverse relationship between Alb and body weight indicates that the scarcity of factors that cause the APR might have a positive impact on weight gain in neonatal lambs.

There was no statistical difference in mean Glb and TP concentrations throughout the study. However, it was determined that both Glb and TP concentrations were higher in male lambs than in females. It has been reported that increases in the Glb fraction are generally due to increases in immunoglobulins (Busher, 1990) and serum immunoglobulin concentration used as an indicator of adequate passive immunity (Alves *et al.*, 2015; Kilpi, 2015). Therefore, higher serum Glb concentration might be an indication that the passive immunity of male lambs is relatively higher than that of females and that adequate passive immunity contributes positively to lamb weight gain.

***Conclusion*:** This study described, for the first time, serum concentrations of Hp, SAA, Cp, Alb, Glb, and TP and the relationship of these proteins to lamb weights in neonatal Awassi lambs. These proteins could clarify the neonatal period of this breed and generally in sheep. It has been suggested that changes in APPs, not always related to a disease, during the neonatal period have been associated with the physiological development of neonates. However, in order to better and accurately understand the neonatal period, there is a requirement for more comprehensive studies that include factors such as colostrum content, absorption and evaluate their effects on physiological development separately.

***Conflict of Interest*:** The Authors declare that there is no conflict of interest.

***Authors' Contribution Statements*:** NP designed the study and collected samples for analysis and ensured the preparation of the article. EEÖ, AŞ, and MK collected samples for analysis. OM and KB carried out performed the laboratory analysis. All authors read and approved the final version of the article.

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Ethical Statement: The study was carried out at the Experimental Animal Application and Research Center (HDAM) Farm Animals Unit, with the permission of Harran University Animal Experiments Local Ethics Committee (HRUHADYEK) numbered 2021/001/02.

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