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Research Article

Mating Season Influences Placental Characteristics, Plasma *IGF* Concentration, and mRNA Expression without Affecting Lamb Birth Weight in Akkaraman Ewes

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Seasonal variations significantly impact lambs' birth weight and post-natal growth of lambs, yet the underlying physiological mechanisms remain insufficiently explored. Therefore, this study examined the effects of mating season on lamb birth weight, placental characteristics, maternal concentrations of insulin-like growth factors (IGFs) in plasma, and placental mRNA expression levels of *IGFs* in Akkaraman ewes reared under extensive conditions. Ewes were mated in the breeding season (September; $n = 35$) and out-of-breeding season (April; $n = 27$). Blood samples were taken from the jugular vein of all ewes using a vacutainer in sterile heparin tubes every month from mating to parturition. Post-lambing, both dam and lamb weights were recorded, and placental characteristics were documented within 12 hours of parturition in both seasons. The number and weight of cotyledons were higher ($P < 0.05$) in ewes mated during the breeding season than those mated during the out-of-breeding season. Also, ewes mated during the breeding season demonstrated thicker large cotyledons ($P < 0.05$). Although placental and cotyledonal efficiencies were similar in both mating seasons, volumetric cotyledon efficiency was higher ($P < 0.05$) in ewes mated during the out-of-breeding season than in those bred during the breeding season. Additionally, ewes mated in the breeding season exhibited higher plasma concentration of IGF-I at three months of gestation than ewes mated in the out-of-breeding season (123.8 ± 9.8 vs. 99.3 ± 11.4 ng/ml, respectively; $P < 0.05$). Although the mating season did not affect the *IGF*-I gene's placental mRNA expression levels, the *IGF*-II gene's expression level was notably higher ($P < 0.05$; fold change: 2.84) in ewes mated during the breeding season. The results suggest that mating season influences placental characteristics, maternal plasma IGF-I concentrations, and *IGF-II* gene expression levels of cotyledons due to alterations in cotyledon numbers and size on the surface of the placenta.

1. Introduction

Generally, Akkaraman ewes adhere to a conventional mating season extending from September to October (early autumn), with lambing taking place during the last months of winter (February and March) [1]. Nevertheless, lambs are also born outside this traditional breeding window because rams are reared with ewes in one flock throughout the year, which is characteristic of extensive Akkaraman breeding [1, 2]. While year-round mating offers advantages in lamb meat production, the seasonal conditions may cause differences in lambs' pre- and post-natal growth and

development [3]. The consequences of lambing during diverse seasons are particularly pronounced in extensive farming conditions, where environmental factors, notably the availability of vegetation, play a pivotal role. Therefore, inadequate management of nutritional resources during "out-of-seasonal" lambing can lead to adverse outcomes in meat production [4, 5]. Previous studies reported that lambs born in the autumn or winter had lower birth weights than spring-born lambs [6, 7]. The quality and nutrient content of the pasture varies in different seasons due to the annual intensity of sunlight, temperature, and precipitation differences [3, 7–10], causing maternal nutrition supply to change throughout the year. For example, seasonal fluctuations in forage supply on natural pastures or rangelands can cause malnutrition among pregnant ewes, reducing lamb birth weights [6, 7]. However, differences in maternal nutrition levels do not entirely account for the seasonal effect on birth weight. Seasonal changes in reproductive activity are strongly influenced by changes in photoperiod and temperature, affecting the secretion patterns of hormones from the pineal and pituitary glands, such as melatonin, prolactin, and growth hormone [5, 7, 11, 12]. These seasonal fluctuations in reproductive hormones also affect placental and foetal growth and development.

The placenta is a temporary organ that develops from the blastocyst shortly after implantation [13, 14]. The placenta contributes primarily to the intrauterine environment for foetal growth and development [15, 16]. The placenta contains structures known as cotyledons, which exchange respiratory gases, nutrients, and metabolic waste between the maternal and foetal bloodstreams [13]. Although the foetal genome plays a vital role in growth potential, the placenta's size and cotyledon's number significantly influence nutrient transfer capacity from the dam to the foetus, influencing the pre-natal growth trajectory [13, 17]. Placental characteristics are one of the leading indicators affecting the post-natal survival of offspring in small ruminants [14, 16, 18, 19]. Mellor and Stafford [20] reported that the post-natal viability of newborns is associated with placental growth and development during gestation. The exchange capacity of the mammalian placenta between maternal and foetal systems depends on the placental size and number of placentomes [13, 17]. Therefore, the placenta size, cotyledons number, and density, which are related to the nutrient transfer capacity, play a pivotal role in determining the pre-natal growth trajectory of the foetus and, hence, birth weight and post-natal viability [13, 16, 17]. Placental efficiency (PE), a marker of placental function, is commonly assessed by the ratio of birth weight to placental weight [21]. Generally, high PE values associated with averaged-sized foetuses represent placentas with a greater nutrient transport capacity. In contrast, low PE values linked to growth-restricted foetuses represent placentas with a reduced nutrient transport capacity or a failure to adapt. To enhance our understanding of PE, new productivity parameters, cotyledon efficiency (CE) and volumetric cotyledon efficiency (VCE), are used to measure the efficiency of cotyledons [18, 22]. CE and VCE are used as newly adopted methods to measure the individual and total surface area of all cotyledons on the placenta of each animal rather than simply determining the total number and weight of cotyledons. Many studies have observed the effects of maternal nutrition level during pregnancy on placental development [16, 17, 23, 24]. Therefore, differences in maternal feed intake during pregnancy, depending on seasonal pasture quality in a pasture-based rearing system, may affect the development of the placenta and the foetus.

IGFs are nutritionally sensitive proteins that regulate foetal and placental growth and development [25–27]. Previous studies reported that the expression and secretion of *IGFs* increase cell proliferation and mitogenesis and regulate apoptosis [13, 25]. Also, IGFs function as cell cycle promoters, stimulating DNA synthesis and cell differentiation in cultured embryos and numerous foetal cell lines [28]. Studies on sheep have demonstrated that IGFs, whether transferred to the foetus or mother, influence the transfer and distribution of glucose and amino acids between foetal and uteroplacental tissues [27, 29]. *IGF-I* stimulates foetal growth; *IGF-II* may indirectly affect the foetus by modifying the placenta's ability to provide nutrients and promote growth [13, 25, 30].

Previous studies have investigated the effect of season on diverse aspects such as placental development, lamb's daily weight gain, meat quality, birth weight, and mortality rate [3, 4, 7, 11, 31]. However, knowledge about the effects of seasonal differences on placental development, placental mRNA expression level of *IGFs*, and maternal plasma IGF concentration, especially in indigenous breeds, could be improved. Therefore, this study aimed to examine the mating season's effect on the lamb's birth weight, placental characteristics, placental mRNA expression level of *IGFs*, and maternal plasma IGF concentration in Akkaraman ewes.

2. Materials and Methods

The animal study protocol was approved by the Local Animal Care and Ethics Committee of Kirsehir Ahi Evran University, Kirsehir, Türkiye (protocol code 68429034/14 and January 27, 2018), ensuring compliance with EC Directive 86/609/EEC for animal experiments. The study used 100 adult Akkaraman ewes with an average body weight of 54.1 ± 2.7 kg and body condition score of 2.5 ± 0.3. All ewes had given birth at least twice. For mating purposes, two three-year-old fertile and healthy Akkaraman rams with an average body weight (73.6 ± 4.2 kg) and body condition scores (2.9 ± 0.1) were selected. All ewes and rams were reared at a private farm in Kirsehir, Türkiye (38° 55′ 56.8″N, 34° 10′ 45.6″E, and 985 m above sea level), under extensive conditions. In the study, continuous mating (uncontrolled mating) was applied. The rams were allowed to interact freely with ewes day and night. Rams were coreared with ewes throughout the year, and the ewes were introduced to Akkaraman rams for the first time in September. The mating seasons of ewes were determined according to the birth seasons. The ewes mated in the breeding season gave birth between February and March, and the ewes mated during the out-of-breeding season gave birth between September

and October. The study examined only the characteristics of ewes giving birth to singleton males. Consequently, thirty-eight ewes that gave birth to twins ($n = 21$) or females ($n = 17$) were excluded from the study to avoid litter size and sex effects on placental characteristics. The final analysis was conducted on sixty-two Akkaraman ewes, which gave birth to a singleton male lamb. These ewes were divided into mated during the breeding season (between September and October; $n = 35$) and out-of-breeding season (between April and May; $n = 27$). The ewes mated in breeding and out-of-breeding seasons exhibited similar body weights (55.1 ± 2.5 kg and 52.8 ± 2.9 kg, respectively) and body condition scores (2.6 ± 0.2 and 2.5 ± 0.4, respectively).

Monthly averages of outdoor temperature (°C) and rainfall rate (kg/m^2) throughout the experimental period were obtained from the Turkish State Metrological Service and are presented in Figure 1. The ewes were freely grazed in the pasture for at least five hours daily during both the first and second trimesters of pregnancy. The grazing areas were natural grasslands with a very low legume composition. The Poaceae species, *Festuca ovina*, *Hordeum murinum*, and*Aegilops triuncialis* and the species belonging to other families *Avena barbata*, *Mentha arvensis*, and*Thymus* sp. are the most common plant species in these areas [10]. Additionally, each ewe was offered 50 g/day of concentrates (89.5% DM, 23.4% crude protein, and 10.9 MJ·ME/kg DM) and 0.5 kg/day of wheat straw (91.2% DM, 4.6% crude protein, and 4.18 MJ·ME/kg DM) during the pregnancy's first and second trimesters. During the third trimester of gestation, the ewes were allowed to pasture and also received 100 g/day of concentrates and 1 kg/day of wheat straw. The daily dietary regimen was divided into two meals, administered at 08:30 and 16:30. This feeding protocol aimed to ensure optimal nutrition and well-being for ewes throughout the different stages of pregnancy.

2.1. Placental Measurements and Sample Collection. Following lambing, the lamb birth weight (LBW) was determined, and in both seasons, the naturally expelled placenta was collected. Placental weight (PW) was determined with discharged placental fluid before weighing. The numbers (TCN) and weights (TCW) of cotyledons, which were dissected from the chorioallantois, were also recorded. The length (CL), width (CWi), and depth (CDe) of all cotyledons were measured using an electronic digital compass. The subsequent classification of cotyledons based on length (CL) categorised them as small (<20 mm), medium (20–25 mm), and large (>25 mm). Only one randomly selected cotyledon from each size was covered with aluminium foil immediately following lambing frozen in liquid nitrogen and stored at −80°C until the mRNA expression analysis of the *IGFs*.

The cotyledons surface area (CSA) was calculated after the measurements of all cotyledons in the individual placenta as cm^2 with the following equation [14]:

$$\mathrm{CSA} = \left(\frac{(\text{cotyledon width} + \text{cotyledon length})}{4}\right)^2 \times 3.14\,(\pi). \tag{1}$$

Placental efficiency (PE) and cotyledon efficiency (CE) were calculated as the ratio of LBW to the PW and the CSA, respectively. These ratios reflect grams of lambs produced per gram of placenta and surface area of cotyledon. Additionally, the cotyledon volume (CV), volumetric cotyledon efficiency (VCE), and cotyledon density (CD) were calculated using equations (2)–(6), respectively [14].

$$\mathrm{CV} = \mathrm{CSA} \times \mathrm{CDe}, \tag{2}$$

$$\mathrm{PE} = \frac{\mathrm{LBW}}{\mathrm{PW}}, \tag{3}$$

$$\mathrm{CE} = \frac{\mathrm{LBW}}{\mathrm{CSA}}, \tag{4}$$

$$\mathrm{VCE} = \frac{\mathrm{CV}}{\mathrm{PW}}, \tag{5}$$

$$\mathrm{CD} = \frac{\mathrm{TCN}}{\text{per gram PW}}, \tag{6}$$

where CV: cotyledon volume, CSA: cotyledons surface area, CDe: cotyledon depth, PE: placental efficiency, LBW: lamb birth weight, PW: placental weight, CE: cotyledon efficiency, VCE: volumetric cotyledon efficiency, CD: cotyledon density, and TCN: total cotyledon number.

2.2. Blood Sample Collection and the Plasma IGF Assays. Blood samples (~10 ml) were taken from the jugular vein of all ewes every month, from mating to parturition, using a vacutainer in sterile heparin tubes. Blood samples were centrifuged at 3400 rpm for 10 minutes at 4°C, and plasma samples were stored at −20°C until analysis of plasma IGF concentration.

The plasma IGF concentrations of ewes in both seasons were determined using the commercial ELISA kit (MyBioSource, sheep IGF-I, and IGF-II ELISA Kit, San Diego, USA). IGF concentrations in plasma samples were measured using an ELISA reader device (Thermo Scientific, Renfrew, UK) adjusted to 450 nm wavelength. In the reading process, the device was standardised according to the control (blind) wells, and then the measurement process was performed. The concentration of IGFs in plasma samples was calculated according to the concentration of the standards and their respective OD (optical density) values using the standard linear regression curve equation. In the assay, the standard concentrations of IGF-I and IGF-II were 600, 300, 150, 75, and 37.5 ng/mL. The R^2 values of IGF-I and IGF-II standard graphs were found to be 0.978 and 0.987, respectively. Assay sensitivity was 0.1 ng/ml with a coefficient of variation of 5% for IGFs.

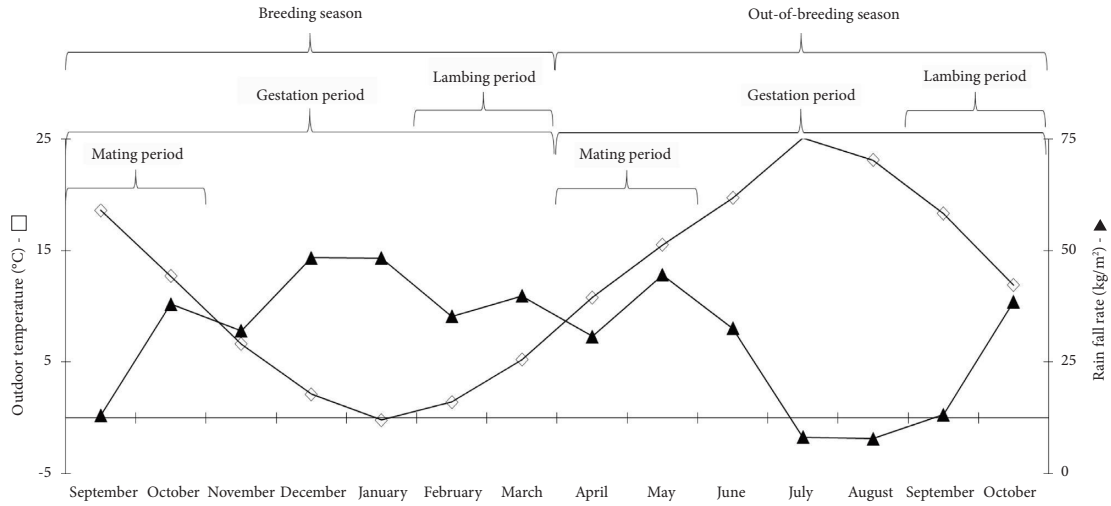

FIGURE 1: Monthly averages of outdoor temperature (°C) and rainfall rate (kg/m^2) during the experimental period.

2.3. Total RNA Isolation, Synthesis of cDNA, and qRT-PCR Analyses. The cotyledons of different sizes obtained from each ewe in the experiment were pooled in a mortar and powdered with liquid nitrogen. The total RNA was extracted from the cotyledon samples using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Genomic DNA was eliminated by digestion with RNase-free DNase I (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The purity and concentration of isolated RNA were evaluated by the A260/A280 ratio using a NanoDrop™ 2000/2000c spectrophotometer (Thermo-Scientific, Renfrew, UK), and all RNA samples showed A260/A280 values within the range of 2.01 to 2.08 and A260/A230 values above 2. The integrity was verified by electrophoresis on a 1% agarose gel. The total RNA was resuspended in 10 mL of buffer solution and stored at −80°C until use in the qRT-PCR assay. Primers used for the amplification of genes were designed using the primer-BLAST tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the related ovine gene sequences (Table 1).

The RNA samples were reverse-transcribed using the commercial cDNA kit (Bio-Rad iScript cDNA, 1708890) according to the manufacturer's Thermal Cycler (Bio-Rad) device instructions. A Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) assay was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA).

The qRT-PCR was conducted in a reaction system with a total volume of 10 μL containing the following components: 5 μL the 2X powerUpTM SYBRTM Green Master Mix (Thermo Fisher Scientific), 0.4 μL forward primer (10 μmol/L), 0.4 μL reverse primer (10 μmol/L), 0.2 μL 50 × ROX Reference Dye, 3 μL 0.1% DEPC water, and 1 μL of cDNA (derived from 25 ng of total RNA) as a template. The PCR protocol included an initial step at 50°C for 2 min (one cycle), followed by 95°C for 2 min (one cycle), and forty cycles of denaturation at 95°C for 15 s and of denaturation at 60°C for 1 min, with a single fluorescence measurement at the extension step. Upon completion of amplification, a final melting curve analysis (Tm) was followed by generating a thermal gradient from 60°C to 95°C with a ramp rate of 0.5°C/s. Standard curves were made from serial dilutions of cDNA ranging from 50 to 5×10^{-4} ng, equivalent to total RNA. Hence, reactions were optimised to maximise the IGFs' amplification efficiency (>90%). The GAPDH gene served as an internal reference gene for normalising the expression of the *IGFs* [25]. Three independent biological replicates of each treatment group were performed. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta Ct}$ method [25].

2.4. Statistical Analysis. The effects of the mating season, treated as a fixed main effect, on placental characteristics, maternal IGF plasma concentration, and placental mRNA expression of *IGFs* genes were analysed using a completely randomised design by the one-way ANOVA procedure of the SPSS package program. Significant differences between means were evaluated using Duncan's test, and results were computed as mean ± standard error of the mean (SEM) [32]. Statistical significance was determined at a threshold of $P < 0.05$. Relationships between birth weight and placental characteristics were investigated through Pearson correlation analysis at the 95% confidence interval. The Fisher Z transformation test was applied to assess the significance of the differences between correlation coefficients for LBW and placental characteristics in both seasons [16].

3. Results

Table 2 presents the post-lambing body weights and body condition scores of ewes mated in breeding and out-of-breeding seasons. The body weights and body condition scores of ewes mated in both seasons were similar at the beginning of the study, but the post-lambing weight of ewes mated in the breeding season exhibited a tendency to be higher (excluding for body condition scores) than those mated in out-of-breeding seasons ($P = 0.083$). The weight of total cotyledons was lower for ewes mated during the out-of-

Table 1: Details of primer pairs used for ovine RT-PCR reactions.

Genes	Primer sequence (5′–3′)	FS (bp)	AN
IGF1	F-GAGACCCTCTGCGGGGCTGA R-CTGCTCGAGCCGTACCCCGT	98	NM_001009774.3
IGF2	F-AGCCCGCAGAGACATCAATG R-AAGTGAGCCAAAGTGTCGTAAT	84	NM_001009311.1
GAPDH	F-TCTCAAGGGCATTCTAGGCTAC R-GCCGAATTCATTGTCGTACCAG	151	NM_001190390.1

FS = fragment size; AN = accession number.

breeding season compared to ewes mated during the breeding season ($P < 0.05$), which was represented by the difference in the large cotyledon weight between the seasons (Table 2). The total number of cotyledons in the placenta of the ewes mated in the breeding season was higher than those mated during the out-of-breeding season (68.3 ± 2.3 vs. 60.9 ± 3.1; $P < 0.05$). No differences were found in the numbers of small (27.3 ± 2.4 vs. 21.4 ± 2.8), medium (29.2 ± 1.5 vs. 25.3 ± 2.1), and large cotyledons (11.8 ± 1.1 vs. 14.2 ± 2.4) between seasons. Although PE, CE, CD, CV, and CSA were similar in both mating seasons, VCE was higher ($P < 0.05$) for ewes mated during out-of-breeding compared to ewes bred in the breeding season (Table 3). Interestingly, while no significant differences were detected between the mating seasons in terms of the measurement characteristics of the cotyledons, it was observed that the large cotyledons in the placentas of ewes mated the breeding season were thicker ($P < 0.05$) than those mated during the out-of-breeding season (Table 3).

Pearson correlation coefficients of placental characteristics and birth-related factors in Akkaraman ewes mated during breeding and out-of-breeding season are presented in Tables 4 and 5, respectively. There were no significant correlations between LBW and placental characteristics in either of the seasons. There were positive correlations between PW and TCW, and negative correlations were observed between PW and CD, PE, and VCE for ewes mated during the breeding season. As expected in the study, there were positive correlations between TCN and CD in ewes bred in the breeding season. In addition to negative correlations between TCW and CE, TCW and CD, TCW and PE, and TCW and VCE were calculated in ewes mated during the breeding season. While the positive correlation between CV and CSA was calculated, negative correlations were obtained between CV, CE, and VCE in the breeding season. Positive correlations were found between VCE and CE, CD, and PE, whereas negative correlations were noted between CSA and CE and VCE.

In the out-of-breeding season, positive correlations were found between PW and TCW, CV, and CSA. Negative correlations were observed between PW and CE, CD, PE, and VCE in that season. There were positive correlations between TCN and TCW and CSA, but negative correlations were found between TCN and CV, CE, PE, and VCE for ewes mated during the out-of-breeding season. Although a positive correlation was calculated between CW and CSA, there was a negative correlation between TCW and PE for ewes mated in the out-of-breeding season. As expected, there was a positive correlation between CV and CSA, while negative correlations were calculated between CV and CE and CV and VCE for out-of-breeding season. Similarly, negative correlations between CSA and CE and VCE were obtained. There were positive correlations between CE and PE and VCE in ewes mated during the out-of-breeding season. A positive correlation between PE and VCE was calculated when ewes mated in the out-of-breeding season.

Table 2: Post-lambing body weights and body condition scores of ewes, along with lambs' birth weights and placental traits (means ± SEM).

Traits	Seasons	
	Breeding	Out-of-breeding
Post-lambing weight (kg)*	52.4 ± 3.1	47.1 ± 2.9
Body condition score	2.5 ± 0.2	2.4 ± 0.2
Lamb birth weight (g)	4973.1 ± 107.1	5048.4 ± 192.0
Placental weight (g)	264.7 ± 13.50	241.1 ± 19.51
Total cotyledon weight (g)	85.3 ± 4.6[a]	70.9 ± 6.4[b]
Small cotyledon weight (g)	13.2 ± 1.3	14.2 ± 2.2
Medium cotyledon weight (g)	38.6 ± 2.2	33.6 ± 2.8
Large cotyledon weight (g)	33.6 ± 3.7[a]	23.1 ± 3.3[b]

[a,b]Different superscript letters in the same row indicate a significant difference, $P < 0.05$; *$P = 0.083$.

Interseasonal comparison of correlation coefficients between birth-related traits and placental characteristics is presented in Figure 2. When the calculated correlation coefficients between LBW and placental traits for each mating season were compared, significant differences were observed between seasons. There were substantial differences between breeding and out-of-breeding seasons in terms of LBW and PW (0.097 vs. −0.332; $P < 0.05$), LBW and TCN (0.229 vs. −0.383; $P < 0.01$), LBW and TCW (0.229 vs. −0.111; $P < 0.05$), and LBW and PE (0.338 vs. 0.724; $P < 0.05$) correlation coefficients. Similarly, significant differences between mating seasons were observed in terms of correlation coefficients of PW and TCN (−0.062 vs. 0.407; $P < 0.05$), PW and CSA (0.341 vs. 0.675; $P < 0.01$), and PW and CD (−0.801 vs. −0.580; $P < 0.05$). The correlation coefficients of TCN and TCW with other placental characteristics significantly differed between mating seasons in terms of TCN and TCW (−0.062 vs. 0.509; $P < 0.01$), TCN and CSA (−0.347 vs. 0.508; $P < 0.01$), TCN and CE (−0.383 vs. −0.685; $P < 0.05$), TCN and PE (0.146 vs. −0.537; $P < 0.01$), TCN and VCE (−0.150 vs. −0.681; $P < 0.01$), TCW

Table 3: Morphometric characteristics and various efficiency traits of placenta and cotyledons (means ± SEM).

Traits		Seasons	
		Breeding	Out-of-breeding
PE		20.0 ± 1.1	23.3 ± 2.3
CE		18.7 ± 1.1	22.0 ± 2.3
CD		0.3 ± 0.02	0.3 ± 0.02
VCE		42.2 ± 3.6[b]	60.6 ± 7.5[a]
CV (mm^3)		1.8 ± 0.3	1.6 ± 0.2
CSA (mm^2)		4.6 ± 0.6	4.2 ± 0.3
ACWi (mm)		21.6 ± 1.2	20.2 ± 0.7
ACL (mm)		27.1 ± 2.4	26.4 ± 2.8
ACDe (mm)		3.9 ± 1.5	5.6 ± 2.1
CWi (mm)	SC	13.8 ± 0.5	14.5 ± 0.6
	MC	22.8 ± 1.5	19.4 ± 0.7
	LC	28.1 ± 1.5	27.8 ± 1.2
CL (mm)	SC	17.31 ± 0.9	18.5 ± 0.5
	MC	27.9 ± 1.2	25.1 ± 0.8
	LC	36.0 ± 1.2	35.5 ± 1.7
CDe (mm)	SC	2.9 ± 0.2	3.2 ± 0.2
	MC	4.1 ± 0.3	3.9 ± 0.2
	LC	4.6 ± 0.2[a]	3.7 ± 0.2[b]
CSA (mm^2)	SC	53.7 ± 7.9	48.5 ± 7.3
	MC	154.0 ± 35.1	99.4 ± 19.8
	LC	99.6 ± 13.2	104.7 ± 17.8

[a,b]Different superscript letters in the same line indicate a significant difference, $P < 0.05$. PE = placental efficiency, CE = cotyledon efficiency, VCE = volumetric cotyledon efficiency, CV = cotyledon volume, CSA = cotyledons surface area, ACWi = average cotyledon width, ACL = average cotyledon length, ACDe = average cotyledon depth, CWi = cotyledon width, CL = cotyledon length, CDe = cotyledon depth, SC = small cotyledon, MC = medium cotyledon, and LC = large cotyledon.

and CSA (0.205 vs. 0.492; $P < 0.05$), TCW and CD (−0.567 vs. −0.113; $P < 0.05$), and TCW and VCE (−0.640 vs. −0.284; $P < 0.05$). The correlation coefficients between CE and PE (0.364 vs. 0.723; $P < 0.05$), CE and VCE (0.698 vs. 0.925; $P < 0.05$), and CD and VCE (0.428 vs. 0.014; $P < 0.05$) significantly differed between seasons.

The trends in maternal plasma IGF-I and IGF-II concentrations in Akkaraman ewes during gestation are presented in Figures 3 and 4, respectively. Plasma IGF-I concentrations were higher ($P < 0.05$) at the 4th month of gestation (158.5 ± 13.4 ng/ml) and lambing (155.2 ± 12.9 ng/ml) compared to the mating (102.8 ± 9.3 ng/ml), 1st (85.6 ± 6.8 ng/ml), and 2nd (82.4 ± 8.7 ng/ml) months of gestation in ewes mated during the breeding season. Also, the concentrations of plasma IGF-I in ewes mated during the out-of-breeding season were significantly higher ($P < 0.05$) in the 4th month of gestation (138.6 ± 12.8 ng/ml) and at birth (133.4 ± 11.7 ng/ml) compared to the mating period (104.1 ± 11.6 ng/ml). Moreover, a significant difference was observed between ewes mated during breeding and out-of-breeding seasons regarding plasma IGF-I concentration at 3rd month of gestation, being higher for ewes bred in the breeding season (123.8 ± 9.8 vs. 99.3 ± 11.4, $P < 0.05$) (Figure 3). Plasma IGF-II concentration at measurement days did not differ between gestation months and mating seasons (Figure 4).

The placental expression levels of *IGF* mRNA relative to GAPDH are presented in Figure 5. There were no significant differences between mating seasons in terms of placental mRNA expression levels of *IGF-I*, but the expression level of *IGF-II* mRNA was higher (fold change: 2.84) for the ewes mated during the breeding season ($P < 0.05$) compared to the out-of-breeding season.

4. Discussion

The present study indicated that mating season influences some placental characteristics, maternal IGF-I plasma concentrations, and placental mRNA expression levels of *IGF-II* without affecting LBW in singleton-bearing Akkaraman ewes. Significant variations were observed regarding the correlation coefficient between LBW and some placental characteristics between mating seasons. This suggests an interplay between the mating season and placental characteristics, emphasising the need for a comprehensive understanding of these relationships.

In the northern hemisphere, the breeding season of sheep coincides with the early autumn and winter months (September through March) when the quality of pasture decreases [6, 31, 33]. Therefore, inadequate maternal nutrition during the breeding season may decrease reproductive performance. Previous studies have demonstrated the significant role of maternal nutrition levels before and after mating and during gestation in influencing reproductive performance [6, 24, 34–36]. Similarly, previous studies indicated the impact of seasonal differences on placental and foetal development associated with LBW and post-natal growth [11, 37]. LBW stands out as one of the most critical factors affecting post-natal growth and survival until weaning, making it a pivotal concern for the sheep production industry [4, 8, 18, 38]. Even if the sheep exhibit estrus depending on the season, ewes can be mated year-round with various breeding practices (exogenous hormone applications, ram effect, etc.) [12]. The birth weight of lambs exhibits seasonality, reflecting the influence of environmental conditions of each season [4, 8, 11, 37, 39]. Peterson et al. [11] and Reid et al. [39] reported that the birth weight of spring-born lambs tended to be higher than that of autumn-born and spring-born lambs. Also, spring-born lambs had higher growth rates compared to autumn-born lambs during the early post-natal period [11, 39]. However, Sušić et al. [4] reported no significant differences in birth weight between spring- and autumn-born lambs. Similarly, in the present study, lambs of Akkaraman ewes mated during breeding and out-of-breeding seasons exhibited comparable birth weights.

The Akkaraman sheep breed has high adaptability and resistance to adverse environmental conditions [2, 18, 40]. Ewes of this breed have high survivability due to their fat tail and have an essential storage nutrient reserve against insufficient feeding periods [1, 2, 41]. In seasons characterised by abundant and high-quality forage, the fat tissue in the tail is formed and grows resulting in an increase in both size and the number of fat cells. During seasons with insufficient forage, lipolysis occurs. The different weather conditions in seasons can directly affect the herbage quantity and quality

Table 4: Pearson correlation coefficients of placental characteristics and birth-related factors in singleton lambs born from Akkaraman ewes mated during the breeding season.

	PW	TCN	TCW	CV	CSA	CE	CD	PE	VCE
LBW	0.097	0.229	0.229	0.138	0.074	0.089	0.008	0.338	0.060
PW		−0.062	0.601**	0.332	0.341	−0.381	−0.801**	−0.876**	−0.539*
TCN			−0.010	−0.309	−0.347	−0.383	0.537*	0.146	−0.150
TCW				0.268	0.205	−0.462*	−0.567*	−0.500*	−0.640**
CV					0.949**	−0.704**	−0.216	−0.243	−0.770**
CSA						−0.747**	−0.210	−0.255	−0.622**
CE							0.180	0.364	0.698**
CD								0.364	0.428*
PE									0.538*

LBW = lamb birth weight, PW = placental weight, TCN = total cotyledon number, TCW = total cotyledon weight, CV = cotyledon volume, CSA = cotyledon surface area, CE = cotyledon efficiency, CD = cotyledon density, PE = placental efficiency, and VCE = volumetric cotyledon efficiency. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 5: Pearson correlation coefficients of placental characteristics and birth-related factors in singleton lambs born from Akkaraman ewes mated during out-of-breeding season.

	PW	TCN	TCW	CV	CSA	CE	CD	PE	VCE
LBW	−0.332	−0.383	−0.111	0.067	0.126	0.378	0.010	0.724	0.296
PW		0.407	0.536*	0.587*	0.675**	−0.610**	−0.580*	−0.832**	−0.533*
TCN			0.509*	−0.495*	0.508*	−0.685**	0.329	−0.537*	−0.681**
TCW				0.421	0.492*	−0.294	−0.113	−0.446*	−0.284
CV					0.937**	−0.695**	−0.072	−0.401	−0.779**
CSA						−0.740**	−0.143	−0.445	−0.763**
CE							0.091	0.723**	0.925**
CD								0.409	0.014
PE									0.621**

LBW = lamb birth weight, PW = placental weight, TCN = total cotyledon number, TCW = total cotyledon weight, CV = cotyledon volume, CSA = cotyledon surface area, CE = cotyledon efficiency, CD = cotyledon density, PE = placental efficiency, and VCE = volumetric cotyledon efficiency. $^{*}P < 0.05$; $^{**}P < 0.01$.

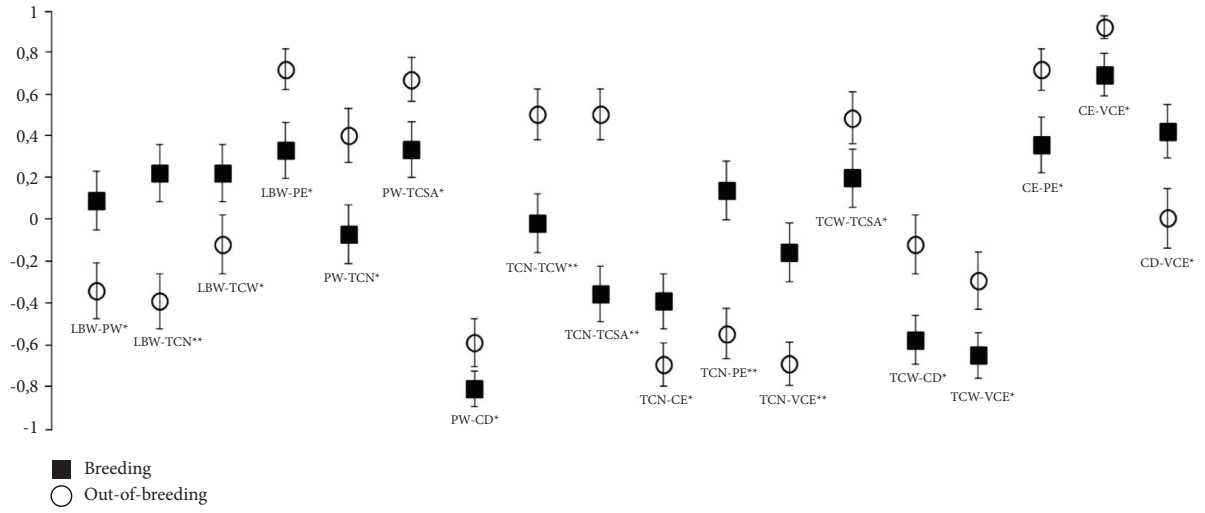

Figure 2: Interseasonal comparison of correlation coefficients between placental characteristics. LBW = lamb birth weight, PW = placental weight, TCN = total cotyledon number, TCW = total cotyledon weight, TCSA = total cotyledon surface area, CE = cotyledon efficiency, CD = cotyledon density, PE = placental efficiency, and VCE = volumetric cotyledon efficiency. $^{*}P < 0.05$; $^{**}P < 0.01$.

of the pasture. Based on the results of the study by Kirbaş [10], the rate of legumes in the pasture areas of the central Anatolia region of Türkiye, where the Akkaraman sheep breed is widely raised, was higher in the breeding season than in the out-of-breeding season. Also, it turns out that this corresponds to the pregnancy of Akkaraman ewes mating out-of-breeding season, which was relatively weaker and coincided with the dry period. Therefore, ewes mated during the out-of-breeding season may experience malnutrition during gestation. Most of the gestation period of ewes mated in the out-of-breeding season coincided with the period with low rainfall.

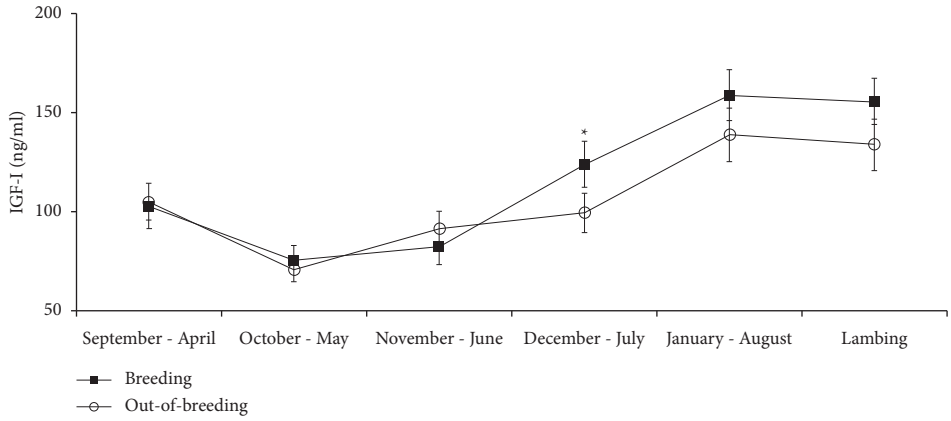

Figure 3: Plasma IGF-I (ng/ml) concentrations in Akkaraman ewes mated during the breeding and out-of-breeding seasons. $^{*}P < 0.05$.

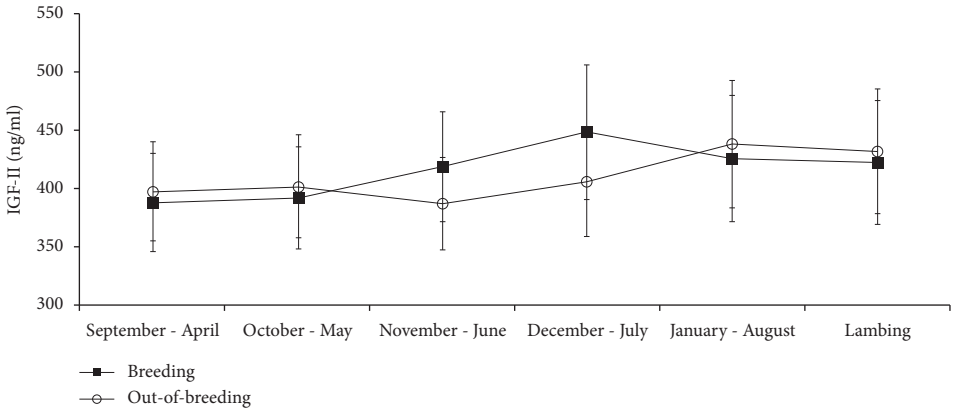

Figure 4: Plasma IGF-II concentrations in Akkaraman ewes mated during the breeding and out-of-breeding seasons.

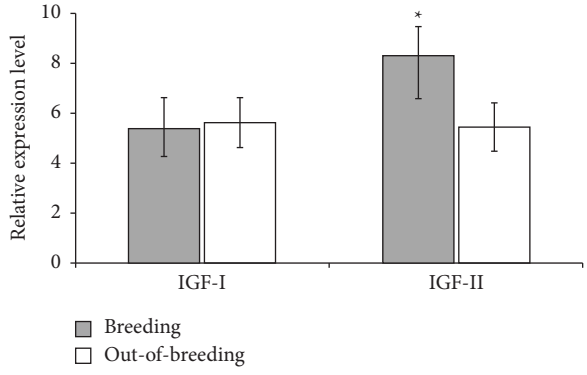

Figure 5: The placental expression levels of *IGF* mRNA relative to GAPDH in Akkaraman ewes mated during the breeding and out-of-breeding seasons. Bars indicate means ± SE. $^{*}P < 0.05$.

In contrast, ewes mated during the breeding season experienced higher rainfall during a significant portion of their gestation period (Figure 1). Despite these environmental influences, the absence of differences in lamb birth weight between mating seasons in the present study was noted. One plausible explanation for this observation may be the occurrence of lipolysis of fatty tissue [42] in the tail during the gestation of Akkaraman ewes mated in the out-of-breeding season.

Moreover, the fat amount of the tail during insufficient feeding periods may support foetal growth and development. Unfortunately, in the present study, the changes in the fat amount of the tail of Akkaraman ewes were not determined during pregnancy. However, there was a notable trend indicating that the decrease in body weight of ewes mated during the out-of-breeding season tended to be more pronounced from mating to the post-lambing period compared to ewes mated the during breeding season. Ewes bred in the out-of-breeding season were approximately 11.3% lighter after lambing than ewes mated in the breeding season. This observation may support the hypothesis that ewes mated in the out-of-breeding season during pregnancy might have utilised fat reserves in their tails.

The placenta undergoes significant growth and development during mid-gestation to support foetal growth and development [13, 43]. In sheep, the placenta reaches maximum size by the 3rd month of gestation, when the foetus gains only 1/10 of birth weight [43, 44]. Previous studies reported a positive relationship between LBW and PW [3, 8, 18, 45, 46]. Therefore, abnormal or insufficient placental development can affect foetal growth and development. In the present study, although there was no difference

between LBW and PW in mating seasons, significant differences were observed in total cotyledon number and weight. The difference in weight of the total cotyledon stemmed from ewes mated in the breeding season having heavier large cotyledons. This, in turn, was attributed to the thicker large cotyledons during the mating season. Although the difference in large cotyledon thickness between mating seasons did not affect the cotyledon volume, it significantly changed the volumetric cotyledon efficiency. Alteration in the volumetric cotyledon efficiency indicates that differences in placental development may occur in different mating seasons. This difference may be influenced by maternal nutrition levels during pregnancy in different seasons [3, 7, 8, 47]. Although the fat tail of Akkaraman ewes supports foetal and placental weight, it may not be sufficient to meet the essential nutrients required for healthy placenta and foetal development. Our results indicated that the correlation coefficients between placental characteristics and LBW differ when comparing the mating seasons, supporting this argument. Although the present study did not investigate the post-natal growth and development of lambs born in different mating seasons, prior studies reported that placental development abnormalities may affect post-natal viability, health, growth, and adult productivity [13, 43, 44]. Therefore, examining the post-natal development and adult productivity of lambs obtained from ewes mating in different seasons can be an essential contributor to revealing the effects of seasonal differences in placental characteristics on the productivity of animals in the adult period.

Previous studies have reported that *IGFs* are expressed and secreted by the sheep uterus and placenta [48, 49]. *IGF-I* and *IGF-II* mRNAs were detected in sheep embryos throughout pre-implantation development from the single-cell stage to the blastocyst stage [50]. Following implantation, maternal uterine *IGF-I* mRNA expression was low, making it unlikely that locally expressed and produced *IGF-I* is essential for placental development [49]. In contrast, expression of *IGF-II* mRNA in the placentome capsule and endometrial stroma continued throughout the first half of pregnancy, and *IGF-II* mRNA expression increased in foetal mesodermal tissues from days 14 to 35 and remained elevated until birth [51]. Overexpression of IGF-II is associated with an enlarged placenta [52]. Gene deletion studies have shown that a deficiency in placental development occurs in embryos carrying null mutations of the *IGF-II* gene, while *IGF-I* gene deletion does not influence placental weight [26, 53–55]. *IGF-II* has a stimulatory effect on the growth of the placenta, but this effect is not mediated through *IGF-I* [56]. However, Osgerby et al. [47] reported that maternal plasma IGF-I concentration positively correlates with the total placentome number, and higher levels of values are observed in ewes in good condition. The results of the present study showed that mating season did not influence the placental mRNA expression level of *IGF-I*. However, increased *IGF-II* mRNA expression was observed in the ewe mated during the breeding season. Interestingly, maternal plasma IGF-II concentrations in ewes mated in both seasons were similar. In contrast, the concentration of IGF-I was higher at the 3rd month of gestation in ewes bred during the breeding season. The higher total number and weight of cotyledons in ewes mated during the breeding season may have resulted from higher placental *IGF-II* mRNA gene expression level and maternal plasma IGF-I concentration [13, 15, 27, 29]. Thus, these observations confirmed that mating season exerts an influence on placental growth and development through alterations in plasma concentration and mRNA expression of *IGFs* [7, 13, 15, 37].

5. Conclusion

In conclusion, the results of the present study imply that mating season influences placental characteristics without affecting lamb birth weight in Akkaraman ewes. Ewes mated during the out-of-breeding season exhibited different placental morphology, potentially leading to alterations in placental sufficiency. Differences in placental characteristics, especially in the total number and weight of cotyledons, of ewes mated in different seasons may be attributed to differences in maternal plasma IGF-I concentration and placental *IGF-II* mRNA expression. Therefore, further studies are needed to understand the effects of seasonal differences in extensive sheep breeding on placental characteristics on lambs' post-natal growth and adult productivity.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

US and ES were responsible for conceptualization, methodology, project administration, and investigation. US, ES, HÖ, CT, and AMY were responsible for validation and data curation. HÖ, CT, AMY, SB, RU, and TLT were responsible for formal analysis. US, ES, and HÖ were responsible for resources. US, SB, HÖ, RU, and TLT were responsible for original draft preparation. ES, HÖ, CT, AMY, SB, RU, and TLT were responsible for review and editing. US, ES, HÖ, SB, RU, and TLT were responsible for visualization. US was responsible for supervision. All authors have read and agreed to the published version of the manuscript.

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