jotaf **Journal of Tekirdag Agricultural Faculty**
Tekirdağ Ziraat Fakültesi Dergisi

Ocak/January 2025, 22(1)
Başvuru/Received: 05/08/24
Kabul/Accepted: 05/01/25
DOI: 10.33462/jotaf.1524295

http://dergipark.gov.tr/jotaf
http://jotaf.nku.edu.tr/

ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Effect of Melatonin Treatment on Reproductive Hormones and Sexual Behaviors in Sheep*

Koyunlarda Melatonin Uygulamasının Üreme Hormonları ve Eşeysel Davranışlara Etkisi

Nesrin ÖZTÜRK[1], Cemil TÖLÜ[2*]

Abstract

In seasonal oestrus animals such as sheep, out-off-season lambing practices and synchronization of oestrus are important in terms of herd management and farm economics. The present study aimed to determine the effects of melatonin implants administered before the breeding season on melatonin, estrogen, progesterone hormone concentrations, and sexual behavior in Tahirova sheep. The study was conducted on 60 Tahirova dairy ewes, aged 1-4 years, and 6 head rams, aged 1-2 years. On June 26, melatonin implants were administered to half of the ewes (18 mg dose/ewe) and rams (54 mg dose/ram). Fifteen days after the implantation of melatonin, all the ewes (530 g/ewe) and rams (660 g/ram) were given supplemental feeding (flushing) with grain feed for a period of 60 days. On August 1, the ewes exhibiting signs of estrus following the introduction of a ram were relocated to individual paddocks, where mating was conducted using the hand-mating method. The frequency of sniffing, touching the ram's scrotum, turning the ram, head-turning, and tail wagging was recorded during the hand-mating procedure. Additionally, the sexual behaviors exhibited by the ram were documented. Furthermore, an investigation was conducted to ascertain whether an interaction occurred within the first minute. The intervals, sexual interaction rate of the ewe, total frequency of sexual interaction, time per sexual interaction, and total sexual interaction time were observed. Blood samples were collected regularly on the initial day, the 21st day, the 42nd day, the 63rd day, the 94th day, and the 129th day for subsequent hormone analysis. Hormonal analyses were conducted using the ELISA method on blood serum samples. The melatonin hormone concentration was observed to be higher in the treatment (MT) group than in the control (C) group on the 63rd day (P=0.0476). The sexual interaction rate of the MT group was found to increase significantly in comparison to the C group (P=0.0045). Additionally, the gestation rate was shorter in the MT group than in the C group (P≤0.05). It can thus be concluded that the melatonin implant, when applied before the breeding season, significantly increases melatonin hormone concentrations on the 63rd day and significantly shortens the periods of gestation in Tahirova dairy sheep.

Keywords: Tahirova sheep, Melatonin, Estrogen, Progesterone, Sexual interaction rate, Gestation, Ewe age.

[1]Nesrin Öztürk, Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Animal Science, Çanakkale and Türkiye. E-mail: nesrinoztuurk@hotmail.com OrcID: 0000-0001-5073-502X
[2]***Sorumlu Yazar/Corresponding Author:** Cemil Tölü, Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Animal Science, Çanakkale and Türkiye. E-mail: cemiltolu@comu.edu.tr OrcID: 0000-0002-6135-4502
Atıf: Öztürk, N., Tölü, C. (2025). Koyunlarda melatonin uygulamasının üreme hormonları ve eşeysel davranışlara etkisi. *Tekirdağ Ziraat Fakültesi Dergisi,* 22(1): 221-232.
Citation: Öztürk, N., Tölü, C. (2025). Effect of melatonin treatment on reproductive hormones and sexual behaviors in sheep. *Journal of Tekirdag Agricultural Faculty,* 22(1): 221-232.

*This study was summarized from the Nesrin ÖZTÜRK MSc thesis.

Öz

Koyun gibi mevsime bağlı kızgınlık gösteren hayvanlarda, mevsim dışı kuzulatma uygulamaları ve östrusların senkronizasyonu sürü yönetimi ve işletme ekonomisi açısından önem taşımaktadır. Bu çalışmada, aşım sezonu öncesi uygulanan melatonin implantının Tahirova koyunlarında melatonin, östrojen ve progesteron hormon seviyeleri ve eşeysel davranışlara etkisi belirlenmiştir. Çalışmada 1-4 yaşları arasında 60 baş Tahirova ırkı koyun ve 1-2 yaşlı 6 baş koç kullanılmıştır. 26 Haziran'da koyun (18 mg doz/koyun) ve koçların (54 mg doz/koç) yarısına melatonin implantı uygulanmıştır. Melatonin implantından 15 gün sonra koyun (530 g/koyun) ve koçların (660 g/koç) tamamına klasik yöntemle dane yemle ek yemleme (flushing) uygulaması 60 gün süreyle gerçekleştirilmiştir. 1 Ağustos'ta koç katımı ile kızgınlıkları tespit edilen koyunlar bireysel bölmelere alınarak elde aşım yöntemiyle çiftleştirmeleri gerçekleştirilmiştir. Elde aşım sırasında koyunlarda koklama, koçun skrotumuna dokunma, koçun etrafında dönme, koça dönüp bakma ve kuyruk sallama davranış sıklığı alınırken, koça ait olan eşeysel davranışlarda kaydedilmiştir. Ayrıca gözlemlerde 1 dakikalık aralıklarla etkileşimin olup olmadığı, koyunların eşeysel etkileşim oranı, toplam eşeysel etkileşim sıklığı, eşeysel etkileşim başına düşen süre ve toplam eşeysel etkileşim süresi belirlenmiştir. Çalışmada başlangıç, 21., 42., 63., 94. ve 129. günlerde kan alımı yapılmıştır. Kan serum örneklerinde Elisa yöntemi ile hormon analizleri ile yapılmıştır. Çalışmada 63. günde melatonin hormon konsantrasyonu uygulama (MT) grubunda kontrol (C) grubundan daha yüksek olmuştur ($P=0,0476$). Koyun eşeysel etkileşim oranı kontrol grubuna göre uygulama grubunda önemli ölçüde artırmıştır ($P=0,0045$). Koç katımı ile gebe kalma oranı uygulama grubunda kontrol grubundan daha kısa gerçekleşmiştir ($P\leq0,05$). Sonuçta, Tahirova süt koyunlarında aşım sezonu öncesi melatonin implantı uygulaması 63. günde melatonin hormon konsantrasyonunda önemli ölçüde artış sağlamış ve gebe kalma süresini ise önemli düzeyde kısaltmıştır.

Anahtar Kelimeler: Tahirova koyunu, Melatonin, Östrojen, Progesteron, Eşeysel etkileşim oranı, Gebe kalma, Koyun yaşı

1. Introduction

Fertility is the most sustainable income on a livestock farm. In seasonal oestrus animals such as sheep, out-off-season lambing practices and synchronization of oestrus are important in terms of herd management and farm economics. Although the use of hormone-impregnated sponges in small ruminants is common, due to difficulties in application, ear root implants that secrete melatonin hormone for 3-4 months have recently become common (Sasa et al., 2016; Öziş Altınçekiç and Koyuncu, 2017; Elhadi et al., 2022; Tölü et al., 2022). Melatonin hormone is used for out-off-season lambing. Melatonin hormone is not a hormone used for synchronization of oestrus. Melatonin synthesis, secreted from the pineal gland in the brain, is under the control of neural, hormonal, and enzymatic systems through photoperiodic interaction (Zarazaga et al., 2009). The main purpose of light management is to increase melatonin release by increasing the dark period during the circadian cycle. The use of exogenous melatonin administration is based on imitation of melatonin release over short days. It is known that melatonin levels in sheep vary depending on the time of day (Carcangiu et al., 2013). The values determined during the day are lower than those determined at night (Carcangiu et al., 2013; Sasa et al., 2016). However, an increase in melatonin hormone levels can be observed during the daytime with the effect of the melatonin implant (Lincoln and Ebling, 1985; Sasa et al., 2016; Elhadi et al., 2022). Kisspeptin neurons appear to play a fundamental role in determining reproductive status, as anoestrus is characterized by increased negative feedback from estrogen on GnRH secretion. Kisspeptin cells provide direct synaptic input to GnRH cells, and kisspeptin is a potent stimulator of GnRH secretion (Clarke et al., 2009). It has been determined that melatonin implant in sheep has a positive effect on oestrus period, pregnancy, birth, and offspring productivity (Tölü et al., 2022).

The oestrous cycle in sheep is approximately 16-17 days. The oestrous cycle consists of proestrus, oestrus, metestrus, and diestrus periods and the anoestrus period outside the breeding season. In sheep, the corpus luteum begins to secrete progesterone on the 2nd and 3rd day of the cycle. Progesterone hormone secretion reaches its highest level on the 8th day and continues at this level until the 12th and 14th day. If there is no pregnancy, $PGF2_{\alpha}$ secreted from the uterus regresses the corpus luteum. Progesterone concentration begins to increase from the third day of this period and reaches its peak level on the 8th day. Progesterone concentration, which remains at its peak level until approximately the 11th day, decreases rapidly after this day and decreases again to <1 ng/mL in the last two days of dioestrus (Kalkan and Horoz, 1997).

The changes observed in domestic sheep breeds mating in short photoperiods in terms of gonadal function and the seasonal release of the hormones that control it (GnRH, FSH, and LH) show gender dependency and the direct effect of lighting is lower in males than in females. While sexual activity in females usually stops during long days, it can continue throughout the year in males. In Hu sheep that received subcutaneous injections of melatonin 5 and 10 mg/sheep during the oestrus period, higher levels of melatonin and progesterone hormones were determined in the application groups compared to the control group (Song et al., 2019). Koca and Özbeyaz (2019) reported that estrogen levels varied between 38.66-97.57 pg/mL during the test days, low progesterone levels were necessary to increase ovulation rates, and low oestrus levels and low progesterone levels were necessary to increase the number of lambs. Tahirova sheep genotype, which is crossbred between East Friesian and Kıvırcık sheep, is an important genotype for Türkiye dairy sheep breeding. Tahirova genotype lambs once a year, have a lactation period of 7-8 months and an average lamb yield of 1.6-1.8 lambs per ewe (Sönmez et al., 2009; Tölü et al., 2022). Determining the effect of melatonin application on the reproductive hormone profile of female animals of Tahirova sheep and its relationship with breeding behavior is important in terms of obtaining offspring out of season. In this study, the effect of melatonin implants applied before the breeding season on melatonin, estrogen, and progesterone hormone levels and sexual behavior in Tahirova sheep was investigated.

2. Materials and Methods

The study was conducted at Çanakkale Onsekiz Mart University Faculty of Agriculture Farm Animal Production Application and Research Unit. The unit is in the center of Çanakkale (40°07'41.9"N 26°26'19.3"E).

2.1. Animals and management

In the study, 30 females of melatonin treatment (MT) and 30 females of control (C) group Tahirova sheep were used, which received melatonin implants (18 mg 1 pellet) on June 26. In addition, hand-mating behavior data of 6 rams (3 heads with melatonin treatment, 3 pellets; and 3 heads with control) were also used in the study. Melatonin

subcutaneous implants were applied to the back of the ear. The study was conducted on two groups (MT and C). Ewes were distributed to the groups randomly, considering age, lactation order, milk yield, live weight, and body condition score. While the ages of ewes varied between 1-4 years, the ages of rams varied between 1-2 years. In the MT group, 11 ewes were 4 years old, 8 ewes were 2, 3 and 1 years old, while in the C group, 11 ewes were 4 years old, 7 ewes were 3 years old, 8 ewes were 2 years old, and 9 ewes were 1 year old. Rams with and without melatonin implants were homogeneously distributed and mated according to the MT and C groups of the females.

The ewes included in the experiment were in the 5th month of lactation. Milk yield was 552 mL/day in the MT group and 556 mL/day in the C group, average live weight was 54 kg and average body condition score was 2.70 in both groups. Milking was done in a 2 x 12 automatic parallel milking unit between 7:30-9:00 in the morning and 16:30-18:00 in the evening. Fifteen days after the melatonin implant, supplementary feeding (flushing) of ewes and rams with whole grain feed was carried out for 60 days. The ewes were fed at a level of 1.6 times the living energy need, and rams were fed at a level of 1.2 times the living energy need during the mating period (NRC, 2007). Alfalfa hay (90.32% DM, 18.48% HP), oat straw (92.37% DM, 9.78% HP), and corn silage (90.64% DM, 10.16% HP) concentrated feed in pellet form (90.53% DM, 18.36% HP) was used in the nutrition of ewes and rams. Ewes were given 530 g/ram barley during evening milking, and 660 g/ewe grain barley (90.35% DM, 10.67% HP) was given to the rams in group conditions. The ewes and rams were housed under open shelter conditions in different paddocks with trees providing shade during the daytime.

2.2. Hormone analyses

Blood serum from day 0 before the melatonin implant, and day 21, day 42, day 63, day 94, and day 129 after the melatonin implant was used. The commercial melatonin implant product (Regulin®) contains 18 mg of melatonin hormone and is stated to release melatonin hormone for 3-4 months. In the study, which introduced the aproned ram 36 days after the melatonin implant, hormone levels were determined for a total of 6 periods before the application and for 4 months. The blood samples taken were centrifuged at 3500 rpm for 10 min. and the serum was separated from the blood cells. Blood serums were stored in a deep freezer at -20 ºC until hormone analysis was performed. Hormone analyses were performed by the Enzyme-linked immunosorbent assay (ELISA) method at the department of Animal Science Laboratory of Çanakkale Onsekiz Mart University, Thermo Scientific Multiskan FC Microplate Reader was used in the analyses. Hormone analyses of melatonin (MEL), estrogen (ES), and progesterone (Pg) were performed with sheep-specific commercial hormone kits. The kit (Shanghai Sunred Biological Technology Co.) uses a double-antibody sandwich enzyme-linked immunosorbent assay.

2.3. Mating observation and measurement

In the study, ewes mating took place between the 38th and 74th days after the melatonin implant. Ewes, whose oestrus was detected with aproned rams in the morning and evening hours, were mated homogeneously with rams with and without melatonin implant by hand-mating method (*Figure 1*). Ewes who were detected to be in heat were mated with the assigned ram 8-12 hours later (Tölü and Savaş, 2010). The oestrus search was done with the rams used for mating. However, the rams that were going to mate were not used in the oestrus search during that period. Gestation rates were determined from lambing status at birth. The mating behavior dataset of each ewe was obtained from direct observation and camera recordings. The protocol for testing sexual behavior was carried out in an area separated by iron bars 3 m wide and 4 m long. For direct observations, one observer counted the behaviors and the other recorded them. The behaviors of ewes sniffing (SNF), touching of ram's scrotum (TSC), turning the ram (TR), looking back at the ram (LBR), and tail wagging (TW) were recorded during mating (Tölü and Savaş, 2010; Konyalı et al., 2011). The ram courtship index was calculated from the sum of the frequency of ano-genital sniffing, foreleg kicks, tongue flicks, vocalizations, and lateral approaches (Tölü et al., 2024). Bilateral sexual interaction rate (BSIR) and ewe sexual interaction rate (ESIR) were determined at 1-min. intervals during the 10-min mating period. Total sexual interaction frequency (TSIF), times per sexual interaction (PSI), and total sexual interaction time (TSIT) were determined from camera recordings. Behavioral observations in camera recordings were made by the same person via computer.

Figure 1. Ewes and rams in group C (left; behavior of touching of ram's scrotum) and group MT (right; behavior of head-turning to ram) during hand-mating in Tahirova sheep

2.4. Statistical Analyses

Repeated measures analysis of variance was used to analyze reproductive hormone concentrations. Logarithmic transformation was applied to hormone values before statistical analysis. Group (MT, C), ewe age (1,…4), and group x age interaction were fixed factors, and the hormone value of the individual per experiment was included in the model as a covariate. While the analysis of mating behaviors was performed with the same model (for fixed factors), the ram courtship index (ano-genital sniffing + foreleg kicks + tongue flicks + vocalizations + lateral approaches) was included in the model as a covariate. A square root ($\sqrt{y+10}$) transformation was applied to each of the behaviors. In the analysis of BSIR, ESIR, TSIF, PSI, and TSIT properties measured in hand-mating observations (no transformation was applied), group (MT, C), sheep age (1,…4), group x age interaction were included with fixed factors in the model. In the analysis of the gestation rate and gestation length analyzed on the hormone measurement days, group (MT, C), ewe age (1,…4), group x ewe age interaction were fixed factors, and the body condition score of the ewe at mating was included as covariates. Tukey test was used in post hoc analyses. The animal was included as random effect in all statistical models. All analyses were performed using the SAS (2021) statistical package program.

3. Results

3.1. Hormone analyses

Melatonin (MEL) hormone concentrations of Tahirova sheep, determined according to the group and ewe ages according to the days of melatonin implant, showed significant differences between the groups on the 63rd day after the implant (*Table 1*). The hormone level of the MT group was higher than that of the C group on the 63rd day after implant (P≤0.05). MEL concentrations at the initial, 21st, and 94th days differed significantly according to their ewe age (P≤0.05). The 4-year-old ewe differed significantly from other ewe ages with its low melatonin value in the initial measurement. In the measurement made on the 21st day, 1- and 2-year-old ewes are lower than 3- and 4-year-old ewes. On the 94th day, 1 and 2-year-old ewes and 3- and 4-year-old ewes had higher values (P≤0.05).

The highest MEL level occurred on the 129th day after the implant, while the lowest level occurred on the 42nd day in the MT group (*Figure 2*). The MEL level was high at the beginning and on the 129th day, while it was at the lowest levels on the 21st day and 42nd day in group C.

Estrogen (ES) concentrations were at similar levels across groups (*Table 2*). Estrogen concentrations differed significantly on the 42nd day and the 94th day according to ewe age (P≤0.05). 1 and 2-year-old ewes had lower ES concentrations in the measurements made on the 42nd day and 3- and 4-year-old ewes ES concentrations were higher on the 94th day (P≤0.05).

After the melatonin hormone implant, ES hormone concentrations in groups MT and C were highest on the 63rd day and lowest on the 129th day (*Figure 3*). In the MT group, the values on the 94th day and the 129th day were similar.

Table 1. Least squares mean ± standard error and P values* of MEL hormone concentrations (ng/L) on melatonin implant days in Tahirova sheep according to group (C, MT) and ewe ages

Melatonin, ng/L		Initial	21st day	42nd day	63rd day	94th day	129th day
Group (G)	C	228.5±25.56	136.0±23.84	104.4±23.34	139.9±27.67^{x}	189.5±34.96	258.4±34.01
	MT	196.1±18.55	134.3±21.89	158.6±35.44	172.5±33.65^{y}	154.3±18.88	211.8±28.14
	P	0.7534	0.1119	0.1132	0.0476	0.4337	0.7847
Ewe age (A)	1	224.1±33.95^{a}	64.7±18.59^{a}	147.9±59.22	210.4±51.05	211.8±47.13^{a}	270.6±53.70
	2	215.3±24.16^{a}	70.8±25.30^{a}	89.7±30.90	123.3±26.87	201.5±30.51^{a}	213.8±26.85
	3	267.5±38.02^{a}	215.7±34.99^{b}	151.2±48.60	181.9±44.01	163.2±42.57^{b}	265.7±46.73
	4	145.2±19.35^{b}	163.1±22.28^{b}	137.9±37.55	100.9±40.29	116.7±28.58^{b}	179.8±41.36
	P	0.0305	<0.0001	0.2865	0.5967	0.0093	0.9502
G x A	P	0.1214	0.8405	0.3370	0.0539	0.0067	0.7917
Initial	P	-	<0.0001	<0.0001	<0.0001	<0.0001	0.0001

*Statistical analyses were performed on transformed (log n+30) values. The difference between the means shown with different letters within each group (x-y) and ewe age (a-b) in the same column is statistically significant (P≤0.05).

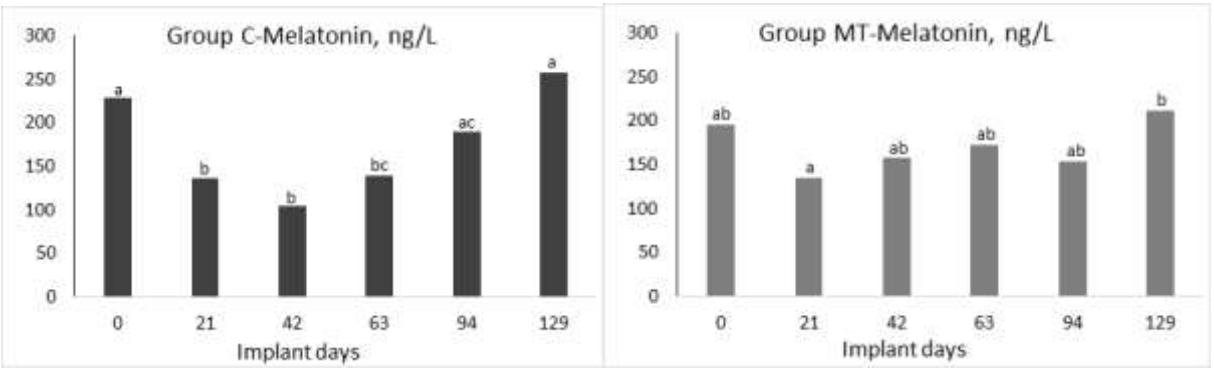

Figure 2. Means of MEL hormone concentrations (ng/L) on melatonin implant days and significance levels between days in Tahirova sheep according to groups (C, MT), (The difference between the means shown with different letters within each group is statistically significant, P≤0.05)

Table 2. Least squares mean ± standard error and P values of ES hormone concentrations (pg/L) on melatonin implant days in Tahirova sheep according to group (C, MT) and ewe ages

Estrogen, pg/L		Initial	21st day	42nd day	63rd day	94th day	129th day
Group (G)	C	12.3±2.50	12.3±1.96	17.1±2.27	24.1±2.09	14.4±2.80	9.5±1.05
	MT	14.7±3.12	15.5±2.55	16.4±2.11	22.5±1.50	11.5±1.49	8.4±1.04
	P	0.1741	0.0835	0.1728	0.9719	0.9335	0.5331
Ewe age (A)	1	17.1±4.92	13.9±4.06	11.5±2.11^{a}	24.8±2.53	17.3±3.11^{a}	10.6±2.06
	2	9.2±2.16	12.7±2.78	11.2±2.72^{a}	23.8±1.82	15.5±2.49^{a}	7.8±0.85
	3	16.2±4.09	16.0±4.00	25.7±2.92^{b}	26.2±3.06	12.0±3.70^{b}	9.4±1.31
	4	10.4±3.64	13.0±2.52	16.7±2.66^{b}	18.5±2.33	7.6±2.16^{b}	7.6±1.45
	P	0.9565	0.1839	<0.0001	0.0660	0.0011	0.9974
G x A	P	0.0520	0.1675	0.3221	0.0982	0.3174	0.4907
Initial	P	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Statistical analyses were performed on transformed (log n+5) values. The difference between the means shown with different letters within and ewe age (a-b) in the same column is statistically significant (P≤0.05).

Progesterone (Pg) hormone concentrations in Tahirova sheep were at similar levels according to groups as in ES hormone (*Table 3*). Pg levels on the 42nd day and 63rd day were slightly higher in the MT group than in the C group (P≤0.10). Pg hormone levels on the 21st day and the 129th day differed according to the age of ewe (P≤0.05). While 1 and 2-year-old ewe had lower values than 3- and 4-year-old ewe in the measurements made on the 21st day, 4-year-old ewe differed from other ewe age groups with its lower values on the 129th day (P≤0.05).

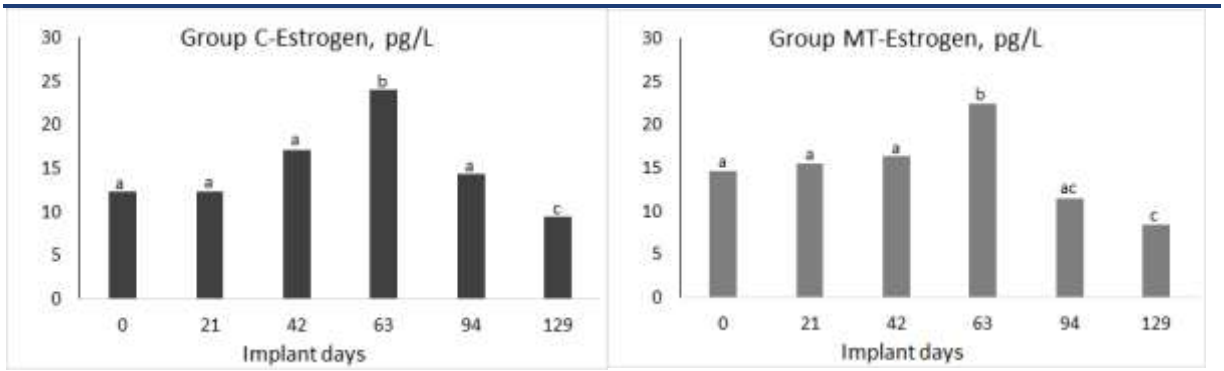

Figure 3. Means of ES hormone concentrations (pg/L) on melatonin hormone implant days and significance levels between days in Tahirova sheep according to groups (C, MT), (The difference between the means shown with different letters within each group is statistically significant, P≤0.05)

Tablo 3. Least squares mean ± standard error and P values* of Pg hormone concentrations (ng/mL) on melatonin implant days in Tahirova sheep according to group (C, MT) and ewe ages

Progesterone, ng/mL		Initial	21st day	42nd day	63rd day	94th day	129th day
Group (G)	C	2.4±0.51	3.8±0.59	3.9±0.46	5.0±0.53	5.0±0.43	5.4±0.41
	MT	2.2±0.51	2.9±0.39	4.2±0.53	5.4±0.61	4.7±0.44	4.7±0.35
	P	0.3297	0.5615	0.0814	0.0715	0.3945	0.2966
Ewe age (A)	1	3.4±0.97	2.3±0.89[a]	4.6±0.88	5.6±1.01	4.3±0.35	5.5±0.50[a]
	2	1.6±0.39	2.6±0.45[a]	4.7±0.52	5.2±0.44	4.5±0.46	5.3±0.43[a]
	3	2.6±0.75	4.7±0.76[b]	5.4±0.70	5.9±0.69	5.9±0.72	5.7±0.66[a]
	4	1.5±0.54	3.3±0.45[b]	2.7±0.53	4.0±0.86	4.5±0.67	3.8±0.45[b]
	P	0.5381	<0.0001	0.0691	0.6923	0.0613	0.0393
G x A	P	0.6352	0.9483	0.5547	0.1887	0.3288	0.0571
Initial	P	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Statistical analyses were performed on transformed (log n+3) values. The difference between the means shown with different letters within each group and ewe age (a-b) in the same column is statistically significant (P≤0.05).

The means of Pg hormone concentrations on melatonin implant days and significance levels between days in Tahirova sheep by groups are shown in *Figure 4*. Pg hormone concentrations in the groups followed an increasing course as the days progressed. While Pg levels differ between the initial day (0 days), the 21st day, and the 42nd, 63rd, 94th, and 129th day, the values between the other days were similar in the MT group. While the initial Pg hormone concentration in group C differed significantly from the other days with its low value, there was a significant difference between the values on the 129th day and the values on the 21st and 42nd days (P≤0.05).

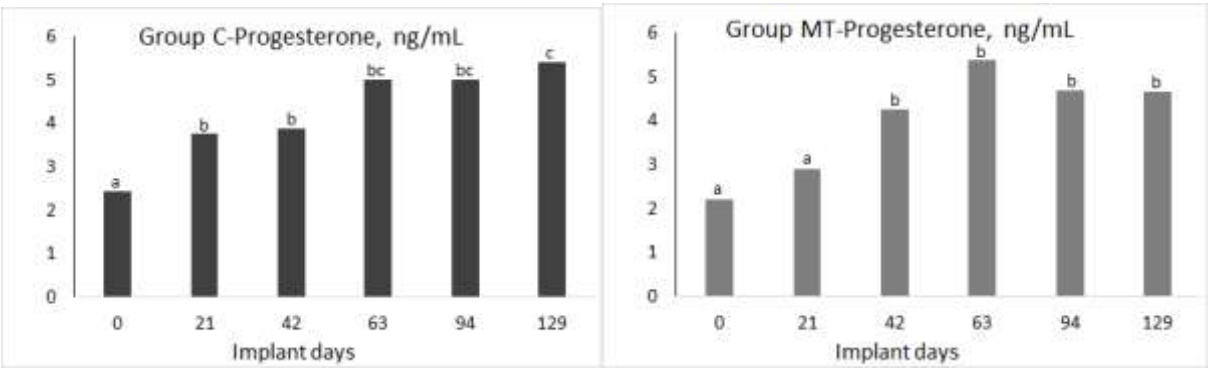

Figure 4. Means of Pg hormone concentrations (ng/mL) on melatonin implant days and significance levels between days in Tahirova sheep according to groups (C, MT), (The difference between the means shown with different letters within each group is statistically significant, P≤0.05)

3.2. Mating observation and measurement

The sexual behaviors obtained in the ewe in the MT and C groups during the hand-mating observations that took place for 10 min. in the study are presented in *Table 4*. Sexual behaviors were similar according to group and ewe age (P>0.05). The behaviors of ewes sniffing (SNF), touching of ram's scrotum (TSC), and tail wagging (TW) behaviors were numerically slightly higher in the MT group. Turn of the ram (TR) and head-turning (HT) behaviors were slightly higher in the control group. Similarly, 1-year-old ewe had slightly higher behavioral frequencies other than TR behavior (P>0.05). It is noteworthy that a lower frequency of TR behavior in 3- and 4-year-old ewes than in 1- and 2-year-old ewes (P>0.05). The ram courtship index (RCI) value, which is included as a covariate in the model, did not have a significant effect on the behaviors considered (P>0.05).

Table 4. Mean ± standard error and P values* of sexual behavior during hand-mating on melatonin implant days in Tahirova sheep according to group (C, MT) and ewe ages

Behavior, times / ewe /10 min.		SNF	TSC	TR	HT	TW
Group (G)	C	2.6±0.56	5.0±0.64	1.4±0.80	7.6±1.15	36.4±5.14
	MT	2.9±0.58	5.7±0.88	0.9±0.31	7.5±1.13	41.8±5.27
	P	0.7912	0.4912	0.4393	0.8268	0.4328
Ewe age (A)	1	3.4±0.94	7.3±1.43	2.0±0.61	5.5±1.18	42.1±7.06
	2	2.9±0.83	5.4±0.63	2.25±1.73	5.3±1.20	41.8±9.32
	3	2.9±0.98	5.1±1.15	0.5±0.40	9.1±2.05	41.3±6.82
	4	2.0±0.56	4.1±0.87	0.3±0.09	9.7±1.65	33.4±5.86
	P	0.7564	0.4074	0.2824	0.3301	0.8446
G x A	P	0.2894	0.3938	0.2868	0.6675	0.9968
RCI	P	0.3868	0.9919	0.5158	0.3974	0.5935

SNF: Sniffing, TSC: Touching of ram's scrotum, TR: Turn of ram, HT: Head-turning, TW: Tail wagging; RCI (ram courtship index): Ano-genital sniffing + foreleg kicks + tongue flicks + vocalizations + lateral approaches. *Square Root (√y+10) transformation was applied to the data.

In the hand-mating observations made for 10 min. in Tahirova sheep, ewe sexual interaction rate (ESIR) differed significantly (P = 0.0045) according to the groups, while other measurements were similar according to the groups (*Table 5*). Ewes in the MT group had a higher rate of ESIR than ewes in the control group (P≤0.05). While total sexual interaction frequency (TSIF) and times per sexual interaction (PSI) differed significantly according to ewe ages, other observations were similar according to ewe ages. While TSIF differed from 3- and 4-year-old ewe with its lower frequency in 2-year-old ewe, a significant difference was observed in PSI between 1- and 4-year-old ewe (P≤0.05).

Table 5. Mean ± standard error and P values of mating measurements during hand-mating on melatonin implant days in Tahirova sheep according to group (C, MT) and ewe ages

Behavior, ewe /10 min.		BSIR, %	ESIR, %	TSIF, times	PSI, sec.	TSIT, min.
Group (G)	C	90.2±2.89	30.5±3.15^{x}	12.6±0.70	25.8±1.50	5.3±0.32
	MT	87.4±3.10	44.2±3.38^{y}	13.1±0.75	27.2±1.61	5.7±0.34
	P	0.5139	0.0045	0.6123	0.5070	0.3080
Ewe age (A)	1	88.9±4.12	35.3±4.49	11.9±1.00^{ab}	30.9±2.14^{a}	6.1±0.46
	2	84.6±4.58	35.8±4.99	10.8±1.12^{a}	28.8±2.37^{ab}	5.3±0.51
	3	94.6±4.58	38.3±4.99	15.0±1.12^{b}	24.0±2.37^{bc}	5.9±0.51
	4	87.3±3.62	40.0±3.94	13.8±0.88^{b}	22.4±1.87^{c}	4.8±0.40
	P	0.4621	0.8564	0.0374	0.0165	0.1901
G x A	P	0.7876	0.1389	0.9823	0.4100	0.4262

BSIR: Bilateral sexual interaction rate, ESIR: Ewe sexual interaction rate, TSIF: Total sexual interaction frequency, PSI: Times per sexual interaction, TSIT: Total sexual interaction time. The difference between the means shown with different letters within each group (x-y) and ewe age (a-c) and ewe age in the same column is statistically significant (P≤0.05).

While no pregnancy was observed in group C on the 42nd day after the implant, pregnancy was observed in 1 ewe in the MT group. On the 63rd day after the implant, an average pregnancy rate of 65.38% and an average

gestation period of 6.00 days were determined in the C group, and an 88.00% pregnancy rate and an average gestation period of 10.72 days were determined in the MT group (P≤0.05; Data not shown).

4. Discussion

In Tahirova sheep that received a melatonin implant before the breeding season, it was determined that the melatonin implant significantly increased the MEL hormone concentration on the 63rd day (*Table 1*). At 63 days post-implant, 172.5±33.65 ng/L MEL hormone concentration was determined in the MT group, while 139.9±27.67 ng/L MEL hormone concentration was determined in the C group. The MEL hormone level, which decreased to the lowest levels in the C group on the 42nd day, remained at similar values in the MT group (*Figure 2*). Group C started to increase on the 63rd day after the introduction of ram on the 36th day. In a study conducted with different sheep breeds, higher levels of melatonin hormone were determined in the application group than in the control group in measurements made on the 43rd day after the implant (Sasa et al., 2016). Elhadi et al. (2022) determined that melatonin implants in early lactation Lacaune and Manchega sheep increased the melatonin hormone concentration in both breeds. In the study in which subcutaneous melatonin injections of 0, 5, and 10 mg/ewe were applied to Hu sheep during the oestrus period; Melatonin hormone level was reported as 458.69 ± 48.40 pg/mL in ewe administered 5 mg melatonin, 458.09 ± 60.60 pg/mL in ewe administered 10 mg, and 393.37 ± 51.53 pg/mL in the control group (Song et al., 2019).

It is known that melatonin, whose daily release increases as the days shorten, stimulates GnRH release in sheep showing seasonal oestrus (Lincoln and Clarke, 1997; Casao et al., 2008). With the increase in GnRH secretion, gonads are stimulated, and oestrus develops in animals. In this study, ewes with melatonin implants came into oestrus in a shorter time and became pregnant earlier than ewes without melatonin implants. This situation seems to occur in the MT group with the effect of the MEL hormone. It is stated that the MEL hormone is used to reduce heat in sheep out of season or to an early period and that it also has a positive effect on embryo development (Tamarkin et al., 1985; Çevik and Yurdaydın, 1998). In the present study, the melatonin hormone, which varies depending on the age of the ewe, differed from each other in 1- and 2-year-olds and 3- and 4-year-olds, although it changed from day to day. Carcangiu et al. (2013) determined that the melatonin level, which varies significantly depending on the age of the ewe, is lower in 3–4-year-old ewes than in 16–18-month-old ewes. Significant interactions between group and age were observed for MEL concentration at days 63 and 94, respectively (*Table 1*). The MEL trends of the groups were different on different days (*Figure 1*). The age of the ewe seemed to affect MEL hormone levels on days 63rd and 94th.

While no significant change in the ES hormone was observed in the study in the days after the melatonin implant, the ES level on the 63rd day was significantly higher than the other days in both groups (*Table 2*; *Figure 3*). In Awassi sheep, higher estrogen hormone levels were determined in the melatonin-implanted group than in the control group (Al-Tai et al., 2021). The estrogen hormone was between 3.50 and 8.42 pg/mL, and the lowest estrogen level was measured on the 15th day in the measurements made between 0 and 18 days in Akkaraman sheep (Arsoy and Sağmanlıgil, 2018). The fact that ES values on the 63rd day of the study were determined to be higher than the other days may be since the ewes' oestrus periods and pregnancy periods occur within this date range. So, estrogen hormone levels in sheep can reach their highest levels during the oestrus period (Hafez, 1993; Goodman and Inskeep, 2015).

While Pg concentrations were similar between groups in the study, the MT group had slightly higher Pg concentrations than group C on the 42nd and 63rd days (*Table 3*). In addition, while the Pg level tended to increase continuously until the 42nd day, it continued at similar values within the group after today (*Figure 4*). It can be said that progesterone concentrations were slightly high in the study in which there was no pregnancy until the 63rd day (except for 1 pregnant ewe on the 42nd day in the MT group). In sheep, it was determined as 0.17 ng/mL during oestrus, 6.50 ng/mL in dioestrus, 0.04 ng/mL in anoestrus, 7.32 ng/mL in early pregnancy, and 11.34 ng/mL in mid-term pregnancy (Alaçam, 2007). Uyanık et al. (2009) determined a progesterone level of 2.02 ng/mL before pregnancy in Morkaraman breed sheep, and they determined a progesterone level between 2.41 ng/mL and 15.92 ng/mL, increasing every week between the 5th and 21st weeks, respectively. In the study, Pg hormone levels were above 2 ng/mL even on the days when mating had not yet started. This situation suggests the possibility that some ewes in Tahirova sheep are in an oestrus cycle. It can be said that out-of-season oestrus monitoring and oestrus

synchronization studies are needed to clarify this situation in the Tahirova sheep genotype obtained by the crossbred method.

In the present study, Pg hormone levels may have been higher in the MT group because the ewes in the MT group came into oestrus and became pregnant in a shorter time than the ewes in the C group. Studies have shown that melatonin implantation can affect pregnancy status when rams are introduced. Therefore, the conception and oestrus of the animals can affect the levels of progesterone hormone. In the measurements made on the 7th and 20th day after melatonin implantation (18 mg/ewe), it was found that the progesterone hormone administration group was higher than the control group in Kıvırcık sheep (Yılmazer et al., 2018). In Border Leicester x Scottish Blackface sheep, it was reported that melatonin-implanted sheep had slightly higher progesterone hormone levels than the control group, and the difference between groups was significant when measured at the 8th week of pregnancy (McEvoy et al., 1998). In Rasa Aragonesa sheep, on the 5th and 8th days after the 18 mg melatonin implant, significantly higher progesterone hormone levels were measured in the treatment group than in the control group sheep (Abecia et al., 2006).

The frequencies of behaviors were similar between groups in hand-mating observations made for 10 min. in Tahirova sheep. However, the ewe sexual interaction rate (ESIR) was higher in the MT group than in the C group (*Tables 4*, *5*). Total sexual interaction frequency (TSIF) and time per sexual interaction (PSI) differed significantly according to ewe age (*Table 5*). In the study, it can be said that the melatonin implant causes an increase in reproductive hormones as well as an increase in the frequency of mating behavior. Studies examining the effects of exogenous melatonin applications on sexual behavior were examined on rams rather than ewes (Rekik et al., 2015; Abecia et al., 2018; Kleemann et al., 2021; Tölü et al., 2024). No study has been found examining the effect of exogenous melatonin application on sexual behavior in females. It can be said that the melatonin implant, which has been found to contribute positively to the increase in sexual activity in male sheep, also increases sexual activity in female sheep in a similar way.

5. Conclusions

Ear subcutaneous melatonin implant application, which is applied just before the breeding season and is expected to increase hormone secretion to higher levels during the breeding season; It significantly increased the melatonin hormone level in Tahirova sheep, especially within the expected date ranges (for 3-4 months). Melatonin implant, which causes an increase in reproductive hormones in ewes during mating intervals, also causes sexual behaviors to occur at a slightly higher frequency and rate during mating. In other words, the melatonin implant seems to increase sexual activity in ewes. So, ewes in the melatonin treatment group had a significantly higher rate of sexual interaction than ewes without melatonin implant treatment.

Melatonin implant applied just before the breeding season (June) increased sexual activity and resulted in oestrus and pregnancy in ewes in the treatment group in a shorter time than in the control group. However, it would be more useful to test the melatonin implant used to provide off-season oestrus in Tahirova sheep in different seasons other than the breeding season.

Acknowledgment

The authors thank Agriculture Engineer Nazif Yazgan, Dr. Hande Işıl Akbağ and Dr. Türker Savaş's contribution to the study. The authors wish to acknowledge the Scientific Research Projects Coordination Unit of Çanakkale Onsekiz Mart University for supporting this study (project number: FYL-2019-3063).

Ethical Statement

All animal handling and experimental procedures were performed by the Committee on Animal Research and Ethics of Çanakkale Onsekiz Mart University (Turkey) on animal use (no. 2018/05-11).

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Cemil, T.; Design: Nesrin, Ö., Cemil, T.; Data Collection or Processing: Nesrin, Ö., Cemil, T.; Statistical Analyses: Cemil, T.; Literature Search Nesrin, Ö., Cemil, T.; Writing, Review and Editing: Nesrin, Ö., Cemil, T.

References

Abecia, J. A., Araya J., Chemineau, P., Palacios, C., Keller, M. and Delgadillo, J. A. (2018). Photoperiod-melatonin-induced, sexually-activated rams increase pregnancy rate and number of lambs per ewe in a ram effect. *Large Animal Review,* 24: 31-35.

Abecia, J. A., Palacin, I., Forcada, F. and Valares, J. A. (2006). The effect of melatonin treatment on the ovarian response of ewes to the ram effect. *Domestic Animal Endocrinology,* 31: 52-62.

Alaçam, E. (2007). *Production of a radioimmunoassay kit for blood progesterone hormone measurements in cows, sheep and dogs and its use in various physio-pathological cases.* Ankara Üniversitesi Bilimsel Araştırma Projesi (2005-08-10-070) Project Report, Ankara (In Turkish).

Al-Tai, S. N. N., Alkalby, J. M. A. and Al-Shwilly, H. A. J. (2021). Profile Of reproductive hormones secretion during estrus and pregnancy associated with different estrus synchronization methods in Awassi ewes in non-breeding season. *Natural Volatiles & Essential Oils,* 8(6): 1934-1947.

Arsoy, D. and Sağmanlıgil, V. (2018). Reproductive cycles in white Karaman ewes: comparison of ovarian hormone secretion and reproductive behavior in non-pregnant and pregnant ewes in semi-intensive conditions. *Acta Scientiarum. Animal Sciences,* 40: e39908.

Carcangiu, V., Mura, M.C., Parmeggiani, A., Piccione, G., Bini, P. P., Cosso, G. and Luridina, S. (2013). Daily rhythm of blood melatonin concentrations in sheep of different ages. *Biological Rhythm Research,* 44(6): 908-915.

Casao, A., Vega, S., Palacin, I., Perez-Pe, R., Lavina, A., Quintin, F. J., Sevilla, E., Abecia, J. A., Cebrian-Perez, J. A., Forcada, F. and Muino-Blanco, T. (2008). Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in Rasa Aragonesa Rams. *Reproduction in Domestic Animals,* 45: 425-432. https://doi.org/10.1111/j.1439-0531.2008.01215.x

Clarke, I. J., Smith, J. T., Caraty, A., Goodman, R. L. and Lehman, M. N. (2009). Kisspeptin and seasonality in sheep. *Peptides,* 30(1):154-63. https://doi.org/10.1016/j.peptides.2008.08.022

Çevik, M. and Yurdaydın, N. (1998). Effect of photoperiodism on fertility in domestic animals. *Lalahan Hayvancılık Araştırma Enstitüsü. Dergisi,* 38(1): 69-78. (In Turkish).

Elhadi, A., Salama, A. A. K., Such, X. and Caja, G. (2022). Responses to melatonin of 2 breeds of dairy ewes in early lactation under autumn photoperiod conditions. *Journal of Dairy Science,* 105: 2587-2596.

Goodman, R. L. and Inskeep, E. K. (2015). Control of the Ovarian Cycle of the Sheep. In: Knobil and Neill's Physiology of Reproduction 4th Edition Volume 2. Eds: Plant T. M. and Zelenik, A. J., Elsevier, London, U.K.

Hafez, E. S. E. (1993). Reproduction in Farm Animals. 6th Edition. Lea & Feibeger., Philadelphia, USA.

Kalkan, C. and Horoz, H. (1997). Pubertal and Sexual Cycles. In: Parturition and Infertility In Domestic Animals. Eds: Alaçam, E., Medisan, Ankara, Türkiye. (In Turkish).

Kleemann, D. O., Kelly, J. M., Arney, L. J., Len, J., Tilbrook, A. J. and Walker, S. K. (2021). Sexual behaviour, semen quality and fertility of young Border Leicester rams administered melatonin during spring. *Animal Reproduction Science,* 231: 106804 https://doi.org/10.1016/j.anireprosci.2021.106804

Koca, A. and Özbeyaz, C. (2019). The effects of progesterone and estrogen hormone levels on some reproductive characteristics of the Akkaraman sheep. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi,* 22(Suppl 2): 424-430.

Konyalı, A., Tölü, C., Ayağ, B. S. and Akbağ, H. I. (2011). Observations on hand-mating behaviors, several physiological and hematological parameters in Turkish dairy goats. *Animal Science Journal,* 82: 251-258.

Lincoln, G. A. and Clarke, I. J. (1997). Refractoriness to a static melatonin signal develops in the pituitary gland for the control of prolactin secretion in the ram. *Biology of Reproduction,* 57: 460-467.

Lincoln, G. A. and Ebling, F. J. P. (1985). Effect of constant-release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. *Journal of Reproduction & Fertility,* 73: 241-253.

McEvoy, T. G., Robinson, J. J., Aitken, R. P. and Robertson, I. S. (1998). Melatonin treatment of embryo donor and recipient ewes during anestrus affects their endocrine status, but not ovulation rate, embryo survival. *Theriogenoiogy,* 49: 943-955.

NRC (2007). Nutrient Requirements of Small Ruminants, National Research Council of the National Academies, Washington, DC., U.S.A.

Rekik, M., Taboubi, R., Ben Salem, I., Fehri, Y., Sakly, C., Lassoued, N. and Hilali M. E. (2015). Melatonin administration enhances the reproductive capacity of young rams under a southern Mediterranean environment. *Animal Science Journal,* 86: 666-672. https://doi.org/10.1111/asj.12350

Öziş Altınçekiç, Ş. and Koyuncu, M. (2017). Comparison of effects of CIDR and prostaglandin applications on the reproductive performance of Kıvırcık ewes in anestrous. *Journal of Tekirdag Agricultural Faculty,* 14(01): 9-15 (In Turkish).

SAS (2021). Statistical Analysis System, SAS/STAT® 9.3 User's Guide. Cary, NC.

Sasa, A., Rodrigues, P. A., Nonaka, K. O., Balieiro, J. C. de C. and Coelho, L. A. (2016). Plasma melatonin and progesterone profiles of Suffolk and Romney Marsh ewes implanted with melatonin during anoestrus season at lower latitudes in Southern Hemisphere. *Acta Scientiae Veterinariae,* 44(1): 1378.

Song, Y., Wu, H., Wang, X., Haire, A., Zhang, X., Zhang, J., Wu, Y., Lian, Z., Fu, J., Liu, G. and Wusiman, A. (2019). Melatonin improves the efficiency of super-ovulation and timed artificial insemination in sheep. *Peer J,* 7: e6750. https://doi.org/10.7717/peerj.6750

Sönmez, R., Kaymakçı, M., Eliçin, A., Tuncel, E., Wassmuth, R. and Taşkın, T. (2009). Sheep breeding studies in Turkey. *Türkiye Koyunculuk Kongresi,* 12-13 February, p. 7-24, İzmir, Türkiye (In Turkish).

Tamarkin, L., Baird, C. J. and Almedia, O. F. (1985). Melatonin: A coordinating signal for mammalian reproduction? *Science,* 227: 714-720.

Tölü, C. and Savaş, T. (2010). Comparison of Gökçeada, Maltese and Turkish Saanen goat genotypes for reproduction traits. Journal of *Tekirdağ Agricultural Faculty,* 7(2): 113-121 (In Turkish).

Tölü, C., Yazgan, N., Akbağ, H. I. and Savaş, T. (2024). Effects of melatonin implant on hormone profile and mating behaviour in rams. *Hayvansal Üretim,* 65(1): 29-36. https://doi.org/10.29185/hayuretim.1473580

Tölü, C., Yazgan, N., Akbağ, H. I., Yurtman, İ. Y. and Savaş T. (2022). Effects of melatonin implants on reproductive performance of dairy sheep and dairy goats. *Reproduction in Domestic Animals,* 57: 665–672. https://doi.org/10.1111/rda.14107

Uyanık, F., Güvenç, K., Gültekin, M. and Gürbulak, K. (2009). Serum leptin and progesterone levels during gestation in ewes. *Journal of The Faculty of Veterinary Medicine Erciyes University,* 6(1): 31-36 (In Turkish).

Yılmazer, C., Çevik, M. and Koçyiğit, A. (2018). Effects of subcutaneous melatonin implants and short-term intravaginal progestagen treatments on estrus induction and fertility of Kıvırcık ewes on seasonal anestrus. *Polish Journal of Veterinary Sciences,* 21(2): 353-359.

Zarazaga, L. A., Gatica, M. C., Celi, I., Guzman, J. L. and Malpaux, B. (2009). Effect of melatonin implants on sexual activity in Mediterranean goat females without separation from males. *Theriogenology,* 72: 910-918.