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Onur Bahan

onurbahan@gmail.com

Yozgat Bozok Universitesi <https://orcid.org/0000-0003-0878-6338>

Gökhan Doğruer

Hatay MKÜ: Hatay Mustafa Kemal Universitesi

Ahmet Gözer

Hatay MKÜ: Hatay Mustafa Kemal Universitesi

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Effects of melatonin exposure duration and estrous cycle phase at the initiation of estrus synchronization on reproductive performance in Awassi ewes during the breeding season

Onur Bahan ^a, Gokhan Dogruer ^b, Ahmet Gozer ^b

^a Yozgat Bozok University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Yozgat, 66010, Turkiye

^b Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Hatay, 31040, Turkiye

Onur Bahan ORCID No: 0000-0003-0878-6338

Gokhan Dogruer ORCID No: 0000-0002-8000-0646

Ahmet Gozer ORCID No: 0000-0001-8658-5916

Corresponding author: Onur Bahan

E-mail address: onurbahan@gmail.com

Abstract

The present study evaluated the effects of melatonin exposure duration and estrous cycle phase at the initiation of estrus synchronization on reproductive performance in Awassi ewes during the breeding season. A total of 120 multiparous Awassi ewes were randomly allocated into four groups (n = 30 per group). Melatonin implants were administered 20 days before synchronization (MEL20), 10 days before synchronization (MEL10), or at synchronization initiation (MEL0), while the control group received no melatonin. Estrus was synchronized using intravaginal medroxyprogesterone acetate sponges for 12 days, followed by eCG and PGF₂ α administration at sponge removal. The estrous cycle phase at sponge insertion was also evaluated in relation to estrus expression and subsequent reproductive outcomes. Melatonin treatment did not significantly affect estrus rate, estrus onset, mating completion, progesterone concentrations at mating and day 18 post-mating, embryonic loss, pregnancy rate, multiple birth rate, lambing rate, lamb yield, or birth weight (P > 0.05). Estrous cycle phase influenced the timing of estrus expression at specific observation times but did not affect mating completion or pregnancy rate. Overall, melatonin exposure duration did not improve reproductive performance, and estrous cycle phase at synchronization initiation did not affect subsequent fertility outcomes under breeding-season conditions.

Keywords: Awassi, ewe, melatonin, breeding season, fertility.

1. Introduction

Ewes are seasonally polyestrous species whose reproductive activity is primarily regulated by photoperiod. Changes in day length modulate pineal melatonin secretion, which in turn regulates the hypothalamic–pituitary–gonadal axis and determines the timing of reproductive activity (Bartlewski et al. 2011; Shi et al. 2013). Although this physiological adaptation ensures optimal lambing under natural conditions, it also contributes to variability in fertility outcomes even during the breeding season. Therefore, improving reproductive efficiency and increasing the number of viable lambs per ewe remain critical for the economic sustainability of sheep production systems (Bartlewski et al. 2011; Reyes-Ramirez et al. 2021).

Exogenous melatonin administration has been widely used to advance cyclic activity during the anestrus period (Kouimtzis et al. 1989; Mura et al. 2017; Waqas and Tibary 2025). Beyond its role in seasonal transition, melatonin has been shown to influence follicular development, steroidogenesis, and luteal function (Williams 1984; Abecia et al. 2019; Duan et al. 2025). These effects are thought to be mediated through enhanced progesterone synthesis, delayed luteolysis, and improved embryo viability, potentially reducing embryonic loss and improving fertility outcomes (Abecia et al. 2002; Zuniga et al. 2002). However, its effectiveness during the breeding season remains inconsistent (Uzabacı and Ustuner 2024). Some studies have reported improved pregnancy rates or increased prolificacy following melatonin treatment (Palacios et al. 2010; Abecia et al. 2019), whereas others have observed limited or no reproductive benefit (Buffoni et al. 2014). These inconsistencies may be attributed to differences in synchronization protocols, breed, nutritional status, and environmental conditions. In addition, it has been suggested that exogenous melatonin administered during the breeding season may interact with endogenous melatonin rhythms, potentially modifying its physiological effects (Buffoni et al. 2014). This indicates that the reproductive response to melatonin may be highly context-dependent.

In this context, the timing of melatonin administration relative to estrus synchronization—and consequently the duration of exposure during critical stages of early embryonic development—may represent a key but insufficiently explored factor. While short-term melatonin exposure has been associated with stimulation of ovarian activity, prolonged exposure may differentially affect luteal function and embryo viability (Williams et al. 1992; Abecia et al. 2019; Vlčková et al. 2022). Nevertheless, the extent to which variations in pre-synchronization melatonin exposure duration influence reproductive outcomes during the breeding season remains unclear.

In addition to melatonin exposure, the ovarian status of ewes at the initiation of estrus synchronization may influence subsequent follicular dynamics and ovulatory responses. The number of small follicles present in the ovary before treatment has been positively associated with subsequent ovulation rate, suggesting that ovarian follicular status at the time of gonadotropic stimulation may contribute to variation in reproductive responses to synchronization protocols (Dott et al. 1979; Driancourt 1987). Previous studies have therefore examined whether the phase of the estrous cycle at the beginning of progestagen-based synchronization, particularly the presence or absence of corpora lutea and the characteristics of antral follicles, influences follicular development and ovulation. Freitas et al. (1996) reported that ovarian structures present at the beginning or end of progestagen treatment did not significantly affect the timing of estrus onset or ovulation rate, although a slight increase in ovulation rate was observed in association with the number of previous corpora lutea. Subsequent studies further indicated that the ovarian structures present at the onset of synchronization may influence the emergence and characteristics of follicular waves, with the presence of corpora lutea and large antral follicles being associated with differences in the growth pattern of subsequent ovulatory follicles (Oliveira et al. 2016a, b). These findings suggest that the phase of the estrous cycle at the initiation of synchronization may represent another source of variation in ovarian responses to synchronization, although its relevance to subsequent reproductive performance remains incompletely understood.

Therefore, the present study aimed to evaluate the effects of melatonin exposure duration prior to estrus synchronization and the phase of the estrous cycle at synchronization initiation on reproductive performance in Awassi ewes during the breeding season. Specifically, the effects of different melatonin exposure durations and non-luteal or luteal phase status at sponge insertion were evaluated in relation to estrus response, estrus onset, luteal function, embryonic loss, and overall fertility. We hypothesized that variation in the duration of melatonin exposure and the ovarian status at the initiation of estrus synchronization may differentially affect reproductive responses in Awassi ewes during the breeding season.

2. Material and methods

2.1. Study location

The study was conducted in Yeşilova District of Reyhanlı, Hatay Province (36°15'04.5"N, 36°32'06.8"E). The study took place during the regional breeding season, from May 24 to November 29, 2021. During this period, average ambient temperatures ranged from 18.9 °C at night to 30.6 °C during the day.

2.2. Study materials

A total of 120 healthy multiparous Awassi ewes, aged 2–5 years and weighing 45–60 kg, were included. All animals were non-lactating and selected based on systemic, clinical, and reproductive examinations. Ewes that were nulliparous, currently ill, or not exhibiting estrus were excluded from the study. Body Condition Scoring (BCS) was performed according to Russel (1984), on a scale of 1–5. The average BCS of the ewes was 2.72 ± 0.13 . Ewes were maintained under an extensive management system, grazed on pasture twice daily, and animal care was provided by the farmer.

2.3. Grouping, estrus induction and mating

Ewes were randomly assigned to four groups of 30 animals each. A single subcutaneous melatonin implant (Regulin®, Ceva, Istanbul, Turkey; 18 mg) was inserted in the left auricular base region 20 days before the initiation of estrus synchronization (MEL20), 10 days before the initiation of estrus synchronization (MEL10), or on the day estrus synchronization was initiated (MEL0). Ewes that did not receive melatonin implants served as the control group (CONTROL).

All ewes received intravaginal cylindrical polyurethane sponges (Esponjavet®, HIPRA Animal Health, Turkey) containing 60 mg medroxyprogesterone acetate, inserted with a specialized speculum for 12 days. Upon sponge removal, all ewes were administered 500 IU eCG (Oviser500®, HIPRA Animal Health, Turkey) and 0.075 mg $\text{PGF}_2\alpha$ (d-cloprostenol, Gestavet®Prost, HIPRA Animal Health, Turkey) intramuscularly.

The same fertile rams were used for estrus detection and natural mating and were introduced 12 h after sponge removal at a ram-to-ewe ratio of 1:10. Rams were allowed to mate with the ewes twice daily for 2-h periods. Estrus was monitored at 12-h intervals (12, 24, 36, 48, 60, and 72 h after sponge removal), and ewes exhibiting estrus and successfully completing mating were separated and housed in a separate pen. Following the initial 72-h mating period, the rams remained with the ewes to allow natural mating during the subsequent estrous cycle.

2.4. Collection of blood samples and determination of serum progesterone concentrations

Blood samples were collected from the jugular vein at three time points: at sponge insertion, at the time of mating, and 18 days post-mating. Samples were centrifuged at 3000 rpm for 10 min, and sera were stored at -20°C until analysis.

Serum progesterone concentrations were measured using a commercial Electrochemiluminescence Immunoassay (ECLIA) kit (Atellica IM Analyzer, Siemens Healthineers), with a detection range of 0.21–60 ng/mL.

2.5. Pregnancy diagnosis

Pregnancy diagnosis was first performed by transabdominal B-mode ultrasonography (Pie Medical, Scanner 100 LC VET) using a 6–8 MHz convex transducer 50 days after mating. A follow-up examination was performed 17 days later (67 days after the first mating) to identify pregnancies resulting from subsequent natural mating. The presence of an embryo with a detectable heartbeat was considered indicative of pregnancy.

2.6. Collection of birth data

Parturition was monitored in pregnant ewes, recording the number of ewes giving birth, number of lambs (single, twins, triplets), and lamb birth weights. The experimental design and timeline of melatonin implantation are shown in Fig. 1.

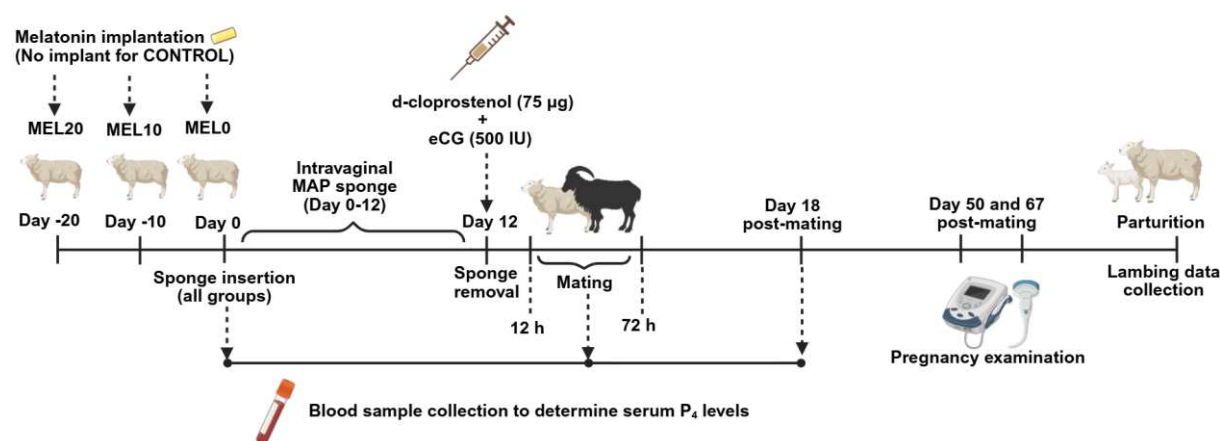


Fig. 1 Schematic view of the experimental design and timeline of melatonin implantation and estrus synchronization in ewes. Melatonin implants were administered 20 days (MEL20), 10 days (MEL10) before, and on the day of sponge insertion (MEL0). Control ewes did not receive melatonin implants. Intravaginal MAP sponges were inserted on Day 0 and removed on Day 12, followed by administration of d-cloprostenol (75 µg) and eCG (500 IU). Mating occurred 12–72 h after sponge removal. Blood samples were collected at sponge insertion (Day 0), at mating, and on Day 18 post-mating for serum progesterone (P₄) analysis. Pregnancy diagnosis was performed on Days 50 and 67 post-mating. Lambing and birth data were recorded at parturition

2.7. Evaluated parameters

The following parameters were analyzed:

Estrous cycle phase at sponge insertion was determined based on serum progesterone concentrations (<1 ng/mL = non-luteal phase; >1 ng/mL = luteal phase), as described by Pinto-Santini and Ungerfeld (2019).

Estrus was defined as acceptance of mounting by the ram. Ewes that allowed successful mating were considered to have completed mating.

Estrus rate, estrus onset timing, and mating completion rate were recorded.

Serum progesterone concentrations were measured at mating, and their relationship with pregnancy outcome was evaluated.

Serum progesterone concentrations at 18 days post-mating were used for early pregnancy detection; values >2 ng/mL were considered indicative of pregnancy.

Embryonic loss was defined as progesterone concentrations >2 ng/mL on day 18 post-mating followed by absence of pregnancy at the day-50 ultrasonographic examination.

Pregnancy rate and conception rate were calculated.

Pregnancy at the subsequent estrus among ewes that failed to conceive following the synchronized estrus was also evaluated.

Lambing rate, single and multiple birth rates, lamb yield, and average lamb birth weight were calculated.

Fertility indices were calculated using the following formulas:

Estrus rate (%) = (Number of ewes exhibiting estrus / Total number of ewes) $\times$ 100

Mating completion rate (%) = (Number of ewes completing mating / Total number of ewes) $\times$ 100

Pregnancy rate (%) = (Number of pregnant ewes / Total number of ewes) $\times$ 100

Conception rate (%) = (Number of pregnant ewes / Number of mated ewes) $\times$ 100

Lambing rate (%) = (Number of ewes that gave birth / Number of pregnant ewes) $\times$ 100

Single birth rate (%) = (Number of ewes giving birth to single lambs / Number of ewes that gave birth) × 100

Multiple birth rate (%) = (Number of ewes giving birth to multiple lambs / Number of ewes that gave birth) × 100

Lamb yield = Number of lambs born / Number of ewes that gave birth

Average lamb birth weight (kg) = Total weight of lambs / Total number of lambs

2.8. Statistical analysis

Categorical variables were presented as frequencies and percentages, and quantitative variables as mean ± standard deviation (SD). Normality of quantitative data was assessed using the Kolmogorov–Smirnov test, and homogeneity of variances was evaluated with Levene’s test. One-way ANOVA followed by Tukey’s post hoc test was applied to compare body condition score (BCS), day 18 progesterone levels, lamb yield, and lamb birth weight among groups. Two-way ANOVA was used to evaluate the effects of pregnancy, group, and pregnancy × group interactions on progesterone levels, as well as the effects of lamb yield, group, and lamb yield × group interactions. Associations between study groups and reproductive parameters—including estrous cycle phase, estrus onset, estrus rates, mating completion, pregnancy, conception, embryonic loss, and lambing rates—were analyzed using Pearson’s chi-square or Fisher’s exact tests. In addition, Fisher’s exact chi-square test was specifically applied to assess the relationships between estrous cycle phase and both the timing of estrus onset and pregnancy rates. Statistical significance was set at $P < 0.05$. All analyses were conducted using IBM SPSS Statistics software version 22.0.

3. Results

3.1. Baseline characteristics of the experimental groups

Baseline body condition scores did not differ among the groups (Table 1, $P > 0.05$). At the time of sponge insertion, 66.7% of ewes were in the non-luteal phase and 33.3% in the luteal phase (Table 2).

Table 1 Body condition scores (BCS) of the experimental groups at the beginning of the study

	Groups								<i>P</i>
	MEL20		MEL10		MEL0		CONTROL		
	<i>n</i>	Mean ± SD	<i>n</i>	Mean ± SD	<i>n</i>	Mean ± SD	<i>n</i>	Mean ± SD	
BCS	30	2.70 ± 0.15	30	2.73 ± 0.11	30	2.72 ± 0.13	30	2.72 ± 0.14	0.755

Table 2 Distribution of estrous cycle phases at the time of sponge insertion

Estrous cycle phase	Groups					Total (%)
	MEL20 (<i>n</i>)	MEL10 (<i>n</i>)	MEL0 (<i>n</i>)	CONTROL (<i>n</i>)		
Non-luteal phase	16	27	15	22		80 (66.7%)
Luteal phase	14	3	15	8		40 (33.3%)
Total	30	30	30	30		120 (100.0%)

3.2. Estrus response and mating dynamics

Estrus response following synchronization did not differ among the experimental groups (Table 3, $P > 0.05$). Estrus onset distribution differed significantly between non-luteal and luteal phases at sponge insertion ($P < 0.05$), with differences mainly observed at 48 and 60 h after sponge removal (Table 4, $P = 0.034$). However, estrous cycle phase did not affect overall mating completion or pregnancy rates ($P > 0.05$). Melatonin treatment did not significantly affect estrus onset timing or mating completion rates (Table 5, $P > 0.05$). Although numerical differences in estrus onset timing were observed among the treatment groups, these differences were not statistically significant.

Table 3 Estrus response of ewes following estrus synchronization

	Groups					<i>P</i>
	MEL20	MEL10	MEL0	CONTROL	Total	
Number of ewes (<i>n</i>)	30	30	30	30	120	0.166
Ewes in estrus (<i>n</i>)	30	28	30	27	115	
Estrus rate (%)	100	93	100	90	95.8	

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198 **Table 4** Estrus onset according to the estrous cycle phase at sponge insertion (a,b: Different superscripts indicate
 199 a significant difference in estrus onset distribution between estrous cycle phases ($P < 0.05$))

Estrous cycle		Estrus onset							<i>P</i>
		12 h	24 h	36 h	48 h	60 h	72 h	Non-mated Total	
Non-luteal	<i>n</i>	0	5	22	32 ^a	16 ^a	0	5	80
phase	%	0.0	6.3	27.5	40.0	20.0	0.0	6.3	100.0
Luteal phase	<i>n</i>	0	1	10	24 ^b	3 ^b	2	0	40
	%	0.0	2.5	25.0	60.0	7.5	5.0	0.0	100.0
Total	<i>n</i>	0	6	32	56	19	2	5	120
	%	0.0	5.0	26.7	46.7	15.8	1.7	4.2	100.0

200

201 **Table 5** Estrus onset according to treatment groups

Estrus Onset	Groups				<i>P</i>
	MEL20	MEL10	MEL0	CONTROL	
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	
12 h	-	-	-	-	0.094
24 h	3 (50.0)	1 (16.7)	2 (33.3)	-	
36 h	8 (25.0)	12 (37.5)	7 (21.9)	5 (15.6)	
48 h	11 (19.6)	13 (23.2)	13 (23.2)	19 (33.9)	
60 h	7 (36.8)	2 (10.5)	7 (36.8)	3 (15.8)	
72 h	1 (50.0)	-	1 (50.0)	-	
Non-mated	-	2 (40.0)	-	3 (60.0)	

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203 3.3. Progesterone concentrations and early embryonic outcomes

204 Progesterone concentrations at mating did not differ among treatment groups or according to pregnancy
 205 status (Table 6, $P > 0.05$). However, progesterone levels exceeding 1 ng/mL at mating were observed in three

ewes, all of which were in the MEL20 group. At 18 days post-mating, progesterone concentrations were significantly higher in pregnant ewes than in non-pregnant ewes (Table 7, $P < 0.05$), but no differences were observed among treatment groups (Table 7, $P > 0.05$). Embryonic loss rates were similar across groups (Table 8, $P > 0.05$). Progesterone concentrations at day 18 were higher in ewes that subsequently gave birth to multiple lambs than in those that subsequently gave birth to a single lamb (Table 9, $P < 0.05$), although this parameter was not affected by treatment (Table 9, $P > 0.05$)

Table 6 Serum progesterone concentrations at mating according to pregnancy status in the treatment groups

Pregnancy status	Groups								<i>P</i>		
	MEL20		MEL10		MEL0		CONTROL		Pregnancy	Group	Pregnancy x Group
	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD			
Non-pregnant	15	0.62 $\pm$ 0.36	7	0.34 $\pm$ 0.16	12	0.35 $\pm$ 0.09	11	0.41 $\pm$ 0.12	0.916	0.140	0.174
Pregnant	15	0.50 $\pm$ 0.28	23	0.47 $\pm$ 0.19	18	0.33 $\pm$ 0.11	19	0.39 $\pm$ 0.09			
Total	30	0.56 $\pm$ 0.32	30	0.44 $\pm$ 0.19	30	0.34 $\pm$ 0.10	30	0.40 $\pm$ 0.10			

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Table 7 Serum progesterone concentrations 18 days post-mating according to pregnancy status in the treatment

groups

Pregnancy status	Groups								<i>P</i>		
	MEL20		MEL10		MEL0		CONTROL		Pregnancy	Group	Pregnancy x Group
	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD	<i>n</i>	Mean $\pm$ SD			
Non-pregnant	15	1.12 $\pm$ 1.54	7	0.78 $\pm$ 1.19	12	1.87 $\pm$ 2.83	11	1.82 $\pm$ 2.97	0.001	0.568	0.435
Pregnant	15	6.94 $\pm$ 2.91	23	7.97 $\pm$ 2.81	18	6.75 $\pm$ 2.49	19	8.18 $\pm$ 2.46			
Total	30	4.03 $\pm$ 3.74	30	6.29 $\pm$ 3.98	30	4.80 $\pm$ 3.55	30	5.85 $\pm$ 4.06			

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221 **Table 8** Embryonic loss rates in the treatment groups

	Groups					<i>P</i>
	MEL20	MEL10	MEL0	CONTROL	Total	
Pregnant ewes on day 18 (<i>n</i>)	17	24	20	22	83	0.784
Pregnant ewes on day 50 (<i>n</i>)	15	23	18	19	75	
Embryonic loss rate (%)	11.76%	4.16%	10.0%	13.63%	9.63%	

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224 **Table 9** Serum progesterone concentrations 18 days post-mating according to litter size in the treatment groups

Number of lambs	Groups									<i>P</i>		
	MEL20		MEL10		MEL0		CONTROL		Overall			
	Mean ±		Mean ±		Mean ±		Mean ±		Mean ±	Pregnanc y	Grou p	Pregnanc y x Group
	<i>n</i>	SD	<i>n</i>	SD	<i>n</i>	SD	<i>n</i>	SD	SD			
Single lamb	13	6.68±3.04	14	6.37±1.49	7	5.31±0.65	8	7.86±1.99	6.57±2.18	0.030	0.645	0.270
Multiple lambs	2	8.65±0.78	9	10.46±2.58	4	9.09±2.76	7	9.06±3.09	9.60±2.63			

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226 3.4. Pregnancy outcomes

227 Pregnancy and conception rates at the first mating did not differ among treatment groups. Pregnancy
 228 rates at the subsequent estrus and overall pregnancy rates, including both the first mating and subsequent estrus,
 229 were likewise comparable across groups (Table 10, $P > 0.05$).

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233 **Table 10** Pregnancy outcomes following the first mating and subsequent estrus in the treatment gorups

		Groups				
		MEL20	MEL10	MEL0	CONTROL	Total
First mating						<i>P</i>
Mated ewes (<i>n</i>)	30	28	30	27	115	
Pregnant ewes (<i>n</i>)	15	23	18	19	75	
Non-pregnant ewes (<i>n</i>)	15	7	12	11	45	
Pregnancy rate (%)	50.0	76.6	60.0	63.3	62.5	
Conception rate (%)	50.0	82.1	60.0	70.3	65.2	0.064
Subsequent estrus						
Non-pregnant ewes at first mating (<i>n</i>)	15	7	12	11	45	
Pregnant ewes (<i>n</i>)	5	3	2	1	11	<i>P</i>
Non-pregnant ewes (<i>n</i>)	10	4	10	10	34	
Pregnancy rate (%)	33.3	42.8	16.6	9.0	24.4	0.293
Total pregnancy outcomes						
Pregnant ewes (<i>n</i>)	20	26	20	20	86	<i>P</i>
Non-pregnant ewes (<i>n</i>)	10	4	10	10	34	
Pregnancy rate (%)	66.6	86.6	66.6	66.6	71.6	0.218

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235 3.5. Lambing performance

236 Lambing rate, multiple birth rate, lamb yield, and lamb birth weight did not differ among treatment
 237 groups (Table 11, $P > 0.05$).

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243 **Table 11** Lambing outcomes in the treatment groups

	Groups					
	MEL20	MEL10	MEL0	CONTROL	Total	
Ewes (<i>n</i>)	30	30	30	30	120	
Pregnant ewes (<i>n</i>)	15	23	18	19	75	
Lambing ewes (<i>n</i>)	15	23	18	19	75	<i>P</i>
Single lambs (<i>n</i>)	13	14	14	12	53	
Twin lambs (<i>n</i>)	2	8	4	7	21	
Triplet lambs (<i>n</i>)	0	1	0	0	1	
Lambs born (<i>n</i>)	17	33	22	26	98	
Lambing rate (%)	100.0	100.0	100.0	100.0	100.0	
Multiple birth rate (%)	13.3	39.1	22.2	36.8	29.3	0.275
Lamb yield	1.13	1.43	1.22	1.37	1.30	0.324
Lamb birth weight (kg. mean ± SD)	4.47 ± 0.77	4.33 ± 0.94	3.68 ± 0.82	3.93 ± 0.90	4.16 ± 0.90	0.086

244

245 4. Discussion

246 Previous studies indicate that melatonin administration prior to estrus synchronization does not
 247 consistently modify estrus expression during either the anestrous or breeding season (Buffoni et al. 2014;
 248 Mansoor et al. 2020; El-Mokadem et al. 2020; Tölü et al. 2022; Akbulut et al. 2025). In agreement with these
 249 reports, pre-synchronization melatonin implantation for varying durations did not alter overall estrus rates in the
 250 present study. These findings indicate that under breeding-season conditions, short-term exogenous melatonin
 251 exposure does not substantially modify estrus responsiveness.

252 The estrous cycle phase at sponge insertion has been proposed to influence follicular dynamics and
 253 ovulatory responses; however, its impact on synchronization success remains controversial (Dott et al. 1979;
 254 Driancourt 1987; Freitas et al. 1996; Oliveira et al. 2016a, b). Although differences in estrus expression were
 255 observed at specific time points, cycle phase at initiation did not affect overall mating completion or pregnancy
 256 rates, indicating that ovarian status at the onset of progestagen-based synchronization did not significantly

influence final fertility outcomes under the breeding-season conditions of the present study. Similarly, melatonin modulates follicular development through its effects on steroidogenesis and granulosa cell proliferation, although its influence on follicular wave dynamics and estrus timing remains inconsistent (Adriaens et al. 2006; Picinato et al. 2008; Berlinguer et al. 2009; Maganhin et al. 2014; Manca et al. 2014; Lima et al. 2015; El-Mokadem et al. 2020; Mansoor et al. 2020). In the present study, melatonin implantation for varying durations prior to synchronization did not significantly alter estrus onset time ($P = 0.094$). Although treated groups showed a higher cumulative proportion of matings at earlier observation times, this numerical difference was not associated with differences in fertility outcomes.

Melatonin has also been reported to exert luteotrophic effects and to modulate luteolysis through its influence on progesterone secretion (Wallace et al. 1988; Lassoued et al. 1997; Noel et al. 1999; Abecia et al. 2006), although findings remain inconsistent (Vázquez et al. 2009). In the present study, mean serum progesterone concentrations at mating were numerically higher in the group with longer melatonin exposure (MEL20). Progesterone concentrations exceeded 1 ng/mL at estrus in only three ewes, all of which were from the MEL20 group; however, the overall progesterone concentrations did not differ significantly among groups and were not associated with improved pregnancy rates.

Serum progesterone concentrations measured 18 days post-mating exceeded 2 ng/mL in all ewes subsequently confirmed as pregnant, supporting the use of this threshold for early pregnancy diagnosis. Higher concentrations in ewes carrying multiple fetuses were consistent with previous reports (Boscós et al. 2003; Gür et al. 2011). Progesterone levels at day 18 did not differ among groups, and melatonin treatment did not affect pregnancy maintenance. Similarly, embryonic loss—defined as elevated progesterone at day 18 followed by absence of pregnancy at day 50 ultrasonography—did not differ among treatments ($P = 0.78$). However, the use of progesterone concentrations at day 18 to assess embryonic loss should be interpreted with caution because individual variation in estrous cycle length may affect the luteal phase at this time point. Although melatonin has been reported to enhance reproductive processes through its antioxidant properties and its regulatory effects on follicular development, steroidogenesis, luteal function, and early embryogenesis (Forcada et al. 2006; Cruz et al. 2014; Maganhin et al. 2014; Abecia et al. 2019; El-Mokadem et al. 2020; Duan et al. 2025), no clear effect was observed under the present breeding-season conditions. This finding is consistent with previous reports indicating limited or inconsistent effects during this period (Vázquez et al. 2009). Overall, the present findings indicate that melatonin supplementation during the breeding season did not significantly influence pregnancy maintenance or embryonic loss.

Exogenous melatonin has been reported to increase ovulation rate in ewes, either directly or by reducing follicular atresia and thereby increasing the number of ovulatory follicles (Robinson et al. 1991; Forcada et al. 1995; Noel et al. 1999; Vlčková et al. 2022). Although higher twinning rates have been attributed to this effect, improved embryo survival mediated through enhanced luteal support may also contribute (Abecia et al. 2019). Despite these reported benefits, several studies and a recent meta-analysis indicate that the *in vivo* effects of melatonin on ovulatory response are inconsistent and limited (Vázquez et al. 2009; Buffoni et al. 2014; Chen et al. 2022). In this context, Buffoni et al. (2014) suggested that although melatonin can decrease follicular atresia and increase the pool of growing follicles, this does not necessarily result in a corresponding increase in ovulation rate. In the present study, multiple birth rates did not differ among groups, although the lowest rate was observed in the longest-exposure group (MEL20). This observation is noteworthy in the context of the variable effects of melatonin on follicular and ovulatory dynamics reported in previous studies.

Lambing rate reached 100% across all groups, and lamb yield did not differ significantly. The lower lamb yield observed in the group with prolonged melatonin exposure (MEL20) was consistent with the lower multiple birth rate observed in this group. Previous studies have reported similarly variable results regarding lamb output during the breeding season (Abecia et al. 2007; Vlčková et al. 2022; Barbanoj and Abecia 2022; Tölü et al. 2022). Collectively, these findings indicate that melatonin supplementation did not significantly improve lambing performance under the conditions of the present study.

Melatonin has been reported to enhance fetal development by improving uteroplacental hemodynamics and fetal oxygenation, potentially increasing neonatal birth weight (Lemley et al. 2012; Brockus et al. 2016; Sales et al. 2019; Abdelnaby 2020; Flinn et al. 2020). In small ruminants, however, birth weight appears primarily determined by maternal body condition, litter size, and fetal sex (Kassim et al. 2019; Tölü et al. 2022). In the present study, birth weight did not differ significantly among groups, although the highest mean value was observed in the longest-exposure group (MEL20). This finding, together with the reported influence of maternal body condition, litter size, and fetal sex on birth weight, suggests that fetal growth may be influenced by multiple interacting factors under the conditions of the present study.

Beyond hormonal interactions, breed characteristics, physiological status, and sample size may have contributed to the observed outcomes. Awassi ewes have relatively modest reproductive performance (Talafla and Ababneh 2011; Allabban and Erdem 2020), and the below-recommended body condition scores at study onset (Vatankhah et al. 2012; Sarracini et al. 2022), combined with the absence of flushing and limited sample

size, may have limited the ability to detect treatment-related differences. These factors highlight the importance of metabolic, management, and experimental conditions when evaluating the reproductive response to melatonin.

5. Conclusion

Under breeding-season conditions, exogenous melatonin administration had limited effects on reproductive outcomes in Awassi ewes. Melatonin treatment did not significantly alter estrus response, estrus onset, progesterone concentrations at mating, pregnancy maintenance, embryonic loss, lambing performance, or birth weight. The phase of the estrous cycle at the initiation of synchronization influenced estrus expression at specific observation times but did not affect subsequent mating completion or pregnancy rate. Overall, neither melatonin exposure duration nor estrous cycle phase at synchronization initiation resulted in improved reproductive performance under the conditions of the present study. These findings indicate that reproductive responses to melatonin and synchronization may be context-dependent and influenced by physiological, nutritional, and management conditions. Further studies using optimized treatment protocols and more comprehensive assessments of endocrine and environmental factors are warranted.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Onur Bahan, Gökhan Doğruer and Ahmet Gözer. The first draft of the manuscript was written by Onur Bahan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

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

Declarations

Statement of animal rights All procedures performed on ewes had been approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee with the approval number 2019/10-12.

Conflict of interest All authors declare that they have no conflict of interest.

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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