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Endogenous Circannual Cycles of Ovarian Activity and Changes in Prolactin and Melatonin Secretion in Wild and Domestic Female Sheep Maintained under a Long-Day Photoperiod[1]

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ABSTRACT

The present study examines the ovulatory activity of wild and domesticated ewes subjected to either a constant photoperiod of long days (16L:8D) or natural changes in daily photoperiod for 16 mo. The aim was to determine whether an endogenous reproductive rhythm controls seasonal reproductive activity in these sheep, and how the photoperiod might affect this. The effects of long-day photoperiods on long-term changes in prolactin and melatonin secretion were also evaluated. The two species showed changes in reproductive activity under the constant photoperiod conditions, suggesting the existence of an endogenous rhythm of reproduction. This rhythm was differently expressed in the two types of ewe ($P < 0.05$), with the domestic animals exhibiting much greater sensitivity to the effects of long days. A circannual rhythm of plasma prolactin concentration was also seen in both species and under both photoperiod conditions, although in both species the amplitude was always lower in the long-day animals ($P < 0.01$). The duration of the nocturnal melatonin plasma concentrations reflected the duration of darkness in both species and treatments. The peak melatonin concentration did not differ between seasons either under natural or long-day photoperiods.

melatonin, ovarian cycles, ovulatory cycle, photoperiod, prolactin, seasonal reproduction, wild and domestic ewes

INTRODUCTION

The restriction of breeding activity to a certain time of year is common among wild mammalian species [1] and is mainly regulated by changes in the photoperiod. This natural adaptive mechanism ensures the birth of offspring at a time of year that is optimal for their survival. The adult females of many wild ruminant species have a short annual period of ovulatory activity followed by a long anestrous period [2–4]. In contrast, for domesticated species this environmental pressure has become much reduced (to the extent that it may no longer exist—a result of artificial selection), and the duration of the ovarian activity period has expanded [5]. Certainly, domestic sheep show great variability in terms of the onset and duration of anestrus and differ from their wild cousins in many aspects of their reproductive physiology [6]. In the mid- and high latitudes of temperate regions, the ewes of most domestic sheep breeds enter the breeding season in the autumn, when the duration of daylight is becoming shorter. If they do not become pregnant they have regular ovulatory cycles until mid-winter, when day length is again increasing. Ovulation then ceases, and the animal remains anovulatory during the long days of spring-summer [7]. There is experimental evidence that at these latitudes the seasonal reproductive cycle of the ewe is the product of an endogenous circannual rhythm [8, 9] that is not directly driven by the photoperiod; rather, it is synchronized by it [10, 11] through its control of the circadian rhythm of melatonin secretion [12–14]. It has been shown that by acting at the premammillary hypothalamus and thus regulating LHRH pulsatile activity [15], the duration of elevated melatonin levels over the long days of spring-summer provides the signal for day length to synchronize the circannual rhythm of reproductive neuroendocrine activity [11, 16]. The shortening days between the summer solstice and the autumn equinox are the critical signal involved in timing the end of reproductive activity in mid-winter [12, 17], which contributes to ensuring the proper duration of the breeding season [18, 19].

In Mediterranean regions, great variability exists between and within sheep breeds in terms of the timing and duration of their seasonal reproductive cycles [20–22]. However, studies on the physiological mechanisms underlying the photoperiod control of seasonal breeding activity at these latitudes have been limited [23, 24], and the extent to which these breeds of sheep use the photoperiod to regulate their annual reproductive cycle remains unclear.

The European mouflon (*Ovis orientalis musimon*) is a wild sheep native to the islands of Corsica and Sardinia. It is closely related to the domestic Spanish Manchega (*Ovis aries*) breed of sheep [25], which is thought to be the ancestor of our current domestic breeds [26]. Recently, substantial differences have been found between the mouflon and the Manchega in terms of the temporal organization of reproduction, despite both being of Mediterranean origin: mouflons begin their ovarian cycles 4 mo later than Manchega ewes and finish 2 mo later [27].

Since genetic variation affecting photoresponsiveness has been clearly demonstrated among populations of photoperiod-sensitive rodents [28], certain domestic breeds of sheep [29], and wild ruminants [30], it has been suggested that the differences between mouflons and Manchega sheep in the timing and duration of breeding might be due to genetic

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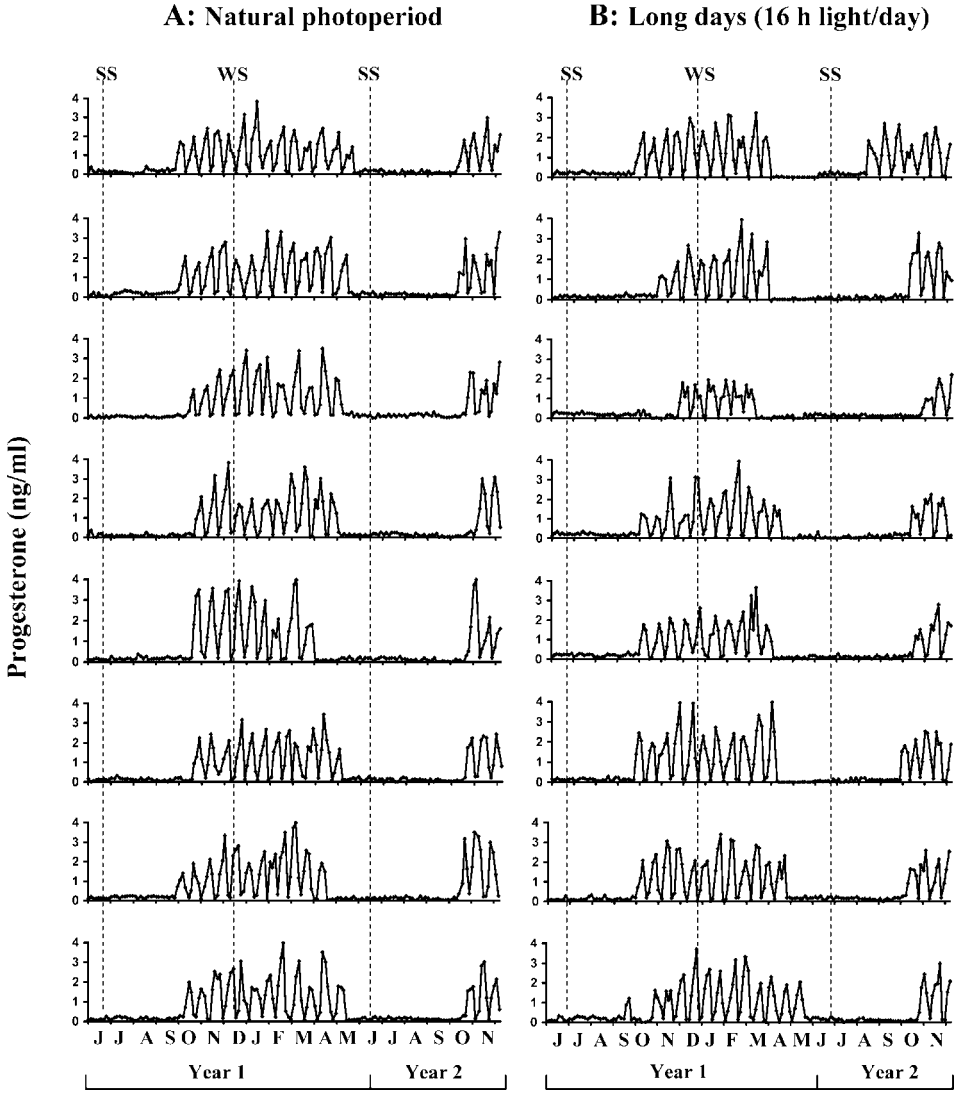

FIG. 1. Ovulatory activity of each of the eight mouflon ewes, all born and raised at latitude 40°25′N and maintained under natural (**A**) or long days (16L:8D; **B**) for 16 mo between 22 June 2002 (indicated with a "J," Year 1) and 31 October 2003 (indicated with an "O," Year 2). Ovulatory activity was assessed by measuring the plasma progesterone concentration in blood samples obtained twice weekly. WS, Winter solstice; SS, summer solstice.

differences governing the nature of their response to the same photoperiod cues [27].

The present study examines the ovulatory activities of adult mouflon and Manchega ewes subjected to either a constant photoperiod of long days (16L:8D) or natural changes in day length at a Mediterranean latitude (40°25′N) for 16 mo; the aim was to determine how much of the difference between their reproductive seasons is due to differences in their responses to the same photoperiod cues. The present study tries, therefore, to determine whether an endogenous reproductive rhythm controls seasonal reproductive activity in these sheep and how the photoperiod might affect this. The effects of the long-day photoperiod on annual changes in plasma prolactin and melatonin concentrations were also evaluated.

MATERIALS AND METHODS

All experiments were performed in accordance with the Spanish Animal Protection Policy RD1201/2005, which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

Animals

The experimental animals were 16 mouflons and 16 Manchega ewes aged 4–8 yr. The mouflon stock originally came from the "El Hosquillo" National Wildlife Reserve in Cuenca, Spain (latitude 40°6′N); the ewes used in the present study were part of a homogenous group of adult females born and housed in captivity at the Animal Reproduction Department of the Instituto Nacional de Investigaciones Agrarias (INIA), Madrid, Spain (latitude 40°25′N). These animals were isolated from males and kept in an open, sand-floor stable (250 m^2) with partial roof cover under natural conditions. The Manchega ewes were part of the experimental flock kept by the same Animal Reproduction Department; these were maintained under similar conditions in an adjacent enclosure. Mean live weight at the beginning of the experiment was 28.3 (±2.2) and 69.0 (±3.1) kg for the mouflon and Manchega ewes, respectively. All animals were fed every morning with Visan K59 (which provides a balanced diet; Visan Ind. Zoot. Madrid, Spain) supplemented with barley grain, barley straw, and dry alfalfa. All animals had free access to water and mineral blocks.

Photoperiod Treatments

Two groups were prepared, each with eight mouflon and eight Manchega ewes. The members of the first group, the natural photoperiod group (NP), were maintained in an open, sand-floor stable (250 m^2) under ambient temperature conditions and under the variations in day length that naturally occur at 40°25′N (from 10 h 17 min to 16 h 3 min of light per day [winter to summer solstices, respectively], including twilight). The other sheep, those of the long-day group (LP), were placed in a separate, open, sand-floor stable and exposed to long days of 16L:8D (equivalent to the photoperiod of the longest day, i.e., the summer solstice) for 16 mo between 22 June 2002 (Year 1) and 31 October 2003 (Year 2). To achieve this, artificial light was provided from 0500 to 0800 h and from 1600 to 2000 h. This photoperiod was regulated using an electric clock that operated fluorescent tubes (positioned laterally to the animals' eyes) providing a light intensity of approximately 350 lux.

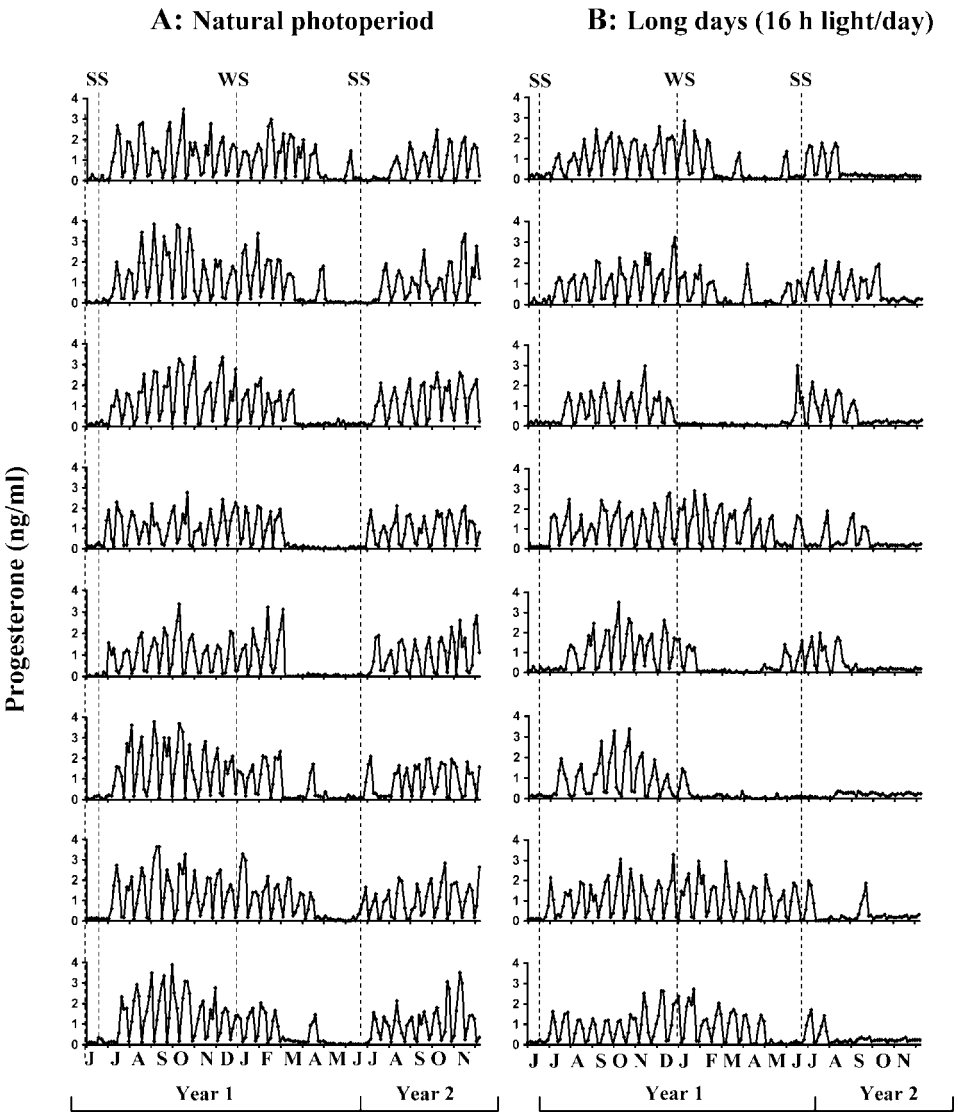

FIG. 2. Ovulatory activity of each of the eight Manchega ewes, all born and raised at latitude 40°25′N and maintained under natural (**A**) or long days (16L:8D; **B**) for 16 mo between 22 June 2002 (Year 1) and 31 October 2003 (Year 2). Ovulatory activity was assessed by measuring the plasma progesterone concentration in blood samples obtained twice weekly. WS, Winter solstice; SS, summer solstice.

Blood Samples

Blood samples (5 ml) were collected from all animals twice weekly at the same time of day (between 1000 and 1100 h) from 1 June 2002 (Year 1) to 30 November 2003 (Year 2), and the plasma progesterone concentration was determined. The plasma prolactin concentration was monitored in one of the samples recovered each week.

During the first year, the plasma melatonin concentration was determined in samples collected on the day of each season change, i.e., at the autumn equinox (22–23 September), the winter solstice (22–23 December), the spring equinox (21–22 March), and the summer solstice (21–22 June). Blood was taken from all animals every 3–4 h overnight, as well as 1 h before and after sunset and sunrise in the NP animals, and 1 h before and after the lights were turned off in the LP animals. The precise timing of dawn and dusk under natural conditions at 40°25′N was kindly provided by the National Observatory of Astronomy, Madrid. At night, blood samples were collected alternatively from the right and left jugular veins. A dim, red light torch (<3 lux) provided light for this operation; care was taken to avoid any direct illumination of the animals' eyes.

All animals in each experimental group were physically restrained and confined in small enclosures (6 m^2) to facilitate blood collection. All blood samples were obtained by venipuncture of jugular veins into heparinized tubes (Vacutainer; Systems Europe, Beckton Dickinson). Plasma was immediately separated by centrifugation at 1500 × *g* for 15 min and stored at −20°C until use.

Hormone Assays

Progesterone. The plasma progesterone concentration was determined in duplicate in 200-μl aliquots of plasma (following extraction with 3 ml fresh hexane) according to the radioimmunoassay method of López-Sebastián et al. [31]. The assay sensitivity was 0.12 ng/ml, and the intra- and interassay coefficients of variation were 7.4% and 10.6%, respectively. The mean extraction efficiency was 94.6%.

Prolactin. Prolactin concentrations were measured in duplicate in 50-μl plasma aliquots using the double-antibody radioimmunoassay [32], employing anti-ovine prolactin serum (anti-o-PRL-DJB-073), NIAMDD-o-PRL-AFP-2060-C for radio-iodination, and NIAMDD-o-PRL-14 as a reference standard. All samples were analyzed in a single radioimmunoassay. The assay sensitivity was 0.2 ng/ml and the intraassay coefficient of variation was 8.6%.

Melatonin. Melatonin concentrations were determined in duplicate in 100-μl aliquots of plasma using the radioimmunoassay of Fraser et al. [33] and employing the antibody raised by Tillet et al. [34]. All samples were analyzed in a single radioimmunoassay. The sensitivity of the assay was 4 pg/ml and the intraassay coefficient of variation was 6.8%.

Ovulatory Activity

The occurrence or nonoccurrence of ovulatory activity was determined by measuring the plasma progesterone concentration in the blood samples collected twice weekly from 1 June 2002 (Year 1) to 30 November 2003 (Year 2). Ovulation was confirmed when two to three consecutive samples showed progesterone concentrations of >0.5 ng/ml. Ovulation was deemed not to have occurred when four or more consecutive results of <0.5 ng/ml were returned.

The beginning and duration of cyclical ovulatory activity was determined in each animal by the appearance of regular cycles of progesterone secretion. The first sample date before the progesterone concentration rose above 0.5 ng/ml for two consecutive plasma samples—in at least three consecutive progesterone cycles—was taken as the onset of cyclical ovulatory activity. The end of

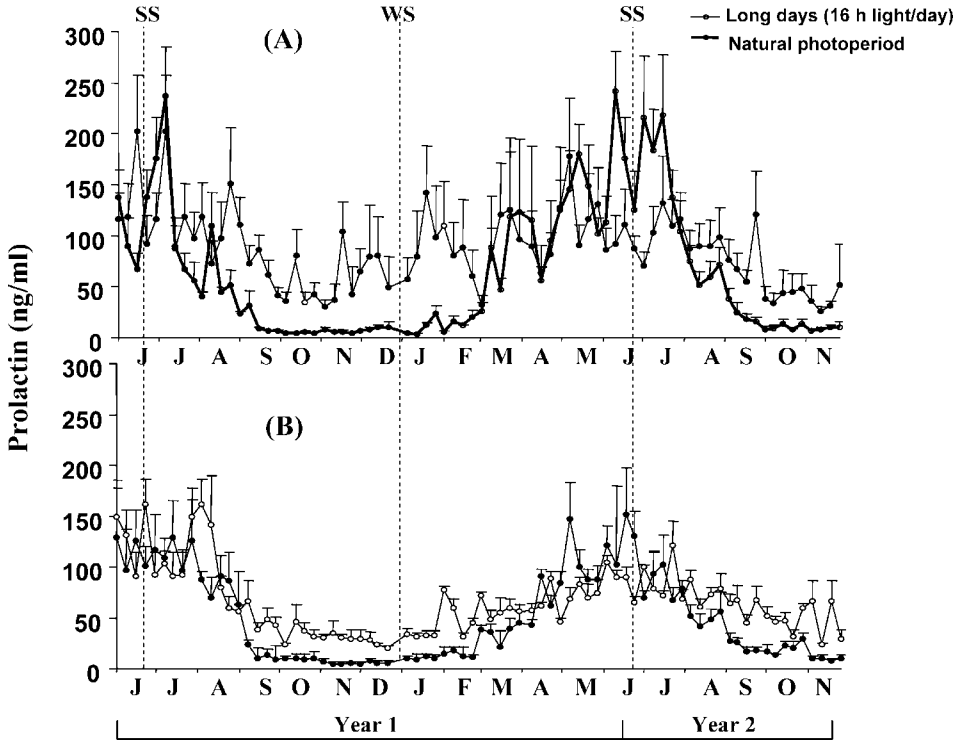

FIG. 3. Changes (mean ± SEM) in plasma prolactin concentration (ng/ml) in the mouflon (**A**) and Manchega (**B**) ewes subjected to natural (black circles) changes in day length (n = 8) or continuously exposed to long days (white or open circles; 16L:8D; n = 8) for 16 mo from 22 June 2002 (Year 1) to 31 October 2003 (Year 2). The plasma prolactin concentrations were determined in blood samples collected once per week.

ovarian cyclicity was taken as the first sample date on which the plasma progesterone concentration fell below 0.5 ng/ml for at least four or more consecutive plasma samples. The dates of transition from one reproductive state to the next were calculated on the basis of these definitions. The length of the luteal phase was determined as the number of days the progesterone concentration exceeded 0.5 ng/ml in consecutive samples.

Statistical Analyses

All statistical procedures were performed using Statistica 6.0 Software for Windows (StatSoft, Inc., Tulsa, OK). Data for the patterns of ovarian activity were subjected to ANOVA; season, photoperiod treatment, species, and the interactions of these variables were included in these analyses. Within species, group differences for the times of onset and offset of the cyclic ovulatory activity, the duration of cyclical activity, the duration of the anestrus, the mean number of ovarian cycles, and the length of the luteal phase were all examined using the Mann-Whitney *U*-test. Differences in the variability of the ovulatory activity onset and offset dates of animals within groups (NP and LP animals) were examined using the *F*-test.

Data on prolactin and melatonin profiles were analyzed by repeated-measures ANOVA; season, photoperiod treatment, species, and the interactions of these variables were included in analyses. Within species, changes in the concentrations of prolactin and melatonin over time were analyzed by repeated-measures ANOVA, with season as a within-factor variable and photoperiod treatment as a between-factor variable for each year (Years 1 and 2). When the photoperiod effect and/or the interaction season × photoperiod was significant, comparisons of the data for NP and LP animals at each sampling point were performed using one-way ANOVA. Similarly, when ANOVA revealed a seasonal effect, mean differences between seasons (spring, summer, autumn, and winter) were compared by one-way ANOVA. The plasma prolactin and melatonin concentrations showed a skewed distribution and were therefore log-transformed before analysis to correct for the heterogeneity of variance.

Where appropriate, the results of both ovarian activity and plasma prolactin and melatonin concentrations are presented as means ± SEM. Significance was set at $P < 0.05$.

RESULTS

Ovulatory Activity

The LP treatment affected the pattern of ovulatory activity in both the mouflon ($P < 0.05$ compared with NP animals) and Manchega ewes ($P < 0.01$ compared with NP animals); the interaction species × photoperiod was significant ($P < 0.05$).

A marked annual rhythm of ovulatory activity appeared in the NP mouflon ewes (Fig. 1A) and in their LP counterparts (Fig. 1B). The long days did not modify the timing of the onset of cyclical ovulatory activity in Year 1 (7 October ± 4 days vs. 9 October ± 7 days for NP and LP animals, respectively). However, in Year 2, a trend ($P = 0.08$) towards an early onset in cyclical ovulatory activity was seen in the LP animals (28 September ± 8 days) compared to the NP mouflon ewes (13 October ± 6 days). In both periods, the timing of the onset of ovulatory activity was more variable among the LP than the NP mouflons (Year 1: $P = 0.08$, $F = 4.06$; Year 2: $P < 0.05$, $F = 8.5$). The range for the NP animals was: Year 1, 24 September though 16 October; Year 2, 4–28 October, whereas for the LP

TABLE 1. Seasonal changes in prolactin concentration (ng/ml) in NP (natural photoperiod) and LP (16L:8D) mouflon ewes.[a]

	Year 1				Year 2	
Treatment group	Summer (July–September)	Autumn (October–December)	Winter (January–March)	Spring (April–June)	Summer (July–September)	Autumn (October–November)
LP mouflon ewes (n = 8)	121.0 ± 10.5	56.6 ± 6.5[b]	84.1 ± 7.9[b]	102.5 ± 10.1	98.0 ± 4.5	51.6 ± 7.0[b]
NP mouflon ewes (n = 8)	95.0 ± 16.0	7.8 ± 2.0[c]	11.4 ± 1.9[c]	105.4 ± 12.1	126.5 ± 18.0	14.2 ± 2.6[c]

[a] Values are the mean plasma PRL concentrations from samples collected once weekly in each season throughout the study and are expressed as means ± SEM.
[b,c] Different superscripts within each column indicate significant differences ($P < 0.001$).

TABLE 2. Seasonal changes in prolactin concentration (ng/ml) in NP (natural photoperiod) and LP (16L:8D) Manchega ewes.[a]

	Year 1				Year 2	
Treatment group	Summer (July–September)	Autumn (October–December)	Winter (January–March)	Spring (April–June)	Summer (July–September)	Autumn (October–November)
LP Manchega ewes (n = 8)	111.4 ± 9.8	37.6 ± 3.1[b]	37.9 ± 5.1[b]	64.6 ± 3.6	83.8 ± 4.8	51.2 ± 4.3[b]
NP Manchega ewes (n = 8)	101.9 ± 5.8	9.4 ± 1.4[c]	10.5 ± 1.1[c]	67.8 ± 9.8	85.8 ± 9.5	17.5 ± 2.0[c]

[a] Values are the mean plasma PRL concentrations from samples collected once weekly in each season throughout the study and are expressed as means ± SEM.
[b,c] Different superscripts within each column indicate significant differences ($P < 0.001$).

animals it was Year 1, 21 September through 20 November; Year 2, 6 August through 18 October (Fig. 1). The LP mouflon ewes became anestrous 1 mo earlier than their NP counterparts (7 April ± 4 days compared to 1 May ± 6 days; $P < 0.05$), but no significant difference was seen ($P = 0.4$, $F = 1.71$) in the variability of the onset of anestrus between the NP mouflon ewes (range: 1 April through May 20) and their LP counterparts (range: 8 March through 17 May). The length of cyclical ovulatory activity tended to be shorter in the LP mouflon ewes than in the NP animals (180 ± 12 days and 206 ± 8 days, respectively; $P = 0.09$). The mean interval between the cessation of cyclical ovulatory activity in Year 1 and the subsequent onset in Year 2 (anestrus) was similar in the NP and LP mouflon ewes (174 ± 10 days and 165 ± 7 days, respectively). During the experimental period (1 June 2002, Year 1, to 30 November 2003, Year 2), neither the number of ovarian cycles (15.0 ± 0.9 and 13.8 ± 0.7 cycles) nor the length of the luteal phase (9.9 ± 0.2 and 10.2 ± 0.2 days) differed between the NP and LP mouflons, respectively ($P > 0.05$).

The ovulatory activity of the LP and NP Manchega ewes showed marked differences over the course of the experiment (Fig. 2). Under the NP conditions (Fig. 2A), these ewes showed well-defined seasonality in their ovulatory activity. During the first year, all females showed ovarian cycles from July 3 (±2 days) to March 17 (±7 days); thereafter, they experienced an anestrous period of 111 ± 9 days during the spring months (April to June), and started cyclicity again by 7 July (±5 days) in Year 2. All ewes were cycling at the end of the study in November of Year 2. In contrast, the ovulatory activity of the LP Manchega ewes was very variable (Fig. 2B). In these animals, the onset of ovulatory activity in the first year of study was no different to that seen for their NP counterparts, (2 and 3 July, respectively). However, the end of ovarian cyclical

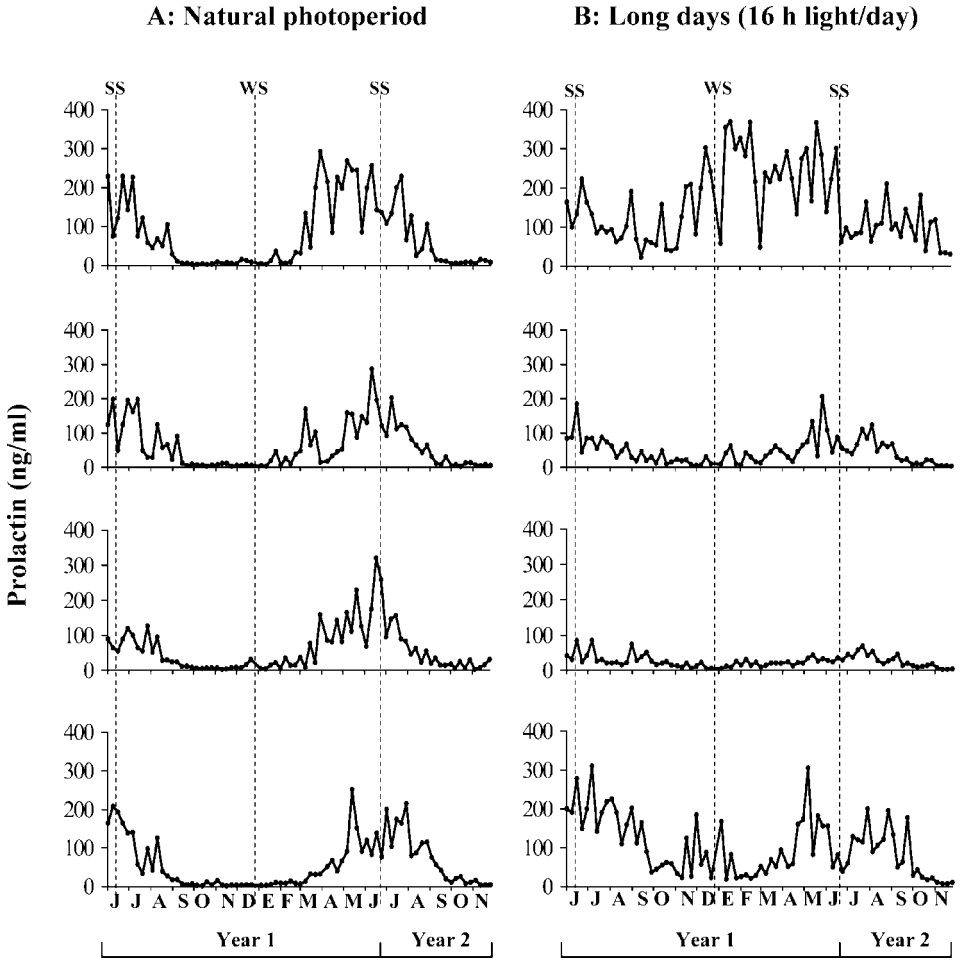

FIG. 4. Changes in plasma prolactin concentration (ng/ml) in four representative mouflon ewes maintained under a natural photoperiod (**A**) or continuously exposed to long days (16L:8D; **B**) for 16 mo from 22 June 2002 (Year 1) to 31 October 2003 (Year 2). The plasma prolactin concentrations were determined in blood samples collected once per week.

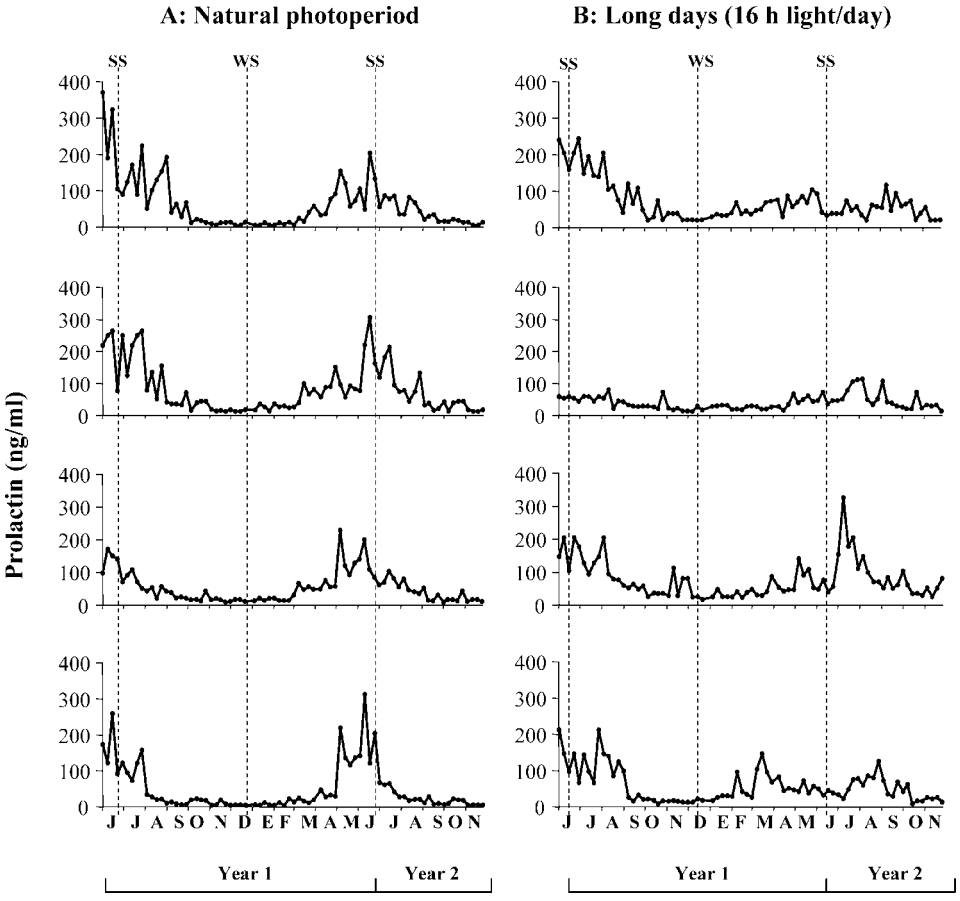

FIG. 5. Changes in plasma prolactin concentrations (ng/ml) in four representative Manchega ewes maintained under a natural photoperiod (**A**) or continuously exposed to long days (16L:8D; **B**) for 16 mo from 22 June 2002 (Year 1) to 31 October 2003 (Year 2). The plasma prolactin concentrations were determined in blood samples collected once per week.

activity, and the subsequent behavior of these animals in the following cycle, showed important variation over the course of the study. Five ewes ended their cyclical ovulatory activity between 21 December and 18 February in Year 1 (24 January ± 11 days). Then, after an anestrous period of 196 ± 13 days (range 91–164), four of these animals started ovarian cycles by 23 May before stopping again by 31 August (Year 2) and then remaining anestrous until the end of the experiment in November. The fifth ewe remained anestrous from January (Year 1) until the end of the experiment. The remaining three ewes showed continuous cyclical ovulatory activity during the first year of the study, but stopped cyclicity by July at the onset of the second Year. Thereafter, one of the ewes experienced progesterone cycles at irregular intervals until September before becoming definitively anestrous, whereas the other two remained anestrous for 153 days from July until the end of experiment.

The mean number of cycles during the 18 mo (1 June 2002, Year 1, to 30 November 2003, Year 2) of the study was 23.9 ± 0.4 (range 23–26 cycles) for the NP ewes and 17.4 ± 1.6 (range 9–22 cycles) for the LP ewes ($P < 0.05$). The length of the luteal phase was similar for the NP and LP ewes (9.8 ± 0.1 and 9.9 ± 0.2 days, respectively).

Prolactin Secretion

For both species, ANOVA revealed a significant ($P < 0.001$) effect of season on the plasma prolactin concentration under both photoperiod conditions. The interaction season × photoperiod was significant ($P < 0.001$). The interaction species × LP photoperiod also had a significant effect ($P < 0.01$).

Marked seasonal changes in prolactin concentration were seen in both the NP and LP mouflons (Fig. 3A). During the two years of the study, the highest prolactin concentrations were observed around the summer solstice and the lowest around the autumn equinox and winter solstice in both groups. However, the amplitude of these changes was smaller ($P < 0.001$) in the LP than in the NP mouflons. The plasma prolactin concentrations recorded in the spring and summer months were similar in both photoperiod groups, but became significantly greater ($P < 0.001$) in the LP mouflons (compared to the NP mouflons) during autumn and winter (Table 1).

A seasonal pattern of prolactin secretion was also seen in both the NP and LP Manchega ewes (Fig. 3B). However, the LP treatment caused a reduction in the amplitude of the seasonal changes in prolactin secretion ($P < 0.001$); the prolactin concentration fell during the autumn and winter months, but the values remained higher ($P < 0.01$) than in the NP ewes (Table 2). During the autumn and winter months, the differences in prolactin concentration between the LP and NP animals were greater in the mouflons than in the Manchega ewes ($P < 0.01$). A great individual variability in the pattern of prolactin secretion existed in both the LP mouflons and Manchega ewes (compared to the NP animals), in which prolactin profiles were similar for all animals (Figs. 4 and 5).

Melatonin Profiles

At each sampling period, a marked day-night rhythm in melatonin concentration was seen, with very low or undetectable concentrations during the day and sustained high levels throughout the night. This was true of both the LP and NP mouflon (Fig. 6) and Manchega (Fig. 7) ewes. In all these animals the duration of nocturnal melatonin secretion closely

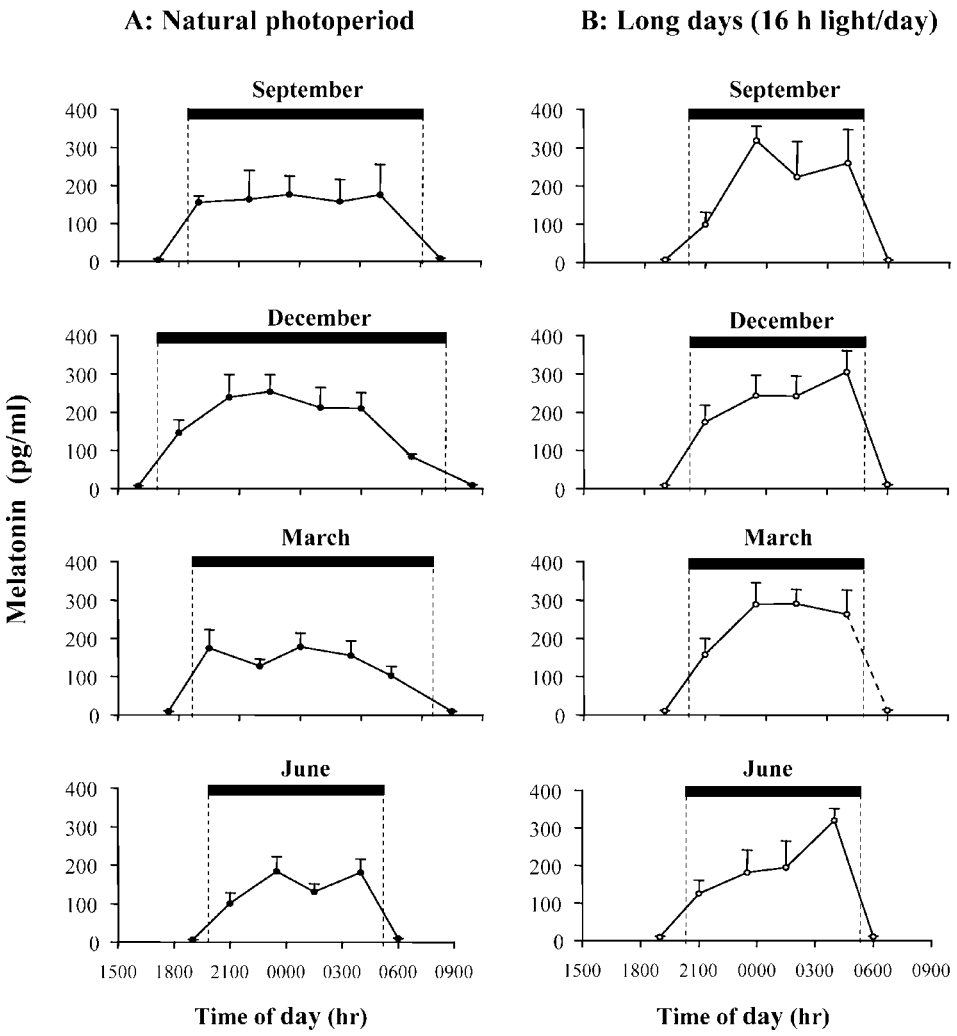

FIG. 6. Mean (±SEM) daily plasma melatonin concentration (pg/ml) at the autumn equinox (22–23 September), the winter solstice (22–23 December), the spring equinox (21–22 March), and the summer solstice (21–22 June) of Year 1 in mouflon ewes (n = 8) maintained under a natural (**A**) or long day (16L:8D) photoperiod (**B**) for 16 mo from 22 June 2002 (Year 1) to 31 October 2003 (Year 2). The black horizontal bars indicate the daily period of darkness.

reflected the duration of darkness. In the NP animals, the duration of the night-time peak differed between seasons (lasting longer with longer nights; $P < 0.01$), whereas in the LP animals, the duration of the melatonin peak was similar in all seasons (because the days were always artificially long). No species-specific differences were found in the mean night-time concentration of melatonin at the different times of the year in either of the two photoperiod treatment groups. However, compared to the NP mouflons, the LP mouflons showed a trend ($P < 0.1$) towards higher night-time melatonin concentrations at each solstice and equinox (Fig. 6).

DISCUSSION

Differences in reproductive seasonality among mammalian species are commonly viewed as having a genetic basis and as reflecting the optimization of phenotypes with respect to their environments. Mammals can regulate their reproductive responses to seasonal change via two mechanisms: through a direct response to an environmental cue (or cues) marking the time of year (mainly changes in photoperiod), and/or through an endogenous circannual rhythm synchronized by photoperiod cues. In many rodent species at least one reproductive transition is a direct result of the photoperiod [35, 36], whereas in other species, including the ground squirrel [37], sheep [9], mink [38], and woodchuck [39], each reproductive transition is generated endogenously—the reflection of a circannual reproductive rhythm synchronized by photoperiod.

The results of the present study help explain the way in which the reproductive patterns of different types of sheep born and raised at similar latitudes are affected by interactions between their genes and the annual photoperiod cycle. When the present study was designed, the timing of the reproductive transitions in the mouflon and Manchega ewes (all of which were maintained in a Mediterranean area at 40°25′N) [27] were known. In the Manchega sheep, and under normal conditions, the onset of cyclic ovulatory activity always started with the onset of long days (June–July) and ended when the days became relatively short (February–March). In the mouflon ewes, and under normal conditions, the onset of ovarian activity always began in October, when the photoperiod is short, and the onset of anestrus always occurred in April-May, when the photoperiod becomes relatively long. The present results show, for the first time, that both these species experience changes in ovulatory activity over the year when subjected to a constant photoperiod of long days. This suggests the existence of an endogenous rhythm of reproduction similar to that observed in other sheep breeds from higher latitudes when maintained under constant short (8L:16D) or 12L:12D photoperiods [9, 40–42]. Further, this rhythm was differently expressed in the two species, with the Manchega ewes showing much more sensitivity to the constant long-day photoperiod than the mouflon ewes.

It should be noted that the reproductive cycles of the mouflon and Manchega sheep were affected differently by the long-day photoperiod. In the LP Manchega ewes, the annual

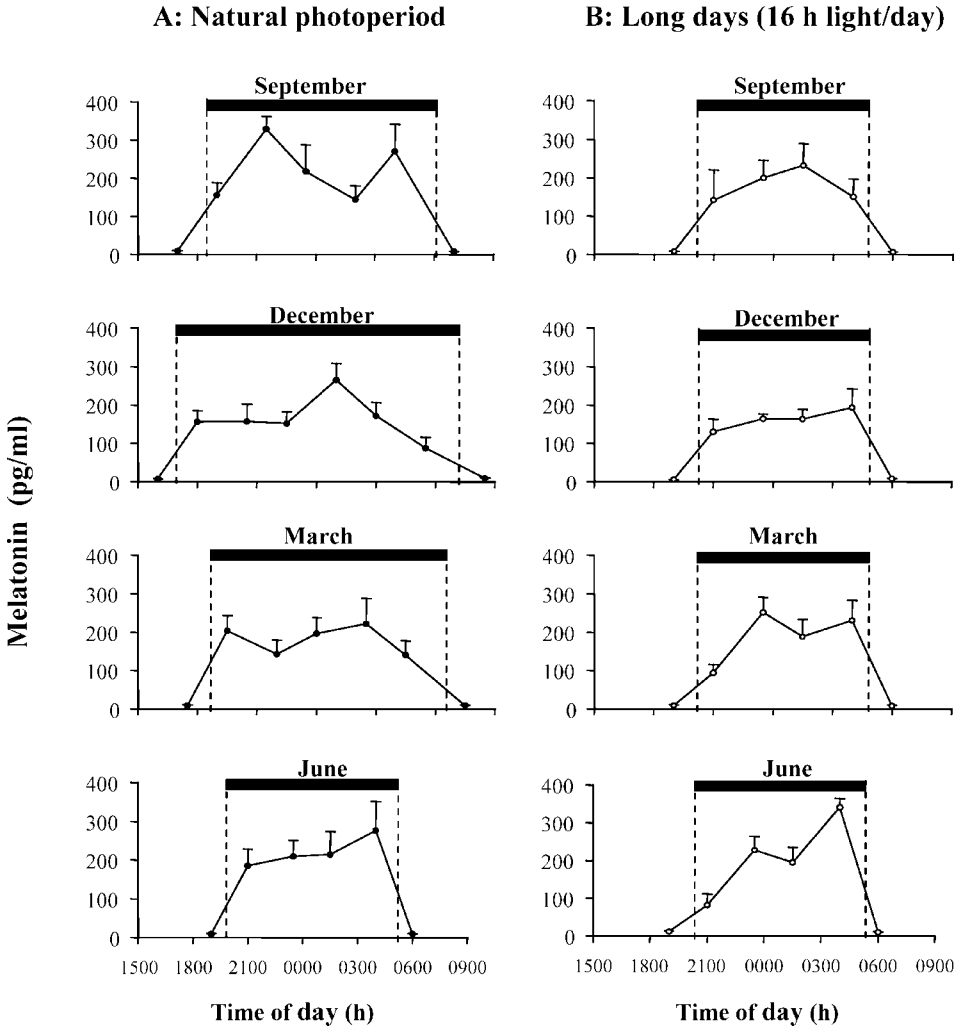

FIG. 7. Mean (±SEM) daily patterns of plasma melatonin concentrations (pg/ml)) for the autumn equinox (22–23 September), the winter solstice (22–23 December), the spring equinox (21–22 March), and the summer solstice (21–22 June) of Year 1 in Manchega ewes (n = 8) maintained under a natural (**A**) or long day (16L:8D) photoperiod (**B**) for 16 mo from 22 June 2002 (Year 1) to 31 October 2003 (Year 2). The black horizontal bars indicate the daily period of darkness.

reproductive rhythm varied greatly among individuals, as well as with respect to that seen in the NP ewes (Fig. 2), whereas this variability was less evident in the LP mouflon ewes, in which the seasonal cycles of ovulatory activity were slightly desynchronized among animals and very similar to that seen in the NP mouflons (Fig. 1). This lower dependence on changing photoperiod in the mouflon might indicate that other environmental factors prevail over or interact with the photoperiod to determine the synchronization of the endogenous reproductive rhythm. It is well known that environmental influences other than light (e.g., nutrition, social interactions, and temperature) can have profound effects on reproductive activity and that these can interact with the photoperiod to synchronize the seasonal reproductive cycle [43]. Nutrition has more important effects in some breeds than in others [44, 45]. Social cues can contribute to the synchronizing of the seasonal reproductive cycle in feral sheep that are not responsive to photoperiodic signals [44] as well as in those capable of perceiving photoperiod information [46, 47]. In the present study, nutritional inputs were held constant, and all the ewes were kept away from males. Under these conditions, the two species showed significant differences in their reproductive patterns under both natural and long-day conditions (Figs. 1 and 2). Thus, for the mouflon, it is improbable that reproductive seasonality in the field can be explained by nutrition and/or social cues prevailing over photoperiod cues. Temperature might also help induce or synchronize the seasonal reproductive cycle in the mouflon; certainly, a dependence of circannual rhythms on ambient temperature has been recorded in the dormouse [48] and in feral sheep [44] in which photoperiod cues have been blocked. In the present study temperature was not controlled. However, changes in the ambient temperature between summer and winter in the natural habitat of the mouflon are not very pronounced; therefore, temperature is unlikely to dictate seasonality in this species. However, temperature may play some role in the expression of the circannual rhythms in both the studied species, as has been suggested for other species [49]. The lower dependence on changing photoperiod in the mouflon might be also be due to this species simply having a strongly expressed endogenous rhythm that does not require other environmental cues.

In contrast to the mouflons, not all Manchega ewes under constant long-day photoperiod conditions showed a clear rhythm of ovulatory/anovulatory activity over the study period. Rather, the annual reproductive rhythm was highly variable among individuals. In domestic sheep, genetics factors have been shown to affect the characteristics of reproductive seasonality [50, 51]. Therefore, the fact that some LP ewes did not show a clear rhythm of reproductive activity might be due to the existence of great genetic variation among the females of this breed with respect to photoperiod cues, a result of the selection process to which domestic sheep breeds are subject. Further, the present experimental conditions (the animals were maintained for only 16 mo under constant long-day photoperiod conditions) were not the most optimal for

the detection of the underlying reproductive rhythm in all animals. A study period of 3 yr under constant long-day photoperiod conditions might have shown circannual fluctuations of ovulatory/anovulatory activity in all the ewes.

Therefore, the difference seen in the timing of the breeding seasons in the mouflon and Manchega ewes under their natural environmental conditions may be a consequence of their genetic differences in the photoperiodic requirements for the synchronization of the circannual reproductive rhythm [52]. The mechanism determining these differences must be the consequence of selection, which in the Manchega sheep must have modified the synchronization of the endogenous ovulatory cyclicity for an earlier onset of reproductive fertility (therefore allowing a longer lambing season). In addition, it is possible that, as seen in the sheep breeds of temperate latitudes, the long days of spring might be critical for resetting the endogenous reproductive rhythm in both types of present sheep, though in the Manchega ewes there is a shorter lag time (about 4 mo shorter) between the perception of the photoperiod signal and the onset of the breeding season. Finally, these differences could be related to differences in the way the photoperiod synchronizes the endogenous rhythm of reproduction. Further studies are needed to determine the species-specific variations in the photoperiodic regulation of seasonal reproductive activity in these sheep.

Although the mechanisms that regulate the breeding season are insufficiently understood to provide a satisfactory explanation for the differences in the timing of reproductive transition between mouflon and Manchega ewes, it has been suggested [53] that they may be explained by differences in the central neuroendocrine mechanisms relaying the effects of day length and controlling the secretion of gonadotrophic hormones. Because the pineal body relays the effects of day length through the seasonal pattern of melatonin secretion [54], and because the nocturnal melatonin concentration reached is under strong genetic control [55, 56], differences between the present sheep species at this level might be expected to affect their response to the photoperiod. However, the mouflon and Manchega ewes had very similar melatonin profiles. The duration of the night-time melatonin peak and the mean nocturnal plasma concentrations at the solstice and equinox periods were similar in both species under both photoperiod conditions. Thus, the physiological explanation for the differences in reproductive seasonality between the mouflon and Manchega sheep does not reside in the systems controlling pineal function. Therefore, it is likely that the genetics of these animals influences the way that the melatonin signal is transduced in the central nervous system rather than the way it is produced. The same conclusion has been drawn in other studies comparing the melatonin profiles of the males [24, 57] of different sheep breeds.

Exposure of the mouflon and Manchega ewes to the long-day treatment altered their seasonal rhythms of prolactin secretion in a similar fashion. In both types of sheep the amplitude of the change in annual prolactin production was smaller than that seen in the NP animals. These findings are comparable to those of previous studies [58, 59] and show the persistence of a circannual rhythm of plasma prolactin concentration (with only a small variation) in ewes exposed to a constant photoperiod. The great individual variability in the annual pattern of prolactin secretion in both of the present species under LP conditions has also been noted both within and between the results of previous investigations in rams [60, 61] and ewes [9, 58, 59]. This suggests that, like reproductive activity, the annual prolactin rhythm in the two species appears to be endogenously generated.

The present results indicate that the pattern of prolactin secretion is probably not involved in the regulation of cyclical ovulatory activity in either of the species studied. In fact, under the NP conditions, the prolactin concentrations of the Manchega sheep were high when they started to cycle (June–July), but they were very low when the mouflons started their ovulatory activity (mid-October). Furthermore, when animals of both species were subjected to LP conditions, some animals with low prolactin levels throughout the study expressed alternating cycles of ovulatory activity, whereas others experienced no ovarian cycles despite having shown robust circannual changes in their prolactin concentrations. This uncoupling of reproductive activity from the prolactin rhythm agrees with the results of other authors [62], indicating that the prolactin concentration is unlikely to be related to reproductive activity; rather, they are probably independently regulated [9, 42].

The present results also agree with those of previous studies involving wild and domestic rams [57], further suggesting that differences in reproductive seasonality between the mouflon and Manchega ewes cannot be attributed to differences in the pattern of prolactin secretion.

In summary, this study provides novel information on the reproductive and endocrine response to long days in a wild (mouflon) and a domestic (Manchega breed) type of Mediterranean sheep. The results show for the first time that these species, which have different patterns of reproductive seasonality under their natural environmental conditions, experience changes in reproductive activity under a constant long-day photoperiod. This suggests that they possess an endogenous circannual rhythm that may be responsible for reproductive transitions irrespective of the natural photoperiod. In addition, Manchega ewes appear to be much more sensitive to changes in the photoperiod than mouflon ewes. The results also suggest that the circannual ovarian cycle, but not the prolactin or melatonin pattern, is differently expressed in these species when subjected to a continuous long-day photoperiod.

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