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What Photoperiodic Signal Is Provided by a Continuous-Release Melatonin Implant?[1]

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ABSTRACT

The purpose of this study was to evaluate whether the insertion of a continuous-release melatonin implant into ewes provides a short-day photoperiodic signal or acts as a functional pinealectomy (provides no specific photoperiodic signal but renders ewes incapable of responding to changes in photoperiod). Ewes primed with 60 long days (18L:6D) during the spring were moved to intermediate day length (13L:11D) for 66 days and then given one of five treatments: 1) short-day control, second drop in photoperiod to 8L:16D; 2) intermediate-photoperiod control, kept on 13L:11D; 3) pinealectomy and kept on 13L:11D; 4) melatonin implant and kept on 13L:11D; 5) melatonin implant and moved to 8L:16D. Mean number of estrous cycles per group and total duration of reproductive activity were determined. Ewes in all groups began to exhibit estrous cycles after the initial reduction in photoperiod. The number of estrous cycles and duration of reproductive activity differed among groups. The number of estrous cycles and duration of reproductive activity was extended in ewes receiving the second drop in photoperiod compared to that of the intermediate-photoperiod controls. Pinealectomized ewes had a number of estrous cycles and duration of reproductive activity similar to those of ewes maintained on the intermediate photoperiod. Melatonin implants increased the number of estrous cycles and prolonged reproductive activity in ewes maintained on the intermediate photoperiod; melatonin implants did not prevent the extension of reproductive activity in ewes receiving the second photoperiodic drop to the short daylength. These findings support the hypothesis that constant-release melatonin implants can act as a short day, rather than as a functional pinealectomy, in prolonging the breeding season.

INTRODUCTION

Seasonal breeding activity in ewes is regulated mainly by photoperiod [1, 2]. Photoperiodic information is transduced to the reproductive neuroendocrine system via circadian secretion of melatonin from the pineal gland [3–5]. Daily melatonin administration to anestrous ewes in the afternoon from early summer has been used to advance the breeding season. The exogenous melatonin, administered either by timed injection [6] or oral ingestion [7, 8], combines with endogenous melatonin and thus extends the time during which circulating melatonin is elevated each day. The resulting pattern of circulating melatonin is similar to that secreted on a short day, although ewes continue to be exposed to longer natural photoperiods characteristic of the anestrous period. More recently, continuous-release implants or rumen-degradable boluses containing melatonin have been used to advance the breeding season [8–11]. Unlike daily administration, these implants or boluses maintain elevated circulating concentrations of melatonin throughout the 24-h day for extended periods of time [8, 12, 13].

There has been no definitive test of the nature of the photoperiodic signal produced by a melatonin implant. It has generally been assumed that such implants convey a short-day photoperiodic signal. This observation is based on comparisons of the effect of exogenous melatonin and exposure to short photoperiod on the time of reproductive transitions [9, 13–15] and prolactin concentrations in blood [11, 16, 17]. An alternative hypothesis, however, is that a continuous elevation in circulating melatonin above baseline does not provide a photoperiodic signal in itself, but renders the ewe insensitive to its own melatonin pattern, such that the animal is unable to perceive the inhibitory photoperiod of summer. This hypothesis is suggested by the reproductive response to pinealectomy, a procedure that renders the ewe incapable of responding reproductively to natural photoperiod [5]. Specifically, pinealectomy at the summer solstice advances the breeding season of ewes [18]. Thus, disruption of the ability to respond to photoperiod at that time of year has much the same effect as treatment with a continuous-release melatonin implant.

The objective of this study was to examine whether a continuous-release melatonin implant imparts a specific photoperiod signal, or whether it acts as a functional pinealectomy and blocks transduction of photoperiodic cues. To distinguish between the possible modes of action of melatonin implants, it was necessary to use an experimental model that could differentiate the effect of short days from that of pinealectomy. The model selected was based

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on the ability of photoperiod to regulate the duration of reproductive activity.

MATERIALS AND METHODS

Experimental Animals and Treatments

The experiment was performed with sexually mature Galway ewes at the University College Dublin farm in Ireland (latitude 53°N). Ewes were maintained indoors in light-sealed rooms under artificial photoperiod. Day length was regulated by electronic timers operating fluorescent bulbs that provided light levels of approximately 350 lux lateral to the head of the sheep during periods of illumination. A dim colored light, producing less than 2 lux at 1 m from the light source and mounted 2 m above the sheep, remained on continuously to facilitate stock inspection and nighttime collection of blood samples. The ewes were fed hay plus supplementary concentrates. Concentrate intake was adjusted monthly to maintain body weight. Water was available ad libitum.

Ewes were pinealectomized using a modification [19] of the procedure of Roche and Dziuk [20]. Pineal glands were removed in one piece. The completeness of removal was initially established by visual inspection of the gland and the site of pineal attachment in the brain. This was subsequently confirmed by the absence of a nighttime rise in melatonin. Melatonin implants were prepared as described previously [8] and were inserted s.c. in the region of the axilla. All implants were recovered at the end of the experiment.

Design

To test the hypothesis that melatonin implants act as a short day, it was necessary to differentiate between the response to a short day and the response to rendering ewes nonphotoperiodic. The experimental model was based on findings that a reduction in photoperiod near the time of reproductive onset can extend the duration of reproductive activity [21], whereas disruption of the ability of the ewe to perceive photoperiod by pinealectomy at a similar time does not [18, 22].

The design is illustrated in Figure 1. The ewes were moved from natural photoperiod (12.5L:11.5D including civil twilight) to long days (18L:6D; lights-on, 0800 GMT) on March 10 and maintained on long days for 60 days to establish a state of reproductive inhibition [23]. On May 9 (Day 0), they were transferred to an intermediate photoperiod (13L:11D; lights-on, 0800 GMT) for a further 66 days and then allocated at random to one of the following five treatments (8–10 ewes per group): 1) short-day control, second drop in photoperiod to 8L:16D; 2) intermediate-photoperiod control, kept on 13L:11D; 3) pinealectomy between Days 60 and 66, kept on 13L:11D; 4) melatonin implant inserted on Day 66, kept on 13L:11D; 5) melatonin implant inserted on Day 66, moved to 8L:16D. Reproductive activity was monitored by measurement of serum progesterone concentrations.

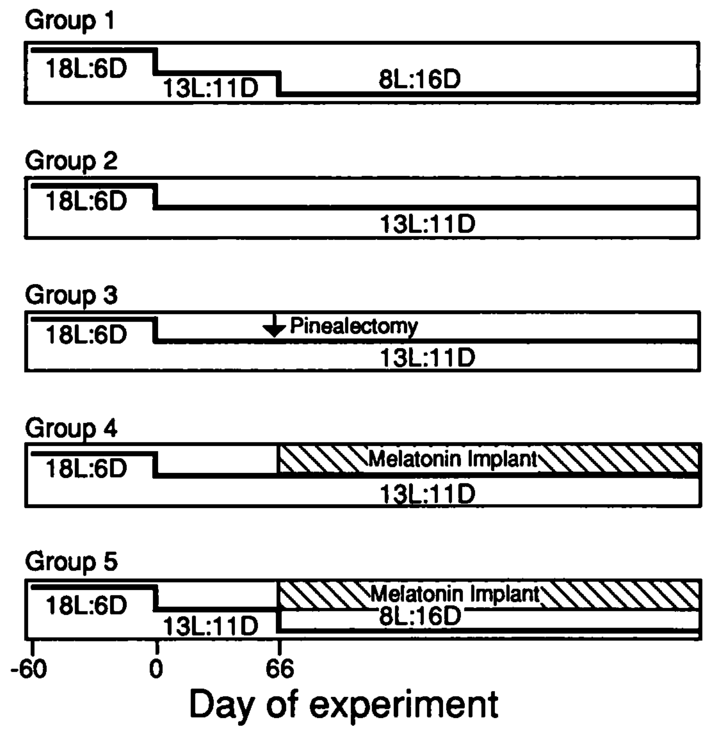

FIG. 1. Experimental design and treatment details. Ewes were exposed to long days (18L:6D) for 60 days, transferred to an intermediate photoperiod (13L:11D; Day 0) for a further 66 days, and then allocated to one of five treatments: 1) short-day control, second drop in day length to 8L:16D; 2) intermediate-photoperiod control, kept on 13L:11D; 3) pinealectomized between Days 60 and 66; 4) melatonin implant inserted on Day 66 and kept on 13L:11D; 5) melatonin implant inserted on Day 66 and moved to 8L:16D (n = 8–10 per group).

Blood Sampling and Assays

Blood was sampled once per week by jugular venipuncture into evacuated tubes, kept at room temperature for 1 h after collection and at 4°C overnight, then centrifuged at 700 × *g* for 20 min and stored at −20°C. Serum progesterone was determined by use of the RIA of Dieleman and Schoenmakers [24] as modified by Ronayne et al. [8]. Interassay CVs for two serum pools with mean values of 1.0 and 3.3 ng/ml, determined four times in each assay, averaged 18.6 and 12.9%, respectively. Intraassay CVs for the same two serum pools were 6.6 and 3.3%, respectively. Sensitivity (two standard deviations from the buffer controls) was 0.2 ng/ml.

To confirm completeness of pinealectomy and to monitor the effect of the implant on circulating melatonin, additional blood samples were obtained at 1-h intervals over a 24-h period on Day 95 of the experiment (n = 5/treatment plus all pinealectomized animals). Melatonin concentrations were determined in duplicate 200-μl samples of serum using a slight modification of the method of Fraser et al. [25] as described by Ronayne et al. [8]. Interassay CVs for three pools of serum with mean concentrations of 36.5, 324, and 433 pg/ml were 18.8, 10.4, and 9.0%, respectively. Intraassay CVs for the same pools were 15.0, 10.5, and 4.7%, respectively. Sensitivity was 16 pg/ml.

Data Analyses

The beginning of reproductive activity was defined as the time of the first progesterone value above 0.5 ng/ml, in a series of two or more consecutive samples above 0.5 ng/ml or one sample above 1 ng/ml. The end of reproductive activity was defined as the time of the last progesterone value above 0.5 ng/ml in a series of two or more consecutive samples above 0.5 ng/ml or one above 1 ng/ml, before four or more consecutive samples below 0.5 ng/ml. Because some animals failed to show any estrous cycles and others had intermittent cycles, the period of reproductive activity identified is presented in two ways: mean number of estrous cycles per group and the total duration of reproductive activity (interval between onset of first cycle and end of last cycle) in animals that had estrous cycles. Differences in the mean number of estrous cycles per ewe and the duration of reproductive activity were compared among groups using Kruskal-Wallis and Mann-Whitney nonparametric tests. Values were considered different at a significance level of $p < 0.05$.

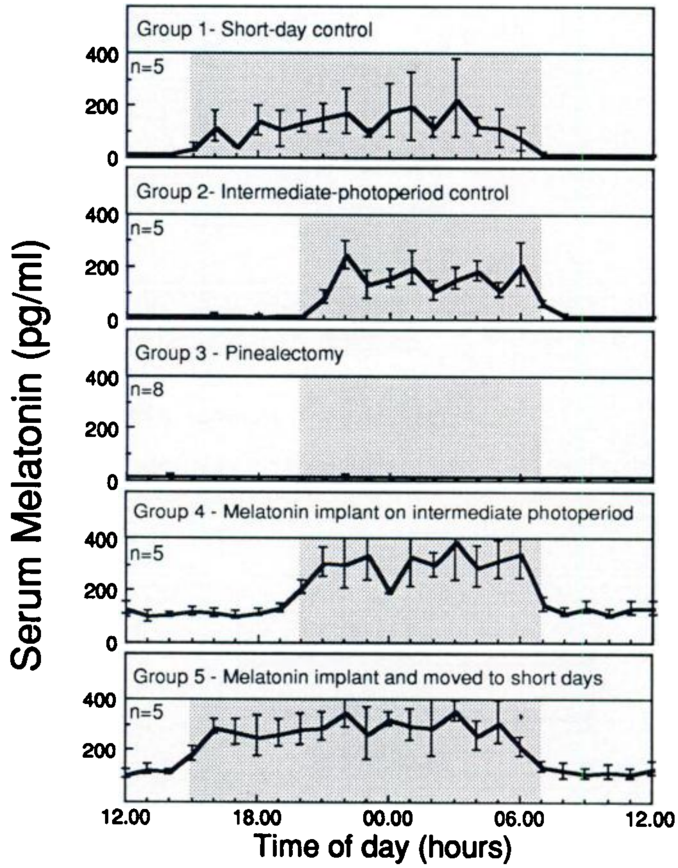

FIG. 2. Mean (±SEM) serum melatonin concentrations in ewes on Day 95 of the experiment. Shaded area indicates time of lights-off.

RESULTS

Melatonin Patterns

Serum melatonin concentrations on Day 95 of the experiment are presented in Figure 2. Melatonin concentrations in ewes on short or intermediate photoperiod rose above baseline within 1 h of lights-off and remained at between 100 and 200 pg/ml until at or near lights-on. Melatonin concentrations in pinealectomized ewes did not rise above baseline in any ewe. Ewes that had received continuous-release melatonin implants had serum melatonin concentrations of around 100 pg/ml during daylight hours; these concentrations increased by 100–200 pg/ml during the hours of darkness.

Reproductive Response

After transfer from the 18L:6D to 13L:11D photoperiod, mean onset of ovarian cyclicity occurred on Day 133 ± 2, with no significant difference among treatments. As illustrated in Figure 3, the second decrease in day length from 13L to 8L on Day 66 increased the number of estrous cycles by 2.4 per ewe ($p < 0.03$) and extended the total duration of reproductive activity by 20 days ($p < 0.05$) compared with that in ewes kept on the intermediate photoperiod of 13L:11D (group 1 vs. group 2). The number of estrous cycles and the duration of reproductive activity in ewes pinealectomized around Day 66 (group 3) was similar to that in ewes maintained on the intermediate photoperiod (group 2). The number of estrous cycles was less ($p < 0.02$) and the duration of reproductive activity shorter ($p < 0.05$) in pinealectomized ewes than in ewes exposed to the second decrease in photoperiod (group 1). Administration of a melatonin implant on Day 66 (group 4) increased the number of estrous cycles ($p < 0.05$) and extended the duration of reproductive activity ($p < 0.05$) in ewes maintained on the intermediate photoperiod (group 2 vs. group 4). The number of estrous cycles and the duration of reproductive activity in ewes receiving both a melatonin implant and the second drop in photoperiod (group 5) was not different from that in ewes given only the second drop in photoperiod (group 1) and was similar to that in implanted ewes given just the single drop to the intermediate photoperiod (group 4).

Individual ovarian cycles, depicted by changes in progesterone concentrations, are shown in Figures 4 and 5. All ewes exposed to a two-step drop in photoperiod had between 4 and 7 estrous cycles (Fig. 4, left panel). In contrast, two ewes maintained on 13L:11D (#1003 and #1068) had no progesterone cycles, and four others had fewer than 4 cycles, with two of these having irregular cycles (Fig. 4,

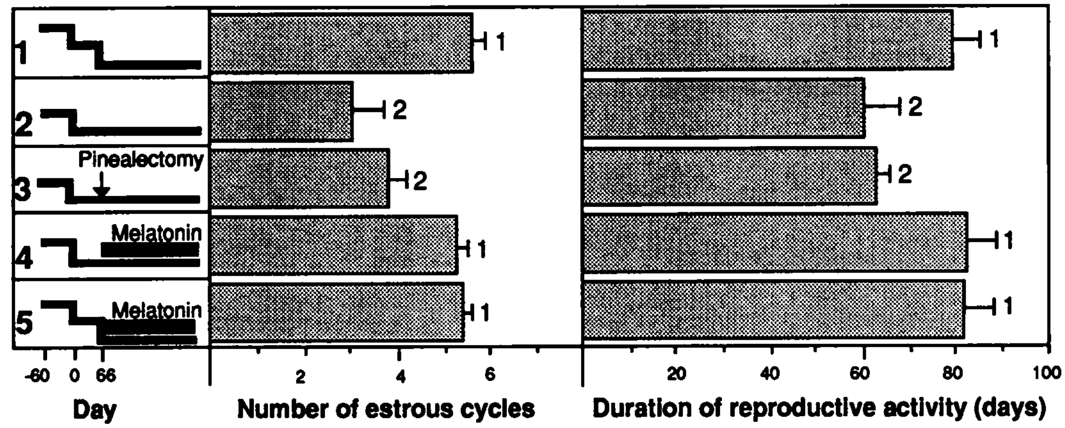

FIG. 3. Mean (±SEM) number of estrous cycles and duration of reproductive activity in ewes. [1,2]Means without a common superscript are significantly different ($p < 0.05$).

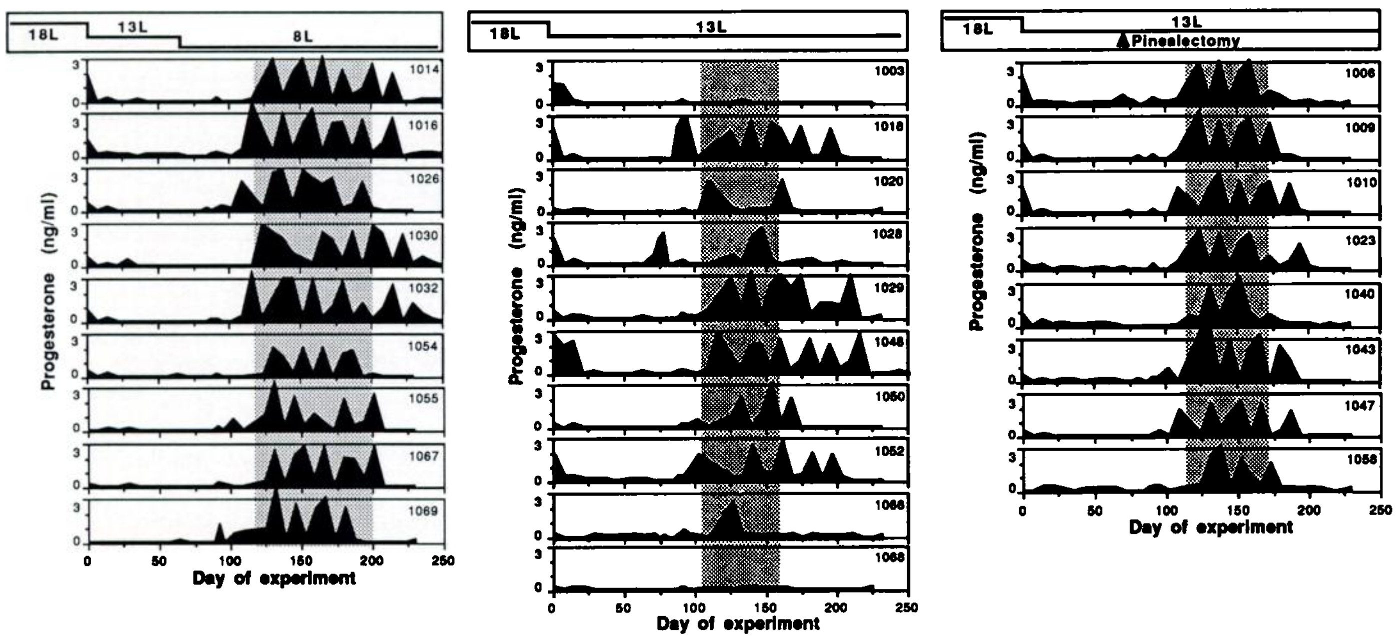

FIG. 4. Weekly serum progesterone of individual ewes. Left panel; group 1, ewes exposed to a second drop in photoperiod from 13L:11D to 8L:16D on Day 66 of the experiment. Middle panel: group 2, ewes maintained on 13L:11D. Right panel: group 3, ewes maintained on 13L:11D and pinealectomized (black triangle) between Days 60 and 66 of the experiment. Shaded area: mean period of reproductive activity for each group.

middle panel). Ewes pinealectomized and maintained on 13L:11D had between 2 and 5 estrous cycles (Fig. 4, right panel). Ewes in the two groups with melatonin implants had between 3 and 7 estrous cycles, with 68% having 5 or more cycles (Fig. 5). Data from one ewe (group 1) were excluded because she had constantly elevated progesterone concentrations (>1 ng/ml) from Day 75 to the end of the experiment, with no evidence of cyclicity.

DISCUSSION

Our results suggest that continuous-release melatonin implants can influence the timing of seasonal reproduction in the ewe by mimicking the effect of short photoperiod, rather than by acting as functional pinealectomy. Specifically, insertion of melatonin implants prior to reproductive onset prolonged the period of reproductive activity, as did the transfer from an intermediate to a short photoperiod. Our findings complement the results of earlier studies that suggested that continuous delivery of melatonin provides an endocrine signal that mimics a short day. For example, Lincoln and Ebling [14] demonstrated that Soay rams exposed to a long photoperiod and implanted with continuous-release melatonin implants have a rapid onset of reproductive development, similar to that produced by exposure to short days. These authors, however, also observed that melatonin implants prevent maximal testicular

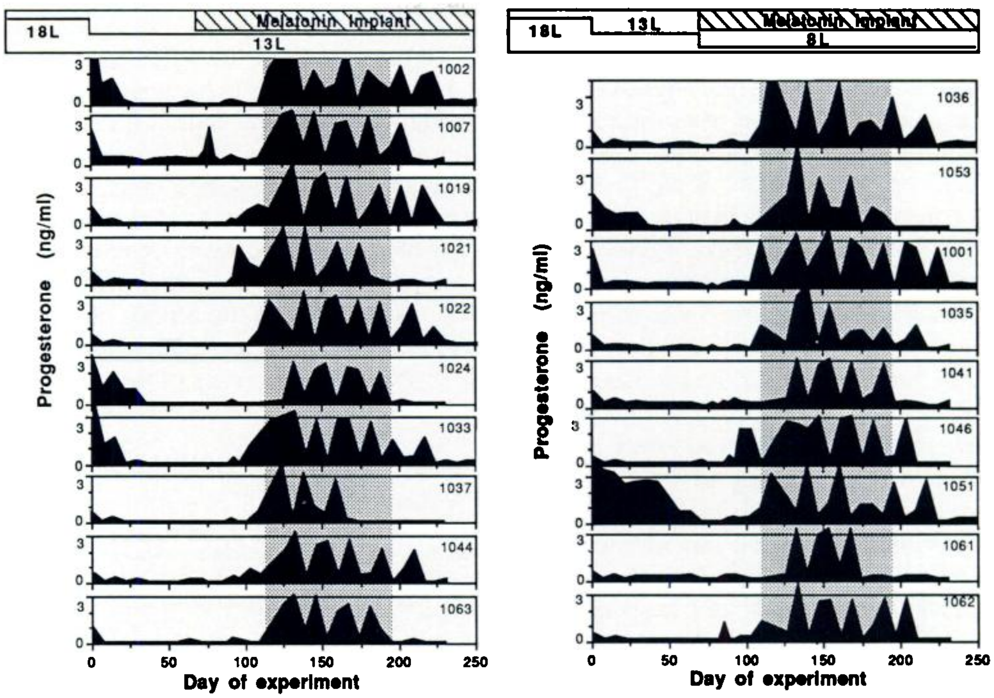

FIG. 5. Weekly serum progesterone of individual ewes. Left panel: group 4, ewes implanted with melatonin on Day 66 of the experiment and maintained on 13L:11D. Right panel: group 5, ewes exposed to a second drop in photoperiod from 13L:11D to 8L:16D on Day 66 of the experiment. Shaded area: mean period of reproductive activity for each group.

growth in response to changes in photoperiod, thus suggesting that implants might also render rams nonphotoperiodic. The conclusion that melatonin implants provide a stimulatory short-day signal to ewes was also reached by Poulton et al. [11], who used onset of the breeding season as an index of reproductive response. Although earlier observations are supportive of the present conclusion, the possibility that continuous delivery of melatonin is perceived as a short day had not previously been demonstrated in a fully controlled study.

It is of interest to consider the mode of action of a continuous-release melatonin implant with respect to current concepts concerning the specific feature of the melatonin pattern that is critical to the measurement of day length [26]. One possibility is that there is a circadian rhythm of sensitivity to melatonin, and the reproductive response is determined by coincidence of elevated melatonin secretion with the sensitive phase of the rhythm. Alternatively, the duration of the melatonin elevation each 24 h may provide the signal, with long durations being stimulatory. Although attempts to distinguish between these hypotheses in sheep have favored duration [27, 28], either mode of action of melatonin is consistent with the conclusion that a continuous-release melatonin implant can act as a short day. On the one hand, the duration of elevated melatonin each day would essentially be maximal; on the other hand, a constantly elevated melatonin level could not but strike any sensitive phase that might exist. It has recently been suggested that the reproductive response to a specific photoperiod is related to the melatonin-free interval between periods of elevated melatonin [29]. If this is the case, it is more difficult to explain how a continuous-release melatonin implant could provide a specific photoperiodic signal, because melatonin is continuously elevated, albeit at varying levels, throughout the 24-h day.

The increased number of estrous cycles and the extended duration of reproductive activity in controls exposed to a second drop in photoperiod prior to the onset of reproductive activity (group 1) confirms and extends the previous findings of Malpaux et al. [21], who showed that a two-step decrease in day length can prolong the period of reproductive activity. The magnitude of this prolongation in our study was less than that of Malpaux et al. [21], but the experimental designs were different. In particular, our study monitored estrous cycles in ovary-intact ewes whereas Malpaux et al. [21] monitored LH in ovariectomized ewes treated with estradiol. Further, the time of the second decrease in photoperiod relative to the onset of reproductive activity was different in the two studies, being within approximately 1–3 wk of reproductive onset in the experiment of Malpaux et al. [21] and within 8–10 wk of reproductive onset in our study. Thus, the time of the second photoperiodic drop relative to reproductive onset may be important in determining the extent to which a short photoperiod can prolong the breeding season.

With regard to the strategy of the present study, our experiments were designed to evaluate the response to melatonin implants in terms of extending reproductive activity. This effect of melatonin on the duration of the breeding season may be one of many roles of melatonin in regulation of reproductive activity. In this regard, it should be stressed that for practical applications, melatonin implants are more commonly used to advance the onset of reproductive activity. Our findings cannot be interpreted in terms of how melatonin implants act to advance the breeding season. It is important to point out that the design of the present study was employed because a short-day effect in advancing reproductive activity is difficult to demonstrate definitively. This is the case because short photoperiod, melatonin implants, and pinealectomy all can advance the transition from anestrus to the breeding season [9, 18]. In addition, evidence is accumulating to indicate that short days may not actually be involved in timing reproductive onset in natural conditions; rather, increasing photoperiod appears to synchronize an endogenous rhythm that generates the onset of the breeding season [30]. It may be prudent, therefore, not to extend our conclusions to the action of melatonin in advancing the breeding season, as this appears not to be a short-day-induced phenomenon. It would be of interest to evaluate whether, in conditions other than those tested in the present study, melatonin implants also act as a short day or whether they render animals nonphotoperiodic.

At a more practical level, melatonin administration has been used in attempts to extend the breeding season. Daily injections of melatonin given in the afternoon starting from the winter solstice prolonged the breeding season [6], but continuous-release melatonin implants beginning in late winter were without effect [31]. The melatonin treatment in the latter case, however, was initiated shortly before termination of the breeding season, at which time the ewes were likely to have been less responsive to the effects of short days [32]. An influence of time of year on the response to a melatonin implant is documented by studies in sheep and red deer. For example, insertion of melatonin implants in June advanced the breeding season of ewes, whereas treatment in April and May did not [9]. The breeding season was advanced in red deer stags implanted with melatonin in late spring, but treatment in late winter delayed reproductive development [33]. Fisher et al. [34] demonstrated that the time of initiation of melatonin treatment influences the response of red deer hinds in terms of summer moult and winter-coat growth. The reproductive response to pinealectomy of ewes is also dependent on season. For example, pinealectomy around the spring equinox delays the subsequent reproductive season, whereas pinealectomy at the summer solstice advances it [18]. Thus, the time of year can have a profound effect on the response to procedures that alter the circulating pattern of melatonin.

In conclusion, the present studies provide evidence that continuous-release melatonin implants can convey a signal similar to that imparted by a short photoperiod in prolonging the breeding season of the ewe. Such implants were found not to act simply as a functional pinealectomy, merely abolishing the response to photoperiod. It will be of interest to determine the extent to which the present conclusions apply when the actions of melatonin implants are tested in other photoperiodic conditions or when implants are given at different times of the annual reproductive cycle.

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