

Photoperiodic Versus Metabolic Signals as Determinants of Seasonal Anestrus in the Mare¹

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ABSTRACT

The objectives of this study were to compare the timing and mechanisms controlling the onset of anestrus in young and mature mares treated either continuously with melatonin and in those that remained untreated. Changes in body weight, subcutaneous body fat measured to provide an estimate of total body fat, and circulating concentrations of leptin were compared throughout the 1-yr experimental period. The results demonstrate that in young mares the timing of anestrus occurs significantly earlier in the year than in mature mares and that mature mares are more likely to exhibit continuous reproductive activity during the nonbreeding season. The propensity of mature mares to exhibit this phenomenon is not modified by continuous treatment with melatonin but is associated with higher mean circulating concentrations of leptin, body weight, and estimated percent of body fat. In both young and mature mares, body weight, percent of body fat, and circulating concentrations of leptin are higher during summer than winter months. We conclude that, in the mare, the reproductive response to a decrease in photoperiod or a presumptive inhibitory melatonin signal is modified by energy availability, which may be signaled to the hypothalamus-pituitary axis via a change in the circulating concentration of leptin. An additional observation confirmed that the prolactin axis is responsive to continuous treatment with melatonin but that a suppression of prolactin secretion is limited to the spring months.

leptin, melatonin, prolactin, seasonal reproduction

INTRODUCTION

In most mammalian species that live in temperate zones, seasonal reproductive activity is timed by the annual change in photoperiod. This strategy ensures birth of offspring at a time of year that is optimal for their survival. In some short-lived species, however, an increase in food availability and, therefore, a positive energy balance, may lead to reproductive activity during a nonstimulatory photoperiod, a phenomenon referred to as opportunistic breeding (for a review, see [1]). The occurrence of reproductive activity during the nonbreeding season is generally considered to be uncharacteristic of long-lived species of seasonal breeders. One exception is the mare, because several in-

dependent laboratories have reported continuous reproductive activity during the nonbreeding season [2–5].

The mechanisms that account for reproductive activity in mares during the nonbreeding season are not yet understood; however, the phenomenon is more commonly observed in mature (aged > 5 yr) rather than young mares [3]. On one hand, therefore, it is conceivable that aging modifies the response to photoperiod; on the other hand, it has also been reported that, regardless of age, pregnancy and subsequent lactation invariably lead to a cessation of reproductive activity during the winter months [3]. Pregnancy and lactation constitute an energetic challenge. In view of this consideration, it is plausible to suggest that reduced energy availability, as a consequence of lactation, increases the likelihood of a cessation of reproductive activity associated with nonstimulatory photoperiod.

A propensity for a greater proportion of mature, nonbred mares to exhibit estrous cycles during the winter months, compared with young mares, may similarly reflect differences in energy availability. Mature mares have been reported to accumulate body fat when maintained nonpregnant in successive years [6]. In this laboratory, the mares comprising the research herd for reproductive research are kept nonbred and, from unpublished observations, we have noted that mature but not young mares exhibit estrous cycles during the nonbreeding season. Furthermore, mature mares appear to be fatter than young mares, which supports the proposal that energy stored as body fat may modify seasonal reproductive activity in this species.

In view of these considerations, the objective of this study was to examine and compare the timing of the onset of anestrus in young versus mature mares in response to a decrease in ambient photoperiod or a continuous inhibitory melatonin signal provided by a subcutaneous implant. Constant treatment of mares with melatonin is believed to be perceived as an inhibitory photoperiodic signal because the advancement of the onset of the breeding season associated with exposure to an artificially extended photoperiod is blocked [7]. An additional objective of the study was to investigate the hypothesis that the availability of stored energy or subcutaneous body fat may be associated with differences in the timing, duration, or both, of the anestrus period in young versus mature mares. Therefore, we compared annual changes in body weight; subcutaneous body fat; and a hormonal product of adipocytes, namely leptin, in young, lean mares and mature, fat mares. Leptin is considered to provide information to the brain on energy status and, conceivably, may serve as a metabolic gate to the reproductive axis [8]. On the basis of our findings we suggest that in the mare, the occurrence of seasonal anestrus is determined, at least in part, by metabolic signals.

MATERIALS AND METHODS

Animals

Twelve mature mares (>10 yr of age) and 11 young mares (2–5 yr of age) of mixed, predominantly thorough-

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bred breeding were employed for this study. The mares were maintained as a single herd on pasture at the University of Kentucky research farm (38°N latitude). Their diet was supplemented with hay, and they had free access to water and trace-mineral salt blocks.

Experimental Design

Beginning 15 June 1998, blood samples were collected thrice weekly from all mares via jugular venipuncture between 1400 and 1500 h. On June 22, 6 mature and 6 young mares were randomly selected and each was implanted with 3 melatonin implants (Regulin, Sanofi, France). The remaining mares in each category remained untreated. Each implant contained 18 mg of melatonin and was designed to release melatonin constantly for 3 mo. Accordingly, the mares were reimplanted before the suggested expiration of the implant on the following occasions: 12 September, 10 December, and 12 March to provide a total treatment duration of approximately 1 yr. The experimental period ended on 29 May 1999. On each occasion of implantation the selected implant site in either the left or right neck/flank region was clipped and scrubbed with a 2% iodine solution followed by alcohol rinsing. The implants were injected s.c. using a trochar gun provided by the manufacturer.

At 3-wk intervals, beginning 26 June, the mares were weighed using an electronic scale and the percent of body fat was determined by ultrasonography. To estimate the percent of body fat, hair covering the right side of the croup was clipped approximately 20 cm anterior of the tailhead and 20 cm off the midline. Fat depth was estimated at a predetermined site located 11 cm anterior to the tailhead and 10 cm off the midline. The measurement of fat depth (cm) by ultrasonography was converted to an estimate of percent of body fat using a prediction equation [9]. Briefly, the algorithm, $Y = 5.47 (\text{ultrasound fat depth in cm}) + 2.47$, where Y is the estimated percent of body fat, was developed following indirect measurement of the fat depth at several predetermined sites at the croup by ultrasonography and directly following euthanasia of the experimental animals, as described elsewhere [9]. In that study, total body fat was determined from selected freeze-powdered subsamples following grinding of the empty body carcass.

The occurrence or absence of ovulation was determined by measurement of progesterone in 6-ml blood samples that were collected 3 times per week. These same samples were also assayed for prolactin concentration as an additional measure of the efficacy of the melatonin implant [7]. An additional 10-ml blood sample was collected weekly, beginning 26 June, and was employed to determine melatonin and leptin concentrations in selected samples encompassing the experimental period. All blood samples were allowed to clot and were kept overnight at 4°C. The next day, serum was harvested and stored frozen at -20°C, until assayed for hormone determination.

Radioimmunoassay

Concentrations of prolactin were measured in serum by a previously described radioimmunoassay (RIA) [5]. The assay employed an anti-porcine prolactin antiserum (LSU R4 EPRL, supplied by Dr. D. Thompson, Jr., Louisiana State University, Baton Rouge, LA) and equine prolactin (AFP7730B, supplied by Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA) as sources for iodination and as reference preparations. The sensitivity of the assay was

0.4 ng/ml; the interassay and intraassay coefficients of variation were 16.7% and 13.8%, respectively.

Melatonin was measured in selected samples by a commercial RIA kit (Alpco, Windham, NH) described previously [10] and validated by our laboratory for measurement of melatonin in equine serum [11]. A 1-ml serum aliquot was extracted according to the manufacturer's directions and reconstituted in a buffer solution that was provided. Aliquots of the extracted samples (50–400 μ l) were assayed in duplicate. Each assay included determination of melatonin concentration in a charcoal-stripped equine serum pool, and an equine serum pool loaded with low (2.5 pg/ml) and high (15 pg/ml) concentrations of melatonin. The charcoal-stripped serum pool routinely provided concentrations of melatonin that were undetectable; interassay and intraassay coefficients of variation for the low concentration pool were 7.2% and 16%, respectively. For the high concentration pool, the interassay and intraassay coefficients of variation were 5.5% and 5.2%, respectively. Recovery estimates for the respective pools were 90% and 107%. Serial dilutions of equine serum exhibited parallelism to the reference preparation. The sensitivity of the assays averaged 0.5 pg/ml.

Leptin was measured using a commercial kit (Linco, St. Charles, MO) that has been validated for measuring leptin in equine serum or plasma. The antibody used was guinea pig anti-human leptin at a working dilution of 1:8000. In the absence of a purified equine leptin preparation, results are reported as human equivalents of immunoreactive leptin (ir-leptin HE). Serial dilution of equine serum containing ir-leptin exhibited parallelism to the human leptin reference preparation. The intraassay coefficients of variation for 2 serum pools, designated as low and high leptin concentrations, and which contained 3.3 and 23.3 ng/ml, respectively, were 10.5% and 10.3% ($n = 9$ assays). Similarly, the interassay coefficients of variation for the respective pools were 10.6% and 7.6%. The limit of detection for the leptin assay was 0.9 ng/ml.

Analysis of Data

Changes in mean circulating concentrations of leptin, melatonin, prolactin, body weights, and estimated percent of body fat were compared between and within groups by ANOVA for a repeated measures design in the Statistical Analysis System using the PROC MIXED [12]. Prolactin values for individual mares were averaged for each month and, for statistical analysis, the values were pooled within treatment groups.

In individual mares, the onset and cessation of anestrus was identified by the maintenance of a low (<0.5 ng/ml) or raised (>0.5 ng/ml) circulating concentration of progesterone in at least 6 successive blood samples collected 3 times weekly. A Fisher exact test was employed to compare the proportion of anestrus mares between groups. The relationship between body fat and ir-leptin and between body weight and body fat in young and mature mares was determined by a covariance procedure using PROC mixed. Where appropriate, the results are presented as the mean \pm SEM.

RESULTS

The mean date of anestrus onset in young control and melatonin-treated mares was November 6 and 9, respectively. In 2 young mares (1 untreated control, 1 melatonin-treated), ovulation ceased in August; subsequently, 8 mares

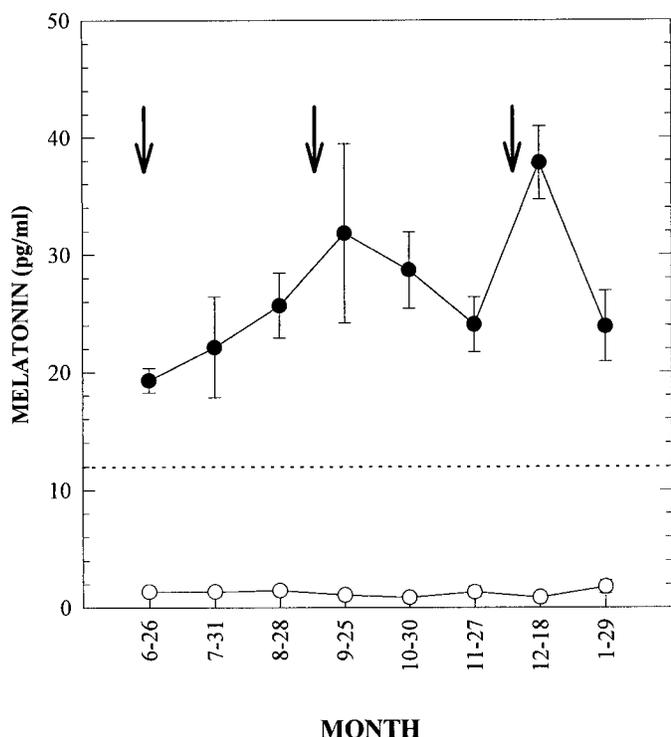


FIG. 1. Changes in mean (\pm SEM) circulating concentrations of melatonin in selected samples collected from mares treated constantly with a melatonin implant (solid circles, $n = 12$) or in those that remained untreated (open circles, $n = 11$). Arrows indicate the timing of implantation.

became anestrus between October and early January; the remaining mare exhibited continuous reproductive activity despite constant melatonin administration throughout the experimental period. Overall, within the young mares, treatment with melatonin did not modify the timing of the onset of anestrus. In contrast to the high proportion of young mares (7 of 11) that were considered to be anestrus by the beginning of December, all mature mares continued to exhibit estrus cycles at the same time of year. This difference in the proportion of young and mature mares exhibiting anestrus was significantly different ($P < 0.001$). During the experimental period, only 5 of 12 mares ceased reproductive activity during the winter months and the timing of the onset of anestrus occurred during the first week of January ($n = 4$) or on March 1 ($n = 1$). Similar to young mares, in mature mares, constant administration of melatonin did not modify the timing of the onset of anestrus.

The duration of anestrus in young mares was similar in melatonin-treated and untreated mares (174 ± 37 days vs. 176 ± 30 days, respectively) and the mean date of first ovulation was May 2 and 3, respectively. For the mature mares, 1 melatonin-treated mare that exhibited anestrus died in early February and, for the remaining mares, the duration of anestrus was similar in melatonin-treated and untreated mares (65 ± 19 days and 75 ± 18 days, respectively). Overall, the duration of anestrus in young mares was longer ($P < 0.05$) than it was for mature mares (175 ± 22 days vs. 69 ± 11 days; $n = 10$ and 4, respectively).

Circulating concentrations of melatonin in untreated and melatonin-treated mares as determined in selected samples encompassing the experimental period are shown in Figure 1. The melatonin implants significantly increased circulating daytime melatonin levels and the observed values were

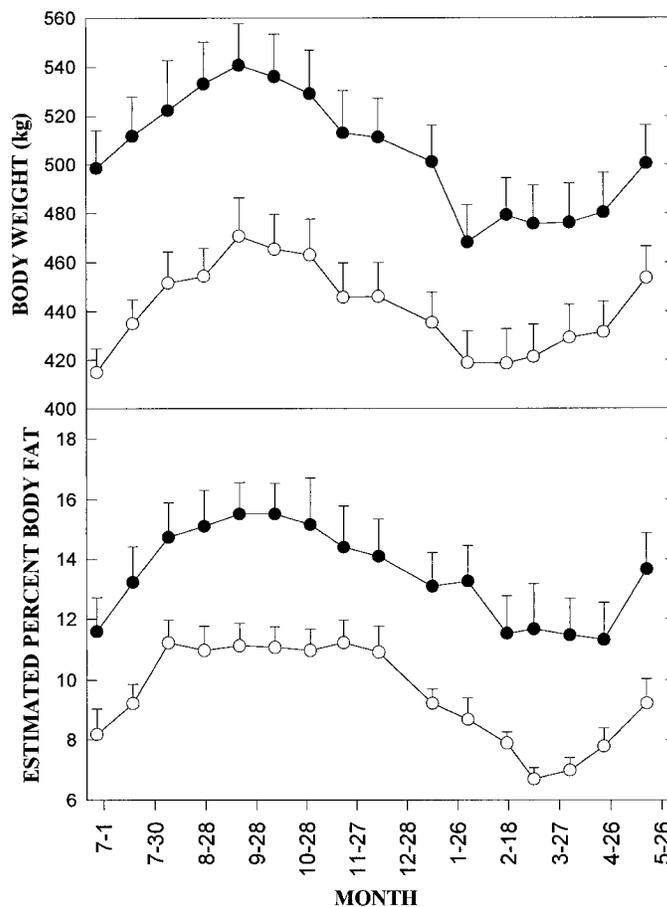


FIG. 2. Changes in mean (\pm SEM) body weight (upper panel) and the estimated percent of body fat (lower panel) in mature (solid circles, $n = 12$) and young (open circles, $n = 11$) mares determined at 3-wk intervals throughout the experimental period.

comparable to nighttime values observed by our laboratory [11].

Changes in mean body weight in young and mature mares are depicted in Figure 2. Mean body weight was significantly different between groups ($P < 0.01$) and, in both groups, changed with time of year ($P < 0.001$). At the beginning of the experimental period (26 June), mean body weight of the young mares increased ($P < 0.01$) from 415 ± 9.6 kg to 470 ± 15.7 kg by mid-September. Subsequently, during the fall and winter months, body weight gradually declined and reached a nadir (418 ± 13.1 kg) in early February. A low body weight was maintained during March but increased gradually during April. At the end of the observation period (in May) the young mares had regained the body weight they had lost during the winter months and mean body weight was greater ($P < 0.01$) than when the study was initiated a year earlier (415 ± 9.6 kg vs. 453.6 ± 12.6 kg; $P < 0.001$). The mean body weight of the mature mares also increased during summer and decreased during fall and winter, before again increasing in spring. At the end of the observation period the mean body weight of the mature mares was marginally but significantly ($P < 0.05$) greater than that observed at the beginning of the study.

The estimated percent of body fat also was significantly different between groups ($P < 0.001$) and, as noted for changes in body weight, body fat increased during summer months and declined during fall and winter ($P < 0.001$). The observed increases and decreases in body weight over

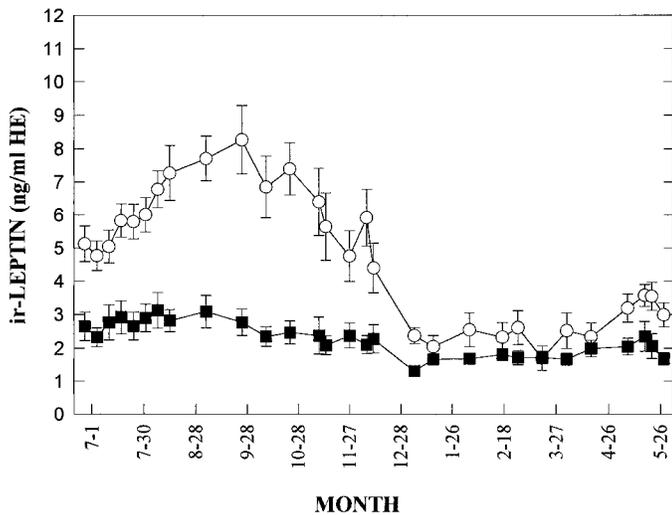


FIG. 3. Patterns of ir-leptin in mature (open circles, $n = 11-12$) and young mares (solid squares, $n = 11$) determined in selected samples encompassing the experimental period.

time were significantly correlated to changes in body fat ($P < 0.001$ for mature and young mares). In addition, in both groups, changes in body fat were highly correlated to changes in circulating concentrations of ir-leptin ($P < 0.001$). The greater estimated percent of body fat in mature versus young mares was mirrored by significantly higher circulating concentrations of ir-leptin throughout the summer and fall months (Fig. 3). In mature mares, concentrations of ir-leptin increased ($P < 0.001$) from 4.77 ± 0.45 ng/ml at the initiation of the study to reach a peak of 8.27 ± 1.02 ng/ml in mid-September, coincident with the time of maximum mean body weight and estimated body fat. In contrast, circulating concentrations of ir-leptin in young mares during this period remained unchanged until late winter, when values declined ($P < 0.05$). For mature mares, a precipitous decline in ir-leptin was observed between early December and January, when values decreased from 5.92 ± 0.86 ng/ml to 2.38 ± 0.28 ng/ml. During the period January–March, concentrations of ir-leptin were not different between mature and young mares, but in April and May, concentrations again increased in mature mares and values were significantly greater ($P < 0.05$) than in young mares.

Although neither reproductive activity, body weight, body fat, nor leptin were disturbed by constant administration of melatonin, the pattern of prolactin secretion was altered. Constant melatonin administration attenuated the seasonal rise in prolactin during the spring months and a modest but significant suppression was observed at the beginning of the study, in July (Fig. 4).

DISCUSSION

The annual change in photoperiod is considered the primary environmental cue that synchronizes seasonal reproductive activity in mares. This premise has been developed largely on the basis of 3 primary observations. First, a majority of mares cease reproductive activity during winter months when day length is short [13]; second, the onset of the breeding season occurs in association with an increase day length; and third, the timing of the onset can be advanced by exposure to an abrupt, artificial increase in day length [14]. Whereas increasing day length provides a robust signal to synchronize the timing of the onset of the

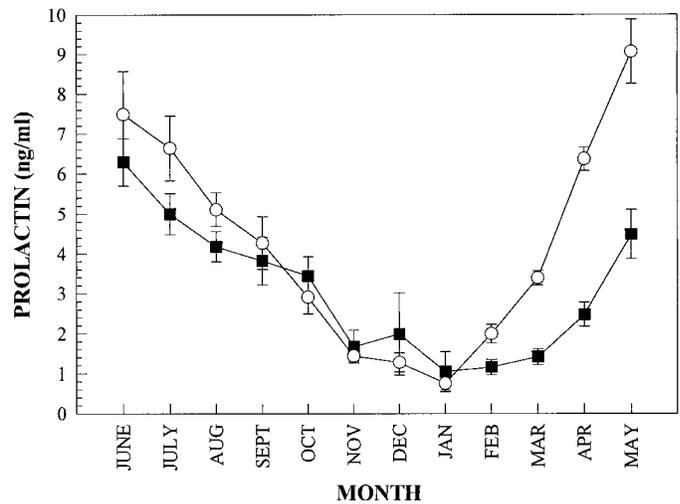


FIG. 4. Comparison of monthly mean (\pm SEM) concentrations of prolactin in melatonin-treated mares (solid squares, $n = 11-12$) or those that remained untreated (open circles, $n = 11$). The dashed line depicts the average nighttime melatonin concentration in untreated mares as determined by this laboratory (McManus and Fitzgerald, unpublished observations).

breeding season, the timing of the onset of anestrus is less rigorously controlled. For example, exposure to an abrupt decrease in day length does not consistently advance the timing of the onset of anestrus [15]. In addition, this manipulation of the photoperiod is accompanied by a proportion of mares that fail to cease reproductive activity, which is similar to the reproductive response of mares maintained in a natural photoperiod [16]. The results of our study clearly support the contention that during winter months, a proportion of mares exhibit continuous reproductive activity. The results also confirm that young mares are less likely than mature mares to exhibit this phenomenon [3].

Continuation of reproductive activity during winter months, predominantly in older mares, may reflect an age-related modification of the processes involved in the recognition or translation of the photoperiodic signal. To address this possibility we previously compared the nighttime elevation of melatonin secretion in mares, which continued or ceased reproductive activity during winter months [4]. The results of that study failed to provide convincing evidence to support or refute the hypothesis that continuation of reproductive activity reflects a failure to generate the characteristic nocturnal rise in melatonin. In addition, we have found that both young and mature mares exhibit similar increased concentrations of melatonin during darkness (unpublished observations). At the present time, therefore, our observations do not suggest that reproductive activity during winter months reflects a failure to recognize a reduction in day length; however, the possibility that continuation of reproductive activity may reflect a failure to correctly process the melatonin signal, which codes for day length, has not been directly addressed for this species. Currently, therefore, we cannot entirely exclude the possibility that continuous reproductive activity during the nonbreeding season reflects modification of the detection and translation of photoperiodic cues. We consider this unlikely in view of our earlier findings that this phenomenon cannot be attributed to an individual propensity to cycle unseasonally, or to the occurrence of a cycle during the preceding year. Thus, we contend that any change in photoperiod detection or translation would be increasingly evident from 1

year to the next, but this trend has not been established from our studies.

That constant administration of melatonin failed to modify the timing of anestrus raises an important question; namely, whether this signal provided a nonstimulatory photoperiodic cue or whether it simply blocked recognition of the photoperiod and thus provided no signal. In other species, both responses to constant administration of melatonin have been reported [17, 18]. Unfortunately, this issue could not be determined from the experimental design of the current study, but several considerations suggest that constant administration of melatonin does provide a photoperiodic cue. In this regard, previous studies have shown that constant administration of melatonin blocks the advancement of the onset of the breeding season, which is associated with an abrupt, artificial increase in day length [19]. In addition, using an identical implant to that employed in our studies, but with treatment beginning in July, results from an earlier study demonstrated that the onset of anestrus was advanced in young mares, compared with controls. In that study, however, constant treatment with melatonin failed to modify the timing of anestrus in mature, lactating mares [20]. It is interesting that, whereas the timing of the onset of the breeding season in response to an abrupt, artificial increase in day length can be delayed by constant melatonin, the timing is not modified in melatonin-treated mares exposed to natural increasing day length. Thus, constant treatment with melatonin appears to exert an inhibitory action, but there is considerable variation in the response between various studies. Collectively, the findings from several studies suggest that in certain experimental conditions, constant melatonin provides an inhibitory photoperiodic cue, but this response may be dependent on other interacting factors.

The identity of the factors that may modify the response to melatonin and photoperiod in the mare have yet to be established. The results of this study suggest that, at the onset of anestrus, body condition or fatness, and a hormonal product of fat cells, leptin, may play a role. In mature mares, increased adiposity was associated with higher circulating concentrations of leptin, supporting the widely held view that leptin concentrations reflect body fat mass [21–23]. Furthermore, in the present study, high circulating concentrations of leptin were associated with a significantly greater proportion of mares exhibiting reproductive activity during winter months. The latter observation strongly suggests that leptin, acting as a metabolic signal, may interact in some manner to modify the reproductive response to inhibitory photoperiod. This suggestion is further supported by the finding that in mature mares, ovulation ceased only when leptin levels had fallen during late winter and, at that time, values were comparable to those observed in young, anestrus mares. This observation is the first demonstration for the horse that a decrease in endogenous leptin secretion is associated with cessation of reproductive activity. In addition, the present results are in agreement with a recent study in Siberian hamsters (*Phodopus sungorus sungorus*) that elegantly demonstrated that serum leptin concentrations are linked to gonadal responsiveness to short day lengths [24]. The design of the present study, however, did not permit direct evaluation of whether the decline in leptin was directly related to a decrease in day length. In addition, continuous administration of melatonin did not modify leptin concentrations and, therefore, in the mare, the decline in leptin may reflect changes in energy availability and depletion of stored body fat. This finding also helps to explain

how mares, which are normally considered to be reproductively responsive to increasing day length, may cease reproductive activity during the period January–March when day length is increasing. Our findings do not, however, address whether a critical level of leptin is necessary for the cessation of reproductive activity, nor do they address whether a critical level of leptin is required for the reestablishment of reproductive activity. Indeed, there is accumulating evidence to suggest that leptin indirectly modifies reproductive activity by interaction with other metabolic signals (for a review see [25]). Thus, based on several recent studies, it is unlikely that body fatness and leptin directly modify reproductive activity, but instead may indirectly exert an action, perhaps by modifying the availability of oxidizable metabolic fuels to the brain [26].

In both mature and young mares, circulating concentrations of leptin fluctuated over time and reflected a decline in values during winter months. In both age groups, this decline was mirrored by a decline in body weight and estimated body fat. In consideration of the well-established relationship between adiposity and leptin, the observed reduction in body fat may be causative to the observed decrease in leptin. A reduction in body fat appears unlikely to fully account for the precipitous decline in leptin observed in mature mares during December and early January. In this regard, whereas the decline in leptin was associated with a parallel decrease in body fat, leptin but not body fat levels were similar between mature and young mares between January and early April. The similarity in leptin levels but not percent body fat during this time period suggests that additional factors may contribute to the observed reduction in leptin concentrations. One candidate may be reduced ambient temperature, because several studies have demonstrated a relationship between leptin and low temperature [22]. In addition, photoperiod may also play a role because in the ewe, exposure to short days led to a decrease in leptin secretion, independent of feed intake and body fatness [27]. Similarly, in Djungarian hamsters, short photoperiod reduced leptin gene expression in adipose tissue independently of ambient temperature [28]. In the present study, however, constant administration of melatonin was unaccompanied by a change in the timing of the suppression of leptin secretion during the short day lengths of winter months. At the present time, therefore, an explanation to account for this observation is not clear and warrants further investigation.

In agreement with our earlier findings, constant administration of melatonin modified the secretion of prolactin [11]; specifically, treatment suppressed the increase in prolactin during spring months. In addition, the present results confirm that the suppression of prolactin by exogenous melatonin is restricted to spring months, a finding that we have interpreted to suggest that the prolactin axis develops refractoriness to melatonin treatment. It is currently unknown how the prolactin axis of the mare regains sensitivity to melatonin. It is also unknown whether the reproductive axis similarly develops refractoriness to melatonin, although it has been suggested that this may occur [20].

In summary, the results of this study implicate the involvement of metabolic signals in the regulation of the cessation of reproductive activity in the mare. The identification of an apparent relationship between leptin and reproductive activity suggests that this hormone may play an important role in equine reproduction and further studies are needed to clarify this possibility. Finally, these studies clearly bring into question the importance of a reduction in

day length as a primary signal for the termination of seasonal reproductive activity in mares.

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