

Original article

**Seasonal changes in ovulatory activity,  
plasma prolactin, and melatonin concentrations,  
in Mouflon (*Ovis gmelini musimon*)  
and Manchega (*Ovis aries*) ewes**

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**Abstract** — Seasonal changes in ovulatory activity, plasma prolactin and melatonin concentrations were monitored in a wild (Mouflon) and a domesticated (Manchega) breed of sheep, both originating and living under similar latitudes (40° N). Mouflons express ovarian cycles significantly later than Manchega ewes (October vs. July,  $P < 0.001$ ); however, they ended cycling one month later than Manchegas (April vs. March,  $P < 0.05$ ). While prolactin concentrations were high when Manchega ewes started to cycle, they were at their lowest concentrations when Mouflons started cycling. Overall, mean prolactin concentrations were higher ( $P < 0.001$ ) in Mouflons than Manchegas throughout most of the year. Within the limits of sampling frequency, the duration of melatonin secretion was similar in both groups during the solstice and equinox periods; however, the amplitude was lower ( $P < 0.01$ ) in Mouflons than Manchegas during the solstice periods. The significant breed differences in the seasonal hormonal changes may be attributed to a genetic influence in the endocrine responses to the same photoperiodic cues.

**Mouflon / sheep / breeding season / melatonin / prolactin**

**Résumé** — Variations saisonnières de l'activité ovulatoire, de la prolactine et de la mélatonine plasmatiques chez la femelle Mouflon (*Ovis gmelini musimon*) et la brebis Manchega (*Ovis aries*). Les variations saisonnières de l'activité ovulatoire et des concentrations plasmatiques de prolactine et de mélatonine ont été suivies dans une race ovine sauvage (Mouflon) et une race ovine domestique

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(Manchega), toutes deux originaires et élevées sous la même latitude (40° N). Les Mouflons commencent leur activité cyclique significativement plus tard que les Manchegas (octobre vs. juillet,  $P < 0,001$ ) ; cependant, elles terminent leur activité cyclique un mois plus tard que les Manchegas (avril vs. mars,  $P < 0,05$ ). Alors que les concentrations plasmatiques de prolactine sont élevées quand les Manchegas commencent leur activité sexuelle, elles sont à leurs minima quand les Mouflons débute leurs ovulations. Tout au long de l'année, les concentrations plasmatiques moyennes de prolactine sont plus élevées chez les Mouflons que chez les Manchegas ( $P < 0,001$ ). Avec la méthode de prélèvement utilisée, la durée de sécrétion de mélatonine est identique entre les deux groupes aux solstices et aux équinoxes ; toutefois l'amplitude de la sécrétion de mélatonine nocturne est plus faible chez les Mouflons que chez les Manchegas aux deux solstices ( $P < 0,01$ ). Les différences significatives entre les deux groupes peuvent être attribuées à un effet du patrimoine génétique, puisque toutes les femelles perçoivent le même environnement photopériodique.

#### **Mouflon / ovin / saison sexuelle / ovulation / mélatonine / prolactine**

### **1. INTRODUCTION**

It has long been accepted that the photoperiod is the primary environmental cue controlling seasonal breeding in sheep [11, 20, 47]. The photoperiodic signal is transduced by the pineal gland into a pattern of melatonin secretion which, in turn, provides a critical endocrine signal to regulate secretion of other hormones involved in the onset and termination of the annual breeding season [4, 16]. There is also direct evidence that an endogenous circannual rhythm exists and that the duration of melatonin in circulation synchronises this intrinsic rhythm with the external photoperiod [45].

Marked seasonal changes in the plasma concentrations of prolactin occur in wild and domesticated sheep, with the highest concentrations in the summer and lowest in the winter [23]. Prolactin acts on many target tissues in addition to the mammary gland, and there is experimental evidence indicating a functional role for the seasonal changes in prolactin in the control of gonadotrophin secretion, gonadal activity and sexual behaviour [22, 30, 31]. In closely related species of the deer (red deer, *Cervus elaphus* and Père David's deer, *Elaphurus davidianus*), the earlier mating season of the David's deer is accompanied by a significant advance of the seasonal rhythm of prolactin secretion relative to red deer [26].

However, the presence or extent of causative relationships between the seasonal variation in reproduction and the concentration of prolactin is not clear [9].

In sheep, there is considerable variation in the annual anestrus season depending on the geographical origin of the breed; those breeds that are typically adapted to high latitudes have longer anestrus periods as an adaptive feature to their natural environment. However, breed differences in the duration of the mating season have been reported even at similar latitudes [15]. Genetic selection for a longer mating season and greater prolificacy has produced a wide range of breeds which differ from the wild type in their reproductive physiology [24]. It has long been known that wild sheep have a restricted mating season. The Mouflon is the European wild sheep (*Ovis gmelini musimon*), which originated in the Mediterranean area (Sardinia and Corsica islands) at 40° N, and represents the wild ancestor of domesticated sheep [8, 42]. Although it is known that Mouflons have a short mating season [7, 23] in comparison with most breeds of sheep, the duration of their season of ovulatory activity has not yet been precisely estimated by progesterone.

Considering that genetic adaptation to the natural photoperiod determines the duration of the mating season, the European Mouflon could be a valuable animal to

study. One might assess the influence of the genetic component in the transduction of photoperiodic information by comparing the endocrine responses to the photoperiodic cue and the mating season of the Mouflon with those of highly domesticated breeds of sheep. Thus, the aims of the present study were to characterise the endocrine changes at the onset and end of the mating season in the Mouflon, to compare the duration of the mating season in the Mouflon with that in a domesticated breed originating and living under similar latitudes (40° N), and to compare the seasonal changes in prolactin and melatonin secretion when both genotypes were raised under the same environmental conditions. The Manchega breed was selected for study because it exhibits a short anoestrus season [14] and originates from the Mediterranean area, at a latitude similar to that of the Mouflon.

## 2. MATERIALS AND METHODS

### 2.1. Experimental design and animals

The Mouflon is a small wild sheep (mean adult weight 29 kg) closely related to the Asiatic Mouflon which is the progenitor of all domesticated sheep. This species has been introduced as a wild animal in many European countries. Manchega is a Spanish milking sheep breed. These ewes are medium in size (mean adult weight 60 kg) and are distributed mainly in central Spain around 40° N in latitude.

Eight adult (2–6 years of age) female Mouflons were maintained under natural daylength conditions at a latitude of 40° 25' N, in a 250 m<sup>2</sup> enclosure for 13 months starting on 1 October and finishing on 31 October of the second year. The Mouflons originally came from the “El Hosquillo” National Wildlife Reserve (Cuenca, 40° 6'). A group of 8 adult Manchega ewes (2–6 years of age) were maintained under similar conditions in an adjacent enclosure. Blood samples were collected twice weekly

during 13 months, by jugular venipuncture and centrifuged at 1 500 *g* for 15 min. The plasma was separated and stored at –15 °C until required for progesterone and prolactin analysis. The prolactin analysis was performed only in samples of 4 animals from each group.

In December, 3 Mouflons and 3 Manchega ewes were added to the experimental groups. The pattern of melatonin secretion was assessed in the 11 females from each group, during the winter (22–23 December, 9L:15D) and summer (21–22 June, 15L:9D) solstices and during the spring and fall equinoxes (21–22 March and 22–23 September, 12L:12D). The frequency of blood sampling to define changes in melatonin secretion was every 3 h during the night, and 1 h before and after the onset of darkness and sunrise. A higher frequency in the blood sampling was limited in the wild species because it could induce a major stress. Animals were physically restrained and confined in a small enclosure (6 m<sup>2</sup>) to permit the collection of blood. All dark-phase samples were taken with the help of a dim red torch (< 3 lux), avoiding light in the animals eyes. Blood was collected alternatively, throughout the night, from both right and left jugular veins. The National Observatory of Astronomy in Madrid kindly provided the precise time of dawn and dusk.

### 2.2. Hormone assay

Progesterone determinations were performed in duplicate aliquots of plasma by radioimmunoassay (RIA) according to López-Sebastián et al. [25]. The inter- and intra-assay coefficients of variation were 13.6% (*n* = 6) and 10.4% (*n* = 8) respectively, and the limit of detection of the assay was 0.16 ng·mL<sup>-1</sup>. The mean extraction efficiency was 84.3 ± 3.2% (*n* = 10).

Plasma concentrations of prolactin were determined by RIA in duplicate 100 μL aliquots by a previously described method [14]. The samples were analysed in a

single assay. The assay sensitivity was  $0.2 \text{ ng}\cdot\text{mL}^{-1}$  and the intra-assay coefficient of variation was 9.3% ( $n = 8$ ).

Melatonin concentrations were estimated in duplicate aliquots of  $100 \mu\text{L}$  of blood plasma by radioimmunoassay using the technique of Fraser et al. [13] with an antibody raised by Tillet et al. [39]. The sensitivity of the assay was  $4 \text{ pg}\cdot\text{mL}^{-1}$  of plasma. The inter- and intra-assay coefficients of variation, estimated from plasma pools every 50 unknown samples, were 15% and 9% respectively.

### 2.3. Analysis

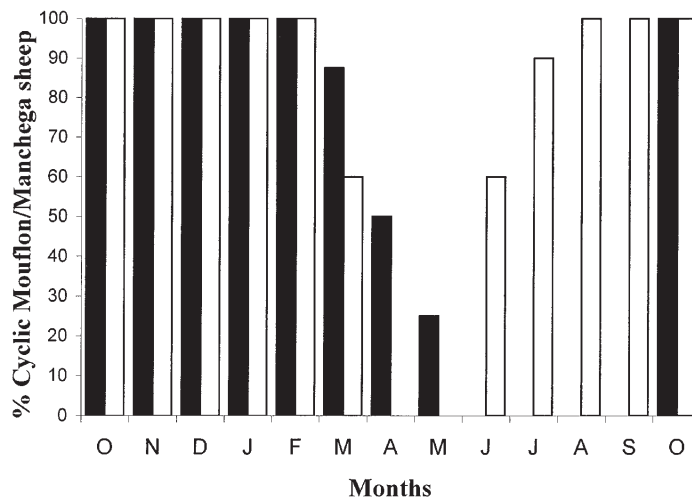
The beginning and duration of ovulatory activity were determined by the appearance of regular cycles of progesterone secretion throughout the experiment. The date of the onset of ovarian cyclicity was the sample date before progesterone rose above  $0.5 \text{ ng}\cdot\text{mL}^{-1}$  in two successive serum samples [12]. The time of the onset and cessation of cyclicity was initially calculated as days relative to 1 January, and was used to determine the mean value for each group; this was then converted to the date with s.e expressed in days. The significance of the

differences between breeds for the times of the onset and cessation of cyclic ovulatory activity, and the duration of the cyclic activity, was compared by ANOVA. Prolactin and melatonin concentrations had a skewed distribution and were therefore transformed to a log scale before analysis; mean hormonal concentrations and differences between groups were compared by ANOVA. The time of the peak and nadir (basal level) of the annual cycle in the concentration of prolactin was calculated for each animal using a three-point moving average [23]. The timing of the increase and attainment of basal levels in prolactin was assessed by comparison with the respective levels at the nadir using a *t*-test. All statistical procedures were performed with the BMDP, Statistical Software, Inc.

## 3. RESULTS

### 3.1. Ovarian cyclical activity of Mouflon and Manchega ewes

The percentage of animals with ovulatory activity, estimated from plasma progesterone measurements, is given in Figure 1. There were significant differences in the



**Figure 1.** Ovarian cyclical activity in Mouflon (■) ( $n = 8$ ) and Manchega sheep (□) ( $n = 8$ ) determined by progesterone concentrations in blood samples collected twice weekly.

mean dates ( $\pm$ s.e.) of the onset of cyclic ovulatory activity between Mouflon and Manchega ewes (October  $18 \pm 4$  days vs. July  $7 \pm 7$  days,  $P < 0.001$ , respectively) as well as in the end of their cyclic activity (April  $9 \pm 12$  days vs. March  $12 \pm 3$  days,  $P < 0.05$ ). However, the end of ovarian cyclical activity in the Mouflon group showed important individual variations, ranging from 24 February until 30 May. The duration of the mating season was shorter in Mouflons than in Manchega sheep ( $173 \pm 13.8$  days vs.  $248 \pm 8.8$  days,  $P < 0.001$ , respectively).

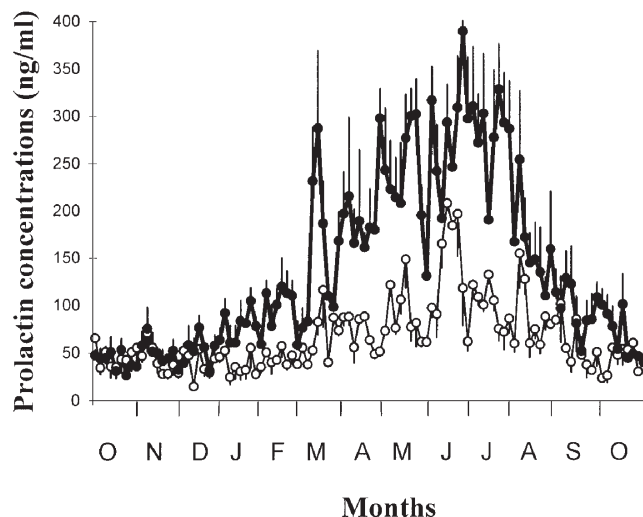
### 3.2. Prolactin concentrations

The seasonal profiles in concentrations of prolactin are illustrated in Figure 2, and followed a trend that was roughly parallel to daylength in both breeds. However, mean concentrations were significantly ( $P < 0.001$ ) higher in Mouflon than in Manchega ewes for every month studied, except for the months of October and November, in which no differences were observed. There were differences between Mouflons and Manchega ewes in the timing of the increase

(January vs. March) and attainment of basal levels (October vs. September). Mean circulating levels were significantly affected by season ( $P < 0.001$ ). In Mouflon, the highest values (mean  $\pm$  s.e) occurred during the spring ( $243 \pm 9.8$  ng·mL<sup>-1</sup>) and summer ( $167.8 \pm 10.7$  ng·mL<sup>-1</sup>), decreasing in the autumn ( $49.8 \pm 2.6$  ng·mL<sup>-1</sup>) and increasing again in the winter ( $102.3 \pm 6.8$  ng·mL<sup>-1</sup>). In Manchega, the highest values occurred during the spring ( $102.7 \pm 7.1$  ng·mL<sup>-1</sup>) and summer ( $73 \pm 5.7$  ng·mL<sup>-1</sup>) and the lowest values were recorded in the autumn and winter ( $44.1 \pm 2.5$  and  $49.3 \pm 3.5$  ng·mL<sup>-1</sup>, respectively).

### 3.3. Melatonin concentrations

The plasma concentrations of melatonin during daytime hours were similar in both groups, and close to the limit of detection of the assay. A pronounced elevation from low daytime levels was recorded 1 h after sunset in both groups, remained elevated during the hours of darkness and declined to daytime levels 1 h after dawn. In Manchega ewes, no difference was found between the mean night-time concentrations of melatonin during the different times of the year.



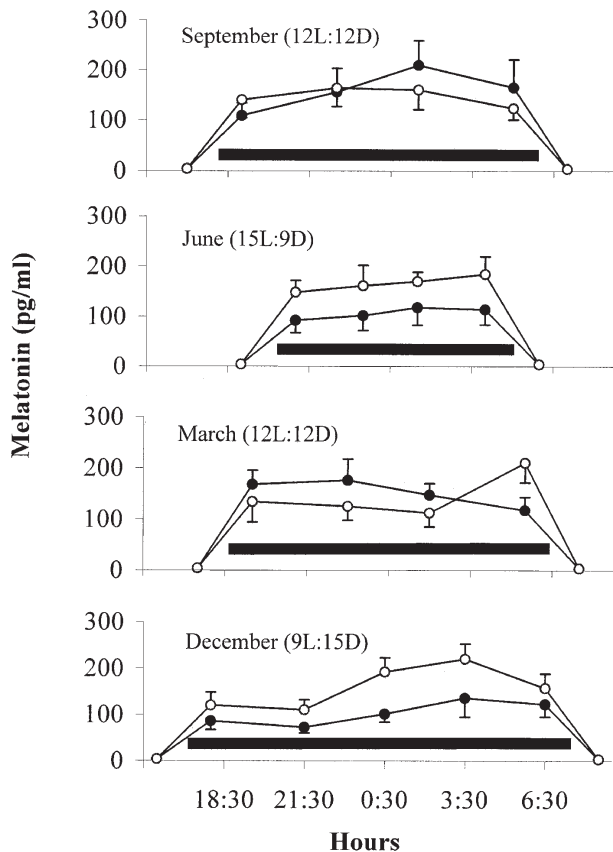
**Figure 2.** Changes in plasma concentrations of prolactin (mean  $\pm$  s.e) throughout the year in Mouflon (●) ( $n = 4$ ) and Manchega sheep (○) ( $n = 4$ ). (Blood samples collected twice weekly.)

For the Mouflon group, however, the amplitude of the nocturnal rise changed with the seasons (Fig. 3), but with important individual variations. The mean night-time concentrations were significantly higher at the spring equinox than during the summer ( $P < 0.05$ ) and winter solstices ( $P < 0.01$ ). This difference also resulted in significantly lower ( $P < 0.01$ ) mean plasma concentrations of melatonin in Mouflons than Manchega ewes for the solstice periods but not for the equinox periods.

#### 4. DISCUSSION

The present study characterised the breeding season of the Mouflon, revealing the wide difference that exists in ovarian

activity between a wild-type and a domesticated breed of sheep, despite the fact that they originate from and live at a similar latitude. The length of the mating season in the Manchega ewe was in agreement with previous studies for this breed [14], and similar to most Spanish breeds originating from the Mediterranean Basin, whose mating season extends from early summer to late winter [40]. The onset of cyclic activity was about three months earlier in the Manchega ewe than in the Mouflon ewe, coinciding with long days. During the course of domestication, selection for an earlier onset of breeding, a trait with reasonably high heritability [2, 36], may have led to an advance of the onset of the mating season in Mediterranean domesticated sheep relative to their wild ancestors. However, it should be noted



**Figure 3.** Day/night patterns of plasma melatonin levels (mean  $\pm$  s.e) in Mouflon (●) ( $n = 11$ ) and Manchega sheep (○) ( $n = 11$ ) during different seasons (summer/winter solstice and autumn/spring equinox). The central area depicts the interval between dusk and dawn. Note that high serum melatonin is only seen during the dark period.

that the dates of onset and offset of the mating season in the Manchega ewe and most breeds of the Mediterranean Basin widely differ from what has been described in the literature for most breeds of sheep from northern latitudes [15], whose mating seasons last from early autumn to late winter.

The onset and cessation of ovulatory cyclic activity in the Mouflon, maintained in a Mediterranean area at its original latitude ( $40^{\circ}$  N), are similar to those of other breeds originating from higher latitudes ( $\geq 50^{\circ}$  N), such as the Scottish Blackface and Finnish Landrace [44]. The relationship between the timing of the mating season and the environment is also illustrated by the breeding patterns in different species of wild sheep; those living at higher latitudes have late rutting seasons [21, 38]. In the Mouflon, the mating season has been established from October until December in latitudes higher than  $40^{\circ}$  N [3, 32]. Our results show that raising the Mouflon under lower latitudes provokes a delay in the onset of the mating season.

In the present experiment, differences in the timing of the reproductive transitions between Mouflons and Manchega ewes exposed to identical photoperiodic condition, clearly suggest the existence of a genetic basis for photoresponsiveness. However, the mechanisms operating in the brain to regulate the breeding season are insufficiently understood to provide a satisfactory explanation. Lincoln et al. [24] suggest that the differences between wild and domestic breeds of sheep may be explained by differences in the central neuroendocrine mechanisms relaying the effects of daylength and controlling the secretion of gonadotropic hormones. Photoperiod is the primary cue for seasonal breeding activity, mediated via the pineal gland and melatonin secretion [1, 5, 16]. Because the pineal gland relays the effects of daylength through the temporal pattern of melatonin secretion and because nocturnal melatonin amplitude was demonstrated to be under a strong genetic

influence [48, 49], differences between genotypes might be expected in this component of the photoperiodic relay. In fact, our results showed that the mean plasma concentrations of melatonin were significantly lower in Mouflon than in Manchega ewes for the solstice periods, but not for the equinox periods, even though plasma concentrations of melatonin varied considerably among individuals as expected at least in sheep [48]. Thus, the differences in the amplitude of nocturnal secretion of melatonin, throughout the year, between Mouflon and Manchega sheep could be a part of the genetic mechanisms of the transduction of the photoperiodic message. This may contribute to the asymmetry between changes in photoperiod and the onset of reproductive response.

In contrast, measurements of the 24-h profiles in the concentrations of melatonin in different breeds studied have shown that there were no differences in the duration of the period of increased melatonin secretion, which reflects the period of darkness (e.g. Merino [17], Ile de France [29], Suffolk [34]). In the current study, in both species, plasma concentrations showed a pronounced elevation from low daytime levels 1 h after sunset, that persisted during the hours of darkness and a decrease to daytime levels 1 h after dawn. These data indicate that it is not the duration of the melatonin signal that differs between breeds. Therefore, it is suggested that a post-pineal mechanism is responsible for the different lag periods between the photoperiodic signal and reproductive activity.

The present results also revealed that plasma prolactin concentrations were consistently higher in Mouflons than Manchega ewes, except for the months of October and November. Prandi et al. [28] and Schillo et al. [35] reported that heat stress may increase prolactin secretion, therefore breeds may differ in the response of prolactin to high ambient temperature. In contrast, other authors have reported higher concentrations

of prolactin in rams of various domesticated sheep than in Mouflon rams [23]. Several factors could explain the discrepancy with this earlier study. Among others, female steroids are known to influence the release of prolactin [10], thus, gender may be a source of variation for prolactin. Also, melatonin can suppress prolactin secretion [27, 41] and Manchega ewes had higher melatonin concentrations during the summer solstice. Low prolactin concentrations that coincide with the onset of ovarian activity have been demonstrated in many studies [18, 37], but there are reports of ovarian activity in the presence of high prolactin values induced by photoperiodic manipulation [46]. Our results show that while prolactin concentrations were high when Manchega ewes started to cycle, they were at low concentrations when Mouflons started cycling, and support the idea that fluctuations in circulating prolactin are not implicated in the onset of cyclic ovulatory activity. Whether prolactin is related to cyclic activity has been quite controversial, and although most investigations have not found a clear relationship [43, 46], the timing of prolactin secretion manipulation may be crucial for an effect on reproductive activity. Indeed, when prolactin was suppressed towards the end of the estrous season, the season was found to be extended [6], whereas suppression of prolactin during the anestrus period did not affect the timing of onset of cyclic activity [19, 33].

In conclusion, this study compared the mating season and the seasonal changes in prolactin and melatonin of a wild and a domesticated breed of sheep, both originating from the Mediterranean Basin. The wide differences in reproductive patterns between Mouflon and Manchega ewes probably results from genetic selection for an earlier mating season. The significant breed differences in the seasonal changes in prolactin and melatonin may be attributed to genetic influences on the transduction of the photoperiodic cues.

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