

Melatonin treatment in winter and spring and reproductive recovery in Sarda breed sheep

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1 **Melatonin treatment in winter and spring and reproductive recovery in Sarda breed sheep**

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12

## 14 **Abstract**

15           This study aimed to evaluate the effect of melatonin treatment carried out between late  
16 winter and early spring on reproductive response in Sarda breed sheep and whether the photo-  
17 refractoriness can influence this reproductive response. The study was conducted on 3,200 adult  
18 ewes, aged 3 to 6 years old, with body condition score (BCS) 2.5-4.0, from 16 commercial sheep  
19 farms in Northern Sardinia. In each farm 200 animals were enrolled and subdivided into 2 groups (n  
20 = 100 each): Group M (treated with one 18 mg melatonin implant), and group C (untreated). The  
21 melatonin treatments were performed on 10<sup>th</sup> February; 10<sup>th</sup> March; 10<sup>th</sup> April and on 10<sup>th</sup> May each  
22 time in 4 different randomly selected farms. Adult males, treated with 3 melatonin implants 1 week  
23 before females, were introduced in each flock 35 days after ewes' treatment, and removed after 45  
24 days of cohabitation with females. Pregnancy was determined by transabdominal ultrasonography  
25 examination between 45<sup>th</sup> and 90<sup>th</sup> day after ram introduction. Data showed that melatonin  
26 treatment increased the fertility rate significantly ( $P<0.05$ ), with the higher fertility rate in the ewes  
27 treated in April and May. The average time in days from male introduction to lambing was shorter  
28 in treated than in control ewes ( $P<0.05$ ). Further, at the 160<sup>th</sup> and 170<sup>th</sup> day after male introduction  
29 the group M showed a higher number of lambed ewes compared to C ( $P<0.01$ ). This effect was  
30 observed at 180<sup>th</sup> and 190<sup>th</sup> days after ram introduction, also, but with lower significance ( $P<0.05$ ).  
31 In conclusion, melatonin treatment improved reproductive efficiency and advanced breeding season  
32 in Sarda sheep, especially when ewes were treated in spring.

33

34 Keywords: Sarda sheep, seasonal reproduction, melatonin implant, fertility

35

## 36 **Introduction**

37           Reproductive seasonality of dairy sheep at the Mediterranean basin latitudes is a major  
38 constraint for production (Chemineau et al., 2007). Furthermore, these areas have a mild climate  
39 during winter and autumn, rainfall mainly concentrated in spring and a very dry summer. Thus, the

40 sheep production cycle is closely linked to pasture growth which occurs predominantly in autumn  
41 and spring (Carcangiu et al., 2011). Therefore, to increase the length of lactation (6-7 months), it is  
42 necessary to mate sheep in spring to obtain lambing in autumn. The timing of reproductive  
43 seasonality in many small ruminant species is controlled by the shortening of photoperiod  
44 (Chemineau et al., 1992) i.e., while the long photoperiods inhibit, the short photoperiods stimulate  
45 the reproductive activity (Bittman and Karsh 1984; Boden and Kennaway, 2006). Melatonin shows  
46 a low blood-concentration during daylight and a high concentration during darkness, thus it can be  
47 considered as an organic informer of the annual photoperiodic trend (Carcangiu et al., 2005,  
48 Carcangiu et al., 2013). Melatonin subcutaneous implants are used in Europe and other parts of the  
49 world, to advance reproductive activity of sheep in the spring (Abecia et al., 2006; Papachristoforou  
50 et al., 2007; deNicolo et al., 2008; Carcangiu et al., 2012). At high latitudes, the administration of  
51 melatonin by slow-release implants is usually performed around the summer solstice (Haresign et  
52 al., 1990), whereas, in the Mediterranean areas, around the spring equinox (Chemineau et al., 1996;  
53 Forcada et al., 1999). Several experiments have been carried out to determine which is the best time  
54 for treatment with melatonin, but the responses vary with breed (Staples et al., 1992; Abecia et al.,  
55 2007). The Sarda sheep (more than 3.0 million ewes farmed in Sardinia) is the main dairy breed in  
56 Italy and its production is parallels the pasture growth pattern (Carta et al., 2009). It exhibits a short  
57 anoestrous period, generally from early February until late March (personal observation).  
58 Administration of melatonin implants has been reported to improved fertility and advance lambing  
59 (Carcangiu et al., 2012; Luridiana et al., 2015). However, even in Sarda sheep the reproductive  
60 responses after treatment with melatonin are sometimes unsatisfactory, probably due to treatment  
61 fallen in the period of refractoriness (Abecia et al., 2007; Sweeney et al., 1997). This study aimed to  
62 evaluate the effect of melatonin treatment carried out between late winter and early spring on  
63 reproductive response in Sarda breed sheep and to determine whether photorefractoriness influences  
64 this response.

65

## 66 **2. Materials and Methods**

### 67 **2.1 Experimental design**

68 This study was conducted on 3,200 Sarda breed ewes from 16 farms, located in North  
69 Sardinia (40°N). In order to minimize the farm effect, farms were selected according to comparable  
70 features such as total number of animals, milk production, flock health, veterinary  
71 recommendations (deworming, vaccinations and use of other pharmacological treatments), nutrition  
72 and reproductive management. All farms were located in the same area, within a 50 km radius of  
73 Sassari. During the day the animals grazed on legumes and grasses and they also received 300 g of  
74 commercial concentrate feed per head (crude protein 20.4% and 12.5 MJ ME/kg DM) during  
75 milking. The sheep were penned during night, and received hay (crude protein 11.1% and 7.2 MJ  
76 ME/kg DM) and water *ad libitum*. Each farm reared approximately 400 sheep, and 200 clinically  
77 healthy ewes were chosen from each farm for the study, accounting for a total of 3,200 head. Ewes  
78 enrolled in the study were lactating, 3 to 6 years old, with body condition score (BCS) 2.5-4.0 and  
79 with a single lamb born between 1<sup>st</sup> November and 10<sup>th</sup> December, 2013. The individual rumen  
80 bolus number was recorded, and each ewe was individually marked with a numbered collar to avoid  
81 mistakes in the animals' identification.

82

### 83 **2.2 Treatment and registration data**

84 On each farm the enrolled animals were randomly subdivided into 2 groups, each containing  
85 100 ewes. The ewes in group M received one melatonin implant (18 mg) (Melovine, CEVA Sanità  
86 Animale, Agrate Brianza, Milano) in the left retro-auricular area; the other group of ewes served as  
87 control and remained untreated (group C). In the first 4 randomly selected farms melatonin implants  
88 were administered on 10<sup>th</sup> February, in the second 4 on 10<sup>th</sup> March, in the third 4 on 10<sup>th</sup> April and  
89 in the last 4 on 10<sup>th</sup> May; controls and treated animals were kept together all the time. Adult rams  
90 (male/female ratio 1/20) were introduced on each farm 35 days after treatment of ewes and  
91 remained in the flock for 45 days. Before ram introduction, ewes were separated from rams for at

92 least 3 months. Rams received 3 melatonin implants, in the left retro-auricular area, 1 week before  
93 ewes. Gestation was diagnosed starting from 45 days and up to 90 days after the rams introduction  
94 by transabdominal ultrasonography examination using Esaote Piemedical Tringa linear equipment  
95 (Esaote Europe B.V., Maastricht, The Netherlands) provided with a 5.0 – 7.5 MHz multiple  
96 frequency linear probe. For each ewe the lambing date and number of new-born lambs were  
97 recorded from 150<sup>th</sup> to 190<sup>th</sup> day after ram introduction.

98

### 99 **2.3 Statistical analysis**

100 All statistical analysis was performed using the computing environment R, version 3.3.2 (R Core  
101 Team, 2016). A logit-link Hierarchical Linear Model (HLM) appropriate for binomial (lambing/not  
102 lambing) data was used to analyse the fertility of different treatment time. Variables considered  
103 were treatment and treatment time. To analyse the effect of treatment time on period in days from  
104 male introduction to lambing a HLM procedure according to the following model was performed.

$$105 Y_{ijk} = \mu + T_m(Pe)_{ij} + \varepsilon_{ijk}$$

106 where  $Y_{ijk}$  is the period in days from male introduction to lambing,  $\mu$  is the overall mean,  $T_m$  is the  
107 fixed effect of treatment,  $Pe$  is the nested time effect within treatment, and  $\varepsilon_{ijk}$  is the error effect.

108 The same model was used to analyse the litter size. A P value <0.05 was considered statistically  
109 significant. Multiple comparisons of the means were performed using Tukey's method.

110

### 111 **3. Results**

112 The number of ewes diagnosed as pregnant differed from the number of lambed ewes of  
113 about 3%. Melatonin treatment increased the fertility rate significantly in the four treatment periods  
114 compared to control group ( $P < 0.05$ ). The higher fertility rate was seen in the ewes treated in April  
115 and May compared to those treated in the other two periods ( $P < 0.05$ ) (Table 1). The average time in  
116 days from male introduction to lambing was shorter in the treated animals compared to controls, in  
117 all the experimental periods (Table1). Melatonin treatment did not affect litter size, and no

118 difference was observed between the treated groups and controls in different periods of observation.  
119 By daily observation of lambing trend, melatonin treated ewes lambbed approximately ten days  
120 earlier compared to controls. At the 160<sup>th</sup> and 170<sup>th</sup> day from male introduction, in all four treatment  
121 periods the treated group showed a higher number of lambbed ewes compared to control (P<0.01).  
122 At the 180<sup>th</sup> and 190<sup>th</sup> day from male introduction the treated ewes exhibited a higher number of  
123 lambbed ewes compared to controls (P<0.05), although the difference between treated and control  
124 group was smaller than in the first 20 days of lambing. In all four treatment times, the lambing peak  
125 was registered at day 170, in treated ewes, and at day 180 from male introduction, in controls (Table  
126 2).

127

#### 128 **4. Discussion**

129 The results from this study show that melatonin was able to advance the reproductive  
130 activity in a dairy sheep. This finding is in agreement with other observations where melatonin  
131 improves the reproductive efficiency in different sheep breeds (Chemineau et al., 1991; Haresign,  
132 1992; Staples et al., 1992; Carcangiu et al., 2012). However, in many experiments melatonin  
133 treatments were performed from the end of April till September, and thus are not completely  
134 comparable to our experiment (Haresign et al., 1990; Forcada et al., 1995; Scott et al., 2009).

135 However, our data indicate that the reproductive response is influenced by the treatment  
136 time; in fact, fertility was higher when the animals were implanted in April and May (early spring)  
137 than in February-March (late winter). This result does not agree with the findings of Abecia et al.  
138 (2007) in three sheep breeds reared in Spain, where fertility of the Rasa Aragonesa breed was not  
139 improved when the administration of melatonin was performed in January and February (winter)  
140 compared to April and May (spring). This finding from Abecia et al. (2007) is also in agreement  
141 with previous study carried out by Forcada et al. (2002) and Zuniga et al. (2002) always on the  
142 Rasa Aragonesa breed .

143 In the Assaf breed melatonin administration in January, February (winter) and May (spring)  
144 increased fertility, but not when it was administered in April (spring). In the same experiment the  
145 administration of melatonin in the Merino breed in February (winter) and April (spring) led to  
146 increased fertility but not in January (winter) and May (spring) (Abecia et al., 2007). This finding is  
147 presumably due to the different sensitivity to photoperiod in the different sheep breeds. Indeed, after  
148 a short-day period, in several sheep breeds, a photo-refractory period occurs, corresponding to the  
149 deep anestrous that can have a different length among sheep breeds (Chemineau et al., 2010).  
150 Therefore the breed differences in response to melatonin and the male effect will impact on  
151 reproductive. Our results show that in the four treatment times, reproductive response was different,  
152 the more effective treatments being those performed in spring, compared to those performed in  
153 winter.

154 The onset of the breeding season is a consequence of the number of long days experienced  
155 by the ewes (Malpaux et al., 1989), and it is possible that our Sarda sheep experienced a number of  
156 long or increasing days sufficient to respond to a short-day treatment such as that resulting from  
157 melatonin implants (O'Callaghan et al., 1994; Malpaux et al., 1997). Sweeney et al. (1997)  
158 postulated that ewes are able to reinitiate reproductive activity in response to 35 long days  
159 (photorefractoriness period) followed by short days or melatonin treatment at any stage between the  
160 winter and summer solstices. In fact in the present study the first melatonin treatment was  
161 performed on February 10, thus approximately 50 days after the winter solstice. It is therefore  
162 plausible to think that the ewes were already at a late-stage of their photorefractoriness period. It is  
163 reasonable to think that the progressive increase in the fertility rate in the other three doses was due  
164 to an increasing sensitivity of the reproductive system to melatonin.

165 In different experiments the reproductive response to the male effect in melatonin treated  
166 ewes was affected by the farm/flock as well (Chemineau et al., 1991; Haresign, 1992; Scott et al.,  
167 2009).



168           The differences in fertility rate found among treated and control ewes are mainly mediated  
169 by the direct effect of melatonin on the ovary (where are located melatonin receptors) (Yie et al.,  
170 1995), as well as by its effect on gonadotropin secretion at pituitary-hypothalamic level (Viguié et  
171 al., 1995). Moreover, in all four treatment times, implanted sheep exhibited a reduced number of  
172 days from ram introduction to lambing than the controls. Subsequently, the treated animals had a  
173 more ready response to the male effect compared to controls. In our experiment a difference  
174 between the melatonin-treated and untreated groups was registered in the lambing periods. That  
175 difference was attributable to the fact that treated ewes were mated within the first 17 days of the  
176 mating period, and had a higher proportion of cyclic ewes at ram introduction than the untreated  
177 group. Then the administration of melatonin was able to stimulate the resumption of reproductive  
178 activity, and this result agrees with the findings from Abecia et al. (2006). Lambing records showed  
179 that the melatonin treated animals exhibited the onset of reproductive activity approximately one  
180 week before the untreated ones. In fact, at the 160<sup>th</sup> and 170<sup>th</sup> day after male introduction the  
181 number of lambed ewes was much higher in treated than in control animals. Conversely, at the 180<sup>th</sup>  
182 and 190<sup>th</sup> day from male introduction the number of lambed ewes increased considerably in control  
183 compared to treated group. This trend probably is related to the "carry-over" effect, that occur when  
184 the treated and control animals are kept together (Abecia et al., 2006). When melatonin treated and  
185 untreated ewes are kept together the onset of reproductive activity occurred earlier in control ewes  
186 as well compared to isolated controls (Kennaway et al. 1987). Moreover, the introduction of estrus  
187 ewes, exposed to controlled photoperiod, to a flock of anestrous ewes triggers reproductive activity,  
188 indicating that female—female social interactions can influence the timing of reproductive  
189 transitions in ewes (O’Callaghan et al., 1994). Establishing the number of sheep in the flock to be  
190 treated to obtain the triggering effect on reproductive activity could be of considerable importance  
191 for farmers, since the treatment of a part of the flock is able to improve the reproductive efficiency  
192 of the entire flock. However, even without an economic calculation, it is clear that in the present  
193 research, the treated ewes had an advance in the onset of lactation compared to controls, and this

194 results in an economic profit for farmers. In fact, the treated animals exhibited on average 10 days  
195 shorter time between male introduction and lambing than controls.

196 The same litter size was observed in treated and control animals in all four treatment times  
197 in this study, as established also in our previous works (Carcangiu et al., 2012; Luridiana et al.,  
198 2015). Our data did not agree with Abecia et al. (2002) and Scott et al. (2009), who found an  
199 increase in litter size in the treated animals. Some other studies found no effect of melatonin  
200 treatment on litter size (Rajkumar et al., 1989; Schoeman and Botha, 1995; Gates et al., 1998).  
201 Moreover, Abecia et al. (2007) after melatonin implants found an increased litter size in Rasa  
202 Aragonesa, but not in Assaf breed. Thus, since Sarda and Assaf are both dairy breeds, we can  
203 hypothesize that the absence of melatonin effect on litter size could be due to the genetic features of  
204 these breeds.

205

## 206 **5. Conclusion**

207 Melatonin treatment improved the reproductive efficiency and advanced the breeding season  
208 in dairy sheep. These effects were more pronounced when ewes were treated in spring than in  
209 winter. So, to obtain an optimal reproductive efficiency in Sarda ewes melatonin implants should be  
210 placed after the spring solstice. The present data also indicate that the period of photorefractoriness  
211 in this breed could be confined between January and early February since the melatonin  
212 administration on February 10 is able to stimulate the reproductive activity.

213

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221

## 222 **Conflict of interest**

223 None of the authors have any conflicts of interest to declare.

224

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Table 1

Fertility rate, litter size and distance in days from rams introduction to parturition (DRIP) in the treated (M) and control (C) ewes in the four period (total ewes n = 3,200).

	M	C		M	C	M	C	
Time	Fertility rate	Fertility rate		Litter size	Litter size	DRIP	DRIP	
10 <sup>th</sup> Febr.	68% <sup>a</sup>	61% <sup>a</sup>	*	1.29	1.18	164.96	175.57	*
10 <sup>th</sup> March	75% <sup>a</sup>	66% <sup>a</sup>	*	1.20	1.22	165.66	174.77	*
10 <sup>th</sup> April	81% <sup>b</sup>	72% <sup>b</sup>	*	1.18	1.17	165.62	175.07	*
10 <sup>th</sup> May	83% <sup>b</sup>	76% <sup>b</sup>	*	1.19	1.22	165.90	175.02	*

Means within a column with different lowercase are significantly different (P<0.05); In group comparison means within a row with \* differ significantly for P<0.05.



Table 2

Cumulative number of lambed ewes from 150<sup>th</sup> to 190<sup>th</sup> day after rams introduction (every 10 days) in the treated (M) and control (C) Sarda breed sheep, in the four observed periods (total ewes n=3,200).

	150-160 DARI <sup>a</sup>			161-170 DARI <sup>a</sup>			171-180 DARI <sup>a</sup>			181-190 DARI <sup>a</sup>		
	M	C		M	C		M	C		M	C	
10 <sup>th</sup> February	83	17	**	213	57	**	253	177	*	272	244	*
10 <sup>th</sup> March	77	21	**	230	68	**	276	201	*	300	264	*
10 <sup>th</sup> April	83	22	**	246	75	**	217	226	*	324	288	*
10 <sup>th</sup> May	83	20	**	248	78	**	302	231	*	332	304	*

<sup>a</sup>DARI = days from rams introduction to parturition. In group comparison means within a row with

\* or \*\* differ significantly for P<0.05 and for P<0.01 respectively.

**Conflict of interest**

None of the authors have any conflicts of interest to declare.