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# OESTRUS INDUCTION IN PRIMIPAROUS LACTATING RABBITS BY A 48 HOURS MOTHER-LITTER SEPARATION: ENDOCRINE AND BEHAVIOURAL RESPONSES

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**Abstract:** Primiparous does express low mating acceptance during lactation and different oestrus induction methods are employed to improve sexual receptivity. The aim of this work was to study the effect of a biostimulation, a 48 h doe-litter separation (DLS), in primiparous lactating rabbits on mating acceptance, external characteristics of the vulva and plasma concentrations of estradiol, testosterone, progesterone and cortisol. Seventy primiparous does from local population were divided on day 1 after kindling into 2 equal groups: control (C, n=35), where does always had free access to the nest-box, and DLS (n=35), where free suckling was adopted, except from day 9 to day 11 post-partum (PP). Litter size was equalised to 6-7 kits. Before mating, blood samples were obtained from 10 does per group on 9$^{th}$ and 11$^{th}$ d post-partum (dpp). The results showed that the DLS treatment improved the mating acceptance on 11$^{th}$ dpp (88.5 *vs.* 54.2%; $P<0.01$) and the frequency of red or pink and turgid vulva (54.3 *vs*. 20%; $P<0.01$). The sexual receptivity of DLS does depended on the time for which the female was presented to the male: 60% refused mating before opening the nest-box, while 80.9% of them accepted the male after the nursing episode. Plasma levels of estradiol, testosterone and progesterone in DLS group rose from 9$^{th}$ to 11$^{th}$ dpp (+44.4; +59.2 and +82%, respectively; $P<0.01$) compared to C group (+6.7; –3.9 and +33.4%, respectively; $P>0.05$). In contrast, cortisol plasma levels were not affected by the DLS treatment (–12.4 and –14.4% for control and DLS group; $P>0.05$). At day 11 PP, higher plasma E2 and P4 levels were described in DLS in comparison to C, although no significant difference was found, while plasma testosterone concentrations tended to be higher in DLS (165.5±27.1 *vs.* 114.9±31.9 pg/mL, for DLS and C, respectively; $P=0.075$). In summary, the 48 h doe-litter-separation acted as an efficient oestrus inductor in primiparous lactating rabbits. Insemination of biostimulated does must occur after the 1$^{st}$ suckling episode following the separation. This biostimulation method did not modify cortisol secretion of the rabbit doe.

**Key Words:** reproduction, biostimulation, rabbit does, doe-litter separation, steroid hormone.

## INTRODUCTION

Lactating rabbits does have low sexual receptivity and reproductive performance, due to the negative effects of prolactin on the ovarian function (Mc Neilly *et al.*, 1982; Rodríguez *et al.* 1989). These negative effects become particularly obvious in the semi-intensive reproductive rhythm. For this reason, hormonal treatment with gonadotropin (eCG) is largely used for the induction of oestrus in commercial European rabbit meat production. To find an alternative to the employment of exogenous hormones, over the last decade "biostimulation" methods for oestrus induction in lactating does have been assayed. These non-hormonal methods (Boiti*,* 1998; Theau-Clément *et al.*, 1998) are essentially based on a transient doe-litter separation or a controlled nursing before insemination. The 48 h doe-litter separation (DLS) or a 2 d controlled nursing on 9$^{th}$ d post-partum (dpp) improved sexual receptivity and, consequently, fertility of the lactating does, and a similar efficiency to eCG has been reported (Alvariño *et al.,* 1998; Maertens*,* 1998; Rebollar *et al.,* 2008). The consequence of DLS is the removal of one suckling episode, since the does usually visit and nurse their litter once a day (Zarrow *et al.,* 1965). The physiological mechanisms whereby this method improves

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sexual receptivity have not yet been clearly defined, and the endocrine response in particular has not been fully explored. On day 11 PP, does stimulated by DLS showed a higher number of follicles greater than 1 mm (Rebollar *et al.,* 2000). The transient separation enhanced estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) plasma concentrations (Ubilla *et al.,* 2000a; Cano *et al.*, 2005) and improved the LH surge after exogenous gonadotropin-releasing hormone (GnRH) injection (Ubilla *et al.,* 2000b), while effects on prolactin blood level lead to contradictory results (Ubilla *et al.*, 2000b; Rebollar *et al.,* 2004b; Cano *et al.*, 2005; Rebollar *et al.,* 2006).

Although sexual receptivity in rabbits is known to be influenced by androgen (Beyer and McDonald, 1973), the effect of DLS on testosterone (T) secretion has not been explored yet. No study has investigated the effect of DLS on adrenal activity, since temporary separation of the mother from her litter could generate social stress in the doe. On the other hand, the effect of DLS on sexual receptivity has rarely been studied in natural mating and the majority of works scored the sexual receptivity on the basis of the external vulvar characteristics and not on that of the mating, which is considered the most reliable method.

Thus, the aim of this work was to determine the effects of DLS on E2, T, progesterone (P4) and cortisol (CORT) plasma levels and on sexual receptivity in primiparous lactating does submitted to natural mating.

## MATERIAL AND METHODS

### *Animals and experimental design*

The trial was conducted in the experimental rabbitry of the National Veterinary School of Algiers. A total of 70 nulliparous does and 18 bucks from a local rabbit population were used. The animals were housed in a naturally ventilated building, maintained under natural light-dark photoperiod. The average ambient temperature varied from 17 to 22°C, and the relative humidity from 62 to 77%. The rabbits were fed *ad libitum* with a commercial pellet diet and had free access to water, with nipple drinkers. The does were reared in individual flat-deck cages in wire mesh (46.5×62×29 cm height) with a closable wooden nest-box (43×26×26 cm height) hung outside the mother's cage. The nulliparous does, weighing about 3.1±0.2 kg, were first mated at the age of 4.5 to 5 mo. On 1$^{st}$ dpp, the litter size were equalised to 6-7 kits, and the does were randomly allotted to one of the 2 experimental groups, with 35 females in each group: Control group (C), where does always have free access to the nest box; 48 h doe-litter separation group (DLS), where free suckling was adopted, except during the 48 h doe-litter separation. The DLS nest-box entry was closed from 11:00 a.m. of 9$^{th}$ dpp to 11:00 a.m. of 11$^{th}$ dpp.

Litter size and weight were determined at 1$^{st}$ dpp after equalisation and at day 9 before treatment. At 11$^{th}$ dpp, all does were presented to a maximum of 3 males until they accepted the mating. To test the influence of suckling on sexual behaviour, DLS does were presented to a maximum of 2 males before the nursing and, if not receptive, again to another buck immediately after nursing. Non receptive does from both groups were presented to bucks again on 12$^{th}$, 13$^{th}$ and 14$^{th}$ dpp until they accepted the mating. Before presentation to the buck, the colour and turgidity of the vulva were checked by visual evaluation. Four colours were attributed (red, pink, purple and white) and 2 levels for turgidity (yes or no), accordingly to the recommendations of the IRRG (2005). The mating frequency for each buck was fixed at 2 matings per day maximum, every 3 or 4 d.

### *Blood sample collection and hormone assays*

Blood samples (4 mL) were collected on 10 females per group, from the marginal ear vein into heparinised tubes and immediately centrifuged at 1000×g for 10 min. Plasma was aliquoted and stored at –20°C until assay. Collection of blood samples was carried out between 10:00 and 11:00 a.m. at 9$^{th}$ and 11$^{th}$ dpp, before presentation to the male, and before the opening of the nest-box in DLS does. In each experimental group, the blood samples were obtained from the same females on 9$^{th}$ and 11$^{th}$ dpp. Plasma E2, P4, T and CORT concentrations were measured in duplicated samples by radioimmunoassay (RIA) methods, using commercial $^{125}$I RIA kits (Immunotech, Beckman Coulter Company: A21854, IM1188, IM1119 and IM1841 for E2, P4, T and CORT respectively). The detection limits were 6 pg/mL for E2, 0.025 ng/mL for T, 0.05 ng/mL for P4 and 10 nM/L (3.62 ng/mL) for CORT. Intra- and inter-assays coefficients of variation (%) were below or equal to 12.1 and 11.2 for E2, 14.8 and 15 for T, 5.8 and 9 for P4, and 5.8 and 9.2 for CORT.

### *Statistical analysis*

All analyses were performed using GraphPad InStat® v.3.10 (2009). Comparison of the 2 groups before experimental treatment (doe weight at kindling, litter size and weight on 1st and 9th dpp) was analysed by Student's t-test. Differences between groups of proportional data were analysed using the Fischer's Exact Test (mating acceptance on 11th dpp, on 12-14th ddp and on 11-14th dpp, percentage of high plasmatic P4 level on 11th dpp, and percentage of functional *corpora lutea* on 11th dpp) and the Chi-square test (distribution of vulva appearance on 11th dpp, and frequency of turgid vulvas on 11th dpp). For the statistical analysis, external vulva characteristics were grouped into 4 categories: 1=red or pink and turgid vulva; 2=red or pink and no turgid vulva; 3=purple vulva; 4=white vulva. Mating acceptance rate was calculated as the number of does accepting the mating per number of observed does×100. The relationships between vulva appearance (the 4 previously named categories) and mating acceptance (accepting the mating or not) were tested using a Chi-square test. The high plasmatic progesterone level was considered for P4>1 ng/mL (Theau-Clément *et al.*, 2008) and the functional *corpora lutea* for P4>2 ng/mL (Mollo *et al.*, 2003). Non-parametric tests for mean comparisons were used for plasma hormone level in each group (Wilcoxon matched-pairs signed ranks test) and between groups (Mann-Whitney Test). Difference were considered significant at $P<0.05$. The values of plasma hormone levels were presented as mean±standard error of the mean (SEM).

## RESULTS AND DISCUSSION

The results showed that before experimental treatment does of both groups nursed similar litters from 1st to 9th dpp (Control: 6.46, 351.2 g and 6.23, 811.4 g; DLS: 6.49, 352.7 g and 6.17, 818.0 g, for litter size and weight on 1st and 9th dpp, respectively).

### *Mating acceptance*

The does of control group showed a low mating acceptance rate at 11th dpp (Table 1). This result is in agreement with previous reports on sexual receptivity in primiparous lactating does at 11th dpp (Maertens, 1998; Szendrö *et al.,* 1999; Theau-Clément *et al.*, 2008). During lactation, primiparous does, in comparison to multiparous ones, display a lower sexual behaviour, perhaps related to a higher negative energy balance (Xiccato, 1996, Castellini *et al.*, 2006). In primiparous lactating does, the highest levels of sexual receptivity are obtained on 1st and 2nd dpp (Díaz *et al.,* 1988). The 48 h doe-litter separation significantly improved the mating acceptance at 11th dpp (88.5 *vs.* 54.2% for DLS and C, respectively; $P<0.01$). Maertens (1998) already reported that 40 h DLS increases the sexual receptivity in primiparous lactating does at 11th dpp (77.8 *vs.* 47.5 %).

In this experiment, mating acceptance of DLS does depended on the moment at which the female was presented to the male: 21 (60%) refused mating before opening the nest-box, while 17 (80.9%) of these 21 females accepted the male immediately after the nursing episode. Bonanno *et al.* (1999) observed that, in does separated 44 h from their litter, the mating acceptance was higher just after nest-box opening. The milk accumulation in the mammary gland

Table 1: Mating acceptance rate and vulva characteristics in primiparous lactating does.

| | Control | DLS |
|---|---|---|
| No. of primiparous does | 35 | 35 |
| Mating acceptance (%): | | |
| 11th dpp | 54.2[a] | 88.5[b] |
| 12-14th dpp | 43.7 | 75 |
| Vulva characteristics at 11th dpp (%): | | |
| Red or pink and turgid vulva | 20.0[a] | 54.3[b] |
| Red or pink, no turgid vulva | 25.7 | 17.1 |
| Purple vulva | 5.7 | 8.6 |
| White vulva | 48.6[a] | 20.0[b] |
| Turgid vulva | 27.5[a] | 54.3[b] |

Different letters in the same row indicate significant difference at $P<0.05$.
Control: free access to the nest box; DLS: 48 h doe-litter separation; dpp: days post-partum.

over 48 h was likely to cause a feeling of discomfort (Rebollar *et al.,* 2004a), which can reduce the libido of the doe. All the DLS does had nursed their litter immediately after the opening of the nest-box. These results confirm that, after a 48 h doe-litter separation, it is better to inseminate the does soon after the suckling period, also in respect to female welfare.

We found that between 12th and 14th dpp the sexual receptivity was more frequent in DLS than in C females, although the difference was not significant, probably due to the low number of does. The contact with the bucks during the previous mating attempts could have stimulated the sexual behaviour of non-receptive DLS and C does. Overall, from 11th to 14th dpp, 97.1% (n=34) of DLS does accepted the mating *vs.* 74.3% (n=26) of C ($P<0.05$). These results confirm that the 48h DLS method acts as an efficient oestrus inductor and synchroniser in primiparous lactating does, so that the majority of the does are in oestrus after this biostimulation.

### *External characteristics of the vulva and relationships with mating acceptance*

At 11th dpp, the vulvar characteristics of C and DLS were different ($P<0.001$; Table 1). The percentage of red or pink and turgid vulva was greater in DLS in comparison to C. The observance of turgid vulva was also more frequent in DLS, while the does of C presented the highest percentage of white vulva. The mating acceptance was maximal when the vulva was red or pink and turgid and minimal when it was white (96.9 *vs.* 22.9 %, respectively; $P<0.001$; Figure 1). Rabbits with red, pink and no turgid vulva, or rabbits with purple vulva, presented a medium sexual receptivity (50 to 65.1%). Based on these results, rabbit sexual receptivity was categorised into 3 levels: high (96.9% of mating acceptance for red or pink and turgid vulva), medium (50-65.1% for red, pink or purple vulva) and low (22.9%) for white vulva). Figure 2 shows that the percentage of does with medium sexual receptivity was not different ($P>0.05$) between C and DLS groups. Díaz *et al.* (1988) observed that mating acceptance was more frequent in lactating does with red and turgid vulva and that the percentage of rejection was high among females with white vulva. If we take into account only the red or pink and turgid vulva as oestrus signs, the sexual receptivity was clearly lower than the real mating acceptance.

### *Hormone assays*

Plasma hormone concentrations at 9th and 11th dpp for both groups are reported in Table 2. Statistical analysis showed that E2, T, P4 and CORT plasma concentrations of C and DLS groups did not differ at 9th dpp ($P>0.05$).

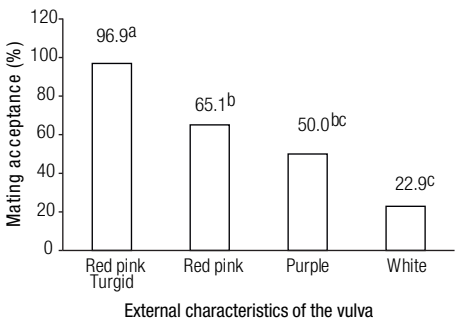


Figure 1: Influence of vulva appearance on mating acceptance in primiparous does at 11th-14th days post-partum (109 observations on 70 does of control and DLS groups.). Control: free access to the nest box; DLS: 48 h doe-litter separation. Values affected with different letters are different at $P<0.05$.

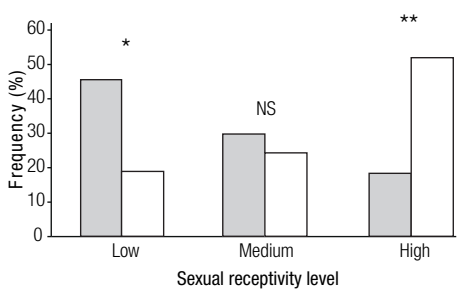


Figure 2: Sexual receptivity (SR) levels at 11th day post-partum in C and DLS groups (low SR for ≤22.9% of mating acceptance; medium SR for 50-65.1% of mating acceptance; high for ≥96.9% of mating acceptance). *: $P<0.05$; **: $P<0.01$; NS: not significant. ▨ Control: free access to the nest box; □ DLS. 48 h doe-litter separation.

Table 2: Estradiol, testosterone, progesterone and cortisol plasma levels in primiparous lactating rabbit does (mean±SEM).

| | 9th dpp | 11th dpp |
|---|---|---|
| Control (n=10) | | |
| Estradiol (pg/mL) | 216.4±30.6 | 230.9±30.2 |
| Testosterone (pg/mL) | 119.6±30.7 | 114.9±31.9 |
| Progesterone (ng/mL) | 0.74±0.12 | 0.99±0.21 |
| Cortisol (ng/mL) | 101.3±6 | 88.7±5.9 |
| DLS (n=10) | | |
| Estradiol (pg/mL) | 200.4±23.6[a] | 289.3±43.2[b] |
| Testosterone (pg/mL) | 103.9±20.9[a] | 165.5±27.1[b] |
| Progesterone (ng/mL) | 0.65±0.08[a] | 1.18±0.26[b] |
| Cortisol (ng/mL) | 111.8±11.2 | 95.6±13.2 |

Control: free access to the nest box; DLS: 48 h doe-litter separation. Different letters in the same row indicate significant difference ($P$<0.05). SEM: standard error of the mean; dpp: days post-partum.

### *Estradiol*

Plasma E2 levels observed in this work were higher than those described throughout lactation period by others authors (11.7±0.3 pg/mL; Ubilla and Rebollar, 1994, 1995). However, they were consistent with those reported previously by Rebollar in lactating rabbit does (Rebollar *et al.,* 2004b, 2006). Plasma E2 levels in DLS group rose from 9th to 11th dpp (+44.4%; $P$<0.01) compared to C (+6.7%; $P$>0.05). Ubilla *et al.* (2000a) and Rebollar *et al.* (2004b, 2006) described similar events in lactating multiparous does. The increased E2 concentrations likely reflect greater follicular steroidogenic activity (Ubilla *et al.,* 2000b). In rabbits, as in the majority of mammal females, oestrogens enhance sexual receptivity. On the other hand, Min *et al.* (2002) noted that estradiol-17-β improved the blood flow in the genital tract of the rabbit doe. This effect probably exerts hyperaemia in the vulva, responsible for its reddish colour and turgid appearance (Plá *et al.,* 1984).

On day 11 PP, higher plasma E2 levels were described in DLS in comparison to C, although the difference was not significant, probably due to the low number of samples analysed. In addition, Rebollar (1992) related that in rabbits a determined plasma E2 concentration was not able to predict sexual receptivity.

### *Testosterone*

At 9th dpp, the does of C and DLS groups had similar T levels (119.6 and 103.9 pg/mL, respectively; $P$>0.05). González-Mariscal *et al.*, (1994) observed similar values in lactating does during the post-partum period. Between 9th and 11th dpp, the DLS does had a significant rise in plasma T concentration (+59.2%; $P$<0.01) compared to C (–3.9%; $P$>0.05). On 11th dpp, and although no significant differences were obtained, values for plasma T levels were higher in DLS in comparison to C ($P$=0.075). To our knowledge, the effect of temporary doe-litter separation on androgen secretion has not been explored in the past. In the rabbit doe, T is produced by the internal theca of the ovarian follicle (Erickson and Ryan, 1976), by the interstitial glands of the ovary (Hilliard *et al.*, 1974) and by the cortical area of the adrenals (Kolanowski *et al.,* 1985). In the rabbit, ovarian venous T output was increased after LH injection (Bahr, 1978). T plays a part in the regulation of follicular development (Armstrong *et al.*, 1978). In rabbit females, androgens induce sexual receptivity through their conversion to oestrogens (Beyer and McDonald, 1973). Thus, the rise in plasma T level we found here after the DLS may have helped improve the mating acceptance.

### *Progesterone*

The mean P4 values recorded in our study at 11th dpp were similar to those obtained by Arias-Álvarez *et al.* (2010) in primiparous lactating does on 11th dpp after 24 h DLS (0.90 ng/mL). Plasma P4 levels on 11th ddp were not statistically different between C and DLS. However, the DLS does showed a significant increase ($P$<0.01) in plasma P4 levels from day 9 to day 11 PP, compared to C does (Table 3). These results are not in agreement with those reported by Ubilla *et al.* (2001) who did not find any change in P4 plasma levels in multiparous does. Our results may be partially attributed to the does with "high P4 syndrome" (P4>1 ng/mL), which is more frequent in primiparous

than in multiparous does (32.5 *vs.* 4.0%, respectively; Theau-Clément *et al.,* 2005) and more frequently observed in lactating primiparous does than in non-lactating ones (36.5 *vs.* 18.9%, respectively; Theau-Clément *et al.,* 2008). In this study, the mean plasma P4 levels recorded in C and DLS were however lower than those described by Theau-Clément *et al.* (2008) in primiparous lactating does on 11th dpp (0.99±0.21 and 1.18±0.26 *vs.* 2.5±3.8 ng/mL, respectively).

In the present work, the percentage of does which had high P4 plasma levels at 11th dpp was 40% for C and 30% for the DLS, but this difference was not significant ($P>0.05$). In both groups, the percentage of does with functional *corpora lutea* (P4>2 ng/mL; Mollo *et al.*, 2003) at 11th dpp was 10%. The high P4 levels at mating depress reproductive performance of the rabbit doe. Sexual behaviour is highly related to P4 plasma levels and shows a significant decrease for P4 levels greater than 6 ng/mL (Theau-Clément *et al.,* 2008). The origin of high plasma P4 in non-inseminated lactating does is likely due to spontaneous ovulation (Boiti *et al.,* 1996). An alternative explanation is that mild stress activates the hypothalamus-pituitary-adrenal axis to cause a small progesterone release by the adrenal (Boiti and Canali, 1988; Boiti *et al.*, 2006). The DLS technique may probably act as mild stressor for the lactating doe, thus inducing progesterone secretion. According to what we know, no studies have described the behaviour of rabbit does during a DLS. In our study, the majority of the females attempt to open the nest-box immediately after and throughout the separation.

### *Cortisol*

On 9th dpp, the plasma levels of CORT in the C and DLS groups were similar (101.3 and 111.8 ng/mL, respectively). These concentrations were higher than those reported in non lactating rabbits (Szeto *et al.,* 2004). In rabbits, CORT follows a circadian rhythm with a peak occurring at 12:00 a.m. and a nadir at approximately 06:00 a.m. Corticosterone and CORT represent a good index of the stress response in rabbit (Szeto *et al.,* 2004). In our study, blood CORT levels did not vary in any group between 9th and 11th dpp (–12.4 and –14.4%, for C and DLS groups, respectively; $P>0.05$). Mean plasma CORT concentrations observed in both groups on 11th dpp were also similar. Thus, the stress induced by DLS was not effective in modifying cortisol secretion in biostimulated does. Several studies report hyporesponsiveness to stressful stimuli in lactating females, particularly in rat and human (Slattery and Neumann, 2008). The stress response and the sensitivity of the hypothalamic-pituitary-adrenal axis have not been fully explored in lactating rabbits.

## CONCLUSION

The present results showed that DLS increases the mating receptivity of primiparous lactating does (+34.3%) submitted to a semi-intensive reproduction rhythm. After 48-h doe-litter separation, the presentation to the buck must occur after the nursing episode to improve the mating acceptance rate, while also respecting female welfare.

Between 9th and 11th dpp, the DLS does had a significant rise in plasma T and E2 concentrations compared to C. As with oestrogens, even androgen could play an important part in the oestrus induction after transient doe-litter separation. In this work, the does of both groups showed, at the end of the treatment, an increase in P4 plasma concentration that was significant in the biostimulated does. It is important to note that under our experimental conditions no significant difference in plasma concentrations of E2 and P4 was observed in the various groups at day 11 PP, while T plasma levels tended to be higher in DLS. Surprisingly, the DLS did not modify plasma CORT level. Further research is needed to explore the effect of the DLS on the adrenal response of the doe.

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