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PITUITARY AND OVARIAN HORMONES: IS THEIR PLASMA CONCENTRATION AFFECTED BY LITTER SIZE IN PRIMIPAROUS LACTATING RABBIT DOES?

FERNÁNDEZ-PACHECO C.*, MILLÁN P.*, RODRÍGUEZ M.†‡, FORMOSO-RAFFERTY N.†, VELASCO B.†, REBOLLAR P.G.†

*Department of Physiology, Veterinary Faculty, Complutense University of Madrid, Avda. Puerta de Hierro s/n, 28040, Madrid, Spain.
†Department of Agrarian Production, ETSIAAB, Polytechnic University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
‡Department of R+D & projects, PigCHAMP Pro Europa S.L., Segovia, Spain.

Abstract: Genetic selection in commercial rabbit lines based on litter size has positively improved the number of kits suckling, presumably to weaning. Although it has been proven that the energetic balance of primiparous does is due to the need to satisfy pregnancy, lactation and growth requirements, litter size adjustment from 7 to 12 kits is applied as a routine in commercial rabbit farms. The suckling stimulus provokes a prolactin (PRL) secretion, which in turn can modulate the preovulatory release of luteinising hormone (LH) and, consequently, the ovulatory and productive responses of the does. This study aimed to determine if litter size of prolific primiparous rabbit does during lactation [Group HL, with high litter density (10-12 kits; n=21) and Group LL, with low litter density (7-9 kits; n=29)] influences plasma concentration of PRL. Blood samples from lactating does were taken weekly throughout lactation starting on day 4 post-partum, until day 32 post-partum, before and immediately after suckling. In addition, the does were re-inseminated after weaning (day 32 post-partum), and sampled at 0 and 60 min after induction of ovulation to determine whether litter size affected the peak of LH, progesterone (P4) concentrations and the main productive parameters of their second pregnancy. All hormones were determined by enzyme immunoassay. Statistical analysis of the results revealed that the PRL concentrations of hyperprolific rabbit does before and immediately after a suckling stimulus from 7-9 or 10-12 kits were significanltly different, as we only detected basal levels, with a rise after weaning in both groups. More studies are necessary, delaying blood sampling to later periods of time after the suckling stimulus, in order to conclude whether the peak release of this hormone is altered or not. There were also no differences in plasma LH and progesterone levels after artificial insemination, or in productive performance of these females after their second pregnancy. In conclusion, the litter size adjustment of prolific primiparous rabbits with 7 to 12 kits determines adequate pituitary, ovarian and reproductive responses at second parturition if the does are inseminated after weaning.

Key Words: rabbit, doe, litter size, prolactin, progesterone, LH, productive performance.

INTRODUCTION

Lactation is a critical moment in cuniculture production, as the future viability and performance of the kits are directly influenced by milk intake during their first weeks of life (Lebas, 1972). The onset of maternal behaviour in rabbit does occurs before parturition (progesterone decreases, and oestradiol rises), while oxytocin and prolactin (PRL) are secreted (McNeilly and Friessen, 1978). Hudson and Distel (1989) noted that the nursing events of the does follow a circadian periodicity and occur most frequently between midnight and 4:00 a.m., depending on a threshold of suckling stimulation (González-Mariscal *et al.*, 2013). Circadian rhythms in the doe and her litter are synchronised, so that body temperature of kits rises before suckling, and they are able to anticipate the mother coming, increasing their activity in order to prepare for the competitive situation that suckling represents (Jilge *et al.*, 2000). Oxytocin and

Correspondence: P.G. Rebollar, *pilar.grebollar@upm.es.* Received *February 2021* - Accepted *July 2021.*
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PRL are secreted in high concentrations as a consequence of suckling and, thereby, milk production is also affected by maternal hormonal levels inducted by suction reflex (Freeman *et al.*, 2000), whose magnitude could be different depending on the number of lactating offspring.

Genetic selection programmes for reproductive traits have improved the prolificacy of artificially inseminated (AI) rabbit hybrids used in commercial farms (Pascual *et al.*, 2013). Rabbit females have increased their capability to avail body reserves in order to guarantee their offspring nutrition, although metabolic and inflammatory changes affect them during the lactation period (Minuti *et al.*, 2015). As the doe only has ten mammary glands, the adoption of young kits after parturition to homogenise litter size is routine practice in commercial rabbit farms. This procedure is necessary to ease the load on the most prolific rabbits (Lebas *et al.*, 1991), to reduce the mortality of young rabbits, particularly those with lower birth weight in large litter sizes (Szendrő and Maertens, 2001), and to achieve homogeneous slaughter weights (Arrington and Kelley, 1984). Usually, even in non-industrial farms, the litters can be homogenised to different sizes if necessary, especially from 7 to 9 kits (González-Redondo *et al.*, 2010) or from 10 to 11 kits (Rodríguez *et al.*, 2018).

Furthermore, lactation is a highly challenging period for rabbit does in terms of physiology (Castellini, 2007), particularly in primiparous ones. The feed intake capacity of primiparous does is lower than in multiparous females, creating a negative energy balance in the first lactation that increases replacement rates (Xiccato, 1996). Intensive or semi-intensive reproductive rhythms (AI on day 4 or 11 post-partum) applied to these lactating females leave them unable to cover the needs for lactation and growth (Castellini *et al.*, 2003; Rebollar *et al.*, 2006). Previous studies have shown that applying an extensive production system (AI after weaning) in primiparous rabbit does considerably improves body condition of the does, thus achieving an optimal metabolic state to endure the following pregnancies (Arias-Álvarez *et al.*, 2009; Rebollar *et al.*, 2009).

The reproductive hormone concentrations such as PRL, LH and oestradiol of the rabbit have previously been described at mating (Ubilla *et al.*, 2000a) in lactating females (González-Mariscal *et al.*, 1994; Ubilla and Rebollar, 1995; Ubilla *et al.*, 2000b). In addition, progesterone concentrations have also been occasionally described in certain periods of pregnancy (Ubilla *et al.*, 1992; Rodríguez *et al.*, 2018) and across pregnancy and lactation (González-Mariscal *et al.*, 1994). Plasma concentrations of PRL at mating can affect the ovulation rate (OR) of does, and females with OR>15 corpora lutea have lower PRL and higher LH concentrations than females with OR≤15 (Muelas *et al.*, 2008). However, it has not been studied whether this pituitary response could change in prolific lactating rabbits with a prolificacy higher or lower than ten kits. On the other hand, although the most important physiological roles of PRL in mammals are milk synthesis and mammary gland development, this hormone promotes a great variety of functions because it is not only synthetised in the pituitary gland, but also within the central nervous system, the immune system, the uterus and its associated tissues at conception, and even in the mammary gland itself (reviewed by Freeman *et al.*, 2000). This implies that PRL release can be linked to a plethora of stimuli other than suckling, such as light, variations of day length in seasonal mammals, specific sounds, olfactory stimuli and stress.

Thus, our target was to determine whether a litter size of 7-9 or 10-12 kits during the first lactation of prolific primiparous rabbit females could have any effect on: 1) PRL plasma concentrations before and immediately after suckling, and after weaning. 2) LH and progesterone plasma concentrations after the ovulation induction at second AI after weaning. 3) Productive performance in the second productive cycle.

MATERIAL AND METHODS

The study was performed according to the Spanish Policy for Animal Protection RD533/2013 and specifically assessed and approved by the Polytechnic University of Madrid Committee of Ethics in Animal Research and by the Community of Madrid (PROEX 302/15).

New Zealand x California rabbits were fed *ad libitum* with a commercial diet (2400 kcal of digestible energy/kg, 35% neutral detergent fibre and 16% crude protein, NANTA, Madrid, Spain) throughout the study. The experimental design is shown in Figure 1. At first parturition, in a total of 50 primiparous does, prolificacy (liveborn and stillborn) and litter weight were recorded. These does were allocated into two different experimental groups according to a litter density adjustment, adding or removing no more than two kits per litter without homogenisation of the kits' weights:

- Group HL (high litter density; n=21): dams with a prolificacy at first parturition of 11.80±0.33 born alive, 0.19±0.09 stillborn and a litter weight of 527.90±17.70 g, whose litter size was adjusted to 10-12 kits.

- Group LL (low litter density; n=29): dams with a prolificacy at first parturition of 9.1±0.45 born alive, 0.34±0.10 stillborn and a litter weight of 430.3±18.6 g, whose litter size was adjusted to 7-9 kits.

Dams and litters were checked daily, recording the number of does or kits dead during the lactation period and the litter weight at weaning.

During lactation, in a random subsample of 12 animals (6 HL and 6 LL) plasma PRL concentrations were measured in blood samples collected from the central ear artery (2.5 mL) with 23 G needles before and after suckling. Plasma was obtained after centrifugation at 1200 *g* for 10 min at 4°C and stored at –20°C until analysed. Samples were taken weekly at day 4, 11, 19, 26 and 32 post-partum. To this end, the nest was closed overnight and reopened after the first sampling, allowing kits to suckle and taking the second sample when it was over. As indicated in previous studies (Fuchs *et al.*, 1984 and Ubilla *et al.*, 2000a), plasma PRL concentrations do not reach peak values until 1-5 min after suckling, which was the time we estimated that sequentially passed since the mothers voluntarily left the nest after suckling, we extracted them from their cage, put them in a block, and finally, obtained the blood sample. In these same 6 animals, in order to maintain the litter size during the study, dead kits were replaced by others of the same age taken from dams of the same farm.

At day 32 post-partum, kits were weaned and, immediately after that, all does were artificially inseminated (AI) with fresh diluted semen (MA 24, Ovejero, León, Spain) and ovulation was induced with gonadorelin (20 µg/doe, i.m.; Inducel-GnRH, Ovejero, León, Spain). At this point, two blood samples were taken at 0 and 60 min after AI from the same 12 rabbit females (6 HL and 6 LL) to determine plasma LH surge, according to the method described by Rebollar *et al.* (2012), and plasma progesterone using a commercial kit (Demeditec Diagnostics GmbH, Germany), respectively. Later, does were sampled again on days 1 and 5 post-weaning or post-AI. Plasma PRL levels were determined in all samples taken throughout the study using a commercial immunoassay kit (Rabbit PRL CEA846Rb, Cloud-Clone Corp, CCC, USA), the detection range of the assay ranging from 2.47 ng/mL to 200 ng/mL, with assay sensitivity 0.92 ng/mL and the inter-assay coefficient of variation (CV) 7.2%. For LH and progesterone, the detection ranges were 5 to 125 ng/mL and 0.3 to 40 ng/mL, the assay sensitivity scores were 0.78 ng/mL and 0.045 ng/mL, and the inter-assay CV were 5.9 and 5.7%, respectively.

After second parturition, the following productive parameters were recorded in both experimental groups: fertility [(number of pregnant does/number of artificially inseminated) x 100)], prolificacy (liveborn and stillborn), litter size and litter weight at weaning.

For the statistical analysis of the results, the SAS software was used (Statistical Analysis System Institute Inc; Cary, NC, USA, 2001). The experimental unit was the rabbit doe. Productive parameters at the end of first lactation (weaned

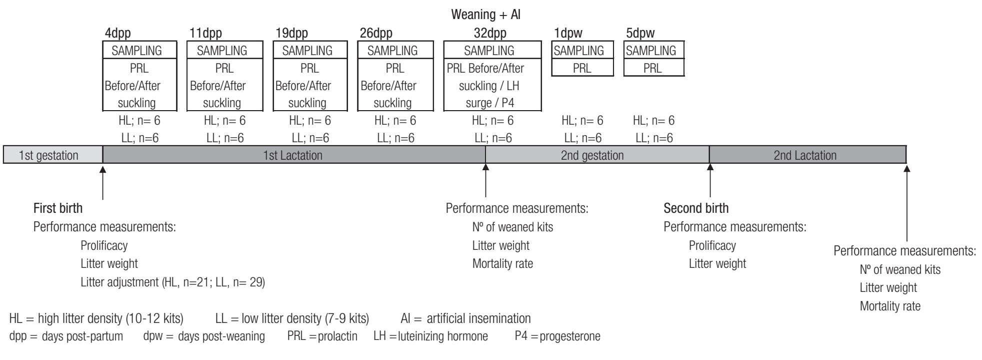

Figure 1: Timeline representation of the experimental design.

kits, litter weight, mortality rate) were analysed considering the litter size adjustment (HL and LL) as the main effect. To study PRL concentrations, a repeated-measures analysis of variance was performed, considering as main effects the treatment (litter size), the time (before and after suckling on days 4, 11, 19, 26, and 32 of lactation, and days 1 and 5 after weaning), and the interaction between both effects. For LH and P4, the main effects considered were the treatment (litter size) and the time (minute 0 and 60 after AI). To analyse productive parameters at second parturition, the main effect considered was the litter size during the previous lactation. If significant main effects were detected, a *t-Student test* was used to compare the means, considering the existence of significant differences for a *P*-value<0.05.

RESULTS

At the end of the first lactation, as a result of litter adjustment, HL does weaned more kits than LL ones (11.22±0.21 and 7.55±0.39 kits, respectively; *P*<0.0001), with higher litter weight (6294±155 and 4655±251 g, respectively; *P*<0.0001), but lighter kits (560.1±15.4 and 637.5±25.9 g, respectively, *P*=0.024). Likewise, the mortality rates at the end of the study were 4.5 and 12.5% of the kits (*P*=0.037) in HL and LL groups, respectively. Furthermore, three does from the HL group and one of the LL group died at the end of the experiment.

The interaction between the two studied effects (litter size and time) on PRL concentrations is shown in Figure 2. During lactation, no significant differences were observed regarding the plasma concentrations of PRL before and immediately after suckling (*P*=0.2520), and the litter size did not affect the plasma concentrations of PRL of the mothers either (*P*=0.7977).

On the other hand, we detected significant differences over time (*P*=0.0003), as in days 1 and 5 after weaning, PRL concentrations in the mothers increased in relation to the pre-weaning period.

Litter size did not affect plasma concentrations of LH (*P*=0.9948) and P4 (*P*=0.8600), but there were differences in relation to the time of sampling (0 *vs*. 60 min after AI) in both hormones (LH; *P*=0.0383 and P4; *P*=0.0138, respectively) (Figure 3).

According to the litter size during the first lactation, results regarding the productive performance of the second productive cycle are shown in Table 1. Litter size did not entail significant differences between groups in any of the productive parameters recorded.

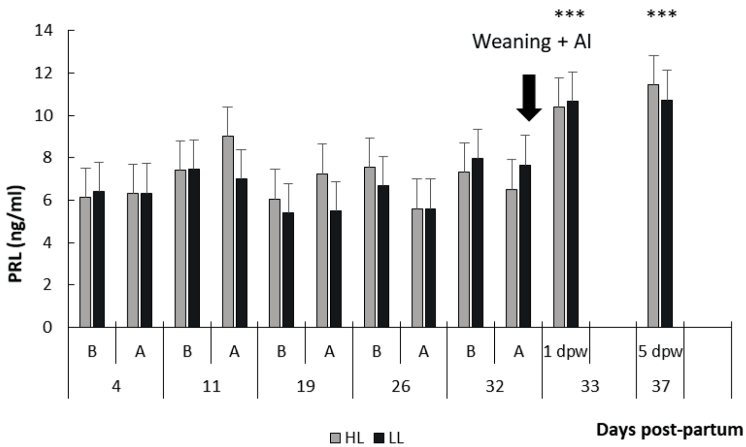

Figure 2: Prolactin (PRL) plasma concentrations before (B) and after (A) suckling of lactating primiparous rabbit does with 10-12 kits (HL; n=6) and 7-9 kits (LL; n=6) and after weaning. AI: artificial insemination. HL: high litter density (10-12 kits). LL: low litter density (7-9 kits). dpw: days post-weaning. ***: *P*=0.0003.

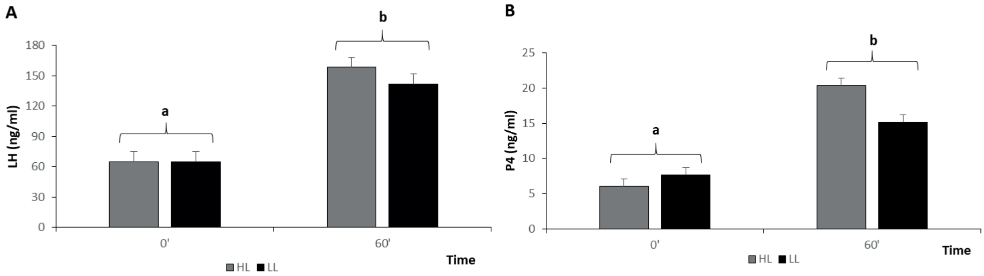

Figure 3: A) Luteinizing hormone (LH) and B) progesterone (P4) plasma concentrations 0 and 60 min after artificial insemination of rabbit does with 10-12 kits (HL; n=6) and with 7-9 kits (LL; n=6) in their previous lactation. a,b: indicate differences between 0 and 60' (*P*<0.05). HL: high litter density (10-12 kits). LL: low litter density (7-9 kits).

DISCUSSION

The differences in litter size and litter weight at first weaning between HL and LL groups were due to the adjustment applied at the start of the lactation period. Mortality rate of the kits during lactation was higher in the LL than in HL group. Although it would be supposed that in LL group, their feeding might have been better due to a less competition between the kits, Poigner *et al.* (2000) determined that heterogeneous birth weights within the litter could have an important influence on mortality rate of kits during the lactation period. In this sense, in the current study, prior to the adjustment, mean±standard deviation of birth weights of kits from HL does was 45.2±6.86 g and from LL does was 49.1±9.48 g, respectively. This implies that in the current study, by not considering the homogenisation of the weights of the kits within the litter, the mortality rate could be affected between groups beacuse the weight differences between the kits of the LL group was greater than that of the HL group kits.

Concerning the mothers' mortality, it could be due to metabolic diseases. Minuti *et al.* (2015) studied the metabolic and biochemical status of does selected for high prolificacy, comparing two groups of does according to the litter size (with >10 or <10 kits), and observed evident differences between them before parturition, which disappeared 4 and 12 d post-partum. Other authors (Xiccato *et al.*, 1999; Mocé *et al.*, 2004) described that energy depletion in mothers could lead to liver diseases and gestational toxaemia when lactation and gestation overlap. Although it was not the case in our study because does were only lactating, negative energy balance and consequent metabolic disease could explain the death of three does in the HL group, which were probably

Table 1: Productive performance (mean±standard error of mean) at 2nd parturition of rabbits with high (HL: 10-12 kits) and low litter size (LL: 7-9 kits) in their previous lactation and inseminated after weaning.

	HL	LL	*P*-value
n	21	29	
Fertility (%)	66.70±11.29	67.90±9.060	0.9348
Prolificacy			
Liveborn	12.64±0.73	11.44±0.61	0.2177
Stillborn	0.00±0.37	0.81±0.31	0.1068
Weaned	10.64±0.61	10.40±0.51	0.7442
Litter weight (g)			
At birth	786.93±43.72	702.70±36.25	0.1506
At weaning	7562±443.55	7086±350.65	0.4079
Mortality[1] (%)	19.05±7.74	10.71±6.71	0.4200

[1]Percentage of dead kits per litter.

in a more demanding condition than those of the LL group. Primiparous does undergo a complex energy balance between day 25 of gestation and the 3rd day of lactation (Xiccato and Trocino, 2010) which, in healthy animals, should be overcome entirely on day 4 post-partum (de la Fuente and Rosell, 2012). Considering that the deaths of does occurred after weaning in the HL group, it could be assumed that there may be underlying diseases that went unnoticed and did not allow the females a full recovery of their body condition and energy balance during lactation.

The results obtained in this study for PRL concentrations confirm that prolific rabbit females have a similar pituitary response immediately after the suckling stimulus elicited for 7-9 kits or for 10-12 kits. Rebollar *et al.* (2008) determined similar PRL concentrations using transitory separation of the litter 24 h before AI to improve ovarian activity as an oestrus synchronisation method. This procedure does not seem to modify pituitary PRL secretion, nor the apparent synchrony between doe and kits. Besides, Fuchs *et al.* (1984) already determined that while oxytocin levels rise quickly during suckling and decline to basal level shortly thereafter, the increase in plasma PRL concentrations is delayed until 1-5 min after suckling has ceased at the beginning of lactation, and in mid-lactation PRL reaches peak levels which plateau for 2-3 h. Although in the current study the sampling was designed and applied following these premises, we were not able to determine the increase in plasma PRL concentrations cited by Fuchs *et al.* (1984), and only basal levels were detected. Therefore, more studies are needed in which the blood sampling is carried out later with respect to the time of suckling and, in this way, to be able to detect differences in the response of mothers with different numbers of offspring.

An increase in plasma PRL after weaning was observed. If females had been pregnant at weaning (in case AI had been performed after parturition), milk production would have abruptly ceased on day 26 of lactation, and the does would have stopped entering the nest (Lincoln, 1974). However, in the current experiment, lactation and gestation did not overlap, resulting in the continuity of maternal behaviour until day 32 post-partum. According to González-Mariscal *et al.* (2016), maternal conduct continues up to day 40 post-partum if the kits keep suckling. Although in our study the suckling stimulus suddenly disappeared on day 32 post-partum (weaning day), PRL continued to be elevated on days 33 and 37, which suggests that maternal actions would have still been present. On the other hand, it is known that PRL is involved in stressful situations (Seggie and Brown, 1975 and Kant *et al.*, 1983). In the current study, the weaning and AI were applied on day 32 post-partum, and Argente *et al.* (2014) have described high cortisol concentrations in rabbit does at the end of lactation as a stress indicator, which could explain the rise in PRL detected on days 33 and 37.

The preovulatory peak of LH and progesterone concentrations determined after AI were similar to those described in previous experimental studies (Rebollar *et al.*, 2012, 2014). The application of AI post-weaning in primiparous does as previous studies suggest (Arias-Álvarez *et al.*, 2009) has, consequently, afforded a satisfactory productive performance of these prolific primiparous rabbit does, similar to those described in previous works (Rebollar *et al.*, 2008).

CONCLUSIONS

In conclusion, these results suggest that the suckling stimuli of litters with 7-9 or 10-12 kits in primiparous does during the first lactation could have the same consequences on plasma LH and P4 during ovulation, and on productive performance in the second parturition. However, to detect an effective suckling-induced stimulus and evaluate whether differences in litter size are associated (or not) with putative differences in PRL secretion, more delayed periods of sampling after suckling (>5 min) are needed. The main key for a suitable productive performance is the application of an extensive production rhythm which fits the energy needs and physiological situation of prolific lactating rabbits.

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