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IMPACT OF CONCURRENT PREGNANCY AND LACTATION ON MATERNAL NEST-BUILDING, ESTRADIOL AND PROGESTERONE CONCENTRATIONS IN RABBITS

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Abtract: We evaluated the impact of concurrent pregnancy and lactation on: nest-building (i.e., digging, straw-carrying, hair-pulling), food intake, milk output, body weight, and the concentration of estradiol and progesterone in blood. Digging was lower in pregnant-lactating (PL) rabbits, compared with pregnant-only (PO) does, on 21-23 d (52±64 *vs.* 104±86 g, respectively; mean±SD; $P<0.05$). Straw-carrying was also reduced in PL does on 24-26 d (9±27 *vs.* 79±94 g; $P<0.005$), 27-29 (27±56 *vs.* 99±77 g; $P<0.005$), and in the total amount of material introduced into the nest box (132±167 *vs.* 286±217 g; $P<0.02$). Hair-pulling was expressed by practically all animals. Food intake declined in PO does on the three days preceding parturition ($P<0.01$) and increased markedly during lactation; this increase was much larger in PL than in lactating-only (LO) rabbits ($P<0.01$). Milk output was similar between PL and LO does during the first 21 d of lactation but a marked decline in this parameter occurred in PL does from then until 30 d. The differences in nest-building between PL and PO rabbits may be related to the concentrations of estradiol and progesterone on specific days of pregnancy. PL does showed significantly higher estradiol levels than PO animals on pregnancy 1 d (33±13 *vs.* 23±4 pg/mL; $P<0.02$) and 21 (34±19 *vs.* 24±6 pg/mL; $P<0.05$) and also higher levels of progesterone on pregnancy 1 d (4±5 *vs.* 1±2 ng/mL; $P<0.05$). However, PL rabbits had lower levels of progesterone on 7 d (6±3 *vs.* 9±2 ng/mL; $P<0.02$) and 14 d (8±3 *vs.* 11±3 ng/mL; $P<0.005$) than PO does. Our results indicate that the unique endocrine milieu of PL rabbits has a direct bearing on specific behavioral and physiological phenomena that impact productivity on the farm.

Key Words: nest building, maternal nest, lactation, food intake, milk output.

INTRODUCTION

In late pregnancy rabbits build a nest to deliver and nurse their young (González-Mariscal *et al*., 1994; Zarrow *et al*., 1963). Nest-building consists of digging a burrow (or into a substrate), carrying straw (into the burrow or nest box), and plucking body hair to line the straw nest. The onset and termination of these activities is controlled by the changing levels of estradiol, progesterone, and prolactin throughout pregnancy (González-Mariscal *et al*., 1994). However, in both wild and farmed animals, pregnant-only (PO) rabbits are rare as, following parturition, does have a post-partum estrus (PPE) at which they can mate (Beyer and Rivaud, 1969; Hammond, 1925). They can therefore start a new pregnancy at the same

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time as they begin nursing the newborn litter. Consequently, the endocrine milieu of pregnant-lactating (PL) rabbits differs from that of PO does and lactating-only (LO) rabbits (not mated at post-partum estrus). Indeed, Fortun *et al*. (1993) found differences in the concentrations of estradiol and progesterone between PO and PL rabbits at specific stages of pregnancy. However, the impact of such differences in specific reproductive parameters on the endocrinology of PO, PL, and LO rabbits has not received a great deal of attention. Regarding lactation, several works have consistently found that milk production is reduced in PL *vs.* LO rabbits (Hudson *et al*., 1996; Lebas, 1972; Lincoln, 1974; Partridge *et al*., 1986). On the other hand, as the nest-building activities of PO and PL does have never been compared, in the present work we quantified: nest-building, body weight, food intake, and the blood concentration of 17β-estradiol and progesterone throughout pregnancy in PL does (i.e., mated at their first PPE) and PO rabbits. Following delivery we compared milk output, body weight, and food intake of PL and LO rabbits.

MATERIALS AND METHODS

Housing and mating

Virgin New Zealand white adult female rabbits (3.5-4.5 kg body weight) bred in our colony were used for the tests. They were kept in individual wire mesh cages (62 cm long × 42 cm wide × 41 cm high) inside the rabbit colony under controlled light (14L:10D; lights off at 21:00 h) and natural temperature (13-25 °C) conditions. Rabbits were given 300 g/d during pregnancy or 400 g/d during lactation of Purina rabbit pellets and water *ad libitum*. Females were mated with sexually active bucks of the same strain inside a round (1 m in diameter) wire mesh arena. Each doe received three services, after which it was returned to its home cage. The day of mating was considered 0 d of pregnancy. Throughout this work animal care and surgical procedures (see below) complied with the Law for the Protection of Animals (Mexico).

Quantification of nest-building, body weight, and food intake

On pregnancy 21 d rabbits were transferred to large, wire mesh, maternal cages (180 cm long × 42 cm wide × 42 cm high). The three components of maternal nest-building (i.e., digging, straw-carrying, and hair-pulling) were quantified from then until parturition, as described in earlier studies (González-Mariscal *et al*., 1994). A wooden box (50 cm long × 30 cm wide × 32 cm high) with a round (24 cm diameter) opening in the front (and no door) was placed in the cage of each female. To quantify digging a piece of compressed cardboard (1.4 cm thick; cut to the size of the floor) was weighed, placed on the floor of the nest box, left inside it for 24 h, removed, and weighed again. The difference in the weight of the cardboard indicated the amount of digging performed in a day. The thickness and texture of the cardboard used allowed the animals to dig into it and effectively remove variable quantities of the substrate, such that changes in the intensity of this behaviour have been reliably determined with this method (González-Mariscal *et al*., 1994). Straw-carrying was quantified by placing 100 g of straw inside the cage (but outside the nest box) and weighing, 24 h later, the straw introduced by the female into the nest box. Hair-pulling was determined by daily inspecting each female's home cage and determining the presence of hair tufts inside or outside the nest box. When found, hair tufts were removed to allow the detection of new hair tufts on subsequent days. In the last third of pregnancy and throughout lactation rabbits were weighed daily and their food intake was determined by measuring the difference in weight between the pellets provided 24 h earlier and those remaining in the dish.

Quantification of nursing behavior and milk output

Starting on pregnancy 30 d, females were spot-checked at intervals throughout the day to determine the approximate time of delivery. At parturition mothers were left undisturbed for 5-8 h, after which, the kits

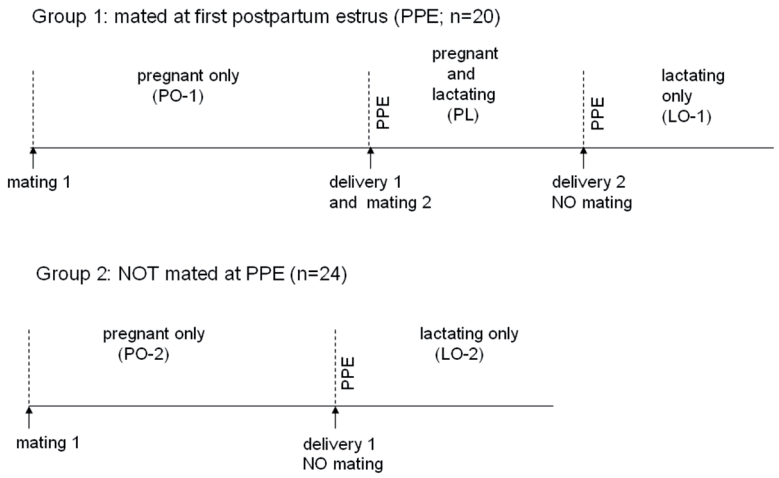

Figure 1: Experimental design showing the different mating schedules used for Groups 1 (mated at the first PPE) and 2 (not mated at PPE). These differences led to the specific reproductive conditions explored in this work, namely: pregnant-only (PO-1 and PO-2), pregnant-lactating (PL), and lactating-only (LO-1 and LO-2).

were removed and counted. The number of liveborn kits varied between eight and ten but, for purposes of the present experiment, litter size was adjusted to five. Following culling, kits were kept away from the doe, inside a box containing paper shavings, under a mild heat source. As shown in Figure 1 some of the females (Group 1; n=20) were mated again at their first PPE and others were not presented to the male (Group 2; n=24). On the following day (i.e., postpartum 1 d) the kits were weighed and placed inside the nest box to record the doe's behaviour. During lactation the following parameters were quantified as described earlier (González-Mariscal *et al*., 1994): a) duration of nursing, which is the time elapsed between the entrance of the doe into the nest box and its exit after suckling the litter and b) milk output. Each behavioral test lasted from the time kits were introduced into the nest box until the mother jumped out of it, or for 1 h if a doe refused to enter the nest box. After completion of the test the kits were weighed again: the difference in their body weight (with respect to the one shown before nursing) indicated milk output.

Blood sampling

On 1, 7, 14, 21, and 28 d of pregnancy 2 mL of blood were drawn from the lateral ear vein by using a gauge 21 needle. In the PL group this procedure was performed immediately after the end of the nursing bout. Blood was left to coagulate at room temperature, serum was removed with a pipette, and then centrifuged at 5000 rpm. The supernatant was removed and immediately frozen at −20 °C until ready for assay.

Determination of serum concentrations of 17β-estradiol and progesterone.

A solid-phase competitive chemiluminescent enzyme immunoassay was used for the quantitative measurement of 17β-estradiol and progesterone in serum. The Immulite® diagnostic kit (Siemens, Los Angeles, CA) was used following the methodology described by Kohen *et al*. (1986). In brief, a solid phase (bead) is coated with rabbit anti-estradiol or anti-progesterone polyclonal antibodies. Reagent containing alkaline phosphatase (bovine calf intestine) conjugated to estradiol or progesterone will compete with the corresponding hormone in the serum sample. Finally, chemiluminiscent substrate is added and a signal

is generated in proportion to the bound enzyme conjugated to estradiol or progesterone. Intra-assay and inter-assay coefficients of variation for estradiol at 180 pg/mL did not exceed 8.0%. For progesterone the coefficients of variation did not exceed 12%. The assay sensitivity for estradiol was 15 pg/mL and for progesterone 100 pg/mL.

Statistical analysis

A repeated measures ANOVA was used to determine whether significant changes in a specific response occurred in each group through time. When pertinent, this was followed by a Wilcoxon test to compare differences in the magnitude of the response between two time points within an experimental group (e.g., food intake on 27-29 d *vs.* 30-32 d). A t-test was used to compare the magnitude of a specific parameter (e.g., amount of substrate dug) on a particular time period (e.g., 21-23 d of pregnancy) between the two experimental groups. The SPSS statistics software package was used for all calculations.

RESULTS

Figure 2 shows the amount of digging performed by PO (PO-1 plus PO-2 groups combined) and PL does during the last third of pregnancy. Significant differences between these groups were found only in 21-23 d, when PL rabbits dug less than the PO females (52±64 *vs.* 104±86 g, respectively; mean±standard deviation; $P<0.05$). However, the total amount of substrate dug during pregnancy 21-29 d was similar between both groups. Straw-carrying was also expressed less intensely by PL does (Figure 3). Significant differences with respect to the PO does (groups PO-1 and PO-2 combined) were found on: 24-26 d (9±27 *vs.* 79±94 g, respectively; $P<0.005$), 27-29 (27±56 *vs.* 99±77 g, respectively; $P<0.005$), and also in the total amount of material introduced into the nest box (132±167 *vs.* 286±217 g, respectively; $P<0.02$). Hair-pulling was expressed by practically all animals of both experimental groups at parturition or on postpartum 1 d. Only two does of the PL group failed to display this behavior in both first and second pregnancies.

Body weight remained practically unchanged throughout pregnancy in both experimental groups. PL does were slightly lighter (4.1±0.5 kg) than the pregnant-only ones (4.4±0.5 kg; for 21-31 d; groups PO-1 and PO-2 combined) but these differences were not statistically significant ($P>0.05$). Food intake was significantly larger on pregnancy 21-23 d in PO-1 than in PO-2 does (363±117 *vs.*

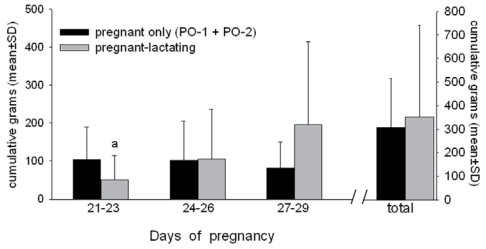

Figure 2: Quantification of digging on specific days of pregnancy in PO does (groups PO-1 + PO-2 combined) and in pregnant-lactating rabbits. Results are presented as the sum of substrate dug in a period of three days and as the total amount dug through 21-29 d.
[a]$P<0.05$.

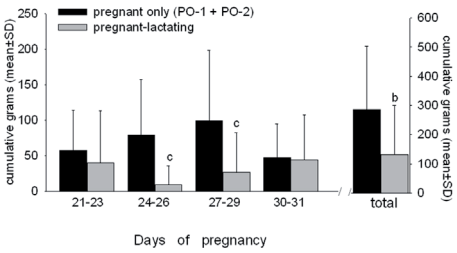

Figure 3: Quantification of straw-carrying on specific days of pregnancy in PO does (groups PO-1 + PO-2 combined) and in pregnant-lactating rabbits. Results are presented as the sum of straw carried into the nest box in a period of three days and as the total amount of straw carried through 21-31 d.
[b]$P<0.02$, [c]$P<0.005$.

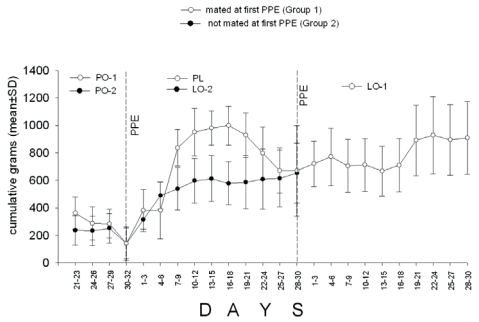

Figure 4: Food intake in rabbits that were: pregnant-only (groups PO-1 and PO-2), pregnant-lactating (PL), or lactating-only (LO-1 and LO-2). Results are presented as the sum of food consumed in a period of three days. See text for levels of significance in specific comparisons within a group and between groups.

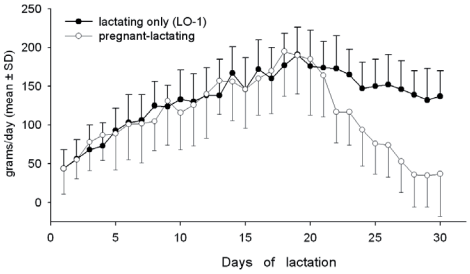

Figure 5: Amount of milk produced by rabbits of Group 1 during their first (i.e., as PL) and second lactation (as lactating only; LO-1).
$P<0.001$ between conditions on 22-30 d.

237±107 g, respectively; $P<0.02$). These differences, however, did not persist for the remainder of pregnancy. In both groups of PO does, food intake significantly declined on the three days preceding parturition (from 285±105 to 142±111 g in group PO-1 and from 252±108 to 140±122 g in group PO-2; $P<0.01$ for both cases; Figure 4). A marked increase in food intake occurred during lactation in PL and LO does with respect to the values seen during pregnancy. The amount of grams ingested per day was consistently higher in PL than in LO does from lactation 7-9 to 22-24 d ($P<0.01$; Figure 4). As their second parturition approached, food intake decreased markedly in PL rabbits (929±162 g on lactation 19-21 d to 670±329 g on 28-30 d; $P<0.001$). Because these animals were not mated after their second parturition, they engaged in their second lactation without being pregnant (LO-1). Under these conditions, food intake remained unchanged on 1-18 d of lactation but significantly increased between 16-18 (710±194 g) and 19-21 d (892±256 g; $P<0.002$) and remained unchanged thereafter.

In the first three weeks of lactation, milk output was indistinguishable between the two reproductive conditions explored in does from Group 1, i.e., as LO (LO-1) and as pregnant-lactating (PL; Figure 5). A steady increase in milk production was evident on 1-21 d but, from then onwards, a precipitous drop in

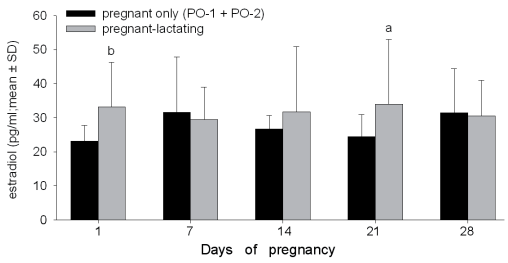

Figure 6: Concentration of estradiol in the blood of rabbits that were: pregnant-only (groups PO-1 + PO-2 combined) or pregnant-lactating.
[a]$P<0.05$, [b]$P<0.02$.

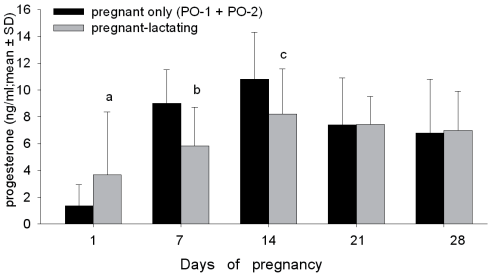

Figure 7: Concentration of progesterone in the blood of rabbits that were: pregnant-only (groups PO-1 + PO-2 combined) or pregnant-lactating.
[a]$P<0.05$, [b]$P<0.02$, [c]$P<0.005$.

milk output occurred in PL does, while only a gradual decline occurred when rabbits were LO ($P<0.001$ on 22-30 d).

The concentration of estradiol in serum did not significantly vary throughout pregnancy in either PO (groups 1 and 2 combined) or PL rabbits. Yet, when comparing specific days, PL does showed significantly higher estradiol levels than PO animals on pregnancy 1 d (33±13 *vs.* 23±4 pg/mL, respectively; $P<0.02$) and 21 (34±19 *vs.* 24±6 pg/mL, respectively; $P<0.05$; Figure 6). In contrast, the concentration of progesterone increased in PO (groups 1 and 2 combined) and in PL does from the minimal levels seen on pregnancy 1 d to the maximal ones observed on 14 d, after which progesterone concentration gradually declined (Figure 7). PL does showed significantly higher levels of progesterone than did PO animals on pregnancy 1 d (4±5 *vs.* 1±2 ng/mL, respectively; $P<0.05$) but lower levels of this hormone on 7 d (6±3 *vs.* 9±2 ng/mL, respectively; $P<0.02$) and 14 d (8±3 *vs.* 11±3 ng/mL, respectively; $P<0.005$).

DISCUSSION

The present results are the first to document that the concurrence of pregnancy and lactation modifies specific aspects of maternal nest-building in rabbits. Thus, the amount of digging displayed by PL females was significantly smaller than that observed in PO animals on pregnancy 21-23 d, but the total quantity of substrate dug throughout pregnancy did not differ between these groups. As digging is stimulated in PO rabbits by the presence of progesterone on a background of estradiol (González-Mariscal *et al*., 1994) differences in the intensity of this behavior between PO and PL does can be explained by the lower concentrations of progesterone found in the latter on pregnancy 7 and 14 d. Our results on steroid measurements agree with the single study that has compared estradiol and progesterone concentrations between PL and PO rabbits. Thus, Fortun *et al*., (1993) reported that PL rabbits had similar levels of estradiol as did PO does but lower concentrations of progesterone on 7 and 17 d but not on 1 or 28 d. Therefore, lower levels of progesterone during the first half of pregnancy might have been a weak stimulus to promote the initiation, but not the progress, of digging in PL does. Indeed, this possibility agrees with our finding that a clear dose-response relationship exists between the dose of progesterone injected into ovariectomized (ovx), estrogen primed rabbits and the intensity of digging (González-Mariscal *et al*., 1996). Straw-carrying was also reduced in PL does: this effect was evident on pregnancy 24-26 d, 27-29 d and in the total amount of straw carried. Since this behavior depends on the decline of progesterone in PO rabbits (González-Mariscal *et al*., 1994) a less steep reduction in the concentration of this hormone in PL does was probably a less potent stimulus for promoting straw-carrying. Indeed, we found a clear dose-response relationship between the dose of progesterone given to ovx estrogen-primed rabbits and the amount of straw carried following progesterone withdrawal (González-Mariscal *et al*., 1996). Interestingly, hair-pulling was practically not affected by the concurrence of pregnancy and lactation, as practically all rabbits showed this behavior. However, we may have missed quantitative differences between PL and PO does, as we did not weigh the amount of hair pulled.

Milk production in Group 1 was indistinguishable between both experimental conditions (i.e., PL *vs.* LO-1) during the first 21 d of lactation. However, from then onwards, a steep decline occurred when does were PL as opposed to the slow decrease observed when they were LO. These results show a remarkable coincidence with those reported earlier by several groups using different breeds of rabbits (Hudson *et al*., 1996; Lebas, 1972; Lincoln, 1974; Partridge *et al*., 1986), thus emphasizing the consistency of the phenomenon, (recently reviewed by Maertens *et al*., 2006). To date, little is known about the mechanisms underlying this dramatic decline in milk output in the last third of lactation as a consequence of concurrent pregnancy. There is evidence that a "critical" balance between progesterone and prolactin is essential to allow the concurrence of pregnancy and lactation in rabbits. Thus, in estrous does, milk secretion induced by prolactin injections is antagonized by the simultaneous administration of progesterone. Conversely,

high doses of prolactin can overcome such inhibitory action of progesterone (Meites and Sgouris, 1954). In LO rabbits the amount of prolactin released following suckling is fairly constant across the first 20 d of lactation. However, from then onwards, suckling-induced prolactin release is markedly reduced (Fuchs *et al*., 1984). From this evidence we propose that milk secretion is abundant in PL does in the first two thirds of lactation due to the high levels of prolactin seen during that period plus the lower concentrations of progesterone found in PL than in PO rabbits (Fortun *et al*., 1993; present work). In the last third of lactation, when progesterone concentrations are similar in PL and PO rabbits, and suckling-induced prolactin release is lower than in early and mid-lactation, the inhibitory action of progesterone prevails and milk secretion declines steeply in PL animals. This hypothesis should be confirmed by measuring prolactin release throughout lactation in PL does.

In addition to the "hormone balance" mechanism proposed above, the decline in milk output seen in PL does may be related with the steep decline in food intake observed in this group a few days before their second delivery (Lebas, 1972; Martínez-Gómez *et al*., 2004; present work). That is, even if hypercaloric diets are provided (Partridge *et al*.,1986) PL rabbits reduce their food intake in late pregnancy (as do PO does; González-Mariscal *et al*., 1994; Martínez-Gómez *et al*., 2004) and show a reduced milk output. These results suggest that changes in specific factors during late pregnancy reduce food intake which, in turn, impacts milk output. The close relationship among food intake, fertility, and milk production has certainly been well documented in rabbits (Boiti, 2004; Castellini, 2007; Fortun-Lamothe and Lebas, 1996), but the pathways that link these processes together are largely unknown. The control of food intake and the release of energy in mammals involves a complex network of protein and steroid hormones, peptides, olfactory and visual stimuli, plus signals emanating from the gastrointestinal tract (for review see: Schneider and Watts, 2002). An important signal that modulates the decline in food intake in PO rabbits is progesterone: a clear correlation exists between the declining concentrations of this hormone in blood and the reduction in food intake in late PO does (González-Mariscal *et al*., 1994). Moreover, this effect can be provoked in ovx estradiol-primed rabbits by injecting progesterone for several days and then withdrawing it (González-Mariscal *et al*., 1996). Investigating how steroid hormones and prolactin determine food intake and milk output in rabbits that are concurrently pregnant and lactating remains a major challenge for the future.

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