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CHANGES IN RESPONSIVENESS TO KIT ODOURS ACROSS PREGNANCY: RELEVANCE FOR THE ONSET OF MATERNAL BEHAVIOUR

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Abstract: Virgin does are indifferent to foster kits but lesions to the main or accessory olfactory systems allow them to behave "maternally" (i.e., they crouch over the litter and allow suckling). This suggests that kit-derived olfactory cues are aversive to virgins but not to lactating does. We hypothesised that the valence of such olfactory cues changes throughout gestation so that, at parturition, does are attracted to the newborn and can then show placentophagia, clean the kits and nurse them. To explore this hypothesis we exposed does to 2 nest boxes containing a variety of pup-derived *vs*. "neutral" odours, quantifying the number of sniffs and entrances to each box over 60 min. Virgins, confronted with 2 different types of contrasts, showed no significant differences in the number of sniffs or entrances directed at any of the 2 boxes. Pregnant rabbits sniffed the "kit-odour" box significantly more than the "neutral" one as early as gestation day 7, depending on the animals' experience with the experimental setup and kit odours as virgins. The number of sniffs declined in late pregnancy in all groups. Entrances into the "kit-odour" box were few and significantly higher than those shown towards the "neutral" box only in 1 group. Our findings agree with a correlation between a shift in the valence of kit-derived olfactory cues and the hormonal changes known to occur throughout pregnancy. The relevance of this phenomenon for the onset of maternal responsiveness at parturition is discussed.

Key Words: olfactory cues, pregnancy, maternal responsiveness, rabbit, parturition.

INTRODUCTION

Maternal behaviour in rabbits begins in late pregnancy as the doe engages in nest-building (González-Mariscal *et al.*, 1994). This process (which involves digging an underground burrow and lining it with straw and body hair) is tightly regulated by the changes in the concentrations of estradiol, progesterone and prolactin throughout pregnancy (González-Mariscal *et al.*, 1994, 1996). Following parturition, maternal behaviour towards the kits is restricted to a single nursing bout per day, which occurs inside the maternal nest, with circadian periodicity (Jilge 1993, 1995; González-Mariscal *et al.*, 2013a), and lasts around 3 min (Zarrow *et al.*, 1965; González-Mariscal *et al.*, 2013b). Ovarian steroids are essential for stimulating the onset of maternal behaviour because: a) while intact virgin estrous does are totally indifferent to foster kits, they can be induced to behave maternally (i.e., to crouch over the young and allow suckling) by lesioning the main (Chirino *et al.*, 2007) or accessory (González-Mariscal *et al.*, 2004) olfactory systems; b) ovariectomy abolishes this response. These findings suggest that kit-derived olfactory cues are aversive to virgins and that removing the perception of such signals allows the expression of maternal behaviour under the influence of ovarian hormones. Yet, already at parturition, most primiparous does ingest the placentas and respond maternally to their newborn without becoming anosmic (González-Mariscal *et al.*, 1998; Melo and González-Mariscal, 2003). Moreover, primiparous mothers -even before the first nursing- show many more c-Fos-immunoreactive cells (indicative of enhanced brain excitability) in specific forebrain nuclei related with maternal behaviour regulation (e.g. preoptic area, paraventricular nucleus, lateral septum) than virgins do (González-Mariscal *et al.*, 2009). These

findings suggest that the hormonal changes occurring throughout pregnancy and the pre-partum period (González-Mariscal *et al.*, 1994) could modify the processing of kit-derived olfactory cues in such a way that, at parturition, they would be interpreted as "attractive" rather than as "aversive". This shift in the olfactory valence of kit odours would promote the mother's approach to the litter and her engagement in kit-oriented behaviours. Thus, in Experiment 1 we hypothesised that pregnant rabbits would change their behavioural responses to kit-derived olfactory cues on specific days of gestation while estrous does would not.

In rats, the rearing environment modifies the responsiveness of adult virgins to foster pups. Specifically, co-habitation of young females with lactating dams and their offspring (inside the same vivarium) reduces their latency to respond maternally to pups in adulthood (Moretto *et al.*, 1986). From this background we hypothesised, in Experiment 2, that pregnant does exposed to kits as virgins would show earlier or greater responses to kit odours (but not to neutral olfactory cues) than pregnant rabbits not given such pre-exposure.

MATERIALS AND METHODS

Animals

Virgin New Zealand white rabbits (3.5-4.5 kg body weight, aged 4-6 mo) bred in our colony were used. They were kept in large wire mesh maternal cages (178×60×40 cm; length×width×height) inside the rabbit colony under controlled lighting (14L:10D; lights off at 21:00) and natural temperature (13-25°C, daily variation) conditions. Water and pelleted feed (Purina™) were provided *ad libitum*. Throughout this work, animal care complied with the Law on Protection of Animals (Mexico).

Experiment 1: Responsiveness of virgin and pregnant rabbits to kit odours

Two wooden nest boxes (50×29×27) with a round opening on one side (24 cm in diameter) were introduced into the cage of virgin rabbits, as this procedure allows us to test a female rabbit's behavioural reactions to 2 different types of contents of the nest boxes (González-Mariscal and Gallegos, 2007). Placing of the 2 boxes within a female's cage (i.e., right or left side) was randomised. The contents of the 2 boxes, as summarised in Table 1, were: Group A (n=15): nothing *vs.* neutral material (i.e., clean straw and synthetic hair), to determine if rabbits preferred a box with "anything" over an empty one. Group B (n=15): nothing *vs.* maternal nest material (i.e., straw and rabbit hair taken from the maternal nests of lactating does in our colony), to establish if maternal and kit olfactory cues were more or less attractive than absence of olfactory stimulus. We quantified, for 60 min/d, 2 behavioural responses as indicators of "interest in" or "aversion to" the contents of the nest boxes: a) the number of sniffs and b) the number of entrances into each box. At the end of the test, the nest boxes remained inside the females' cages but the materials inside them were removed. These procedures were performed on five consecutive days. Two to four weeks after the end of the above tests, females from group B were mated with sexually active bucks of the same strain inside a round (1 m in diameter) wire mesh arena. Pregnant does were then assigned to group C (n=15), which was exposed to boxes that contained: nothing *vs.* only kits (3-4; aged 2-5 d; see Table l). The boxes remained inside the female's cage after the 60 min test described above (only kits were withdrawn). This test was repeated in early- (day 7), mid- (days 14 and 21), and late- (days 28, 29, and 30) pregnancy.

Experiment 2: Effect of experience with kit odours as virgins on the responsiveness of pregnant does to kit-derived cues

Table 1 summarises the setups for each of the 3 experimental groups used. Virgin rabbits from Group D (n=15) were exposed for 1 h, for 5 consecutive days, to 2 nest boxes that contained: neutral material *vs.* maternal nest material (see Table 1). The number of sniffs and entrances into the nest box were quantified for 60 min/d, as above. Virgins from Group E (n=15) did not receive any experience with kits, nest material or nest boxes. Virgins from Group F (n=15) were exposed for 1 h, over 5 consecutive days, to 2 nest boxes that each contained only neutral material. Rabbits from the 3 groups were mated as above; this occurred 4 d after completion of the "exposure" tests (in Groups D and F). On pregnancy days 7, 14, 21, 28, 29, and 30 we introduced neutral material *vs.* maternal nest material plus

kits into the nest boxes (already present in Groups D and F; introduced on day 7 for Group E). The number of sniffs and entrances into each of the boxes was counted for 60 min, as above, after which the boxes remained inside the females' home cages.

Statistical analysis

To compare, within a group, the number of sniffs or entrances towards each of the 2 boxes over all the test days, a repeated measures ANOVA was performed on each behaviour. This was followed by the post-hoc Wilcoxon test to compare, on a single day, the number of sniffs or entrances directed towards each of the boxes. To compare, between 2 different groups, the number of sniffs or entrances directed at one particular type of box on a specific day, Mann-Whitney's U-test was used (Siegel and Castellan, 1988).

RESULTS

Experiment 1

Figure 1 shows that, over the 5 days of testing, virgin rabbits confronted with 2 different types of contrasts (see Table 1; Groups A, B) showed no significant differences in the number of sniffs they directed at the contents of any of the 2 nest boxes placed in their home cage. A similar pattern was observed regarding entrances into the nest boxes (Figure 1). In contrast, pregnant rabbits of Group C, faced with a high contrast setup, showed significantly
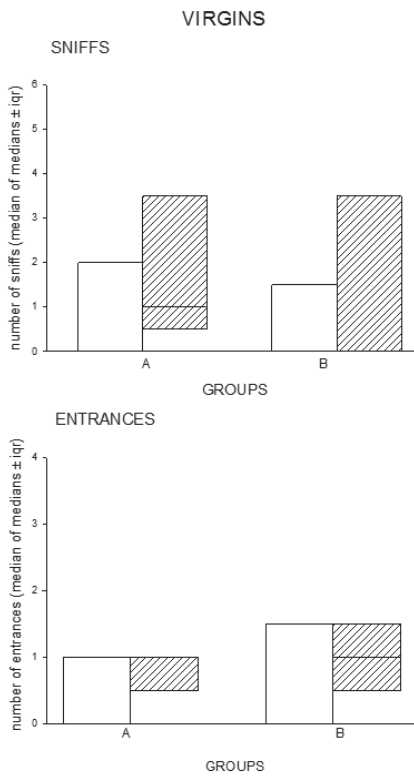

Figure 1: Number of sniffs and entrances shown over 60 min towards an empty box (☐) vs. one containing neutral material (Group A) or nest material (Group B; ▨) by virgin does throughout the 5 d of testing.
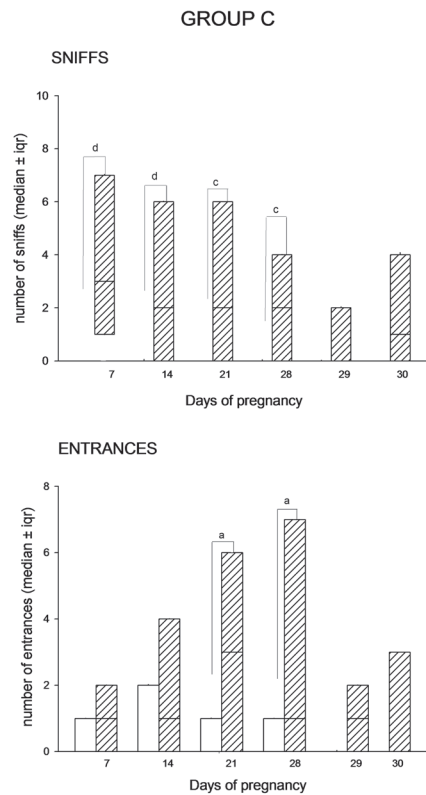
Figure 2: Number of sniffs and entrances shown over 60 min towards an empty box (☐) vs. one containing kits (▨), on specific days of pregnancy, by does in Group C. [a]$P<0.05$, [c]$P<0.01$, [d]$P<0.005$.

more sniffs towards the nest box containing only kits than towards the empty one on pregnancy days 7, 14, 21, and 28 (Figure 2). Indeed, the magnitude of the differences in sniffing remained almost unchanged over time and, consequently, differences were not significant (P=0.109). Does also went into the box containing kits more times than into the empty one. These differences, however, were significant only on pregnancy days 21 and 28 (Figure 2; P=0.076).

Experiment 2

Before mating, virgins from Group D showed no significant differences in the number of sniffs or entrances they directed at any of the nest boxes on any day. Specific values (expressed as medians, interquartile range in brackets) of number of sniffs directed towards "neutral" *vs.* kit odours, through days 1-5 were: 7 (4,9) *vs.* 8 (7,13); 0 (0,4) *vs.* 2 (1,5); 1 (0,2) *vs.* 2 (2,5); 3 (1,4) *vs.* 2 (0,4); 0 (0,2) *vs.* 1 (0,2). The corresponding values for number of entrances were: 0 (0,3) *vs.* 1 (0,5); 0 (0,1) *vs.* 1 (0,2); 0 (0,1) *vs.* 1 (0,3); 1 (0,4) *vs.* 1 (1,2); 1 (0,1) *vs.* 1 (0,2). This pre-exposure to kits, however, promoted a greater responsiveness to kit cues on pregnancy day 7, as these does displayed significantly more sniffs towards the box containing nest material+kits than did females from Group C (compare Figure 2 *vs.* Figure 3; P<0.001). Additionally, pre-exposed does from Group D also showed significantly more sniffs towards the box with kit cues than towards the one containing neutral material on days 7, 14, and 21 (Figure 3; ANOVA: df=5; F=8.949; P=0.0001). In
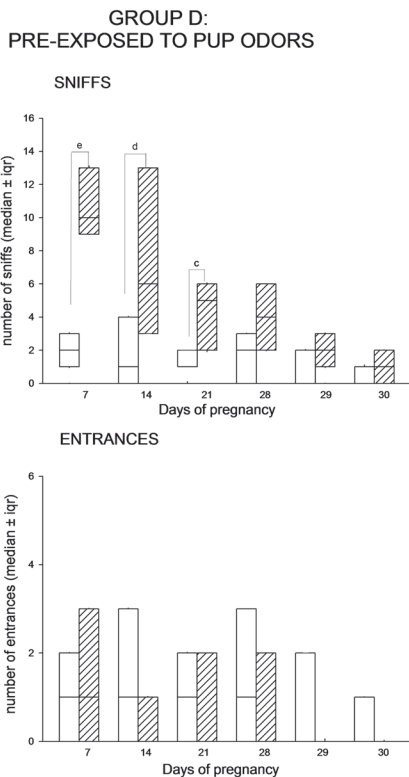

Figure 3: Number of sniffs and entrances shown over 60 min towards a box containing neutral material (☐) *vs.* one containing nest material+kits (▨), on specific days of pregnancy, by does in Group D. [c]P<0.01, [d]P<0.005, [e]P<0.001.
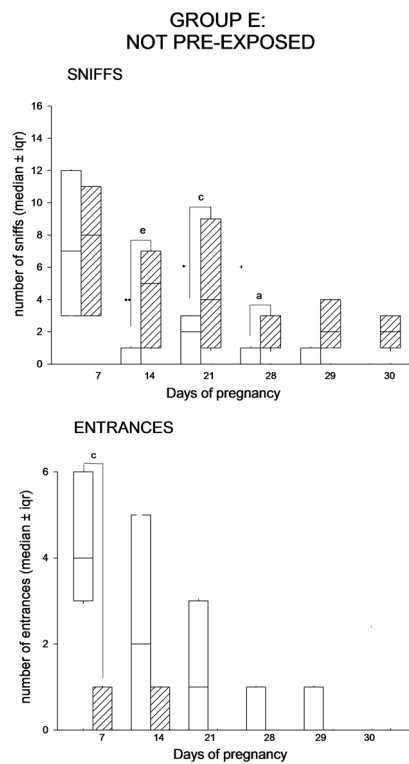
Figure 4: Number of sniffs and entrances shown over 60 min towards a box containing neutral material (☐) *vs.* one containing nest material+kits (▨), on specific days of pregnancy, by does in Group E. [a]P<0.05, [c]P<0.01, [e]P<0.001.

contrast, entrances into any of the nest boxes were scarce in does from Group D (Figure 3; *P*=0.510). Females that lacked pre-exposure to pup odours and nest boxes (Group E) showed significantly more sniffs towards the nest box containing maternal nest material+kits only on pregnancy days 14, 21 and 28, but not on day 7 (Figure 4; *P*=0.006). Entrances into the nest boxes were also scarce in these does, but they were significantly more frequent towards the neutral box on day 7 (Figure 4; *P*=0.0001). Experience with the nest box and neutral material (but not with kit odours, Group F) before mating caused an effect similar to that seen in Group D, i.e., a larger number of sniffs directed at the box containing maternal nest material+kits already on pregnancy day 7 (Figure 5). These differences persisted into late pregnancy (*P*=0.058). Moreover, these does from Group F showed a larger number of sniffs towards the "kit-cue" box than did females from Group C on pregnancy day 7 (compare Figure 2 *vs.* Figure 5; *P*<0.01). Interestingly, pre-exposure to the boxes as virgins also promoted a significantly larger number of entrances into the neutral box on pregnancy days 7 and 14 (Figure 5; *P*=0.118).

DISCUSSION

The present results show that, as in rats (Bauer, 1983; Fleming *et al.*, 1989; Kinsley and Bridges, 1990), pregnancy-associated factors facilitate the responsiveness of doe rabbits to kit-derived odours. Thus, virgins did not sniff more or enter more times a particular box under any of the contrast conditions used. Pregnant does, however, directed more sniffs at the box containing kits under a variety of contrasts used. The time of onset and the magnitude of this difference were influenced by 2 factors: a) experience with the experimental setup; b) experience with kit odours as virgins. Thus, in Groups C, D, and F the preference for sniffing the box containing kits was already evident on pregnancy day 7, while in Group E (not pre-exposed to anything) the preference was delayed to pregnancy day 14.

Figure 5: Number of sniffs and entrances shown over 60 min towards a box containing neutral material (☐) *vs.* one containing nest material+kits (▨), on specific days of pregnancy, by does in Group F. [a]*P*<0.05, [b]*P*<0.02, [d]*P*<0.005.

Towards the end of gestation, most does showed a decline in the number of sniffs directed at the kit-containing box, coinciding with the time when the estradiol/progesterone ratio changes (González-Mariscal *et al.*, 1994), prolactin is released (McNeilly and Friessen, 1978) and parturition approaches. This decrease in sniffing in the pre-partum period contrasts with the results found in rats, a species in which maximal levels of attraction to pup odours are evident close to delivery (Bauer, 1983; Fleming *et al.*, 1989; Kinsley and Bridges, 1990). However, the correlation between changes in sniffs towards the "pup box" and variations in the hormonal milieu throughout pregnancy coincides with evidence showing that hormones modulate particular aspects of olfactory function in rodents (Pietras and Moulton, 1974; Yu *et al.*, 1996a; Halem *et al.*, 1999, 2001; Baum and Keverne, 2002; Xiao *et al.*, 2004) and carnivores (Kelliher *et al.*, 1998). Moreover, oxytocin action on the olfactory bulb is associated with the induction of maternal behaviour in estrogen-primed virgin rats (Yu *et al.*, 1996b) and sheep (Kendrick *et al.*, 1987; Lévy *et al.*, 1995). Although, to our knowledge, hormonal effects on specific aspects of olfactory function have not been investigated in rabbits, our results support the possibility that the attraction to kit odours, observed only in pregnant does, is a consequence of the changing and unique hormonal milieu characteristic of particular gestational stages.
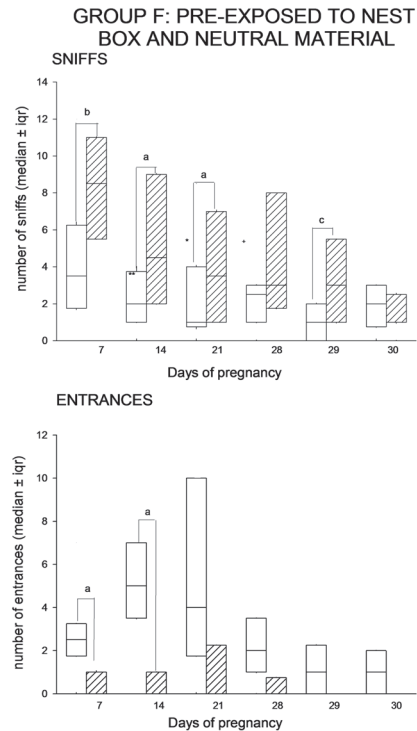
In contrast to sniffing, entrances into the "kit box" were scarce in most experimental groups. Only does from Group C entered this box significantly more times than the "neutral box" on pregnancy days 21 and 28. Perhaps exposure to the maternal nest material as virgins, two-four weeks before mating, facilitated this response. In the remaining groups, however, we believe that the scarce entrances into the "kit box" may indicate fear of kits (or their odour), as has been proposed for rats (Fleming *et al.*, 1989). Accordingly, doe rabbits would be avoiding entrance into this box because it is "risky". Future studies should explore these possibilities.

CONCLUSIONS

Pre-exposure to kit odours and a nest box before mating allows an earlier sniffing response towards these olfactory cues throughout pregnancy and favours entrances into the nest box even before parturition. These findings could be used to promote a good maternal responsiveness in primiparous does, many of which do not show adequate behavioural responses to the kits at parturition and throughout the first lactation, failures that decrease productivity.

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