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Olfactory Preference for Own Mother and Litter in 1-Day-Old Rabbits and Its Impairment by Thermotaxis

ABSTRACT: *We investigated the ability of rabbit pups to display preferences towards various elements of their postnatal environment during the stage of confinement in the nest. Subjects were submitted to a two-choice test during the first week after birth to assess if they could detect and discriminate between does, litters of pups, or nesting materials of the same developmental stage. On D1 and D7, pups were attracted to any lactating doe, litter, or nest when compared to an empty compartment. When two stimuli were opposed, pups preferred their own nest to an alien one on D1 and D7 but not their mother nor their siblings when opposed to alien does or pups. However, additional tests indicated that this lack of preference for kin conspecifics resulted from a predominant attraction to thermal cues over individual odors. Indeed, pups were strongly attracted to a warm compartment (37°C) than to a cooler one (20°C) and once thermal cues were controlled for in the testing situation, the pups were specifically attracted to odors of their own mother's hair and of their siblings. No preference was observed towards the mother's uterine secretions. In conclusion, pups can recognize olfactory cues emanating from their mother and their siblings the day after birth. The preference for nesting materials would reflect in major part the combined attraction to maternal and sibling odors present in the nest. © 2008 Wiley Periodicals, Inc.* Dev Psychobiol 50: 542–553, 2008.

Keywords: *neonatal; smell/olfaction; social; early preferences; rabbit*

INTRODUCTION

In altricial species, mother–young interactions are organized within the nest, a particular ecological niche which prolongs intrauterine thermotactile comfort. During the first few days or weeks after birth, the nest is the only environment that the neonates are permanently in contact with. In Belding's ground squirrel (*Spermophilus beldingi*), females rear their litter alone in underground burrows for about 25 days after birth and defend their natal burrow against intrusion from conspecifics (Holmes, 1990). Under both field and laboratory conditions, the young distinguish between familiar and unfamiliar dams only at the age when they would emerge above ground for the first time in the field (approximately at 22 days) and litter-mate preferences are not fully developed at that stage either (Holmes, 1997; Mateo, 2006). Similar results have been reported for altricial rats *Rattus norvegicus* (Leon & Moltz, 1971; Carr, Marasco, & Landauer, 1979), house mice *Mus musculus* (Breen & Leshner, 1977) and Mongolian gerbils *Merius unguiculatus* (Gerling & Yahr, 1982), thereby suggesting that the process of recognition develops at a stage when the young have to seek their mother more actively and initiate suckling out of the nest. Although the ability to discriminate familiar conspecifics in regards to ecological constraints was described in these species, the time course for the development of such social discrimination remains to be elucidated in regards to the early olfactory learning abilities of altricial neonates.

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The rabbit pup (*Oryctolagus cuniculus*) is a particularly interesting subject for studying the development of early preferences toward elements of the postnatal environment. Firstly, from an eco-ethological point of view, the rabbit is the only mammal to use its body hair to build a maternal nest in addition to other materials from the surrounding vegetation (Gonzalez-Mariscal & Rosenblatt, 1996). Therefore, for the first postnatal weeks, pups have the opportunity to develop preferences towards several elements of their environment: the nest materials and the siblings with which they huddle constantly until they emerge out of the burrow, as well as the mother's abdomen encountered during each short daily nursing episode. Yet there is still very limited knowledge about the development of early social discrimination in rabbits (Mykytowycz & Ward, 1971). Secondly, from a developmental point of view, rabbit pups are able to display exploration behavior in an arena, detect the presence of adult animals and display olfactory preferences in a two-choice test. In such a test, 2- to 3-day-old pups discriminate between olfactory cues emanating from abdominal areas (Coureaud, Schaal, Langlois, & Perrier, 2001) and display the strongest attraction to lactating females, compared to males or nonlactating, nonpregnant or even pregnant females (Coureaud & Schaal, 2000). Nonetheless, under the same conditions Patris, Schaal, and Coureaud (2004) failed to demonstrate a preference for the mother's abdomen in comparison to that of an alien doe at the same physiological state. Curiously, they are on the other hand quite able of early olfactory learning under experimental situations using artificial odorants. One-day-old rabbit pups can rapidly be conditioned to associate specific odors spread on their mother's ventrum with suckling and to respond specifically to that same odorant when re-exposed to it on the following day (Hudson, 1985; Hudson, Labra-Cardero, & Mendoza-Soylovna, 2002; Kindermann, Hudson, & Distel, 1994). This is also the case in rat pups, which can develop preference for artificial odors in response to tactile stimulation (Woo & Leon, 1987).

The contrast between discriminative responses by pups obtained with the use of biological or artificial odorants and the lack of it when tested with live mothers or live stimuli pups led us to hypothesize that supra-individual cues are more potent attractants to neonatal rabbits than individual olfactory signatures. Warmth could well be a major supra-individual attractant to altricial neonates born with poor thermal insulation. Pacheco-Cobos, Rosetti, Distel, and Hudson (2003) demonstrated that rabbit pups have the capacity to detect and orient to thermal gradients; they come to rest when their body is positioned over an area between 35 and 41°C. In the present work we examined the hypothesis that attraction to thermal cues biases the choice made by newborn pups when confronted with two females of the same physiological state (Patris et al., 2004; Val-Laillet & Nowak, 2008) and that a preference for familiar olfactory cues of nest, littermates or the mother, could be displayed when pups were tested at temperatures not inducing thermal attraction (i.e., below 35°C). Experiment 1 examined the preference displayed by newborn pups towards (i) lactating females and (ii) litters of pups in two-choice tests including thermal cues emanating from bodies of familiar and alien animals. Experiment 2 tested the preference displayed towards nest materials (including mainly maternal hair and litter odors), which do not produce any warmth. Experiment 3 assessed the capacity of pups to detect a thermal gradient under exactly the same experimental conditions. Last, Experiment 4 evaluated the capacity of newborns to discriminate between familiar and alien cues from their mother and siblings in two-choice tests excluding thermal cues. We hypothesized that pups would not discriminate between familiar and alien cues in tests including thermal cues whereas they would do so in tests excluding the thermal cues.

GENERAL METHODS

Breeding and Housing Conditions

Thirty breeding does of the New Zealand white strain (Station ''BioAgresseurs, Santé et Environnement'', INRA, Nouzilly, France) were kept under a 13:11 hr light:dark cycle (light on at 7.00 a.m.) while ambient temperature was maintained at 20°C. Water and food pellets (Huttepain Bouix, Piace, France) were provided ad libitum. All the animals were maintained in individual cages (70 cm × 46 cm and 60 cm high). A nest box (46 cm × 14 cm and 24 cm high) containing wood shavings as bedding was inserted into the cage 3 days prior to parturition.

Test Apparatus and Stimulus Animals or Substrates

Experiment 1: Neonatal Detection and Discrimination Between Own and Alien Mother and Siblings in Two-Choice Tests Including Thermal Cues We submitted the pups to two kinds of test for a specific type of stimulus: (1) the choice between a stimulus (familiar or unfamiliar) and an empty compartment to check that the stimulus was detected and whether it was attractive or aversive, (2) the choice between a familiar and an alien stimulus to determine the existence of a preference. When pups were given the choice between an empty compartment and a stimulus (familiar or not), ten pups per group were tested, in the second one we doubled the number of animals used due to greater variability of the behavioral response.

Experiment 1a: detection of the mother and an alien female and discrimination between own and alien mother. The orientation response towards the mother or an alien doe at the same lactational stage was investigated by using a double-U-shaped device (36 cm × 31 cm × 13 cm high) made of polyvinyl chloride (PVC) derived from the methods used by Coureaud and Schaal (2000) (Fig. 1). The two side-by-side chambers (36 cm × 15 cm and 13 cm high) were intended to accommodate stimulus mothers in a supine position. Individual pups were tested in a rectangular polypropylene box (31 cm × 21 cm and 13 cm high) designed to sit on the side walls of the double-U-shaped device. The arena had a double wire mesh floor (mesh size: 1 mm^2) spaced 1 cm apart so that when the box was sitting on top of the does, pups could not sense any tactile stimulation from them. The test arena was supported on the side walls of the stimulus chambers enclosing a section of the abdominal region of the mothers, the zone of interaction during nursing, but excluded ano-genital and inguinal regions. Ten does used as stimulus females were anesthetized 10 min before testing by an intramuscular injection of 1 ml levopromazine (Nozinan, 25 mg/ml, Aventis, Paris, France), followed 10 min later by an intramuscular injection of 2 ml ketamine (Clorketam 1000, 100 mg/ml, Vétoquinol S.A., Lure, France). After a doe was anesthetized, she was laid supine in a chamber of the double-U device. She was returned to her home cage once a testing session was accomplished, which usually took less than an hour.

Experiment 1b: detection of the siblings and alien pups and discrimination between own and alien pups. The test apparatus used to study the preference for own or alien litter of pups of the same age was a rectangular PVC box (42 cm × 32 cm and 15 cm high) divided in two by a median wall (Fig. 2). The top part of the box was a platform made of a double layer of wire mesh (mesh size: 1 mm^2) spaced 1 cm apart which could be lifted up to insert stimulus pups into the chambers. Stimulus pups were put in small plastic containers (19 cm × 14 cm and 9.7 cm high) filled with clean wood shavings. Each chamber of the experimental box could contain two containers with the same total number of pups (between 2 and 3/container depending on their size). The containers were installed so that the pups were in physical contact with the double layer of wire mesh. PVC walls (42 cm × 32 cm and 9 cm high) were then added onto the wire mesh platform and delimited the discrimination area. Pups were immediately put back in their nest after a series of tests was finished.

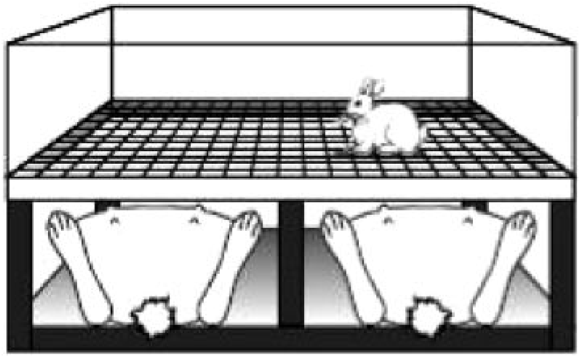

FIGURE 1 Two-choice device testing the preference of a pup toward its own mother's abdomen and an alien mother's abdomen.

Experiment 2: Neonatal Detection and Discrimination Between Own and Alien Nest Materials in Two-Choice Tests Excluding Thermal Cues The nest is a complex structure made of maternal hair while the presence of pups enriches the chemosphere by adding up their own odors. In the peri-partum period it contains also uterine fluids, blood and placental fragments. Therefore, the nest appears as being mostly made of elements from the mother and the pups. The test apparatus was the same as in Experiment 1b (Fig. 3). Pups were submitted to two kinds of test: (1) the choice between the stimulus (own or alien nest) versus an empty compartment, (2) the choice between their own nest versus an alien nest. A sample of nest containing maternal hair, uterine secretions and wood shavings was collected and put in Petri dishes (9.5 cm × 9.5 cm) just before a test began. Each chamber of the experimental box contained three Petri dishes of a specific stimulus.

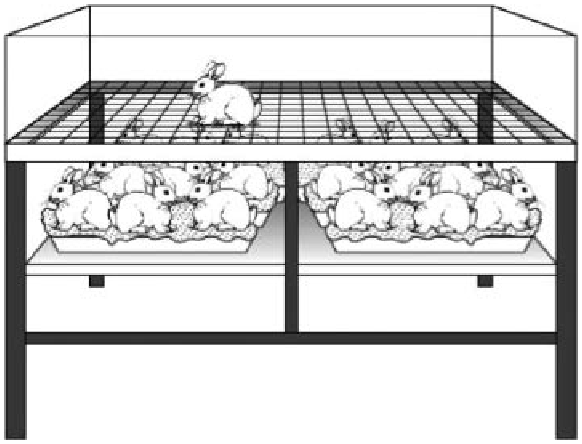

FIGURE 2 Two-choice device testing the preference of a pup toward its own siblings and alien pups of the same age.

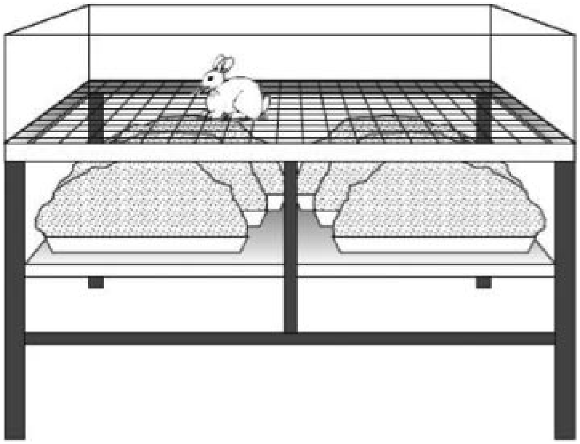

FIGURE 3 Two-choice device testing the preference of a pup toward its own and alien nest materials.

Experiment 3: Preference for Thermal Cues A soft plastic water bottle (36 cm × 16 cm) was put in each compartment of the two-choice apparatus described in Experiment 1b. One was filled with water heated to 35°C, that is, the average temperature recorded via an electronic thermometer (EcoScan) on adult rabbits' abdomen and in a cluster of pups in the nest. The warm water was replaced systematically at the end of each test to ensure a stable temperature across the experiments. The other bottle did not contain any water and was presented at room temperature (20°C).

Experiment 4: Discrimination Between Own and Alien Cues From Mother or Siblings in Two-Choice Tests Excluding Thermal Cues The following set of experiments aimed at testing the orientation response of newborn pups towards olfactory cues from their mother or siblings that could be perceived during the two-choice tests described in Experiments 1a and 2. The aim was to test the response of pups to three types of odors without being exposed to the strong thermal cues normally associated with them. We tested the response to maternal hair, to body odors carried by the litter and to intrauterine elements, which are known to be attractive to newborn pups (Coureaud, Schaal, Hudson, Orgeur, & Coudert., 2002) and likely to carry olfactory cues from the mother as well as from the litter.

Experiment 4a: preference for olfactory cues from the mother. The nest built by the mother provided an easily accessible source of maternal hair. However, after parturition the nest is contaminated with odors from the litter and uterine secretions. To take a sample of noncontaminated hair, samples had to be collected before parturition. As the mother starts building her nest 1 day or 2 before giving birth (Gonzalez-Mariscal & Rosenblatt, 1996), samples could be collected approximately 24 hr before parturition. Each sample was put into an individual plastic bag, labeled, and stored at room temperature so that it was kept under the same conditions as the hair left in the nest box. The testing conditions were the same as in Experiment 1c.

Experiment 4b: preference for olfactory cues from the litter. Four pups per litter were transferred into an artificial nest made of clean wood shavings and cotton wool. They remained in it for 24 hr at room temperature under the same conditions as the rest of the litter left in the nest box. Tested pups were given the choice between a sample of cotton wool in which they had remained for 24 hr with three siblings and a sample of cotton wool from an unfamiliar litter.

Experiment 4c: preference for olfactory cues from the intrauterine environment. Samples of intrauterine elements (blood, fetal fluids, and pieces of placenta) were collected in the nest within minutes after parturition. Each sample was stored in a plastic bag for 24 hr at room temperature under the same conditions as the elements left in the nest box. Pups were given the choice between intrauterine substrates from their mother and from an unfamiliar female. Due to the small amount of the samples collected (the amount of amniotic fluids is minute and the mother ingests most of the placenta), the pups were tested in the small arena described in Experiment 1a.

Test Animals

In order to determine the optimal testing age of our pups, 104 subjects from fifteen litters were checked daily in a preliminary study. The day of birth was designated as postnatal day 0. Each morning (at 8:00 a.m.) the number of individuals having at least one eye open or being seen out of the nest was noted. Results showed that 64.7% of them were initially observed to open at least one eye on postnatal day 9 and that 95.2% had both eyes open on the following day (10). A few rabbit pups were first seen out of their nest on postnatal day 13. In the following week, the number of pups seen outside the nest box increased but was extremely variable both between and within litters, as pups moved constantly in and out. By postnatal day 22, all the pups were seen out of their natal nest.

Based on these observations and on the fact that social recognition may improve with age (Mykytowycz & Ward, 1971; Val-Laillet & Nowak, 2008), we chose to test the rabbit pups on Day 1 (they were aged between 24 and 36 hr) and on Day 7. Each pup was tested once only either on Day 1 or on Day 7. To limit litter effects, pups were allocated at random to one of the 11 experimental groups, with one or two subjects of a same litter at the most contributing to each group. Pups were deprived of nursing the night prior to testing (by closing the entrance of the nest box) to optimize responsiveness to maternal and non maternal stimuli and standardize their motivational state (Coureaud et al., 2001).

Test Procedure

At the beginning of the test, the pup was placed in the testing arena length wise on the midline separating the two chambers. The test started once the pup was released and the cumulative amount of time spent on each side of the testing arena was recorded during a 5 min period by observers blind to what pup was being tested. A pup was considered to be in a specific half of the arena once its whole head was beyond the midline separating the two chambers. Each test was carried out under ambient temperature (20°C) since preliminary studies had shown that subjects tested under heated conditions (35°C) fell rapidly asleep. In addition, it avoided temperature gradient as an orientation cue. The tested pup was immediately put back in its nest at the end of the test. In order to remove any olfactory traces left by the previous pup, after each test, the arena and the wire mesh were washed with pure water, 95% ethyl alcohol, then water again and finally dried. In addition, the testing device was rotated 180° every four trials.

Statistical Analyses

The behavior of individual pups was quantified by measuring the total time spent on either side of the medial line of the testing arena. To assess whether the data sets from each experiment followed a normal distribution, they were submitted to a Lilliefors test. In all cases, normal distribution was assumed. As their performance did not differ significantly from a normal distribution, parametric statistical tests were used to compare behavior among groups and results were presented as means. The time spent by pups during the test on the side of the apparatus with the relevant stimulus (*a* stimulus in the case of tests vs. an empty compartment, the *familiar* stimulus in the case of choices between the familiar and an alien stimulus) was analyzed. The group means of the time spent by pups above the relevant stimulus were submitted to one-sample *t*-tests. We tested this variable "time" against the null hypothesis of random choice (hypothesized mean = 150 s) which represents no preference and random exploration. Therefore when the performance of a given group differed statistically from random, it indicated a preference in favor of the stimulus on which pups stayed more time. To evaluate the possible change in performances between D1 and D7, a second analysis was conducted using unpaired *t*-tests since tests were performed with different animals. We assessed whether the percentage of time spent over the relevant stimulus differed across groups of pups at these different ages. Unilateral *p*-values less than .05 were considered as statistically significant.

Ethical Note

Animal care and experimental procedures were in accordance with directive A37801 of the French Ministry of Agriculture and the experiments were approved by a local Ethic Committee (Comité Régional d'Ethique pour l'Expérimentation Animale, Provence). Anesthesia of the does was performed under the direct supervision of a veterinarian. The females received the injection in their home cage in order to limit stress of manipulations and were only removed from it once deeply asleep. No adverse effects of anesthesia were observed on the does which usually woke up after an hour. However, access to the nest box was not permitted for another 2–3 hr, until complete recovery in order to avoid inadequate maternal care. Neither pup nor adult mortality was affected by anesthesia or testing procedures.

Experiment 1: Neonatal Detection and Discrimination Between Own and Alien Mother and Between Siblings in Two-Choice Tests Including Thermal Cues

Experiment 1a: preference for mothers. Pups had the choice between (i) the abdomen of their anesthetized mother and an empty compartment ($N = 10$ both at D1 and D7), (ii) the abdomen of an anesthetized unfamiliar doe of the same lactational stage and an empty compartment ($N = 10$ both at D1 and D7), (iii) the abdomen of their anesthetized mother and that of an anesthetized unfamiliar doe of the same lactational stage ($N = 19$ on D1 and $N = 20$ on D7).

Compared to an empty compartment, own and alien does were clearly and rapidly detected. The pups spent more time exploring the area over their mother (238.5 ± 70.5 s) than in the hypothesis of random choice on D1 (random: 150 s; One sample *t*-test: $t = 3.968$, $N = 10$, $p = .003$; Fig. 4) and on D7 (mother: 230.5 ± 50.6 s; One sample *t*-test: $t_9 = 5.028$, $p < .001$; Fig. 4). A similar phenomenon was observed when an alien doe (208.7 ± 12.4 s) was pitted against an empty compartment on D1 (One sample *t*-test: $t_9 = 4.732$, $p = .001$; Fig. 4) and on D7 (alien doe: 200.4 ± 54.5 s; One sample *t*-test: $t_9 = 2.925$, $p = .017$; Fig. 4). On the other hand, the time spent by pups over the mother's abdomen or over that of the alien doe did not differ from random neither on D1 (mother's

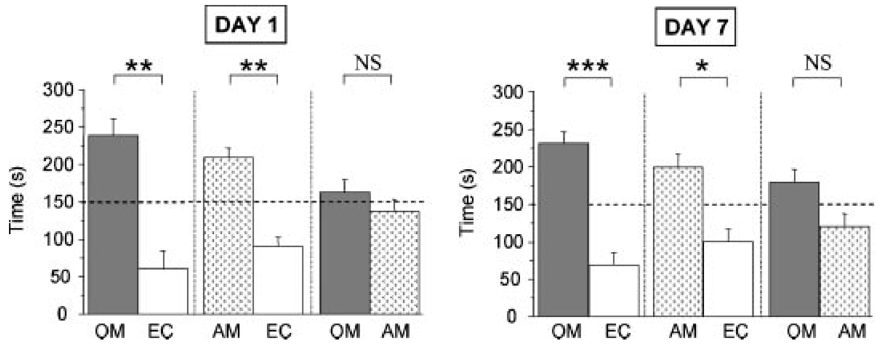

FIGURE 4 Mean orientation time (±SEM) of 1-day-old (a) and 7-day-old (b) pups over the following paired stimuli: Own Mother's abdomen (OM) versus Empty Compartment (EC); Alien Mother's abdomen (AM) vs. Empty Compartment (EC); Own Mother's abdomen versus Alien Mother's abdomen (AM). The horizontal dashed line at 150 s indicates random level of pup's orientation; we assessed the difference between the durations that pups stayed over either stimulus against random using one-sample *t*-tests. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, NS = not significant. No differences were found between D1 and D7.

abdomen: 163.2 ± 17 s; One sample *t*-test: $t_{18} = .777$, $p = .447$; Fig. 4) nor on D7 (mother's abdomen: 179.4 ± 75.1 s; One sample *t*-test: $t_{19} = 1.750$, $p = .096$; Fig. 4). No change in the pups' performance was detected between D1 and D7 in the time spent over a specific stimulus (D1 vs. D7) (i) own mother/empty compartment: Unpaired *t*-test: $t_{18} = .525$, $p = .606$; (ii) alien mother/empty compartment: Unpaired *t*-test: $t_{18} = .391$, $p = .700$; (iii) own mother/alien mother: Unpaired *t*-test: $t_{37} = -.556$, $p = .582$; Fig. 4). Thus, at both ages, pups spent significantly more time over a lactating doe than over an empty compartment but they did not spend more time over their mother than over the alien female. Even though rabbits have a distinct individual olfactory signature (Mykytowycz & Dudzinski, 1972), the mother was not more attractive than the alien doe suggesting that individual odors are not relevant in this experimental situation.

Experiment 1b: preference for litters of pups. Pups had the choice between (i) their siblings and an empty compartment ($N = 10$), (ii) alien siblings of the same age and an empty compartment ($N = 10$), (iii) their own and alien siblings of the same age ($N = 20$). Tests were carried out at D1 and D7 using different pups.

When given the choice between an empty compartment and their siblings, the pups stayed significantly longer over their own siblings (212.7 ± 50.2 s) than at random (150 s) on D1 (One sample *t*-test: $t_9 = 3.948$, $p = .003$; Fig. 5) and on D7 (own siblings: 207.8 ± 10.1 s; One sample *t*-test: $t_9 = 18.141$, $p < .0001$; Fig. 5). They also preferred alien litters to the empty compartment on D1 (alien litters: 191.9 ± 37.6 s; One sample *t*-test: $t_9 = 3.524$, $p = .007$; Fig. 5) and on D7 (alien litters: 227.5 ± 44.2 s; One sample *t*-test: $t_9 = 5.543$, $p < .001$; Fig. 5). The newborns did not treat the two stimuli differently when siblings (135.9 ± 86.6 s) were paired with alien pups of the same age neither on D1 (One sample *t*-test: $t_{19} = -.728$, $p = .475$; Fig. 5) nor on D7 (siblings: 168.5 ± 72.5 s; One sample *t*-test: $t_{19} = 1.141$, $p = .268$; Fig. 5). No change in the pups' performance was detected between D1 and D7 in the time spent over a specific stimulus (D1 vs. D7) (i) siblings/empty compartment: Unpaired *t*-test: $t_{18} = .303$, $p = .766$; (ii) alien pups/empty compartment: Unpaired *t*-test: $t_{18} = -1.940$, $p = .068$; (iii) siblings/alien pups: Unpaired *t*-test: $t_{38} = -1.291$, $p = .205$; Fig. 5). Although rabbit pups detected the presence of other young of the same age when pitted against an empty compartment, they did not discriminate between own and alien litters. This suggests that the two litters were equally attractive.

Experiment 2: Neonatal Detection and Discrimination Between Own and Alien Nest Materials in Two-Choice Tests Excluding Thermotaxis Forty pups tested on D1 and 40 on D7 had the choice between (i) a sample from their own nest and an empty compartment ($N = 10$), (ii) a sample from an alien nest of the same postpartum stage and an empty compartment ($N = 10$), (iii) samples from their own and an alien nest of the same postpartum stage ($N = 20$).

Pups spent more time over the sample of their familiar nest (234.4 ± 66.2 s) than in the hypothesis of random exploration (150 s) on D1 (One sample *t*-test: $t_9 = 4.030$, $p = .003$; Fig. 6) and on D7 (familiar nest: 232.6 ± 29.1 s; One sample *t*-test: $t_9 = 8.984$, $p < .0001$; Fig. 6). A similar attraction was displayed towards the alien nest (205.1 ± 44 s) versus the empty compartment (94.9 ± 44 s) on D1 (One sample *t*-test: $t_9 = 3.957$, $p = .003$; Fig. 6) and on D7 (alien nest: 245.6 ± 31.2 s; One sample

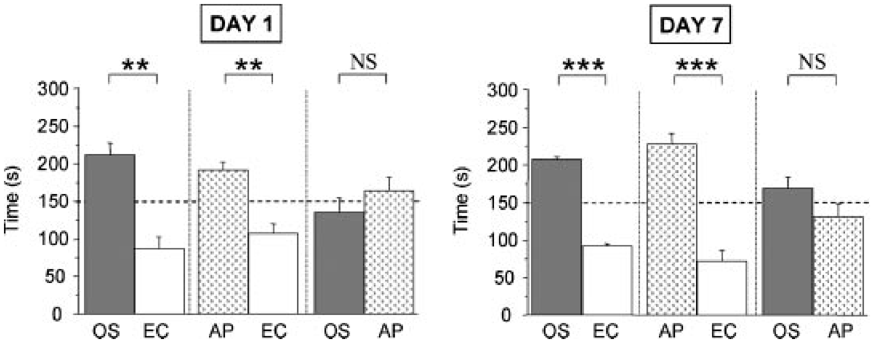

FIGURE 5 Mean orientation time (±SEM) of 1-day-old (a) and 7-day-old (b) pups over the following paired stimuli: Own Siblings (OS) versus Empty Compartment (EC); Alien pups (AP) versus Empty Compartment (EC); Own Siblings (OS) versus Alien Pups (AP). The horizontal dashed line at 150 s indicates random level of pup's orientation; we assessed the difference between the durations that pups stayed over either stimulus against random using one-sample *t*-tests. $^{**}p < .01$, $^{***}p < .001$, NS = not significant.

t-test: $t_9 = 9.689, p < .0001$; Fig. 6). In addition, in the test opposing two nest samples, pups oriented longer toward the side of the arena located above their own nest (180.6 ± 55 s) than in the case of random exploration. This was significant both on D1 (One sample *t*-test: $t_{19} = 2.483$, $p = .023$; Fig. 6) and on D7 (own nest: 192.1 ± 54.3s; One sample *t*-test: $t_{19} = 3.469$, $p = .003$; Fig. 6). Although there was no age effect on the performance of the animals in the percentage of time they spent over their own nest when given the alternative choice of an empty compartment (Unpaired *t*-test: $t_{18} = .079$, $p = .938$; Fig. 6), the time spent by pups on the alien nest when pitted against the empty compartment was more important on D7 than on D1 (Unpaired *t*-test: $t_{18} = -2.373$, $p = .029$; Fig. 6). No change in the performance was observed between D1 and D7 when own and alien nests were compared (Unpaired *t*-test: $t_{38} = -.668, p = .508$; Fig. 6). Therefore, postnatal attraction to the nest and discrimination between own and alien nest is established very early in the pup's life. The two nests present sufficient olfactory differences to allow a pup to discriminate and prefer its own from an alien one.

Experiment 3: Preference for Thermal Cues The results of Experiments 1 and 2 indicated that pups preferred their nest, but not their mother, nor their siblings when these were presented in two-choice tests including thermal cues emanating from the bodies of the stimulus animals. Experiment 3 assessed the relative importance of thermal cues on the orientation response during such two-choice tests.

We tested 20 pups on D1 and 20 others on D7. When given the choice between a warm and an empty bottle, pups spent significantly more time over the warm

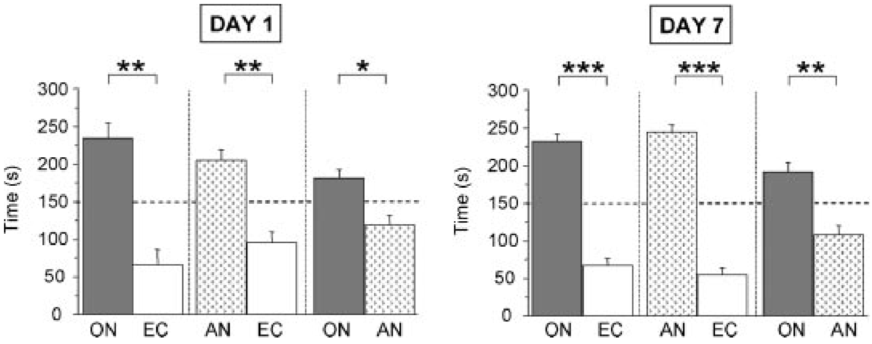

FIGURE 6 Mean orientation time (±SEM) of 1-day-old (a) and 7-day-old (b) pups over the following paired stimuli: Own Nest (ON) versus Empty Compartment (EC); Alien nest (AN) versus Empty Compartment (EC); Own Nest (ON) versus Alien Nest (AN). The horizontal dashed line at 150 s indicates random level of pup orientation; we assessed the difference between the durations that pups stayed over either stimulus against random using one-sample *t*-tests. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

compartment (262.4 ± 35.4 s) than at random (150 s) both on D1 (One sample *t*-test: $t = 14.214$, $N = 20$, $p < .0001$; Fig. 7) and on D7 (warm bottle: 251.2 ± 57.6 s; One sample *t*-test: $t = 7.863$, $N = 20$, $p < .0001$; Fig. 7). No difference in the time spent over the warm bottle was noted between D1 and D7 (Unpaired *t*-test: $t_{38} = .741$, $p = .463$; Fig. 7). Most of the tested animals spent more than 80% of their time over the heated bottle, demonstrating their ability to orient appropriately using thermal cues alone. Thus, the warmth emanating from the mother and the siblings could well be more attractive than their individual olfactory signatures. Because thermotaxis may have influenced the orientation of the pups in Experiments 1a and 1b, we excluded thermal cues emanating from bodies of stimulus animals in the next experimental procedures.

Experiment 4: Discrimination Between Own and Alien Cues From Mother or Siblings in Two-Choice Tests Excluding Thermotaxis Experiment 2 provides evidence that in a situation comparing an empty compartment versus nest materials excluding thermotaxis, pups can detect and orient towards the olfactory stimulus presented. Therefore in Experiment 4 testing the preference for various olfactory cues present in the nest this experimental situation was no longer tested and pups were only submitted to a choice between familiar and unfamiliar olfactory cues.

Experiment 4a: preference for olfactory cues from the mother. Twenty pups tested on D1 had the choice between a sample from their own mother's hair and one from an alien preparturient female. No tests were performed on D7. Pups oriented for significantly longer over their mother's hair (170.8 ± 39.5 s) than in the hypothesis of random exploration (150 s) (One sample *t*-test: $t = 3.353$, $N = 20$, $p = .030$; Fig. 8). Clearly, if the dams' olfactory signatures are not associated with thermal cues, rabbit pups can discriminate between the odor of their mother's hair and that of an alien doe the day after they are born.

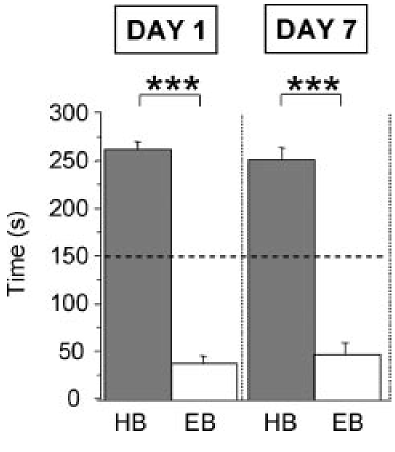

FIGURE 7 Mean orientation time (±SEM) of 1-day-old (a) and 7-day-old (b) pups over a Hot water Bottle (HB = 35°C) and an Empty Bottle (EB = 20°C). The horizontal dashed line at 150 s indicates random level of pup's orientation; we assessed the difference between the durations that pups stayed over either stimulus against random using one-sample *t*-tests. $^{***}p < .001$.

Experiment 4b: preference for olfactory cues from the litter. When given the choice between their own cotton nest and that of alien pups, the amount of time spent by 1-day-old pups over their own cotton wool nest (183.5 ± 11 s) differed significantly from random (150 s) (One sample *t*-test: $t = 3.038$, $N = 20$, $p = .007$; Fig. 8). This experiment provides evidence that once they are no longer influenced by thermal cues newborn pups can discriminate between familiar odors from their litter and odors from alien pups of the same age class.

Experiment 4c: preference for olfactory cues from the intrauterine environment. When given the choice between the uterine secretion of their own mother and that of an alien doe, pups spent 166.4 s ± 10.9 s over the mother's uterine elements. This value did not differ from those for a random exploration (One sample *t*-test: $t = 1.506$, $N = 20$, $p = .149$; Fig. 8). Under our conditions,

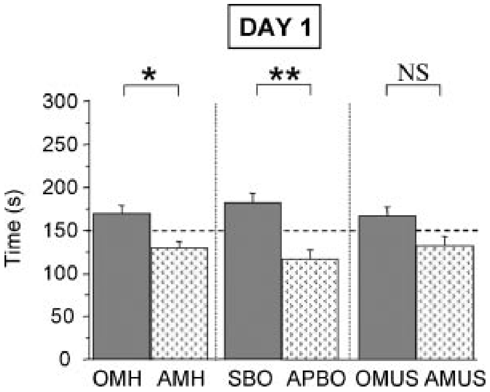

FIGURE 8 Mean orientation time (±SEM) of 1-day-old pups over the following paired stimuli: Own Mother's Hair (OMH) versus Alien Mother's Hair (AMH); Sibling's Body Odor (SBO) versus Alien Pups' Body Odor (APBO); Own Mother's Uterine Substrates (OMUS) versus Alien Mother's Uterine Substrates (AMUS). The horizontal dashed line at 150 s indicates random level of pup orientation; we assessed the difference between the durations that pups stayed over either stimulus against random using one-sample *t*-tests.$^{*}p < .05$, $^{**}p < .01$, NS = not significant.

intrauterine elements do not take part in the preference for own nest.

GENERAL DISCUSSION

Lactating females and young litters emit supra-individual signals that are detectable and attractive to pups. In the present work warmth was a major attractant and can explain the contrast between on the one hand the lack of discrimination between own and alien mother or age mates in Experiments 1a and 1b, and on the other hand the preference for nest odors in Experiment 2 or odors of their familiar partners in Experiments 4a and 4b. Our results clearly demonstrate that newborn pups can discriminate between cues from familiar and unfamiliar conspecifics of the same physiological or developmental stage once attractive thermal cues are no longer associated to the social stimulus. The contribution of a favorable thermal environment to growth and survival in altricial neonates is of utmost importance (Bautista, Drummond, Martinez-Gomez, & Hudson, 2003) and may well explain the indiscriminate responses by newborn pups to the bodies of their mothers in previous studies (Patris et al., 2004; Val-Laillet & Nowak, in press).

Though Garay, Maldonado, and Hudson (2002) had shown that 1-day-old rabbit pups could orient toward a nest by smell, the present study went a step further by providing the very first demonstration of a preference for familiar biological odors found in the nest. Under laboratory conditions, the nest is a structure mostly composed of maternal hair and litter's odor. In the peripartum period it also contains intrauterine fluids, blood and placental traces (Hudson, Bilkó, & Altbäcker, 1996). Because the chemosensory cues detected by pups originate partly from the mother and partly from the litter, the preference for their own nest in Experiment 2 reflects a preference not only for conspecifics in general, but also for kin conspecifics. The olfactory signals that are recognized still remain to be identified. In adult rabbits, excretion and secretion of olfactory substrates depend on specialized glands that are mostly spread over the ventral surface (chin, inguinal and anal areas; Goodrich & Mykytowycz, 1972; Cerbon, Camacho-Arrayo, Gamboa-Dominguez, & Gonzalez-Mariscal, 1996). By recording respiratory responses in juveniles, Mykytowycz and Ward (1971) found that wild rabbits learned the specific odor of their mother's anal and inguinal glands during the first week of life. Whether these odors are present in the hair used in nest building and are recognized the day after birth remains to be elucidated. Urine could be involved as it is a widespread signal used in interindividual recognition by mammals (Halpin, 1986). Although there is no indication in the literature that mothers urinate in their nest, the pups on the other hand do so simultaneously after a nursing bout (Hudson & Distel, 1982). This is followed by vigorous digging and burrowing under nest materials for approximately 15 min after which pups reaggregate. We also know that rabbit mothers leave fecal pellets in the nest shortly before giving birth and then daily for several days (Hudson et al., 1996). They have been reported to be involved in the transmission of information about mothers' diet (Altbäcker, Hudson, & Bilkó, 1995; Bilkó, Altbäcker, & Hudson, 1994) but whether they also carry their olfactory signature is unknown. Consequently, an olfactory blend made of urine and body odors, in combination with maternal hair and pellets, may contribute to the individual olfactory signature of the nest and possibly of the litter.

By contrast, uterine and fetal by-products did not play any role in the preference for the nest in the present study. There is evidence that rabbit neonates are attracted to placentas; however the only work performed so far focused on neonatal detection of alimentary aromas ingested by pregnant females and present in the placenta (Coureaud et al., 2002). Under such conditions newborn pups preferred a placenta of a female which had been fed the same diet as their mother to the placenta of a female fed a different regimen. The fact that in our experiments all the does received the same diet may explain the lack of preference by pups for their own prenatal substrates.

Although we anticipated an improvement in pups' performance between D1 and D7, this was not observed, suggesting that learning was already well established on the first day after birth, that is to say after one or two suckling episodes. This appears to be congruent with studies on other mammalian species using artificial odorants which show that neonatal olfactory learning is facilitated by reinforcers associated with parturition or maternal care such as expulsion of the fetus (Varendi, Porter, & Winberg, 2002), thermal or tactile stimulation (Alberts & May, 1984; Sullivan & Hall 1988; Sullivan et al., 1991), suckling or milk infusion (Brake, 1981; Sullivan & Hall, 1988). Newborn rabbit pups can be conditioned to respond to a novel odorant spread on their mother's ventrum during a single 3-min nursing episode. When tested on the following day, pups display orientation responses towards the same odorant (Hudson, 1985; Hudson et al., 2002). Postnatal preferences for natural odors emanating from the nest, the litter, or the mother could well establish during the very first suckling episode, somewhat parallel to what has been found in newborn lambs (Nowak, 2006). Additional reinforcers such as uterine contractions at parturition, and thermotactile stimulation provided by the cluster of siblings could participate as well. The repetitive occurrence or the continuous presence of some rewards (suckling, warmth) may even reinforce early learning and maintain olfactory preferences over time.

Another nonexclusive hypothesis to explain these early preferences would be that all the postnatal stimuli have some degree of similarity with their prenatal environment. In this case, prenatal olfactory learning would be responsible for the expression of postnatal olfactory preferences. There is wide evidence of a transnatal chemosensory continuity between the prenatal and the postnatal environment (Schaal, 2005). Rabbit pups can detect the odors of placenta or colostrum; however they do not display any preference when placenta and colostrum are presented in a two-choice test (Coureaud et al., 2002). Similar responses were obtained in human babies who had been given the choice between their amniotic fluids and colostrum (Marlier, Schaal, & Soussignan, 1998). In all likelihood, the development of olfactory preferences towards elements of the postnatal environment could derive from a combination of prenatal and postnatal learning processes. A certain degree of similarity between uterine fluids and the postnatal ecological niche would help the rabbit pup to build a rapid and dynamic olfactory image of its environment, the importance of postnatal experience predominating over time.

The early preferences for mother, litter of pups and nest by rabbit pups are remarkably different from what was observed in other altricial species. In Belding ground squirrel (Holmes, 1988, 1990), there is a clear relationship between the emergence of parent-offspring preferences and the time in development when alien conspecifics are encountered for the first time. According to Holmes, the initial expression of preferences will coincide temporally with the *need* to distinguish familiar from unfamiliar individuals (Mateo, 2006; Sherman, Reeve, & Pfenning, 1997). There is no clear explanation for the differences between the rabbit pup (a lagomorph) and altricial rodents in the establishment of social preferences. One possibility might be that unlike altricial rodents, rabbit does do not retrieve their pups which stray from the nest (Ross, Denenberg, Frommer & Sawin, 1959). Thus the *need* to discriminate familiar odors would emerge earlier in the rabbit, because the pup cannot rely on its mother to find its nest and the familiar odors associated with it. These are vital since they provide thermal comfort and the unique daily nursing episode.

To conclude, our data show that learning of individual signatures can take place in 1-day-old rabbit pups, possibly providing the basis for the display of interindividual recognition at the time of emergence from the burrow. Our findings also question whether similar results could be found in other altricial species, should similar methodological precautions be taken. It seems logical to assume that, whatever the species, the expression of preferences for familiar olfactory stimuli will be influenced by the *immediate* biological needs of the young, like thermal needs. Supra-individual cues will play a major attractive role in the first stage of development, ensuring immediate benefit for the young in terms of survival. Concurrently, individual cues will become more salient, until they predominate once the young is thermoregulatory independent and starts to interact socially with conspecifics. Thermal and olfactory cues emanating from the mother and siblings, and consequently from the nest, will act synergistically to fulfill a triple function: they ensure appropriate thermoregulation, they contribute to the location of nesting materials and the aggregation with litter mates, and they facilitate learning of individual signatures essential for the establishment of future social interactions at the emergence of the nest.

NOTES

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