

# Post-oral and Perioral Stimulations during Nursing Enhance Appetitive Olfactory Memory in Neonatal Rabbits

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## Abstract

Nursing–suckling interactions facilitate olfactory learning in newborns as long as suckling and the olfactory stimulus temporally overlap. We tested the hypothesis that olfactory preferences would develop even with a long delay between odor presentation and nursing. Thyme was presented to 2-day-old rabbit pups by placing an odorized plate 2 cm above their nest box. Duration and time of nursing were controlled and occurred before, during, or after odor presentation. Controls were not nursed. When exposed to the odor for 15 min, control pups preferred thyme to a novel odor in a 2-choice test immediately after exposure but not 3 and 22 h later. When pups were nursed immediately before thyme exposure or during exposure, they preferred the familiar odorant until 22 h later. Identically, when nursing occurred 30 min before odor exposure, a preference for thyme was maintained up to 22 h. This was not observed when nursing occurred 60 min before odor presentation. We concluded that enhancement of olfactory memory occurs in neonates during nursing but also after post-oral stimulation by postprandial internal state.

**Key words:** conditioning, newborn rabbit, nursing, olfactory learning, postprandial state, suckling

## Introduction

In a variety of altricial mammals, experience with olfactory cues during suckling has been documented to influence the development of subsequent olfactory preference, nipple attachment, diet preference, as well as reproductive behavior (Blass 1990; Alberts 1994; Smotherman and Robinson 1994). The rapid shift from inborn or prenatal cues to post-natal cues reflects the remarkable abilities of neonates to form and retain new association. This has provided opportunities to understand how suckling interactions help the infants learn about their surroundings (Blass et al. 1977; Hudson 1985). Various experimental procedures that mimic mother–young interactions as they seem to occur naturally have readily obtained appetitive olfactory conditioning in infant rats and rabbits.

Three classes of maternal stimuli encountered during suckling can cause long-term behavioral changes that are olfactory guided. One class concerns maternal excitation exemplified in rats by anogenital stimulation. As the mother enters her nest, she arouses her infants by treading on them, moving them about, and licking their anogenital area. This state of arousal induced by experimental tactile stimulation mimicking mater-

nal licking facilitates learning of novel olfactory cues in the mouse (Bouslama et al. 2005) and in the rat (Sullivan and Hall 1988). In the rabbit, arousal induced by the entrance of the mother into the nest does not seem to be a major reward in olfactory learning (Hudson et al. 2002).

A second class of maternal stimuli concerns the oral stimulation provided by the nipple itself and its integration with the motor pattern of nonnutritive sucking. Rat and rabbit pups are capable of associating the rewarding aspect of nonnutritive sucking by attaching to the nipples of an anesthetized female with a novel olfactory stimulus and hence develop a preference for this odor (Brake 1981; Hudson et al. 2002). Nonlactating nipples have also been shown to provide strong incentive for initiating and maintaining contact with the mother in an operant conditioning paradigm when the reward was the opportunity to attach to a nipple of an anesthetized dam (Amsel et al. 1976; Kenny and Blass 1977).

Events surrounding milk letdown constitute a third broadly defined class of rewarding stimuli. Classical and instrumental learning paradigms have successfully shown that neonates establish preferences for initially aversive or neutral

odors when they are presented contingently with milk intake. Using small infusions of milk via an intraoral cannula as a reward, several authors have described a preferential orientation toward a novel odor in rat pups (Johanson and Hall 1979, 1982; Johanson and Teicher 1980; Brake 1981; Johanson et al. 1984). Hudson et al. (2002) suggested that milk could facilitate olfactory learning in rabbit pups as well, and recently Coureaud et al. (2006) provided the demonstration that a mammary pheromone isolated from milk could act as a primary reinforcing agent in olfactory learning.

In most experiments reported so far in appetitive conditioning, the conditioned stimulus (CS) such as a novel odor is presented a few seconds before and then overlaps with the reward or unconditioned stimulus (US) (Johanson and Teicher 1980; Brake 1981; Johanson and Hall 1982; Johanson et al. 1984; Hudson 1985; Sullivan and Hall 1988; Kindermann et al. 1994; Hudson et al. 2002). In rabbit pups, even a temporal dissociation as short as 1 s between odor and mammary pheromone presentation is sufficient to prevent the development of an olfactory conditioned response (Coureaud et al. 2006). However, the concomitant presentation of a novel stimulus and a reward is not the sole rule leading to appetitive learning. Several authors report that rat pups are also capable of trace conditioning. Trace conditioning includes an interval devoid of any apparent stimulation between CS and US presentation. Border and Spear (2005) showed that learning was possible in 1-day-old pups when lemon odor (CS) was paired with saccharine (US) even though both stimuli were separated by up to 120 s. Newborns (only 3 h old) and fetuses are also capable of acquiring a form of trace conditioning with a gustatory US (Varlinskaya et al. 1997; Cheslock et al. 2000). This requires that the subject maintains some sort of memory of the CS between its cessation and the onset of the US in order to develop a conditioned response. In all the cases reported so far, the CS–US intervals are rather short and do not exceed 2 min. Recently, Valiante et al. (2006) reported that 2- to 3-day-old neonates had a better memory for spoken words 30 min after being fed (breast milk or formula) than before. This drastic effect was specific to memory and did not include attention or rate of learning. The main differences with trace conditioning is that the CS (words) follows the US (feeding) and that the interval between cessation of the US and onset of the CS is far more important. Although this result does not refer to chemoreception like in altricial animal models, it does point out that neonatal learning is possible even though feeding and a novel stimulus are completely disconnected. According to Valiante et al. (2006), it is the postprandial state that enhances memory. Whether this also applies to olfactory learning remains to be discovered.

Among altricial mammals, 3 remarkable traits make rabbit pups particularly suitable to study the effects of delayed nursing on the development of early olfactory learning. 1) Placed in a 2-choice arena, 1-day-old rabbit pups detect the presence of odors and display olfactory preferences for familiar cues (Serra and Nowak 2008). 2) Rabbit pups

rapidly associate odors with suckling in a single conditioning trial (Hudson 1985; Kindermann et al. 1994; Hudson et al. 2002). 3) Mother–young interactions in rabbits are limited to one daily nursing episode, which lasts only for a few minutes (Lincoln 1974; Drewett et al. 1982; González-Mariscal 2007). All this provides an ideal situation to explore the involvement of peri- and post-oral stimulation on early olfactory learning because the stimulus can be presented in a standardized manner at various moments relative to nursing.

The present study was therefore designed to investigate whether novel olfactory information acquired by rabbit pups at various times around nursing or after a long delay following nursing would gain hedonic valence. The odor was presented on a plate on top of the pups so that it could be disconnected from nursing. The pups were tested for olfactory preference in a 2-choice arena designed by Serra and Nowak (2008) immediately after odor presentation, 3 h later, and the day after. Experiment 1 investigated the role of the duration of odor exposure on the maintenance of olfactory preference in the absence of any reward from the nursing–suckling interaction. Based on this outcome, Experiment 2 evaluated the enhancement effect of suckling on olfactory learning when paired in close association with odor exposure (perioral determinants). Experiment 3 examined olfactory learning after a long delay between presentation of the odor and suckling (post-oral determinants). From the results obtained in human neonates (Valiante et al. 2006), we hypothesized that post-oral determinants would facilitate olfactory learning as easily as perioral determinants.

## Materials and methods

### Subjects and housing conditions

Thirty white New Zealand multiparous does and 6 males of strain INRA 1077 constituted the nucleus of dams and sires. All the animals were reared in the Station “BioAgresseurs, Santé et Environnement” (INRA, Nouzilly, France), and maintained in individual cages (70 × 46 and 60 cm high). A nesting box (46 × 14 and 24 cm high) that contained wood shavings as bedding was inserted into the cage 3 days prior to parturition. Animals were kept under a constant 13:11 h light:dark cycle (lights on at 0700), and ambient temperature was maintained at 20 °C. Water and food pellets (Huttepain Bouix, Piace, France) were provided ad libitum. The day of birth was designated as postnatal day 0. Litter size was not adjusted, it varied between 6 and 8 pups, and the proportion of males and females was unknown at the time of testing. The pups remained in their natal nest except for the odorization procedure and during testing.

### Treatments

#### *Odorization procedure*

On day 2, the pups were transferred from their natal nest into a training nest where they were exposed to the odorant. The

training nest was a plastic container the bottom of which was filled with clean wood shavings and a sample of their own mother's hair that provided the same characteristics (size, texture, and temperature) as their natal nest. This procedure was chosen for 2 reasons: first, the duration of odor presentation could be controlled as it was limited to the time spent by the pups in the training nest and second the natal nest materials were not contaminated by the odorant. The odorant used was essential thyme oil diluted in vegetable oil (10%). Thyme odor was chosen because it has been reported as readily accepted by rabbit pups: 1) after 24 h of familiarization to the thyme oil, 2-day-old pups prefer an odorized to a nonodorized nest (Nowak and Serra 2005) and 2) pups born from thyme-fed does eat more thyme at weaning than those born from control-fed mothers (Hudson and Altbäcker 1995). Two milliliters of the solution were uniformly spread over a sheet of filter paper that was then positioned on a rectangular PVC plate (32 × 21 × 0.5 cm). The plate was tipped upside down so that the odorized filter paper was placed 2 cm above the pups and covered the whole training nest. With such a procedure, the pups could perceive the odor while contamination of their body was very limited. The odorization procedure lasted for 15 min except in Experiment 1 where one group of pups was exposed to thyme for 1 h. The pups were transferred back into their natal nest at the end of the odorization procedure, and the content of the training nest was discarded. Transferring the pups between the 2 nests took only a few seconds.

#### *Control of the period of nursing*

Nursing was controlled from the day of birth. After parturition, the entrance to the nest box was kept closed except for a daily 7-min period, which mimicked the natural nursing rhythm of the species and fulfilled the nutritional needs of the pups. Nursing was set at specific periods relative to the moment of odor exposure for each litter.

#### **Testing procedure**

##### *Odorants and test apparatus*

The tests consisted of a choice between 2 odorants: thyme and cinnamon. The odorants were diluted in vegetable oil at 10% for essential thyme oil and at 1% for essential cinnamon oil after a preliminary study demonstrated that these concentrations were neither attractive nor aversive for naive pups. The test apparatus was a rectangular PVC box (42 × 32 and 15 cm high) divided into 2 by a median wall. A platform made of a double layer of wire mesh (mesh size: 1 mm<sup>2</sup>) spaced 1 cm apart covered the top part of the box. Nine-centimeter high PVC walls were added onto the sides of the platform and delimited the testing arena (42 × 32 cm). Two milliliters of the odorants were evenly spread over a sheet of absorbent paper so that each stimulus was in contact with the platform while the double layer of wire mesh pre-

vented the tested pups from having direct contact with the paper. The median wall of the box avoided mixing of air and blending of odors between the 2 stimuli and constituted the midline separating the 2 compartments of the testing arena.

##### *Tests monitoring*

Two-choice tests were conducted at 3 different moments: immediately after the end of odor exposure (T0), 3 h later (T3), and 22 h later (T22). Each pup was tested once only. To limit litter effects, pups were allocated at random to 1 of the 3 times of test with 1 or 2 subjects of a same litter at the most contributing to each group. The remaining pups were not used. At the beginning of a test, the pup was placed in the testing arena lengthwise just on the midline separating the 2 chambers. The test started once the pup was released. The cumulative time spent on each side of the testing arena was recorded during a 5-min test. A pup was considered to be in a specific half of the arena once its whole head was over the midline separating the 2 chambers. After each test, to remove any olfactory traces left by the previous pups, the box and the wire mesh were washed with pure water, 95% ethyl alcohol, then water again, and finally dried. In addition, the sheet of absorbent paper was changed after each test, and the arena was rotated 180° every 4 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 are presented as means. Differences between groups were evaluated by an analysis of variance (ANOVA) (age × treatment). Where appropriate, post hoc comparisons were made using *F*-tests for simple main effects. In addition, the group means of the time spent by pups above thyme odor were submitted to 1-sample *t*-tests. We tested this variable "time" against the null hypothesis (hypothesized mean = 150 s), which represents no preference or random choice. Therefore, when the performance of a given group differed statistically from random, it indicated a preference in favor of the stimulus over which pups spent more time. *P* values less than 0.05 were considered as statistically significant.

## **Results**

### **Experiment 1: influence of the duration of thyme exposure**

Food-deprived pups were exposed to the odor of thyme for either 15 min or 1 h. These 2 groups were chosen to investigate 1) if pups could establish olfactory learning without

any reward from the nursing–suckling interaction and 2) if they did whether such learning relied on the duration of odor exposure. For experimental purposes, these animals had been deprived of the morning nursing. Because feeding deprivation could not exceed 24 h without affecting the nutritional needs of the pups, the mother was allowed to nurse her litter immediately after T3.

The preference for thyme during the choice tests was significantly affected by the treatment ( $F = 4.226$ , degrees of freedom [df] = 1/54,  $P = 0.045$ ), but no time effect and no interactions could be found. Subsequent 1-sample  $t$ -tests revealed in both groups that 2-day-old pups spent significantly more time over thyme at T0 than in the hypothesis of a random choice (1 h: thyme =  $207.2 \pm 47.6$  vs. 150 s for random,  $t_9 = -3.799$ ,  $P < 0.001$ ,  $n = 10$ ; Figure 1A; 15 min: thyme =  $211.1 \pm 49.4$  s,  $t_9 = -3.910$ ,  $P < 0.01$ ,  $n = 10$ ; Figure 1B). This preference persisted in pups exposed to thyme for 1 h at T3 (thyme =  $213.2 \pm 60.7$  s,  $t_9 = -3.291$ ,  $P < 0.01$ ,  $n = 10$ ) and at T22 (thyme =  $217.2 \pm 57.1$  s,  $t_9 = -3.724$ ,  $P < 0.01$ ,  $n = 10$ ; Figure 1A). By contrast, pups exposed to thyme for 15 min did not express a preference for thyme neither at T3 (thyme =  $162.8 \pm 60.9$  s,  $t_9 = -0.664$ ,  $P = 0.523$ ,  $n = 10$ ) nor at T22 (thyme =  $175.2 \pm 56.3$  s,  $t_9 = -1.416$ ,  $P = 0.190$ ,  $n = 10$ ; Figure 1B). Thus, if all the pups displayed a preference for thyme immediately after the familiarization procedure, only those exposed to the odor for 1 h maintained a clear preference 3 and 22 h later.

### Experiment 2: influence of nursing

In the present experiment, we were primarily interested in the concomitant association between nursing and thyme odor presentation in order to test the enhancing effect of nursing (combining perioral and post-oral stimulation) on olfactory

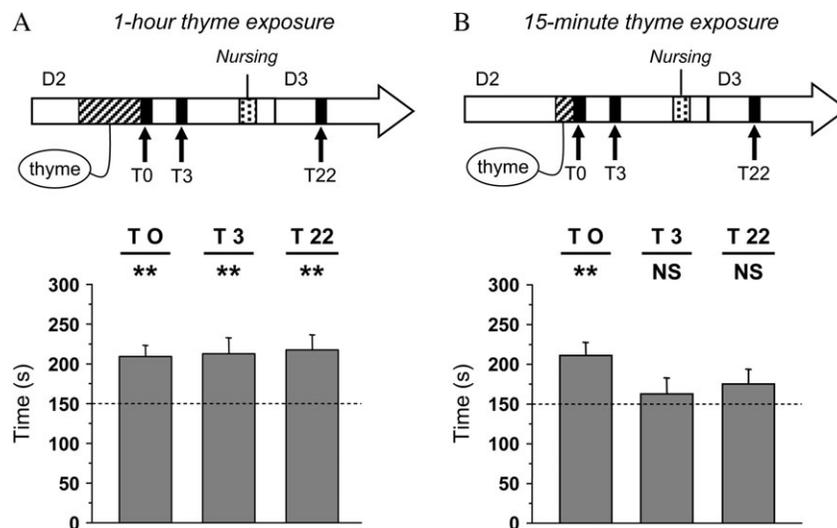
learning. Based on the outcome from Experiment 1, we exposed the pups to the odor of thyme for 15 min only.

### Nursing immediately after exposure to thyme

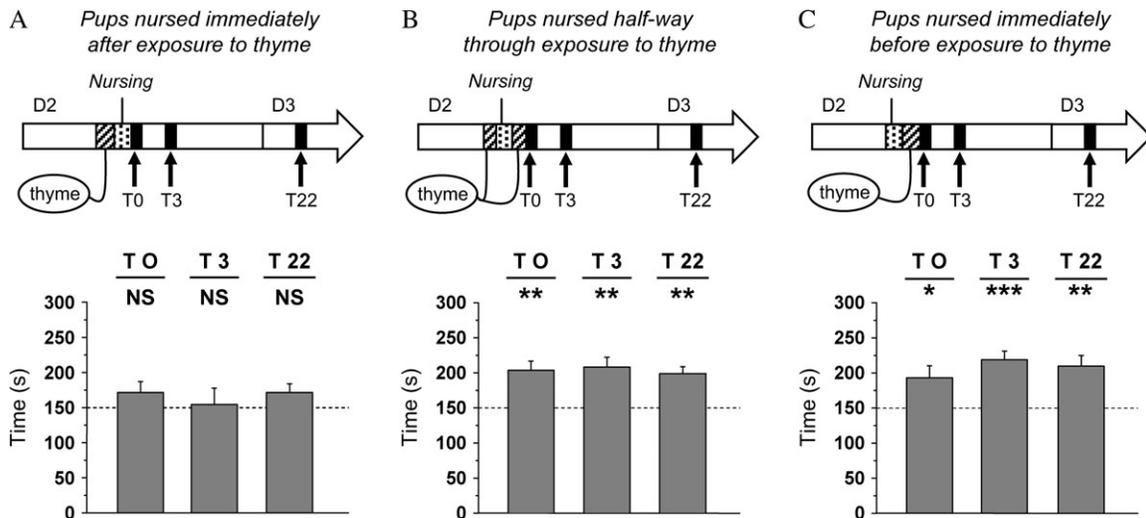
Pups were first exposed to thyme odor for 15 min in the training nest. Immediately after, they were transferred into their own nest, the entrance of which was opened, and the doe was allowed to nurse her litter for 7 min. The nest box was closed once the doe left it, and the pups were immediately tested in the arena. The amount of time spent over thyme and over cinnamon at the 3 periods of tests did not differ from random (T0: thyme =  $169.9 \pm 52.6$  vs. 150 s for random,  $t_9 = 1.197$ ,  $P = 0.262$ ,  $n = 10$ ; T3: thyme =  $153.3 \pm 76$  s,  $t_9 = 0.137$ ,  $P = 0.894$ ,  $n = 10$ ; T22: thyme =  $170.5 \pm 40.8$  s,  $t_9 = 1.589$ ,  $P = 0.147$ ,  $n = 10$ ; Figure 2A). We conclude that when nursing occurs immediately after the end of thyme exposure, rabbit pups do not express a preference for the familiar odor.

### Control for nursing immediately after exposure to thyme.

Nursing the pups after thyme exposure implied that the first testing session could not take place immediately after the odorized plate was removed (T0) like for the pups tested in Experiment 1 but 7 min later (T0 + duration of nursing). Could this delay between odor exposure and testing be responsible for the lack of olfactory preference in the arena? A control group of pups was added to the experiment and submitted to the 2-choice test after a delay of 7 min following exposure to thyme but without being nursed. Under such conditions, the choice displayed by pups did not differ from random (T0 + 7 min delay: thyme =  $159.3 \pm 60.2$  vs. 150 s,  $t_9 = -0.488$ ,  $P = 0.637$ ,  $n = 10$ ). Therefore, the short temporal discontinuity between odor exposure and testing



**Figure 1** Mean orientation time over thyme in a 2-choice test by pups exposed to this odor for 1 h (A) or 15 min (B). Pups were tested immediately after exposure (T0), 3 h later (T3), or 22 h later (T22). The horizontal dashed line at 150 s indicates the 0 (random) level of pup orientation; we assessed the difference between the durations that pups stayed over thyme against this 0 level using 1-sample  $t$ -tests. \*\* $P < 0.01$ ; error bars indicate standard error of mean.



**Figure 2** Mean orientation time over thyme in a 2-choice test by pups exposed to this odor for 15 min and (A) nursed immediately after exposure, (B) nursed halfway through exposure, or (C) nursed immediately before exposure. Pups were tested immediately after exposure (T0), 3 h later (T3), or 22 h later (T22). The horizontal dashed line at 150 s indicates the 0 (random) level of pup orientation; we assessed the difference between the durations that pups stayed over thyme against this 0 level using 1-sample *t*-tests. NS = not significant; error bars indicate standard error of mean.

explains the lack of preference for thyme found in the previous experiment.

#### Nursing halfway through exposure to thyme

Exposure to thyme odor was divided in 2 half-sessions of 7.5 min each. Immediately after the first exposure in the training nest, the pups were transferred into their own nest box, and the doe was allowed to nurse them for 7 min. The pups were then put back in the training nest for a further 7.5 min. In the choice test, the pups spent more time over thyme than in the case of random exploration at the 3 periods of tests (T0: thyme =  $203.7 \pm 42.4$  vs. 150 s for random,  $t_9 = -4.006$ ,  $P < 0.01$ ,  $n = 10$ ; T3: thyme =  $207.9 \pm 44$  s,  $t_{10} = -4.367$ ,  $P < 0.01$ ,  $n = 11$ ; T22: thyme =  $199.1 \pm 36.5$  s,  $t_{10} = -4.464$ ,  $P < 0.01$ ,  $n = 10$ ; Figure 2B). Therefore, if nursing is made possible halfway through the odorization procedure, newborns develop and maintain a preference for the odorant until 22 h later.

#### Nursing immediately before exposure to thyme

The does were allowed to nurse their young for 7 min just before exposing the pups to thyme odor for 15 min in the training box. In the arena opposing thyme to cinnamon, the pups oriented for longer toward the side located above thyme than in the case of random exploration at the 3 periods of tests (T0: thyme =  $192.8 \pm 58$  vs. 150 s for random,  $t_{11} = -2.560$ ,  $P = 0.027$ ,  $n = 12$ ; T3: thyme =  $218.8 \pm 43.3$  s,  $t_{11} = -5.508$ ,  $P < 0.001$ ,  $n = 12$ ; T22: thyme =  $209.3 \pm 53.8$  s,  $t_{11} = -3.812$ ,  $P < 0.01$ ,  $n = 12$ ; Figure 2C). We conclude that if pups are nursed immediately before being exposed to thyme, they develop and maintain a preference for this odorant until 22 h later.

#### Comparison between groups

The control group for nursing immediately after exposure to thyme was excluded from the analysis as pups were not tested at T3 and T22. Comparison of the 3 other groups revealed that the preference for thyme was significantly affected by the treatments ( $F = 6.529$ ,  $df = 2/88$ ,  $P = 0.002$ ), but no time effect and no interactions could be found. Pups nursed immediately after odor exposure spent significantly less time over the side located above thyme than pups nursed halfway through ( $P = 0.005$ ) or just before odor exposure ( $P = 0.001$ ).

#### Experiment 3: delayed effect of nursing

In the present experiment, we were interested in the temporal dissociation between nursing and thyme odor encountered in the training nest, in order to test the enhancing effect of post-oral stimulation on olfactory learning. By testing the delayed effects of nursing on olfactory learning, we excluded the influence of perioral sensory stimulation because olfactory learning is not possible when novel odor presentation and perioral reward are delayed (Sclafani 1995; Coureaud et al. 2006).

#### Nursing 30 or 60 min before exposure to thyme

In this experiment, we exposed the pups to thyme either 30 or 60 min after nursing. Like in Experiment 2, pups were nursed in their own nest box for 7 min and exposed to the odor for 15 min in the training box. Once in the test arena, pups exposed to thyme 30 min after being nursed oriented for longer over thyme than in the case of random exploration at the 3 periods of tests (T0: thyme =  $234.1 \pm 42.9$  vs. 150 s for random,  $t_{11} = -6.789$ ,  $P < 0.0001$ ,  $n = 12$ ; T3: thyme =  $204.3 \pm 71$  s,

$t_{11} = -2.650$ ,  $P = 0.023$ ,  $n = 12$ ; T22: thyme =  $193.9 \pm 63.7$  s,  $t_{11} = -2.389$ ,  $P = 0.036$ ,  $n = 12$ ; Figure 3A). Pups given a 60-min delay oriented for significantly longer over the thyme compartment at T0 (thyme =  $231.5 \pm 41.5$  s,  $t_9 = -6.210$ ,  $P < 0.001$ ,  $n = 10$ ; Figure 3B) but no difference could be detected at T3 (thyme =  $171.3 \pm 62.5$  s,  $t_9 = -1.078$ ,  $P = 0.309$ ,  $n = 10$ ; Figure 3B) nor at T22 (thyme =  $191.5 \pm 77.1$  s,  $t_7 = -1.522$ ,  $P = 0.172$ ,  $n = 8$ ; Figure 3B). These results show that olfactory learning can take place even after a 30-min delay between nursing and odor presentation but that a 60-min delay is not followed by such an effect.

#### Control for nursing 30 min before exposure to thyme

The present control group was added to this experiment to evaluate if the state of arousal elicited when the mother enters her nest could favor the development of delayed olfactory learning. The procedure consisted in waking up the pups by stroking gently the whole litter by hand, opening the nest box, and letting the doe enter and stay in her nest for 7 min. Nursing was prevented by covering the nest with wire mesh, but this permitted to expose the entire litter to the mother's ventral odors. Pups were alert and expressed nipple-search behavior as long as the doe sat on top of the nest. The pups were exposed to the odor of thyme 30 min later. In the 2-choice test, the pups explored the thyme compartment significantly more than at random at T0 (thyme =  $184.3 \pm 46.1$  vs. 150 s for random,  $t_9 = -2.352$ ,  $P = 0.043$ ,  $n = 10$ ; Figure 4) but not at T3 (thyme =  $164.3 \pm 55.6$  s,  $t_9 = -0.813$ ,  $P = 0.437$ ,  $n = 10$ ) nor at T22 (thyme =  $172.3 \pm 54.2$  s,  $t_9 = -1.302$ ,  $P = 0.225$ ,  $n = 10$ ). It appears that the arousal state elicited

by the mother's presence does not have any impact on olfactory learning when pups are exposed to thyme 30 min later.

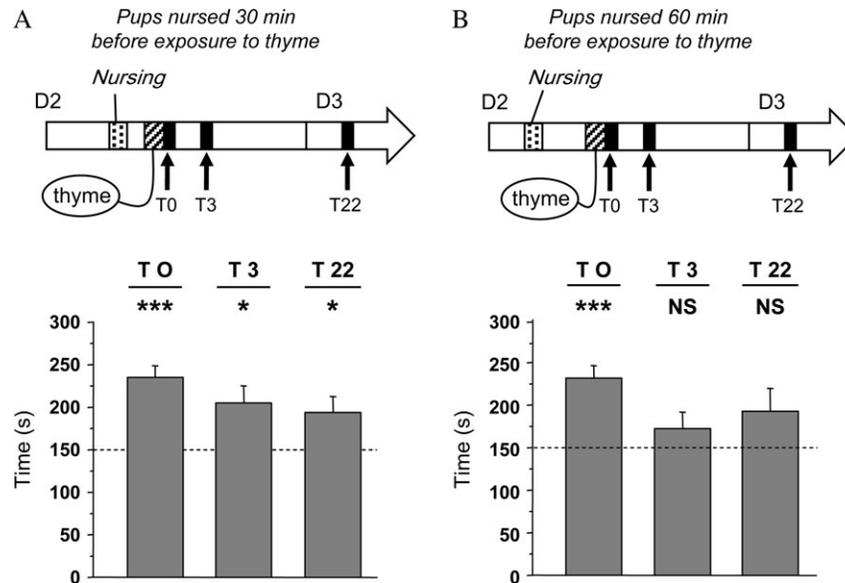
#### Comparison between groups

The ANOVA revealed a strong tendency for a treatment effect ( $F = 2.929$ ,  $df = 2/85$ ,  $P = 0.058$ ) and a significant time effect ( $F = 3.496$ ,  $df = 2/85$ ,  $P = 0.035$ ), but no interaction was found. Control pups in contact with their mother's ventrum spent significantly less time over the side located above thyme at T0 than pups nursed either 30 or 60 min before being exposed to the odor ( $P < 0.02$  in both cases). A significant effect persisted at T3: pups in contact with their mother's ventrum and pups nursed 60 min before thyme exposure spent significantly less time over thyme than pups nursed 30 min before ( $P < 0.05$  in both cases). No intergroup differences were found at T22.

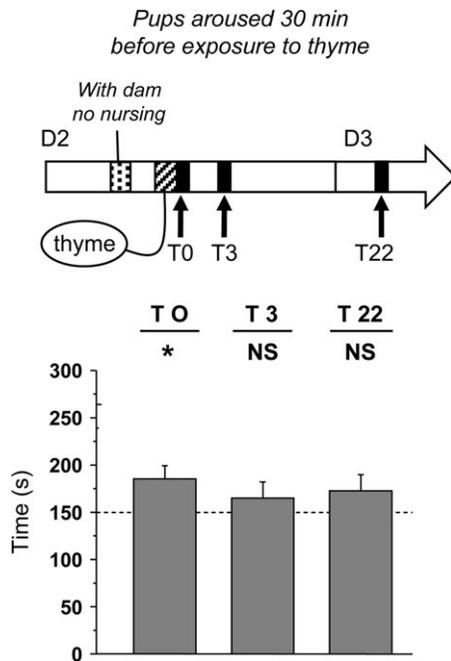
#### Discussion

The findings of this study indicate that a combination of perioral and post-oral determinants results in a preference to odorants encountered in the natal environment. In Experiments 2 and 3, regardless of whether nursing occurred around thyme exposure or 30 min before, rabbit pups developed and maintained a preference for thyme until 22 h later. This set of experiments demonstrates that olfactory learning can be established in one trial only and results in robust olfactory memory even though suckling (US) is temporally disconnected from the novel odor (CS).

In most previous experiments performed on appetitive learning in rat and rabbit pups, when authors followed



**Figure 3** Mean orientation time over thyme in a 2-choice test by pups exposed to this odor for 15 min and nursed either 30 (A) or 60 min (B) before exposure. Pups were tested immediately after exposure (T0), 3 h later (T3), or 22 h later (T22). The horizontal dashed line at 150 s indicates the 0 (random) level of pup orientation; we assessed the difference between the durations that pups stayed over thyme against this 0 level using 1-sample  $t$ -tests. NS = not significant,  $*P < 0.05$ ,  $***P < 0.001$ , error bars indicate standard error of mean.



**Figure 4** Mean orientation time over thyme in a 2-choice test by pups exposed to this odor for 15 min. Pups were aroused and exposed to their mother's belly without being nursed 30 min before exposure to thyme. The horizontal dashed line at 150 s indicates the 0 (random) level of pup orientation; we assessed the difference between the durations that pups stayed over thyme against this 0 level using 1-sample *t*-tests. NS = not significant, \**P* < 0.05, error bars indicate standard error of mean.

a classical conditioning procedure, the conditioned olfactory stimulus preceded and overlapped the unconditioned stimulus (suckling or intraoral infusion of milk) (Johanson and Teicher 1980; Brake 1981; Johanson and Hall 1982; Johanson et al. 1984; Hudson 1985; Sullivan and Hall 1988; Kindermann et al. 1994; Hudson et al. 2002). By and large, appetitive learning relies on a temporal contiguity between CS and US, and often, several trials are necessary for successful conditioning (e.g., 10 trials in rat pups, Johanson and Hall 1979, 1982). Only work on trace conditioning has shown that 1-day-old rats could form associative learning even though the CS and the US do not overlap. With this procedure, the period free of stimulus, called the trace interval, must not exceed 300 s (Border and Spear 2005). Results from Experiment 3 go against the appetitive odor conditioning paradigms described so far: when rabbit pups were nursed 30 min before thyme exposure, they oriented to the odorant for significantly longer 3 and 22 h later. To our knowledge, this is the first evidence that a backward presentation between the US (nursing) and the CS (thyme) can result in robust olfactory memory when both stimuli are separated by such a length of time. Obviously, the arousal state of the litter elicited by the entrance of the mother into the nest 30 min before exposing the pups to thyme cannot explain this behavioral outcome because it was not sufficient to induce olfactory learning (Experiment 3b). When nursing

was allowed 60 min before thyme exposure, enhancement of olfactory memory seems to have failed because the performances of the pups were not as good at T3 as those of pups nursed 30 min before. At first sight, the formation of appetitive olfactory learning with a delay between the reward (US, nursing/suckling) and the presentation of the novel odorant (CS, thyme) appears unusual and is restrained to a narrow time window (up to 30 but not 60 min after nursing). In the literature, backward sequences are reported to be ineffective with appetitive conditioning whether the rat pups are exposed to the novel odor immediately after milk infusion (Brake 1981) or 20 min later (Johanson and Hall 1982). The fact that olfactory memory was enhanced in the present work even though nursing appears disconnected from the novel odor suggests that odor was not associated with the suckling bout itself but with its postprandial consequences. We hypothesize that the post-oral consequences of suckling induced an internal state during odor exposure that facilitated the maintenance of odor memory. Suckling is a very composite stimulus, 2 of which may exert their effects through post-oral route: gastrointestinal distension and the nutrients present in milk. This is supported by the studies on learned flavor preferences in adult and weanling rats. Postingestive actions of nutrients, either within the intestinal lumen or postabsorptively, have a powerful reinforcing effect on conditioned flavor preference (Myers and Sclafani 2006). Although the nature of the postingestive rewarding signals in rabbit pups is still an unresolved issue, some hypotheses can be put forward. For instance, results obtained on other species suggest that it is the satiating actions of nutrients or gastrointestinal peptides that facilitate learning (Rozin and Vollmeche 1986; Morley et al. 1992; Oomura et al. 1993; Sclafani 1995; Nowak et al. 2007). In addition, preference conditioning has also been attributed to energy signals generated by nutrients (Mehiel and Bolles 1988). Whether this applies to our case remains to be investigated and needs to be tested in experimental situations involving intragastric infusion of milk or various nutrients. Serum metabolites like free fatty acids, glucose, and proteins seem to remain relatively stable across the internursing period (Escobar et al. 2000), but no one has ever investigated in detail the physiological changes around nursing.

Our results are consistent with 2 other findings suggesting a role of the internal state on neonatal learning. The first one is reported in human babies. Two- to 3-day-old newborns develop better memory for spoken words 30 min after feeding than before (Valiante et al. 2006). The authors hypothesized that the enhanced memory after feeding is mediated by several mechanisms including plasma glucose and the release of gastrointestinal factors. The second finding is reported in rabbit pups by Pongrácz and Altbäker (1999) and Csatádi et al. (2007). They found that human handling of pups during the first week of life, shortly after the daily nursing bout, can significantly increase rabbits' affinity toward a human experimenter at weaning. In addition, a well-defined sensitive

period appears for successful handling, starting 15 min before and ending 30 min after nursing and more interestingly, not 60 min later, suggesting a role of the internal state (Pongrácz and Altbäker 1999). The internal state, as reported by these authors, may not be specific to olfactory learning and could facilitate various forms of memory.

Our finding in Experiment 3 cannot exclude the additive long-term reinforcing effect of perioral stimulations. In particular, the mammary pheromone isolated from rabbit's milk is known to promote learning of neutral odorants paired with the pheromone in single and short trials (Coureaud et al. 2006). The pheromone-induced learning is efficient from birth and supports successive acquisition of distinct odorants. Despite its powerful reinforcing properties in olfactory learning, the mammary pheromone has to overlap with the novel odor for conditioning to occur. When newborns are exposed to a novel odorant for 2.5 min and then exposed to the mammary pheromone 1 s later, they do not learn the odorant. Of course, this does not exclude the additional effect of the mammary pheromone in Experiment 3 as suckling occurred before and not after exposing the pups to the novel odor, and backward pairing of the mammary pheromone with a novel odorant has never been tested. Non-nutritive sucking or the taste of milk through some of its components could be other rewards to take into account. Nonetheless, should additive effects between peri- and post-oral stimulations take place when rabbit pups suckle by their dam, this does not change our conclusions about the importance of a specific post-ingestive internal state on olfactory memory enhancement.

In contrast to Experiment 3, olfactory learning through the concomitant occurrence of suckling and thyme exposure in Experiment 2 is likely to result from the rewarding effect of perioral determinants. Several factors have been shown to contribute to the change in olfactory-guided behaviors: chemosensory signals released by the nipple (Hudson 1985; Kindermann et al. 1994), nonnutritive sucking (Brake 1981; Hudson et al. 2002), milk (Brake 1981; Johanson and Teicher 1980; Johanson et al. 1984), or the mammary pheromone found in the milk (Coureaud et al. 2006). Nonetheless, an additive influence of post-oral determinants is possible especially in Experiment 2C when nursing precedes thyme exposure. In this case, post-ingestive rewarding circuits (e.g., gastrointestinal distension) may be activated within minutes after milk is consumed and therefore be concomitant to odor exposure.

Inversely, when nursing was permitted immediately after odor exposure, rabbit pups did not show a preference at any of the 3 testing times. Our results do not align with the literature on trace conditioning with rat pups and do not appear logical at first sight. This temporal sequence precluded any chance of association even though they received equivalent thyme exposure and nursing to pups that were nursed either halfway through or immediately before being exposed to thyme. The postnursing state does not explain the

lack of preference for thyme because a mere delay of 7 min without nursing leads to the same outcome. In addition, pups nursed halfway through exposure to thyme were likely to be in a similar postnursing state; yet, they did develop olfactory learning. One explanation would be that olfactory learning can only establish if nursing precedes or overlaps with odor presentation supporting reinforcement by the mammary pheromone as demonstrated by Coureaud et al. (2006) or by perioral sensory stimulation as described by Hudson et al. (2002).

Though it is clear that a combination of perioral and post-ingestive determinants enhances olfactory memory in rabbit pups, Experiment 1 shows that 2-day-old pups can also develop olfactory preferences within the nest in the absence of suckling. The establishment of such preferences at this time point relies then on a longer duration of exposure. In food-deprived pups, the preference for thyme was maintained over 22 h as long as exposure lasted for 1 h. Enhancement of olfactory memory in this group may result from stimulus familiarity (Janus 1989) but also from associative learning. Indeed, thyme odor may well have been associated during the 1-h exposure with various nonnutritive rewards found in the natal environment such as warmth, natural odors, or tactile stimulations from conspecifics.

In all studies conducted so far on appetitive classical conditioning in altricial mammalian neonates, it is the contingency between odor presentation and nursing or milk infusion that was necessary for learning to establish (except for the delay reported in the literature on trace conditioning). Our results provide the very first demonstration that olfactory learning can take place when nursing and odor presentation are completely dissociated over time, suggesting that the post-ingestive state enhances olfactory memory. This suggestion will require further empirical tests to be conclusive by using intragastric infusion of milk instead of nursing as a reward. Nonetheless, our results fit with the current body of literature and provide additional evidence for memory enhancement in infants through a specific internal state induced by feeding.

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