The mammary pheromone of the rabbit: from where does it come?

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Newborn rabbits, *Oryctolagus cuniculus*, are directed to their mother's nipples by specialized odour cues. Previous investigations have suggested that these cues are released from the doe's abdominal surface from structures located around the nipple. We tested pups with samples of various cutaneous tissues or fluids collected from lactating females to determine the location of the source of the odour cues. After finding that the nipples from lactating does were more attractive than those of virgin females, we conducted three experiments using skin samples collected at increasing distance from the nipples, dermal and mammary tissues taken below the nipples, and milk collected at different levels of the mammary pathway. These different substrates were assessed for their ability to elicit searching/grasping responses in pups. Efficient odour cues were released only from the nipples, whereas the dermal or mammary tissues sampled beneath the nipples were behaviourally inefficient, and milk became behaviourally active only after it had flown through the nipple. These results suggest dual exocrine sources of active factors from the nipple of lactating rabbits: cues released within the nipple that render milk behaviourally active and cues distributed over the nipple epidermis.

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To survive, newborn mammals depend on their ability to locate the mother, find the mammary area, grasp a nipple and suck as soon as possible after birth. In general, mammalian mothers help their offspring by standing still, close to or above them, adopting the nursing posture and directing the infants' heads towards the mammaries (Stern 1989, 1996; Hennessy & Jenkins 1994; Gonzalez-Mariscal & Poindron 2002). However, it is the newly born infants' ultimate decision to mouth and grasp the nipple, suck and swallow colostrum, a liquid to which they have never been directly exposed before. The postparturient female provides a range of cues to arouse and guide the infant (Hofer et al. 1976; Blass & Teicher 1980; Rosenblatt 1983;

Vince & Ward 1984; Schaal et al. 2001). Among these cues, textural and thermal features of the skin and perimammary odours, predominate (Rosenblatt 1983; Vince 1993). Experimental alteration of these odour cues (through washing or masking with novel odours), and impairment of the corresponding sensory systems (through anaesthesia or lesion), lead to suckling failure, starvation and finally often to the newborn animal's death (e.g. Teicher & Blass 1976; McChelland & Cowley 1982).

Among mammals, rabbit, *Oryctolagus cuniculus*, newborns meet particularly parsimonious nursing conditions. Rabbit females make one 3–4-min nursing visit a day during the first 2 postnatal weeks (Zarrow et al. 1965; Lincoln 1974). Pups then have to seek out a nipple in the doe's abdominal fur while competing with littermates. They are, however, highly efficient at reaching the nipple from the very first nursing opportunity, using limited senses coupled to a specialized motor pattern producing head-searching movements and oral grasping (Schley 1977; Drewett et al. 1982; Hudson & Distel 1983).

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The ability of newborn rabbits to reach and grasp a nipple is controlled mainly by chemosensory cues released from the female's abdomen. (1) The initiation and maintenance of the typical nipple searching–grasping behaviour depends on the olfactory system (Schley 1977, 1981; Distel & Hudson 1985). (2) The effectiveness of the abdominal odour cues is tied to the female's physiological state, lactating females having a more effective signal than males, virgin does or pregnant does (Müller 1978; Hudson & Distel 1984; Coureaud & Schaal 2000; Gonzalez-Mariscal et al. 2000). (3) Experimental masking of the skin around the nipple has led to the hypothesis of a gradient of the active odour peaking on the nipple and decreasing on the surrounding skin (Müller 1978; Hudson & Distel 1983; Coureaud et al. 2001). (4) Undefined odorants that affect pups' behaviour occur in milk (Müller 1978; Keil et al. 1990; Coureaud et al. 2001, 2002). Finally, (5) these milk cues are as attractive to newborn pups as are the cues emitted from the abdominal skin (Coureaud et al. 2001), suggesting that either the same signal or different signals with similar functions are released in both secretions.

Schley (1976, 1977, 1981), Drewett et al. (1982), Hudson & Distel (1983) and Coureaud et al. (2001) hypothesized that rabbit pups are directed to the nipples through a specialized scent signal. This has recently been corroborated by an investigation of the volatiles emitted from fresh rabbit milk. Among the 150–160 molecular compounds detected in rabbit milk, one substance, 2-methylbut-2-enal, is highly active in releasing the typical searching and oral grasping responses in pups (Coureaud et al. 2003; Schaal et al. 2003). As this substance is excreted in milk and meets the definition of the concept of pheromone as applied to mammals (Beauchamp et al. 1976; Johnston 2000), it has been named mammary pheromone (MP).

Several studies have suggested that the abdomen, the mammary area, the nipple surface, or milk could be loci of secretion or excretion of the signal (Schley 1976; Müller 1978; Hudson & Distel 1983, 1995; Keil et al. 1990; Coureaud & Schaal 2000; Coureaud et al. 2001, 2003; Schaal et al. 2003). In this study, we attempted to localize the source of the MP more precisely.

We carried out a series of experiments to evaluate the response induced in newborn pups by various histological or secretory substrates collected from rabbit females. In experiment 1, we assessed the relative behavioural activity of nipples from lactating and virgin does. In this experiment we aimed to verify whether the small skin patch restricted to the nipple could account for the observed attractiveness of the whole abdomen of lactating females. In experiment 2 we examined whether the nipple was the only skin region releasing the odour cue or whether it was distributed over the neighbouring skin surface. In experiment 3 we sampled various tissues of epidermal, dermal or mammary origins to investigate whether the whole mammary tract of lactating females generated the odour cues. In a final experiment we evaluated whether milks collected at various levels of the mammary tract elicited searching and oral grasping responses in pups.

GENERAL METHODS

Animals and Housing Conditions

Rabbits of the New-Zealand × Californian cross-breed (strains Grimaud GD 14 and GD 24 for females and males, respectively) were housed in the breeding unit of the Etablissement National d'Enseignement Supérieur Agricole of Dijon. Females and males were kept in individual cages (62.5 × 46.5 cm and 27.5 cm high and 76 × 46.5 cm and 30 cm high, respectively). For pregnant does, a nestbox (42 × 25 cm and 25 cm high) was attached to the cage 2 days before parturition. In total, 575 newborn rabbits (from 109 litters), two virgin females (aged 16 weeks) and 38 multiparous lactating females (days 1–4 postpartum) were involved. On the day of birth (designated postnatal day 0), the litters were culled to 10–12 pups for reasons of pup and female welfare. Culled pups were fostered to lactating females that had smaller litters or were known to be good milk producers. Exceptionally, (ca. 0.3% of live newborns), very small pups were killed by an intraperitoneal lethal injection of pentobarbitol. Several investigations indicate that increasing litter size results in higher incidence of pups missing sucking, and therefore in increased suffering and losses (Fortun-Lamothe & Gidenne 2000). The animals were kept under a constant 14:10 h light:dark regime (lights on at 0700 hours) and ambient temperature of 20–22°C. Water and pelleted food (Stabipro, Glon-Sanders, France) were provided ad libitum.

General Test Conditions

The pups were tested on postnatal days 1–5. To limit litter effects, only five or six pups were tested per litter. They were tested up to 1 h before a suckling episode. To prevent the females entering the nestbox between the test periods, we limited the does' access to the nest to a 15-min period in the morning. This nursing duration and inter-nursing interval of nearly 24 h corresponds to the natural timing of mother–young relationships in this species (Zarrow et al. 1965; Lincoln 1974; Jilge 1995). Controlled nursing has a positive impact on welfare as assessed by behavioural and husbandry variables (Verga et al. 1986; Coureaud et al. 2000). All behaviour testing was conducted in the breeding unit to avoid disrupting the animals' habitual environment. The breeding unit and related procedures are approved by the Ministry of Agriculture, and the Centre des Sciences du Goût is authorized for animal experimentation by the Ministries of Agriculture and of Research & Technology. The experimenters were either authorized to study live mammals or were operating under the supervision of an authorized scientist.

Stimuli Collection

The stimuli used in the four experiments included various histological and secretory samples. We collected skin samples from lactating (days 1–4 postpartum) and

virgin, oestrous females that were killed for regular meat marketing, or after husbandry problems (failures at two consecutive insemination trials) or sanitary problems (including severe partum-related complications, localized abscesses, respiratory infections and occasional diarrhoea). The prevalence of these problems in our rabbitry is in line with those habitually noted in rabbit breeding units. The animals were stunned by electronarcosis (voltage: 90 volts; duration: 5 s), by experienced technicians, and then bled. We collected milk samples from either live or dead lactating females (cf. experiment 4).

The MP was used as a positive control to gauge for normal chemoperceptive abilities of the pups in experiments 1, 2 and 3. It was presented on a glass rod at a dilution of 10^{-6} g/ml in water (Schaal et al. 2003; Coureaud et al. 2004). This additional test was run with all pups in experiment 1 and, to limit disturbance, with a subset selected at random in experiments 2 and 3. In several cases, the same animals were assayed with different stimuli. (1) The pups were exposed to the MP in alternation with another stimulus, the order of presentation of both stimuli being counterbalanced (experiments 1–4). (2) To check for the homogeneity of the behavioural effectiveness of nipples collected from different locations along the nipple-line, we exposed individual pups to all four types of nipples in counterbalanced order (experiment 1). Finally, (3) to see whether tissue from lactating females contains attractive odour cues, we exposed a group of pups to the nipple of a lactating female in alternation with nonmammary tissue (experiment 3).

Behavioural Assay and Variables

We conducted the behavioural assays in the rabbitry between 0800 and 1400 hours. Individual pups were taken from the nest in the gloved hand of the experimenter, with their head left to move freely. When the pups were calm (absence of general agitation, relative immobility of the head), a stimulus was presented 5 mm in front of the muzzle without contact for 10 s. The tissue samples were held with Kocher forceps (length: 11 cm) and the liquid samples (milk or MP) were presented on glass rods (length $\times$ diameter: 15×0.3 cm). Preliminary tests indicated that neither device elicited searching/oral grasping responses in rabbit pups. Between each test, glass rods and forceps were rinsed twice with pure ethanol and pure water, and then dried. The pups were never tested with samples collected from their own mother. Individual pups were visually marked with a scentless, nontoxic colour mark (Pigmax, Sakura, Japan) the day before testing.

We recorded the occurrence of searching-like head movements or oral grasping of the stimulus by the pups in each assay. Searching movements consist of vigorous, low-amplitude horizontal and vertical, very rapid scanning movements of the head, displayed after maximal stretching of the neck towards the stimulus. Searching is generally followed by the pup seizing the substrate in its mouth, which can be repeated one or several times during the 10-s presentation of a given stimulus. However, such repetitions of oral seizing were not recorded in the present study. Pups could seize the device only in the glass rod assays. In the assays presenting skin samples, we prevented the pups grasping the sample to avoid contamination with saliva. In these tests, the pups were considered to express oral activity when they opened their mouth in an attempt to take hold of the sample, which always occurred before actual seizing in the glass rod test. If a pup seized a solid stimulus, we discarded it and replaced it with a fresh one for the next test. In principle, the same tissue sample was presented consecutively to 10 pups. We recorded the proportion of pups expressing the typical searching or oral grasping in response to a given stimulus.

Statistical Analyses

The frequencies of pups from independent groups displaying searching with or without grasping responses to different stimuli were compared using the χ^2 test (with Yates correction if necessary). The frequencies of searching and oral grasping responses of pups of the same group exposed to different olfactory stimuli were compared using the MacNemar χ^2 test. We used the Statistica software (Statsoft, Tulsa, U.S.A.).

EXPERIMENT 1

In this experiment we examined whether the odour emitted from the surface of the nipple could account for the olfactory attractiveness of the entire abdomen noted previously in lactating females. We also examined whether nipples taken from different positions on the nipple-lines differ in their behavioural activity for pups.

Methods

Abdominal skin tissues were excised immediately after the death of the animal. To avoid expressing milk or other skin secretions, we took the tissue samples, especially the nipples (sampled at thoracic, abdominal and inguinal locations), by taking hold of neighbouring tissue, with pliers. We took several precautions to circumvent odour contamination between samples, or between the experimenters and samples: the abdominal hair was roughly shorn before the incisions (leaving approximately 0.2 mm of hair); the scalpel blades were changed between each sampling; and the experimenters wore gloves (Euromedis, France) which were changed between each sampling as well. The tissue samples were snap-frozen ($-80\,^{\circ}$C) immediately after excision and stored at this temperature until testing, which took place within 2–3 weeks of collection. A preliminary assay ($N = 20$ pups, aged 3 days, from four litters, five per litter) ascertained that nipples (from one lactating female) stored at $-80\,^{\circ}$C for 3 weeks were as effective as freshly collected nipples (from one lactating female) (searching: 100% for fresh and frozen nipples; grasping: fresh nipple: 100%; frozen nipple: 92%; $\chi^2_1 > 2.1$, NS in all cases). Just before the tests, the tissues were cut while still frozen to obtain same-sized samples

(approximately 1 cm^2 of surface). They were presented to the pups using the oral activation assay after 1 min of thawing at ambient temperature (see General Methods section).

The stimuli came from three lactating and two virgin females. The New-Zealand × Californian females possess at least eight functional nipples (two thoracic, four abdominal and two inguinal nipples) and one or two supplementary functional nipples (de Rochambeau et al. 1988; Coisne 2000). To check for homogeneity in the behavioural effectiveness of the nipples taken at different locations on the nipple-line, we exposed 24 pups (from four litters, six per litter) to four nipples (one thoracic, two abdominal, one inguinal) of a lactating female in counterbalanced order. To compare the effectiveness of nipples from lactating and from virgin females (including surrounding skin within an area of 3 mm in radius and depth), we exposed pups (N = 36, from six litters, six per litter) to two excised nipples from either category of females. Finally, to ascertain the chemosensory integrity of the pups, we exposed them to the MP using the glass rod assay.

Results and Conclusion

Nipples collected from different positions on the nipple-lines of lactating females did not elicit differential rates of responding by pups (searching: $\chi_3^2 = 3.03$, NS; grasping: $\chi_3^2 = 2.04$, NS; Fig. 1a). All nipples were thus considered equivalent in terms of behavioural activity and used thereafter regardless of their position on the nipple-line.

The nipples from lactating females released more searching and oral grasping responses than those from virgin females (searching: lactating: 97.2%; virgin: 8.3%; $\chi_1^2 = 32$, $P < 0.001$; grasping: lactating: 94.4%; virgin: 8.3%; $\chi_1^2 = 31$, $P < 0.001$; Fig. 1b). The high level of response to MP ascertained that the pups had a normally working nasal chemosensation. Nipples from lactating females were nearly as active as the MP (searching: lactating: 97.2%; MP: 100%; grasping: lactating: 94.4%; MP: 94.4%; $\chi_1^2 > 1$, NS, for both responses; Fig. 1b).

Regardless of their position on the nipple-lines, the nipples from lactating females were more effective than those from virgin females at releasing the searching and oral grasping responses of pups. Thus, as lactation begins, rabbit pups appear to react to quantitative or qualitative alterations of the volatile signal(s) released from the nipple and its immediate surroundings.

EXPERIMENT 2

In this experiment we investigated whether the odour cue is limited to the nipple itself, or is emitted from, or spread over, the adjacent skin. Two previous experiments had noted decreasing attraction or searching responses of rabbit pups as a function of increasing distance from the nipple, and the authors conjectured that this may be caused by a progressive decrease in concentration of the active cue (Hudson & Distel 1983; Coureaud et al. 2001).
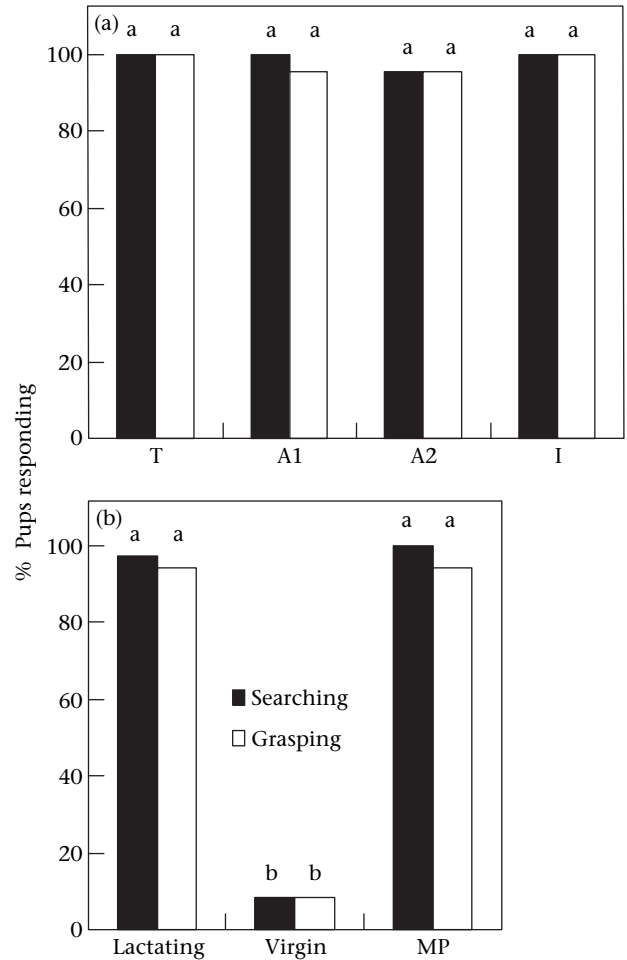
Figure 1. Percentage of newborn rabbits displaying searching and oral grasping in response to: (a) nipples taken from different locations on a lactating female (one thoracic (T), two abdominal (A1 and A2) and one inguinal (I) nipple) (N = 24 pups, four per litter from six litters, aged 2–5 days); (b) nipples from lactating and virgin females, and the mammary pheromone (MP) (N = 12 pups/ stimulation, six per litter from two litters, aged 2–5 days). All samples presented through oral activation assays; letters a and b indicate statistical differences between groups for the corresponding behaviour at the $P < 0.001$ level.

This notion of a graded signal around the nipple is further addressed here.

Methods

The pups were exposed to the odour of skin samples from two lactating females. The stimulus series was composed of five elements: (1) a nipple without adjacent skin; (2) 1 cm^2 of skin immediately adjacent to the nipple (0–1 cm from the nipple's edge, 1 mm depth, hair roughly shorn); (3–5) three samples of 1 cm^2 of skin at 1-cm intervals from the nipple's edge (1–2, 2–3 and 3–4 cm from the nipple's border, 1 mm depth, hair shorn). Five independent groups of pups (N = 24 per stimulation, from four litters, six per litter) were exposed to the various

skin samples as described in the General Methods. To verify their chemosensory integrity, we randomly chose 24 of the 120 pups in this experiment to be exposed to the MP.

Results and Conclusion

Among the five samples of nipple and non-nipple skin, only the nipple itself elicited behavioural responses (Fig. 2). All non-nipple skin samples were nearly or completely inactive on pups (searching and grasping: nipple: 87.5%; non-nipple skin: <8.3%; $\chi_1^2 > 27.05$, $P < 0.001$ for all comparisons and for both variables). The distinct non-nipple skin samples did not release differential searching–mouthing activity (searching and grasping: non-nipple skin 0–1, 1–2, 2–3 and 3–4 cm: $\chi_3^2 = 4.2$, NS, for all comparisons and for both variables).

Otherwise, the nipples were as efficient as the pure MP at releasing the typical searching and grasping responses ($\chi_1^2 < 1.4$, NS for both variables; Fig. 2). The group of pups exposed to the non-nipple skin areas showed little responsiveness. However, this result cannot be explained by low chemosensory faculties of the pups as they appeared to be normally responsive to the MP ($\chi_1^2 > 33.4$, $P < 0.001$ for all comparisons and for both variables).

These results indicate that the main emission site of the active odour cues in lactating females is the nipple itself. Since neonatal pup responsiveness dropped sharply to nearly zero right beside the nipple, we conclude that there is no spreading of the cues further than the nipple skin itself.
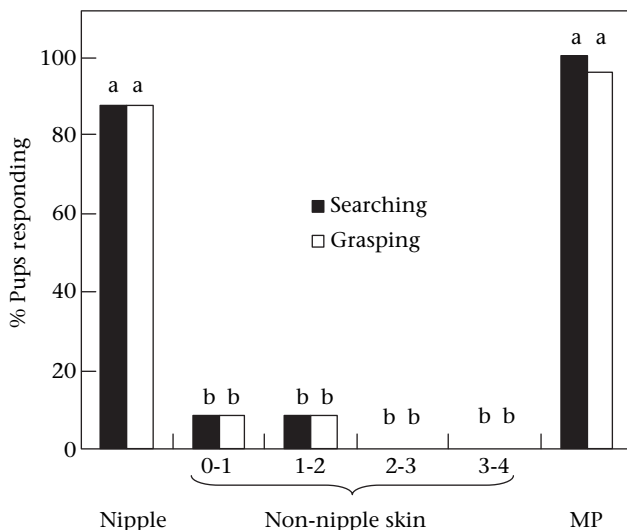
Figure 2. Percentage of newborn rabbits displaying searching and oral grasping in response to the nipples, 1-cm² skin sample immediately adjacent to the nipple (0–1) and 1-cm² skin samples collected at increasing distance from the nipple (1–2, 2–3, 3–4 cm), and to the mammary pheromone (MP). All samples collected from lactating females and presented by oral activation assays ($N = 24$ pups/stimulation, six per litter from four litters, aged 2–5 days). Letters a and b indicate statistical differences between groups for the corresponding behaviour at the $P < 0.001$ level.

EXPERIMENT 3

In this experiment we sampled representative tissues vertically along the mammary tract to evaluate whether the odour cues present on the nipple are specific to its epidermis or whether they are also present in the layers of dermal or secretory mammary tissues underlying the nipple, or in any tissue from lactating females.

Methods

We obtained three mammary-related samples from two lactating females: (1) the nipples themselves without any surrounding skin (the nipple was excised at its base); (2) 1 cm² (0.5 cm depth) of dermal tissue immediately beneath the nipple; and (3) 1 cm² (0.5 cm depth) of mammary tissue about 2 cm under the nipple. These tissues were presented to three independent groups of newborn pups ($N = 20$ per group; from four litters, five per litter). As in the previous experiments, 20 pups were randomly chosen from these three groups to ascertain normal chemosensory responsiveness (to the MP). Finally, to see whether the odour of any tissue from lactating females (i.e. a tissue unrelated to mammary function) was effective, we exposed another group of pups ($N = 20$, from four litters, five per litter) consecutively to a nipple and a control tissue (anterior leg muscle) from a lactating female.

Results and Conclusion

The dermal and mammary tissues taken beneath the nipple were inefficient relative to the nipple in releasing, searching and grasping (nipple: 90%; dermal tissue: <5%; mammary tissue: 0%; nipple versus dermal tissue: $\chi_1^2 > 29$, $P < 0.001$, for both responses; nipple versus mammary tissue: $\chi_1^2 = 29.2$, $P < 0.001$, for both responses; Fig. 3). Searching and oral grasping were released in less than 5% of the pups for both non-nipple, mammary tissues ($\chi_1^2 < 1.03$, NS, for both responses). These two mammary tissues were as effective as a tissue sample that had no functional link with lactation (searching and grasping: dermal tissue, mammary tissue and muscular tissue: <5%; $\chi_1^2 < 1.03$, NS, for all comparisons and for both responses). The nipple was as efficient as the MP (searching and grasping: nipple: 90%; MP: >95%; $\chi_1^2 < 2.1$, NS, for both responses). The samples of mammary or nonmammary tissues collected did not induce significant responses in the pups. In contrast, the pups were highly reactive to the nipple, suggesting that the localization of the active odour cues may be restricted to its surface, and that they may not originate from, or be present in, the underlying tissues.

EXPERIMENT 4

In this experiment we tested whether the nipple is attractive because it secretes the odour cues itself, or because the cues are produced in the mammary tract and are carried to the nipple by the flow of milk. Rather
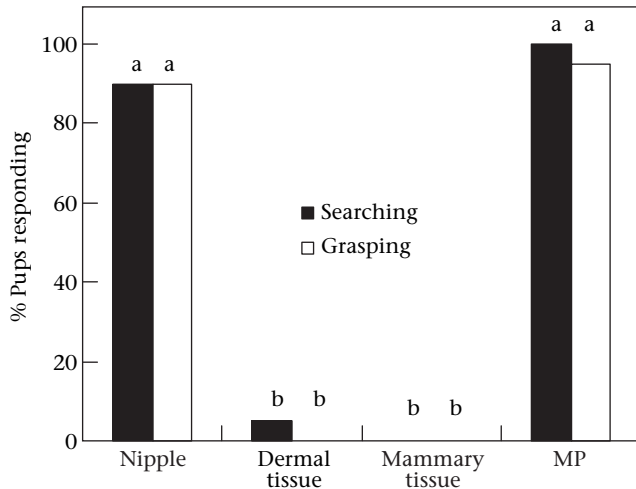
Figure 3. Percentage of newborn rabbits displaying searching and oral grasping in response to the nipple and samples of dermal and mammary tissues from lactating females, and to the mammary pheromone (MP). All samples presented through oral activation assays ($N = 20$ pups/stimulation, five per litter from four litters, aged 2–5 days). Letters a and b indicate statistical differences between groups for the corresponding behaviour at the $P < 0.001$ level.

than directly using histological samples from the mammary tract, we collected and assayed the fluid they excrete or that flows across them during lactation.

Methods

Milk samples were obtained at three loci of the mammary pathway: the alveolae, the main ducts beneath the nipple, and after ejection. We collected milk by milking (ejected milk), by puncture (tubular milk) in live animals, and by post mortem incision of the mammary tissue (alveolar milk). Milking was carried out by holding the female prone on one experimenter's lap, while another experimenter softly squeezed the nipples to collect several drops of milk. The females ($N = 16$, postpartum days 1–4) were habituated to such handling and at the moment of milk collection (1 ml/female) they showed no signs of stress. We ensured that the drops of milk pearled at the extreme tip of the nipple to avoid contamination from secretions of the skin gland on the columnar or basal parts of the nipple. This ejected milk was assayed immediately after collection. Tubular milk (3–4 ml/female) was collected by puncturing the mammary ducts (0.5–1 cm) below the nipple (Barone et al. 1973) and was tested immediately after collection. Females ($N = 10$, postpartum days 1–4) habituated to the procedure were locally disinfected and anaesthetized with a spray of xylocain. During the procedure, the female was held supine by one experimenter while a trained technician collected the milk. If blood contamination occurred, the milk samples were discarded. The procedures had no adverse consequences for the females or for their litter. Finally, we obtained alveolar milk by incising the mammary lobes of females that were killed and bled. This milk sample (2–3 ml/female) was immediately snap-frozen ($-80°C$) and stored at that

temperature until testing which took place within 3 weeks. Preliminary experiments have shown that these conditions of milk conservation preserve its behavioural activity (Coureaud 2001). Care was taken to avoid blood contamination of the milk samples. We used only two females (day 3 postpartum), which had to be slaughtered because of husbandry problems, for this method of milk collection. These samples were presented to independent groups of pups for the assessment of differential behavioural activity. Alveolar milk was presented to 40 pups (aged 1–5 days, from eight litters, five per litter). Milk punctured from the enlarged tubules was presented to 90 pups (aged 1–4 days, from 18 litters, five per litter). Ejected milk was presented to 165 pups (aged 1–4 days, from 33 litters, five per litter) after 1 min of thawing.

Results and Conclusion

The alveolar and tubular milks were inefficient at releasing, searching and oral grasping (<9% of responding pups for both samples), with no significant difference between them ($\chi^2_1 < 1.7$, NS, for both responses; Fig. 4). The rates of responding triggered by the ejected milk were clearly higher than those induced by the alveolar and tubular milks (searching: alveolar milk: 2.5%; ejected milk: 82.4%; $\chi^2_1 = 89.2$, $P < 0.001$; grasping: alveolar milk: 5%; ejected milk: 60%; $\chi^2_1 = 36.8$, $P < 0.001$; searching: tubular milk: 9%; ejected milk: 82.4%; $\chi^2_1 = 125.1$, $P < 0.001$; grasping: tubular milk: 4.5%; ejected milk: 60%; $\chi^2_1 = 72.4$, $P < 0.001$; Fig. 4). Although the ejected milk was strongly active, it appeared to be less powerful than
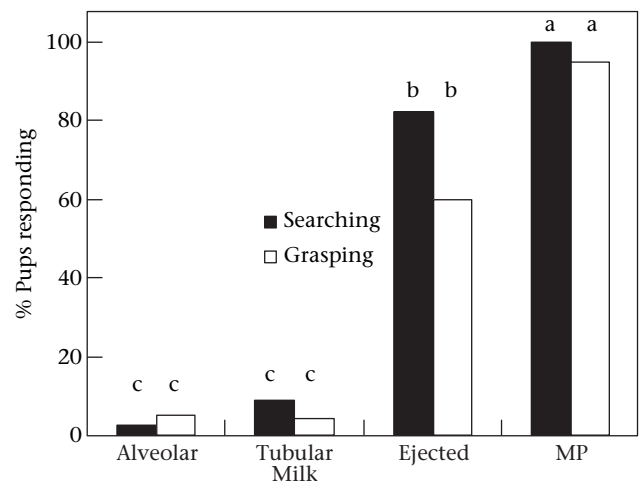
Figure 4. Percentage of newborn rabbits displaying searching and oral grasping in response to milk samples collected from postpartum females and to the mammary pheromone (MP) (alveolar milk: $N = 40$ pups, five per litter from eight litters, aged 1–5 days; tubular milk: $N = 90$ pups, five per litter from 18 litters, aged 1–4 days; ejected milk: $N = 165$ pups, five per litter from 33 litters, aged 1–4 days; MP: $N = 20$ pups, five per litter from four litters, aged 2–5 days). All tests used the oral activation procedure. Letters a, b and c indicate statistical differences between groups for the corresponding behaviour at the $P < 0.001$ level; a versus b indicate a difference at the $P < 0.05$ level.

the pure MP (searching: ejected milk in experiment 4: 82.4%; MP in experiment 3: 100%; $\chi_1^2 = 4.2$, $P < 0.05$; grasping: ejected milk: 60%; MP: 95%; $\chi_1^2 = 9.46$, $P < 0.05$; Fig. 4).

These results make clear that the milk becomes behaviourally active only after its passage through the ducts at the level of the nipple.

DISCUSSION

Our results show that the nipple itself is the source of the cues that elicit searching and oral grasping responses in newborn rabbit pups. That the nipple of female rabbits has special properties for pups, not shared by adjacent skin surfaces, has been noted in several studies (Schley 1976; Müller 1978; Hudson & Distel 1983; Gonzalez-Mariscal et al. 2000; Coureaud et al. 2001). Hudson & Distel (1995) hypothesized that odour cues are distributed following a gradient of concentration which is maximal on the nipple and decreases as a function of distance from it. Our results do not verify this hypothesis, as the skin immediately adjacent to the nipple was ineffective in eliciting pup responses (experiment 2). The skin samples we used were partly shorn, excluding hair as possible dispensers of the secretions of attached skin glands (Albone 1984). However, the role of abdominal hair in eliciting pup searching–grasping responses is thought to be minimal (Müller 1978). Regardless of the presence or absence of hair, the evidence points to the cues being present on the surface of the nipple.

Only odour cues from the nipples of lactating does (relative to those of virgin oestrous does) were efficient releasers of searching–grasping responses in pups (experiment 1), a result raising at least two issues. (1) Is the release of the odour cues under the same endocrine control as lactation itself? (2) Why did oestrous does emit cues releasing negligible (10%) pup responsiveness in our experiments (excised nipples), whereas the nipples of oestrous does release a non-negligible level of pup response (50%) when presented in situ (in the context of the abdominal fur; e.g. Hudson & Distel 1984)? As to issue 1, it is clear that the endocrine factors of lactation are involved in the production of the odour cues from nipples. The hormonal balance at lactation onset, particularly high levels of prolactin concurrent with low levels of progesterone (McNeilly & Friessen 1978; Gonzalez-Mariscal et al. 1994a; Negatu & McNitt 2002), or the injection of prolactin in conjunction with oestradiol priming (in ovariectomized females), stimulate the maximum discharge of the odour cues (Gonzalez-Mariscal et al. 1994b). Furthermore, progesterone (injected on a background of oestrogen in pregnant or ovariectomized females) promotes alveolar growth and releases high responsiveness in pups, whereas its antagonization has the reverse effects (Gonzalez-Mariscal et al. 1994b). This indicates that progesterone is critical for the production of the cues. Advantage should now be taken of the fact that one compound (the MP) of the odour cues is chemically known (Coureaud 2001; Schaal et al. 2003) to delineate the hormonal conditions that are optimal for its production.

Concerning issue 2, although different studies may have considered different behavioural criteria, the apparent difference between our results and those of other studies may be caused by pups exposed to nipples on the abdominal skin facing a wider set of odour stimuli unrelated to lactation (e.g. dietary aromas, self-licking cues, bacterially altered skin secretions) in conjunction with tactile and temperature features of the fur known to be intrinsically active (Distel & Hudson 1985). In contrast, the 'ex situ nipple' test used here presents only local odour cues without tactile noise (hair shorn). Searching tests carried out on the abdominal fur may overestimate the behavioural activity of the nipple odour by confounding tactile cues from the fur and multiple, basic odour cues. It is thus reasonable to conclude that the ex situ nipple activity represents a more reliable approximation of the power of the volatiles it carries.

Through which sources does the rabbit female's nipple gain behavioural activity for pups? Three possibilities may be considered: (1) skin glands distributed over the surface of the nipple; (2) glands situated at the entry of the galactophorous ducts; and (3) sources located in deeper structures of the nipple and externalized through milk. While possibility (1) accounts for the instantaneous activity of the nipple, it does not integrate the role of milk as a source of cues. Possibilities (2) and (3) would imply that the same source is involved in the behavioural activity of the nipple. Possibility (2) would imply that the source is located at the ostium of the galactophorous ducts, releasing its secretion both into the air and into the flowing milk. Possibility (3) would imply that milk collects a discharge from the lumen of the galactophorous ducts and that the cues are sent outside as milk oozes before nursing. These different possibilities can be settled only by systematic histological analyses. Experiments 3 and 4 favour both possibilities (3) and (4) that one source of the cues is in direct contact with the flow of milk. To our surprise, milk became behaviourally active for pups only after it flowed through the ultimate portion of the mammary tract, suggesting that the internal source of the active compound is highly localized. This may be the result of at least four mechanisms.

(1) Substrates produced by skin glands at the very tip of the nipple may be dissolved in milk. Skin glands surrounding the mammary orifices have been described in various mammalian females where they bear multiple functions linked with epidermal lubrication, protection from corrosive saliva, prevention of entry by pathogens, and female-to-pup communication (Albone 1984; Schaal & Porter 1991). They have been found in the female rat, *Rattus norvegicus*, (Toyoshima et al. 1998a, b), but not in the rabbit (A. S. Moncomble, A. Quennedey, B. Quennedey, G. Perrier, R. Brossut & B. Schaal, unpublished data). Their existence in the rabbit would raise the possibility that the nipple-surface cues and the milk cues are confounded.

(2) Sebaceous or apocrine structures located deeper in the nipple dermis may liberate their secretions into the lumen of the galactophorous ducts. There is no anatomical evidence for such structures; however, the

lumen of the galactophorous ducts is extremely circum-voluted (A. S. Moncomble, A. Quennedey, B. Quennedey, G. Perrier, R. Brossut & B. Schaal, unpublished data), indicating that it may function as a surface of exchange.

(3) Symbiotic microorganisms in the galactophorous ducts may liberate volatile compounds from nonvolatile precursors. Microbial action in the production of semi-ochemicals has been reported in various mammals (e.g. Albone 1984). There is evidence for a commensal micro-flora in the mammary gland and in milk from healthy pregnant and lactating females (Martin et al. 2004), but the contribution of these milk bacteria to the odorant profile of ejected milk is not known.

(4) The odour cues may be secreted in the acini of the mammary gland, bound to a proteinic or lipid carrier, this carrier–ligand complex being sensorily ineffective until the ligand is liberated. Many carrier proteins have been characterized in mammals (Akerstrom et al. 2000). Among these, Odorant Binding Proteins (OBP) are hypothesized to favour the transfer of the lipophilic odorant molecules to the receptor sites of the olfactory cilia or vomeronasal villosities (Pelosi 2001). Such OBPs have been located in the nasal mucus of numerous adult mammals (Tegoni et al. 2000), including rabbits (Garibotti et al. 1997). Such carrier molecules are also often emitted in the emission sites of the semiochemically active compounds (Pelosi 2001). For example, in the hamster, *Mesocricetus auratus*, the protein aphrodisin is secreted in the vaginal discharge concurrently with an unidentified ligand (Singer & Macrides 1992; Briand et al. 2004); in humans, the axillary odorant *E*-3-methyl-2-hexenoic acid is produced at the same time as its carrier, apolipoprotein D (Zeng et al. 1992). Milk contains a variety of proteins, the functional properties of which resemble those of OBPs (e.g. β-lactoglobulin, α-lactalbumin, serum albumin), particu-larly with regard to their strong affinity for small lipo-philic ligands (Sawyer & Kontopidis 2000; Tromelin & Guichard 2003). To become behaviourally active, the ligand has to be split from its carriers by enzymatic or physicochemical processes (e.g. the odorant can be re-leased by acidification; Zeng et al. 1992). Although we have found no data on particular enzymatic processes in mammalian nipples or teats, there is evidence that the physicochemical properties of milk change rapidly during its passage through the teat (e.g. Allen & Neville 1983). If such a process applies to our case, behaviourally inactive rabbit milk collected from the alveolae could become active after physicochemical treatment.

In conclusion, our results suggest that the active odor-ants from the nipple of lactating rabbits potentially derive from dual exocrine sources, surface cues distributed over the nipple epidermis and cues released within the nipple which render milk olfactorily powerful. While the 'outer cues' are functionally important to newly born pups for guidance at suckling initiation and for relocation when they switch nipples during a suckling bout, the 'inner cues' may provide a strongly reinforcing signal to main-tain pup attachment to the nipple (Blass 2003). Future analyses should settle whether these outer cues and inner cues are based on the same or different odorants. Present evidence suggests that both cues are functionally alike

because pups behave in a very similar way when they are exposed to nipples in vivo on the lactating female's abdomen (Schley 1977; Müller 1978; Hudson & Distel 1983; Coureaud et al. 2001), to freshly excised nipples (present study), to fresh milk (Müller 1978; Keil et al. 1990; Coureaud et al. 2001, 2002; present study) or to the pure MP (Coureaud et al. 2003; Schaal et al. 2003; present study). Nevertheless, if the source of the MP is in the galactophorous ducts, the tip of the nipples may be contaminated by milk so that a single source may well be responsible for the attractiveness of the milk and of the epidermal tip of the nipple. Thus, although the MP has been isolated from milk, it may also be one key compound among the mixture of volatiles accounting for the re-leasing effect of surface secretions from the nipple. This needs to be ascertained by chemical analyses.

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