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Newborn Rabbit Responsiveness to the Mammary Pheromone is Concentration-dependent

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Abstract

The effect of the intensity of odour signals has rarely been investigated in the regulation of odour-guided behaviour in young mammals. This series of experiments used the mammary pheromone (MP) of the female rabbit to assess the influence of stimulus concentration on neonatal pup responsiveness. The MP is a single compound isolated from rabbit milk that releases in pups the typical head searching and oral seizing behaviour. The pups ($n = 621$) were exposed to graded concentrations of the MP in bioassays varying in stimulus delivery conditions. Experiment 1 demonstrated that in aqueous dilutions the MP efficiently elicits behavioural responses only within a limited range of concentrations (from 2.5×10^{-9} to 2.5×10^{-5} g/ml). Experiment 2 yielded the same outcome with highly purified MP delivered in dynamic conditions with a gas chromatograph. Finally, Experiment 3 used deodorized milk as the solvent of the MP; despite this change in the physico-chemical context of stimulation, similar results were reached.

Key words: milk, mother–infant relation, olfaction, pheromone, rabbit (*Oryctolagus cuniculus*), suckling.

Introduction

Stimulus intensity has been recognized as a major property of the sensory environment that organizes early development, but little is known about its effects on early behaviour. Schneirla (1965) proposed that the approach/withdrawal responses of very young organisms are mainly regulated by stimulus intensity, mild stimuli leading to approach responses and high intensity stimuli leading to withdrawal responses. While this rule has been well documented in the auditory and visual modalities (e.g. Turkewitz *et al.*, 1983), little evidence exists for olfaction so far. Studies that have addressed psychophysical aspects of chemosensory development in young mammals are few. One may mention pioneering works in human infants (e.g. Engen *et al.*, 1963; Rovee, 1969; reviewed in Schaal, 1988) and in the rat pup (Alberts and May, 1980) which revealed that: (i) the amplitude of response increases as a function of stimulus intensity; (ii) sensitivity improves in an age-related manner as a function of experience and/or chemosensory maturation; and (iii) the developmental courses of sensitivity to artificial, pure odorants and to biologically relevant, complex odorants do not differ. The generalizability of these results remains limited, however, because they were based on a narrow range of odorants and because they did not take into account internal variables known to influence stimulus receptivity (e.g. motivational state, species-specific sensory biases). Thus, notions such as the 'effective stimulus intensity' or the 'optimal intensity range' have rarely been described for chemosensory stimuli.

The present experiments aim at examining further the impact of intensity in the regulation of behaviour in a young mammal. Rabbit newborns constitute an interesting model system for developmental olfactory psychophysics as they are mainly governed by olfaction and they display a repeatable, stereotyped behaviour pattern in response to volatile cues originating from the female's abdomen and milk (Schley, 1979, 1981; Hudson and Distel, 1983; Keil *et al.*, 1990; Coureaud and Schaal, 2000; Coureaud *et al.*, 2001, 2002). Recently, a single volatile isolated from rabbit milk was shown to elicit at a given concentration the typical head-searching–oral-grasping responses in >90% of pups (Schaal *et al.*, 2003), that is, as efficiently as milk itself (searching > 80%, grasping > 60%; Keil *et al.*, 1990). This compound, 2-methylbut-2-enal, met the five stringent criteria used to define pheromones in mammals (Beauchamp *et al.*, 1976) and was named 'mammary pheromone' (MP; Schaal *et al.*, 2003). It is active in a highly selective fashion based on odour quality. None of a series of reference odorants (namely, 20 identified in rabbit milk and 20 selected for their absence in rabbit milk) against which the MP was tested were behaviourally effective. Therefore, its behavioural impact cannot be assigned to non-specific arousal or novelty effects (Coureaud *et al.*, 2003; Schaal *et al.*, 2003).

The qualitative selectivity of the MP seems, however, to be concentration-dependent. In two preliminary experiments (Coureaud *et al.*, 2003; Schaal *et al.*, 2003), newborn pups were exposed to a dilution series composed of 5–6 steps ranging from 10–^{10} to 10^{0} g/ml of the MP in water. Their responses were distributed along what can be named (from a descriptive point of view) a 'bell-shaped curve', with no response for the lowest and highest dilutions and optimal responsiveness between 10^{-8} and 10^{-6} g/ml. The present experiments aimed to expand these first results for a more fine-grained understanding of the psychophysical properties of the MP. In Experiment 1, the MP dilution series was refined in terms of interval between dilution steps to reach more accurate values for (i) the thresholds for behavioural 'activation' and 'deactivation' (as defined in the Materials and methods section) to the MP, and (ii) its optimal concentration range. Moreover, the commercial 2-methylbut-2-enal used in the above-mentioned investigation contained impurities which, although behaviourally inactive by themselves (Schaal *et al.*, 2003), might have had an influence on its concentration–response curve. Therefore, in Experiment 2, the rabbit pup sensitivity to the MP was assessed in conditions of maximal purity after separation in a chromatograph equipped with a sniffing port (Schaal *et al.*, 2003). Finally, as the MP had been administered in a physico-chemical context which differed from that prevailing in milk, its natural solvent, we examined in Experiment 3 its concentration–response curve when dissolved in rabbit milk previously treated to inactivate the natural MP.

Materials and methods

Breeding conditions

The animals (New Zealand and New Zealand–Californian breeds) originated from Station de Pathologie Aviaire et de Parasitologie (strains Inra 2066 and 1077 for males and females, respectively; Inra, Nouzilly) and Cunifrance, (strain Génia 77; Epeigné/Dème, France; for full description of the housing conditions, see Coureaud *et al.*, 2001, 2002). In total, 621 pups born from 101 multiparous females were used. The day of birth was designated as postnatal day 0.

Stimulations and experimental groups

In all experiments, the different concentrations of the MP were prepared from an initial stock of 2-methylbut-2-enal purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). In Experiment 1, the MP was diluted in distilled water. In Experiment 2, the pure MP was delivered in gas phase after separation from the volatile impurities with a gas chromatograph (GC). In Experiment 3, the MP was diluted in milk.

Experiment 1

To define how rabbit pups respond to the MP according to its concentration, 440 pups (from 73 litters, age = day 2) were exposed to it diluted in water. For each dilution, 20 pups (from four litters, 5/litter) were tested using the oral activation test (described below). Twenty-two levels of dilution were used ranging from 10^{-11} to 2.5×10^{-3} g/ml, with reduced inter-dilution intervals (2.5) in the two windows where inflexions in the concentration–response curve were noted in Coureaud *et al.* (2003) and Schaal *et al.* (2003). All other dilution steps were separated by a factor of five or ten. All dilutions were made in 10 ml of distilled water, and kept in polypropylene tubes (Falcon, 15 ml; Becton Dickinson). The four data sets of pup responses corresponding to dilution steps 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} g/ml were taken from the study by Schaal *et al.* (2003). Distilled water, used as control stimulus, released negligible behavioural activity (<5%).

Experiment 2

In Experiment 1, a commercial sample of the MP that included some impurities was used. In the present experiment, the presentation of the MP through a device coupling GC and olfactory sampling (GCO) permitted the recording of pup responses to the pure MP in the absence of any interferences from the impurities. Moreover, this method may allow an increase in the accuracy of the concentration–response analysis, as the sniff-port of the GC delivered the MP at a dynamic concentration, with a constant velocity and in a warmed and humidified airstream. We investigated the behavioural responses induced by 11 aqueous solutions of the MP ranging from 10^{-8} g/ml to 10^{-2} g/ml and including concentrations of 2.5×10^{-8}, 5×10^{-8}, 10^{-7}, 10^{-6}, 3×10^{-6}, 2.5×10^{-5}, 5×10^{-5}, 10^{-4} and 10^{-3} g/ml. All dilutions were made in dichloromethane (purity 99.8%; SDS, Peypin, France). For each level of dilution, 1 μl of the MP solution was injected into the GC (see below). Under our conditions, 1 μl of mammary pheromone injected into the GC between 2.5×10^{-8} and 10^{-3} g/ml corresponded to air concentrations (calculated) situated between 1.2×10^{-11} and 6×10^{-8} g/ml, respectively, at the sniff-port. The MP was presented to 110 pups (from 15 litters, age = day 2), 10 of them (2/litter) being tested for each concentration. Control assays made with dichloromethane (1 μl injected, 20 pups tested) released no behavioural activity.

Experiment 3

Chemically simple compounds differ perceptually as a function of the complexity of the solvent in which they are presented (e.g. Land, 1970; Prindiville *et al.*, 1999; Tandon *et al.*, 2000). The same rule may apply to the MP which may interact with proteins and lipids in milk and give off behavioural effects that differ from aqueous solutions. To test this hypothesis, the natural content of the MP in milk was removed through a deodorization procedure involving the passage for 2 h of a stream of nitrogen (100 ml/min, quality HP45; Carboxyque Française, Vénissieux, France) through fresh milk (32 g) via the porous double-bottom of a 500 ml flask placed in a 30°C water bath. The elimination of the MP from milk was ascertained by GC/MS and by the pup responses in the glass-rod test (Schaal *et al.*, 2003). The pup responses were then assessed with a series of 10 dilutions of the MP in the deodorized milk (range = 10^{-10}–10^{-1} g/ml; inter-dilution interval = 1 log unit). The dilutions were made before testing in aliquots (5 ml) from deodorized milk (obtained from four females on postpartum day 2) and kept at 4°C. Seventy-one pups (from 13 litters, age = day 2) were exposed to these stimulations with the glass-rod test. For practical reasons, 41 newborns (from seven litters, 4–7/litter) were successively exposed (minimal intertrial time = 8 min) to the six dilutions between 10^{-10} and 10^{-5} g/ml, and 30 pups (from six litters, 5/litter) to the four dilutions between 10^{-4} and 10^{-1} g/ml. The dilution series were presented in a different randomized sequence for each litter.

Testing devices and procedures

Two assays that capitalized upon the expression of the searching–oral-grasping movements by the pups were used.

The glass-rod test

The glass-rod test was validated in previous experiments (i.e. Keil *et al.*, 1990; Coureaud *et al.*, 2002, 2003; Schaal *et al.*, 2003). The pups were held in one gloved hand of the experimenter, so that only the head was left free. The stimuli were presented before the pups' muzzles on a glass-rod (length × diameter: 15 × 0.3 cm). The tip of the rod was positioned 0.5 cm in front of the animals' nares. Each stimulus presentation lasted 10 s. A test was considered positive when the stimulation elicited searching movements and/or the oral grasp of the glass-rod. The tests were run by two experimenters: A prepared the stimuli out of sight of B who, blind to their concentration, presented them to the pups. Experimenters A and B alternated so that eventual differences in pup responses were not attributable to differences in the experimenter's individual skills.

The gas chromatography–olfaction (GCO) test

The stimulating device was the same as the one used to separate chemically the pheromonal signal from the other volatiles of milk (Schaal *et al.*, 2003). It included a GC modified for the perceptual assessment of aromas eluting from the injected mixtures. At the end of the GC column, the stream of gas carrying the separated odorants was split into two equal subflows directed towards (i) the flame ionization detector of the GC and (ii) an external sniff-port (for full description of the device and conditions of chromatography, see Schaal *et al.*, 2003). This device allowed us to match in time the physical detection of the effluent by the GC and the biological detection by the nose of the pups. For each level of concentration, 1 µl was injected into the GC. The progress of the elution was followed on the paper trace of the recorder by one experimenter. Pups were positioned by two alternating experimenters (who were blind to the dilution step being injected) at the sniff-port 15 s before the expected elution time of the MP (known through preliminary assays). Each newborn was exposed to a single concentration of the MP by being maintained in front of the sniff-port during the minute that covered the duration of the MP elution (which ranged from 6 to 45 s for the lowest and highest concentrations). The GCO test was interrupted after the complete elution of the MP. The next stimulus was injected 5 min after the elution of the compound. Every 10 injections, the oven was heated to 200°C (10°C/min) to purge possible impurities that might have stuck to the GC column.

Behavioural analyses

The searching and oral-grasping movements of the pups directed towards the glass-rod or the sniff-port of the GC were recorded as responses. Searching movements consist of vigorous, low amplitude horizontal and vertical scanning actions of the head. Oral-grasping movements consist in more or less deep oral seizing action of the glass-rod or of the GC sniff-port. The oral-grasping response is preceded by the sequence of head searching. The two tests were run immediately before suckling which occurred every day at fixed hours. In both tests, the threshold for behavioural activation was considered to be reached when the frequency of pups responding by searching and/or oral grasping raised to or above 50%. Inversely, the threshold for behavioural deactivation was considered to be reached when the frequency of reactive pups decreased to or below 50% (Davis, 1973; Slotnick and Ptack, 1977; Passe and Walker, 1985).

Statistical analyses

The results were considered at the level of the individual pup. To control for litter effect, the number of pups exposed per litter to a given dilution or series of dilutions was limited (see above). In Experiments 1 and 2, the pups were exposed to only one dilution step of the MP. Therefore, the frequencies of searching and oral-grasping responses elicited by the different dilution steps of the MP were compared two-by-two using the χ^2 test (with Yates correction when necessary). When the same pups were exposed successively to different dilution steps of the MP (Experiment 3), the frequencies of their responses to the different dilutions were compared two-by-two with the χ^2 test of McNemar. The analyses were made using Systat software (Evanston, IL).

Results

Experiment 1: concentration–response relationship of the mammary pheromone diluted in water

The response frequencies had an approximately bell-shaped distribution with an extended zenith (Figure 1). The MP concentration–behaviour curve can thus be divided into an activation region, a plateau region and a deactivation region.

Activation region

At the highest dilution steps, namely 10^{-11} and 10^{-10} g/ml, the proportion of pups responding by searching was very weak (5%; Figure 1). The searching rate increased then progressively to 35% at step 10^{-9}, after which a concentration increment of 2.5 (2.5×10^{-9}) was sufficient to boost the proportion of responding pups from 35 to 90%. The threshold for searching was thus between steps 10^{-9} and 2.5×10^{-9}. The percentage of pups responding with oral grasping tended to increase more progressively between steps 10^{-11} and 10^{-9} as compared to searching (non-significant difference; Figure 1). But, as already noted for the searching response, crossing from step 10^{-9} to step 2.5×10^{-9} corresponded to a sharp increase in the percent of pups responding by oral grasping (from 25 to 75%), suggesting that the threshold for this motor pattern was reached.

Plateau region

Both responses were released at a stable, high level of frequency between steps 2.5×10^{-9} and 2.5×10^{-5}. For searching, the plateau level was maintained between 90 and 100%. For oral grasping, it peaked between 70 and 80%.

Deactivation region

With still increasing concentrations, the proportion of searching pups decreased progressively (Figure 1). The drop to the deactivation threshold occurred between steps 2.5×10^{-4} and 5×10^{-4} for which pups expressed searching in 55 and 20% of the assays, respectively. Finally, the proportion of searching pups continued to decrease to nearly zero from step 7.5×10^{-4}. The oral-grasping response followed the same trend, the proportion of responding pups starting to diminish (non significantly) at dilution step 2.5×10^{-5} and reaching deactivation threshold at step 5×10^{-5}. At this latter dilution step, pups expressed grasping less frequently than searching ($\chi^2 = 3.9$, $P < 0.05$), suggesting that the two responses may have different thresholds for deactivation. The grasping response vanished around 7.5×10^{-4} g/ml (Figure 1).

The findings extend those of our previous studies (Coureaud *et al.*, 2003; Schaal *et al.*, 2003). The threshold for searching and oral grasping is located within the same window of values in the present (10^{-9}–2.5×10^{-9} g/ml) and

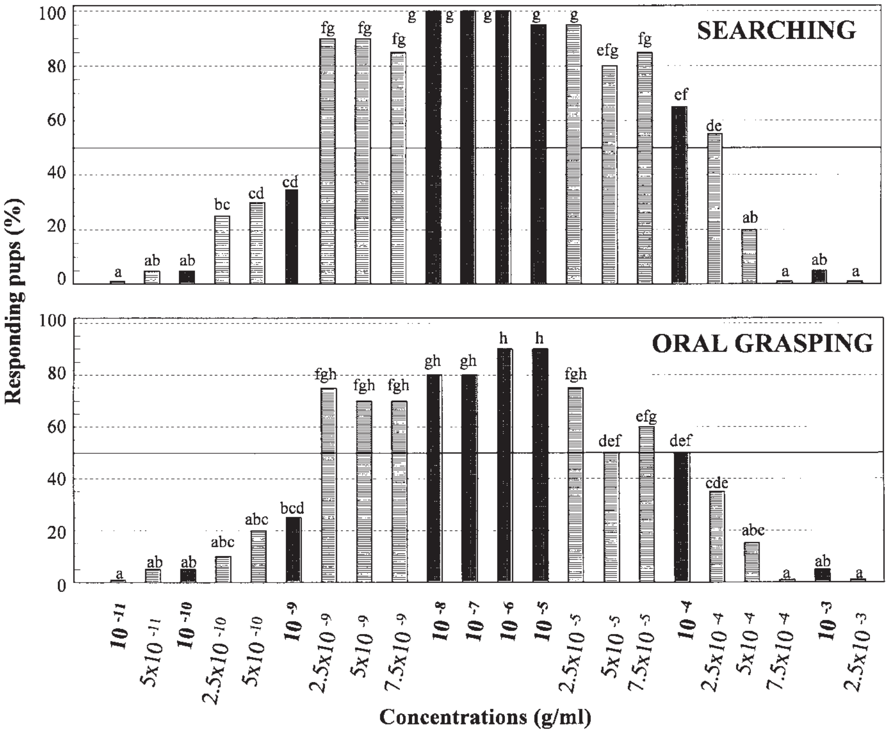

Figure 1 Frequency of rabbit pups (%) responding by searching and oral grasping as a function of the concentration of the MP diluted in distilled water and presented through the glass-rod test. Statistical analyses of the responses illustrated in the two graphs are done independently. Within each graph, distinct digits indicate statistical differences at the $P < 0.05$ level (χ^2 test; for each dilution step, n pups tested = 20, from four litters; age, day 2).

previous studies (10^{-10}–10^{-8} g/ml). The results show additionally that (i) the thresholds for activation of both responses are reached very abruptly after a small increment of the concentration (×2.5) and (ii) the rate of oral grasping is decreasing more rapidly than the rate of searching.

Experiment 2: concentration–response relationship of the mammary pheromone in pure form

As in Experiment 1, the curves of both behavioural responses were approximately bell-shaped (Figure 2).

Activation region

The searching responses followed an increasing slope between steps 10^{-8} and 3×10^{-6}. The threshold for the release of searching was located between steps 2.5×10^{-8} and 5×10^{-8}. Following the same trend, oral grasping increased between steps 10^{-8} and 5×10^{-8}, its threshold being attained at step 2.5×10^{-5}, but never crossed (Figure 2).

Plateau region

The searching responses became maximal (90–100%) between steps 3×10^{-6} and 5×10^{-5} (Figure 2), while the oral grasping reached a stable ceiling situated around 30–40% of responding pups between steps 5×10^{-8} and 3×10^{-6}.

Deactivation region

The proportion of searching pups descended from maximal to threshold values when the concentration of the injected solution was increased from step 5×10^{-5} to step 10^{-4}. A further increase in concentration reinforced this reduction in searching (Figure 2). Although they remained low at the plateau phase, oral-grasping responses decreased non significantly between steps 2.5×10^{-5} and 10^{-4} and vanished completely by step 10^{-3} (Figure 2). A comparison of the deactivation thresholds for the searching response obtained in GCO and in glass-rod assays points to a similar range of values (GCO, 5×10^{-5}–10^{-4}; glass-rod, 2.5×10^{-4}–5×10^{-4} g/ml). The same tendency was noted for oral grasping, although the peak level for this response was almost two times lower in the GCO than in the glass-rod test.

In sum, concentration–response curves for the MP obtained with the GCO test follow roughly the same shape as those obtained with the glass-rod assay (Experiment 1). Thus, although the administration conditions of the stimulus differ radically in the two tests, they release the same patterns of response as a function of stimulus concentration. However, the stimulation through the GCO clearly influenced the plateau level of oral grasping (as compared to the glass-rod assay), while it did not affect the frequency of searching by pups.

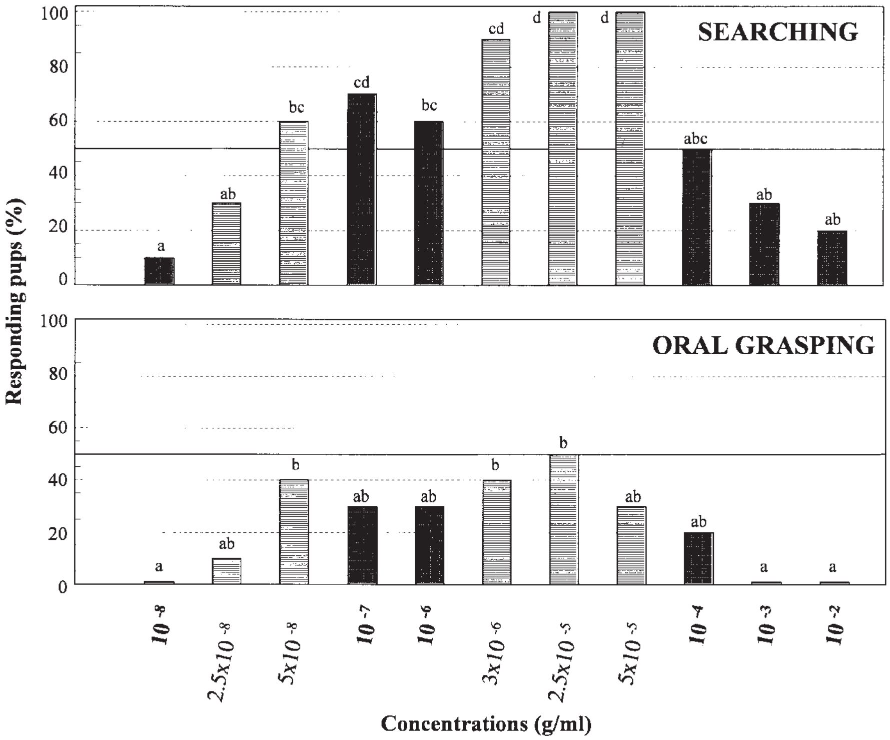

Figure 2 Frequency of rabbit pups (%) responding by searching and oral grasping as a function of the concentration of pure MP delivered through the gas chromatography-sniffing test. Statistical analyses of the responses illustrated in the two graphs are done independently. Within each graph, distinct digits indicate statistical differences at the $P < 0.05$ level (χ^2 test; or each dilution step, n pups tested = 10, from five litters; age, day 2).

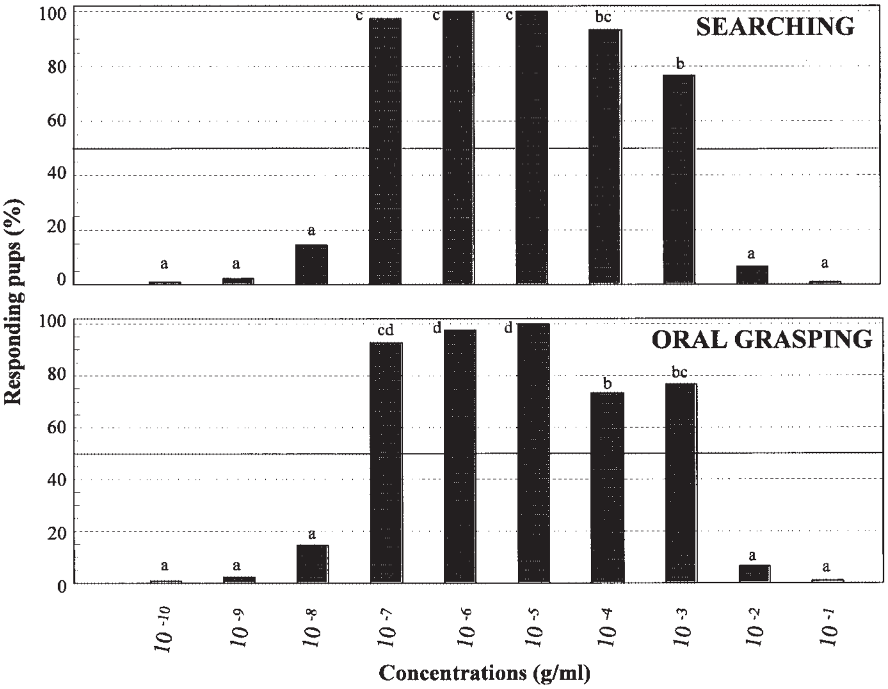

Figure 3 Frequency of rabbit pups (%) responding by searching and oral grasping as a function of the concentration of the MP diluted in deodorized milk and presented through the glass-rod test. Statistical analyses of the responses illustrated in the two graphs are done independently. Within each graph, distinct digits indicate statistical differences at the $P < 0.05$ level (χ^2 test or McNemar χ^2 test, for independent or dependent comparisons, respectively; for each dilution step included between 10^{-10} and 10^{-5} g/ml, *n* pups tested = 41, from seven litters, age, day 2; and for each other dilution step, *n* pups tested = 30, from six litters, age, day 2).

Experiment 3: concentration–response curve of the mammary pheromone diluted in deodorized rabbit milk

Again, the course of the two behavioural responses as a function of concentration might be described as bell-shaped (Figure 3).

Activation region

The proportion of pups responding by searching or oral grasping was null or low for concentrations $\leq 10^{-8}$ g/ml. The threshold for both responses was between steps 10^{-8} and 10^{-7}, the latter step releasing very high responsiveness (98% of responding pups) for both variables (Figure 3).

Plateau region

A stable, optimal level of response (situated between 90 and 100% responding pups) was maintained between steps 10^{-7} and 10^{-4} for searching, and between steps 10^{-7} and 10^{-5} for oral grasping.

Deactivation threshold

The frequency of searching dropped significantly (–25%), but remained above the threshold, when the concentration increased from 10^{-5} to 10^{-3} g/ml. The deactivation threshold for searching was crossed with a further concentration increase of 1 log unit, with an additional drop of 70% of responding pups. Above step 10^{-2}, searching was absent (Figure 3). A similar threshold value was observed with the oral-grasping response (Figure 3).

Comparison of the concentration–response curves of the mammary pheromone dissolved in milk and water

Figure 4 makes clear that the pup responsiveness was shifted towards the higher concentrations when the MP was dissolved in milk. While the activation thresholds for both responses to the MP were between steps 10^{-9} and 10^{-8} for water solutions, they were between steps 10^{-8} and 10^{-7} for milk dilutions. The same shift was noted at the other end of the curve, with deactivation thresholds for both responses situated between steps 10^{-5} and 10^{-4} in water dilutions, and between steps 10^{-3} and 10^{-2} in milk dilutions.

To sum up, both behavioural responses had similar values for the activation and deactivation thresholds. The shift of the concentration–response curves of one log unit as a function of the solvent (water versus milk) can be explained either by a pup effect or by a solvent effect. A potential pup effect may result from repeated testing of the same pups with different dilution steps of the MP. However, a detailed analysis of the pup responses to the first presentation of the MP in a series comprising varying dilutions of MP in milk revealed that (i) the threshold values for activation were crossed between steps 10^{-8} and 10^{-7} (9–100% of responding pups, respectively) and (ii) the threshold values for deactivation were crossed between steps 10^{-3} and 10^{-2} (90–20%). In other words, repeated testing did not have an impact on pup responses and the shift in the concentration–response curves probably resulted from the solvent.

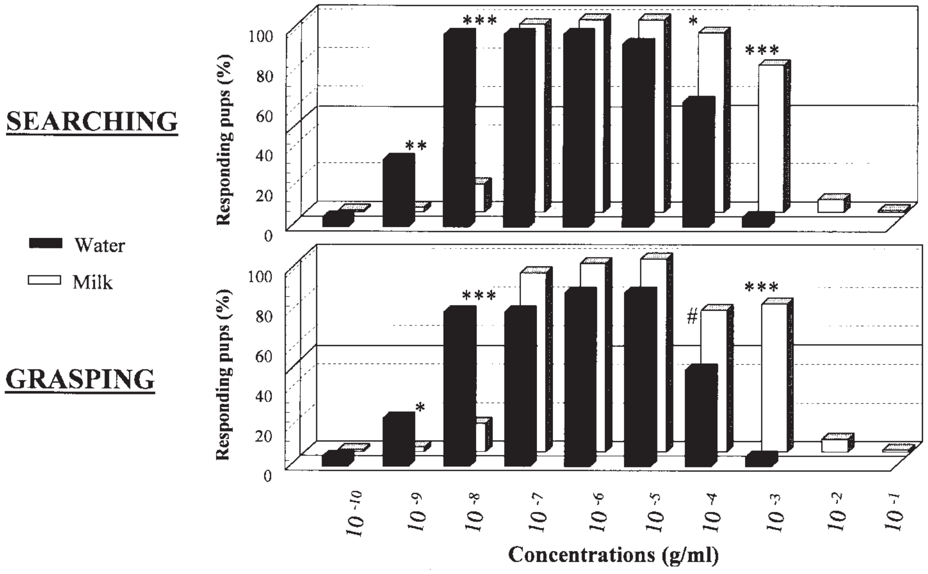

Figure 4 Comparative frequency of rabbit pups (%) responding by searching and oral grasping to the MP diluted either in distilled water (Water; results of Experiment 1) or in deodorized milk (Milk; results of Experiment 3) and presented through the glass-rod test ($0.1 < \# < 0.05$; *$P < 0.05$; ***$P < 0.001$; χ^2 test).

Discussion

Before going into details it may be useful to point out a provisional assumption that is running through the following discussion: the pheromonal signal under study is more likely to be processed through the main, rather than through the accessory, olfactory pathway. Several facts incline us to think this way for the moment, including (i) unaffected responsiveness to the females' abdominal odour in vomeronasalectomized pups and total disruption of responses in pups lesioned at both vomeronasal and olfactory levels (Schley, 1981; Hudson and Distel, 1986) and (ii) absence of 2-deoxyglucose uptake in the accessory olfactory bulb after stimulation with the MP, but strong uptake in the main olfactory bulb (Astic, Saucier, Coureaud, Gigot, Sicard and Schaal, unpublished data). Nevertheless, the following comments on the MP concentration–pup response relationship may apply regardless of the chemoreceptive subsystem(s) involved.

The concentration–response curve of the mammary pheromone in the newborn rabbit

The concentration–response curve of the MP is not linear: pups are inactive at the lowest concentrations, reach a plateau of maximal activity that extends for 5 log-units at mid-range concentrations and cease responding at the highest concentrations. The activation threshold for both variables recorded is clearly located by a sharp increase in response rate (+55% for searching and +50% for oral grasping) after a moderate increase in MP concentration (between steps 10^{-9} and 2.5×10^{-9} g/ml). The attainment of the deactivation threshold is more progressive and depends on the response considered (searching, between 2.5×10^{-4} and 5×10^{-4} g/ml; oral grasping, between 2.5×10^{-5} and 2.5×10^{-4} g/ml).

The threshold for behavioural activation in response to the MP can be located within tight limits of the concentration–response curve, i.e. 10^{-9}–2.5×10^{-9} g/ml in water (1.2×10^{-8}–3×10^{-8} M). But these values define the content of the MP in the solvent, not in the vapour phase inhaled by the pups; the latter being lower than the former, the above response threshold values are certainly underestimated. Reference publications on olfactory acuity in mammalian newborns or on thresholds for pheromone action in adult organisms are lacking (for an exception, see Alberts and May, 1980). Psychophysical approaches in various mammals with non-pheromonal odorants, such as the widely used amyl acetate (see Passe and Walker, 1985), indicate, however, that the behavioural acuity level of rabbit pups to the MP does not appear exceptional.

A surprising observation on the psychophysical function of the MP was the very abrupt drop in pup responses above certain concentration levels (namely, 2.5×10^{-5}–5×10^{-4} g/ml in water and 10^{-3}–10^{-2} g/ml in milk). Here again, the highest stimulus values of pheromones are rarely documented in mammals and no comparative base can be referred to. However, one may cite the work of Bronson (1971) who described a situation where male urine odour is more effective at lower than at higher concentrations on oestrus induction in female mice. Such data are more common in invertebrates. For example, if male *Argyrotaenia velutinana* (Lepidoptera) express the typical wing fluttering in response to a given ratio of the female sexual pheromone, *Z*- and *E*-11-tetradecenyl acetate, their response is inhibited when concentrations are increased (Akers and O'Connell, 1988). Thus, it may not be uncommon in pheromonal chemoreception that optimal information transfer is reached only within a relatively precise range of signal concentrations. This phenomenon is probably of more general occurrence, as it has also been noted with conventional odorants (e.g. Soni and Finch, 1979).

If pheromone intensity is encoded, it seems to be over a wide range of concentrations. Rabbit pups evince here their highest response level within a range of concentrations extending over 5 log units. This corroborates a previous result indicating that rabbit milk remains behaviourally active after dilution by factor 10^4 (Keil *et al.*, 1990). A wide range of optimal effectiveness of putative pheromones has also been reported in adult Mammals also. Dimethyl disulfide in hamster vaginal secretions is an effective attractant for males over a range varying from factor 1 to 60 (2–128 ng; Singer *et al.*, 1976; but see Johnston, 1981). In *Antilocapra*, the reaction released by isovaleric acid, the dominant odorant from male subauricular gland, remains stable in form and frequency over a concentration range of four orders of magnitude (Müller-Schwarze *et al.*, 1974). Similarly, beavers do not vary in response to 4-ethylphenol, the major castoreum constituent, within a concentration window of four orders of magnitude (Schulte *et al.*, 1994). In sum, receivers have to incorporate variability in their coding rules, because the intensity of any signal may fluctuate within and between emitting individuals.

Why would the MP release pup responses only at some concentrations and not at others? The lower inflexion of the MP concentration–response curve is very sharp. This may be related to the common observation that olfactory detection threshold is situated within less than 1 log unit of stimulus concentration in humans (Toyota *et al.*, 1978). A similar phenomenon is observed at the olfactory receptor neuron level (Firestein *et al.*, 1993) where the concentration interval between the detection threshold and the maximal unitary response rarely exceeds 1 log unit (Holley and MacLeod, 1977; Duchamp-Viret *et al.*, 2000). While a minimal amount of molecules is necessary to activate neuroreceptors in sufficient number to release behaviour, for given odorants the concentration of the stimulus can be increased by 4 log units before the maximal firing rate is attained (Duchamp-Viret *et al.*, 1989). With still increasing concentration, the deactivation of the behavioural responses occurs. This phenomenon may be accounted for by concentration-dependent odour quality alterations, and/or the recruitment of trigeminal inputs. According to the former alternative, a further increase of the MP concentration may produce distortions in the pattern of activated neuroreceptors because the odorant binds with non-optimal receptor sites (Sicard, 1990; Imamura *et al.*, 1992; Johnson and Leon, 2000; Meister and Bonhoeffer, 2001). Such neural activation changes may be correlated with changes in the odour percept, recognition ability and stimulus meaning (Freeman and Skarda, 1985; Xu *et al.*, 2000), with the ultimate consequence that the organism becomes unable to recognize the odorant at all and consequently ceases to respond to it. Such concentration-dependent perceptual shifts have been described with conventional odorants in human psychophysics (Moncrieff, 1967; Gross-Isseroff and Lancet, 1988; Olson and Cain, 2000) and the present data suggest that they may be generalized to pheromones. In any case, the dependence of the MP–behaviour coupling on concentration makes the rabbit pup a timely model to understand olfactory intensity 'tuning' curves at the molecular, cellular, systemic and behavioural levels of integration.

The context of stimulus presentation and the shape of the concentration–response curve of the mammary pheromone

The relationship between MP concentration and pup responses has been examined for its susceptibility to (i) the odour delivery conditions, (ii) the influence of impurities and (iii) the effect of the solvent. These points are examined below, as follows.

1. As compared to the static conditions of the MP delivery in the glass-rod test, the GCO test used dynamic conditions known to improve stimulus detection (Prah *et al.*, 1995). The rate of responding pups should thus increase in the GCO test relative to the glass-rod test and the response thresholds be shifted towards lower values. However, the effective concentration values in both experiments cannot be directly compared because the amount of MP in the vapour phase is known in the GCO test but obviously not in the glass-rod test. Overall, the GCO test did not appear to facilitate pup responsiveness: the shape of the two tuning curves remained 'bell-shaped' and the optimally effective concentrations remained in a close range of values.
2. The pups responded (especially by searching) in a concentration-dependent manner to the pure MP delivered in the GCO test in following roughly the same curve as that obtained with the non purified compound in the glass-rod test. Thus, the impurities of the commercial MP used in the glass-rod test had no marked effect *per se* or in interaction with the MP (for example, in terms of facilitation through hyperadditivity).
3. The responsiveness to the MP diluted in deodorized-milk mirrored that to the MP in water, but a 1 log unit translation towards higher concentrations was noted. This altered effect of milk as the solvent may probably result from reduced availability of the MP in the headspace due to interactions with proteic, lipidic and glucidic constituents of milk (De Roos, 1997; Fischer and Widder, 1997; Godshall, 1997; Hurst *et al.*, 1998) or to the pH of the matrix (Albone, 1984).

To sum up, the behavioural significance of the MP is not greatly altered by the conditions of the experiments: it remains a very strong releaser of the typical responses of the pups in physico-chemical and social conditions that are far from those they encounter in the natural situation.

The present study confirms that the newborn rabbit responds to the MP in a concentration-dependent manner. The concentration–response curve of the MP recorded in various conditions of stimulus delivery and physico-chemical context shows a relatively stable range of the optimally efficient concentrations. This further reveals a context-invariant pattern of pup responsiveness to the MP that fits well with the stringent definition of mammalian pheromones proposed by Beauchamp *et al.* (1976). From infraliminary concentrations, a very small increase of concentration induces the highest levels of searching and oral grasping. However, the searching rises more progressively as a function of stimulus intensity than the oral grasping. This observation raises the possibility for differential thresholds of the elementary behaviour sequences that contribute to nipple localization.

Several research lines ensue from the above results. First, the optimally efficient concentration range of the pure MP should be compared to its natural levels in milk and eventually in other exocrine secretions of the rabbit female. Secondly, the present study was restricted to the characterization of the concentration–response profile of the MP at a given age, i.e. 2–3 days; ongoing investigations are following up the intensity tuning curve of the MP at different ages in the same animals to look for intra-individual variability in the sensitivity to the MP as a function of psychobiological changes in the growing organism (i.e. onset of non-olfactory sensory modalities; changing alimentary needs).

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