Behavioural Processes 167 (2019) 103913

Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavproc

Attractive and appetitive odor factors in murine milk: Their fade-out time and differential cryo-preservation

Magali Klaey-Tassone*, Bruno Patris*, Karine Durand, Benoist Schaal*, The MILKODOR Consortium[1]

Developmental Ethology & Cognitive Psychology Group, Centre for Smell, Taste and Feeding Science, Dijon, France

ARTICLE INFO

Keywords:
Olfaction
Milk
Mouse (*Mus musculus*)
Neonate
Chemical communication
Parent-infant interactions

ABSTRACT

Murine milk conveys an odor factor that is both attractive and appetitive to conspecific newborns. Up to now, little is known about the temporal dynamic of this odor factor and about the stability of its behavioral activity after milk ejection. We aim to characterize the conditions in which the attractive and appetitive potency of milk to newborns is best conserved and, as a logical outcome, at standardizing conditions in which milk varies in reactogenic potency for newborns. Milk was collected and conserved in two conditions of cold (4 °C, −80 °C) for several durations (3 and 24 h, and 1, 2 and 8 months). The reactogenic potency of milk was assayed in 2 day-old mouse pups. We found that milk remains olfactorily attractive and appetitive to newborns after 3 h of storage at 4 °C, but it completely loses reactogenic potency on newborn pups after 24 h of storage at 4 °C. Storage at −80 °C preserves the behavioral activity of milk up to 1 month, but milk stored for 2 months at this temperature remains appetitive but not attractive to pups. Finally, the reactogenic potency of murine milk in pups is abolished after 8 months of storage at −80 °C. This study highlights that attractive and appetitive factors of milk appear dissociable and, in any case, highly labile. It provides, for two different storage temperatures, a temporal window in which milk remains behaviorally active on pups. These results will allow designing a contrastive chemical approach to identify the reactogenic compounds of milk.

1. Introduction

Once emitted in air, natural odor signals vary in the behavioral or neuroendocrine activity they elicit in conspecifics. Some retain their signaling potency for days or weeks, while others lose it within minutes or hours. Such temporally-variable chemosignals do not bear equivalent communicatory functions, the former advertising more or less stable individual-specific information (*e.g.*, identity, sexual state, quality), whereas the latter transmit some urge to react (*e.g.*, alert, fear, excitement, receptivity). Some examples of the former are: the urinary factor that primes pubertal acceleration in mice which persists up to 3–7 days after voiding (Drickamer, 1986); female mouse urine which elicits ultrasonic vocalizations (USV) in males after standing for 30 days at ambient temperature (Nyby and Zakeski, 1980); or the odor factor in hamster vaginal secretion that is still effective after 100 days (Johnston and Schmidt, 1979). In contrast, other odor factors have been called "ephemeral" (Sipos et al., 1992) because they turn out to be ineffective after short periods of standing: 18–24 h in the case of male mouse urine to elicit interest in other males (Cavaggioni et al., 2008; Hurst et al., 1998); or only 60 min for rabbit milk to release oral grasping responses in rabbit pups (Keil et al., 1990; Schaal et al., 2003). In sum, the signaling potency of scents can be heterogeneous over time, and the same secretion/excretion may function as a sequential mosaic of signaling odorants bearing various fade-out times (*e.g.*, Sipos et al., 1993; Humphries et al., 1999).

In a few cases, chemical correlates were found to explain the reactogenic instability of mammalian secretions/excretions. For example, the ephemeral effect of rabbit milk odor on pup responses was linked to the rapid concentration drop in ejected milk of the pheromone 2-methyl-but-2-enal (Schaal et al., 2003); in mouse urine, the concentration of 3,4-dehydro-exo-brevicomin (DHB), eliciting aggressiveness in males and estrus in females, falls in 30 min after urine sampling (Hurst et al., 1998). When the headspace of mouse urine was measured between 0 and 24 h after voiding, some active compounds were undetectable after 22 min (*i.e.*, DHB, linalool), others were still detected after 24 h (*i.e.*, indole, 4-ethylphenol), and still others (2-sec-butyl-4,5-

* Corresponding authors at: Centre des Sciences du Goût et de l'Alimentation, UMR 6265 CNRS, Université de Bourgogne-AgroSupDijon-Inra, 9Eboulevard Jeanne d'Arc, 21000 Dijon, France.
E-mail addresses: magali.klaey@gmail.com (M. Klaey-Tassone), bruno.patris@u-bourgogne.fr (B. Patris), benoist.schaal@u-bourgogne.fr (B. Schaal).
[1] *Cf.* Note.

https://doi.org/10.1016/j.beproc.2019.103913
Received 30 April 2018; Received in revised form 22 May 2019; Accepted 22 July 2019
Available online 24 July 2019
0376-6357/

dihydrothiazole) increased in concentration relative to fresh urine (Cavaggioni et al., 2008). All in all, the dynamics of biological scents results from complex physicochemical interactions between volatile and non-volatile constituents, differential evaporation and oxidation by enzymes or microbiota, and variations due to solvent content and adherence to the substratum (*e.g.*, Regnier and Goodwin, 1977; Kwak et al., 2013). Therefore, tracking behavioral activity changes of secretions/excretions after they are externalized may help to pinpoint i) the physico-bio-chemical conditions in which their signaling efficacy is optimal, and ii) the compounds that are most effective in the mixture.

The focus of the present study is on murine milk odor and its behavioral activity in mouse pups. As in other mammal species (Schaal, 2010; Schaal and Al Aïn, 2014), murine milk odor bears positive value for conspecific newborns, as they show attraction and appetence to it. Mouse neonates are indeed *attracted* to murine milk odor, as they orient and actively move toward it. Milk odor is also *appetitive* to pups as it stimulates pre-ingestive oral actions, such as mouth opening, licking, oral seizing and sucking (Al Aïn et al., 2012, 2013a,b, 2014, 2015; Logan et al., 2012; Patris et al., 2013). Neonatal responsiveness toward murine milk is observed even prior to any suckling experience (Al Aïn et al., 2014). But milk is highly instable in chemical, biochemical and chemosensory terms, and newborns react to compositional alterations due to various biological or environmental causes [maternal genetic background (Al Aïn et al., 2015) or diet (*e.g.*, Hausner et al., 2008; Mennella and Beauchamp, 1991; Schaal et al., 1994), shifts in lactational physiology (Al Aïn et al., 2012, 2014), or alterations occurring between milk ejection and bioassay (aging at ambient temperature, thermic/cold treatment; Keil et al., 1990)]. Taken together, behavioral studies indicate that an active odor factor is conveyed in murine milk. This active odor factor is: i) embedded in a complex mixture, ii) volatile but effective at a short distance from milk or from lactating females' nipples (Al Aïn et al., 2013b; Hongo et al., 2000), and iii) easily washed away from the nipples using solvents (Hongo et al., 2000). But the chemical identity of the milk volatiles correlating with or responsible for neonatal attraction and/or appetence remains so far unknown.

Olfaction-based behavioral assays have been validated for neonatal mice (*e.g.*, Schaal et al., 2013). These will be seminal for the characterization of the reactogenic odor factor in milk, a substrate composed of more than a thousand mineral and organic compounds bearing variable properties in terms of volatility, polarity, solubility, stability, and bioactivity (Jensen, 1995; Mills et al., 2011). Multiple analytic options can be followed to split milk complexity (such as solvent extraction, fractionation of GC eluent, and headspace analysis) or to pinpoint peaks of interest without fractionating the original mixture [such as metabolomics, gas chromatography (GC) coupled with olfactometry (*e.g.*, Schaal et al., 2003) or GC coupled with electrophysiology (*e.g.*, Lin et al., 2005)]. But all these approaches need first to ensure that milk collection and chemical extraction preserve its original level of behavioral activity on newborns. Breeding facilities being generally at a distance from the chemistry lab, conservative methods must be optimized to ensure that the highest-impact milk is subjected to bioassay as well as to extraction and GC injection. Thus, the **first goal** of the present study is to define conditions for murine milk which best preserve its attraction and appetence release potency in newborns.

The **second goal** is to define conditions in which milk varies in reactogenic potency for neonate mice, from maximal activity in fresh milk to partial or complete loss of activity after various storage conditions. Understanding this variation will enable the design of a contrastive chemical approach to assess which volatile constituents are impactful (as previously applied with mouse urine; *e.g.*, Schwende et al., 1986; Kwak et al., 2013).

So far, mammalian neonates' responses to sensory changes of milk after its post-ejection treatment and storage have been rarely studied. Here, we aim to evaluate the impact of murine milk storage temperature and duration on the murine neonate responsiveness. Two storage temperatures (+4 °C and −80 °C) will be assessed for several storage durations in anoxic conditions, using two behavioral bioassays capitalizing on the olfactory attractiveness and oral appetence of murine milk for 2 day-old mice.

2. Animals, materials, and methods

2.1. Ethics, animals and housing conditions

Applicable national and institutional guidelines for the care and use of animals were strictly followed (Authorization # 2017072815523131).

C57Bl/6 mice (*Mus musculus*, purchased at Charles River, L'Arbresle, France) were housed in standard Plexiglas cages (28 × 17 x 13 cm) in the local breeding unit. Males were left with females to favor their parental contribution and promote conception at postpartum estrous.

The ambient temperature of the room was set at 21 °C (±2 °C) and the light was turned on from 12:00 to 0:00, following a 12/12 h light/dark cycle. The breeding cages were lined with wood sawdust (SAFE, Augy, France). Water and food pellets (SAFE, Augy, France) were offered ad libitum. The pelleted chow was constituted from wheat, corn, wheat bran, barley, extruded soya seeds, soya meal, condensed fish, soluble yeast, calcium carbonate, dicalcium phosphate, and vitamin (A, E and D3) and oligo-element (copper sulfate pentahydrate) premixes [21.4% of proteins, 5.1% of fat, 5.7% of ash (mineral material) and 4% fibers].

Pups were separated from their mother 4 h before the test to maximize their responsiveness to the stimuli. In order to avoid any social stress and hypothermia, the cage was placed on a heating plate (28 x 20 cm; Gestigkeit, Düsseldorf, Germany) set at 37 °C during this 4 h period and the male was kept in the cage.

2.2. Stimuli

Milk was collected on lactation day 2. Donor females were separated from their litter for 4 h and anesthetized by an intraperitoneal injection of ketamine (Imalgène 1000, Vibrac, France; dose: 70 mg/kg in NaCl 0.9%) and xylazine (Rompun 2%, Bayer, Puteaux, France; dose: 14 mg/kg in NaCl 0.9%). To stimulate milk production and let-down, females were injected intraperitoneally with oxytocin (0.15 mL, Intervet, Unterschleissheim, Germany) and gently massaged on the mammary areas. Two minutes later, all 10 nipples of the female were aspirated using a Pasteur pipette. The total amount of collected milk was 0.2 to 0.5 mL per female. During the 15-min milking procedure, the milk was spiked in an argon-filled glass vessel (1.5 mL, 11.6 (diameter) x32 mm (height), VWR) kept in ice. Milk was then either used immediately (so called "fresh milk") or kept in different conditions of cold (4 °C or −80 °C). For each of these conditions of cold, the storage time was progressively extended until no response was observed anymore with our behavioral assays. Accordingly, depending on the experiment, the milk samples were kept in a refrigerator (4 °C) for 3 or 24 h, or frozen at −80 °C for 24 h, or for 1, 2 or 8 months.

On the day of the experiment, the refrigerated or frozen milk samples were thawed by being left on the bench for about 2 min, before being used in a test. To avoid interference with odor stimuli from conspecifics, all the tests were run in a room separate from the breeding room. A pup was never tested with the milk of its own mother. The control stimulus consisted of distilled water kept in an argon-filled vessel at room temperature.

2.3. Behavioral assays and variables

Two behavioral assays were used in the present study to assess distinct behavioral constructs which are valid in the repertoire of 2 day-old mouse pups, *viz.* attraction and appetence (as defined in the Introduction and below). A first assay assessed attraction, operationalized here as the relative duration of pups' lateral head

movements toward simultaneously presented odor stimuli. In the second assay, appetence was assessed by measuring pre-ingestive oral movements expressed ahead of full oral seizing, that is mouth opening and stretching the tongue to lick the odor source. While the attraction assay was run in an ecologically poor setting, it has the advantage to assess pup responsiveness to paired stimuli following a within-subjects design (for each pair of stimuli). In contrast, the oral activation assay is run in ecological conditions, *i.e.* directly on a non-lactating dam's nipple, but it assesses pup responsiveness following a between-subjects design (for each stimulus). Note that each pup was tested with only one of the two assays. Both of these assays are described in detail below.

2.3.1. The relative attraction assay

Test device. The relative attraction assay was used to evaluate the pup's preferential exploration toward two paired odor stimuli (Al Aïn et al., 2012; adapted from Hepper, 1988). Blotting paper (34.5 x 19.5 cm; Tork Universal Wiper 310 Centrefeed Roll, Göteborg, Sweden) was taped to a heating plate (set at 37 °C). Two scentless auto-adhesive labels (0.8 x 1.2 cm; AmpliI Paper, Barcelona, Spain) were fixated bilaterally at 3 mm from the midline on the blotting paper, delimiting two equidistant areas. A drop of 20 µL of stimulus (*viz.* fresh milk, milk stored at 4 °C for 3 or 24 h, milk stored at −80 °C for 24 h, or for 1 or 2 months, or water as the control) was spiked on either label. Both labels as well as the blotting paper were replaced after every test.

Testing procedure. Pups were held between the gloved thumb and index (gloves: VWR International, Leuven, Belgium) and were first moved close to each stimulus for 6 s without contact. This pre-exposure was assumed to render both stimuli familiar and to arouse pups. The side of this pre-exposure was random at first presentation, and then systematically alternated. The pup was then deposited with its sagittal line aligned with the midline of the blotting paper. The test began when both of its nostrils crossed the midline toward one of the two stimuli for the first time and was videotaped for 3 min. The pup's body was gently maintained so that only cephalic motions were possible.

For each experimental group, 7–13 litters were used. To limit a litter effect a maximum of 4 pups were tested per litter in one experimental group. In total, 200 2 day-old mouse pups were tested in this assay, and 10 experimental groups were formed (see Results section).

Behavioral variables. The duration of pup orientation to either stimulus was computed using the Observer software (Noldus, Wageningen, the Netherlands) operated by a coder who was blind as to the nature and lateral position of the stimuli. An animal was considered to be oriented toward a stimulus when both of its nostrils crossed the midline of the device in direction of this stimulus. If a pup did not orient to one odor stimulus 2 min after being positioned on the midline or if it did not orient to both stimuli during the 3-min test, it was excluded from further analyses. From the 200 mouse pups tested, none of them was discarded from our analyses.

2.3.2. The oral activation assay

Test device. The 90-s oral activation test (Al Aïn et al. 2013a; adapted from Teicher and Blass, 1976) was used to assess pups' oral response to a single odor stimulus painted on a nipple. Non-lactating females were used to present the stimulus nipples. Each test was carried out on a heating plate (set at 37 °C) covered by a blotting paper (34.5 x 19.5 cm; Tork Universal Wiper 310 Centrefeed Roll, Göteborg, Sweden).

Testing procedure. Non-lactating stimulus females were sedated by an intraperitoneal injection of ketamine (Imalgène 1000, Vibrac, France; dose: 70 mg/kg in NaCl 0.9%) and xylazine (Rompun 2%, Bayer, Puteaux, France; dose: 14 mg/kg in NaCl 0.9%). One nipple was then painted with 20 µL of one of the stimuli (*viz.* fresh milk, milk stored at 4 °C for 3 or 24 h, milk stored at −80 °C for 24 h, or for 1, 2 or 8 months, or water as control) and gently dried with a cotton-tip to remove the excess of liquid. The anesthetized female was hand-held by the inter-scapular skin. The pup was presented with its muzzle 1 mm from the nipple and held for 90 s (Fig. 1A). Each of the 4 inguinal nipples was presented only once. Each pup was only assayed once and in one stimulus condition. Pups were never tested with their mother's milk as a stimulus.

To evaluate the pup's olfactory integrity or arousal level, a positive control test was carried out right after the assay by presenting the pup to a nipple of a lactating female (known to be highly reactogenic; Al Aïn et al., 2012) who was anesthetized under the same conditions as the non-lactating stimulus females. Pups that did not mouth, lick or grasp this lactating nipple within 90 s were considered as non-responsive and thus discarded from further analyses.

A maximum of 4 pups per litter were tested in any experimental group. In total, 189 2 day-old mouse pups were tested and data from 168 of them were analyzed in 8 experimental groups (see Results section).

Behavioral variables. The test was videotaped and subsequently coded for oral activation along the sequence leading from contact with the mother to eventual sucking. Typical behaviors occur sequentially as shown in Fig. 1: to locate the nipple, the pup starts by moving toward the mother's body showing rooting head movements until oral contacts with the nipple; the pup then stretches its head (Fig. 1B), opens its mouth and vigorously licks the nipple until it erects making its oral grasping easy (Fig.1C-D). The present assay being carried out on non-lactating females, nipples are small and not optimally graspable. Therefore, oral activation was counted as positive when the pup stretched its mouth open to reach the nipple area and started to lick it. The percentage of responding pups and the latency of the first oral response were calculated for each group of pups.

2.4. Statistical analyses

A Shapiro-Wilk's test was used to evaluate the distribution of data. As most groups did not display a normal distribution, non-parametric statistics were used (Statistica 8, Statsoft, Paris, France). Thus, Wilcoxon tests were applied to compare the total time pups spent oriented to either stimulus (fresh milk, stored milk or water) in the choice device in the relative attraction assay.

In the oral activation assay, the proportions of pups responding positively to a nipple painted with fresh milk, stored milk or water were compared two by two using Fisher's exact tests, and the latencies to orally respond were compared with Mann-Whitney tests.

Kruskal-Wallis tests were applied to verify any litter or milk donor effect in both assays. No litter effect or milk donor effect ($2.51 < H < 12.32$, in all cases $p > 0.05$) reached significance in the relative attraction assay. In the oral activation assay, Kruskal-Wallis tests showed no significant litter effect on the proportion and the latencies of pups orally responding over the 90-sec test ($0 < H < 8.77$, in all cases $P > 0.05$), except for proportion of pups responding to a nipple painted with milk refrigerated for 3 h ($H = 13.14$, $P = 0.022$) or milk frozen for 24 h ($H = 9.92$, $P = 0.041$).

3. Results

3.1. Attraction of pups to odors of fresh vs. stored milk

3.1.1. Milk stored at 4 °C

After collection, milk was stored at 4 °C for 3 or 24 h and tested for its olfactory attractiveness against fresh milk and the scentless control stimulus (*viz.* distilled water).

As shown in Fig. 2, 2-day old pups oriented their head significantly longer to the odor of 3-h refrigerated milk than to water ($Z = 2.763$, $P = 0.006$, $n = 20$). When simultaneously exposed to fresh milk and to refrigerated milk, pups did not differentiate between them ($Z = 0.448$, $P = 0.654$, $n = 20$). This indicates that a 3-h refrigeration of milk at 4 °C preserves its attractive potency for 2-day old pups.

When milk was stored at 4 °C for 24 h, pups did no longer show any differential orientation to the odor of milk *vs.* water ($Z = 1.412$,

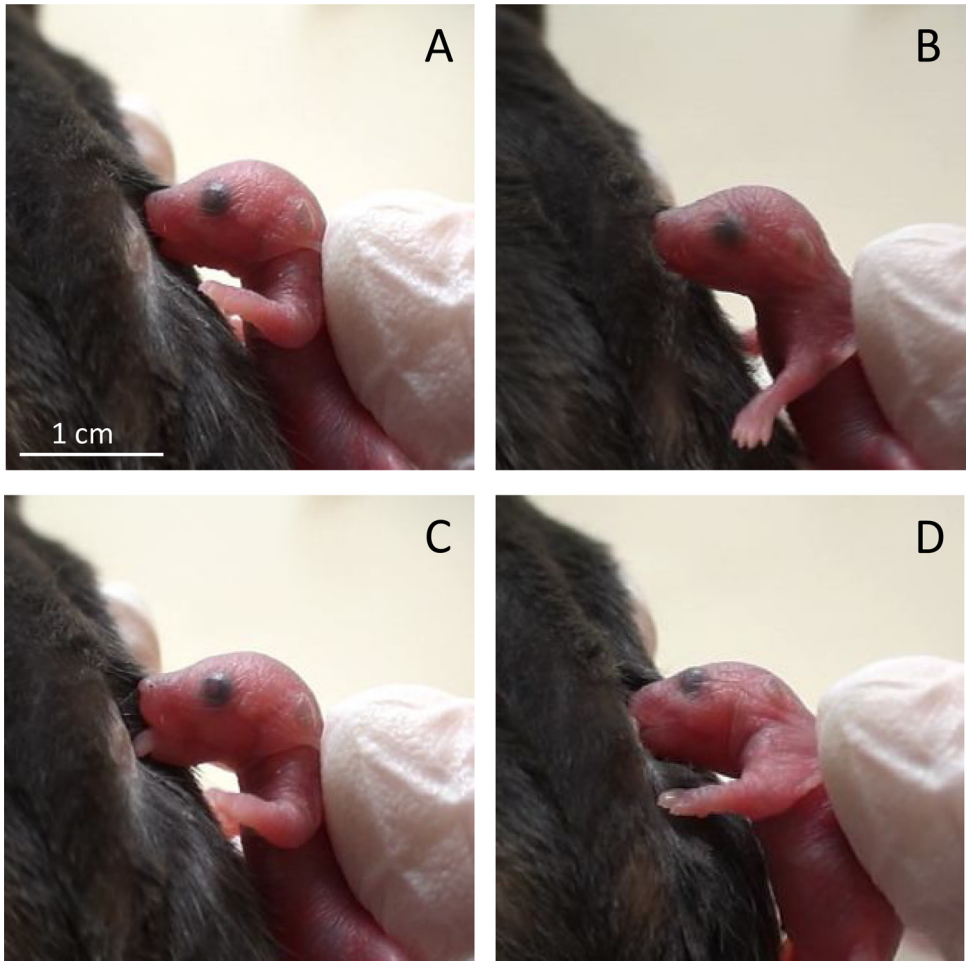

Fig. 1. Typical behavioral response in the oral activation assay. (A) Pup is positioned on the female's abdomen with its muzzle at 1 mm of the nipple. It then moves its head from side to side (rooting) until it contacts a nipple, leading to cephalic stretching (B), mouth opening and vigorous licking of the nipple (C) before grasping it (D).

P = 0.158, n = 22) (Fig. 2). When 24-h refrigerated milk was presented together with fresh milk, pups turned their head longer to the odor of fresh milk than to the 24-h refrigerated milk (Z = 3.337, P < 0.001, n = 17). Thus, after 24 h at 4 °C, murine milk's attractiveness decreases significantly.

3.1.2. Milk stored at −80 °C

Milk was frozen directly after collection and was assayed for the effect of 24-h *vs*. 1-month *vs*. 2-months storage durations on olfactory attractiveness.

After thawing, milk was presented to 2 day-old pups simultaneously

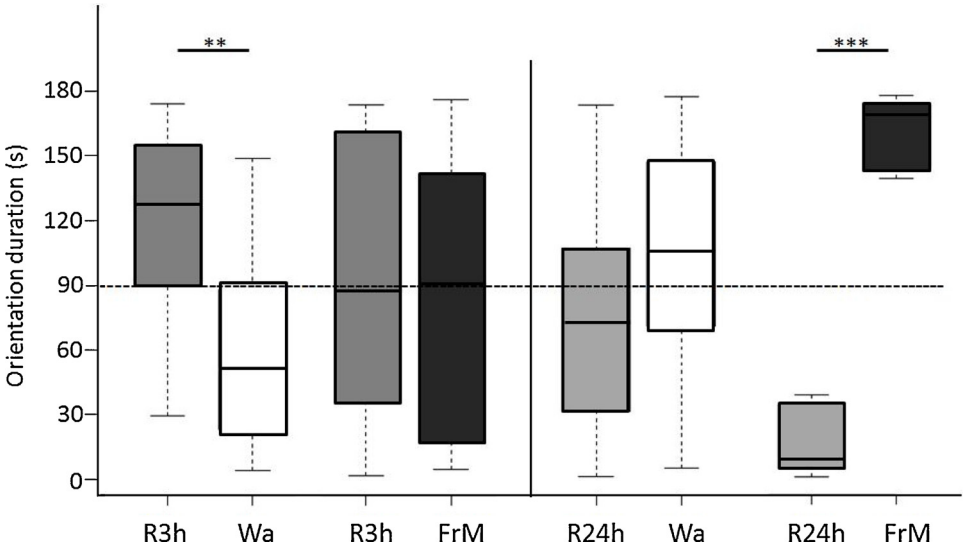

Fig. 2. Differential head-orientation duration (seconds) of 2 day-old pups exposed simultaneously to the odor of 3-h refrigerated milk (R3h) *vs.* water (Wa) or fresh milk (FrM); the odor of 24-h refrigerated milk (R24 h) *vs.* Wa or FrM. The dashed line indicates the theoretical level of random orientation (assay duration: 180 s). Wilcoxon's test: **p < 0.01, ***p < 0.001. The central line within the boxes represents the median; the boxes enclose the interquartile range; whiskers show the first and ninth deciles.

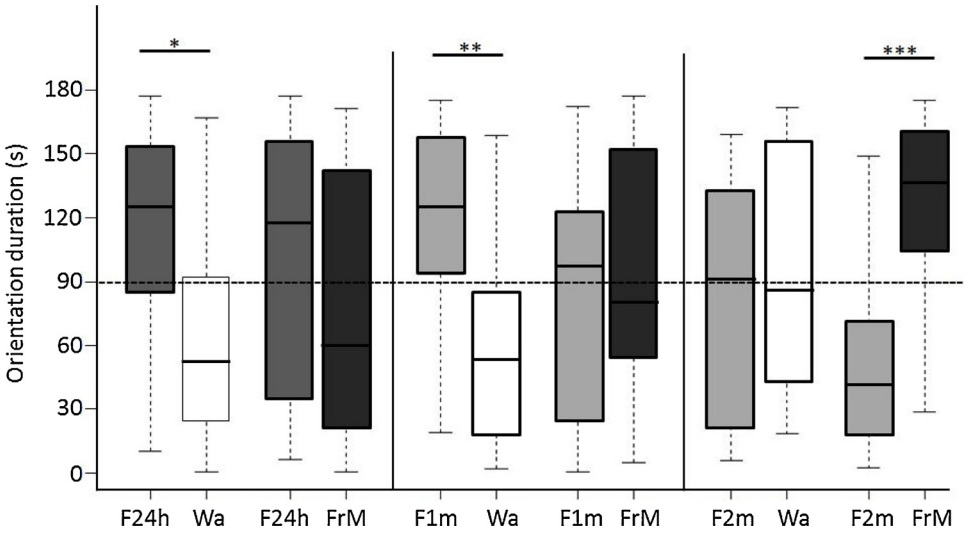

Fig. 3. Differential head-orientation duration (seconds) of 2 day-old pups exposed simultaneously to the odor of 24-h frozen milk (F24 h) *vs.* water (Wa) or fresh milk (FrM); the odor of 1-month frozen milk (F1m) *vs.* Wa or FrM; the odor of 2-month frozen milk (F2m) *vs.* Wa or FrM. The dashed line indicates the theoretical level of random orientation (assay duration: 180 s). Wilcoxon's test: *p < 0.05, **p < 0.01, ***p < 0.001. The central line within boxes represents the median; boxes enclose the interquartile range; whiskers show the first and ninth deciles.

with water. As shown in Fig. 3, 24-h frozen milk was explored significantly longer than water (Z = 2.240, P = 0.025, n = 20). When 24-h frozen milk was presented against fresh milk, pups did not orient their head more to either (Z = 0.355, P = 0.723, n = 17). Thus, after 24 h of −80 °C storage, milk remains olfactorily attractive to 2 day-old pups.

The attractiveness of milk stored at −80 °C for 1 month was examined. Using the same assay, the odor of milk frozen 1 month was explored significantly longer than water by 2 day-old pups (Fig. 3, Z = 3.000, P = 0.003, n = 24). But it elicited equivalent average duration of orientation as the odor of fresh milk (Fig. 3, Z = 0.643, P = 0.520, n = 21). Thus, after 1 month of −80 °C storage, milk still bears its olfactory attractiveness, which is then similar to that of fresh milk.

Milk was then kept frozen at −80° for 2 months. When such milk was paired with water, 2 day-old pups did not turn their head significantly longer to either (Fig. 3, Z = 0.569, P = 0.569, n = 16). However, when milk frozen for 2 months was presented against fresh milk, pups explored significantly more the odor of the latter than the odor of the former milk (Z = 3.376, P < 0.001, n = 23). In sum, milk stored at −80 °C for 2 months does no longer appear to convey olfactory attractiveness to 2 day-old pups with this behavioral assay.

3.2. Neonatal appetence for the odor of fresh vs. stored milk

3.2.1. Milk stored at 4 °C

Fresh milk was collected and stored at 4 °C for 3 or 24 h before being assayed for its ability to trigger responses in a 90-s oral activation test. Both the percentage of pups' oral responses and the latency to respond were assessed.

As shown in Fig. 4, milk stored for 3 h did not differ from fresh milk in terms of proportion of responsive pups (11/19 *vs.* 15/21, P = 0.509) and of latency to respond (mean ± standard error: 52.3 ± 8.2 *vs.* 40.0 ± 7.7 s, U = 152.5, P = 0.198), but it triggered significantly more pups responding (11/19 *vs.* 8/30, P = 0.039) with a shorter latency than to water (52.3 ± 8.2 *vs.*73.4 ± 5.3 s, U = 186, P = 0.021). Milk stored for 24 h induced significantly fewer oral responses (5/19 *vs.* 15/21, P = 0.010) with a longer latency than fresh milk (79.4 ± 4.9 *vs.* 40.0 ± 7.7 s, U = 83.5, P < 0.001). But it did not differ from water in terms of proportion of responding pups (5/19 *vs.* 8/30, P = 1) and latency (79.4 ± 4.9 s *vs.* 73.4 ± 5.3 s, U = 275, P = 0.845) (Fig. 4 A, B).

Thus, after being refrigerated for 3 h, milk retains some of its appetitive potency. However, this potency vanishes after 24 h of refrigeration with a percentage of pups which express oral responses and a latency comparable to those caused by water. These results are consistent with those of the relative attraction assay (Fig. 2).

3.2.2. Milk stored at −80 °C

Freshly collected milk was frozen at −80 °C and stored for 24 h, or 1, 2 and 8 months. A 90-s test was carried out to assess the olfactory appetence of these different milks (Fig. 4 A, B).

Milk frozen for 24 h, and for 1 or 2 months did not differ from fresh milk in terms of the proportion of responsive pups (15/19, P = 0.721; 14/22, P = 0.747; 14/19, P = 1, respectively) and of average latency to respond (37.0 ± 7.5 s, U = 199, P = 1; 54.0 ± 6.9 s, U = 166, P = 0.110; 36.0 ± 6.2 s, U = 192, P = 0.848, respectively). However, each of these stimuli induced significantly more oral responsiveness than water (P < 0.001; P = 0.011; P = 0.003, respectively), and with a shorter latency (U = 119.5, P < 0.001; U = 210, P = 0.013; U = 118.5, P < 0.001, respectively).

Since milk stored for 2 months still triggered oral activation, we increased storage duration to 8 months. Only 7 out of 19 pups responded orally to a nipple painted with such aged milk, with a mean latency of 67.1 ± 7.2 s. This proportion only tended to differ (P = 0.054) with this obtained with fresh milk (15/21), but the latency to orally respond was significantly higher (U = 104.5, P = 0.007). Moreover milk frozen for 8 months did not differ in terms of proportion of orally responsive pups (P = 0.532) and of latency to respond (U = 262.5, P = 0.580) to a nipple painted with water (Fig. 4 A, B).

Thus, the ability of milk to trigger an oral activation response taken as a proxy for appetence is preserved up to 2 months of storage at −80 °C. However, after 8 months of storage, this releasing property is significantly reduced.

4. Discussion

The present study had several goals. *First*, it aimed to find out the best conditions for preserving the highest potency of freshly ejected murine milk to affect the behavior of mouse newborns. *Second*, as a corollary of the previous point, this study aimed to characterize procedures by which murine milk partially or completely loses its potency to release pups' criterion responses. Finally, a subsidiary, more speculative aim hinted to explore preliminarily the functional complexity of the odor factor(s) conveyed to murine pups through conspecific milk.

4.1. Cryo-differentiation of the behavioral activity of milk

When kept for a short period, milk stored at 4 °C remained olfactorily reactogenic to pups. Both behavioral assays consistently revealed that up to 3 h of storage at 4 °C, milk remained as attractive and

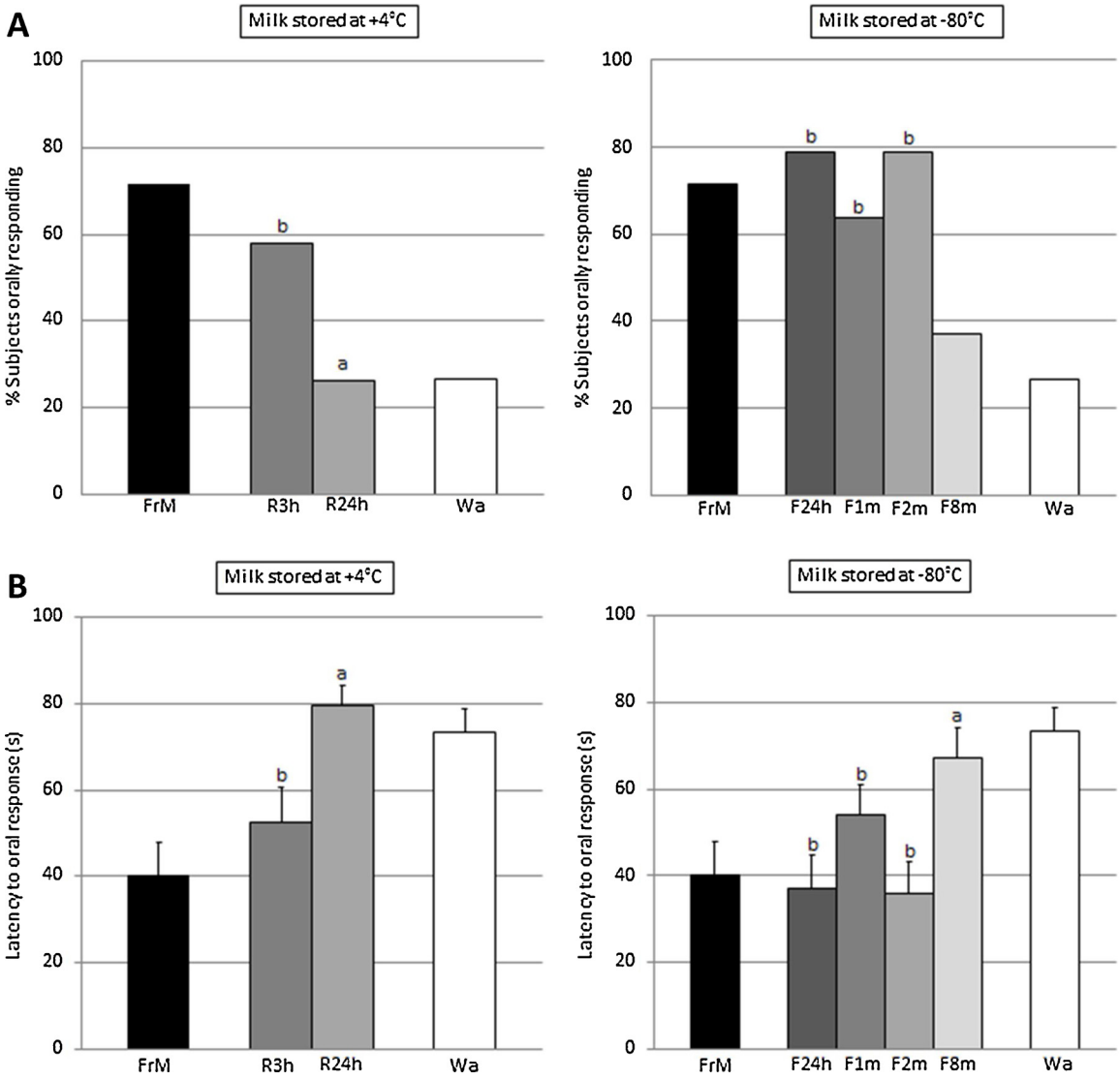

Fig. 4. Oral activation responses of 2-day old pups of a nipple painted with fresh milk (FrM), 3-h refrigerated milk (R3h), 24-h refrigerated milk (R24 h), 24-h frozen milk (F24 h), 1-month frozen milk (F1m), 2-months frozen milk (F2m), 8-months frozen milk (F8m) or water (Wa). (A) Percentage of pups' oral response toward a nipple (Fisher's exact test). (B) Mean latency (error bars are Standard Errors) to the first oral response (s) (Mann-Whitney test). Statistical significant differences ($p < 0.05$) between stored milk and fresh milk or water were indicated with "a" or "b", respectively.

appetitive than fresh milk. This result is practically significant in the context of behavioral assays with fresh milk, which usually take more than 2 h for the completion of an experimental group. Thus, knowing that the milk odor factor is preserved within this range of time is essential to ensure reliable assays.

As expected from previous studies (*e.g.*, Sandgruber et al., 2012), relative to storage at 4 °C, the storage at −80 °C massively increased the preservation time of milk odor behavioral activity. In such deep-freezing conditions the relative attraction assay demonstrated that milk was still attractive after up to 1 month of storage. But somewhere between 1 and 2 months of storage, with this same assay, pups did not discriminate the odor of stored milk from the control stimulus anymore, and the odor of stored milk was significantly less attractive than the reference odor of fresh milk.

However, milk stored at −80 °C for 2 months remained olfactorily appetitive in the oral activation assay, as mouse pups still orally responded to a nipple painted with it. Thus, either both assays may depend on different olfactory thresholds to release the criterion responses or some background stimuli associated with the nipple of a live stimulus female (*i.e.*, background odor, fur, temperature) provide a more ecologically-relevant environment where a smaller amount/intensity of the appetitive odor factor is required to trigger pup's oral activation. Accordingly, some remaining amounts of the reactogenic factor left in milk after a 2-month storage may indeed not suffice to elicit head orientation in 2 day-old pups in the relative attraction assay, but can, together with aforementioned stimuli occurring around a natural nipple, trigger mouthing response in the oral activation assay. Regardless, our study provided a reliable time window in which behavioral testing is effective and in which chemical analyses to characterize the chemical nature of the active milk odor factor should be achieved. To be conservative, however, we will consider that storage at −80 °C for up to 1 month post-ejection ensures that the total reactogenic potency is preserved in murine milk.

4.2. Partial and complete loss of behavioral activity to milk after ejection

Our study also aimed at standardizing conditions in which milk odor loses its reactogenic potency for neonate mice. After 24 h of storage at 4 °C, milk completely lost its reactogenic value for 2 day-old pups. Interestingly, the orientation assay between milk stored 24 h at 4 °C and

the control stimulus suggested that the former's odor did not become repulsive, but rather appeared only having lost its attractive potency.

At −80 °C, the two behavioral assays revealed that the appetitive and attractive properties of milk did not resist storage for the same durations. As mentioned above, this difference could be related to the specific environment of each test setting. However, if non-milk factors are not the cause, these results raise a hypothetical dissociation between attractive and appetitive effects of murine milk odor on mouse neonates. Such functional dissociation of milk semiochemistry may be explained either by a qualitative or by a quantitative effect of milk components on newborn pups. One possibility is that different volatile factors of milk elicit attraction and appetence responses in pups; another possibility is that these different responses are under the control of a same factor but at different thresholds, the elicitation of attraction requiring higher concentration levels than the elicitation of appetence. While a brief storage at 4 °C or a 1-month storage at −80 °C do preserve both behavioral components, a deep-freezing storage for 2 months conserves the appetitive factor but not the attractive factor (or keeps the former at a level sufficient to elicit appetence, but not attraction). Further experimentation is required to test this hypothesis. Finally, extending the deep-freezing storage duration for up to 8 months revealed the complete abolition of both attractive and appetitive components conveyed in murine milk.

Our experimental dissociation over such differential reactogenic potency of murine milk on newborn pups will favor a subtractive chemical strategy to pinpoint candidate odorants that explain the difference in behavioral activity of fresh murine milk *versus* such milk inactivated by storage. Thus, comparing the chemical profiles of reactogenic milks (*viz*. fresh milk, milks stored at 4 °C for 3 h maximum, or at −80 °C for 1 month) *vs*. non reactogenic milks (milks stored at +4 °C for 24 h or at −80 °C for 8 months) should allow us to pinpoint the chemical(s) that might have been lost between the former and the latter (or that are significantly lower in concentration), and which would therefore be indispensable in guiding or motivating the newborns to the mother's nipple. Further, comparing the chemical profiles of milks stored at −80 °C for 2 months and of milk stored so for 8 months should allow characterizing the chemical correlates of attractive *vs*. appetitive components of pups' responses to milk odor.

Although it is an important preparative step in the process of chemical analysis or behavioral bioassay of natural substrates, improving procedures to inhibit or attenuate evaporation or oxidization, storage imposes modifications on milk. Several studies on milk storage have already been carried out for human milk and the impact of heating, lyophilization or refrigeration/freezing on the sensory and chemical properties of milk have been described (*e.g.*, Contador et al., 2015; Spitzer and Buettner, 2010, 2013; Spitzer et al., 2010; Sandgruber et al., 2012). For example, fatty acids in human milk are oxidized into compounds bearing metallic and fishy off-odors when milk is stored at −19 °C for 1–2 months (Spitzer et al., 2010), but not when milk is stored at −80 °C, even for 24 months (Sandgruber et al., 2012). Thus, despite the facts that certain enzymes remain active at low temperatures (Berkow et al., 1984) and that physical structure of milk may be altered (Jensen, 1995), deep-freezing is a good option to preserve the chemosensory and semiochemical properties of milk over long periods, although refrigeration (+4 °C) may be sufficient over shorter terms.

4.3. Chemical stability of murine milk and the communicative functions of its components

The present results lead to the conclusion that murine milk conveys highly labile semiochemical information to murine pups. The temporal window of behavioral activity of fresh murine milk on newborn mice bears resemblance to those natural odor signals that vanish after a brief delay and that function to communicate some critically urgent information (see Introduction), rather than with the more persistent odor cues that transfer more stable species- or individual-specific information. The behavioral activity of murine milk odor is indeed quasi-immediate upon presentation and it rapidly declines as milk ages after ejection. Thus, the olfactory information carried in murine milk compares well with the case of rabbit milk which was shown to be extremely attractive and appetitive for a short period of time after ejection (maximum 60 min at room temperature; Keil et al., 1990; Schaal et al., 2003), conveying a signal that operates instantaneously, but which is rapidly degraded (in the context of milk).

The (bio)chemical processes behind this high lability of murine milk odor is unknown. Whether evaporation, lipolysis or protein denaturation causes the loss of attractive potency is not clear yet. Since murine milk was stored in anoxic conditions (milk samples being stored in vessels pre-filled with inert argon), we preliminarily assume that the loss of reactogenic potency is not due to an oxidative process, but rather to the evaporation of the active factor.

Finally, in addition to the fact that the active odor factor of fresh murine milk appears volatile, it nevertheless needs a very short distance to be effective (Hongo et al., 2000; Al Aïn et al., 2013b). Indeed, mice pups express licking and grasping a nipple of a lactating dam when their muzzle is placed at 1 mm of it. Similarly, they positively orient to milk from a 2-3-mm distance. When quasi-direct contact is required for communication between the source and the receiver, the chemical signal may not need to be of high volatility.

In conclusion, murine milk conveys semiochemicals that elicit both attraction and appetitive responses in murine pups. The volatile compound(s) of milk supporting this behavioral activity in newborns is (are) however highly labile after ejection. They can nevertheless be stabilized by immediate deep freezing. Such cold treatment can indeed sustain the behavioral activity of milk for a 1-month window for the attraction criterion and for a 2-month window for the appetence criterion of response.

Note. The MILKODOR Consortium is composed of Benoist Schaal, Karine Durand, Bruno Patris, Robert Soussignan, Fabrice Damon and Magali Klaey-Tassone (Centre des sciences du Goût, Dijon, France); Nicolas Baldovini (Institut de Chimie de Nice, Nice, France); Carole Prost, Angélique Villière, Catherine Fillonneau, and Sarah Le Roy (Flavor Group, Oniris, Nantes, France); and Evelyne Vigneau and Philippe Courcoux (Chemometry and Statistics Group, Oniris, Nantes, France).

Acknowledgments

The authors are grateful to Ms. Anne Lefranc for her expert care of the animals. Ms. Camille Goudet's help is acknowledged in data collection. Reviewers are thankfully acknowledged for their useful comments. This research was supported by the Agence Nationale de la Recherche (Paris) under grant ANR-15-CE21-0009-01 to BS.

References

Al Aïn, S., Belin, L., Patris, B., Schaal, B., 2012. An odor timer in milk? Synchrony in the odor of milk effluvium and neonatal chemosensation in the mouse. PLoS One 7, 1–7.
Al Aïn, S., Belin, L., Schaal, B., Patris, B., 2013a. How does a newly born mouse get to the nipple? Odor substrates eliciting first nipple grasping and suckling responses. Dev. Psychobiol. 55, 888–901.
Al Aïn, S., Chraïti, A., Schaal, B., Patris, B., 2013b. Orientation of newborn mice to lactating females: identifying biological substrates of semiochemical interest. Dev. Psychobiol. 55, 113–124.
Al Aïn, S., Mingioni, M., Patris, B., Schaal, B., 2014. The response of newly born mice to odors of murine colostrum and milk: unconditionally attractive, conditionally discriminated. Dev. Psychobiol. 56, 1365–1376.
Al Aïn, S., Goudet, C., Schaal, B., Patris, B., 2015. Newborns prefer the odor of milk and nipples from females matched in lactation age: comparison of two mouse strains. Physiol. Behav. 147, 122–130.
Berkow, S.E., Freed, L.M., Hamosh, M., Bitman, J., Wood, D.L., Happ, B., Hamosh, P., 1984. Lipases and lipids in human milk: effect of freeze-thawing and storage. Pediatr. Res. 18, 1257–1262.
Cavaggioni, A., Mucignat-Caretta, C., Redaelli, M., 2008. Mice recognize recent urine scent marks by the molecular composition. Chem. Senses 33, 655–663.
Contador, R., Delgado, F.J., Garcia-Parra, J., Garrido, M., Ramirez, R., 2015. Volatile

profile of breast milk subjected to high-pressure processing or thermal treatment. Food Chem. 180, 17–24.
Drickamer, L.C., 1986. Acceleration and delay of puberty in female mice via urinary chemosignals: age of the urine stimulus. Dev. Psychobiol. 19, 155–161.
Hausner, H., Bredie, W.L., Molgaard, C., Petersen, M.A., Møller, P., 2008. Differential transfer of dietary flavour compounds into human breast milk. Physiol. Behav. 95, 118–124.
Hepper, P.G., 1988. Adaptive fetal learning: prenatal exposure to garlic affects postnatal preferences. Anim. Behav. 36, 935–936.
Hongo, T., Hakuba, A., Shiota, K., Naruse, I., 2000. Suckling dysfunction caused by defects in the olfactory system in genetic arhinencephaly mice. Biol. Neonate 78, 293–299.
Humphries, R.E., Robertson, D.H.L., Beynon, R.J., Hurst, J.L., 1999. Unravelling the chemical basis of competitive scent marking in house mice. Anim. Behav. 58, 1177–1190.
Hurst, J.L., Robertson, D.H.L., Tolladay, U., Beynon, R.J., 1998. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. Anim. Behav. 55, 1289–1297.
Jensen, R.G., 1995. Handbook of Milk Composition. Academic Press, New York.
Johnston, R.E., Schmidt, T., 1979. Responses of hamsters to scent marks of different ages. Behav. Biol. 26, 64–75.
Keil, W., Von Stralendorff, F., Hudson, R., 1990. A behavioral bioassay for analysis of rabbit nipple-search pheromone. Physiol. Behav. 47, 525–529.
Kwak, J., Grigsby, C.C., Preti, G., Rizki, M.M., Yamazaki, K., Beauchamp, G.K., 2013. Changes in volatile compounds of mouse urine as it ages: their interactions with water and urinary proteins. Physiol. Behav. 120, 211–219.
Lin, D.Y., Zhang, S.Z., Block, E., Katz, L.C., 2005. Encoding social signals in the mouse main olfactory bulb. Nature 434, 470–477.
Logan, D.W., Brunet, L.J., Webb, W.R., Cutforth, T., Ngai, J., Stowers, L., 2012. Learned recognition of maternal signature odors mediates the first suckling episode in mice. Curr. Biol. 22, 1–10.
Mennella, J.A., Beauchamp, G.K., 1991. Maternal diet alters the sensory qualities of human milk and the nursling's behavior. Pediatrics 88, 737–744.
Mills, S., Ross, R.P., Hill, C., Fitzgerald, G.F., Stanton, C., 2011. Milk intelligence: mining for bioactive substances associated with human milk. Int. Dairy J. 21, 377–401.
Nyby, J., Zakeski, D., 1980. Elicitation of male mouse ultrasounds: bladder urine and aged urine from females. Physiol. Behav. 24, 737–740.
Patris, B., Al Aïn, S., Schaal, B., 2013. Suckling odours in rats and mice: biological substrates that guide newborns to the nipple. In: In: East, M.L., Dehnhard, M. (Eds.), Chemical Signals in Vertebrates Vol. 12. Springer, New York, pp. 77–85.
Regnier, F.E., Goodwin, M., 1977. On the chemical and environmental modulation of pheromone release from vertebrate scent marks. In: Müller-Schwarze, D., Mozell, M.M. (Eds.), Chemical Signals in Vertebrates. Plenum Press, New York, pp. 115–133.
Sandgruber, S., Much, D., Amann-Gassner, U., Hauner, H., Buettner, A., 2012. Sensory and molecular characterization of the protective effect of storage at -80°C on the odour profiles of human milk. Food Chem. 130, 236–242.
Schaal, B., 2010. Mammary odor cues and pheromones: mammalian infant-directed communication about maternal state, mammae, and milk. Vitam. Horm. 83, 83–136.
Schaal, B., Al Aïn, S., 2014. Chemical signals "selected for" newborns in mammals. Anim. Behav. 97, 289–299.
Schaal, B., Al Aïn, S., Patris, B., 2013. Testing smell when it is really vital: behavioral assays of social odors in the neonatal mouse. In: Touhara, K. (Ed.), Pheromone Signaling: Methods and Protocols. Humana Press-Springer, New York, pp. 349–371.
Schaal, B., Coureaud, G., Langlois, D., Giniès, C., Sémon, E., Perrier, G., 2003. Chemical and behavioural characterization of the rabbit mammary pheromone. Nature 424, 68–72.
Schaal, B., Orgeur, P., Porter, R.H., 1994. Short-term flavor preference induced by dietary flavours transferred into mother's milk. Inf. Behav. Dev. 7 927-927.
Schwende, F.J., Wiesler, D., Jorgenson, J.W., Carmack, M., Novotny, M., 1986. Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. J. Chem. Ecol. 12, 277–296.
Sipos, M., Kerchner, M., Nyby, J., 1992. An ephemeral sex pheromone in the urine of female house mice (Mus domesticus). Behav. Neural Biol. 58, 138–143.
Sipos, M.L., Nyby, J.G., Serran, M.F., 1993. An ephemeral sex pheromone of female house mice (*Mus domesticus*): pheromone fade-out time. Physiol. Behav. 54, 171–174.
Spitzer, J., Buettner, A., 2010. Characterization of aroma changes in human milk during storage at −19°C. Food Chem. 120, 240–246.
Spitzer, J., Buettner, A., 2013. Monitoring aroma changes during human milk storage at -19°C by quantification experiments. Food Res. Int. 51, 250–256.
Spitzer, J., Doucet, S., Buettner, A., 2010. The influence of storage conditions on flavour changes in human milk. Food Qual. Preference 21, 998–1007.
Teicher, M.H., Blass, E.M., 1976. Suckling in newborn rats: eliminated by nipple lavage, reinstated by pup saliva. Science 193, 422–425.