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# 1                    **Chemical identification of ‘maternal signature odours’ in rat**

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15

16

## 17   **Abstract**

18   Newborn altricial mammals need just after birth to locate their mother’s nipples for suckling. In  
19   this precocious behaviour, including for the human baby, maternal odour via the olfactory process  
20   plays a major role. Maternal odour emitted by lactating females or by amniotic fluid (AF) attracts  
21   pups, but the chemical identity of this attractant has not yet been elucidated. Here, using  
22   behavioural tests and gas chromatography coupled with mass spectrometry (GC-MS) techniques,

23 we show that AF extracts from rat pregnant female, nipples, ventral skin, milk and nest extracts of  
24 mother contained 3-6 active substances. AF extracts contained 3 active compounds: ethylbenzene,  
25 benzaldehyde and benzyl alcohol, and their mixture in similar proportions to those found in AF  
26 extracts, in a ratio, respectively, of 1:1:12 (700 ng), attracts pups as putative maternal attractant  
27 substances (MAS). These 3 AF substances have already been identified in milk, nipples, ventral  
28 wash and nest extracts of mother, but not in feces. Moreover, anethole flavour incorporated in  
29 pregnant rat and mother's diet is also detected in AF, nipples, milk and nest extracts and the pups  
30 are attracted to anethole odour, but not in the case of the no-anethole pups. Maternal attractant  
31 substances, combined with diet flavours present in the AF bath, represent olfactory signals as  
32 'maternal signature odours' (MSO) that are learned by foetus and pups. These findings open the  
33 way to improved understanding of the neurobiology of early olfactory learning and of the  
34 importance of evolutionarily conserved survival behaviour in many mammal species.

35

36 **Key words:** odour preference, maternal signature, learning and memory, amniotic fluid, diet,  
37 rodents.

38

### 39 **Introduction**

40

41 For the survival and adaptation process of the species, newborn mammals are confronted, shortly  
42 after birth, with the need to locate, grasp and suck from the nipple of their mother. This is  
43 particularly obvious for the neonate which at birth must be able to use and respond appropriately  
44 to a whole range of new stimuli. In this early adaptation, maternal odour, via the olfactory  
45 mechanisms, plays a major role in early behavioural development, such as orientation to maternal  
46 odour and suckling (Cheal, 1975; Rosenblatt, 1983; Varendi et al., 1994; Porter and Winberg, 1999;  
47 Schaal, 2010; Schaal and Al Aïn, 2014). The role of olfactory signals in the mediation of mother-

48 newborn interactions and precocious attachment has been demonstrated in several species of  
49 rodents (Carter and Marr, 1970; Noirot, 1970; Schapiro and Salas, 1970; Gandelman et al., 1971;  
50 Leon and Moltz, 1972; Moltz and Leon, 1973; Wallace et al., 1973; Devor and Schneider, 1974;  
51 Porter and Doane, 1976; Breen and Leshner, 1977; Hudson and Distel, 1986). In rat, olfaction is  
52 essential in early development (Salas et al., 1970; Gregory and Pfaff, 1971; Singh et al., 1976).  
53 Without maternal assistance, the neonate rat, although blind and deaf, can crawl under its  
54 mother's body, position itself, choose a free nipple, and suckle. Rat pups which have been  
55 rendered insensitive to olfactory cues by olfactory denervation with intranasal ZnSO<sub>4</sub> are unable to  
56 attach to the nipples of an anaesthetized lactating female and when housed with their own un-  
57 anaesthetized mother, lose weight and often die (Singh et al., 1976). Rat pups are also highly  
58 dependent on odour cues from the dam's ventrum for nipple location and attachment (Hofer et al.,  
59 1976; Teicher and Blass, 1976). An odorous attractive substance, released by a lactating female rat  
60 and impregnating a soiled litter (Nyakas and Endröczy, 1970; Salas et al., 1970; Schapiro and Salas,  
61 1970; Gregory and Pfaff, 1971; Galef and Muskus, 1979), as well as another substance called  
62 maternal pheromone (Moltz and Leon, 1973), serves as a powerful attractant to the young and  
63 incites early walking in three-day-old neonatal rats (Jamon and Clarac, 1998). Moreover, the ventral  
64 zone of lactating mothers contains olfactory cues that orient rat pups to finding and attaching to  
65 nipples, but chemical washing of the nipple area eliminated this behaviour (Hofer et al., 1976;  
66 Singh and Hofer, 1978; Blass and Teicher, 1980). The relationship of maternal ventral skin  
67 substances and maternal pheromone is not yet clear. The nature of these putative maternal  
68 attractant substances is as yet unknown. Do these olfactory signals performing different  
69 behavioural functions originate from the same substances?

70 Previous studies in many animal species showed that neonates are capable of perceiving and  
71 recognizing the odour profile of their amniotic fluid (AF). Prenatal exposure to odour substances

72 introduced into the AF (Pedersen and Blass, 1982) or ingested by their pregnant mother may  
73 influence responsiveness to those same cues after birth (Hepper, 1988; Schaal and Orgeur, 1992;  
74 Mennella et al., 2001; Wells and Hepper, 2006). Moreover, some newborn mammals are attracted  
75 to the odour of AF per se (Schaal and Orgeur, 1992; Logan et al., 2012). What are the respective  
76 roles of putative maternal attractant substances (MAS) and of odorant intake of AF?

77 The purpose of the present study is to assess the source and identify volatile active  
78 compounds of the 'maternal signature odours' (MSO) by bioassay and gas chromatography–mass  
79 spectrometry (GC-MS) techniques. We used direct nest contact and Y maze tests to assess  
80 attractive natural and synthetic substances with newborn rats and 12-14 day-old post-natal rats.  
81 Moreover, a substance, anethole, was incorporated in pregnant and mother rats' diet to assess  
82 whether diet flavours could moreover generate a specific olfactory signal as in mice 'maternal  
83 signature odours' (MSO) (Wyatt, 2010, 2014; Logan et al., 2012). Signature odours have been  
84 proposed to underlie kin recognition between ewe and lambs (Wyatt, 2010; Porter et al. 1991).  
85 Previously described individual olfactory signatures act to enable a ewe to distinguish and identify  
86 her individual lambs (Porter et al. 1991). Here we show the presence of 3 active compounds of the  
87 MAS in AF, and the existence of chemosensory cues related to the mother's diet. These findings  
88 suggest that the foetus and pups learn and memorize a specific combination of volatile odours or  
89 'maternal signature odours' (MSO), facilitating suckling just after birth.

90

91

## 92 **Materials and methods**

### 93 **Animals and experimental design**

94 Wistar rats (male, virgin female, gravid female and female with litters) (280-300 g) (Charles  
95 River Company, France) were housed with pine wood shavings and given food (SAFE, A04, route de

96 St Bris, 89290 Augy, France) and water *ad libitum* at a constant temperature (22°C), under a  
97 12h/12h light/dark cycle (lights on at 7:00 a.m.). Each gravid female was individually housed and  
98 the date of parturition (postnatal day 0, P0) was recorded. Male and female pups used were  
99 postnatal day 5 (P5) in direct nest contact tests and postnatal day 12-14 (P12-14) in Y maze tests.  
100 The young rats were isolated from their mothers for at least 1 hour before the tests and put  
101 together in a box maintained at 24°C. Each pup was identified by a number marked on its back.  
102 Pups were naïve to each stimulus and were tested only once for a given odour. For each test, pups  
103 came from 2-3 different litters. The behavioural tasks were completed in a single-blind design in an  
104 experimental room at 24°C. All experiments were carried out in accordance with the European  
105 Communities Council Directive (86/609/EEC). Care was taken to minimize the numbers of animals  
106 used and to maintain them in good general health. Aix-Marseille Université and the Centre  
107 National de la Recherche Scientifique (CNRS) approved the study.

108

109

## 110 **Behavioural tests**

111 ***Direct nest contact test.*** The test apparatus consisted of a white plastic pan (28 cm length x 18 cm  
112 width x 4 cm height), divided into two parts, and separated by about 1.5 cm midline space (Fig. 2A).  
113 One side contained the nest wood shavings of lactating female (~35g) and the other side contained  
114 the nest wood shavings of virgin female or clean nest shavings. Pups (P5), placed in the midline  
115 space, reacted to one type of nest shaving by snout probing, moving the head, crawling, burying in  
116 the nest material to the left or right side. The pup behaviour was recorded on a digital video camera  
117 (Canon Power Shot A95). The choice of a nest shaving was made when pups stopped walking or  
118 moving but stayed in the same nest side at least 5 sec. The choice and amount of time each pup  
119 spent over each type of nest shavings was recorded within a 60-second trial time.

120

121 ***Y maze test***

122 ***Apparatus.*** A Y maze olfactometer in plastic material was composed of a start box (20 cm length, 6  
123 cm width, 4 cm height) and two choice arms, (12 cm length with removable transparent cover  
124 (135° angle between start box and choice arms, 90° angle between each choice arm)) (Fig.2B). The  
125 end of each choice arm was delimited by a removable Plexiglas door with a hole (4 mm) in the  
126 middle. A stimulus box comprising a plastic cylinder tube (6 cm length, 2.6 cm diameter) was  
127 positioned near the end of each choice arm. The choice arm and stimulus box were connected by a  
128 Teflon tube (2 cm x 4 mm in diameter) for delivery of the air and volatile substances. At the other  
129 end of the stimulus box, that was removable, a Teflon tube was attached as an air flow entry. Air  
130 flow used was 480 ml/min. At the junction of the choice arms, a central wall in Plexiglas (4 cm x 4  
131 cm) was placed 3 mm from the screen moving door. This wall enabled the two stimuli air streams  
132 to be conducted to the beginning of the start box where the pups were positioned.

133 ***Test procedure.*** The 3-min test begins when a pup is placed in the start box, in front of the  
134 removable Plexiglas door. The door is then opened, enabling the pup to access the odour stimuli  
135 and make a choice. When the pup's snout has passed through a limit line, traced in each choice  
136 arm, two cm behind the moving door of the start box, the choice was recorded. It was noted "+"  
137 when a stimulus was chosen. When pups did not pass through the line, a no-response was noted  
138 and the pup was excluded from the experiment. Every 5 tests, the stimuli were changed and placed  
139 in the opposite choice arms. The olfactometer was cleaned with detergent, rinsed with distilled  
140 water, then with 95 % ethanol and dried. The performance was noted by an observer blind to the  
141 conditions of the experiments (in particular the nature of the tested compounds).

142

143 ***Olfactory stimuli***

144 Different crude materials, extracts (nipples, milk, AF, nest shavings, feces, diet), synthetic

145 compounds (alone or in a mixture), and polar solvent as control were used as olfactory stimulus.

146

147 **Crude materials** - The stimulus boxes were filled with clean litter, feces or with nest shavings of  
148 gravid, lactating or virgin female (34 ml), free of feces. For the crude amniotic fluid (AF), a filter  
149 paper was impregnated with fluid (50  $\mu$ l), put in the stimuli box and clean filter paper was used as  
150 control.

151

152 **Extracts** – To determine the composition of the maternal chemosensory cues, we used an aprotic  
153 polar solvent (dichloromethane) because most of the substances of biofluids are hydrophilic, as in  
154 the AF and milk. Moreover, the polar solvent is able to extract polar and apolar molecules as fatty  
155 acids.

156 **Organ and tissue extracts (nipples, milk, skin, and amniotic fluid).**

157 Three pregnant (18 days of gestation) and three lactating females (P4) of normal and anethole  
158 groups were anaesthetized with ketamine (5%, Virbac, France) and medetomidine (1 mg/ml,  
159 Janssen), injected subcutaneously (0.33 ml/kg). 500  $\mu$ l milk were collected from the lactating  
160 females, pooled in a cooled vial and extracted in 1 ml solvents. The nipples of gravid and lactating  
161 rats with the addition of surrounding skin (approximately 2 mm<sup>2</sup>) and the skin from the mother's  
162 sides (approximately 4 mm<sup>2</sup>) were removed and soaked in 3 ml of dichloromethane during 1 hr,  
163 then the skin and nipples were removed from the solvents. Amniotic fluid was collected from  
164 amniotic cavities and transferred to a vial at 4°C in a pooled sample and extracted in polar solvent  
165 in the ratio of 500  $\mu$ l of AF for 1 ml of solvent during 1 hr. Then, the milk and AF extracts were  
166 vortexed for 1 min, centrifuged (10,000 tr/min) for 5 min and only the solvent parts were removed.  
167 All extracts were evaporated to 0.5 ml with nitrogen stream, stored in the refrigerator at -20° C  
168 until used for behavioural tests or for GC-MS analysis.

169 **Ventral wash extracts.**



170 Each nipple and ventral surrounding tissue of three anaesthetized lactating females were washed 3  
171 times with cotton swabs impregnated with polar solvent. Each cotton swab was transferred to a vial  
172 containing 3 ml of solvent and soaked for 1 hr. Then the cotton swab was removed from the solvent  
173 and the extracts were evaporated to a final 0.5 ml with nitrogen stream and stored in a freezer at -  
174 20°C prior to use.

175 **Diet and feces extracts.** 200 mg of broken chow or of feces were infused in 500 µl polar solvent for  
176 30 min, centrifuged (10,000 tr/min) for 5 nm and only the solvent parts were removed. These  
177 extracts were stored in the refrigerator at -20° C until used.

178

179 **Extracts and trappings of volatile substances of nests.** To obtain the attractive **substances** from  
180 nests, five nest shavings of lactating females (P7-8), free of feces, were used. The nest materials  
181 were placed in an Erlan Meyer (1 litre), heated to 60°C in a bain-marie. An air stream (250 ml/min)  
182 passed through the nest shavings and trapped the volatile odours in a clean glass tube in U form  
183 (3mm diameter, 16 cm long), cooled with ice + salt as refrigerant. After a 30 min trapping period,  
184 the glass U tubes were rinsed twice with 1 ml of polar solvent. The extracts were pooled and  
185 concentrated to 1 ml with nitrogen stream, stored in the refrigerator at -20° C until used for  
186 behavioural tests or GC-MS analysis.

187

188 **Chemicals** - All chemicals, including the 17 synthetic compounds as candidates for **active**  
189 constituents, were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). They were >98%  
190 pure as determined by GC-MS analysis and were used without further purification. Each compound  
191 was diluted in polar solvent to obtain an adequate quantity for behavioural tests or for chemical  
192 identification with GC-MS. After pilot studies, we used 3 different doses: 500, 800 and 1,000 ng in  
193 1µl dichloromethane unloaded on a filter paper for the Y maze test.

194

195 **Chemical analysis - Gas chromatography-mass spectrometry (GC-MS).**

196 The biological extracts and authentic compounds were analysed by gas chromatography–mass  
197 spectrometry (GC-MS), performed on a Thermo Finnigan PolarisQ Ion trap mass spectrometer with  
198 a Trace GC equipped with a split–splitless injector. An analytical capillary column Thermo Trace TR-  
199 5-MS was used (30m x 0.25 mm i.d., film thickness: 0.25 µm) with helium at 1 ml/mn as carrier gas.  
200 Analyses were performed with a 1 µL splitless injection at 250°C, with an injection time of 1 min.  
201 The temperature program was as follows: 1 min at 50 °C with 5 °C min<sup>-1</sup> to 235 °C, held for 20 min  
202 and increased at a rate of 15 °C min<sup>-1</sup> to 310 °C and finally held for 5 min. The transfer line and the  
203 manifold temperatures were at 280 and 200 °C, respectively. MS acquisition ranged from 50-650 in  
204 full scan mode. Compound identification was based on comparison of retention time and mass  
205 spectra of authentic standards as well as mass spectra comparison with the NIST (National Institute  
206 of Standard and Technology) mass spectral reference library.

207

208 **Anethole exposure**

209 To expose the foetuses and newborns to odorant during the gestation period and after parturition,  
210 we used anethole, a synthetic anisole compound. The novel flavour was incorporated in beverage  
211 water (0.5 µg anethole/1 ml water) and in chow food (1µg / 1gr chow) for three gravid and  
212 lactating females (anethole group). In addition, three gravid and lactating females were given food  
213 without anethole as control group. The nest shavings of lactating females (P7-8), free of feces,  
214 were used either for chemical extraction of active substances using trapping technique or for  
215 attraction stimulus in Y maze test.

216

217 **Statistical analysis.**

218 Differences in behavioural responses to stimuli were tested for statistical significance using  
219 Graphpad Prism6 software. The amount of time spent in direct contact nest was analysed by

220 separate paired t-test (t-tests) in each group across individuals in a litter as statistical unit of  
221 analysis. All analyses were performed on raw performance data, not percentages. Fisher's test was  
222 used for analysis of performances in the direct contact nest and Y maze tests.  $P < 0.05$  was  
223 considered to indicate a significant difference.

224

225

## 226 **Results**

227 To identify the chemical composition of the MSO in rat, we developed consecutive stages to  
228 evaluate the attraction level between potential biological sources of active substances, to  
229 characterize the chemical compounds of these biological extracts, then to test the attraction power  
230 of identified molecules, and finally to investigate whether compounds in the diet can attract the  
231 pups (Fig.1).

232

### 233 **Attraction level between potential biological sources of active compounds**

234 Two types of two choice behavioural tests were performed: the direct nest contact test and the Y  
235 maze test (Fig. 2A, B). In the direct nest contact test, postnatal day 5 (P5) pups were placed in the  
236 midline space between 2 types of nest shavings. The amount of time spent on the lactating female  
237 nest was higher than on clean or virgin female nests (Fig. 2C) (separate paired t-tests,  $P < 0.05$ ).  
238 Significant preference for lactating female nest shavings was found in comparison to clean wood  
239 nests or virgin female nests, and no attraction difference was found between clean and virgin  
240 female nests (Fisher's,  $P < 0.05$ )(Fig. 2D). These data indicate that the pups are attracted to the nest  
241 shavings of lactating females which are familiar to them and spend more time there. In addition, to  
242 refine this result, we performed Y maze tests wherein only the volatile substances emanating from  
243 substrates were submitted to the pups (P12-P14). The volatile substances of lactating female nests  
244 were more attractive for pups when they were opposed to clean, virgin female or male nest

245 shavings (Fisher's,  $P < 0.05$ ) (Fig. 2E). The percentages of attraction responses for virgin nests in  
246 comparison with clean nest did not differ. We also tested the potential attraction of feces. No  
247 significant attraction of feces of lactating female was detected in comparison with that of male and  
248 of virgin female (Fisher's test, NS) (Fig. 2F). Only the lactating female nests contained attractive  
249 volatile substances that can be extracted in solvents for chemical analysis.

250

## 251 **Characterization of the chemical compounds of biological extracts from gravid and lactating** 252 **mothers**

253 Volatile compounds of nest shavings of the lactating mother were extracted with polar  
254 (dichloromethane) and apolar (pentane) solvents. Newborns responded positively to mother's nest  
255 trappings extracted in polar and apolar solvents when paired with control solvents. As polar and  
256 apolar extracts elicit similar responses (Fig. 3A, Fisher's test,  $P < 0.05$ ), only the polar results were  
257 presented. To define the biological sources of these volatile attractive compounds, we investigated  
258 the effect of different tissue extracts from lactating female and from the AF of gravid female on  
259 P12-P14 pups. The volatile compounds of the nipple, ventral wash and milk from lactating females  
260 attract pups in the Y maze test in comparison with solvent as control (Fisher's,  $P < 0.05$ ) (Fig. 3B).  
261 Pups also performed positive responses for nipples and crude AF of gravid rat early on day E18 of  
262 gestation, and for extracts of AF, when they were paired with solvent (Fisher's,  $P < 0.05$ ) (Fig. 3C). In  
263 our experiments, mother's ventral area, nipples, milk and pregnant AF were the sources of the  
264 MSO.

265

266 Then, gas chromatography-mass spectrometry (GC-MS) was used to identify specific or common  
267 molecules from ventral area, nipples, milk and AF extracts, together with extracts of nest and feces  
268 of lactating females, and rodent diet (Fig. 1 and 4). We identified 37 compounds from these

269 different extracts (Table 1). However, some compounds could not be tested or identified because  
270 of uninterpretable mass spectra caused by the lack of synthetic substances or by concentration or  
271 co-elution problems. Among the recognized molecules, 12 compounds were present in the AF and  
272 milk, 2 in the AF but not in milk, and 8 in the milk but not in AF. Four compounds were common to  
273 the diet and milk and only one to diet and AF (Table 1). Seventeen synthetic substances that could  
274 be potential MSO candidates were selected among the recognized compounds (Table 2). These  
275 compounds were used for identification of biological substances by comparison of their mass  
276 spectra. We showed that the spectrum of biological compounds found in tissue extracts was  
277 identical to that of synthetic compounds used for the Y maze (Fig. 5). Other molecules not tested in  
278 behavioural bioassays might be shown in the future to have a role in maternal signature odours.

279

280

#### 281 **Effect of characterized compounds in behavioural tests.**

282 Only the behavioural tests in the Y maze allowed assessment of the biological properties of volatile  
283 active compounds. Among the seventeen molecules, six compounds, compared to solvent,  
284 attracted pups in the Y maze test and are considered as MSO constituents: diacetone alcohol,  
285 ethylbenzene, benzaldehyde, benzyl alcohol, methyl salicylate and vanillin (Table 1, Fig. 6A)  
286 (Fisher's,  $P < 0.05$ ). Gravid AF, nipples, and mother's nipples and milk contained 3 of these identified  
287 active substances in variable proportions: ethylbenzene (2-10%), benzaldehyde (3-10%) and benzyl  
288 alcohol (85-95%), presented with their mass spectrum (Fig.3b). These 3 active compounds could be  
289 produced by gravid and mother rat itself, whereas diacetone alcohol and vanillin seemed to be  
290 acquired from the diet. These two compounds, diacetone alcohol and vanillin, are undetectable in  
291 AF by our systems of analysis, but may be present as well, although in trace quantities. Methyl  
292 salicylate was not detected in AF, and was not used in the blend (see below). These 6 attractive  
293 compounds are found associated with mother's nipple, ventral wash and nest trapping extracts.

294 Adult rat urinary volatile compound, 2-heptanone (Osada et al., 2009), was detected in nest  
295 trappings, but had no effect on pup attraction. Together, these findings suggest that the rat MAS  
296 was composed of ethylbenzene, benzaldehyde and benzyl alcohol, at least, present in AF, nipples  
297 and milk. Moreover, to attempt to define the natural MAS as a whole, we performed a synthetic  
298 MAS blend with the 3 common active compounds found in the AF, nipples and milk. The blend of  
299 these substances was prepared in similar proportions to those found in AF extracts, i.e.  
300 ethylbenzene, benzaldehyde, benzyl alcohol, in a ratio, respectively, of 1:1:12 (700 ng), according  
301 to chemical analysis. In behavioural tests, pups showed significant preference for the MAS blend in  
302 comparison with the control solvent (Fisher's,  $P < 0.05$ ) (Fig. 6B).

303

#### 304 **Attractiveness of an odorant incorporated in pregnant and lactating mother's diet**

305 To attempt to determine the potential role of an odorant intake in MSO, we followed the route of  
306 an odorant, anethole, a synthetic anisole compound incorporated in pregnant and lactating  
307 mother's diet. Using GC-MS analysis, anethole was detected in AF, nipples, milk, and feces extracts  
308 of pregnant female and mother that consumed anethole. In Y maze tests, pups born from the  
309 mothers who consumed anethole were attracted by this aromatic compound (Fisher's,  $P < 0.05$ )  
310 (Fig. 6C). In contrast, no attraction was detected for pups born from the mothers who did not  
311 consume anethole (Fig. 1 and 6C). This experiment shows that previous experience of an odour is  
312 sufficient to elicit pup attraction.

313

314

#### 315 **Discussion**

316

317 The first aim of this study was to examine the maternal attractive substances from rat lactating  
318 females that attract pups for suckling and try to provide new insights concerning the controversial  
319 origin of these attractive substances. The behavioural tests demonstrated that nest, nipples, milk,

320 ventral area of lactating females, amniotic fluid and nipples of pregnant females are effective for  
321 attracting pups, but not feces. These data confirmed previous behavioural studies showing that AF,  
322 mother's ventral area, nipples and milk played a major role in nipple orientation, attachment and  
323 suckling process in newborn mammals (Hofer et al., 1976; Teicher and Blass, 1976, 1977; Singh and  
324 Hofer, 1978; Blass and Teicher, 1980; Rosenblatt, 1983; Varendi et al., 1994; Porter and Winberg,  
325 1999; Beauchamp and Mennella, 2009; Schaal, 2010; Schaal and Al Aïn, 2014). AF is also an  
326 olfactory attraction source for newborns of several mammal species, including man (Schaal et al.,  
327 1995; Varendi et al., 1996; Porter and Winberg, 1999; Logan et al. 2012).

328 The second aim was to identify, for the first time, the chemical composition of volatile MSO. MSO  
329 is a multi-component mixture, composed of 3 to 6 compounds depending on sources: 3 substances  
330 from amniotic fluid and nipples of pregnant females, 4 from ventral skin, 5 from milk and 6 from  
331 nipples, ventral wash and nest trapping of lactating females. These six substances have never been  
332 previously identified in rats as MSO components. The multi-component mixture of pheromone has  
333 already been underscored in many animal species (for review, Wyatt, 2014). In AF, benzyl alcohol is  
334 the major compound with two minor substances, ethylbenzene and benzaldehyde. The level of  
335 positive behavioural performance reaction of the pups to the blend of these three compounds was  
336 very close to that for natural amniotic fluid. We did not find a synergy of the blend in the  
337 attractiveness performance as found for the nematode sex pheromone of *Caenorhabditis elegans*  
338 (Pungaliya et al., 2009; Forseth and Schroeder, 2011; Lim and Sorensen, 2012; Wyatt, 2014). The  
339 positive behavioural performance level of the blend was similar to that for benzyl alcohol (500-  
340 800ng). However, this mixture could be suggested as a blend identity of rat species. These three  
341 individual compounds have been found as pheromone constituents in many animal species,  
342 especially insects (Pherobase) ([www.pherobase.com](http://www.pherobase.com)). Moreover, benzaldehyde and benzyl alcohol  
343 were identified in relatively abundant amounts in human volatile metabolites of blood plasma  
344 collected from mother-infant pairs (Stafford et al., 1976) and in secretions of African wild dogs

345 (Wells and Hepper, 2006; Apps et al., 2012), but the behavioural role of these compounds remains  
346 unknown. Likewise, benzaldehyde is contained in rabbit milk (Schaal et al., 2003) and in human  
347 milk (Hausner et al., 2009), without having a role in attraction of neonate. This compound has  
348 been identified as a pheromone component of more than 1 hundred insect species (Pherobase).  
349 Ethylbenzene has been identified in bovine urine (Kumar et al., 2000) and in *Coffea arabica* and  
350 used as a component of synthetic blend for coffee berry borer *Hypothenemus hampei* (Mendesil et  
351 al., 2009). Furthermore, the 2-heptanone, dimethyl sulfone and benzaldehyde have been identified  
352 as urinary volatile compounds from mouse and rat (Zhang et al., 2007, 2008) and phenol, 4-  
353 ethylphenol or indole (Osada et al., 2009) were also detected in our nest trapping, ventral wash or  
354 nipple extracts. However, these molecules were not attractive for rat pups. Dimethyl disulphide  
355 (DMDS), isolated from estrous hamster vaginal secretion, is found as an attractant for male  
356 hamsters (Singer et al. 1975). In rat, DMDS was shown to be effective in eliciting nipple grasping in  
357 3-5 day-old pups, but with low releasing potency (about 50%) relative to that of olfactory intact  
358 nipples (Pedersen and Blass, 1981; Schaal and Al Aïn, 2014). DMDS is undetectable in rat bioactive  
359 extracts by our chemical system of analysis. While another sulfur compound, dimethyl sulfone, is  
360 identified in all bioactive extracts (table1) but is not attractive for newborn rats. Diacetone alcohol  
361 was identified in diet and detected in nipples, milk, nest trapping or ventral lavage. This  
362 hydroxyketone compound has been identified from anal gland of *Tapinoma simrothi* pheonicium  
363 ant as alarm pheromone (Hefetz and Lloyd, 1983) and from temporal gland secretions of Alpine  
364 marmot *Marmota marmota* as a component of scent-marking substance (Bel et al., 1999). Vanillin,  
365 identified in nipples, ventral wash, nest trapping or faeces, and originating from diet, is utilised by  
366 many insect species in their communication system, and was also detected in human milk  
367 (Buettner, 2007). Methyl salicylate, present at trace level in some rat extracts, is utilised by more  
368 than 80 insect species in their chemical communication systems (Pherobase). In addition, 2MB2, a  
369 volatile pheromone extracted from milk doe which facilitates suckling in young rabbit (Schaal et al.,



370 2003), was not detected in rat MSO. On the same principle as for the first suckling episode in mice  
371 (Logan et al.2012), our data support the existence of 'maternal signature odours' rather than a  
372 classic pheromone that drives a hardwired response to specific or predetermined chemosensory  
373 cues. Our experiment with an exogenous molecule (anethole) incorporated in food is sufficient to  
374 enable pup behaviour (but not in no-anethole pups), which further reinforces this view.

375

376 The highlighting of a rat efficient volatile MSO, present in pregnant female's AF, and then in  
377 mother's nipples and milk, supports the hypothesis that the learning and memory mechanism  
378 processes would be activated *in utero*, as already suggested by Rosenblatt (1983), Porter and  
379 Winberg (1999), Schaal (2010) and Hepper et al., (2013). The relationship of biological rat MAS  
380 with intake of diverse diet odorants during the gestation period establishes the specific chemical  
381 signature of individual mother cues or 'maternal signature odours' (Logan et al.2012). This  
382 association opens new avenues for further investigating the neural mechanism underlying prenatal  
383 chemosensory learning and memory storage in the newborn brain receptors and behavioural  
384 specificity. Unravelling the chemical signals that cause innate attraction and searching for suckling,  
385 nipple attachment, foetal learning and memory in a mammal species is a necessary step in  
386 understanding the neuronal organisation pathways in signal reception and neuro-modulation in  
387 the formation of the new odorant receptors. Such a system may confer survival advantages by  
388 promoting the acquisition of information about safe foods. How does the plasticity process of the  
389 olfactory system operate in modifying its properties for specific environmental adaptation?  
390 Further dissection of the circuits engaged by this MSO should facilitate our understanding of the  
391 neurobiology of this evolutionarily conserved, innate social behaviour.

392

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398

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522 and 3 male pheromones in the rat. *Chem Senses*. 33:611–621.

523  
524

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529

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534

## 535 **Figure Captions**

536

537 **Figure 1 / General schema of 'maternal signature odours' (MSO) identification.** Synopsis of  
538 behavioural tests (BT) and chemical techniques (GC-MS) leading to final chemical 'maternal  
539 signature odours' identification. **A**, nest and feces stimuli for pup attraction; only nests of lactating  
540 female attracted pups. **B**, Behavioural tests and chemical analysis of substrate extracts from gravid,  
541 lactating female, and food. Six compounds and blend of three synthetic compounds found in the  
542 amniotic fluid, milk and nipples attracted pups as putative maternal attractant substances (MAS).  
543 **C**, Anethole, the synthetic aniseed flavour, given in the diet of gravid and lactating rats, affected the  
544 chemical composition of amniotic fluid, milk, mother and gravid nipples. Only pups from 'anethole  
545 gravid rat' showed a chemosensory preference for anethole. In contrast, pups from 'no-anethole'  
546 gravid rat were not attracted by anethole.

547

548 **Figure 2 / Behavioural effects on rat pups of different stimuli from nests and feces.** **A**, schema of  
549 direct contact nest. Neonatal pups (P5) had to head for one of two nests and stay at least 5 sec out  
550 of 60 sec. **B**, schema of the Y maze apparatus (olfactometer). Pups had to choose between two sets  
551 of volatile stimuli issued from nests, feces, biofluids, rat tissue extracts or synthetic compounds,  
552 dispersed by an air flow in the choice arms. The choice was made when pups (P12-14), initially  
553 located in the start box, passed through the limit line of a choice arm over 3 min. **C**, in nest choice  
554 test, pups spend significantly more time in lactating mother nests than in clean nest or virgin  
555 female nest. Mean  $\pm$  S.E.M. **D**, pups preferentially chose lactating nests when paired with clean  
556 nests or virgin female nest. Only lactating female nest attracted pups. Positive responses are  
557 expressed in percentage of total responses. **E**, In Y maze tests, no significant difference was found

558 in pup attraction for crude feces of lactating female, of virgin female or male. Data, i.e. positive  
559 responses, at a stimulus (target odour or solvent), are expressed in percentage of total responses.  
560 **F**, behavioural preference for olfactory stimuli from nests of lactating, virgin females and male.  
561 Pups were preferentially attracted by olfactory stimuli of lactating female. Numbers indicate the  
562 numbers of pups tested. (\*,  $P < 0.05$ ; Fisher's test).

563

564 **Figure 3 / Behavioural effects on rat pups of different stimuli from biofluids, and tissue extracts**  
565 **of rats.**

566 **A**, in the Y maze test, behavioural preference for olfactory stimuli from nest trappings of lactating  
567 females in polar (dichloromethane) and apolar (pentane) solvents. Pups significantly responded  
568 positively and similarly to volatile substances from the nest trappings in comparison to polar and  
569 apolar solvents. **B**, olfactory stimuli from polar extracts (milk, nipple, ventral wash residues) of  
570 lactating female were paired with polar solvent during the Y maze test. Positive responses of pups  
571 were significantly higher for extracts than for solvent. **C**, olfactory stimuli from crude amniotic fluid  
572 (AF) and polar extracts (AF, nipples) of gravid female were paired with polar solvent. Pups were  
573 preferentially attracted to AF and tissue extracts in comparison with solvent. Numbers indicate the  
574 numbers of pups tested. (\*,  $P < 0.05$ ; Fisher's test).

575

576 **Figure 4 / Representative gas chromatography–mass spectrometry profiles of extracts.**

577 Typical chromatograph of extracts of nest trappings (A), of extracts of AF (gravid female) (B),  
578 nipples (C) and milk (D) of lactating female. The numbered GC peaks corresponded to the  
579 compounds in Table 1, listed in order of retention time. Behavioural active compounds were  
580 named on GC peaks. Peaks labelled "x" corresponded to the contaminated compounds such as  
581 complex siloxane or phthalate compounds. RT 17.70-Anethole indicated the retention time of  
582 synthetic anethole added to the diet of gravid female. Three identified compounds are common to

583 extracts: Ethylbenzene, benzaldehyde and benzyl alcohol. Methyl salicylate was found in lactating  
584 female and nipples. Vanillin and diacetone alcohol, found in milk and nipples of lactating female,  
585 were derived from food. Anethole was found in all spectra when the female diet contained this  
586 substance.

587

588 **Figure 5 / Representative spectra of biological and synthetic compounds** common to different  
589 extracts and of anethole. m/z: mass/charge number of ions.

590

591 **Figure 6 / Behavioural effects of the identified compounds on pup attraction.** (A) The 17  
592 compounds (800 ng), present in AF, milk or nipples, were presented to pups in Y maze. They were  
593 paired with polar solvent. Pups significantly responded positively to 6 compounds (underlined):  
594 Vanillin and diacetone alcohol issued from food; 3 compounds, ethylbenzene, benzaldehyde,  
595 benzyl alcohol were found in the AF, milk and nipples; and methyl salicylate was present in milk  
596 and nipples but not in AF. (B) In Y maze, pups were attracted by a blend of the 3 synthetic  
597 compounds found in the AF (ethylbenzene, benzaldehyde, benzyl alcohol, in a ratio respectively of  
598 1:1:12 for 700 ng in all). We suggest that this combination corresponds to natural MAS. (C),  
599 Attraction of pups to anethole that was introduced or not in the diet of rat female during pregnant  
600 and lactating period. Two experimental groups of pups were used: pups related to gravid females  
601 receiving anethole (A) and pups related to gravid females not receiving anethole (N). Only the "A"  
602 group pups were significantly attracted by anethole. This attraction suggests that food compounds,  
603 present in AF, contribute to the MSO and are involved in the prenatal learning and memory  
604 process.

605 Numbers indicate the numbers of pups tested. (\*,  $P < 0.05$ ; Fisher's test)

606

607



608 **Table 1: Compound identification by GC-MS from different extracts of gravid, lactating females**  
609 **and diet.**

610 The pick number indicated the identified compounds on GC profiles of different extracts in order of  
611 retention time (RT). MW: molecular weight. "x" corresponds to compounds identified in different  
612 extracts. **Bold** and *italic*: attractive compounds present in AF or milk or nipples or wash or nest but  
613 not in diet. Regular and *italic*: attractive molecules found in diet. \*: compounds tested in  
614 behavioural tests. RT 17.70-Anethole indicates the retention time of synthetic anethole added to  
615 the diet of gravid female.

616

617 **Table 2: Statistical results for behavioural performance of compounds (500 and 1,000 ng) present**  
618 **in potential 'maternal signature odours'.**

619 **Bold** and *italic*: attractive molecules found in AF or milk nipples or ventral wash but not in diet.

620 These substances potentially consisted of 'maternal signature odours'. Regular and *Italic*: attractive  
621 molecules found in diet. n: number of pups performing in Y maze test. Fisher's test,  $P < 0.05$  is  
622 significant.