



Novel sampling strategy for alive animal volatolome extraction combined with GC-MS based untargeted metabolomics: Identifying mouse pup pheromones

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ABSTRACT

In this study, we identify 11 mouse pup volatiles putatively involved in maternal care induction in adult females. For this purpose, we have adapted the dynamic headspace methodology to extract the volatolome of whole alive animals. Untargeted metabolomic methodology was used to compare the volatolome of neonatal (4–6 days) with elder pups until the age of weaning (21–23 days old). Pup volatolome was analyzed by gas chromatography (GC) coupled to single quadrupole mass spectrometry (MS) using automated thermal desorption for sample introduction. After data processing and multivariate statistical analysis, comparison with NIST spectral library allowed identifying compounds secreted preferentially by neonatal pups: di(propylene glycol) methyl ether, 4-nonenal, di(ethylene glycol) monobutyl ether, 2-phenoxyethanol, isomethyl ionone, tridecanal, 1,3-diethylbenzene, 1,2,4,5-tetramethylbenzene, 2-ethyl-p-xylene and tri(propylene glycol) methyl ether. Palmitic acid was enriched in the volatolome of fourth week youngsters compared to neonatal pups. The results demonstrated the great potential of the new sampling procedure combined with GC-MS based untargeted volatolomics to identify volatile pheromones in mammals.

1. Introduction

Animals use chemical senses for inter-individual communication. In many species this includes the excretion or secretion of pheromones, chemicals delivered by an individual that elicit a stereotyped response (either behavioral or neuroendocrine-developmental) in conspecifics [1]. Rodents, which are commonly used in experimental biomedicine, are macrosmatic mammals, e.g. they display two highly developed nasal chemosensory systems [2] for monitoring the presence of relevant chemicals in their environment, the vomeronasal organ (VNO) and the olfactory epithelium (OE).

In the last thirty years, an intense investigation has partially clarified the chemical nature of mouse pheromones mediating a wide variety of social interactions [3]: intersexual attraction, inter-male or maternal aggression; and other behavioural interactions between adult individuals, male avoidance of female youngsters as sexual partners, male

effects on female sexual maturation, avoidance of ill conspecifics [4] and pup killing by sexually-naïve males [5]. This has uncovered many molecules putatively involved in social communication in mice, which include volatiles such as farnesenes, thiazolines, heptanones, steroids or small formyl peptides, as well as molecules with lower volatility. Surprisingly, there have not been attempts to identify molecules involved in maternal care, a crucial social behavior that ensures offspring survival and promotes proper neurodevelopment of pups thus facilitating mental and bodily health [6,7]. There is solid evidence indicating a major role of olfaction in pup care: anosmic mothers either eat or abandon their pups [8,9]. Together, these findings suggest a key role of chemical stimuli (mainly volatile odorants) in maternal care, although there is a need of identifying the molecules involved. This requires analyzing the volatiles emitted by the whole body of pups.

The analysis of the emitted volatiles can be done by means of different analytical approaches. Among them, metabolomics, the

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“-omics” technique focused on the small molecules or metabolites of a biological sample that changes in response to an internal or external alteration (disease, environmental or simply growth) is a good alternative. Although targeted metabolomics has been more commonly used [10], the potential of facing the study through an unbiased screening global methodology (untargeted approach) seems a good alternative [11,12]. To unravel the biomarkers involved in such changes, statistical tools such as multivariate statistical analysis are employed [13]. Finally, when the study is focused on the volatile or semi-volatile emitted part of the metabolome, the term volatolomics is generally used [14,15].

Gas chromatography coupled to mass spectrometry (GC-MS) is the gold standard for the analysis of the volatile organic compounds (VOCs) as chemical messengers or volatile pheromones [12,14,16]. Compound separation by GC followed by MS detection, traditionally using electron ionization (EI) source, is a sensitive and reproducible combination with a great capability in tentative identification based on the mass spectra search in commercial libraries [12,14,17].

One of the critical aspects when conducting a study of the VOCs that constitute the volatolome is the sampling step. The VOCs are emitted to/from various body fluids and tissues. Their final excretion through physiological fluids and glandular secretion constitutes a matrix of olfactory signals directly related to communication and interaction between animals [14,15,18]. For their extraction from the different biological material, the use of the proper enrichment extraction techniques such as dynamic headspace with sorbent trapping (DHS-P&T) is required. This allows extracting the volatile components from the matrix by dynamic purge of the headspace with, usually, an inert gas and then trapping them in a sorbent. This technique is characterized by its high volatile recovery and pre-concentration factor entailing an enhancement of the sensitivity, along with a good efficiency, low sample manipulation and solventless approach [16,19,20]. Additionally, thanks to the use of a thermal desorption unit (TDU) mounted on a programmed temperature vaporizer injection system the compounds can be directly and efficiently transferred into the GC, further reducing the sample handling and solvent use [14,21].

In our case, however, we are interested in obtaining samples of pup's whole body volatolome and, therefore, animals must be alive and comfortable throughout extraction and the methodology requires repeating sampling in the same animals at different periods of time. This work explores an adaptation of the traditional methodology by using air instead of an inert gas to remove and preserve the intact volatolome of non-stressed, healthy animals. To the best of our knowledge, this approach has never been used before. To do so, the volatolome of 4-6-day old pups, which elicit intense, dedicated care in postpartum females [22], and the one corresponding to 21-24 day old pups, the approximate age of weaning, when pups are largely rejected by the dam, are compared to identify volatolome changes during pup development.

Therefore, the aim of the work was to apply this novel sampling technique combined with untargeted volatolomics approach based on TDU-GC-MS, to identify putative pup pheromones inducing maternal care in mice.

2. Materials and methods

2.1. Chemicals and reagents

Internal standards (IS) 4,4'-difluorobiphenyl, 4-methyl-2-pentanol and methyl octanoate were purchased from Sigma Aldrich (Germany). An internal standard working solution was prepared with the previously mentioned compounds at 19 $\mu\text{g mL}^{-1}$, 100 $\mu\text{g mL}^{-1}$ and 25 $\mu\text{g mL}^{-1}$ respectively in hexane. For identity confirmation, the following compounds were purchased: di(propylene glycol) methyl ether, tri(propylene glycol) methyl ether, di(ethylene glycol) monobutyl ether, 2-phenoxyethanol, 1,2,4,5-tetramethylbenzene, 1,3-diethylbenzene, isomethyl ionone and palmitic acid from Sigma Aldrich (St Quentin Fallavier, France); tridecanol from Alfa Aesar (Karlsruhe, Germany); 2-

ethyl-p-xylene from abcr GmbH (Karlsruhe, Germany) and 4-nonenal from Ambinter (Orléans, France). Alkane standard solution C8-C20 (Sigma-Aldrich, Germany) was used for Kovats Index determination. The organic solvent hexane (trace Analysis quality (AT) GC) was provided by Scharlau (Barcelona, Spain). Tenax® TA glass TD tube, fritted, O.D. 6 mm \times 4 mm (i.d.) \times L 60 mm, preconditioned, 60-80 mesh, used as traps were purchased from Gerstel (Mülheim an der Ruhr, Germany).

2.2. Animals

In this experiment, a total of $n = 4$ female mice of the CD1 strain (Janvier Labs, France) of 10 weeks of age were used. Animals were treated throughout according to the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1). Accordingly, procedures were approved by the Committee of Ethics on Animal Experimentation of the Jaume I University of Castellón where the experiments were performed and, ultimately, by the Valencian *Conselleria d'Agricultura Medi Ambient, Canvi Climàtic i Desenvolupament Rural* (code 2019/VSC/PEA/0049). After mating with an adult male, females were weighted daily to check that they were pregnant and, by day 14-15 of pregnancy, were provided with enough nest material (shredded filter paper) and housed individually in polypropylene cages (145 mm wide, 465 mm long and 215 mm high; Panlab) under controlled temperature (24 ± 2 °C) and lighting conditions (12 h:12 h; lights ON at 8 a.m.), with ad libitum access to food and water. All the precautions were taken to avoid contamination with volatiles coming from the personnel involved in the handling of animals during the experiment (no use of perfumes and/or cosmetics).

2.3. Experimental design for studying volatolome through pup development

Mating was planned so that two females delivered 17 days after the other two. This allowed processing at the same time newborn pups and pups by the age of weaning. When the younger pups were 4 days old and the elder ones were 21-day old, extraction process was started. Volatiles were extracted from animals of the two ages in parallel (see Table 1) in two non-consecutive days, thus obtaining volatiles of pups of 4-6- (first week pups) and 21-23-days old (fourth week pups). One week afterwards, volatiles were extracted again from the younger pups for two additional days, thus getting data from 10- and 12-day old pups (second week pups).

2.4. Purge and trap extraction procedure from whole alive animals

Volatiles were extracted from groups of sibling pups of both sexes. Pups of the same group were gently removed from the nest, weighed and introduced together in a conic flask with a cotton litter (Fig. 1). The number of pups of each group and the volume of the conic flask depended on the postnatal day evaluated: during the 1st week of life, 8 pups in a 150 mL flask (total mass ~ 26 g), in the 2nd week 6 pups (total mass ~ 33 g) and a 250 mL flask and finally for the 4th week 3 pups (total mass ~ 52 g) in a 250 mL flask. The flask was closed with a glass tap with a connection tube for the air entrance, and another as exit that was connected to the sorbent Tenax® TA TDU trap cartridge tube (Fig. 1). The sorbent trap was previously spiked with 10 μL of the internal standards working solution for future extraction deviation correction. For 1st and 2nd week pups, whose thermoregulation capability is much reduced, flasks were put on a heated sand bath at 35-40 °C during volatile extraction to ensure pup comfort and avoid stress that could modify the volatolome. The DHS-P&T extraction was carried out for 90 min with an air flow of 500 mL min^{-1} induced by a vacuum pump device. Immediately after extraction, pups were returned to the nest with their mother, and a second extraction of the same flasks with the soiled-cotton litter was performed with the same conditions except that the temperature was set at 60 °C. Table 1 presents the experimental design used in the different extractions.

Table 1
Experimental conditions of the mice pup volatolome purge and trap extraction.

Experiment	N° of replicates	N° of mice pups	Postnatal week	Postnatal day	Conic flask volume (mL)	Extraction Temperature	
1	4	8	1	4	100	35 - 40 °C	
1_nest	4	Pup soiled-cotton litter after experiment 1					60 °C
2	4	8	1	6	100	35 - 40 °C	
2_nest	4	Pup soiled-cotton litter after experiment 2					60 °C
3	4	6	2	10	250	35 - 40 °C	
3_nest	4	Pup soiled-cotton litter after experiment 3					60 °C
4	4	6	2	12	250	35 - 40 °C	
4_nest	4	Pup soiled-cotton litter after experiment 4					60 °C
5	4	3	4	21	250	Ambient	
6	4	3	4	23	250	Ambient	

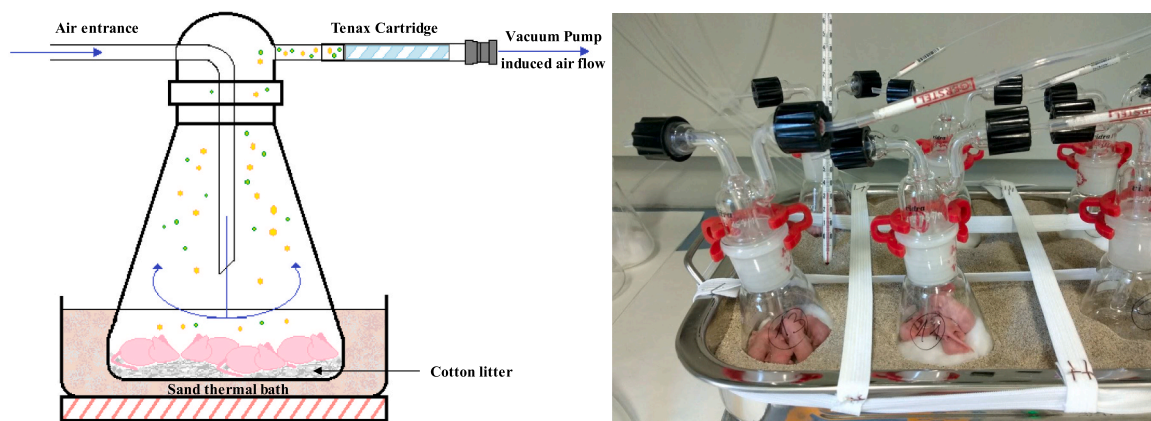


Fig. 1. Experimental design used for alive mice pup volatolome extraction by purge and trap.

Each day of analysis, a blank with cotton litter (cotton litter blank) and an empty flask blank (air blank) were extracted with the same procedure for each extraction batch. After the extraction process the sorbent trap tubes were desorbed in a thermal desorption unit and the analysis was carried out in a GC-EI-MS analytical platform.

2.5. GC-EI-MS analysis

The gas chromatograph Agilent 6890 Plus Series coupled to a quadrupole mass spectrometer, Agilent 5973 N Mass Selective Detector, with an EI source was used for the mice pup volatolome analysis. The MPS 2 autosampler from Gerstel (Mülheim an der Ruhr, Germany) was employed and the injection system involved two parts; TDU and CIS 4 PTV. First, the Tenax® TA sorbent tubes were thermally desorbed with the TDU in splitless mode with a desorption program starting at 50 °C with 1 min equilibrium time, and then heated to 260 °C at 12 °C s⁻¹ and held for 8 min. The CIS 4 PTV was equipped with Tenax® TA packed liner and the temperature program started at 40 °C during 1 min, followed by a temperature ramp at 12 °C·min⁻¹ until 260 °C and held for 8 min. The transfer line temperature was set at 260 °C.

The GC separation was carried out on a 30 m × 0.25 mm DB-5MS (0.25 µm film thickness) capillary column (J&W Scientific, Folsom, CA, USA), with helium at a constant flow of 1 mL min⁻¹ as carrier gas. The oven temperature program started at 70 °C for 3 min; then increased to 300 °C at 10 °C·min⁻¹ and held for 9 min (total chromatographic run 35 min).

2.6. Data treatment

The GC-MS data were converted to *netcdf* file format using Chemstation® (Agilent) to perform the data pre-processing with PARADISE (PARAFAC2 based Deconvolution and Identification System) data

treatment software. Approximately 200 regions of interest (ROIs) along the chromatogram were manually selected taking into consideration not to leave empty spaces between the intervals and being aware of peak presence when visible in the total ion chromatogram (TIC). The software calculated a model with a maximum of 8 components with 50,000 iterations for each ROI, in order to resolve the underlying and/or overlapping compounds. The models for each ROI were optimized selecting a maximum number of compounds while reaching a background removal, maintaining the model fitting and model consistency over 95% and avoiding model overfitting. The spectra of each deconvoluted component were automatically compared with the NIST08 (National Institute of Standards and Technology) mass spectral library and each component was tentatively assigned to the best NIST match. Subsequently, a report in *.xls* format was created with the list of compounds and their peak area in each sample. These areas were normalized based on the area of the closest internal standard (IS) and the total mice pup weight of each extraction, to correct the differences due to the instrumental drift and to adjust the emitter volatolome to the body mass of the pups, respectively.

The statistical analysis was performed using EZinfo 3.0 software (Umetrics, Sweden). A pareto-scaling was applied by EZinfo previous to the evaluation of the samples differences with the multivariate analysis by principal component analysis (PCA), Partial Least Squares – Discriminant Analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA).

3. Results and discussion

3.1. Mice pup conditions during volatolome sampling

The procedure employed for volatolome extraction apparently did not interfere with pup's growth and comfort. In fact, in many instances, young pups were calmly sleeping during volatolome extraction, thus proving the lack of stress and assuring the intact volatolome.

Accordingly, pups were always taken care by the dams when returned to the nest and gained weight according to the standards (Fig. 2).

For volatolome extraction, the number of animals was adjusted according to their age (Table 1), based on the assumption that the volatolome load is proportional to the total mass of the sampled animals. Despite this, since there is a huge increase in the body weight due to exponential growth of the pups (Fig. 1), the total mass sampled was used to normalize the acquired signals.

3.2. Experimental set up for whole volatolome extraction from alive animals

The choice of the volatile extraction technique was based on the previous experience in DHS-P&T in our laboratory [21,23]. The selection of Tenax® TA as the sorbent was based on cited previous works, as good results were obtained for volatiles sampling due to its universality, high retention range and good response to thermal desorption. Nevertheless, the procedure had to be adapted from food matrices to alive mice pups. Firstly, the typical continuous extraction with a flow of an inert gas (as dry N₂) was unfeasible to preserve the mice pups' life. The carrier gas was substituted by ambient air, whose entrance was forced with a vacuum pump device and the flow was set to approximately 500 mL min⁻¹ draw-in air. Although the purging gas used was not inert, it was assumed that the possible oxidations occurred in the volatolome with the ambient air were those that naturally occur and, therefore, the markers detected would be those that the mouse dams actually perceived. Moreover, in order to rule out compounds coming from the air and/or the cotton litter, blanks were also extracted (empty conic flask and conic flask with cotton litter, respectively).

Secondly, it is usual to increase temperature to promote the release of the volatile components from the matrix. In the case of the extractions with the mice pups, increasing the temperature too much could stress pups thus possibly causing unwanted changes in the volatolome. Therefore, the sand bath was set at 40 °C in mice pups volatolome extractions of the first and second week, to reach a temperature of 37–39 °C within the flasks, which corresponds to the comfort temperature for the pups. Extractions from fourth week pups, which are able to thermoregulate, were carried out at room temperature, 24 °C during the experiments. When extracting the volatile compounds that remain in the soiled-cotton litter after the 90 min period of the first volatolome extraction, the temperature was raised to 60 °C to further facilitate release of volatile compounds. This second experiment was carried out in order to obtain complementary information about the behavior of pup pheromones. Specifically, the experiment was aimed to check if a portion of the pheromones emitted by pups is retained in the nest where

pups are living. Nevertheless, this was done only in the two first weeks of study because after the extraction, fourth week mice pups used to defecate abundantly and, therefore, cotton bedding of these pups was discarded because it can be contaminated by bacteria-derived volatiles.

In chromatography-MS-based metabolomics, it is a common procedure to monitor the quality of the results and the analytical process by means of quality controls (QCs, a homogeneous pool of all samples). Moreover, for standardizing the data acquisition process and minimize the bias, it is advisable to randomize as much as possible the samples in the sequence of analysis [12]. Unfortunately, none of these methodologies could be applied as the extractions were carried out as mice pup grew up, therefore there was no way to randomize the samples and, obviously, to make a QC. Instead, each sorbent trap was spiked with a mix of internal standards prior to the extraction process to ensure stability and correct the possible instrumental drift as well as extraction deviation.

3.3. Data processing

The data processing started, as indicated above, with the conversion of GC-MS data to a machine-independent format (.cdf) using Chemstation® (Agilent Technologies).

PARADISE [24,25] was the processing software chosen based on the previous satisfactory experience in our laboratory for the peak picking and retention time alignment in VOCs analysis [21,26].

All sample data were processed simultaneously, and using the interactive visualization of the software, the composite of the total ion chromatograms (TIC) were divided into 199 ROIs. Each interval was then individually PARAFAC2 modelled [27,28], which allows the peak deconvolution based on the intensity and the spectra of the signals. Following Khakimov et al. (2012) [29] recommendations, the validation of each model was conducted. The software calculates models for each ROI from one to eight components, and the optimal number was decided attending a good model fitting and core consistency (both over 95%), low residuals, noise removal and avoiding model overfitting. Only the well resolved peaks with a robust NIST match were selected for statistical processing and those that represents baseline and artefact peak as column bleeding were eliminated.

The optimization of the models from the 199 intervals of the GC-MS raw data resulted into approximately 173 components tentatively identified and recorded with their peak area in a final report as .xls data table.

The capabilities of PARADISE to resolve complex data system with little user interaction, with a data analysis procedure transparent, simple and time effective, allows to obtain consistent data matrix that facilitates

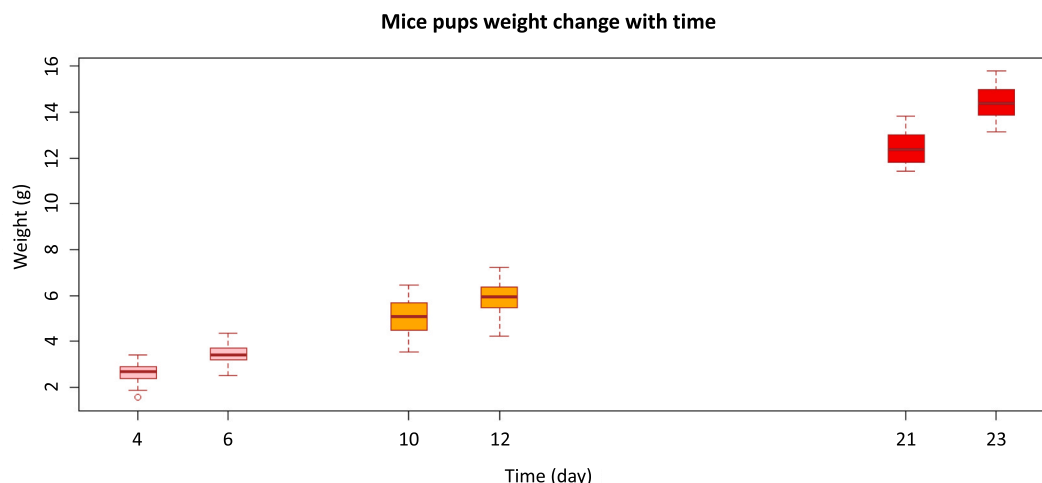


Fig. 2. Mice pups' weight change with time.

the following statistical steps. For further information, this software has been explained in detail in our previous work of Sales et al. (2019) [21].

To correct the possible instrumental deviation, raw data were processed dividing the peak area of each compound by the peak area of the nearest IS in each sample. Then, the data were also corrected by the total weight of the mice pup in each extraction. Prior to multivariate analysis, a pareto-scaling to the obtained peak data was also applied.

3.4. Multivariate statistical analysis

Firstly, the unbiased PCA was performed as exploratory analysis of the data obtained from the volatolome extracted from the pups of different ages. Fig. 3 shows the evolution of the PCA loadings plot after the data corrections mentioned before. Firstly, the main information that can be extracted from the PCA applied to the non-corrected data (Fig. 3a) is that the blanks extraction, both from the empty flask (blank_syst) or the flask containing only clean cotton litter (blank_litter), are grouped and separated from the other groups of samples, evidencing that they are significantly different to the mice pups volatolome extractions. Once this is checked, blank data were removed for subsequent statistical analysis.

Furthermore, it can be observed an intrinsic separation of the samples by post-natal day and also by week, from left to right (COMP.1) from the younger to the oldest mice pups. Since the first principal component of a set of features is the normalized linear combination of the features, this indicates a global, general increase in volatolome size with age. Seven principal components were necessary to explain the 79% of the explained variance, where first and second component only

explain the 29% and 15% of the variance, respectively. After the IS correction (Fig. 3b) the variance explained was 83% with five components, of which 42% corresponds to Comp.1 and 15% to Comp.2. The separation of the groups by day or by week is not significantly improved by the correction. This could be due to the fact that the instrumental drift is not as significant as in other longer analysis runs, and therefore this correction has not a strong impact on the data. Nevertheless, the statistical procedure was continued with the corrected data. Finally, in Fig. 3c it can be observed the effect of correction by the weight. Five components were enough to explain the 82% of the variance, 43% by Comp.1 and 15% by Comp.2. Comp.1 explains the separation by age, more evident if the samples are grouped by week, from right to left from the earlier week to the last week of life analyzed; while Comp.2 apparently finds significant differences between day 4 and day 6 of mice pup life. A possible reason why volatolome could be affected in this way is that during this two-day period the mice pups began to develop fur [30].

The same processing workflow was applied to the data of soiled-cotton litter extraction at 60 °C performed after the mice pup volatolome extraction. The PCAs obtained after correction by internal standard and mice pup weight showed that only three components were necessary to explain the 76% of the total variance and it can be observed a separation between the samples by days of life and by week (Fig. S1).

Subsequently, after the primary examination of the data with PCA, the partial least squares discriminant analysis (PLS-DA) was applied. This supervised multivariate statistical analysis considers additional information about the samples to try to reach a target grouping. Based on the information extracted from the PCA, it was considered that it

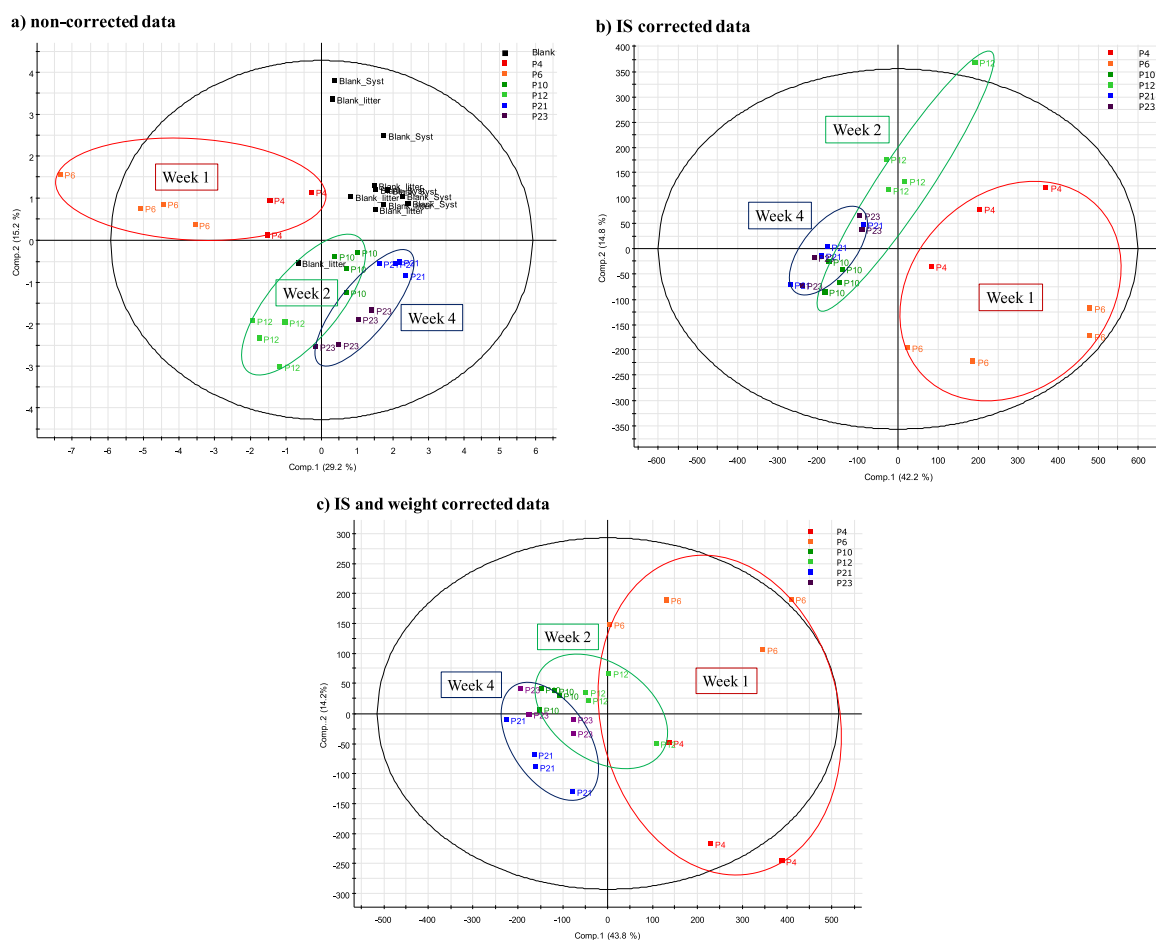


Fig. 3. PCA score plots in the plane Comp.1 vs Comp.2 of the samples of mice pup volatolome extraction: (a) the non-corrected data with blank samples; (b) data corrected the nearest internal standard and (c) data corrected by the nearest IS and subsequently by the total weight of mice pup in each extraction.

might be more interesting to attempt the grouping by weeks. The PLS-DA obtained (Fig. 4 and its corresponding loading plot Fig. S2) reach a successful classification of the samples of mice pup extracted volatolome with 97% of the variance explained and 87% of the predicted with six latent variables. The groups (weeks) are separated along the first latent variable, which explains the 43% of the total variance. Moreover, there is also a separation along the second latent variable (11% of the explained variance) related to the second week of life.

Since the objective was to focus on those volatiles that are present at high concentrations in the volatolome of young pups, an OPLS-DA was applied facing the logically more dissimilar of the mice volatolome extraction groups, week 1 vs week 4. The S-plot from the OPLS-DA permitted to highlight the markers more relevant for this differentiation (Fig. 5): In this plot the markers are distributed according to their discriminatory power between the two groups selected, being the most discriminant those with a $P[\text{corr}]$ closer to 1. It was used as a threshold to select the most relevant marker a $P[\text{corr}] > 0.8$, obtaining 42 compounds which had a higher abundance in week 1 and 2 than in week 4, and 3 compounds with higher abundance in the week 4. It was studied the presence of these features in the blank samples (blank_syst and blank_litter), and 11 out of 45 were discarded as their signal was relevant enough and cast doubt on their validity as markers.

3.5. Elucidation process

Putative identifications of the relevant compounds selected from the S-plot were reviewed. These tentative identifications were obtained thanks to the automatic comparison between the deconvoluted spectra and the NIST EI spectral library (NIST08 version) performed by PARADISE software. Additionally, the Linear Retention Indices (LRIs) were calculated for each compound using a C7–C20 alkane mixture. Those features with NIST match factor lower than 700 and/or a LRIs match with the NIST library below ± 20 were not identified, remaining a total of 24 markers with a reliable tentative identification.

Finally, 15 markers corresponding to 13 different compounds were selected to be purchased according to their discrimination power and

their availability. Following the criteria of our laboratory and the Chemical Analysis Working Group (CAGW) Metabolomics Standards Initiative (MSI) [31], their identities were confirmed by the comparison of spectra and retention time with the reference standard injected under the identical sample analysis conditions. Two putative markers did not match with the retention time of reference standard, and therefore were discarded. Table 2 shows the results of the 13 markers finally identified, with their retention time, molecular formula, the molecular ion, the NIST match and the experimental and theoretical LRI. In the case of the two peaks corresponding to Tri(propylene glycol) methyl ether, no theoretical LRIs were found, but their identities were confirmed with the injection of the commercially available mixture of isomers. Fig. S3 shows intensity variations across the samples for each of these markers using variable trend plots.

3.6. Putative pup volatile pheromones

As shown in Table 2 and Fig. S3, we have identified several volatile compounds that are secreted by pups of the 1st and 2nd weeks of age, when pups elicit intense care in adult females and motivated maternal behavior in lactating dams [32], but their secretion decreases as the pups get older and are very scarce in the volatolome of 4-week youngsters (the age of weaning). Therefore, these compounds can possibly act as chemosignals in the context of mother-pup interactions, thus maybe qualifying as pup-derived maternal behavior-inducing pheromones.

Among the compounds identified in the volatolome of 1st and 2nd week old pups, there are three glycol ethers (di(ethylen glycol) methyl ether, di(propylen glycol) methyl ether and tri(propylen glycol) methyl ether). We have not found previous reports of these compounds being direct metabolites in vertebrates or invertebrates, even if they are commonly used in industry (as solvents and hydraulic fluids) and, as pollutants, their derived metabolites have been analyzed in rodents [33] in the context of their possible toxicity.

On the contrary, 4-nonenal has been reported as a pheromone secreted by females of *Drosophila* [34] and also of the stink bug *Acrosternum aseadum* [35]. As far as we know, no previous reports indicate its

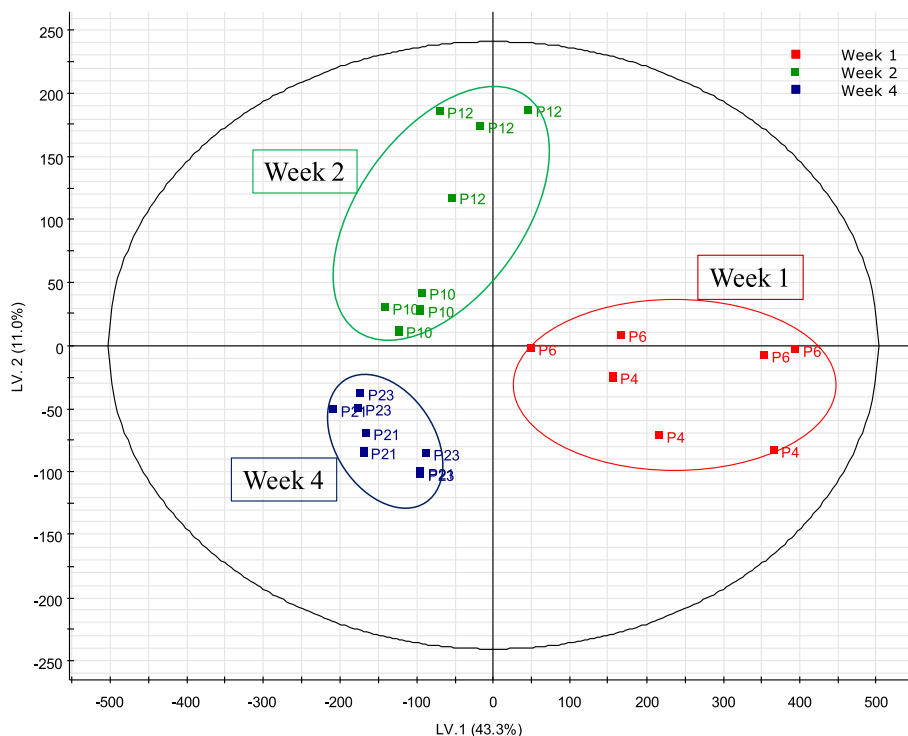


Fig. 4. PLS-DA in the LV.1 vs LV.2 plane score plot of the samples of mice pup volatolome extraction data (internal standard and mice pup the total weight corrected) grouped by weeks of life. From the 97% of the variance explained in this model 43% and 11% corresponds to LV.1 and LV.2 respectively.

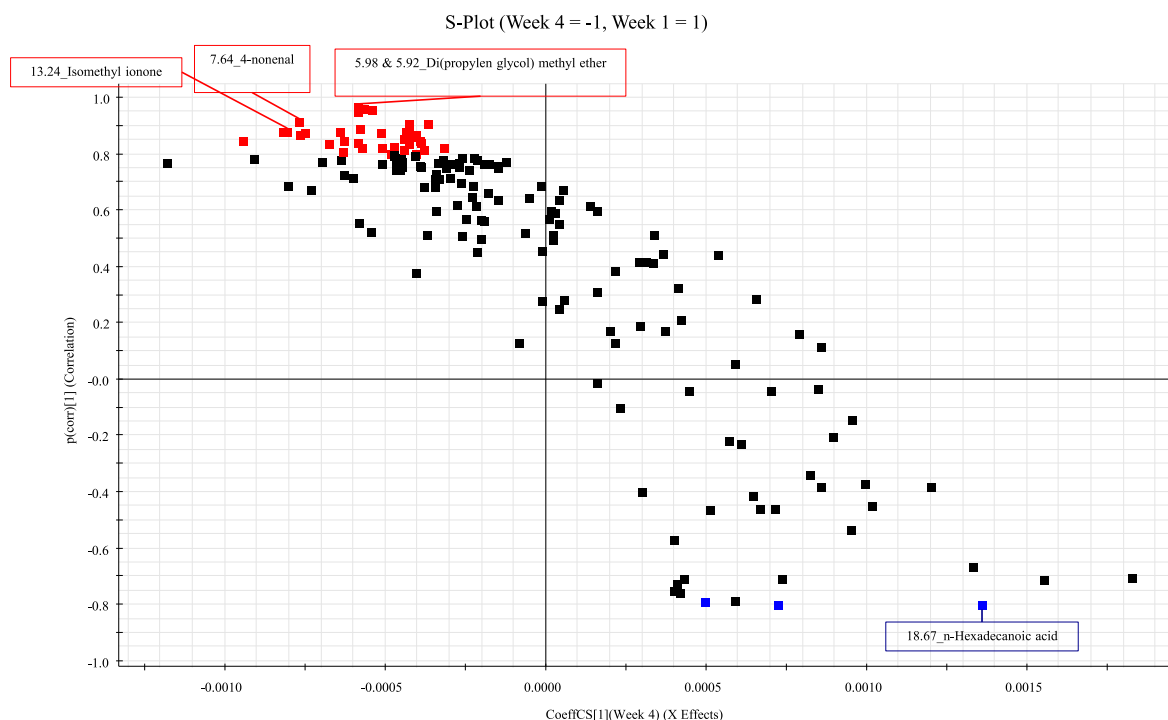


Fig. 5. OPLS-DA S-Plot for individual biomarker highlighting. Markers with a $P[\text{corr}] > 0.8$ are colored in red and blue with higher presence in the week 1 and week 4 respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

GC/MS measurements for the identified pup-derived volatile markers.

P (Corr) value	MARKER	t_R (min)	Molecular Formula	M^+ (m/z)	NIST Match	Linear Retention Index (LRI)		LRI Reported in NIST library ^a
						Experimental		
0.96	Isomer 1 Di(propylen glycol) methyl ether	5.98	C7H16O3	148 ^c	907	1000		981
0.95	Isomer 2	5.92			894	997		
0.91	4-nonenal	7.64	C9H16O	140	893	1095		1096
0.90	Di(ethylene glycol) monobutyl ether	9.14	C8H18O3	162 ^c	860	1189		1189
0.88	2-phenoxyethanol	9.69	C8H10O2	138	869	1222		1221
0.88	Isomethyl ionone	13.24	C14H22O	206	702	1477		1473
0.88	Tridecanal	13.67	C13H26O	198 ^c	903	1511		1513
0.87	1,3-diethylbenzene	7.02	C10H14	134	802	1060		1055
0.87	1,2,4,5-tetramethylbenzene	8.05	C10H14	134	772	1120		1116
0.87	2-ethyl-p-xylene	7.51	C10H14	134	880	1088		1093
0.87	Isomer 1 Tri(propylene glycol) methyl ether	10.88	C10H22O4	206 ^c	928	1302		^b
0.87	Isomer 2 ether	10.95			797	1307		
-0.81	n-Hexadecanoic acid (palmitic acid)	18.67	C16H32O2	256	914	1961		1963

^a The Linear Retention Index (LRI) were obtained for each compound from NIST Library (<https://webbook.nist.gov/>) according to the most similar column and chromatographic conditions.

^b There are not reported LRI in NIST library for this compound.

^c Not present in the EI spectra.

role in chemical communication in vertebrates.

Another of the 1st and 2nd week pup secreted volatiles, 2-phenoxyethanol, was detected in the secretion of the chin gland of adult male rabbits [36], where its concentration rises when the male becomes dominant. However, since this compound is commonly used in cosmetics to fix odorants, it has been postulated that 2-phenoxyethanol may also subserve a similar function in rabbit chemical communication, so that adding it to chin secretion would facilitate odors of dominant males to persist in the environment and not dissipate. Whether a similar function occurs in mouse pups, requires further investigation.

The volatolome of 1-week mouse pups is also enriched in an interesting compound, 1,2,4,5-tetramethylbenzene or durene, the presence of which decreases already in the second week of age. Two other similar compounds (2-ethyl-p-xylene and 1,3-diethylbenzene) show a similar

profile of secretion during pup maturation (Fig. S3). In a pioneer study, Sam et al. (2001) [37] used Ca^{++} imaging to identify compounds that activate specifically mouse vomeronasal neurons *in vitro*. They tested several substances previously suggested to be pheromones, but also mixtures of odorants for which a pheromonal role had not been proposed. Among them, they included camphoric odorants, of which, only duren showed a brisk, specific activation of some vomeronasal neurons. Twenty years afterwards, our work shows that this compound is naturally present in the volatolome of pups, and very enriched in 1-week pups, thus suggesting a possible role for this and related molecules, as pup pheromones eliciting maternal behavior in dams. This will be tested in the near future.

As compared to mouse youngsters at the time of weaning, neonatal pups also secrete several other compounds for which we have not found

previous reference in the literature of acting as chemosignals. This includes 2-ethyl-p-xylene, isomethyl ionone and tridecanal. Some of these compounds are plant-related odorants detected by the olfactory system of insects (1,3-diethylbenzene [38] and tridecanal [39] sometimes used in cosmetics and perfume industry but, to the best of our knowledge, there are no previous reports of they being involved in animal communication.

Finally, there is a single molecule identified as enriched in the volatolome of 4-week youngsters as compared to neonatal pups, hexadecanoic acid (palmitic acid). This fatty acid is very common in many vegetal oils, and it cannot be discarded that its appearance in the volatolome of mouse pups at weaning be related to autonomous feeding of standard food chops, maybe enriched in this nutrient. Nonetheless, its possible role in materno-filial communication should be explored in future investigations.

Fig. S4 shows the variation in the intensity obtained across the weeks and the comparison with the obtained ones in the soiled-cotton litter extraction. It can be observed that only part of the volatolome is retained in the cotton bedding, and even not retained in some case as for isomethyl ionone and 1,3-dimethylbenzene. This suggest whereas the remaining compounds may reach the bedding, as for example if they are excreted in the urine, these two compounds may be released using a different way of secretion/excretion. As hexanoic acid was not found either in the cotton litter in the first two weeks, and the cotton bedding of the fourth week was not analyzed.

4. Conclusions

This work has proven the usefulness and validity of a novel sampling procedure for volatolome extraction of whole, alive mouse pups. Its combination with untargeted metabolomics GC-MS approach has allowed identifying putative pheromones involved in maternal behavior. Investigation on the volatolome of mouse pups from first week (neonatal, receiving maternal care) to fourth week of age (time of weaning) has shown a more rich and abundant volatolome in neonatal pups (receiving more maternal care), supporting previous evidence that suggests a role of chemical communication in maternal behavior. Several of the compounds of neonatal pups are virtually absent in 4-week youngsters and are candidates to pup pheromones eliciting maternal care in adult females. The use of the software PARADISE in combination with NIST spectral libraries have allowed a robust peak detection and an effective tentative identification. Eleven of these putative pheromones identities have been confirmed with the commercially available standard, one of which, 1,2,4,5-tetramethylbenzene, has previously been shown to activate specifically sensory cells of the vomeronasal organ of adult male and female mice. On the other hand, we have identified a single compound, palmitic acid, which is enriched in the volatolome of 4-week youngsters as compared to neonatal pups, although it is not clear yet whether it is related with the change in diet associated to weaning. Further research is needed to clarify the putative role of these compounds in materno-filial communication and the regulation of maternal behavior.

Deontological

These experiments were performed throughout following the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1). Accordingly, procedures were approved by the Committee of Ethics on Animal Experimentation of the Jaume I University of Castellón where the experiments were performed and, ultimately, by the Valencian *Conselleria d'Agricultura Medi Ambient, Canvi Climàtic i Desenvolupament Rural* (code 2019/VSC/PEA/0049).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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