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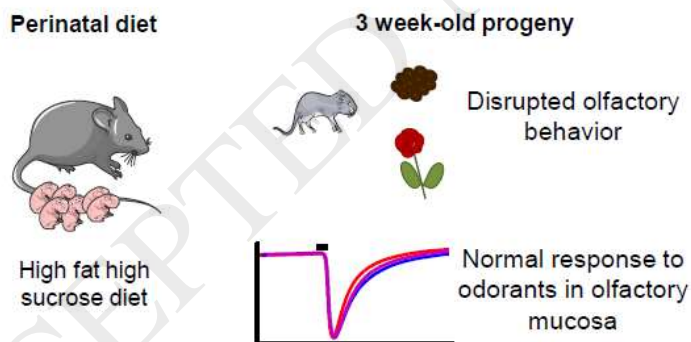
Title: Maternal high fat high sugar diet disrupts olfactory behavior but not mucosa sensitivity in the offspring

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Graphical abstract



Highlights

- Perinatal high fat high sucrose (HFHS) diet decreased olfactory perception at weaning stage
- Perinatal HFHS diet had no impact on olfactory mucosa sensitivity

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- Perinatal HFHS diet induced overweight, increased visceral fat and enhanced circulating leptin
- Additional pre-gestational HFHS diet did not exacerbate the impact on pups' olfactory system

Abstract:

The influence of maternal diet on progeny's metabolic health has been thoroughly investigated, but the impact on sensory systems remains unexplored. Neurons of the olfactory system start to develop during the embryonic life and carry on their maturation after birth. Besides, these neurons are under metabolic influences, and it has recently been shown that adult mice exposed to an obesogenic or diabetogenic diet display reduced olfactory abilities. However, whether or not olfactory function is affected by the perinatal nutritional environment is unknown.

Here we investigated the effect of a high fat high sucrose (HFHS) maternal diet (46% of total energy brought by lipids, 26.6% by sucrose) on progeny's olfactory system in mice.

In male offspring at weaning stage, maternal HFHS diet induced overweight and increased gonadal fat, associated with hyperleptinemia. The progeny of HFHS diet fed dams showed reduced sniffing behavior in the presence of low doses of phenylethanol (an attractive odorant for mice), compared to the progeny of standard diet fed dams. Furthermore, they exhibited increased time to retrieve a piece of breakfast cereals hidden beneath the bedding in a buried food test. Meanwhile, electroolfactogram recordings revealed no change in the sensitivity of olfactory mucosa. mRNA levels for elements of the olfactory transduction cascade were not affected either.

Our results demonstrate that maternal HFHS diet during gestation and lactation strongly modulates olfactory perception in the offspring, without impairing odor detection by the olfactory epithelium. Maternal HFHS diet starting two months before gestation did not induce additional impairments in progeny.

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Keywords: maternal diet, olfactory behavior, plethysmography, electro-olfactogram, olfactory mucosa, weaning

1. INTRODUCTION

Current eating behaviors involve high intake of fat and sugar. This type of diet, associated with sedentary lifestyle, increases the risk of developing obesity and metabolic diseases (Andersen, 2000). According to the World Health Organization (WHO), 39 % of worldwide adults were overweight and 13 % were obese in 2016. Women of childbearing age do not make an exception. The prevalence of overweight and obesity among pregnant women has reached more than 20 % (Lu et al., 2001).

In the early 1990's, Barker and collaborators raised the idea that major disorders in adults, such as obesity, stroke and cardiovascular diseases, might originate from the environment experienced *in utero* additionally to lifestyle, feeding habits and genetic factors (Barker, 1990). The concept of the developmental origins of health and disease relies on fetal programming mechanisms. Fetal tissues and organs are able to adapt to the early nutritional environment throughout their development (Barnes and Ozanne, 2011). In mammals, maternal diet before conception and during gestation influences the intra-uterine environment. Maternal diet also determines the milk composition and therefore the nutritional intake of newborns. As a result, maternal diet and subsequent maternal obesity are likely to modify the perinatal nutritional environment and consequently the susceptibility of progeny to develop diseases in adulthood. Numerous reviews highlight the relationships between maternal metabolic state and diet and the risk for the offspring to develop metabolic syndrome (Ribaroff et al., 2017; Williams et al., 2014), cardiovascular diseases (Dong et al., 2013) and behavioral disorders (Sullivan et al., 2015). However, the potential link between maternal diet and progeny's sensory abilities remains unexplored.

Acute senses are essential for individuals to survive and reproduce in their environment. Olfaction is especially involved in social interactions, avoiding predators and feeding. Odorant molecules are detected in the nasal cavity by olfactory sensory neurons (OSNs) in

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the olfactory mucosa (OM). OSNs convert the chemical stimulation into an electrical signal sent to the olfactory bulb (OB) and other brain structures. The olfactory system develops during embryonic life and pursues its maturation after birth. All the elements composing the olfactory system are thus likely to be affected by maternal diet. In addition, the olfactory system is modulated by metabolic peptides and hormones (Palouzier-Paulignan et al., 2012). Among these actors, insulin and leptin, two major metabolic hormones exerting an anorexigenic effect, can modulate the electrical activity of OSNs (Savigner et al., 2009) and decrease olfactory detection abilities in rodents (Aimé et al., 2012; Getchell et al., 2006; Julliard et al., 2007). Conversely, orexigenic factors such as orexins, ghrelin and neuropeptide Y (NPY) increase odorant detection and sniffing (Julliard et al., 2007; Negroni et al., 2012; Prud'homme et al., 2009; Tong et al., 2011). Because maternal diet can lead to metabolic disturbances in the offspring, their blood levels of metabolic hormones can be modified and at the root of olfactory defects. Besides, both a hyperlipidemic diet generating metabolic syndrome and a high fructose diet inducing diabetes caused anatomical and functional changes in the OM, such as a decrease in electrophysiological responses to odorants, as well as behavioral alterations (Rivière et al., 2016; Thiebaud et al., 2014).

We hypothesize that perinatal exposure to fat and sugar could impair olfaction in young mice. The present study aimed to investigate the impact of a High Fat High Sugar (HFHS) diet administered to females, from the onset of gestation or two months before and until the end of lactation, on their offspring's olfactory behavior and olfactory peripheral system. Nutritional disorders in pregnant and lactating women usually originate from deleterious eating behaviors established months before child conception. Our experimental protocol of maternal exposure to HFHS diet for two months pursued during gestation and lactation was designed to approximate these nutritional conditions. A supplementary protocol – maternal exposure to HFHS during gestation and lactation only – was added to help dissociate the consequences of maternal feeding vs metabolic alterations in dams. We first studied dam's ability to breastfeed, maternal milk composition and the metabolic phenotype of the 3-week old offspring. Maternal HFHS diet induced changes in milk fatty acids composition, and

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overweight, increased visceral adiposity as well as higher blood leptin levels in progeny. Monitoring the sniffing behavior of the weaned progeny using a plethysmograph showed a reduction in their response to low concentrations of an attractive odorant (phenylethanol, PEA). Moreover, HFHS progenies were slower to retrieve a food item using olfactory cues in a buried food test. When investigating pups' OM properties, electroolfactogram (EOG) recordings and gene expression analyses for constituents of the olfactory transduction cascade revealed no impact of maternal HFHS. Our results show that maternal diet did substantially disrupt olfactory behavior of the offspring and suggest alterations in central structures rather than in peripheral olfactory system.

2. MATERIALS AND METHODS

2.1. Animals, diets and experimental design

Gene-targeted *SR1-IRES-tauGFP* mice, expressing the green fluorescent protein under the promoter of the *sr1* (*Olfrr-124*) odorant receptor gene (Grosmaître et al., 2009), were used in all experiments. Mice were housed 2 per cage with food and water *ad libitum*, under constant conditions of temperature and humidity in a 12h light/12h dark inverted cycle. As illustrated on **Supplementary figure 1**, female mice were placed on a standard diet (SD) or a HFHS diet (Safe, **Supplementary Table 1**) during a pre-conception period of 8 weeks. One age-matched male was introduced in each cage to mate with the 2 females, removed one week before expected births, and females were housed individually for the remaining time. One group of mice under SD and all mice under HFHS diet were maintained on their respective diets throughout gestation and lactation (respectively *CTRL* and *HFHS 8w + gest-lact* groups). The other group of females under SD received the HFHS diet from mating to weaning (*HFHS gest-lact* group). One day after birth, litters were standardized (5 to 7 pups per litter) to ensure comparable metabolic demands during lactation. Offspring were weaned on postnatal day (PND) 19 to 24 depending on the experiments. Dam's body weight and food intake were monitored twice a week during the whole procedure (**Supplementary figures 2 and 3**).

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All experiments were conducted in accordance with the guidelines of the European Community for the use and care of laboratory animals (2010/63/EU) and resulting French regulations. Accordingly, all experimental protocols were approved by the French Ministry of Higher Education and Research (reference 01286.02).

2.2. Evaluation of lactation and milk composition analysis

Lactation ability was evaluated at day 10 of lactation (L10). Litters were separated from their mother for 4h while kept warm on a temperature-controlled surface. Litters were then weighted and placed back in their mother's cage, and weighted again 2 hours later. The pups' milk consumption, assessed by the pups' weight gain during the two hour-period, represented the milk production of the dam.

Milk collection was also performed at L10 on another set of dams. As above, the litter was separated from the mother for 4h. Dams were anesthetized via intraperitoneal (i.p.) injection of Ketamine-Xylazine (70-14 mg/kg, Sigma-Aldrich). After an i.p. injection of oxytocin (100 μ L, Intervet, Unterschleissheim, Germany) to induce milk let down, mice were milked using a glass pipette with an inflating bulb (Depeters and Hovey, 2009). Milk samples were stored at -80°C until analysis for lipid, protein and sugar composition.

Lipids were extracted according to the Folch method and analyzed using gas chromatography as described elsewhere (Thierry et al., 2014). Proteins were measured with DC Protein assay (Bio-Rad) following manufacturer recommendations. Lactose was measured via colorimetric assay using 3,5-dinitrosalicylic acid (DNS), an aromatic compound that reacts with reducing sugars upon the application of alkaline conditions and heat to form 3-amino-5-nitrosalicylic acid, which strongly absorbs light at 540 nm (Sumner and Noback, 1924). DNS reagent was prepared in distilled water as follows: 43.2 mM DNS (Alfa Aesar), 1.06 M potassium sodium tartrate (Rochelle salt, $\text{C}_4\text{H}_4\text{KNaO}_6$, MP Biomedicals), 4% v/v sodium hydroxide (NaOH 30%, RP normapur, Prolabo). 20 μ L of DNS reagent was added to 30 μ L of 100 fold diluted milk samples or standard lactose solutions (0-500 $\mu\text{g}/\text{mL}$) and

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mixtures were incubated at 100°C for 5 min. After cooling on iced water, 150 µL of distilled water was added and absorbance was read at 540 nm.

2.3. Metabolic features of the offspring

Litters were weighted on PND 1, and individual weight was followed twice a week during lactation. At weaning, blood was collected from the tail vein without anesthesia and centrifuged to obtain serum samples. Leptin serum levels were determined using a Mouse Leptin ELISA Kit (Crystal Chem, #90030). Mice were dazed with isoflurane inhalation and sacrificed by decapitation to collect and weight the epididymal fat pad.

Tolerance to glucose was assessed in pups fasted for 5h by i.p. glucose tolerance test (ipGTT) as in (Bowe et al., 2014). Glucose i.p. injection (2g/kg body weight) was performed and glycaemia was measured using an Accu-Chek Performa glucometer (Roche) from blood collected from the tail vein before and 15, 30, 45, 60, 90, 120 minutes after glucose injection.

2.4. Behavioral experiments

2.4.1. Buried food test

Global olfactory acuity was assessed in male offspring using a buried food test, which relies on mice natural tendency to use olfactory cues for foraging. We adapted the protocol from Yang and Crowley (Yang and Crowley, 2009) to be suitable for young mice. Chocolate breakfast cereals (Crunch®, Nestlé) were used as food stimulus. Animals were habituated to cereals and test cages (conventional mouse cages (33x19x13 cm) filled with 6 cm of bedding) on three consecutive days before the actual test. We controlled that every mouse ate cereals at least one time during the whole habituation process. On test day, male pups were weaned and kept without food for 5h. Animals were tested for 3 consecutive trials separated by a 3-minute rest time. During the first trial, a piece a cereal was placed on the surface bedding at one randomly chosen spot (out of 8), and the mouse was introduced in the center of the cage. The time to reach and start eating the cereal was measured with a manual timer and used to assess mice motivation to reach a food item. Mice were prevented

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from eating the entire cereal to avoid satiation. The two remaining trials were performed the same way, except that the cereal was buried beneath 2 cm of bedding. Test ended once either the animal found the cereal or did not achieve to find it within 10 minutes. The shortest time to retrieve and eat the cereal between the two trials was used to assess olfactory performance.

To ensure that times of retrieval were due to pups' olfactory abilities and not to changes in exploratory and digging behaviors, we performed a behavioral observation in another set of pups. As above, animals were habituated to cereals, fasted for 5h on test day and the first trial with the cereal on the surface bedding was done. After a 3-minute rest time, the animals were introduced again in the cage without any piece of cereal. Four basic behaviors were monitored for 10 min using J Watcher software: exploration of the surface bedding or the walls and upper part of the cage, digging into the bedding, grooming, staying immobile.

2.4.2. Odor-induced sniffing behavior

A whole-body plethysmograph chamber (Emka Technologies, France) was used to record mice respiratory activity during odor stimulations in an experimental setup adapted from Hegoburu *et al.* (Hegoburu et al., 2011) (**Figure 3A**). Mice breathing induced pressure changes measured by a differential pressure sensor with one port exposed to the animal chamber and the other to the above reference chamber. Constant airflow (2.2 L/min) was provided through the apparatus, and a lateral port was connected to a vial containing the odorant solution. The vial was connected by a plastic tube equipped with a 3-way valve, allowing airflow deviation to deliver odorized air within the animal chamber.

The respiratory signal collected from the plethysmograph was interfaced to a computer equipped with AxoScope (Axon Instruments; sampling rate = 1 kHz). The recordings were analyzed with routines written under Matlab. Inspiration and expiration phases were extracted to calculate respiratory frequency (mean number of respiratory cycles per second) and inspiration amplitude (mean size of inspiration phases). In subsequent analysis, a z-normalization was applied (Guilloux et al., 2011) to integrate these parameters as a sniffing

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index (called “z-sniffing”). For each mouse and each parameter, a z-score was calculated as follows: $z = ((x-\mu)/\sigma)$ where x represents the individual data for the calculated parameter, and μ and σ the mean and standard deviation for the CTRL group during base period, respectively. Then the mean of the two z-score (z-amplitude and z-frequency) was calculated and taken as the sniffing index for each animal. The day before the actual test, mice were placed inside the plethysmograph and stimulations with distilled water inside the vial were performed to habituate mice to airflow deviations. On test day, male pups were separated from their mother for 1h and introduced in the plethysmograph. They were allowed to calm down during 5 minutes before starting the stimulations. Each stimulation lasted 30 seconds, was preceded by a 30 seconds baseline period and followed by another 30 seconds period to allow mice to return to a quiet state. For the baseline period, the experimenter waited for the mouse not to be actively sniffing, in order to avoid bias in the basal breathing due to exploratory sniffing events. The first 3-6 stimulations were performed with distilled water inside the vial, until the mouse did not respond to the stimulation. Then, two consecutive odorant stimulations were performed with PEA (Sigma Aldrich) solutions inside the vial, first at 10^{-5} M then at 10^{-4} M.

From the breathing recordings, two exclusion criteria were established: the animal was excluded from the analysis if 1) the basal respiratory frequency was higher than 7.5Hz during 2 or more stimulations, indicating a failure to reduce stress, 2) the last stimulation with water induced a 10% increase (or more) in respiratory frequency and inspiration amplitude during stimulation time compared to the basal period, indicating a failure in habituation to airflow deviation.

2.5. EOG recordings

EOG recordings were performed on turbinate III from an opened nasal cavity on mouse hemi-heads as previously described (Lacroix et al., 2008). Briefly, the hemi-head was placed under a constant flow of humidified air (~1L/min) delivered through a 7 mm-diameter plastic tube, positioned 2 cm from the epithelial surface. Odorant stimulations were performed by

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blowing air puffs (200 ms, 1 psi) through an exchangeable Pasteur pipette, enclosed in the plastic tube, containing a filter paper impregnated with 10 μ L of odorant solution. PEA and acetophenone (ACE; Sigma Aldrich) were used as odorants. Odorant-free air stimulation (with ultra-pure water) was always performed before any odorant stimulation to record mechanical responses. EOG voltage signals were recorded using a Axoclamp amplifier (Axon Instruments) used in a DC current-clamp configuration ($I=0$), digitized at a rate of 1 kHz, using a Digidata 1440 (Axon Instruments, Molecular Devices) and interfaced to a computer equipped with AxoScope. A reference Ag/AgCl electrode was placed in the OB. Recordings were made with glass micropipettes (8 μ m-diameter) filled with a saline solution (Lacroix et al., 2008). Data was analyzed using Matlab to measure peak amplitude and dose-response curves were fitted with the Hill equation model ($A = A_{max}/(1+10^{-k(C-EC50)})$, where A represents the peak amplitude, A_{max} the maximum response at saturating concentrations, k the Hill coefficient, C the concentration of odorant and EC50 the concentration at which half of the maximum response was reached).

2.6. Quantitative RT-Polymerase Chain Reaction (qPCR)

Mice were dazed with isoflurane inhalation, sacrificed by decapitation and the nose was dissected out to remove the olfactory epithelium attached to the nasal septum and the dorsal recess. The olfactory tissue was immediately frozen into liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was extracted (Nucleospin RNA plus kit, Macherey-Nagel) and cDNA were synthesized from 1 μ g of RNA using reverse transcriptase (PrimeScriptTM RT reagent Kit, Takara) following the manufacturer recommendations. For quantitative PCR, 1 μ L (50 ng) of cDNA templates were added to the 9 μ L reaction mixture containing SYBR Green Master Mix (Life technologies) and 250 nM primers (see **Supplementary Table 2** for primers sequences). The qPCR was performed on a StepOne thermocycler (ThermoFisher Scientific, Applied Biosystems) during 40 amplification cycles consisting of 3 s at 95°C and 30 s at 60°C . Quantification was achieved by calculation the Ct difference between the gene of interest and the reference gene (*Gapdh*) for each mouse.

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2.7. Statistical analysis

All statistical analyses were done using GraphPad Prism software and blind to the experimenter. Data are expressed as mean \pm standard error of the mean (SEM), except for sniffing indexes which were represented as box and whiskers. Normality was systematically tested using Shapiro test to determine parametric or non-parametric statistical plan. One-way ANOVA followed by Tuckey post hoc tests or Kruskal Wallis followed by Dunn's multiple comparison test were used to determine statistical differences between the three groups; except for male body weight evolution during lactation, glucose tolerance test, and sniffing indexes which were analyzed using two-way repeated measures ANOVA followed by Bonferroni multiple comparisons. A probability value of $p < 0.05$ was used as an indication of statistical differences.

3. RESULTS

3.1. Maternal HFHS diet does not compromise lactation but changes milk composition

Before mating, the global weight gain from diet onset was 41% lower in females fed with the HFHS diet compared to females fed with the CTRL diet (**Supplementary figure 2.A.2**, (t,df) = (3.449,35), $p = 0.0015$). However, HFHS diet for two months induced glucose intolerance in dams (**Supplementary figure 2.B.2**, $F(1,21) = 13,25$, $p = 0.0015$). During gestation and lactation, dams body weight evolution differed between the three groups (**Supplementary figure 3.A**, $F(2,49) = 3.462$, $p = 0.0392$) and dams fed with the HFHS diet ingested more energy particularly during mid-lactation (**Supplementary figure 3.B**, $F(2,43) = 8.548$, $p = 0.0007$, CTRL vs HFHS gest-lact: $t = 3.300$, $p < 0.05$ at day 28, $t = 4.669$, $p < 0.001$ at day 32, $t = 3.392$, $p < 0.05$ at day 35, CTRL vs HFHS 8w + gest-lact: $t = 3.961$, $p < 0.01$ at day 25, $t = 5.808$, $p < 0.001$ at day 28, $t = 4.646$, $p < 0.001$ at day 32).

After birth, the number of pups per litter was standardized to avoid any litter size effect and to ensure equivalent milk access for each pup. The milk access was verified: milk intake and

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consequently milk production were similar at mid-lactation (L10) in the three groups (**Table 1**, $F(2,27) = 1.77$, $p = 0.190$).

Milk composition at L10 from CTRL and HFHS groups is depicted in **Table 1**. There were no significant differences in protein or lactose content between groups ($K = 0.987$, $p = 0.611$ for protein content and $K = 0.007$, $p = 0.997$ for lactose content). The total milk fat content did not differ between CTRL and HFHS dams ($K = 4.987$, $p = 0.0826$), however the fat composition changed in the milk of HFHS dams. Milk from HFHS diet fed dams contained 1) less saturated fatty acids (SFA), especially less lauric, myristic and palmitic acids (C12:0, C14:0 and C16:0), 2) much more oleic acid (C18:1 n-9), which is a monounsaturated fatty acid (MUFA), 3) less polyunsaturated fatty acids (PUFA) and a higher n-6 / n-3 ratio, caused by less linoleic acid (C18:2 n-6) and less linolenic and docosahexaenoic acids (C18:3 n-3 and C22:6 n-3) (**Supplementary table 3**).

3.2. Impact of the mother's diet on the progeny metabolic phenotype

To evaluate the impact of maternal HFHS diet on general metabolic features in the offspring, we first analyzed the growth pattern of pups. As shown in **Figure 1**, pups from HFHS diet fed dams exhibited similar birth weight compared to CTRL group (**Figure 1.A**, $K = 2.285$, $p = 0.319$). They became significantly overweight during lactation (**Figure 1.B**, $F(2,175) = 16.81$, $p < 0.001$): by 10.3 % (HFHS gest-lact, $t = 3.421$, $p < 0.01$ compared to CTRL) and 11.5 % (HFHS 8w + gest-lact, $t = 3.174$, $p < 0.05$ compared to CTRL) at PND 15, and by 13.1 % (HFHS gest-lact, $t = 5.452$, $p < 0.001$ compared to CTRL) and 11.1 % (HFHS 8w + gest-lact, $t = 4.021$, $p < 0.001$ compared to CTRL) at weaning. Furthermore, at weaning, the offspring of HFHS diet fed dams presented higher amounts of visceral fat (**Figure 1.C**, $K = 25.08$, $p < 0.001$; x2.2 for HFHS gest-lact vs CTRL, $D = -22.31$, $p < 0.01$, and x2.8 for HFHS 8w + gest-lact vs CTRL, $D = -26.43$, $p < 0.001$). When we investigated the plasma levels of metabolic hormones, we found increased levels of leptin in HFHS pups (**Figure 1.D**, $K = 11.36$, $p < 0.01$; x1.8 for HFHS gest-lact vs CTRL, $D = -8.933$, $p < 0.05$, and x2.0 for HFHS 8w + gest-lact vs CTRL, $D = -9.767$, $p < 0.05$), which is in accordance with increased fat

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mass in these pups. Regarding glucose metabolism, there was a difference neither in basal glycaemia (**Figure 1.E**, $F(2,36) = 2.31$, $p = 0.114$), nor in glucose tolerance (**Figure 1.F**, $F(2,36) = 0.711$, $p = 0.498$) between the three groups.

To summarize, in our experimental design, maternal HFHS diet led to the onset of metabolic disturbances in the offspring.

3.3. The offspring from HFHS diet fed dams exhibit reduced olfactory abilities

General olfactory abilities were first assessed in a buried food test (**Figure 2**). In this food-finding context, the progeny of HFHS diet fed dams exhibited increased time (x1.9) to retrieve the buried cereal (**Figure 2.B**, $K = 12.92$, $p = 0.0016$; HFHS gest-lact vs CTRL: $D = -20.62$, $p < 0.01$; HFHS 8w + gest-lact vs CTRL: $D = -21.76$, $p < 0.05$).

Several non-olfactory factors could explain this behavioral result. In the first trial, when the cereal was visible, all mice displayed similar time (~10 sec) to navigate into the cage, find and eat the food item (**Figure 2.A**, $K = 3.855$, $p = 0.146$). Thus, HFHS progeny were as motivated to find food as CTRL mice. Moreover, retrieving the buried food implies not only to explore the surface bedding, but also to dig deep into the bedding. We performed a spontaneous behavioral observation without hidden cereal to characterize the digging behavior of CTRL and HFHS progenies. Within 10 min, the spontaneous behavioral pattern was similar between the three groups (**Figure 2.C**). More precisely, mice spent around 20 % of total time digging (CTRL: 22.22 %, HFHS gest-lact: 19.95 %, HFHS 8w + gest-lact: 18.83 %, $F(2,46) = 0.341$, $p = 0.713$) and more than 70 % of total time walking or running on the surface bedding (CTRL: 73.12 %, HFHS gest-lact: 71.94 %, HFHS 8w + gest-lact: 72.92 %, $F(2,46) = 0.0371$, $p = 0.964$).

To sum up, maternal HFHS diet did not impact motivation to find food or digging behavior in the offspring, but reduced their olfactory ability towards a food odor.

In order to investigate olfactory perception in freely behaving mice, independently of feeding context or rewarded behavior, we used a whole-body plethysmograph to record mice

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breathing (**Figure 3.A**) and assess odorant perception. For each stimulation, we checked that mice basal breathing was similar between the three groups (non-patterned boxes, diet effect: $F(2,72) = 2.194$, $p = 0.119$, $F(2,72) = 0.00992$, $p = 0.990$, $F(2,72) = 2.193$, $p = 0.119$ for stimulation with water, PEA 10^{-5} M, PEA 10^{-4} M respectively).

During the first non-odorized stimulation, mice did not modify their sniffing behavior (**Figure 3.B**, time effect: $F(1,72) = 0.0322$, $p = 0.858$). When PEA was presented at 10^{-5} M, only CTRL mice increased their sniffing behavior (**Figure 3.C**, time effect: $F(1,72) = 16.69$, $p < 0.001$; CTRL: $t = 5.631$, $p < 0.001$, HFHS gest-lact: $t = 0.4577$, $p > 0.05$, HFHS 8w + gest-lact: $t = 2.086$, $p > 0.05$), whereas the three groups exhibited increased sniffing behavior when PEA was presented at 10^{-4} M (**Figure 3.D**, time effect: $F(1,72) = 62.58$, $p < 0.001$; CTRL: $t = 6.267$, $p < 0.001$, HFHS gest-lact: $t = 5.445$, $p < 0.001$, HFHS 8w + gest-lact: $t = 2.789$, $p < 0.05$). Contrary to CTRL group, HFHS progenies did not react to the lower odorant concentration, which highlights altered odor-induced behavior.

All together, these results showed that maternal HFHS diet impaired olfactory perception in the offspring. Is this impairment correlated with a modulation of odorant detection at the peripheral olfactory level?

3.4. Altered olfactory perception in the progeny is not associated with changes in the sensitivity of the OM

To investigate whether odor detection by the OM was altered in HFHS progenies, we recorded the electrical response of a population of OSNs following odorant stimulations with EOG measurements. Using PEA to perform odorant stimulations, dose-response curves for peak amplitudes largely overlapped between the three groups (**Figure 4.A.1**). Maternal HFHS diet did not modify the dose-response characteristics of OSNs (**Figure 4.A.2**, $K = 4.505$, $p = 0.105$, $K = 0.905$, $p = 0.636$, $F(2,41) = 1.148$, $p = 0.327$ for $-\log(\text{EC}_{50})$, Hill coefficient and A_{max} respectively). When odorant stimulations were performed with acetophenone, Hill coefficient and $-\log(\text{EC}_{50})$ were not affected by maternal HFHS diet

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(**Figure 4.B.2**, $F(2,34) = 2.029$, $p = 0.147$, $F(2,34) = 1.941$, $p = 0.159$ for $-\log(\text{EC}_{50})$ and Hill coefficient respectively), but the average maximum amplitude elicited by saturating concentrations was slightly increased in mice from dams under HFHS diet during 8 weeks, gestation and lactation (**Figure 4.B.2**, $K = 6.457$, $p = 0.0396$; HFHS 8w + gest-lact vs CTRL: $D = -11.10$, $p < 0.05$).

To confirm that OM functioning was not disrupted in HFHS progenies; we analyzed gene expression of four main constituents of olfactory transduction cascade. No difference was found in gene expression of Golf (**Figure 5.A.1**, $K = 2.415$, $p = 0.299$), ACIII (**Figure 5.A.2**, $K = 0.613$, $p = 0.736$), CNG (**Figure 5.A.3**, $K = 0.571$, $p = 0.752$) and PDE (**Figure 5.A.4**, $K = 1.318$, $p = 0.517$).

OM cells (OSNs and supporting cells) also express numerous receptors for metabolic hormones, such as insulin and leptin, that enables a metabolic influence on olfactory perception (Palouzier-Paulignan et al., 2012). In our three groups of mice, gene expression of insulin receptor (Ins-R) was similar (**Figure 5.B.1**, $K = 1.112$, $p = 0.573$), but leptin receptor (Lept-R) was 8.1 % less expressed in HFHS 8w + gest-lact group compared to CTRL group (**Figure 5.B.2**, $K = 9.825$, $p = 0.0074$; HFHS 8w + gest-lact vs CTRL: $D = 9.879$, $p < 0.05$).

4. DISCUSSION

Recent literature has reported deleterious effects of maternal obesity and diet on progeny's health but no insights were provided into the impact on sensory systems in the offspring. In this study we report that maternal HFHS diet disrupts olfactory behavior in the 3-week old progeny. Male mice born from dams fed with the HFHS diet were overweight at weaning stage compared to CTRL mice, and exhibited higher visceral adiposity associated with increased plasma leptin levels. The altered olfactory abilities were not found to be correlated with alterations of the peripheral olfactory system.

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In our experimental design, female mice showed a resistance to diet-induced obesity, a phenomenon which has already been reported (Wahlig et al., 2012). Dams fed with the HFHS diet for two months did not gain weight despite the obesogenic context, but developed glucose intolerance. The effects of maternal HFHS diet feeding on the olfactory system of the progeny were independent of maternal obesity. We can further notice that maternal HFHS exposure during the pre-conception period did not exacerbate the effects of HFHS exposure during gestation and lactation. Indeed, for every parameter measured in the offspring (metabolic or olfactory parameter), the values for HFHS gest-lact group and HFHS 8w + gest-lact group were not statistically different. In our protocol, the metabolic state of females prior to mating (i.e. glucose intolerance) did not induce any additional olfactory defects in progeny. This suggests that the critical period for maternal diet to impact olfaction in the offspring might be found during gestation and/or lactation. Additional groups with diet switching after parturition could be used to investigate in which precise period maternal HFHS diet most influences progeny's olfactory abilities.

Milk from HFHS diet fed dams contained similar amounts of protein, lactose and lipid, but the fatty acids (FA) composition was different. Exposure to a moderate high-fat (HF) diet without obesity has been shown to increase the trafficking of dietary lipids to adipose tissue, liver and mammary gland while subsequently decreasing de novo lipogenesis in these tissues, leading to changes in milk FA composition without affecting the global fat content (Wahlig et al., 2012). In line with previous studies, we highlighted a decrease in SFA content in the milk of HFHS diet fed dams. MUFA and PUFA contents did not vary as expected from other studies which could be explained by the specificities of the diet used in the present study. Milk PUFA composition was shown to be specifically manipulated by the maternal diet composition; meanwhile, milk MUFA content appeared very resistant to manipulation of the dietary FA composition (Oosting et al., 2015). Our data showed that the MUFA and PUFA milk levels reflected the respective quantities from maternal diet. MUFA were almost twice more concentrated in the milk of dams exposed to the HFHS diet than the milk of CTRL dams, as is the HFHS diet compared to the SD. Taken together, our results clearly confirmed that the

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pups' dietary FA intake was modulated by maternal exposure to the HFHS diet. FA are mandatory constituents of neuronal membranes, and the FA composition of the milk consumed by HFHS offspring could have prevented the normal development of neurons involved in olfactory processing.

Our experimental design provides a perinatal exposure to fat and sugar through maternal diet with few consistent effects on offspring metabolic physiology, as already reported by others (Chin et al., 2017). In addition to similar fat, protein and lactose total amounts among maternal milks, we found no difference of milk consumption between the three groups. The weight gain observed in HFHS progenies' from PND 15 to PND 20 could thus result from early independent feeding rather than higher energy intake from milk. Such a phenomenon has been observed in rats: maternal HF diet increased pre-weanling offspring body weight by facilitating early independent feeding, through a nutritive nursing and without affecting suckling parameters (Kojima et al., 2016).

The progeny of dams fed with the HFHS diet exhibited higher amounts of visceral fat and enhanced circulating leptin. We can assume that pups born from HFHS diet fed dams are at very early stages of developing metabolic disturbances. Few studies have investigated the effects of diet on olfactory abilities. Older mice affected by metabolic syndrome or diabetes after direct adult exposure to modified diets also display increased time to retrieve a food item during a buried food test (Rivière et al., 2016; Tucker et al., 2012). In addition to showing decreased olfactory abilities in the buried food test, we demonstrated lower PEA perception, a non-food but attractive odor (Root et al., 2014), using the plethysmograph device. Unlike the studies of Thiebaud and Rivière, our data indicates that impaired olfactory abilities are not correlated with modifications of the OM. We only observed using EOG recordings that the maximum of the dose-response curve after ACE stimulations was higher in HFHS 8w + gest-lact group compared to CTRL group. We assume that this difference might not be relevant regarding OM sensitivity, which would be more correlated to EC50 and dynamic range of the responses (Hill coefficient). The discrepancy with previous studies may

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underlie the complex relationships between metabolic and olfactory systems. The way individuals are exposed to fat and sugar, i.e. direct exposure through adult diet or perinatal exposure through maternal diet, must differently influence olfactory structures.

Olfactory perception does not only rely on odorant detection by the peripheral olfactory system, but also on central integration of the olfactory message. The OB receives inputs from OSNs and sends neuronal projections to the olfactory cortex, which then innervates numerous brain areas (hypothalamus, amygdala, and hippocampus). Although we did not explore all the anatomical and physiological characteristics of the olfactory mucosa, our results suggest that altered olfactory behavior in HFHS progeny would rather derive from modifications in the central nervous system.

Maternal diet has been shown to alter brain anatomy and physiology in the offspring: HF maternal intakes, prior and during gestation, may alter fetal brain development. Cell proliferation, apoptosis and early neuronal differentiation in fetal hippocampus were affected by HF diet-induced maternal obesity in mice (Niculescu and Lupu, 2009). Maternal obesity also increased the proliferation of neuronal precursor cells (Chang et al., 2008) and astrocytes (Dong et al., 2016) in fetal and neonatal hypothalamus. The development of the olfactory system starts as early as embryonic day 10 (E10) (Smart, 1971). In the OB, mitral and tufted cells are entirely produced before birth and OB granule cells arise from E18 to PND 20 (Hinds, 1968). In the central olfactory system (piriform cortex, olfactory tubercle, anterior olfactory nucleus, lateral olfactory tract), fiber growth begins prenatally but maturation and connections mainly occur after birth (Schwob and Price, 1984). Therefore, the olfactory system undergoes pronounced cell proliferation and maturation early during embryonic development and during postnatal life. Processes observed in the hippocampus and hypothalamus after maternal HF diet could also affect the developmental steps and the plasticity of the olfactory system.

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In addition, maternal HF diet might alter astrocytic and neuronal morphology. In adult rats, astrocyte morphology in the hippocampus was modified following perinatal exposure to a HF diet without maternal obesity (Lépinay et al., 2015). Adult offspring from HF diet fed dams further exhibited shorter basal dendrites in hippocampal and basolateral pyramidal neurons (Janthakhin et al., 2017), and a loss of spines in the somatosensory cortex (Hatanaka et al., 2016), leading to synaptic instability and subsequent impaired neuronal processing. Maternal HF diet also decreased both spine density and dendritic length in medial prefrontal cortex of 3 week-old pups (Rincel et al., 2017). Altered olfactory abilities observed in our model could partly derive from such neuro-morphological modifications affecting olfactory neurons in the OB or in the other brain structures integrating olfactory information.

Leptin has proven to be very efficient as a modulator of the olfactory system. Genetically engineered mice lacking leptin physiological regulation (*ob/ob* and *db/db* mice) exhibit improved olfactory abilities compared to wild-type ones (Getchell et al., 2006), and injecting leptin in starved rats decreases olfactory detection ability (Julliard et al., 2007). Besides, leptin has been shown to directly modulate the electrical activity of OSNs (Savigner et al., 2009). Leptin levels were twice higher in the progeny of dams fed with HFHS diet compared to CTRL mice, which is in line with previous findings (Janthakhin et al., 2017; Lépinay et al., 2015). Consequently, the high leptin titers measured in the HFHS group could have contributed to the decreased olfactory perception observed in these animals.

Lept-R was less expressed in HFHS 8w + gest-lact group compared to CTRL group. Because plasma leptin levels were higher in this group, we assume that lower expression of Lept-R in MOE reflects a compensatory mechanism that could have prevented a modulatory effect of leptin at the peripheral level of the olfactory system.

It is well known that leptin has been involved in neurodevelopment. The effects of leptin in the brain are not limited to the neural control of feeding behavior in mature animals: leptin can also influence neurodevelopmental processes in the immature brain. Leptin acts as a neurotrophic agent that promotes neurogenesis, axon growth, synaptic plasticity and

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dendritic morphology (Bouret, 2010). Leptin receptors are expressed in the brain as early as E10.5 (Hoggard et al., 1997; Udagawa et al., 2000), and are found in the adult olfactory system (Baly et al., 2007; Getchell et al., 2006; Shioda et al., 1998). During the leptin surge identified between PND 7 and 10 (Ahima et al., 1998), maternal HFHS diet exposure may have contributed to more elevated leptin levels leading to impairment of olfactory circuitry formation.

Other numerous peptides and hormones are involved in modulating olfaction (Palouzier-Paulignan et al., 2012). These metabolic factors could have participated in the modulation of olfactory abilities in the progeny of HFHS diet fed dams. Insulin is known to exert effects on OSNs and olfactory perception similar to the action of leptin. In our design, the glycaemia of pups from dams fed with the HFHS diet was not different from the glycaemia of CTRL pups, and they presented normal glucose tolerance. Therefore, insulin levels and regulation might not be impaired in these pups, making insulin unlikely to alter their olfactory behavior. However, orexins, ghrelin, glucagon, were not quantified in this study. Further work is needed to better characterize the effects of these other metabolic peptides in pups born to mice fed with a HFHS diet.

To our knowledge, we provide for the first time evidence for a deleterious impact of maternal HFHS diet on progeny's olfactory abilities. Impaired olfaction in the 3-week old progeny was not correlated to modifications of olfactory mucosa functioning. We infer that central processing of olfactory information might be affected by the perinatal exposure to HFHS diet. Because leptin is involved in both modulating the activity of olfactory neurons and brain developmental processes, leptin is a strong candidate to constitute the link between maternal HFHS diet and altered olfactory perception in progeny. When (i.e. during embryonic development or at postnatal stages), where (i.e. in the OB, piriform cortex) and how (i.e. by regulating proliferation, dendritic morphology) leptin could potentially act remain to be determined.

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AUTHOR CONTRIBUTIONS

LM, DJ and XG designed the research. LM, OP, PB, SG, VS and DJ performed experiments. LM and DJ analyzed data and performed statistical analyses. LM, DJ and XG wrote this paper. Graphical abstract and figure 3 were made using images from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License (CC-BY) and available at <https://smart.servier.com/>.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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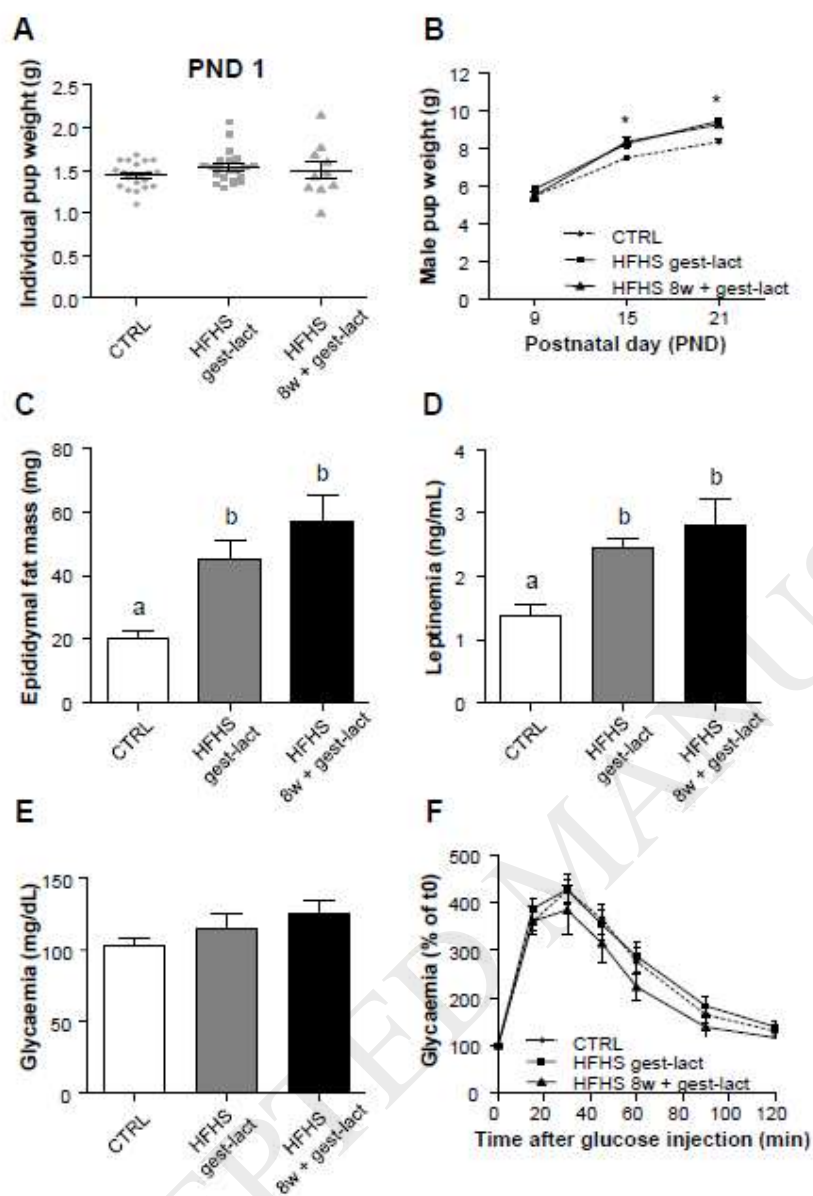


Figure 1

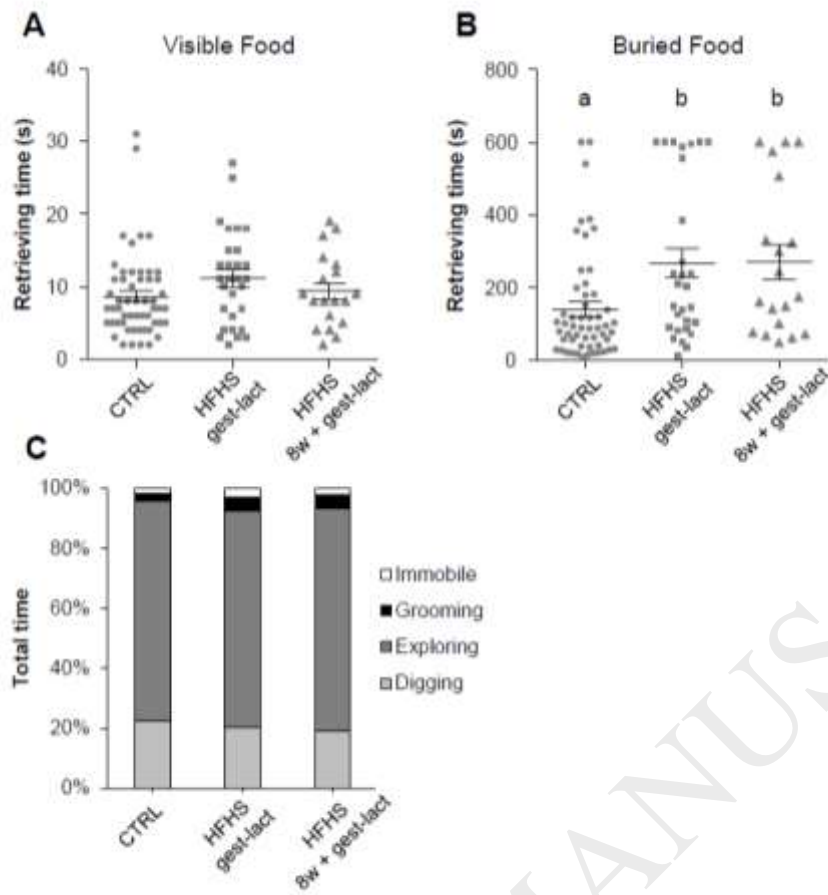


Figure 2

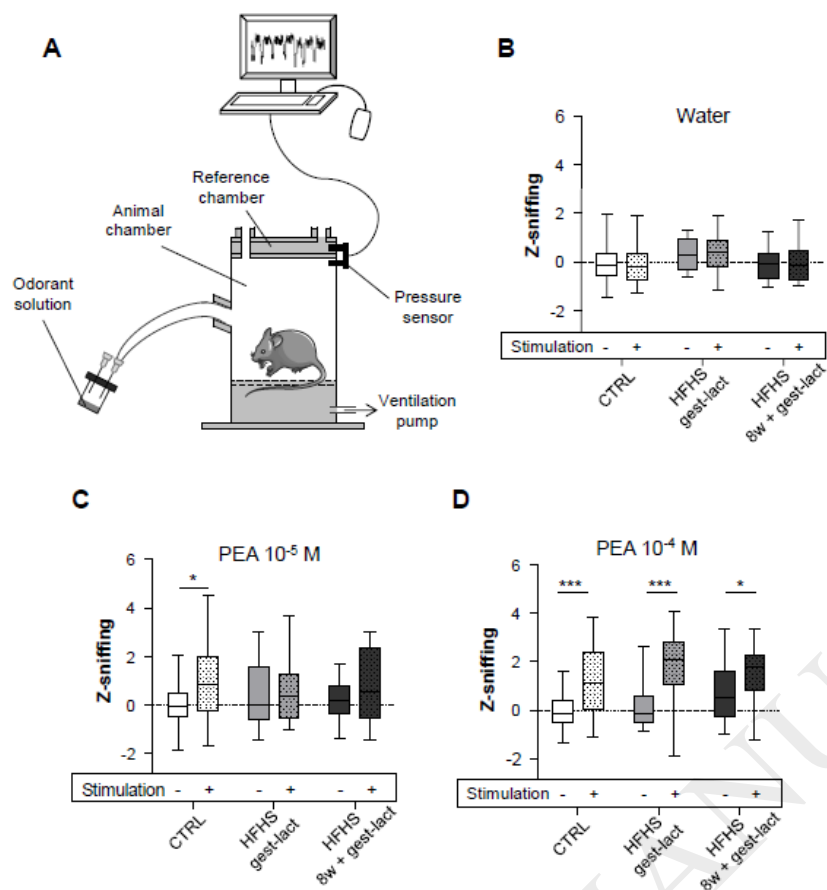


Figure 3

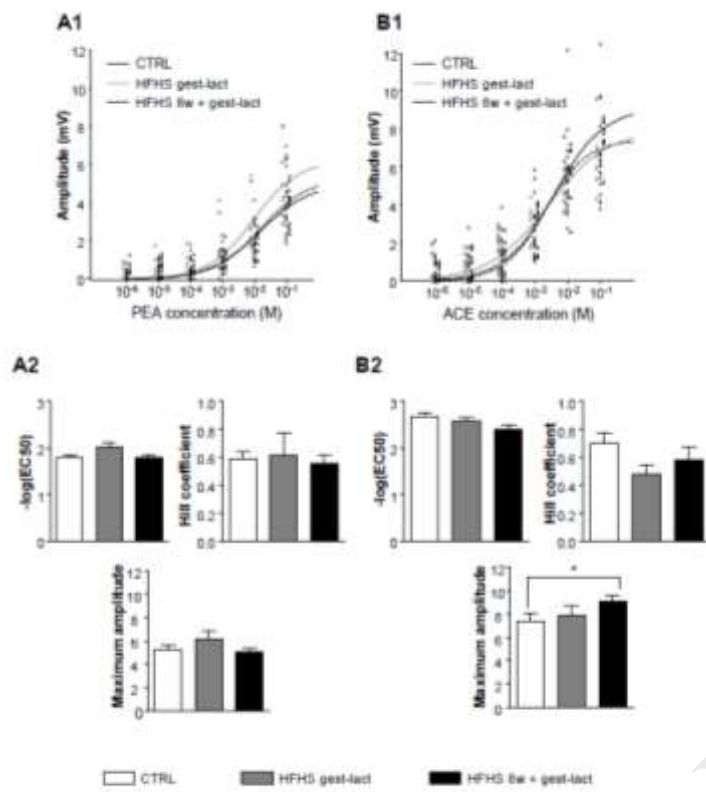


Figure 4

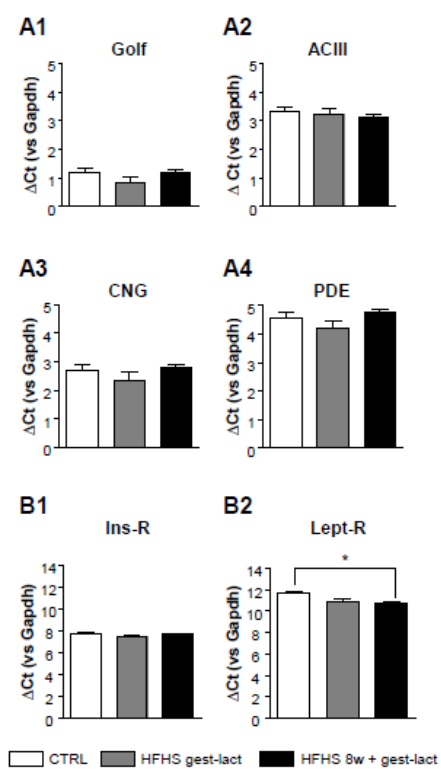


Figure 5

Table 1

	CTRL	HFHS gest-lact	HFHS 8w + gest-lact
Milk production (g/pup)	0.14 ± 0.025	0.19 ± 0.017	0.20 ± 0.016
Milk lipids (% weight)	23.42 ± 0.97	26.63 ± 1.35	25.83 ± 1.45
SFA (% total lipids)	56.07 ± 1.29	48.98 ± 1.57***	43.25 ± 1.58**
MUFA (% total lipids)	25.61 ± 0.99	45.47 ± 1.39***	43.30 ± 1.51**
PUFA (% total lipids)	18.32 ± 0.41	13.55 ± 0.22**	13.46 ± 0.35**
- n-6 (% total PUFA)	16.45 ± 0.38	12.42 ± 0.21**	12.27 ± 0.32**
- n-3 (% total PUFA)	1.78 ± 0.05	1.03 ± 0.02**	1.10 ± 0.31**
- n-6 / n-3 ratio	9.27 ± 0.22	12.10 ± 0.32***	11.22 ± 0.31**
Milk proteins (% weight)	21.58 ± 0.99	20.86 ± 0.53	21.25 ± 1.22
Milk lactose (% weight)	3.10 ± 0.43	2.90 ± 0.37	3.29 ± 0.74