Title

Exposure to environmentally relevant doses of plasticizers alters maternal behavior and related neuroendocrine processes in primiparous and multiparous female mice

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Abstract

Phthalates are organic pollutants frequently detected in the environment. The effects of these substances on male reproduction have been extensively studied but their potential impact on female reproductive behaviors in particular at environmental doses still remains to be documented. In the present study, we examined the effects of chronic exposure to di(2-ethylhexyl) phthalate (DEHP) alone at 5 or 50 μg/kg/d, or in an environmental phthalate mixture on maternal behavior of lactating female mice after a first (primiparous) and a second gestation (multiparous). Exposure of DEHP alone or in a phthalate mixture reduced pup-directed behaviors, increased self-care and forced nursing behaviors and altered nest quality for both primiparous and multiparous dams. In pup-retrieval test, primiparous and multiparous dams exposed to DEHP alone or in a phthalate mixture retrieved their pups more rapidly, probably due to a higher emission of ultrasonic vocalizations by the pups. At lactational day 2 following the third and last gestational period, the neural circuitry of maternal behavior was analyzed. A lower number of oxytocin-immunoreactive neurons in the paraventricular and anterior commissural nuclei was found in dams exposed to DEHP alone or in a phthalate mixture, while no changes were observed in the number of arginine-vasopressin immunoreactive cells. In the medial preoptic area, exposure to DEHP alone or in a phthalate mixture reduced ERα-immunoreactive cell number. Dendritic spine density assessed for DEHP at 5 μg/kg/d was also reduced. Thus, exposure to DEHP alone or in a phthalate mixture altered maternal behavior probably through a neuroendocrine mode of action involving oxytocin and estrogen through ERα, key pathways necessary for neuroplasticity and behavioral processing.

1. Introduction

Phthalates, mainly used as plasticizers, are widely detected in the environment (Gao and Wen, 2016). Among the phthalate family, butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), dicyclohexyl phthalate (DCHP) and di-isobutyl phthalate (DiBP) were identified as endocrine disrupting compounds for human health, and di(2-ethylhexyl) phthalate (DEHP) was recognized to have endocrine disrupting property for both human health and environment (for review: Adam and Mhaouty-Kodja, 2022). Phthalates have mainly been studied for their ability to alter testosterone production and male urogenital tract development (Howdeshell et al., 2008; Rowdhwal and Chen, 2018), but recent studies have shown that they can also induce female reproductive dysfunctions. Epidemiological studies reported associations between phthalates and the age of puberty in girls, ovarian functions, or development of endometriosis (for reviews: Hlisníková et al., 2020; Mesquita et al., 2021). Experimental studies performed on rodents described adverse effects on puberty onset, ovarian function and fertility (Fletcher et al., 2022; Hannon and Flaws, 2015; Hlisníková et al., 2020).

In this context, the potential effects of phthalate exposure on the neural control of female reproduction have been less studied. In particular, few studies have addressed the effects of adult chronic exposure to phthalates at environmentally relevant doses (Adam and Mhaouty-Kodja, 2022). We recently showed that adult exposure to low doses of DEHP alone or in a phthalate mixture reduced lordosis behavior, olfactory preference towards males, and the ability to attract and stimulate the emission of male ultrasonic vocalizations (Adam et al., 2021). These behavioral alterations were associated with reduced progesterone receptor (PR)-immunoreactivity in the neural circuitry underlying female sexual behavior. It remains to be investigated whether adult chronic exposure to phthalates, under similar experimental conditions, alters other reproductive behaviors such as maternal behavior.

Maternal behavior includes all the behaviors elicited by the mother to ensure and improve pup survival and growth. Starting from pregnancy, female rodents engage in an intensive nest building and exhibit increased aggression towards intruders (Numan and Insel, 2003). After birth, dams show a strong interest towards pups by exhibiting licking and nursing behaviors. Dams also display a retrieving behavior in response to ultrasonic vocalizations (USVs) emitted by pups when they are isolated outside the nest. Sensory information from the pups is integrated in the neural circuitry in which the hypothalamic medial preoptic area plays a key role under the control of a timely regulated hormonal sequence (Keller et al., 2019). In particular, the role of the oxytocin signaling pathway induced by estrogens at the end of pregnancy has been largely described (Yoshihara et al., 2017).

In a previous study, Lee et al. (2021) reported that developmental exposure of rats to DEHP at 10 mg/kg/d disturbed pup retrieval behavior. The effects of adult exposure to phthalates were addressed in four studies using an exposure period beginning during gestation and lasting until the lactational period. One study reported that exposure to relatively high doses of DBP (50 or 100 mg/kg/d) altered pup retrieval behavior as well as nest quality (Lee et al., 2020). In contrast, exposure of rats to high DINP doses (300-900 mg/kg/d) or to a phthalate mixture at low doses ranging from 0.2 to 1 mg/kg/d, or exposure of mice to low DEHP doses (5 to 400 µg/kg/d) was not found to disturb pup retrieval or maternal behavior (Boberg et al., 2011; Kougias et al., 2018; Quinnies et al., 2017). The causes of this discrepancy are not clear, but they might be related to differences in the experimental conditions (strain, duration of exposure, lactational period of analyses, etc.).

The present study was designed to perform a thorough analysis of the effects induced by chronic exposure to DEHP alone or in an environmental mixture on maternal behavior and

potential underlying mechanisms. Adult C57BL/6J female mice were exposed to the vehicle (control group), DEHP alone at the tolerable daily intake (TDI) dose (50 μg/kg/d) or at a dose in the range of the environmental exposure (5 μg/kg/d), or to an environmental phthalate mixture containing DEHP (5 μg/kg/d), DBP (0.5 μg/kg/d), BBP (0.5 μg/kg/d), DiBP (0.5 μg/kg/d) and DEP (0.25 μg/kg/d) as previously described (Adam et al., 2021). Dams were exposed for 6 weeks before the first mating and treatment lasted until lactational day (LD) 2 following the third pregnancy and delivery. Primiparous (first litter) or multiparous (second litter) dams were analyzed for spontaneous behaviors including nest quality, pup-directed and self-care behaviors. They were also subjected to pup retrieval testing, and their pups were analyzed for the emission of ultrasonic vocalizations. Brains collected from dams at the end of treatment (LD2 following the third delivery) were analyzed for oxytocin- and arginine vasopressin (AVP)-immunoreactivity in the paraventricular, supraoptic and preoptic nuclei, together with estrogen receptor α (ERα)-immunoreactivity and dendritic spine density in the preoptic area.

2. Material and methods

2.1. Animals and treatments

Studies were performed according to the European legal requirements (Decree 2010/63/UE) and were approved by the "Charles Darwin" Ethical committee (project number 01490-01). Experiments followed the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines.

Mice were obtained from breeding of male and female C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France). They were housed in nest-enriched polysulfone cages with polysulfone bottles, at 22°C under an inverted photoperiod, i.e., dark time began at 1:00 p.m. with a 12:12 h light-dark cycle, with free access to food (A03-10; Safe-diets, Augy, France) and water.

Four treatment groups comprising each 12 adult eight-week old female mice were respectively exposed through the diet to the vehicle, DEHP at 5 µg/kg/d, DEHP at 50 µg/kg/d or DEHP in a phthalate mixture as previously described (Adam et al., 2021; Ahmadpour et al., 2021, 2022) and illustrated in Figure 1A. The DEHP dose of 50 µg/kg/d corresponds to the tolerable daily intake dose established by the European Food Safety Agency (EFSA, 2019). The DEHP dose of 5 µg/kg/d, within the estimated environmental exposure, and the mixture composition and doses of phthalates in this mixture were based on French and European studies showing the co-exposure to these substances (Blanchard et al., 2013) and the presence of their metabolites in urinary samples (Anses, 2015; Dewalque et al., 2014). Altered female estrous cyclicity and sexual behavior were observed for these doses of DEHP alone or in a phthalate mixture (Adam et al., 2021).

Females were weighed to adjust phthalate doses every 5 days. Data on the mean body weight throughout the whole study are indicated in Figure S1A.

After 6 weeks of phthalate exposure, females were mated and treatment was maintained during three subsequent pregnancy and delivery periods (Figure 1B). Protocol details are available in Supplemental Methods. The treatment ended two days after the last delivery, and brains were collected and processed for immunofluorescence and dendritic spine density analyses.

2.2. Behavioral analyses

Tests were conducted 2 h after the beginning of the dark phase under red-light illumination, except for the nest score evaluation that was performed during the light phase. Dams in their home cages were acclimated for 3 h to the experimental room before the beginning of the behavioral observations by a blind experimenter. Analyses were performed during the lactational period following the first and second mating (Figure 1B) The analyses included spontaneous maternal behavior observation, nest score evaluation and pup retrieval tests. Pup ultrasonic vocalizations were also recorded for multiparous dams.

2.2.1. *Spontaneous maternal behavior observation*

The protocol of behavioral assessment was adapted from Palanza et al. (2002). Primiparous and multiparous dams were observed daily from LD 2 to 15 in their home cages for 120 min. Each dam was observed every 4 min, for a total of 30 observations per female. The observer recorded the behavior the dam was displaying at the moment of the observation. The observed behaviors included pup directed-behaviors (nursing, pup licking and nest building), self-care behaviors (eating and drinking, grooming and resting outside the nest), and forced nursing as defined in Supplemental Methods. Data were expressed as the sum of behaviors displayed during the fourteen days of observations.

2.2.2. Nest score evaluation

156 The nest built by each dam was observed during the light phase (10 a.m.) at LD3, LD7, LD11,
157 and LD15 for primiparous and multiparous dams. A nest quality score was given based on the
158 scale of Hess et al. (2008). A score of 0 corresponds to undispersed nesting material while 5
159 corresponds to a complete dome nest.

160 *2.2.3. Pup retrieval test*

161 Pup retrieval tests were performed at LD3, LD7, and LD11 after spontaneous maternal behavior
162 observations. Primiparous and multiparous dams were briefly removed from their home cage
163 and reintroduced after placing four pups in the opposing side of the nest. The cage was recorded
164 for 10 min, and the recordings were analyzed a posteriori. The time taken by the dam from
165 touching the first pup to retrieving the last pup to the nest was measured.

166 *2.2.4. Pup emission of ultrasonic vocalizations*

167 A first recording of emitted ultrasonic vocalizations (USVs) was conducted on PND3 pups born
168 from primiparous females (Figure S2). Analyses of the obtained pup retrieval data motivated
169 further recording of USVs at PND3, PND7 and PND11 for pups born from multiparous dams.
170 Tests were performed at least 30 min after a pup retrieval test.
171 One male and one female from each litter were tested. Each pup was put in a beaker placed on
172 a 25°C heating pad and covered with a sound-reducing box, USVs were recorded for 3 min as
173 previously described (Adam et al., 2021; Capela and Mhaouty-Kodja, 2021; Dombret et al.,
174 2017). The total number and duration of USVs and of each syllable type were analyzed.
175

176 ***2.3. Necropsy and tissue collection***

177 Multiparous dams with a litter after the third mating (vehicle: n = 10; DEHP-5: n = 10; DEHP-
178 50: n = 8; Mix: n = 8) were euthanized at LD2 by pentobarbital injection (120 mg/kg) (Figure
179 1B). Six females from each treatment group were transcardially perfused with a solution of 4%
180 paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains from 5-6 dams were post-

fixed overnight in 4% PFA in PBS and kept in a 0.5% PFA-PBS solution until immunofluorescence analysis was carried out. The remaining brains from 4 dams of the vehicle and the DEHP-5 groups were processed for Golgi-Cox staining; the lower number of lactating females from the DEHP-50 and Mix-groups did not enable us to perform such an analysis.

2.4. Immunofluorescence

Brains collected from multiparous females were sliced into 30-µm thick coronal sections in a vibratome (Leica Microsystems VTC1000S, Wetzlar, Germany). Oxytocin- and vasopressin-immunolabeling was conducted as previously described for incubation with primary rabbit antisera against AVP or OT with 1:4000 dilution (Vacher et al. 2002). ERα immunolabeling using 1/400 diluted polyclonal anti-ERα (Santa Cruz Biotechnology) was detailed in Adam et al. (2021). The detection of the bound primary antibodies was performed for 2 h using the Alexa-Fluor 488-conjugated donkey anti-rabbit secondary antibody (A21206, Life Technologies-Invitrogen) diluted at 1:500.

Brain sections were scanned by a high-resolution NanoZoomer Hamamatsu scanner (Hamamatsu Corporation). The number of oxytocin-, arginine vasopressin- and ERα-labeled cells was quantified from anatomically matched sections identified using the Mouse Brain Atlas of Paxinos et al. (2001) as shown in Figures S3, S4 and S5, respectively. Immunolabeled cells for oxytocin and arginine-vasopressin were analyzed within the paraventricular and supraoptic nuclei (plates 36-41 and 36-39, respectively) and the anterior commissural nucleus of the dorsal preoptic area in Bregma -0.10 (Yoshihara et al. 2017). Data are expressed as the mean of immunoreactive cells per section. The number of ERα-immunoreactive cells was counted in the medial preoptic area within an area of 0.80 mm^2 (plate 30), in the bed nucleus of the stria terminalis within an area of 0.70 mm^2 (plate 30) and in the medial amygdala within an area of 0.56 mm^2 (plate 46). Data are expressed as the total number of immunoreactive cells per area.

2.5. Golgi-Cox staining

Brains collected from multiparous females were fixed for 8 days in Golgi-cox solution and cut into 30-μm thick coronal slices, which were post-impregnated in the same solution as previously described (Jean et al., 2017). Twelve neurons localized in the medial preoptic area were analyzed for each animal. The analyses were performed for each neuron on a dendritic length of 50 μm localized at 30 μm from the soma. Subtypes of dendritic spines were determined by applying the previously described criteria (Harris et al., 1992).

2.6. Statistics

Data expressed as means ± S.E.M were analyzed using GraphPad Prism 6 (GraphPad Software). Shapiro-Wilk test was performed to analyze the normality of data. A student paired t-test was used to analyze the number of dendritic spines. For data passing the normality test, the one-way analysis of variance (ANOVA) was used to analyze the main effect of exposure, followed by Bonferroni's post hoc tests to determine group differences. For data that did not pass the normality test, the Kruskal-Wallis test was used to analyze the effect of exposure, followed by Dunn's post hoc to determine group differences. Two-way ANOVA was used to analyze the pup USVs sex differences. P values less than 0.05 were considered significant.

3. Results

3.1. Effects of exposure to DEHP alone or in a phthalate mixture on reproductive parameters

Table S1 summarizes the reproductive parameters for primiparous and multiparous dams, including the ratio of pregnant and parturient dams out of the initial total number of mated females, gestation duration, litter size and sex of pups, as well as pup survival. No effects of the treatment to DEHP alone or in a phthalate mixture were found on any of these parameters, despite a tendency to decrease of pup survival for multiparous dams from the DEHP-50 group. The body weight of females monitored throughout gestation and lactation was also comparable in the four treatment groups (Figure S1A). Their corresponding pups were also weighed during the period of behavioral analyses at postnatal days (PND) 3, 7, 11 and 15. No treatment effects were observed on the mean body weight of pups either (Figure S1B).

3.2. Spontaneous behaviors elicited by primiparous and multiparous dams exposed to DEHP alone or in a phthalate mixture

Pup-directed versus selfcare behaviors

Maternal behaviors exhibited by primiparous and multiparous dams were observed in their home cages from LD2 to LD15. Figure 2A shows the effect of treatment on the total number of pup-directed behaviors exhibited by primiparous dams ($p = 0.0002$). A lower number of behaviors was observed for DEHP-5 (-26%), DEHP-50 (-34%) and Mix-groups (-21%) compared to the vehicle group. Detailed analyses show an effect of treatment on the number of nursing ($p = 0.001$), nest building ($p = 0.0002$) and pup licking behaviors ($p = 0.027$), with a reduced number of the two first behaviors in all three treatment groups and of the last behavior in the DEHP-5 group (Figure 2B). Figure 2C shows that an effect of treatment was observed on the number of self-care behaviors ($p = 0.003$), with an increased number of events for DEHP-

5 (+25%), DEHP-50 (+23%) and Mix-groups (+21%) compared to the vehicle group. Analyses illustrated in Figure 2D show an effect of treatment on eating-drinking behavior ($p = 0.004$), with increased behaviors for the three treatment groups (+34%, +33% and +35%, respectively) compared to the vehicle group. Resting outside the nest was also affected by the treatment ($p = 0.006$), with increased behaviors for the DEHP-50 and the Mix group (+293% and +225% versus the vehicle group, respectively).

In multiparous dams, there was an effect of treatment on pup-directed behaviors ($p = 0.029$), in particular on nursing ($p = 0.021$) and nest-building behaviors ($p = 0.005$) but not on pup licking despite a tendency to decrease (Figure 2E-F). Lower nursing behavior was observed in the DEHP-50 group (-26%), and nest building behavior was reduced in the DEHP-5 and DEHP-50 groups (-35% and -38%, respectively) compared to the vehicle group (Figure 2F). Treatment effect was also seen on self-care behaviors ($p = 0.041$), with a higher number of behaviors in the DEHP-50 group (+24% versus the vehicle group) (Figure 2G). This was due particularly to the treatment effect on resting behavior ($p = 0.004$), which was increased for DEHP-5, DEHP-50 and Mix groups (+163%, +225% and 189%, respectively) compared to the vehicle group. Eating-drinking behavior was no affected in multiparous females ($p = 0.324$) (Figure 2H).

In both primiparous and multiparous dams, grooming behavior was not affected by the treatment ($p = 0.49$ and $p = 0.63$, respectively) as shown in Figure 2D, H.

Nest score

The nest quality was analyzed at LD3, LD7, LD11 and LD15 by assigning a nest score, following the scale from Hess et al. (2008). For primiparous dams (Figure 3A), an effect of treatment on the nest score was observed at LD7 ($p = 0.024$) with a significant modification in the DEHP-5 group, and at LD15 ($p = 0.017$) with a significant change in the DEHP-50 group compared to the vehicle group. For multiparous dams (Figure 3B), an effect of treatment was

also observed at LD3 ($p = 0.036$), LD7 ($p = 0.019$) and LD15 ($p = 0.014$), with significant score changes in the DEHP-5 group compared to the control group.

Forced nursing

Exposure to DEHP alone or in phthalate mixture had an effect on forced-nursing behavior performed by pups from both primiparous ($p < 0.0001$) and multiparous dams ($p = 0.0004$) (Figure 3C). A higher number of pups exhibited this behavior in the DEHP-50 group of primiparous dams (+582%) and in DEHP-5, DEHP-50 and Mix groups of multiparous dams (+135%, +205% and +187%, respectively) compared to the corresponding vehicle groups.

3.3. Pup retrieval by primiparous and multiparous dams exposed to DEHP alone or in a phthalate mixture and emission of ultrasonic vocalizations by their pups

The ability of primiparous and multiparous dams to retrieve pups placed outside the nest was analyzed at LD3, LD7 and LD11 (Figure 4A-B). In primiparous dams, an effect of treatment on the retrieval duration was observed at LD3, LD7 and LD11 ($p = 0.011$, $p = 0.013$ and $p = 0.036$, respectively) (Figure 4A). A reduced duration was seen for DEHP-5 and DEHP-50 groups at LD3 (-29% and -36% versus the vehicle group, respectively) and for DEHP-50 and Mix groups at LD7 (-49% and -43%, respectively) compared to the vehicle group.

In multiparous dams, treatment affected the retrieval duration at LD3, LD7 and LD11 ($p = 0.007$, $p = 0.017$ and $p = 0.013$, respectively) (Figure 4B). A significant decrease was observed at LD3 for DEHP-5 and Mix groups (-30% and -33%, respectively), and for DEHP-50 at LD7 and LD11 (-39% and -49%, respectively) compared to the vehicle group.

Pup retrieval is elicited by dams in response to the USVs emitted by their pups, so USV emission by isolated pups was recorded. The data were grouped for the two sexes since our analysis did not show sex differences in the number of emitted USVs at any of the postnatal

300 days tested (Figure S6). A first analysis conducted on PND3 pups born from primiparous
301 females shows an effect of treatment on the number ($p = 0.027$) and duration of emitted USVs
302 ($p = 0.002$), with significantly increased parameters in DEHP-5 and DEHP-50 groups (Figure S2).
303 Thorough analysis of pups from multiparous dams shows also an effect of treatment on the total
304 number of emitted USVs at PND3 ($p = 0.014$), PND7 ($p = 0.0004$) and PND11 ($p < 0.0001$)
305 (Figure 4C). Comparison with the vehicle group shows a higher number for the DEHP-5 group
306 at PND7 (+265%) and PND11 (+250), for the DEHP-50 group at PND3, PND7 and PND11
307 (+275%, +238%, +193%, respectively), and for the Mix group at PND7 (+183%). A tendency
308 to decrease was also observed at PND3 for DEHP-50 and Mix-groups as indicated by post hoc
309 analyses ($p = 0.0585$ and $p = 0.0703$, respectively, versus the vehicle group).
310 Similarly, an effect of treatment was observed on the USV duration at PND3, PND7 and PND11
311 ($p = 0.031$; $p = 0.0002$ and $p = 0.001$, respectively) (Figure 4D). A longer USV duration was
312 observed at PND7 and PND11 for DEHP-5 (+411 % and +263%, respectively), DEHP-50
313 (+448% and +156%) and Mix groups (+433%, and 170%, respectively) compared to the vehicle
314 group.
315 A detailed analysis of emitted syllables was performed for pup vocalizations emitted at PND7
316 (Figure S7A). For the simple category, the treatment affected the total number of short ($p =$
317 0.0002) and downward syllables ($p = 0.023$) (Figure S4B), with a higher number of short
318 syllables emitted by pups from DEHP-5 and DEHP-50 groups (+192% and +241%,
319 respectively), and downward syllables emitted by pups from the DEHP-50 group (+141%)
320 compared to the vehicle group. The treatment also affected the total duration of short ($p =$
321 0.0002), flat ($p = 0.007$) and downward syllables ($p = 0.031$) (Figure S7E), with a 200% higher
322 duration of short syllables for all treatment groups and a 600% higher duration of flat syllables
323 for the DEHP-50 group.

For the complex category (Figure S7C, F), the treatment affected the number and duration of both modulated ($p = 0.011$ and $p = 0.001$, respectively) and complex syllables ($p = 0.012$ and $p = 0.04$, respectively), with a higher number of modulated syllables for the DEHP-50 group (+230%) and a higher duration of modulated syllables for all groups (+417%, +533%, +350%, $p = 0.005$) compared to the vehicle group. For the frequency jump category, the treatment altered both the number and the duration of one-jump syllables ($p = 0.002$ and 0.011), with a higher number and duration for the Mix group (+624%, and +1250%) compared to the vehicle group (Figure S7D, G). Interestingly, no pups from the control group emitted frequency-jump syllables. These syllables were emitted by pups from the DEHP- and Mix- treated groups. An effect of treatment was observed on both the number and the duration of frequency-jump syllables ($p = 0.011$ and $p = 0.006$, respectively).

3.4. Neural oxytocin-immunoreactivity in multiparous dams exposed to DEHP alone or in a phthalate mixture

Given the importance of the oxytocinergic system in the expression of maternal behavior, we wished to find out whether the altered behavior of exposed dams was related to modifications in this neuroendocrine system. Analysis of oxytocin immunoreactivity was thus undertaken at LD2 following the third mating/delivery session. Specific cytoplasmic oxytocin-immunoreactivity was detected in the paraventricular nucleus, the supraoptic nucleus and the anterior commissural nucleus (Figures 5A, S3). Quantitative analyses show an effect of treatment on the number of oxytocin-immunoreactive neurons in the paraventricular nucleus ($p = 0.009$) and the anterior commissural nucleus ($p = 0.002$), but not in the supraoptic nucleus ($p = 0.231$) (Figure 5B). Post-hoc analyses showed a lower number of oxytocin-immunoreactive neurons in the paraventricular nucleus of DEHP-5 and DEHP-50 dams (-26% and -27% versus the vehicle group, respectively). A lower number of oxytocin-immunoreactive neurons was also

observed in the anterior commissural nucleus of DEHP-50 and Mix dams (-29% and -28 versus the vehicle group, respectively).

Given the similarities in structure and function between oxytocin and arginine-vasopressin (AVP), we investigated whether the treatment also affected the number of AVP-immunoreactive neurons. Specific cytoplasmic AVP-immunoreactivity was detected in the paraventricular nucleus and the supraoptic nucleus (Figures S4, S8A), but quantitative analyses revealed no effect of treatment on AVP immunoreactivity in these regions (Figure S8B).

3.5. ERα-immunoreactivity and dendritic spine density in the medial preoptic area of multiparous dams exposed to DEHP alone or in a phthalate mixture

The medial preoptic area, the key region involved in processing of maternal behavior, integrates signals from oxytocin under the control of ERα-mediated regulation by estrogens (Champagne et al., 2003; Young et al., 1998). We thus quantified ERα-immunoreactivity in both the medial preoptic area and upstream brain areas including the medial amygdala and bed nucleus of stria terminalis. Figure 6A shows the presence of a specific nuclear signal in these three brain areas. An effect of treatment was found on the number of ERα-immunoreactive neurons in the medial preoptic area ($p = 0.005$), with a lower number in DEHP-50 dams (-38% versus the vehicle group) (Figures 6B, S5). No treatment effects were observed in the two other brain regions.

Previous studies have shown that morphological and functional modifications related to dendritic spine density occurring in the medial preoptic area are important in the retention of maternal behavior in experienced dams (Okino et al., 2020). The density and morphology of spines, which are emanating from neuronal dendrites and known to constitute excitatory post synapses, were thus analyzed in the medial preoptic area of multiparous dams from the vehicle and DEHP-5 groups. For this, brains were processed in Golgi-Cox staining and dendritic spines were analyzed and classified in stubby, thin and mushroom types according to their level of

maturation as previously described (Jean et al., 2017). Figure 6C-D shows a lower number of total spines, which seemed to be mainly due to a reduced number of mushroom spines (-39% versus the vehicle group).

4. Discussion

The present study is the first to show that chronic exposure to low doses of DEHP alone or in an environmental mixture disturbs maternal behavior in both primiparous and multiparous dams. These behavioral alterations were to the observed reduced oxytocin-immunoreactivity in the paraventricular nucleus and ACN, and ERα-immunoreactivity and dendritic spine density in the medial preoptic area, the key brain areas controlling the expression of maternal behavior.

Exposure of females to DEHP alone or in an environmental mixture affected the expression of spontaneous maternal behavior in both naive (primiparous) and maternally-experienced (multiparous) dams. Behaviors directed toward pups (nursing, nest building and pup licking) were reduced while, conversely, self-care behaviors (eating and drinking, outside the nest) were increased compared to the vehicle group. A global diminished nest quality was also observed in primiparous and multiparous dams exposed to DEHP alone or in a phthalate mixture. Despite these effects on maternal behavior, pup growth did not seem to be affected as evidenced by the comparable body weight measured for pups from mothers of the four treatment groups. This was possibly due to the fact that pups from primiparous or multiparous dams exposed to DEHP alone or in a phthalate mixture displayed more forced-nursing behavior. This kind of compensatory behavior exhibited by pups may then counterbalance the reduced nursing behavior of their corresponding mothers.

When pups were placed outside the nest, dams exposed to DEHP alone or in an environmental mixture retrieved their pups with a shorter latency compared to the vehicle group. This may appear contradictory with the reduced spontaneous behavior reported above. However, it is important to stress that pup retrieval behavior is induced in response to USVs emitted by isolated pups. Interestingly, analyses of pup USV emission show that those born

403 from dams exposed to DEHP alone or in a phthalate mixture produced a higher number and
404 duration of USVs. First, this may explain, at least in part, why these pups were retrieved more
405 rapidly by their mothers. Second, these data indicate that pup retrieval test, when used alone,
406 cannot account for the effects on maternal behavior. An overall analysis including also
407 spontaneous behaviors and pup emission of vocalizations is required.
408 The detailed analyses of USVs show that six out of the nine syllables (short, downward,
409 modulated, complex, one-jump, frequency-jump) were disturbed. In particular, pups from the
410 vehicle group did not show any emission of frequency-jump syllables, which were produced
411 by those exposed through their mothers to DEHP alone or in a phthalate mixture. Whether this
412 effect of pup-emitted vocalizations results from direct developmental exposure to phthalates
413 and/or from a reduced maternal behavior remains to be investigated.
414 We recently reported reduced number and duration of USV emission during mating in adult
415 males exposed under similar experimental conditions to DEHP alone or in a phthalate mixture
416 (Capela and Mhaouty-Kodja, 2021; Dombret et al., 2017). To our knowledge, this is the first
417 study showing an alteration of pup vocalizations following maternal exposure to phthalates.
418 Altered pup emission of ultrasonic vocalizations is widely used as a marker of autism-like
419 behaviors in rodents (Fischer and Hammerschmidt, 2011). It is possible that these offspring
420 exhibited deficits in social interaction. In agreement with this hypothesis, previous studies
421 reported altered social behavior following perinatal exposure to phthalates in male and female
422 rats and mice (Kougias et al., 2018; Morová et al., 2021; Quinnies et al., 2017, 2015; Zhang et
423 al., 2021).
424 Overall, the present studies indicate an impact of exposure to low doses of DEHP alone or in a
425 phthalate mixture on maternal behavior. To our knowledge, this is the first study addressing
426 maternal behavior of both primiparous and multiparous dams. The comparable data obtained

for primiparous and multiparous dams indicate that the deleterious effects exerted by phthalate exposure were maintained and did not change with maternal experience.

The lower spontaneous behavior of dams reported in this study contrasts with the unaffected behavior observed at relatively low doses of DEHP in mice (Kougias et al., 2018) and of a phthalate mixture in rats (Quinnies et al., 2017). This could be related to differences in the treatment duration, since in our study the exposure started 6 weeks before the first mating session and lasted for a long period until LD2 following the third delivery, with analyses performed on both primiparous and multiparous dams.

The expression of maternal behavior is regulated by oxytocin neurons located in the paraventricular nucleus (Champagne et al., 2001; Pedersen et al., 1994). These neurons project to the medial preoptic area, where oxytocin acts on its oxytocin receptor to activate the behavior (Okabe et al., 2017). This circuitry is highly sensitive to estrogens, with a high expression of ERα in the medial preoptic area (Champagne et al., 2003; Fang et al., 2018; Ribeiro et al., 2012). It is of great interest that exposure to DEHP alone or in a phthalate mixture induced changes in this circuitry in multiparous dams. Oxytocin-immunoreactivity was selectively reduced in the paraventricular nucleus, while no effects were found on AVP-immunoreactivity. Reduced oxytocin-immunoreactivity was also found in the anterior commissure nucleus, a brain locus containing oxytocin neurons thought to modulate parental care in mammals (Yoshihara et al., 2017). This shows that the oxytocinergic system can be also impacted by adult exposure to phthalates. A previous study reported changes in the expression of hypothalamic oxytocin following developmental exposure of rats to DEHP at 10 mg/kg/d (Lee et al. 2021).

In parallel to these modifications, ERα-immunoreactivity was reduced in the medial preoptic area of dams exposed to DEHP alone or in a phthalate mixture. The behavioral effects and related changes in oxytocin- and ERα-immunoreactivity induced by exposure to DEHP alone

or in a phthalate mixture were observed in maternally-experienced dams. This suggests that these chemicals may impact neuronal connectivity and underlying neuroplasticity processes, which occur in the medial preoptic area and are necessary for behavioral maintenance. Quantification of dendritic spine density using Golgi staining in the medial preoptic area of multiparous dams showed a reduced total number of dendritic spines. In particular, an effect was mainly found on mushroom spines, which represent the most stable and mature spines. It is largely reported that maturation of dendritic spines towards mushroom characterizes learning processes and synaptic strength in the hippocampus and medial preoptic area and are associated with behavioral improvement (Jean et al., 2017; Leuner and Shors, 2004; Mahmmoud et al., 2015). This suggests that exposure to DEHP alone or in a phthalate mixture interfered with neuroplasticity processes possibly involved in long-term maintenance of maternal behavior in the medial preoptic area. Previous studies showed that exposure of to DBP altered adult hippocampal neurogenesis in mice (Lee et al., 2019), while MEHP inhibited excitability and synaptic plasticity of CA3 hippocampal neurons (Lu et al., 2022). The present study indicates that neural processes may be also impacted by phthalate exposure in other brain regions including the hypothalamus.

In conclusion, the present data describing disturbed maternal behavior, together with our previous study showing impaired estrous cyclicity and sexual behavior (Adam et al., 2021), indicate that exposure to DEHP alone or in an environmental mixture may have an important impact on female reproduction in mice. For the majority of analyzed parameters and behaviors, comparable effects were observed for DEHP alone or in a phthalate mixture, suggesting that DEHP drove the effects possibly because it was more abundant than the other phthalates in this mixture.

476 The behavioral and neural effects of DEHP were observed at doses equivalent to or below the
477 TDI of 50 μg/kg/d, established on the basis of reduced fetal testosterone production EFSA,
478 2019, 2005). This is added evidence in favor of a high brain sensitivity to exposure to
479 environmental doses of phthalates. In particular, neuroendocrine pathways involving
480 progesterone receptor (Adam et al., 2021) and oxytocin and estrogen-dependent signaling
481 (present study) were altered by exposure to environmental doses of DEHP alone or in a
482 phthalate mixture. It is possible that these described effects and modes of action in mice may
483 also occur in other mammalian species including humans, given the conserved hormonal
484 regulation of reproductive processes.
485

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The authors declare they have no actual or potential competing financial interests.

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660 **Figure Legends**

661 **Figure 1. Schematic representation of the experimental design. A.** Adult females were
662 exposed to the vehicle, DEHP alone or in a phthalate mixture at the indicated doses per body
663 weight (bw) and per day (d). **B.** Adult females (F0 dams) were exposed for 6 weeks (W) before
664 mating, and exposure was maintained until the end of experiments. Females were mated three
665 times. Maternal behavior was analyzed after the first (primiparous dams) and second delivery
666 (multiparous dams) as indicated. After the third delivery, dams were euthanized at LD2 and
667 brains were processed for immunofluorescence and dendritic spine density analyses. F1
668 offspring born from F0 dams were assessed for body weight and emission of ultrasonic
669 vocalizations (USV). Behavioral tests included observations of spontaneous behaviors, pup
670 retrieval test and recording of pup USV emission.

671

672 **Figure 2. Effects of adult exposure to DEHP alone or in a phthalate mixture on pup-**
673 **directed and self-care behaviors.** Spontaneous maternal behaviors of primiparous (**A-D**) and
674 multiparous dams (**E-H**) dams (n = 8-11/treatment group) exposed to the vehicle (Veh), DEHP
675 at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix). **A, E**. Total number of pup-directed
676 behaviors displayed by dams from LD2 to LD15, expressed ad the sum of nursing, nest
677 building, and pup licking behaviors (**B, F**). **C, G**. Total number of self-care behaviors displayed
678 by dams from LD2 to LD15, expressed as the sum of eating-drinking, resting outside the nest,
679 and grooming behaviors (**D, H**). Data are presented as means ± S.E.M. One-way ANOVA for
680 the treatment effect (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$) followed by Bonferroni's multiple
681 comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the vehicle group) are shown.

682

683 **Figure 3. Effects of adult exposure to DEHP alone or in phthalate a mixture on forced-**
684 **nursing and nest score.** Behaviors were observed for primiparous and multiparous dams

exposed to the vehicle (Veh), DEHP at 5 or 50 μg/kg/d, or to a phthalate mixture (Mix) (n = 8-11/ treatment group). **A-B.** Nest score at lactational days (LD) 3, 7, 11 and 15 for primiparous (**A**) and multiparous (**B**) dams. **C.** Total number of forced-nursing behaviors exhibited by pups towards primiparous (left) and multiparous (right) dams. Data are represented as means ± S.E.M. One-way ANOVA for the treatment effect (#p < 0.05, ###p < 0.001) followed by Bonferroni's multiple comparisons tests (*p < 0.05, **p < 0.01, ***p < 0.001 versus the vehicle group) are shown.

Figure 4. Effects of exposure to DEHP alone or in a phthalate mixture on pup-retrieval by dams and pup vocalizations. A-B. Pup retrieval tests were performed lactational days (LD) 3, 7 and 11 for primiparous (**A**) and multiparous (**B**) dams (8-11/treatment group) exposed to the vehicle (Veh), DEHP at 5 or 50 μg/kg/d, or to a phthalate mixture (Mix). The duration corresponds to the time spent by the dam from touching the first pup until retrieving the last pup to the nest. **C-D.** Ultrasonic vocalizations (USV) emitted by pups (13-19/ treatment group) from multiparous dams were recorded at PND 3, PND7 and PND11. The mean number (**C**) and duration (**D**) of USVs are shown. Data are presented as means ± S.E.M. Kruskal-Wallis analyses for the treatment effect (#p < 0.05, ##p < 0.01, ###p < 0.001) followed by Dunn's multiple comparisons tests (*p < 0.05, **p < 0.01, ***p < 0.001 versus the control group) are indicated.

Figure 5. Effects of exposure to DEHP alone or in a phthalate mixture on oxytocinergic immunoreactivity. A. Representative oxytocin- (OT) immunoreactivity in the paraventricular (PVN), supraoptic (SON) and the anterior commissural nuclei (ACN) of multiparous dams (5-6/treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50 μg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 50 μm for the PVN and

100 μm for the SON and ACN. **B.** Quantitative analysis of the number of OT-immunoreactive (ir) neurons in the PVN, SON and ACN. Data are presented as means ± S.E.M. One-way ANOVA for the treatment effect (##$p < 0.01$) followed by Bonferroni's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, versus the control group) are indicated. 3V, 3rd ventricle; AC, anterior commissure; opt, optic chiasma.

Figure 6. Effects of exposure to DEHP alone or in a phthalate mixture on estrogen receptor (ER) α-immunoreactivity and dendritic spine density in the medial preoptic area. A. Representative ERα-immunoreactivity in the medial preoptic area (MPOA), bed nucleus of stria terminalis (BNST) and medial amygdala (MeA) of multiparous dams (5-6/treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50 μg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 100 μm. **B.** Quantitative analysis of the number of ERα immunoreactive (ir) neurons in the mPOA, BNST and MeA. **C.** Representative image of Golgi-Cox impregnated neurons of the MPOA in dams from the Veh and DEHP-groups. Scale bar = 50 μm. **D.** Quantification of the total number of dendritic spines and subtypes (thin, stubby, mushroom). Data are presented as means ± S.E.M. One-way ANOVA for the treatment effect (##$p < 0.01$) followed by Bonferroni's multiple comparisons test (**$p < 0.01$ versus the vehicle group) for (**B**), and paired Student test for (**D**) (*$p < 0.05$ versus the control group) are indicated. 3V, 3rd ventricle; AC, anterior commissure.

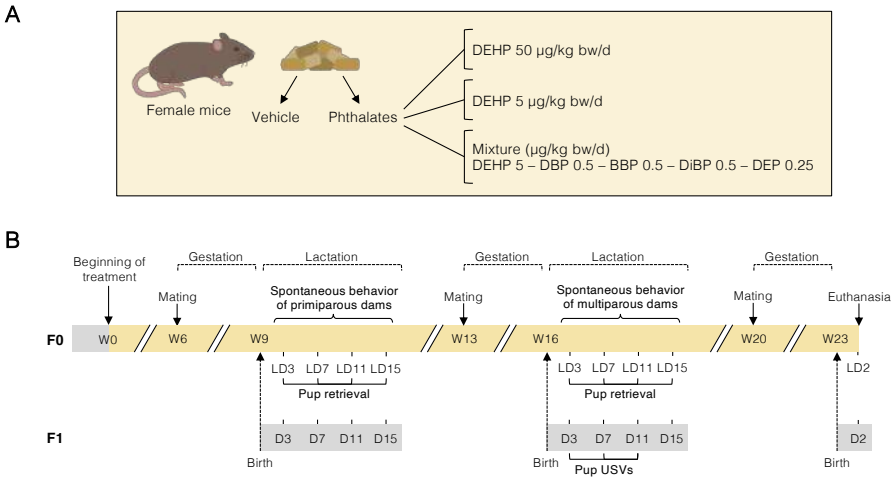


Figure 1

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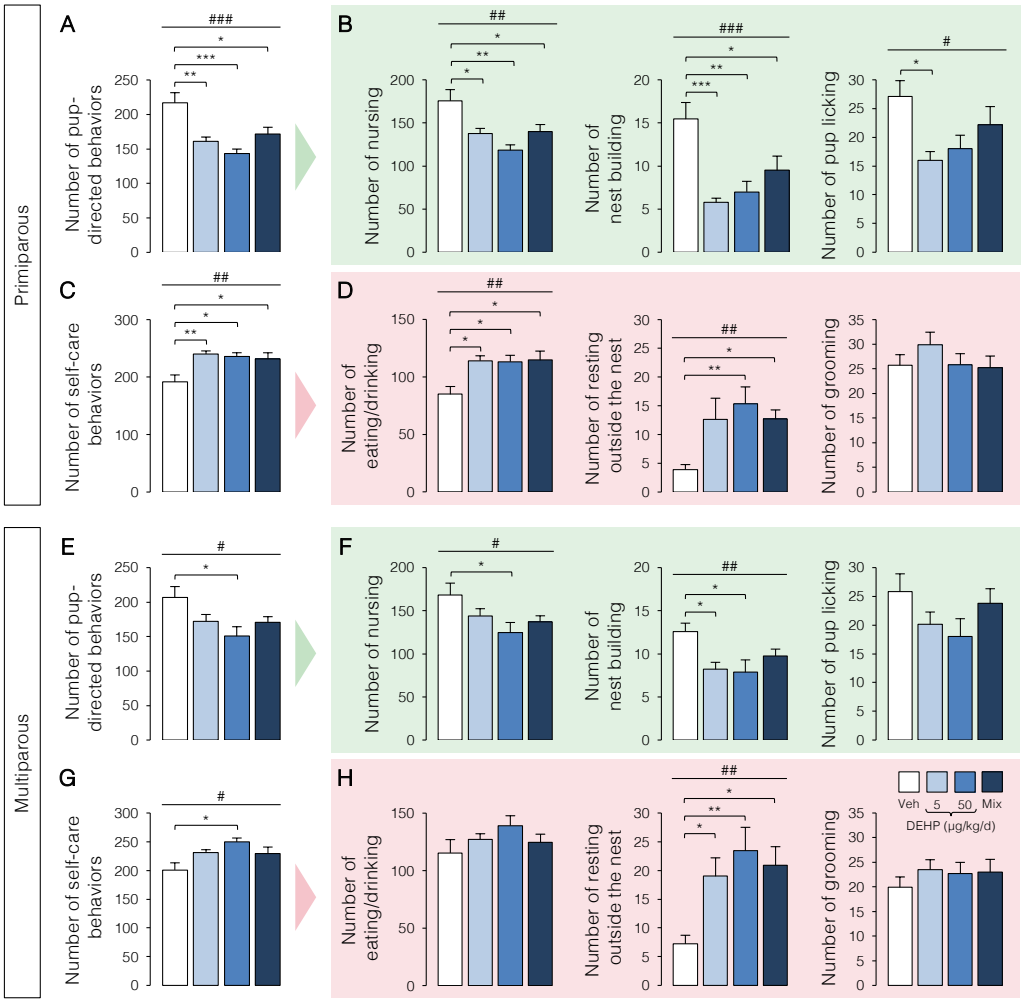

Primiparous
Multiparous
A
B
C
D
E
F
G
H
Number of pup-directed behaviors
Number of nursing
Number of nest building
Number of pup licking
Number of self-care behaviors
Number of eating/drinking
Number of resting outside the nest
Number of grooming
Veh
5
50
Mix
DEHP (μg/kg/d)

Figure 2

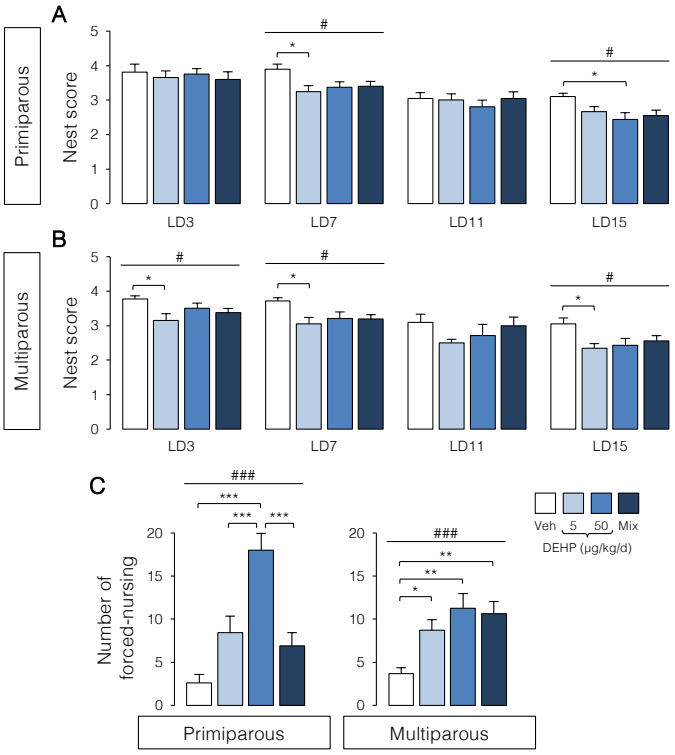


Figure 3

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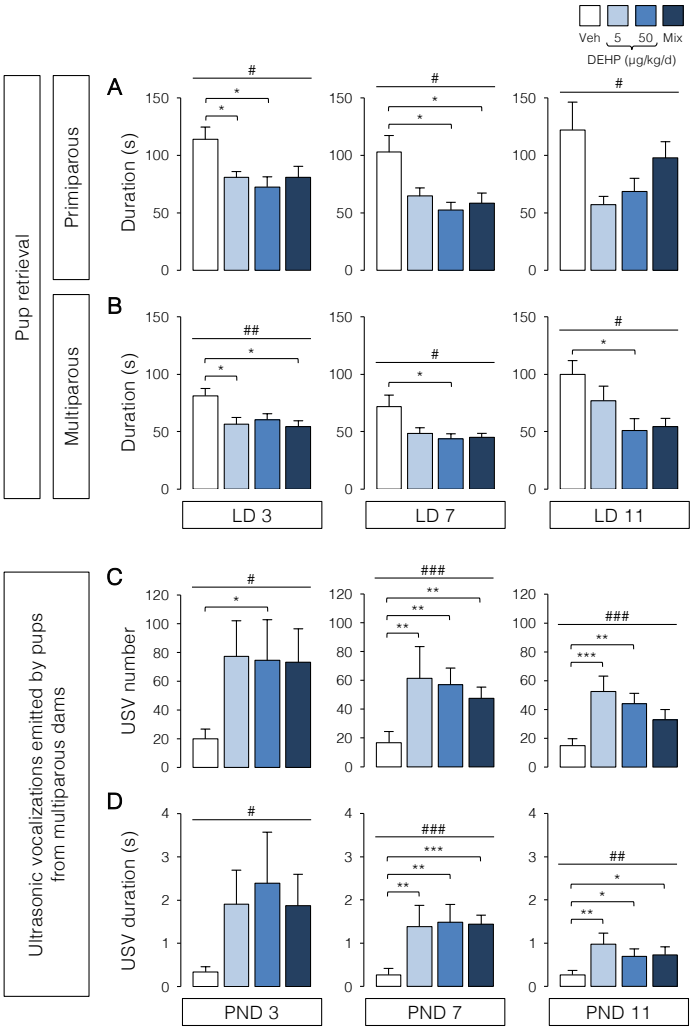


Figure 4

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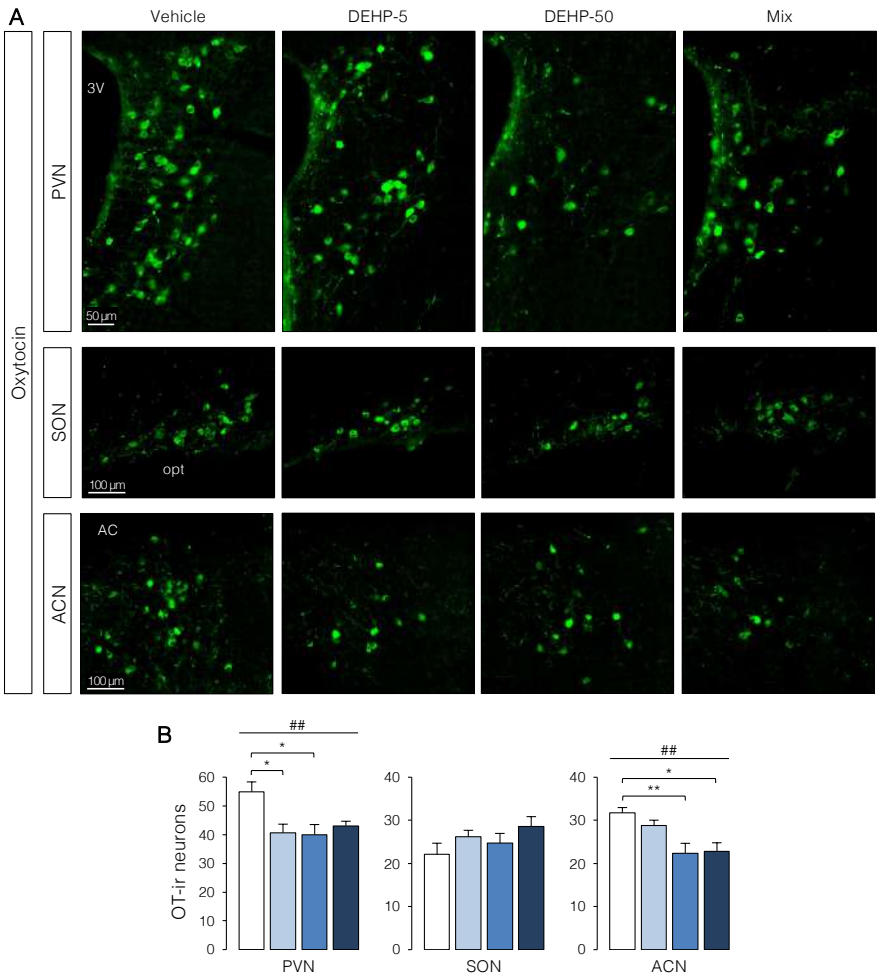


Figure 5

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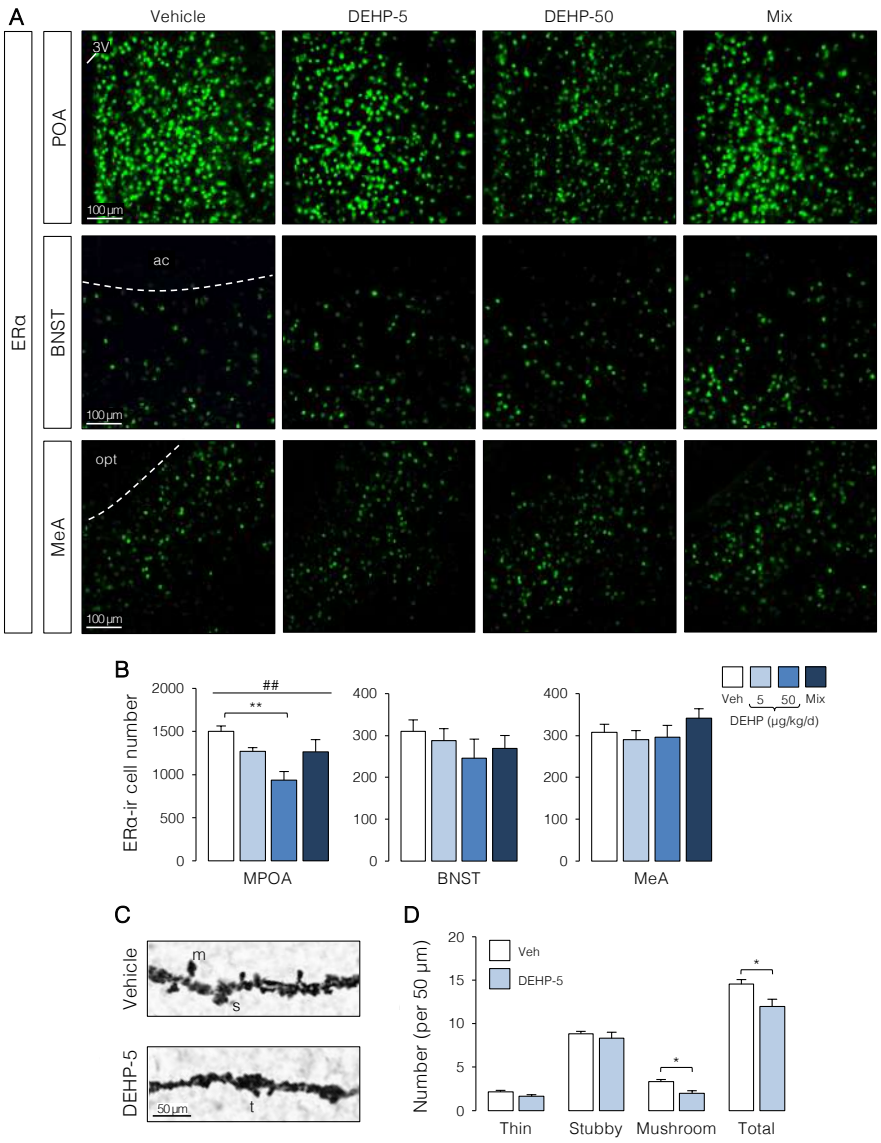


Figure 6

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Supplemental Methods

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Figure S7

Figure S8

Table S1

753 ***Methods***

754 ***Gestational and lactational parameters***

755 After 6 weeks of exposure, females were checked each morning for their estrous cycle stage.
756 Females at the proestrus stage were paired overnight with control C57BL/6J males. They were
757 checked the next day for plugs and the day of the plug was gestational day (GD) 1. Females
758 were then weighed every five days to adjust the phthalate doses. Females that did not gain
759 weight during the first five days were paired with another male. If they did not get pregnant
760 after 4 mating attempts, they were excluded from analyses. Females had a total of three
761 pregnancy and delivery sessions as described in Figure 1B. The gestation duration,
762 corresponding to the number of days from the observed plug until parturition was scored.
763 On postnatal day 1 (PND 1), litter size (number of pups per dam), pup survival (number of live
764 pups divided by the total litter size), and sex of animals were reported. Sex was determined
765 based on Wolterink-Donselaar et al. (2009). Litters were culled to 6 pups per dam, with a sex
766 ratio of 1 when possible. Dams and their pups were weighed on lactational days (LD) 3, LD7,
767 LD11, and LD15.

768

769 **Spontaneous maternal behavior observation**

770 The observed behaviors included:

771 a) Nursing: the dam allowed at least one pup to suckle.

772 b) Licking pups: the dam was licking or grooming at least one pup.

773 c) Nest building: the dam was rearranging her nest from being inside or outside the nest.

774 d) Eating and drinking: the dam was eating its pellet or drinking from the bottle.

775 e) Grooming: the dam was self-grooming.

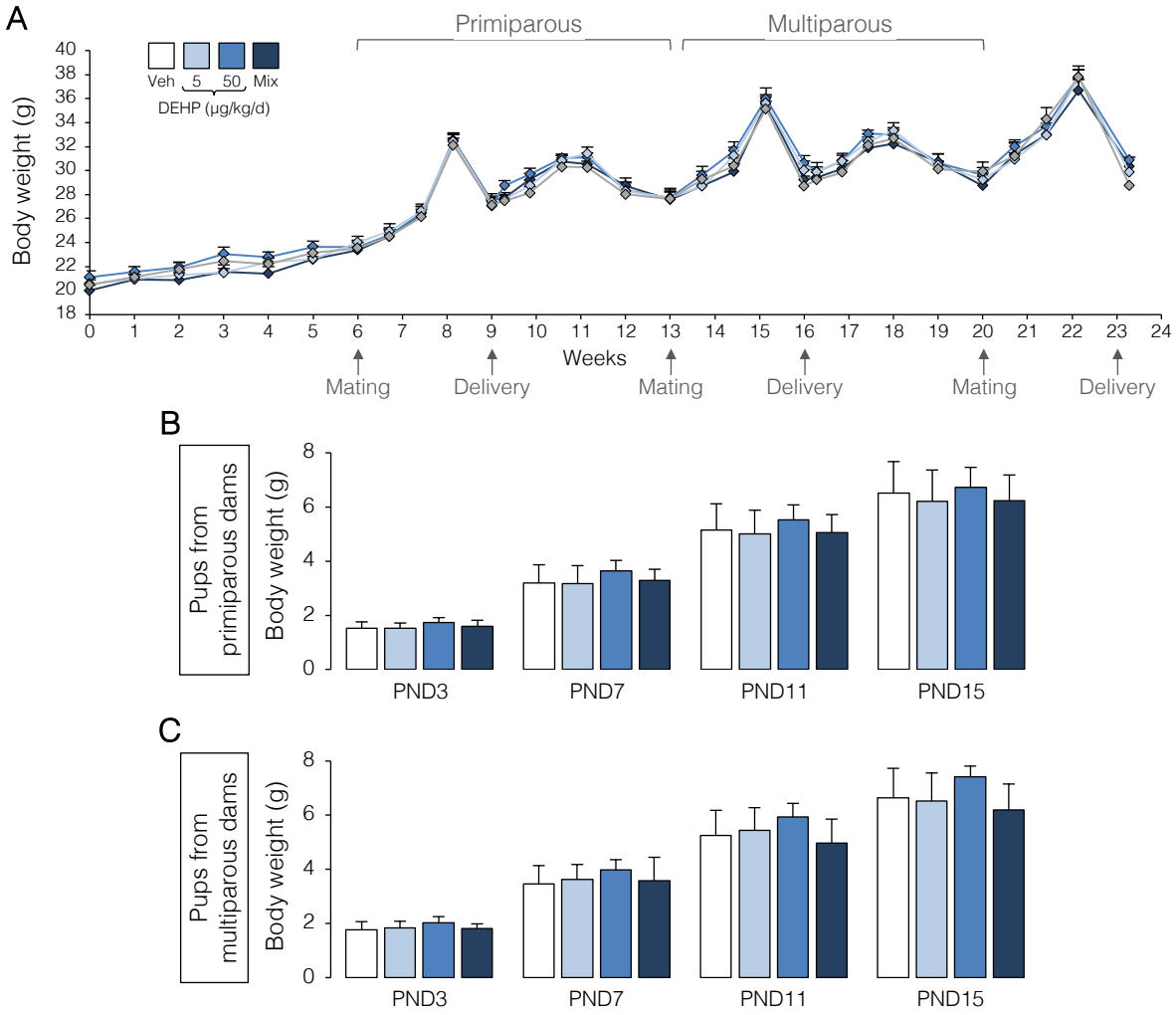
776 f) Resting: the dam was lying in the cage without moving, and without any pups attached
777 to her nipples.

g) Forced-nursing: the dam was engaged in a behavior not directed towards the pups, but at least one pup reached the dam to suckle.

Pup-directed behaviors represent the sum of nursing, licking and nest-building behaviors (a-b-c). Self-care behaviors represent the sum of eating and drinking, grooming, and resting behaviors when performed outside the nest (d-e-f).

References

Wolterink-Donselaar, I.G., Meerding, J.M., Fernandes, C., 2009. A method for gender determination in newborn dark pigmented mice. Lab Anim. 38, 35–38. https://doi.org/10.1038/laban0109-35



789

790 **Figure S1. Effects of exposure to DEHP alone or in a phthalate mixture on body weight**
791 **of dams and pups. A.** Growth curve of dams (n = 8-11 per treatment group) exposed to the
792 vehicle (Veh), DEHP alone at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix) for the total
793 period of treatment and analyses. **B-C.** Body weight of pups (n = 40-49 per group) born from
794 primiparous (**B**) and multiparous (**C**) dams of the four treatment groups at postnatal days (PND)
795 3, 7, 11 and 15. Data are presented as means ± S.E.M.

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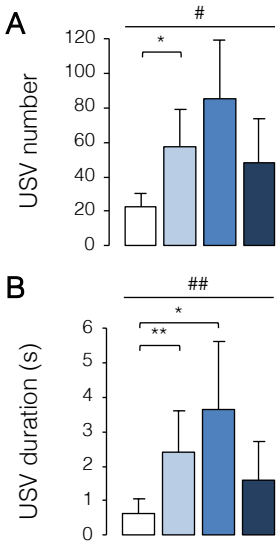


Figure S2. Effects of exposure to DEHP alone or in a phthalate on vocalizations emitted by pups from primiparous dams. A-B. Ultrasonic vocalizations (USV) emitted by pups (13-19 per treatment group) from primiparous dams were recorded at postnatal day (PND) 3. The mean number (**A**) and duration (**B**) of USVs are shown. Data are presented as means ± S.E.M. Kruskal-Wallis analyses for the treatment effect (#$p < 0.05$,) followed by Dunn's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, versus the control group) are indicated.

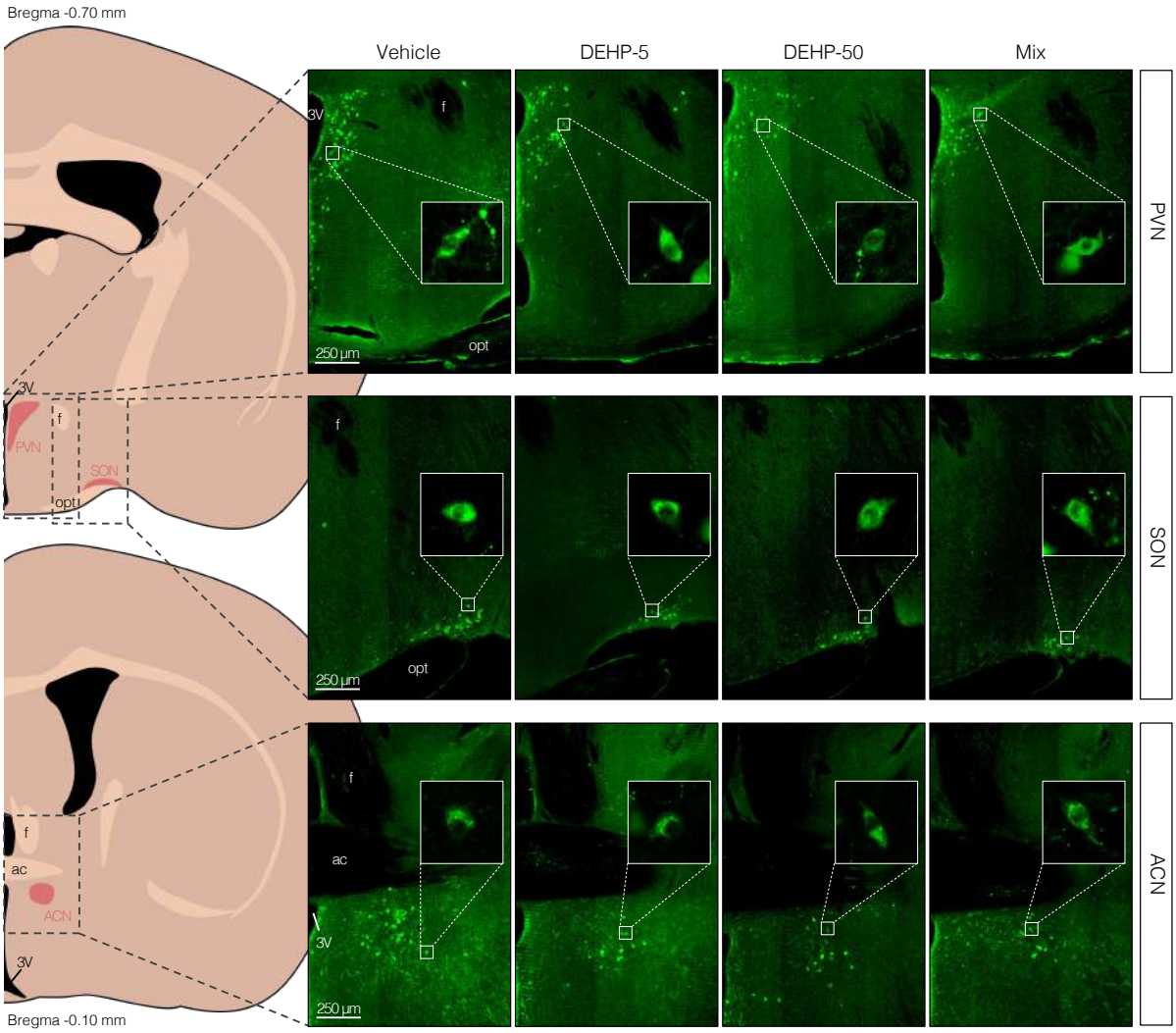


Figure S3. Representative oxytocin-immunoreactivity of anatomically matched sections. Representative cytoplasmic oxytocin-immunoreactivity in the paraventricular nucleus (PVN), supraoptic nucleus (SON) and anterior commissural nucleus (ACN) of multiparous dams exposed to the vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. The insert shows the cytoplasmic immunostaining. Scale bar = 250 µm. ac, anterior commissure; f, fornix; opt, optic chiasma; 3V, 3rd ventricle.

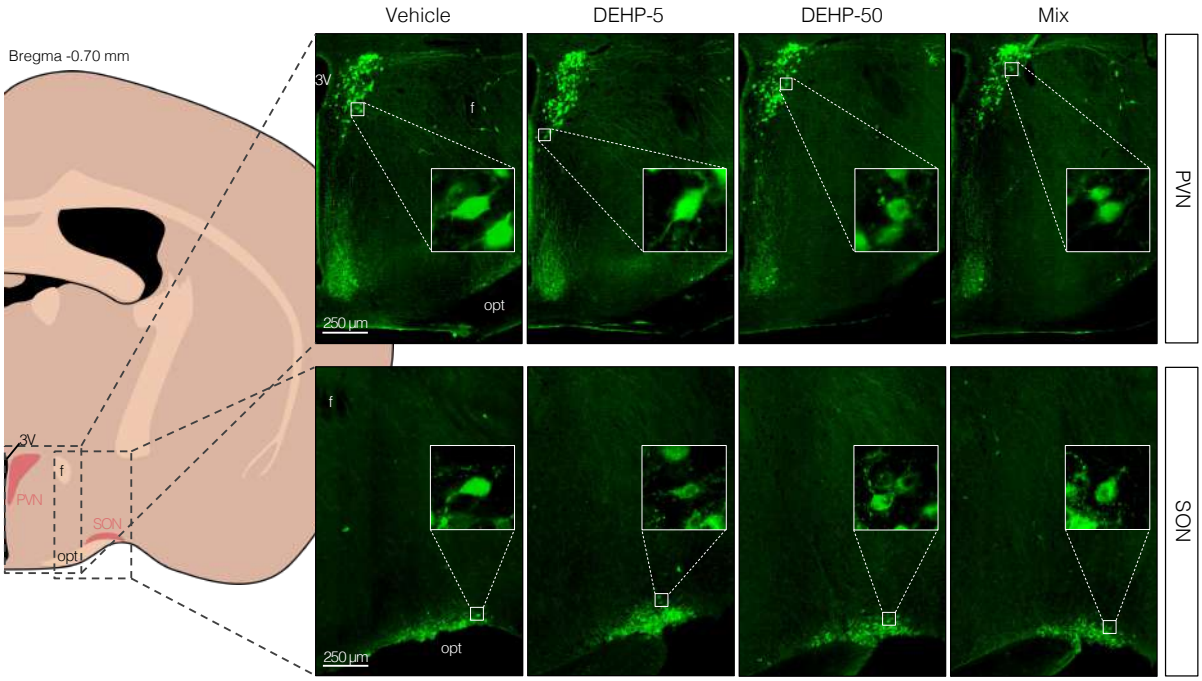


Figure S4. Representative arginine vasopressin-immunoreactivity of anatomically matched sections. Representative cytoplasmic arginine vasopressin-immunoreactivity in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of multiparous dams exposed to the vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. The insert shows the cytoplasmic immunostaining. Scale bar = 250 µm. ac, anterior commissure; f, fornix; opt, optic chiasma; 3V, 3rd ventricle.

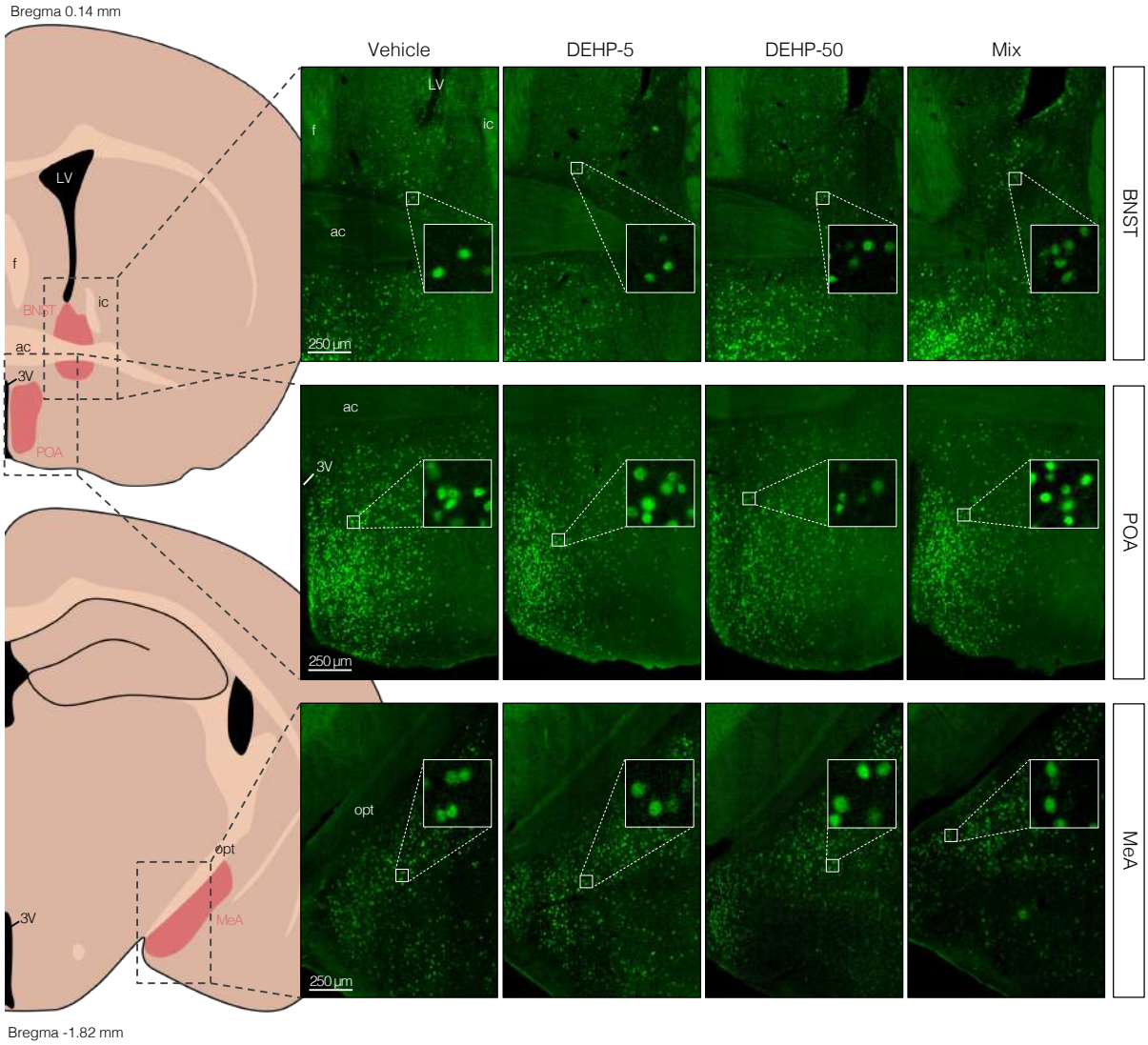


Figure S5. Representative ERα-immunoreactivity of anatomically matched sections. Representative nuclear ERα-immunoreactivity in the bed nucleus of stria terminalis (BNST), medial preoptic area (MPOA) and medial amygdala (MeA) of multiparous dams exposed to the vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. The insert shows the nuclear immunostaining. Scale bar = 250 µm. ac, anterior commissure; f, fornix; ic, internal capsule; LV, lateral ventricle; opt, optic chiasma; 3V, 3rd ventricle.

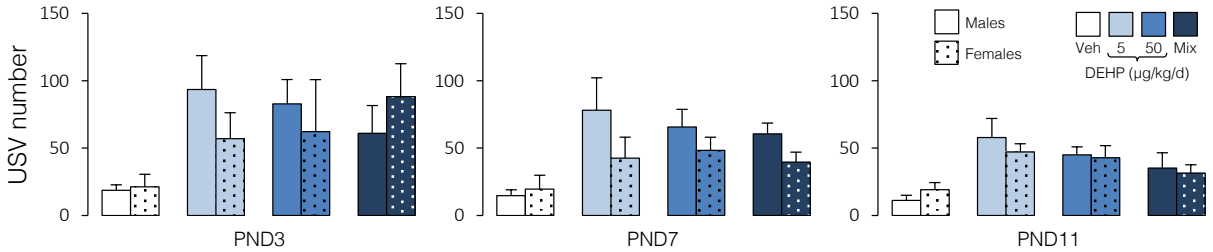


Figure S6. Number of ultrasonic vocalizations (USV) emitted by male and female pups exposed to DEHP alone or in a phthalate mixture. Number of USVs emitted by male and female pups born from multiparous dams exposed to the vehicle (Veh), DEHP alone at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix). Data shown for postnatal days (PND) 3, 7 and 11 are presented as means ± S.E.M (n = 8-13 per treatment group per sex). Two-way ANOVA did not show any effect of the sex at PND 3 ($p = 0.658$), PND7 ($p = 0.085$) and PND11 ($p = 0.703$).

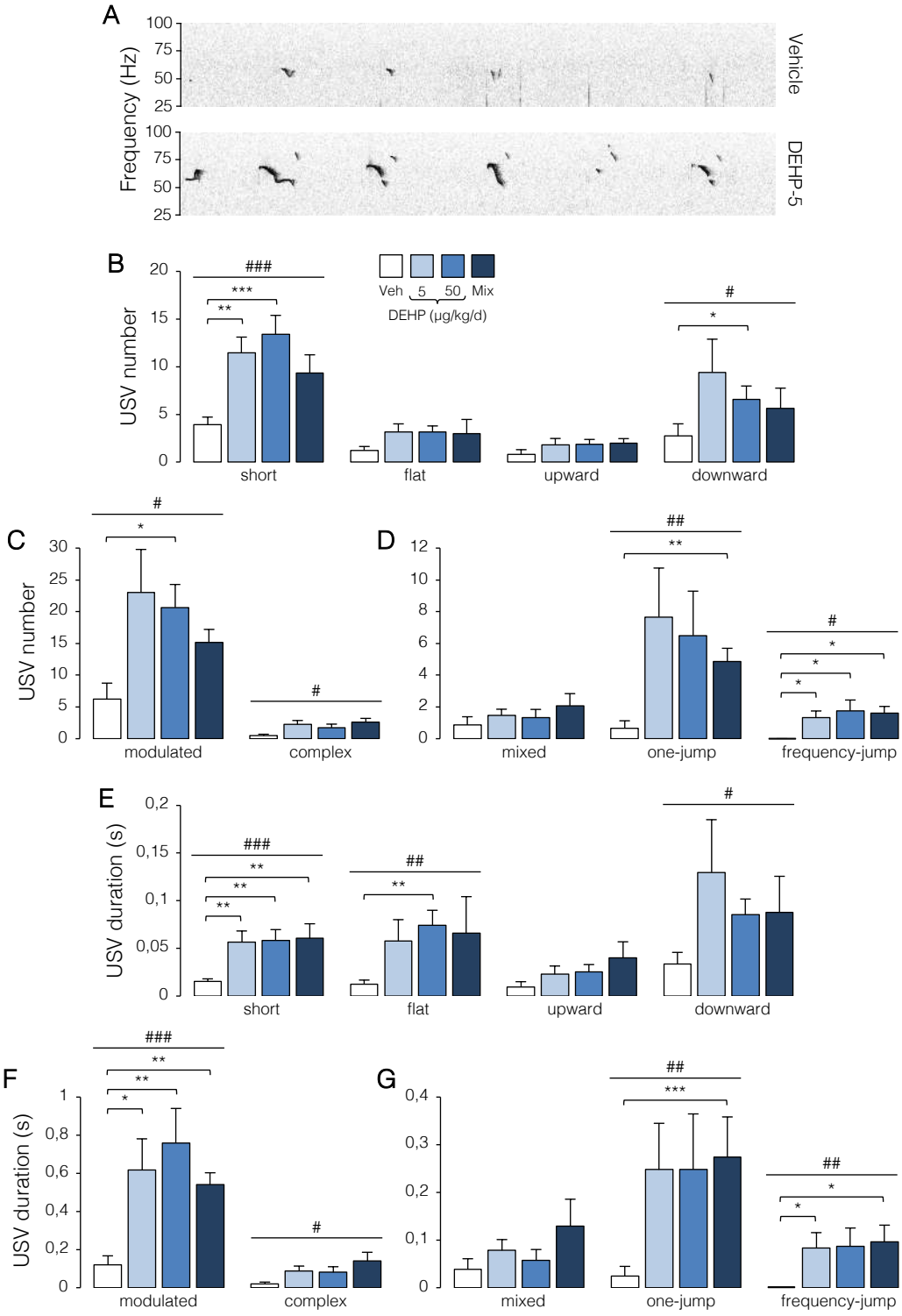


Figure S7. Effects of maternal exposure to DEHP alone or in a phthalate mixture on the type of syllables emitted by pups at postnatal day 7. Ultrasonic vocalizations (USVs) emitted by pups (13-18 per treatment group) born from multiparous dams exposed to the vehicle (Veh), DEHP at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix) are analyzed on lactational day 7. **A.** Representative spectrogram of ultrasonic vocalizations (USV) emitted by pups from the vehicle

862 and DEHP-5 groups. **B-G.** Total number (**B-D**) and duration (**E-G**) of syllables of the simple
863 (**B**, **E**), complex (**C**, **F**) and frequency jump (**D**, **G**) categories. Data are presented as means ±
864 S.E.M. Kruskal-Wallis tests for the treatment effect (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$)
865 followed by Dunn's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the
866 vehicle group) are indicated.
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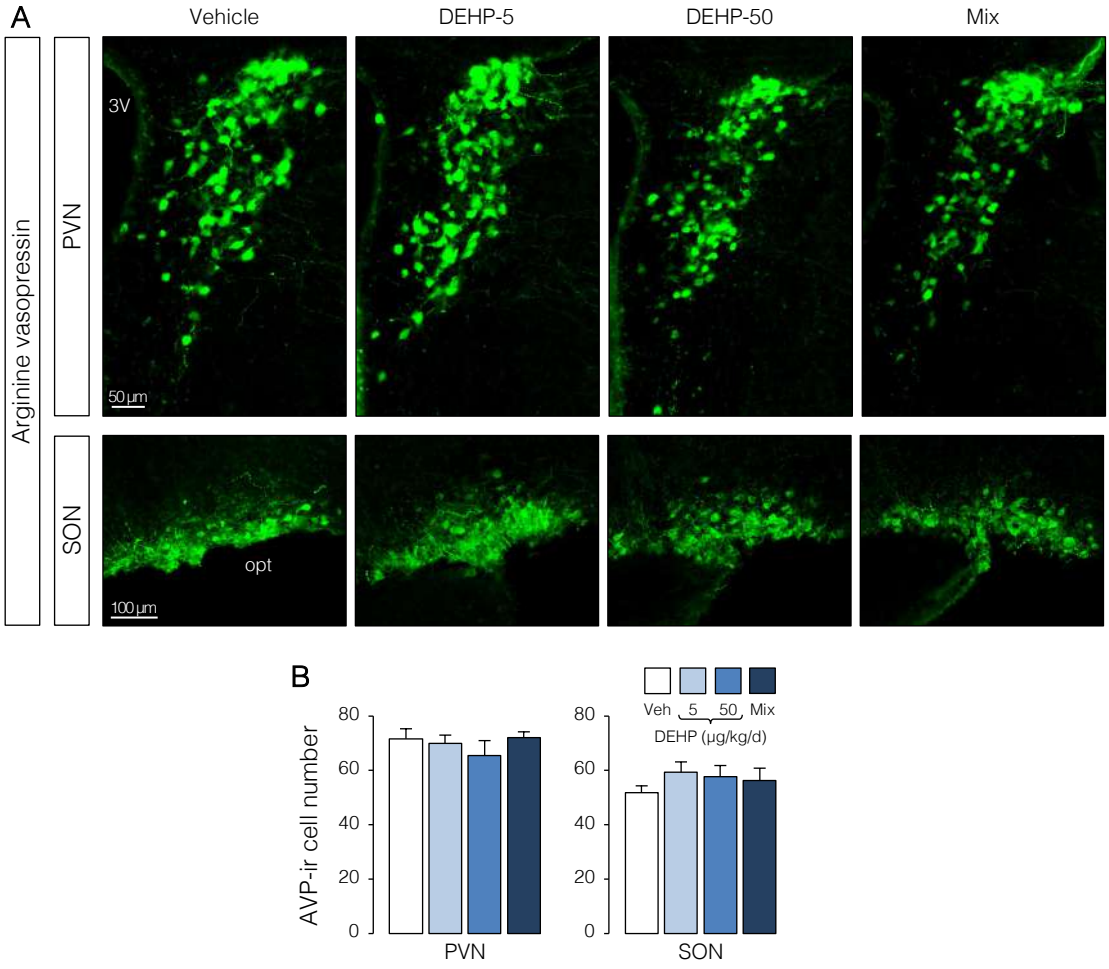


Figure S8. Effects of exposure to DEHP alone or in a phthalate mixture on arginine vasopressin-immunoreactivity. A. Representative arginine vasopressin- (AVP) immunoreactivity in the paraventricular (PVN) and supraoptic (SON) nuclei of multiparous dams (5-6 per treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 50 µm for the PVN and 100 µm for the SON. **B.** Quantitative analysis of the number of AVP-immunoreactive (ir) neurons in the PVN and SON. Data are presented as means ± S.E.M. opt, optic chiasma; 3V, 3rd ventricle.

885 **Table S1.** Reproductive parameters for primiparous and multiparous dams exposed to the

886 vehicle, DEHP alone at 5 (DEHP-5) or 50 μg/kg/d (DEHP-50) or in a phthalate mixture (Mix).

Parameters	Session	Vehicle	DEHP-5	DEHP-50	Mix
Number of pregnant/number of mated females	Primiparous	12/12	10/12	11/12	11/12
	Multiparous	12/12	10/10	11/11	8/11
Number of parturient females with a litter	Primiparous	11/12	10/10	8/11	11/11
	Multiparous	10/12	8/8	8/11	8/8
Gestation duration (days)	Primiparous	19.25 ± 0.13	19.09 ± 0.09	19.09 ± 0.09	19.00 ± 0.00
	Multiparous	19.18 ± 0.12	19.10 ± 0.10	19.64 ± 0.28	19.13 ± 0.13
Litter size	Primiparous	7.75 ± 0.57	8.09 ± 0.58	7.73 ± 0.47	7.82 ± 0.48
	Multiparous	6.75 ± 0.93	8.20 ± 0.53	6.55 ± 0.91	8.00 ± 0.27
Number of males per litter	Primiparous	3.36 ± 0.54	3.73 ± 0.52	3.82 ± 0.50	4.09 ± 0.28
Number of females per litter		4.09 ± 0.51	4.09 ± 0.59	3.64 ± 0.47	3.64 ± 0.39
Number of males per litter	Multiparous	4.40 ± 0.58	5.00 ± 0.49	3.00 ± 0.53	4.88 ± 0.44
Number of females per litter		3.40 ± 0.31	3.00 ± 0.61	4.50 ± 0.87	3.13 ± 0.23
Number of living pups/total number) * 100	Primiparous	82.02 ± 9.51	97.56 ± 1.64	82.21 ± 11.27	98.99 ± 1.01
	Multiparous	83.33 ± 11.24	95.75 ± 3.112	63.64 ± 15.21	93.87 ± 2.34

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