



HAL
open science

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

Nolwenn Adam, Marie-Amélie Lachayze, Caroline Parmentier, Hélène Hardin-Pouzet, Sakina Mhaouty-Kodja

► To cite this version:

Nolwenn Adam, Marie-Amélie Lachayze, Caroline Parmentier, Hélène Hardin-Pouzet, Sakina Mhaouty-Kodja. Exposure to environmentally relevant doses of plasticizers alters maternal behavior and related neuroendocrine processes in primiparous and multiparous female mice. *Environmental Pollution*, 2022, 315, pp.120487. 10.1016/j.envpol.2022.120487 . hal-03862518

HAL Id: hal-03862518

<https://hal.science/hal-03862518>

Submitted on 21 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Title**

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

4

5 **Authors**

6 Nolwenn Adam, Marie-Amélie Lachayze, Caroline Parmentier, Hélène Hardin-Pouzet, Sakina
7 Mhaouty-Kodja

8

9 **Author affiliations**

10 Sorbonne Université, CNRS UMR 8246, INSERM U1130, Neuroscience Paris Seine – Institut
11 de Biologie Paris Seine, 75005 Paris, France.

12

13 **Corresponding author**

14 Sakina Mhaouty-Kodja

15 Sorbonne Université, CNRS UMR 8246, INSERM U1130

16 7 quai St Bernard, Bât A 3ème étage 75005, Paris, France.

17 Tel: +33 1 44 27 91 38

18 email: sakina.mhaouty-kodja@sorbonne-universite.fr

19

20

21 **Abstract**

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

42

43 **1. Introduction**

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

57
58 In this context, the potential effects of phthalate exposure on the neural control of female
59 reproduction have been less studied. In particular, few studies have addressed the effects of
60 adult chronic exposure to phthalates at environmentally relevant doses (Adam and Mhaouty-
61 Kodja, 2022). We recently showed that adult exposure to low doses of DEHP alone or in a
62 phthalate mixture reduced lordosis behavior, olfactory preference towards males, and the ability
63 to attract and stimulate the emission of male ultrasonic vocalizations (Adam et al., 2021). These
64 behavioral alterations were associated with reduced progesterone receptor (PR)-
65 immunoreactivity in the neural circuitry underlying female sexual behavior. It remains to be
66 investigated whether adult chronic exposure to phthalates, under similar experimental
67 conditions, alters other reproductive behaviors such as maternal behavior.

68

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

79 In a previous study, Lee et al. (2021) reported that developmental exposure of rats to DEHP at
80 10 mg/kg/d disturbed pup retrieval behavior. The effects of adult exposure to phthalates were
81 addressed in four studies using an exposure period beginning during gestation and lasting until
82 the lactational period. One study reported that exposure to relatively high doses of DBP (50 or
83 100 mg/kg/d) altered pup retrieval behavior as well as nest quality (Lee et al., 2020). In contrast,
84 exposure of rats to high DINP doses (300-900 mg/kg/d) or to a phthalate mixture at low doses
85 ranging from 0.2 to 1 mg/kg/d, or exposure of mice to low DEHP doses (5 to 400 µg/kg/d) was
86 not found to disturb pup retrieval or maternal behavior (Boberg et al., 2011; Kougias et al.,
87 2018; Quinnies et al., 2017). The causes of this discrepancy are not clear, but they might be
88 related to differences in the experimental conditions (strain, duration of exposure, lactational
89 period of analyses, etc.).

90

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

93 potential underlying mechanisms. Adult C57BL/6J female mice were exposed to the vehicle
94 (control group), DEHP alone at the tolerable daily intake (TDI) dose (50 $\mu\text{g}/\text{kg}/\text{d}$) or at a dose
95 in the range of the environmental exposure (5 $\mu\text{g}/\text{kg}/\text{d}$), or to an environmental phthalate
96 mixture containing DEHP (5 $\mu\text{g}/\text{kg}/\text{d}$), DBP (0.5 $\mu\text{g}/\text{kg}/\text{d}$), BBP (0.5 $\mu\text{g}/\text{kg}/\text{d}$), DiBP (0.5
97 $\mu\text{g}/\text{kg}/\text{d}$) and DEP (0.25 $\mu\text{g}/\text{kg}/\text{d}$) as previously described (Adam et al., 2021). Dams were
98 exposed for 6 weeks before the first mating and treatment lasted until lactational day (LD) 2
99 following the third pregnancy and delivery. Primiparous (first litter) or multiparous (second
100 litter) dams were analyzed for spontaneous behaviors including nest quality, pup-directed and
101 self-care behaviors. They were also subjected to pup retrieval testing, and their pups were
102 analyzed for the emission of ultrasonic vocalizations. Brains collected from dams at the end of
103 treatment (LD2 following the third delivery) were analyzed for oxytocin- and arginine
104 vasopressin (AVP)-immunoreactivity in the paraventricular, supraoptic and preoptic nuclei,
105 together with estrogen receptor α (ER α)-immunoreactivity and dendritic spine density in the
106 preoptic area.

107

108 **2. Material and methods**

109 *2.1. Animals and treatments*

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

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

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

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

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

136

137 **2.2. Behavioral analyses**

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

145

146 **2.2.1. Spontaneous maternal behavior observation**

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

155 **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 transcidentally perfused with a solution of 4%
180 paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains from 5-6 dams were post-

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

185

186 ***2.4. Immunofluorescence***

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

195 Brain sections were scanned by a high-resolution NanoZoomer Hamamatsu scanner
196 (Hamamatsu Corporation). The number of oxytocin-, arginine vasopressin- and ER α -labeled
197 cells was quantified from anatomically matched sections identified using the Mouse Brain Atlas
198 of Paxinos et al. (2001) as shown in Figures S3, S4 and S5, respectively. Immunolabeled cells
199 for oxytocin and arginine-vasopressin were analyzed within the paraventricular and supraoptic
200 nuclei (plates 36-41 and 36-39, respectively) and the anterior commissural nucleus of the dorsal
201 preoptic area in Bregma -0.10 (Yoshihara et al. 2017). Data are expressed as the mean of
202 immunoreactive cells per section. The number of ER α -immunoreactive cells was counted in
203 the medial preoptic area within an area of 0.80 mm² (plate 30), in the bed nucleus of the stria
204 terminalis within an area of 0.70 mm² (plate 30) and in the medial amygdala within an area of
205 0.56 mm² (plate 46). Data are expressed as the total number of immunoreactive cells per area.

206

207 ***2.5. Golgi-Cox staining***

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

214

215 ***2.6. Statistics***

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

224

225 **3. Results**

226 *3.1. Effects of exposure to DEHP alone or in a phthalate mixture on reproductive*
227 *parameters*

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

237

238 *3.2. Spontaneous behaviors elicited by primiparous and multiparous dams exposed to*
239 *DEHP alone or in a phthalate mixture*

240 *Pup-directed versus selfcare behaviors*

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

250 5 (+25%), DEHP-50 (+23%) and Mix-groups (+21%) compared to the vehicle group. Analyses
251 illustrated in Figure 2D show an effect of treatment on eating-drinking behavior ($p = 0.004$),
252 with increased behaviors for the three treatment groups (+34%, +33% and +35%, respectively)
253 compared to the vehicle group. Resting outside the nest was also affected by the treatment ($p =$
254 0.006), with increased behaviors for the DEHP-50 and the Mix group (+293% and +225%
255 versus the vehicle group, respectively).

256 In multiparous dams, there was an effect of treatment on pup-directed behaviors ($p = 0.029$), in
257 particular on nursing ($p = 0.021$) and nest-building behaviors ($p = 0.005$) but not on pup licking
258 despite a tendency to decrease (Figure 2E-F). Lower nursing behavior was observed in the
259 DEHP-50 group (-26%), and nest building behavior was reduced in the DEHP-5 and DEHP-50
260 groups (-35% and -38%, respectively) compared to the vehicle group (Figure 2F). Treatment
261 effect was also seen on self-care behaviors ($p = 0.041$), with a higher number of behaviors in
262 the DEHP-50 group (+24% versus the vehicle group) (Figure 2G). This was due particularly to
263 the treatment effect on resting behavior ($p = 0.004$), which was increased for DEHP-5, DEHP-
264 50 and Mix groups (+163%, +225% and 189%, respectively) compared to the vehicle group.
265 Eating-drinking behavior was not affected in multiparous females ($p = 0.324$) (Figure 2H).

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

268

269 *Nest score*

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

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

277

278 *Forced nursing*

279 Exposure to DEHP alone or in phthalate mixture had an effect on forced-nursing behavior
280 performed by pups from both primiparous ($p < 0.0001$) and multiparous dams ($p = 0.0004$)
281 (Figure 3C). A higher number of pups exhibited this behavior in the DEHP-50 group of
282 primiparous dams (+582%) and in DEHP-5, DEHP-50 and Mix groups of multiparous dams
283 (+135%, +205% and +187%, respectively) compared to the corresponding vehicle groups.

284

285 ***3.3. Pup retrieval by primiparous and multiparous dams exposed to DEHP alone or in a*** 286 ***phthalate mixture and emission of ultrasonic vocalizations by their pups***

287 The ability of primiparous and multiparous dams to retrieve pups placed outside the nest was
288 analyzed at LD3, LD7 and LD11 (Figure 4A-B). In primiparous dams, an effect of treatment
289 on the retrieval duration was observed at LD3, LD7 and LD11 ($p = 0.011$, $p = 0.013$ and $p =$
290 0.036 , respectively) (Figure 4A). A reduced duration was seen for DEHP-5 and DEHP-50
291 groups at LD3 (-29% and -36% versus the vehicle group, respectively) and for DEHP-50 and
292 Mix groups at LD7 (-49% and -43%, respectively) compared to the vehicle group.

293 In multiparous dams, treatment affected the retrieval duration at LD3, LD7 and LD11 ($p =$
294 0.007 , $p = 0.017$ and $p = 0.013$, respectively) (Figure 4B). A significant decrease was observed
295 at LD3 for DEHP-5 and Mix groups (-30% and -33%, respectively), and for DEHP-50 at LD7
296 and LD11 (-39% and -49%, respectively) compared to the vehicle group.

297 Pup retrieval is elicited by dams in response to the USVs emitted by their pups, so USV
298 emission by isolated pups was recorded. The data were grouped for the two sexes since our
299 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.

324 For the complex category (Figure S7C, F), the treatment affected the number and duration of
325 both modulated ($p = 0.011$ and $p = 0.001$, respectively) and complex syllables ($p = 0.012$ and
326 $p = 0.04$, respectively), with a higher number of modulated syllables for the DEHP-50 group
327 (+230%) and a higher duration of modulated syllables for all groups (+417%, +533%, +350%,
328 $p = 0.005$) compared to the vehicle group. For the frequency jump category, the treatment
329 altered both the number and the duration of one-jump syllables ($p = 0.002$ and 0.011), with a
330 higher number and duration for the Mix group (+624%, and +1250%) compared to the vehicle
331 group (Figure S7D, G). Interestingly, no pups from the control group emitted frequency-jump
332 syllables. These syllables were emitted by pups from the DEHP- and Mix- treated groups. An
333 effect of treatment was observed on both the number and the duration of frequency-jump
334 syllables ($p = 0.011$ and $p = 0.006$, respectively).

335

336 *3.4. Neural oxytocin-immunoreactivity in multiparous dams exposed to DEHP alone or* 337 *in a phthalate mixture*

338 Given the importance of the oxytocinergic system in the expression of maternal behavior, we
339 wished to find out whether the altered behavior of exposed dams was related to modifications
340 in this neuroendocrine system. Analysis of oxytocin immunoreactivity was thus undertaken at
341 LD2 following the third mating/delivery session. Specific cytoplasmic oxytocin-
342 immunoreactivity was detected in the paraventricular nucleus, the supraoptic nucleus and the
343 anterior commissural nucleus (Figures 5A, S3). Quantitative analyses show an effect of
344 treatment on the number of oxytocin-immunoreactive neurons in the paraventricular nucleus (p
345 $= 0.009$) and the anterior commissural nucleus ($p = 0.002$), but not in the supraoptic nucleus (p
346 $= 0.231$) (Figure 5B). Post-hoc analyses showed a lower number of oxytocin-immunoreactive
347 neurons in the paraventricular nucleus of DEHP-5 and DEHP-50 dams (-26% and -27% versus
348 the vehicle group, respectively). A lower number of oxytocin-immunoreactive neurons was also

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

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

356

357 *3.5. ER α -immunoreactivity and dendritic spine density in the medial preoptic area of* 358 *multiparous dams exposed to DEHP alone or in a phthalate mixture*

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

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

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

377

378 4. Discussion

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

384

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

397

398 When pups were placed outside the nest, dams exposed to DEHP alone or in an
399 environmental mixture retrieved their pups with a shorter latency compared to the vehicle
400 group. This may appear contradictory with the reduced spontaneous behavior reported above.
401 However, it is important to stress that pup retrieval behavior is induced in response to USVs
402 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

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

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

435

436 The expression of maternal behavior is regulated by oxytocin neurons located in the
437 paraventricular nucleus (Champagne et al., 2001; Pedersen et al., 1994). These neurons project
438 to the medial preoptic area, where oxytocin acts on its oxytocin receptor to activate the behavior
439 (Okabe et al., 2017). This circuitry is highly sensitive to estrogens, with a high expression of
440 ER α in the medial preoptic area (Champagne et al., 2003; Fang et al., 2018; Ribeiro et al.,
441 2012). It is of great interest that exposure to DEHP alone or in a phthalate mixture induced
442 changes in this circuitry in multiparous dams. Oxytocin-immunoreactivity was selectively
443 reduced in the paraventricular nucleus, while no effects were found on AVP-immunoreactivity.
444 Reduced oxytocin-immunoreactivity was also found in the anterior commissure nucleus, a brain
445 locus containing oxytocin neurons thought to modulate parental care in mammals (Yoshihara
446 et al., 2017). This shows that the oxytocinergic system can be also impacted by adult exposure
447 to phthalates. A previous study reported changes in the expression of hypothalamic oxytocin
448 following developmental exposure of rats to DEHP at 10 mg/kg/d (Lee et al. 2021).

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

452 or in a phthalate mixture were observed in maternally-experienced dams. This suggests that
453 these chemicals may impact neuronal connectivity and underlying neuroplasticity processes,
454 which occur in the medial preoptic area and are necessary for behavioral maintenance.
455 Quantification of dendritic spine density using Golgi staining in the medial preoptic area of
456 multiparous dams showed a reduced total number of dendritic spines. In particular, an effect
457 was mainly found on mushroom spines, which represent the most stable and mature spines. It
458 is largely reported that maturation of dendritic spines towards mushroom characterizes learning
459 processes and synaptic strength in the hippocampus and medial preoptic area and are associated
460 with behavioral improvement (Jean et al., 2017; Leuner and Shors, 2004; Mahmmoud et al.,
461 2015). This suggests that exposure to DEHP alone or in a phthalate mixture interfered with
462 neuroplasticity processes possibly involved in long-term maintenance of maternal behavior in
463 the medial preoptic area. Previous studies showed that exposure of to DBP altered adult
464 hippocampal neurogenesis in mice (Lee et al., 2019), while MEHP inhibited excitability and
465 synaptic plasticity of CA3 hippocampal neurons (Lu et al., 2022). The present study indicates
466 that neural processes may be also impacted by phthalate exposure in other brain regions
467 including the hypothalamus.

468

469 In conclusion, the present data describing disturbed maternal behavior, together with
470 our previous study showing impaired estrous cyclicity and sexual behavior (Adam et al., 2021),
471 indicate that exposure to DEHP alone or in an environmental mixture may have an important
472 impact on female reproduction in mice. For the majority of analyzed parameters and behaviors,
473 comparable effects were observed for DEHP alone or in a phthalate mixture, suggesting that
474 DEHP drove the effects possibly because it was more abundant than the other phthalates in this
475 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

486 **Acknowledgments**

487 This work was supported by Sorbonne Université, Centre National de la Recherche Scientifique
488 (CNRS) and Institut National de la Santé et de la Recherche Médicale (Inserm). We thank the
489 IBPS platform for taking care of the animals.

490

491 **CFID declaration**

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

493

494 **References**

495 Adam, N., Brusamonti, L., Mhaouty-Kodja, S., 2021. Exposure of Adult Female Mice
496 to Low Doses of di(2-ethylhexyl) Phthalate Alone or in an Environmental Phthalate Mixture:
497 Evaluation of Reproductive Behavior and Underlying Neural Mechanisms. *Environ. Health*
498 *Perspect.* 129, 017008. <https://doi.org/10.1289/EHP7662>

499 Adam, N., Mhaouty-Kodja, S., 2022. Behavioral Effects of Exposure to Phthalates in
500 Female Rodents: Evidence for Endocrine Disruption? *Int. J. Mol. Sci.* 23, 2559.
501 <https://doi.org/10.3390/ijms23052559>

502 Ahmadpour, D., Mhaouty-Kodja, S., Grange-Messent, V., 2021. Disruption of the
503 blood-brain barrier and its close environment following adult exposure to low doses of di(2-
504 ethylhexyl)phthalate alone or in an environmental phthalate mixture in male mice.
505 *Chemosphere* 282, 131013. <https://doi.org/10.1016/j.chemosphere.2021.131013>

506 Ahmadpour, D., Mhaouty-Kodja, S., Grange-Messent, V., 2022. Effects and underlying
507 cellular pathway involved in the impairment of the neurovascular unit following exposure of
508 adult male mice to low doses of di(2-ethylhexyl) phthalate alone or in an environmental
509 phthalate mixture. *Environ. Res.* 112235. <https://doi.org/10.1016/j.envres.2021.112235>

510 Anses, 2015. Connaissances relatives aux données de contamination et aux expositions
511 par des composés de la famille des Phthalates (Rapport d'étude).

512 Blanchard, M., Teil, M.-J., Dargnat, C., Alliot, F., Chevreuril, M., 2013. Assessment of
513 Adult Human Exposure to Phthalate Esters in the Urban Centre of Paris (France). *Bull. Environ.*
514 *Contam. Toxicol.* 90, 91–96. <https://doi.org/10.1007/s00128-012-0859-5>

515 Boberg, J., Christiansen, S., Axelstad, M., Kledal, T.S., Vinggaard, A.M., Dalgaard, M.,
516 Nellemann, C., Hass, U., 2011. Reproductive and behavioral effects of diisononyl phthalate
517 (DINP) in perinatally exposed rats. *Reprod. Toxicol.* 31, 200–209.
518 <https://doi.org/10.1016/j.reprotox.2010.11.001>

519 Capela, D., Mhaouty-Kodja, S., 2021. Effects of pubertal exposure to low doses of di-
520 (2-ethylhexyl)phthalate on reproductive behaviors in male mice. *Chemosphere* 263, 128191.
521 <https://doi.org/10.1016/j.chemosphere.2020.128191>

522 Champagne, F., Diorio, J., Sharma, S., Meaney, M.J., 2001. Naturally occurring
523 variations in maternal behavior in the rat are associated with differences in estrogen-inducible
524 central oxytocin receptors. *Proc. Natl. Acad. Sci.* 98, 12736–12741.
525 <https://doi.org/10.1073/pnas.221224598>

526 Champagne, F.A., Weaver, I.C.G., Diorio, J., Sharma, S., Meaney, M.J., 2003. Natural

527 Variations in Maternal Care Are Associated with Estrogen Receptor α Expression and Estrogen
528 Sensitivity in the Medial Preoptic Area. *Endocrinology* 144, 4720–4724.
529 <https://doi.org/10.1210/en.2003-0564>

530 Dewalque, L., Charlier, C., Pirard, C., 2014. Estimated daily intake and cumulative risk
531 assessment of phthalate diesters in a Belgian general population. *Toxicol. Lett.* 231, 161–168.
532 <https://doi.org/10.1016/j.toxlet.2014.06.028>

533 Dombret, C., Capela, D., Poissenot, K., Parmentier, C., Bergsten, E., Pionneau, C.,
534 Chardonnet, S., Hardin-Pouzet, H., Grange-Messent, V., Keller, M., Franceschini, I., Mhaouty-
535 Kodja, S., 2017. Neural Mechanisms Underlying the Disruption of Male Courtship Behavior
536 by Adult Exposure to Di(2-ethylhexyl) Phthalate in Mice. *Environ. Health Perspect.* 125.
537 <https://doi.org/10.1289/EHP1443>

538 European Food Safety Authority, 2019. Update of the risk assessment of di-
539 butylphthalate (DBP), butyl-benzyl-phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), di-
540 isononylphthalate (DINP) and di-isodecylphthalate (DIDP) for use in food contact materials.
541 *EFSA J. Eur. Food Saf. Auth.* 17, e05838. <https://doi.org/10.2903/j.efsa.2019.5838>

542 European Food Safety Authority, 2005. Opinion of the Scientific Panel on food
543 additives, flavourings, processing aids and materials in contact with food (AFC) related to
544 Bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. *EFSA J.* 3.
545 <https://doi.org/10.2903/j.efsa.2005.243>

546 Fang, Y.-Y., Yamaguchi, T., Song, S.C., Tritsch, N.X., Lin, D., 2018. A Hypothalamic
547 Midbrain Pathway Essential for Driving Maternal Behaviors. *Neuron* 98, 192-207.e10.
548 <https://doi.org/10.1016/j.neuron.2018.02.019>

549 Fischer, J., Hammerschmidt, K., 2011. Ultrasonic vocalizations in mouse models for
550 speech and socio-cognitive disorders: insights into the evolution of vocal communication.
551 *Genes Brain Behav.* 10, 17–27. <https://doi.org/10.1111/j.1601-183X.2010.00610.x>

552 Fletcher, E.J., Santacruz-Márquez, R., Mourikes, V.E., Neff, A.M., Laws, M.J., Flaws,
553 J.A., 2022. Effects of Phthalate Mixtures on Ovarian Folliculogenesis and Steroidogenesis.
554 *Toxics* 10, 251. <https://doi.org/10.3390/toxics10050251>

555 Gao, D.-W., Wen, Z.-D., 2016. Phthalate esters in the environment: A critical review of
556 their occurrence, biodegradation, and removal during wastewater treatment processes. *Sci.*
557 *Total Environ.* 541, 986–1001. <https://doi.org/10.1016/j.scitotenv.2015.09.148>

558 Hannon, P.R., Flaws, J.A., 2015. The effects of phthalates on the ovary. *Front.*
559 *Endocrinol.* 6, 8. <https://doi.org/10.3389/fendo.2015.00008>

560 Harris, K.M., Jensen, F.E., Tsao, B., 1992. Three-dimensional structure of dendritic

561 spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications
562 for the maturation of synaptic physiology and long-term potentiation. *J. Neurosci. Off. J. Soc.*
563 *Neurosci.* 12, 2685–2705.

564 Hess, S., Rohr, S., Dufour, B., Gaskill, B., Pajor, E., Garner, J., 2008. Home
565 Improvement: C57BL/6J Mice Given More Naturalistic Nesting Materials Build Better Nests.
566 *J. Am. Assoc. Lab. Anim. Sci.* 47, 25–31.

567 Hliseníková, H., Petrovičová, I., Kolena, B., Šidlovská, M., Sirotkin, A., 2020. Effects
568 and Mechanisms of Phthalates' Action on Reproductive Processes and Reproductive Health: A
569 Literature Review. *Int. J. Environ. Res. Public Health* 17, 6811.
570 <https://doi.org/10.3390/ijerph17186811>

571 Howdeshell, K.L., Rider, C.V., Wilson, V.S., Gray, L.E., 2008. Mechanisms of action
572 of phthalate esters, individually and in combination, to induce abnormal reproductive
573 development in male laboratory rats. *Environ. Res.* 108, 168–176.
574 <https://doi.org/10.1016/j.envres.2008.08.009>

575 Jean, A., Bonnet, P., Liere, P., Mhaouty-Kodja, S., Hardin-Pouzet, H., 2017. Revisiting
576 medial preoptic area plasticity induced in male mice by sexual experience. *Sci. Rep.* 7, 17846.
577 <https://doi.org/10.1038/s41598-017-18248-3>

578 Keller, M., Vandenberg, L.N., Charlier, T.D., 2019. The parental brain and behavior: A
579 target for endocrine disruption. *Front. Neuroendocrinol.* 54, 100765.
580 <https://doi.org/10.1016/j.yfrne.2019.100765>

581 Kougias, D.G., Cortes, L.R., Moody, L., Rhoads, S., Pan, Y.-X., Juraska, J.M., 2018.
582 Effects of Perinatal Exposure to Phthalates and a High-Fat Diet on Maternal Behavior and Pup
583 Development and Social Play. *Endocrinology* 159, 1088–1105.
584 <https://doi.org/10.1210/en.2017-03047>

585 Lee, W., Cho, J.-H., Lee, Y., Lee, S., Kim, D.H., Ha, S., Kondo, Y., Ishigami, A.,
586 Chung, H.Y., Lee, J., 2019. Dibutyl phthalate impairs neural progenitor cell proliferation and
587 hippocampal neurogenesis. *Food Chem. Toxicol.* 129, 239–248.
588 <https://doi.org/10.1016/j.fct.2019.04.040>

589 Lee, S.M., Jeon, S., Jeong, H.J., Kim, B.-N., Kim, Y., 2020. Dibutyl phthalate exposure
590 during gestation and lactation in C57BL/6 mice: Maternal behavior and neurodevelopment in
591 pups. *Environ. Res.* 182, 109025. <https://doi.org/10.1016/j.envres.2019.109025>

592 Lee, Y.-J., Lin, H.-T., Chaudhary, M.A., Lee, Y.-C., Wang, D.-C., 2021. Effects of
593 Prenatal Phthalate Exposure and Childhood Exercise on Maternal Behaviors in Female Rats at
594 Postpartum: A Role of Oxtr Methylation in the Hypothalamus. *Int. J. Mol. Sci.* 22, 9847.

595 <https://doi.org/10.3390/ijms22189847>

596 Leuner, B., Shors, T.J., 2004. New Spines, New Memories. *Mol. Neurobiol.* 29, 117–
597 130. <https://doi.org/10.1385/MN:29:2:117>

598 Lu, Y., Wang, H., Yang, J., Jiang, W., Xin, H., Luo, Y., Loya-López, S., Gu, H., Ran,
599 D., 2022. Distinct Role of Mono-2-ethylhexyl Phthalate in Neuronal Transmission in Rat CA3
600 Hippocampal Neurons: Involvement of Ion Channels. *Molecules* 27, 3082.
601 <https://doi.org/10.3390/molecules27103082>

602 Mahmmoud, R.R., Sase, S., Aher, Y.D., Sase, A., Gröger, M., Mokhtar, M., Höger, H.,
603 Lubec, G., 2015. Spatial and Working Memory Is Linked to Spine Density and Mushroom
604 Spines. *PLOS ONE* 10, e0139739. <https://doi.org/10.1371/journal.pone.0139739>

605 Mesquita, I., Lorigo, M., Cairrao, E., 2021. Update about the disrupting-effects of
606 phthalates on the human reproductive system. *Mol. Reprod. Dev.* 88, 650–672.
607 <https://doi.org/10.1002/mrd.23541>

608 Morová, M., Senko, T., Olexová, L., Dzirbíková, Z., Kršková, L., 2021. A Mixture of
609 Diethylhexyl, Diisononyl and Dibutyl Phthalate Decreased Anogenital Distance, Postnatal
610 Testosterone Levels, and Changed Social Behavior in Wistar Rats. *Physiol. Res.* S489–S498.
611 <https://doi.org/10.33549/physiolres.934599>

612 Numan, M., Insel, T.R., 2003. *The neurobiology of parental behavior*. Springer, New
613 York.

614 Okabe, S., Tsuneoka, Y., Takahashi, A., Ooyama, R., Watarai, A., Maeda, S., Honda,
615 Y., Nagasawa, M., Mogi, K., Nishimori, K., Kuroda, M., Koide, T., Kikusui, T., 2017. Pup
616 exposure facilitates retrieving behavior via the oxytocin neural system in female mice.
617 *Psychoneuroendocrinology* 79, 20–30. <https://doi.org/10.1016/j.psyneuen.2017.01.036>

618 Okino, E., Morita, S., Hoshikawa, Y., Tsukahara, S., 2020. The glutamatergic system
619 in the preoptic area is involved in the retention of maternal behavior in maternally experienced
620 female rats. *Psychoneuroendocrinology* 120, 104792.
621 <https://doi.org/10.1016/j.psyneuen.2020.104792>

622 Palanza, P.L., Howdeshell, K.L., Parmigiani, S., vom Saal, F.S., 2002. Exposure to a
623 low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice.
624 *Environ. Health Perspect.* 110, 415–422. <https://doi.org/10.1289/ehp.02110s3415>

625 Paxinos, G., Franklin, K.B.J., Franklin, K.B.J., 2001. *The mouse brain in stereotaxic*
626 *coordinates*, 2nd ed. ed. Academic Press, San Diego.

627 Pedersen, C.A., Caldwell, J.D., Walker, C., Ayers, G., Mason, G.A., 1994. Oxytocin
628 activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial

629 preoptic areas. *Behav. Neurosci.* 108, 1163–1171. [https://doi.org/10.1037//0735-](https://doi.org/10.1037//0735-7044.108.6.1163)
630 7044.108.6.1163

631 Quinnes, K.M., Doyle, T.J., Kim, K.H., Rissman, E.F., 2015. Transgenerational Effects
632 of Di-(2-Ethylhexyl) Phthalate (DEHP) on Stress Hormones and Behavior. *Endocrinology* 156,
633 3077–3083. <https://doi.org/10.1210/EN.2015-1326>

634 Quinnes, K.M., Harris, E.P., Snyder, R.W., Sumner, S.S., Rissman, E.F., 2017. Direct
635 and transgenerational effects of low doses of perinatal di-(2-ethylhexyl) phthalate (DEHP) on
636 social behaviors in mice. *PLOS ONE* 12, e0171977.
637 <https://doi.org/10.1371/journal.pone.0171977>

638 Ribeiro, A.C., Musatov, S., Shteyler, A., Simanduyev, S., Arrieta-Cruz, I., Ogawa, S.,
639 Pfaff, D.W., 2012. siRNA silencing of estrogen receptor- α expression specifically in medial
640 preoptic area neurons abolishes maternal care in female mice. *Proc. Natl. Acad. Sci.* 109,
641 16324–16329. <https://doi.org/10.1073/pnas.1214094109>

642 Rowdhwal, S.S.S., Chen, J., 2018. Toxic Effects of Di-2-ethylhexyl Phthalate: An
643 Overview. *BioMed Res. Int.* 2018, 1750368. <https://doi.org/10.1155/2018/1750368>

644 Vacher, C.-M., Frétier, P., Créminon, C., Calas, A., Hardin-Pouzet, H., 2002. Activation
645 by Serotonin and Noradrenaline of Vasopressin and Oxytocin Expression in the Mouse
646 Paraventricular and Supraoptic Nuclei. *J. Neurosci.* 22, 1513–1522.
647 <https://doi.org/10.1523/JNEUROSCI.22-05-01513.2002>

648 Yoshihara, C., Numan, M., Kuroda, K.O., 2017. Oxytocin and Parental Behaviors, in:
649 Hurlemann, R., Grinevich, V. (Eds.), *Behavioral Pharmacology of Neuropeptides: Oxytocin,*
650 *Current Topics in Behavioral Neurosciences.* Springer International Publishing, Cham, pp.
651 119–153. https://doi.org/10.1007/7854_2017_11

652 Young, L.J., Wang, Z., Donaldson, R., Rissman, E.F., 1998. Estrogen receptor alpha is
653 essential for induction of oxytocin receptor by estrogen. *Neuroreport* 9, 933–936.
654 <https://doi.org/10.1097/00001756-199803300-00031>

655 Zhang, X., Huang, J., Zheng, G., Liang, J., Hu, B., Lou, Z., Li, A., Ding, Y., 2021.
656 Prenatal exposure to di (2-ethylhexyl) phthalate causes autism-like behavior through inducing
657 Nischarin expression in the mouse offspring. *Biochem. Biophys. Res. Commun.* 585, 29–35.
658 <https://doi.org/10.1016/j.bbrc.2021.11.020>

659

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 as 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

685 exposed to the vehicle (Veh), DEHP at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix) (n = 8-
686 11/ treatment group). **A-B.** Nest score at lactational days (LD) 3, 7, 11 and 15 for primiparous
687 (**A**) and multiparous (**B**) dams. **C.** Total number of forced-nursing behaviors exhibited by pups
688 towards primiparous (left) and multiparous (right) dams. Data are represented as means ±
689 S.E.M. One-way ANOVA for the treatment effect (#p < 0.05, ###p < 0.001) followed by
690 Bonferroni's multiple comparisons tests (*p < 0.05, **p < 0.01, ***p < 0.001 versus the vehicle
691 group) are shown.

692

693 **Figure 4. Effects of exposure to DEHP alone or in a phthalate mixture on pup-retrieval**
694 **by dams and pup vocalizations. A-B.** Pup retrieval tests were performed lactational days (LD)
695 3, 7 and 11 for primiparous (**A**) and multiparous (**B**) dams (8-11/treatment group) exposed to
696 the vehicle (Veh), DEHP at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix). The duration
697 corresponds to the time spent by the dam from touching the first pup until retrieving the last
698 pup to the nest. **C-D.** Ultrasonic vocalizations (USV) emitted by pups (13-19/ treatment group)
699 from multiparous dams were recorded at PND 3, PND7 and PND11. The mean number (**C**) and
700 duration (**D**) of USVs are shown. Data are presented as means ± S.E.M. Kruskal-Wallis
701 analyses for the treatment effect (#p < 0.05, ##p < 0.01, ###p < 0.001) followed by Dunn's
702 multiple comparisons tests (*p < 0.05, **p < 0.01, ***p < 0.001 versus the control group) are
703 indicated.

704

705 **Figure 5. Effects of exposure to DEHP alone or in a phthalate mixture on oxytocinergic**
706 **immunoreactivity. A.** Representative oxytocin- (OT) immunoreactivity in the paraventricular
707 (PVN), supraoptic (SON) and the anterior commissural nuclei (ACN) of multiparous dams (5-
708 6/treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-
709 50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 50 µm for the PVN and

710 100 μm for the SON and ACN. **B.** Quantitative analysis of the number of OT-immunoreactive
711 (ir) neurons in the PVN, SON and ACN. Data are presented as means \pm S.E.M. One-way
712 ANOVA for the treatment effect ($\#\#p < 0.01$) followed by Bonferroni's multiple comparisons
713 test ($*p < 0.05$, $**p < 0.01$, versus the control group) are indicated. 3V, 3rd ventricle; AC,
714 anterior commissure; opt, optic chiasma.

715

716 **Figure 6. Effects of exposure to DEHP alone or in a phthalate mixture on estrogen**
717 **receptor (ER) α -immunoreactivity and dendritic spine density in the medial preoptic**
718 **area. A.** Representative ER α -immunoreactivity in the medial preoptic area (MPOA), bed
719 nucleus of stria terminalis (BNST) and medial amygdala (MeA) of multiparous dams (5-
720 6/treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-
721 50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 100 μm . **B.** Quantitative
722 analysis of the number of ER α immunoreactive (ir) neurons in the mPOA, BNST and MeA. **C.**
723 Representative image of Golgi-Cox impregnated neurons of the MPOA in dams from the Veh
724 and DEHP-groups. Scale bar = 50 μm . **D.** Quantification of the total number of dendritic spines
725 and subtypes (thin, stubby, mushroom). Data are presented as means \pm S.E.M. One-way
726 ANOVA for the treatment effect ($\#\#p < 0.01$) followed by Bonferroni's multiple comparisons
727 test ($**p < 0.01$ versus the vehicle group) for (**B**), and paired Student test for (**D**) ($*p < 0.05$
728 versus the control group) are indicated. 3V, 3rd ventricle; AC, anterior commissure.

729

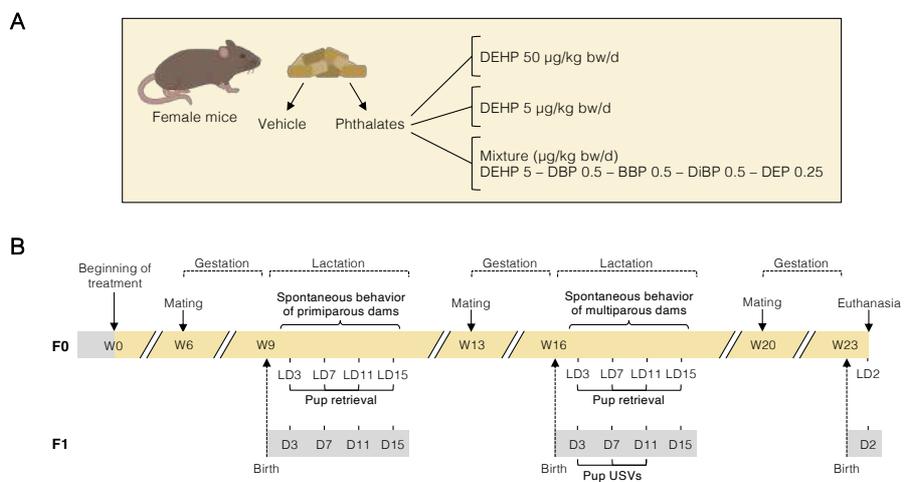


Figure 1

730

731

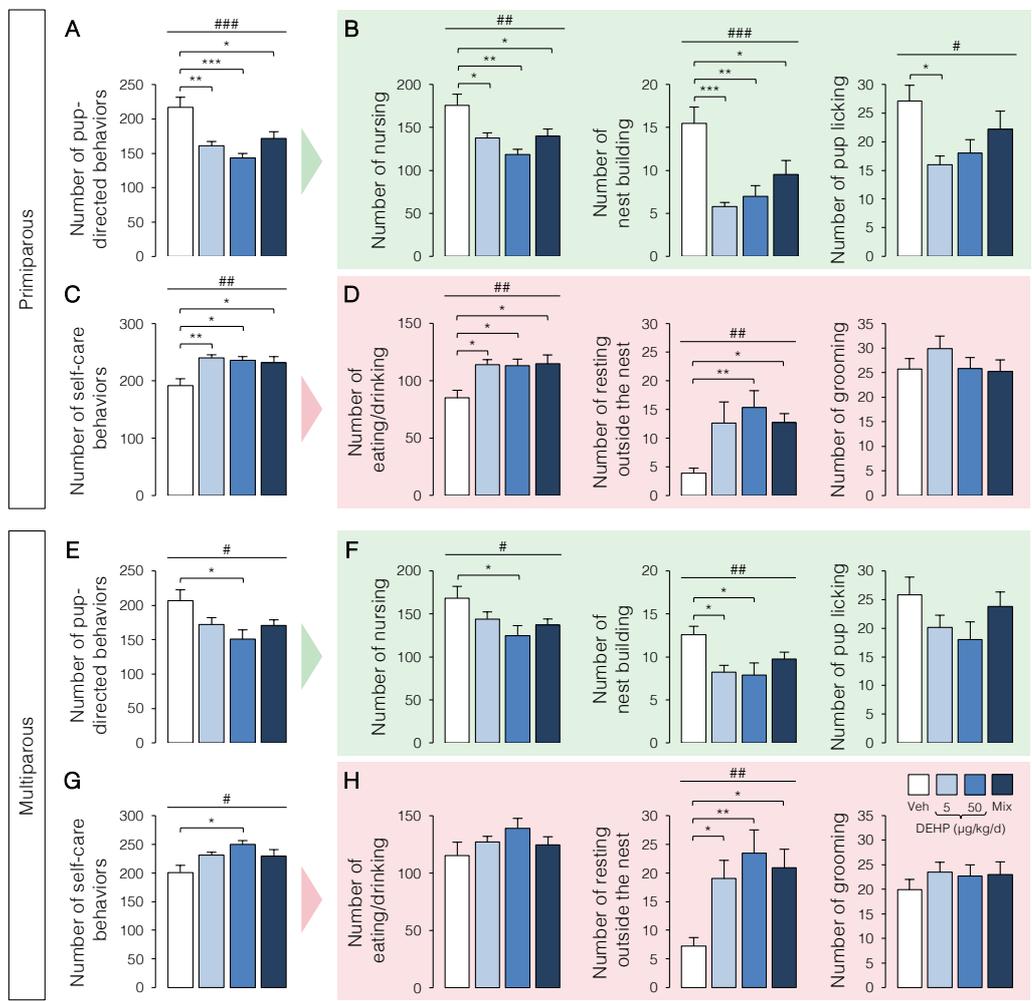


Figure 2

732

733

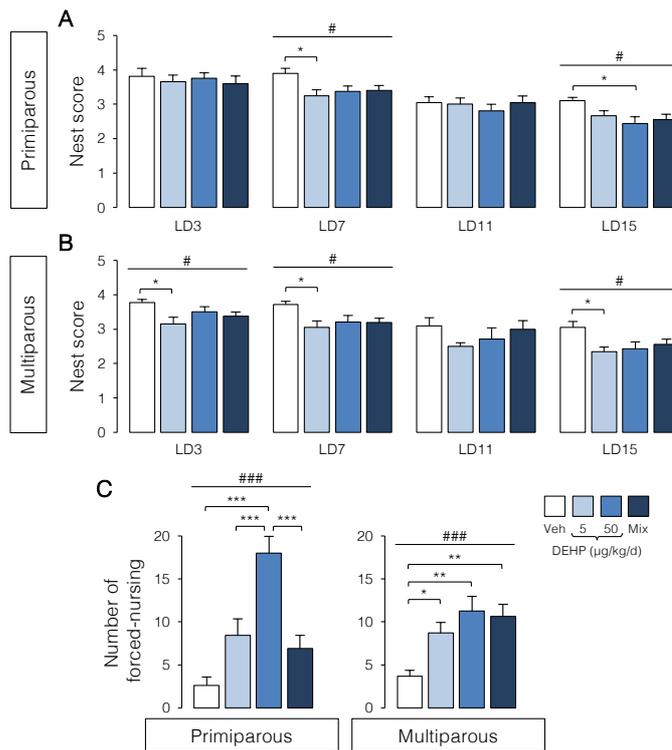


Figure 3

734

735

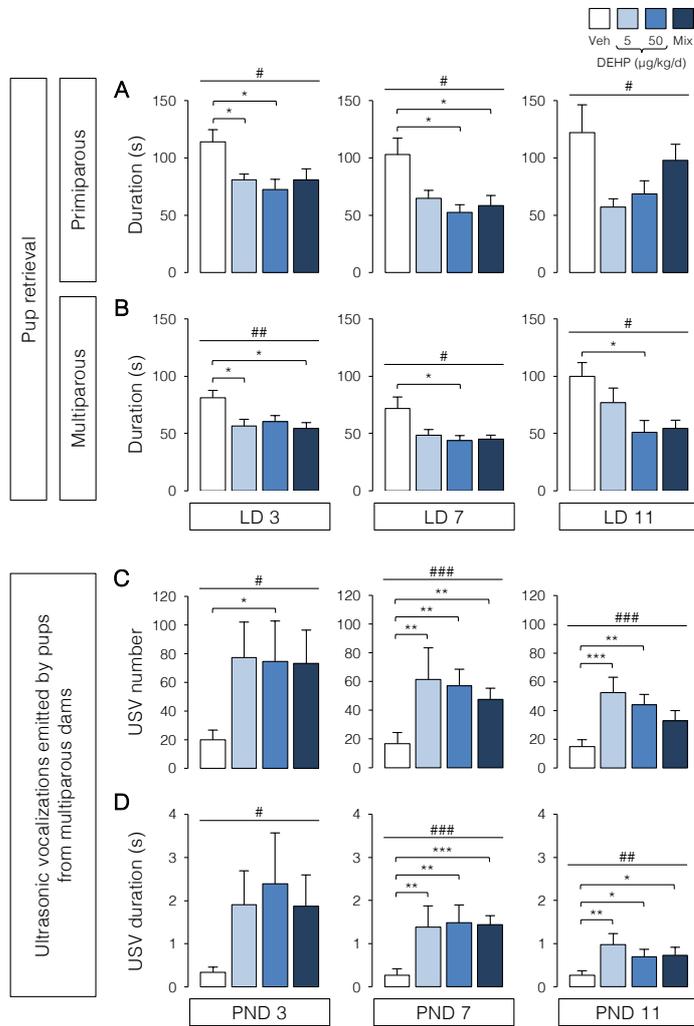


Figure 4

736

737

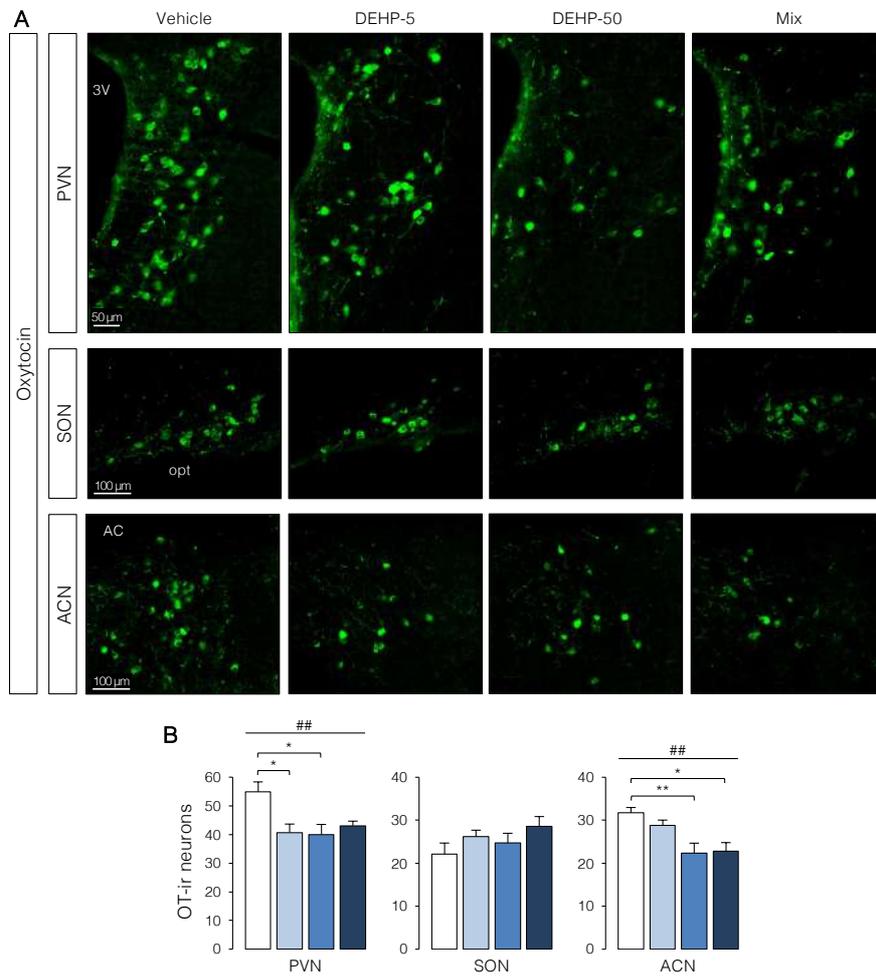


Figure 5

738

739

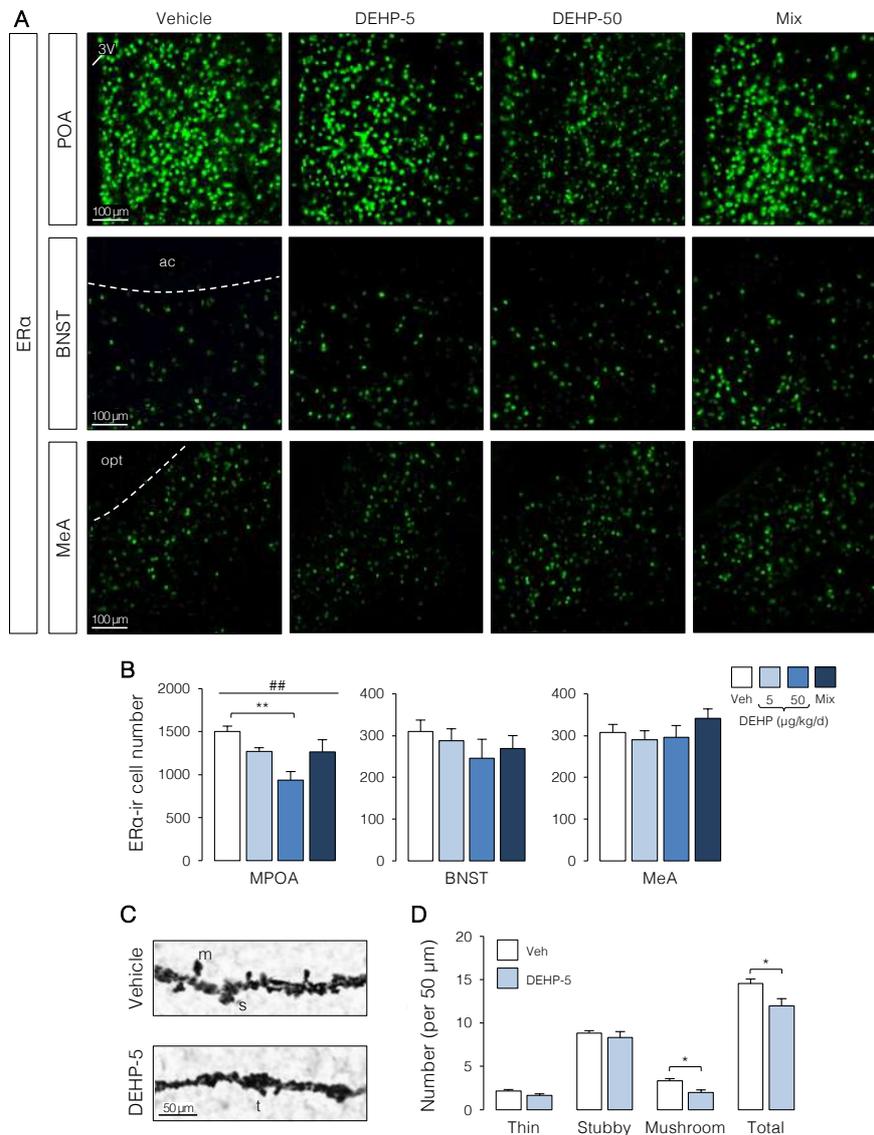


Figure 6

740

741

742 **Supplemental Methods**

743 **Figure S1**

744 **Figure S2**

745 **Figure S3**

746 **Figure S4**

747 **Figure S5**

748 **Figure S6**

749 **Figure S7**

750 **Figure S8**

751 **Table S1**

752

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.

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

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

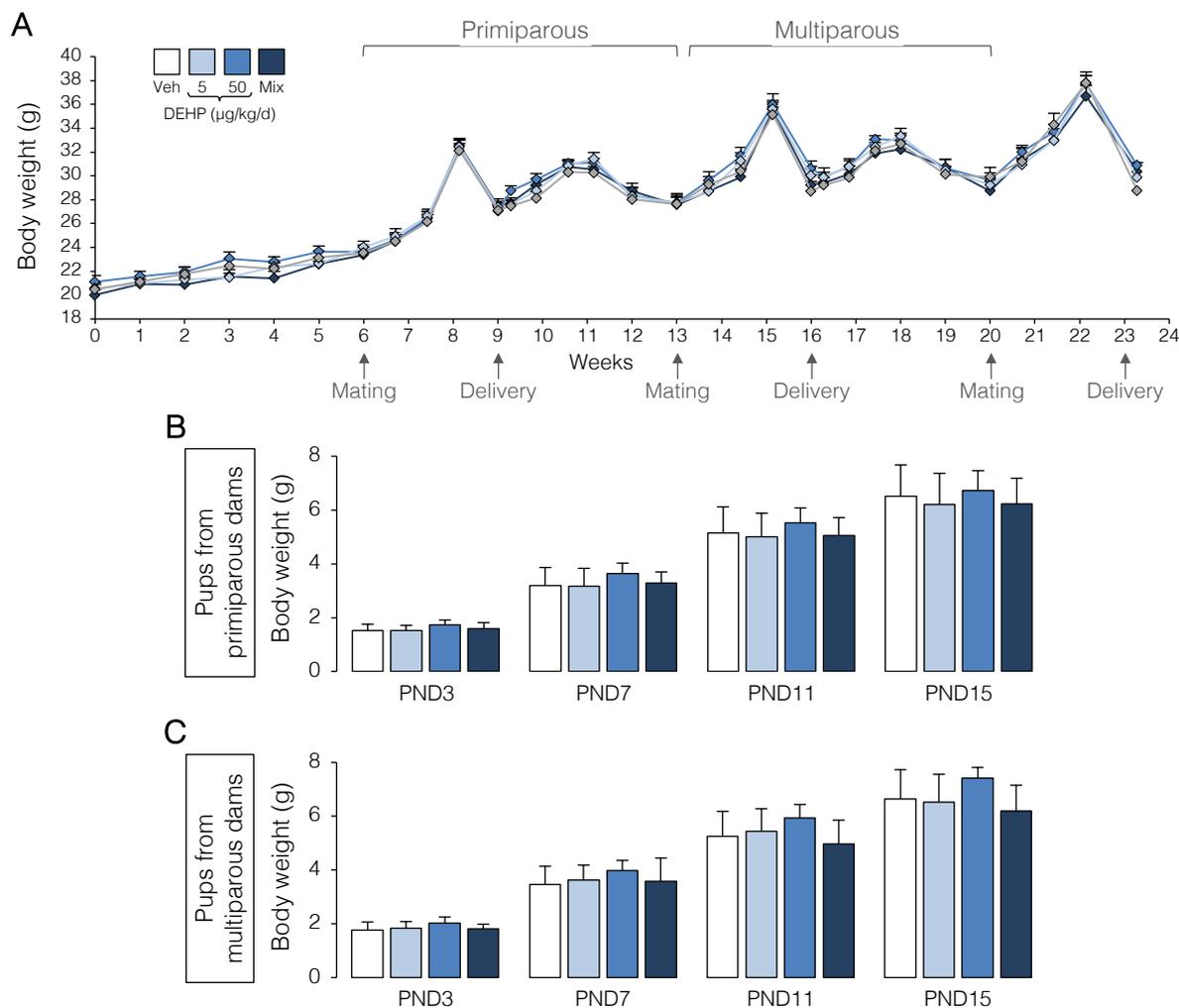
783

784 **References**

785 Wolterink-Donselaar, I.G., Meerding, J.M., Fernandes, C., 2009. A method for gender
786 determination in newborn dark pigmented mice. *Lab Anim.* 38, 35–38.

787 <https://doi.org/10.1038/labani0109-35>

788



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

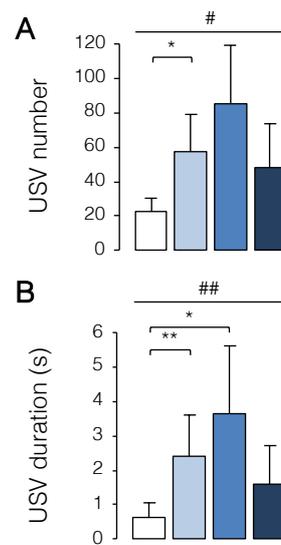
794 primiparous (**B**) and multiparous (**C**) dams of the four treatment groups at postnatal days (PND)

795 3, 7, 11 and 15. Data are presented as means ± S.E.M.

796

797

798



799

800

801 **Figure S2. Effects of exposure to DEHP alone or in a phthalate on vocalizations emitted**

802 **by pups from primiparous dams. A-B.** Ultrasonic vocalizations (USV) emitted by pups (13-

803 19 per treatment group) from primiparous dams were recorded at postnatal day (PND) 3. The

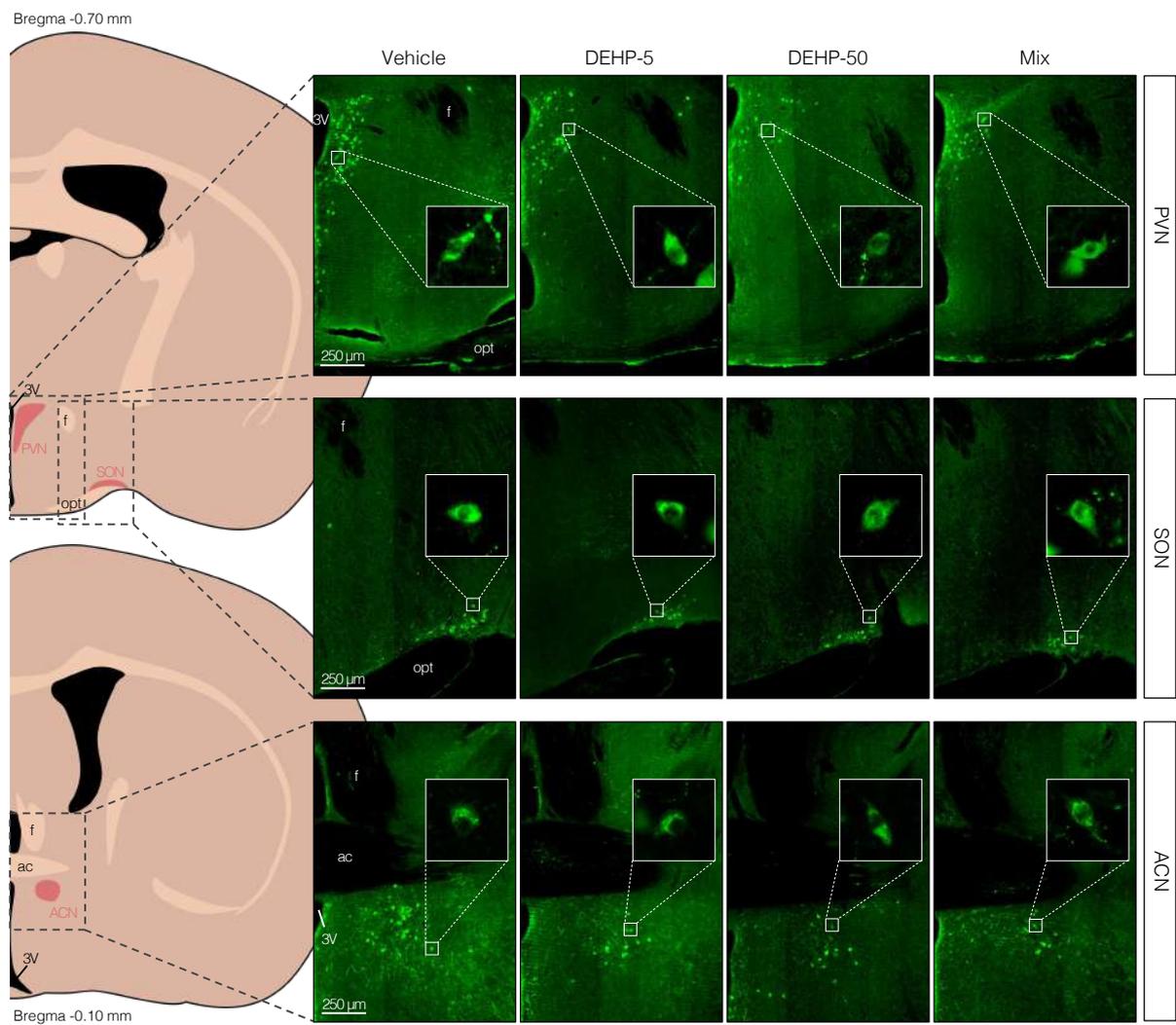
804 mean number (A) and duration (B) of USVs are shown. Data are presented as means \pm S.E.M.

805 Kruskal-Wallis analyses for the treatment effect (# $p < 0.05$.) followed by Dunn's multiple

806 comparisons tests (* $p < 0.05$, ** $p < 0.01$, versus the control group) are indicated.

807

808



809

810

811 **Figure S3. Representative oxytocin-immunoreactivity of anatomically matched sections.**

812 Representative cytoplasmic oxytocin-immunoreactivity in the paraventricular nucleus (PVN),

813 supraoptic nucleus (SON) and anterior commissural nucleus (ACN) of multiparous dams

814 exposed to the vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-50), or to a

815 phthalate mixture (Mix) at lactational day 2. The insert shows the cytoplasmic immunostaining.

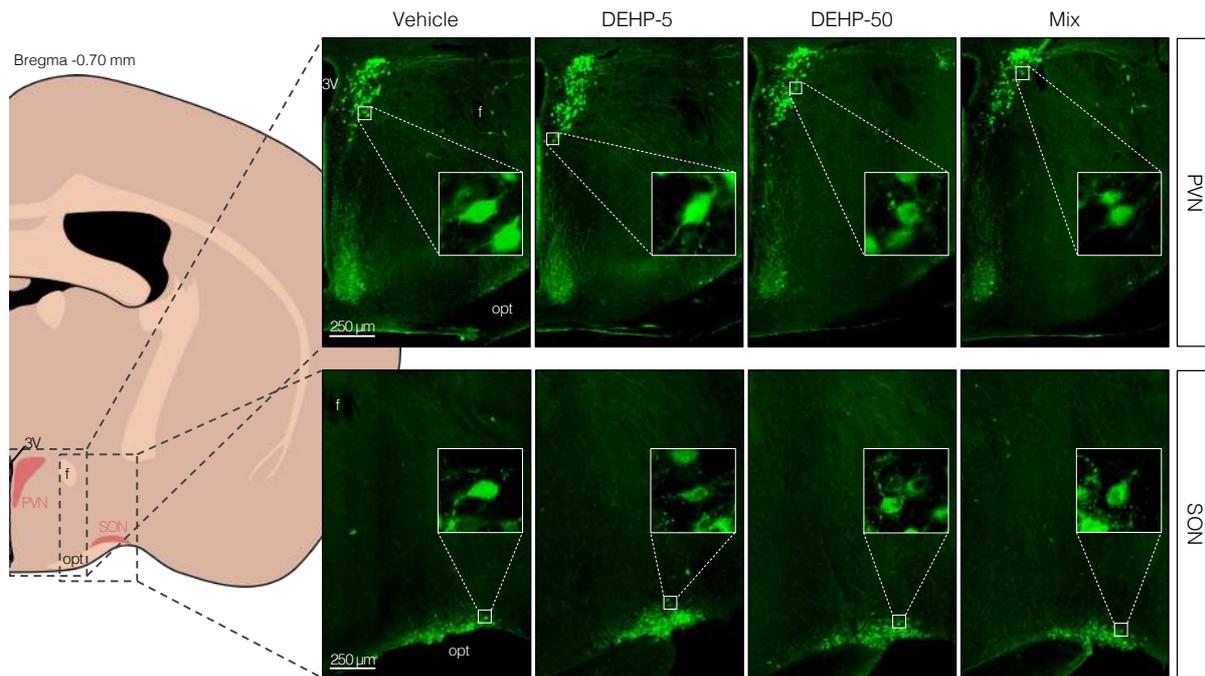
816 Scale bar = 250 μm . ac, anterior commissure; f, fornix; opt, optic chiasma; 3V, 3rd ventricle.

817

818

819

820



821

822

823

824

825

Figure S4. Representative arginine vasopressin-immunoreactivity of anatomically

826

matched sections. Representative cytoplasmic arginine vasopressin-immunoreactivity in the

827

paraventricular nucleus (PVN) and supraoptic nucleus (SON) of multiparous dams exposed to

828

the vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-50), or to a phthalate mixture

829

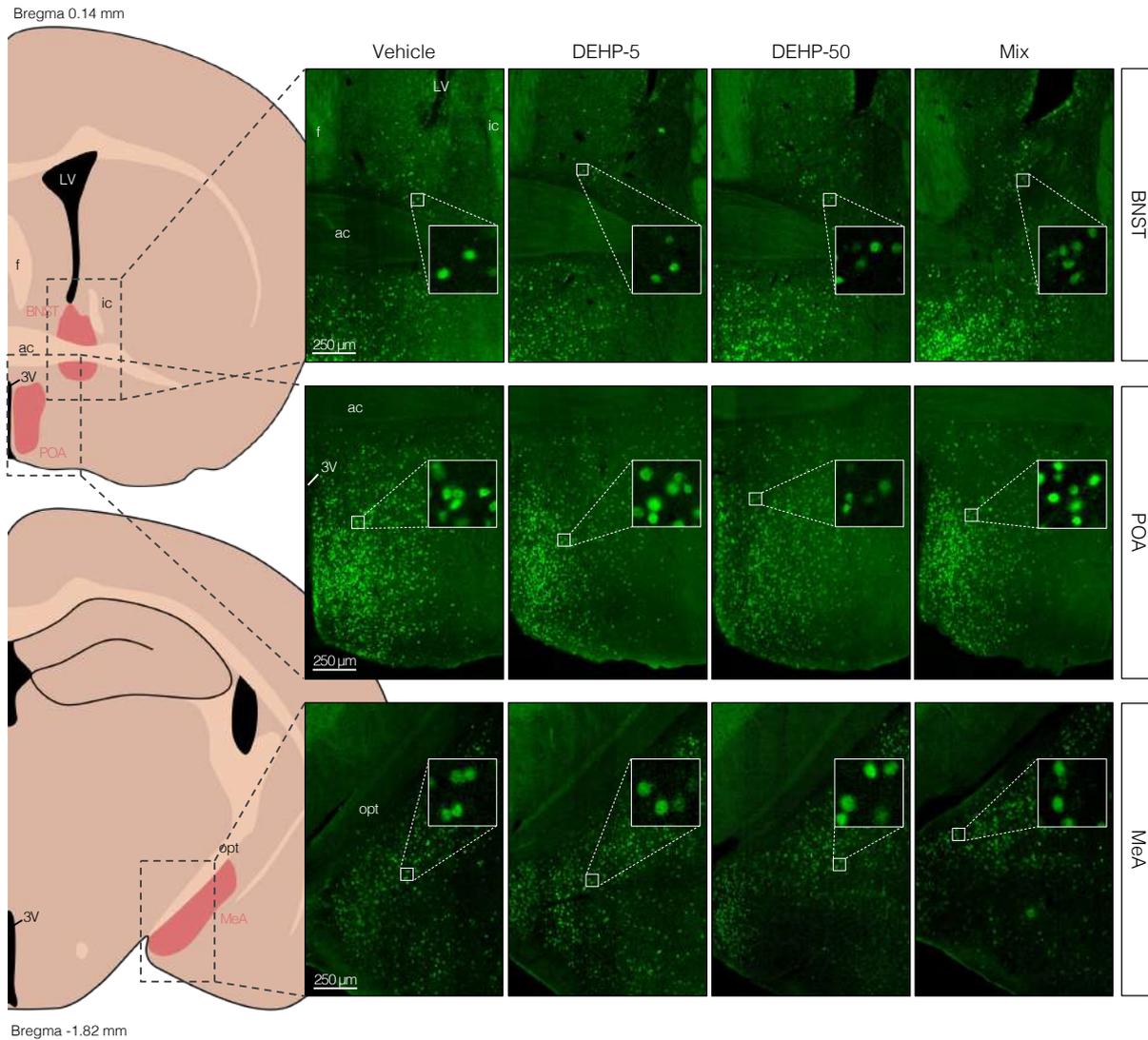
(Mix) at lactational day 2. The insert shows the cytoplasmic immunostaining. Scale bar = 250

830

μm . ac, anterior commissure; f, fornix; opt, optic chiasma; 3V, 3rd ventricle.

831

832



834

835

836 **Figure S5. Representative ER α -immunoreactivity of anatomically matched sections.**837 Representative nuclear ER α -immunoreactivity in the bed nucleus of stria terminalis (BNST),

838 medial preoptic area (MPOA) and medial amygdala (MeA) of multiparous dams exposed to the

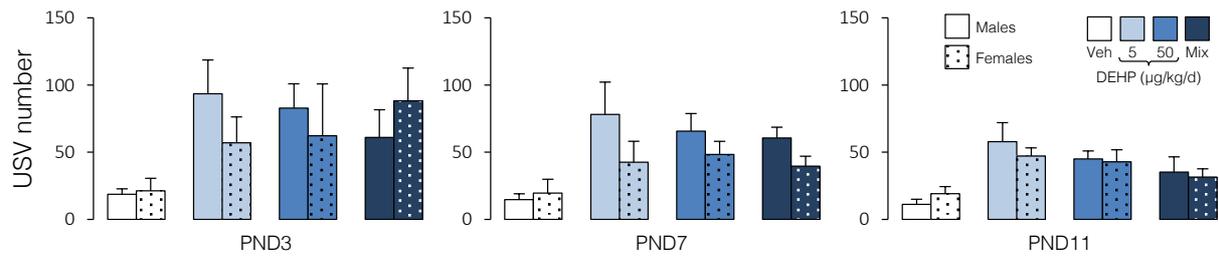
839 vehicle (Vehicle), DEHP at 5 (DEHP-5) or 50 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-50), or to a phthalate mixture840 (Mix) at lactational day 2. The insert shows the nuclear immunostaining. Scale bar = 250 μm .

841 ac, anterior commissure; f, fornix; ic, internal capsule; LV, lateral ventricle; opt, optic chiasma;

842 3V, 3rd ventricle.

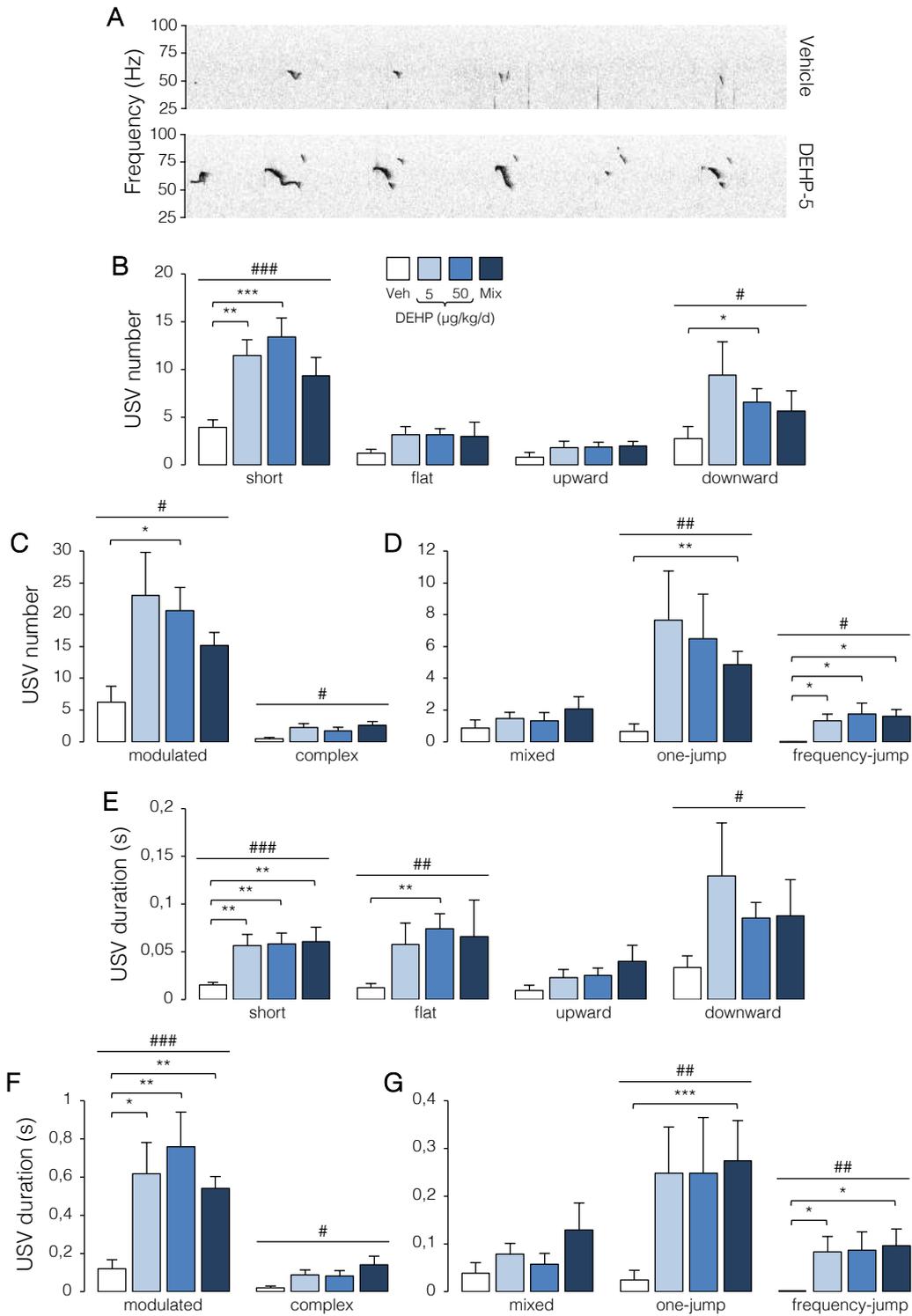
843

844



845
 846 **Figure S6. Number of ultrasonic vocalizations (USV) emitted by male and female pups**
 847 **exposed to DEHP alone or in a phthalate mixture.** Number of USVs emitted by male and
 848 female pups born from multiparous dams exposed to the vehicle (Veh), DEHP alone at 5 or 50
 849 µg/kg/d, or to a phthalate mixture (Mix). Data shown for postnatal days (PND) 3, 7 and 11 are
 850 presented as means ± S.E.M (n = 8-13 per treatment group per sex). Two-way ANOVA did not
 851 show any effect of the sex at PND 3 (p = 0.658), PND7 (p = 0.085) and PND11 (p = 0.703).

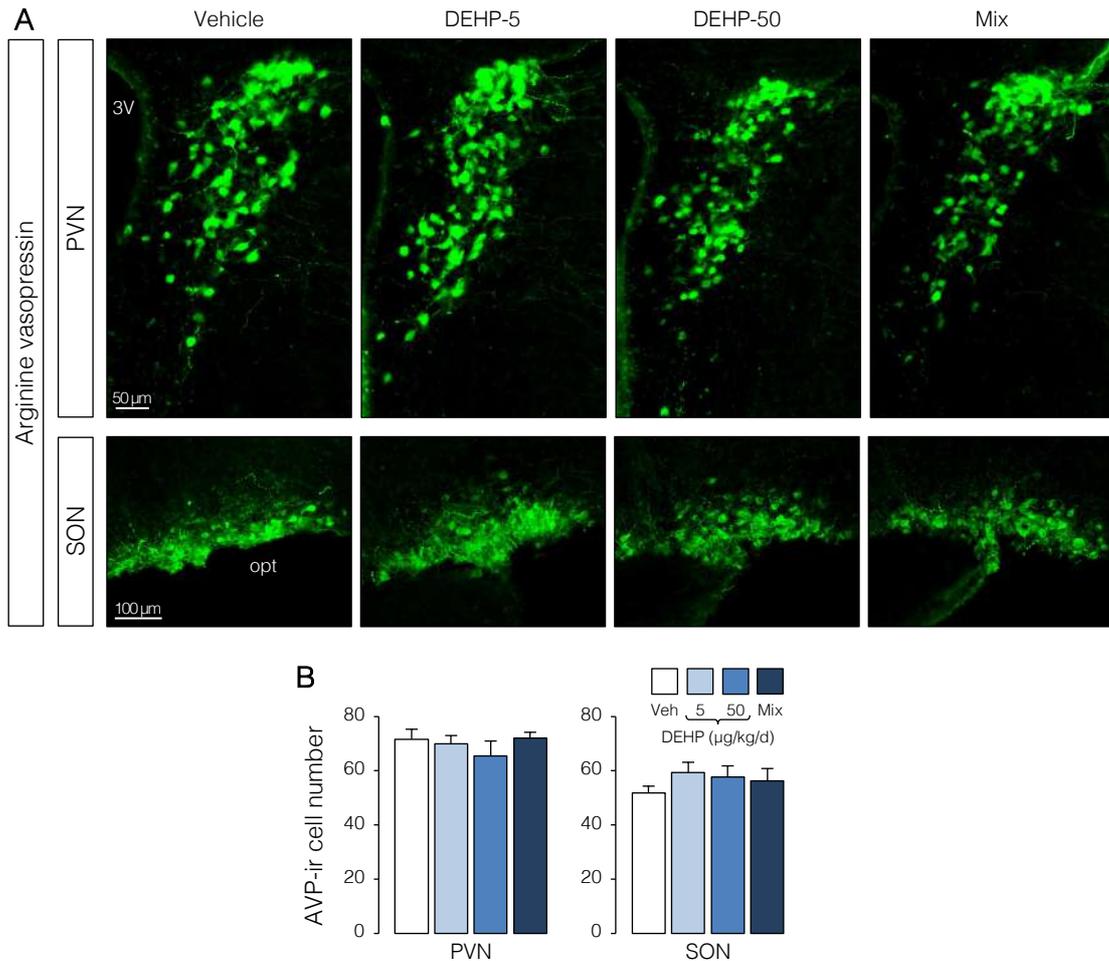
852
 853
 854
 855



856
 857 **Figure S7. Effects of maternal exposure to DEHP alone or in a phthalate mixture on the**
 858 **type of syllables emitted by pups at postnatal day 7.** Ultrasonic vocalizations (USVs) emitted
 859 by pups (13-18 per treatment group) born from multiparous dams exposed to the vehicle (Veh),
 860 DEHP at 5 or 50 $\mu\text{g}/\text{kg}/\text{d}$, or to a phthalate mixture (Mix) are analyzed on lactational day 7. **A.**
 861 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 \pm
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.

867



869
870
871
872
873

Figure S8. Effects of exposure to DEHP alone or in a phthalate mixture on arginine

874 **vasopressin-immunoreactivity.** **A.** Representative arginine vasopressin- (AVP)
875 immunoreactivity in the paraventricular (PVN) and supraoptic (SON) nuclei of multiparous
876 dams (5-6 per treatment group) exposed to the vehicle (Veh), DEHP at 5 (DEHP-5) or 50
877 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-50), or to a phthalate mixture (Mix) at lactational day 2. Scale bar = 50 μm for
878 the PVN and 100 μm for the SON. **B.** Quantitative analysis of the number of AVP-
879 immunoreactive (ir) neurons in the PVN and SON. Data are presented as means \pm S.E.M. opt,
880 optic chiasma; 3V, 3rd ventricle.

881
882
883
884

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

887

888

889