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## Effects of combined exposure of adult male mice to di-(2-ethylexyl)phthalate and nonylphenol on behavioral and neuroendocrine responses

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1 **Title**

2 Effects of combined exposure of adult male mice to di-(2-ethylexyl)phthalate and nonylphenol on  
3 behavioral and neuroendocrine responses

4 **Running title**

5 Effects of co-exposure to DEHP/NP in male mice

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23

24 **Abstract**

25 The present study evaluates the effects of adult exposure to low doses of a mixture of di-(2-  
26 ethylexyl)phthalate (DEHP) and nonylphenol (NP) on reproductive neuroendocrine function and  
27 behavior. The neural circuitry that processes male sexual behavior is tightly regulated by testosterone  
28 and its neural metabolite estradiol. In previous studies, we showed that adult exposure of mice to low  
29 doses of each of these widespread environmental contaminants resulted in altered sexual behavior,  
30 without any effect on the regulation of the gonadotropic axis. Here, adult C57BL/6J male mice were  
31 exposed to DEHP/NP (0.5 or 5  $\mu\text{g}/\text{kg}$  body weight/day) for 4 weeks before starting the analyses. Mice  
32 treated with DEHP/NP at 0.5  $\mu\text{g}/\text{kg}/\text{day}$  show altered olfactory preference, and fewer of them emit  
33 ultrasonic vocalization compared to the other treatment groups. These mice also exhibit a lower  
34 number of mounts and thrusts, increased locomotor activity and unaffected anxiety-state level, along  
35 with unaltered testosterone levels and kisspeptin system, a key regulator of the gonadotropic axis.  
36 Analysis of the neural circuitry that underlies sexual behavior showed that the number of cells  
37 expressing androgen and estrogen receptors is comparable between control and DEHP/NP-exposed  
38 males. The comparison of these data with those obtained in males exposed to each molecule separately  
39 highlights synergistic effects at the lower dose of contaminants of 0.5  $\mu\text{g}/\text{kg}/\text{day}$ . In contrast, the  
40 effects previously observed for each molecule at 5  $\mu\text{g}/\text{kg}/\text{day}$  were not detected. A detailed  
41 comparison of the effects triggered by separate or combined exposure to DEHP and NP is discussed.

42

43 **Highlights**

44 Adult co-exposure to DEHP/NP alters male sexual behavior.

45 DEHP/NP mixture increases locomotor activity and reduces anxiety-state level.

46 The lower dose of DEHP/NP mixture triggers the majority of behavioral effects.

47 No effects of DEHP/NP mixture on the gonadotropic axis.

48 Unaltered number of AR- and ER $\alpha$ -immunoreactive cells in exposed males.

49

50	<b>Keywords</b>
51	Nervous system
52	Endocrine disruptor
53	Phthalates
54	Nonylphenol
55	Behavior
56	Reproduction
57	

## 58 **1. Introduction**

59 Di-(2-ethylhexyl)phthalate (DEHP) and nonylphenol (NP) are abundant organic pollutants found in  
60 the environment. While DEHP is used in the manufacture and processing of plastic products, NP  
61 derives from the degradation of alkylphenol that is used in a wide variety of industrial, agricultural and  
62 domestic applications such as soap, cosmetics, paint, herbicides and pesticides, or plastic fabrication.  
63 Both DEHP and NP were classified by the EU in 2000 as priority substances “presenting a significant  
64 risk to or via the aquatic environment” in the Water Framework Directive 2000/60/EC, which was  
65 updated in 2008 and 2013 (Directive 2013/39/EU of the European Parliament and of the Council of 12  
66 August 2013). These molecules were described as exhibiting, respectively, estrogenic activity for NP  
67 (Laws et al., 2000), and anti-androgenic effects like the reduction of testosterone production for DEHP  
68 (Barakat et al., 2017; Pocar et al., 2012).

69 Recently, we showed that chronic treatment of adult male mice with DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{day}$   
70 induced a sexual alteration. This was evidenced by the reduced emission of ultrasonic vocalizations  
71 and attractiveness, and a delayed initiation of mating and ejaculation (Dombret et al., 2017). These  
72 behavioral modifications were associated with down-regulation of the androgen receptor (AR) in the  
73 neural circuitry that underlies sexual behavior, without any changes in the circulating level of  
74 testosterone or the integrity of the gonadotropic axis. In contrast, chronic exposure of adult male mice  
75 to NP at 5  $\mu\text{g}/\text{kg}/\text{day}$  increased the emission of ultrasonic vocalizations, the number of mounts,  
76 intromissions, and thrusts. This also resulted in delayed ejaculation (Capela et al., 2018). Ten-fold  
77 lower and higher doses of NP (0.5 and 50  $\mu\text{g}/\text{kg}/\text{day}$ , respectively) did not induce any behavioral  
78 changes. Once again, exposure to NP altered neither the gonadotropic axis nor testosterone levels.  
79 However, it modified the levels of AR and the estrogen receptor (ER) in the neural circuitry that  
80 underlies sexual behavior. In this context, whether and how combined exposure to DEHP and NP  
81 affects these neural processes, in particular at doses close to that of environmental exposure, remains  
82 to be studied.

83

84 In the literature, the few reported studies that address the effects of combined exposure to  
85 phthalates and NP focus on peripheral functions. Studies in rats *in vitro* show a negative additive  
86 effect of a mixture containing di-n-butyl phthalate (DBP) or its metabolite mono- butyl phthalate  
87 (MBP) and NP. This causes decreased viability of Sertoli cells, and changes in morphological  
88 parameters and membrane permeability, as well as increased apoptosis (Hu et al., 2012; Li et al.,  
89 2010). In another study, the same group observed an alteration in the morphology of tight junctions  
90 located between rat Sertoli cells, both *in vitro* and *in vivo*, with higher sensitivity to NP than to MBP  
91 (Hu et al., 2014a). A similar mixture also resulted in antagonistic effects on levels of testosterone, LH  
92 (luteinizing hormone), and FSH (follicle stimulating hormone), at doses ranging from 50 to 450  
93 mg/kg/day, indicating competitive interactions between the two chemicals (Hu et al., 2014b). A recent  
94 analysis pertaining to combined treatment of mice with phthalates (DEHP, DBP and benzyl butyl  
95 phthalate (BBP) at 300  $\mu\text{g}/\text{kg}/\text{day}$ ) and alkylphenols (NP and octylphenols (OP) at 50  $\mu\text{g}/\text{kg}/\text{day}$ )  
96 showed changes in the testicular miRNome, together with decreased testicular estradiol, altered  
97 spermatogenesis, and germ cell apoptosis following long-term exposure including gestational,  
98 lactational, prepubertal, and pubertal periods (Buñay et al., 2017).

99

100 The present study was undertaken to characterize the effects of chronic exposure of male mice to  
101 low doses of the two molecules together. The same behavioral and neuroendocrine endpoints  
102 previously tested for each compound alone were analyzed. To this end, male mice were treated orally  
103 with the vehicle alone or with DEHP and NP (5 and 0.5  $\mu\text{g}/\text{kg}/\text{day}$ ) for four weeks. Courtship behavior  
104 of male mice was analyzed by testing their olfactory preference and emission of courtship vocalization  
105 in the presence of receptive females. Latency and frequency of copulatory behavioral events were also  
106 quantified. Furthermore, we measured locomotor activity and the anxiety-state level, which, if altered,  
107 could interfere with the expression of sexual behavior. Testosterone levels and weight of androgen-  
108 sensitive tissues of the genital tract were compared between the three groups. Kisspeptin-  
109 immunoreactive neurons were quantified in the rostral preoptic periventricular nucleus (RP3V) and in  
110 the arcuate nucleus. Kisspeptin acts as a central regulator of the gonadotropic axis and is currently

111 considered to be a key central target of testosterone feedback regulation for GnRH (gonadotropin-  
112 releasing hormone)/LH release (Navarro et al., 2011; Raskin et al., 2009; Ruka et al., 2016; Smith et  
113 al., 2005). Finally, the number of AR- and ER-immunoreactive neurons in the neural circuitry that  
114 underlies sexual behavior was determined.

115



## 116 **2. Materials and Methods**

### 117 **2.1. Animals and treatment**

118 Analyses were performed according to European legal requirements (Decree 2010/63/UE) and were  
119 approved by the “Charles Darwin” Ethical committee (project number 01490-01). Mice of the  
120 C57BL/6J strain (Janvier) were housed in nest-enriched polysulfone cages maintained at 22°C, with a  
121 12:12 h light-dark cycle, and were fed a standard diet with free access to food and water. The numbers  
122 of experimental and control groups are given in the figure legends.

123 To mimic the major route of exposure to NP and DEHP, eight-week-old mice were fed ad libitum a  
124 standard diet containing the vehicle (control group) or DEHP and NP (Sigma-Aldrich). These  
125 compounds were dissolved in an ethanol-water mix and were incorporated into the food, so that  
126 exposure was equivalent to 0.5 µg/kg body weight/day (DEHP/NP-0.5 group) or 5 µg/kg body  
127 weight/day (DEHP/NP-5 group). DEHP and NP doses were calculated for a daily food intake of 5 g  
128 per animal. Mice were weighed weekly and DEHP and NP doses adjusted according to changes in  
129 their body weight. This latter parameter was followed throughout the whole experimental period, and  
130 was similar in the four treatment groups ( $27 \pm 0.3$  g,  $27.8 \pm 0.6$  g,  $25 \pm 0.6$  g for the vehicle,  
131 DEHP/NP-0.5 and DEHP/NP-5, respectively, on the first day of treatment; and  $31 \pm 0.6$  g,  $33.1 \pm 0.8$   
132 g,  $32 \pm 0.8$  g on the last day). Analyses were started after 4 weeks of treatment with DEHP and NP,  
133 conditions that were maintained during the whole experimental period.

134

### 135 **2.2. Behavioral tests**

136 Tests were conducted under red-light illumination 2 h after lights were turned off, and were  
137 videotaped for later analysis as previously described (Capela et al., 2018; Dombret et al., 2017). Four  
138 weeks after exposure to DEHP and NP, naïve male mice were housed individually for 3 days and then  
139 paired with a sexually receptive female for their first sexual experience as described below (Capela et  
140 al., 2018; Dombret et al., 2017). Analyses of recordings were performed by blind observation, since  
141 males were identified by numbers attributed at weaning without any information concerning their  
142 treatment details.

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**2.2.1. Preparation of sexually receptive females**

C57BL/6J female mice used as stimuli were ovariectomized under general anesthetic (xylazine/ketamine) and implanted with SILASTIC implants (Dow Corning, Midland, MI) filled with 50  $\mu$ g of estradiol-benzoate (Sigma-Aldrich) in 30  $\mu$ l of sesame oil. Four to 5 h before the tests, females were given a sub-cutaneous injection of 1 mg of progesterone (Sigma-Aldrich) in 100  $\mu$ l of sesame oil. Female receptivity was verified before the beginning of experiments, using sexually experienced males.

**2.2.2. Olfactory preference test**

Olfactory preference was assessed in an enclosed plexiglass Y-maze. On the day of the test, male mice were offered the choice between an anesthetized sexually receptive female and an anesthetized gonadally intact male. The time spent in chemo-investigation of each stimulus and the number of entries into each arm of the Y-maze were scored over 10 min. The discrimination index was calculated as the time spent in female investigation (F) minus the time spent in male investigation (M) divided by the total time of investigation (F-M/F+M).

**2.2.3. Ultrasonic vocalization recording**

Each male mouse was tested in its home cage, in the presence of a sexually receptive female. After introduction of the female into the cage, vocalizations were recorded for 4 min with a microphone (UltraSoundGate) connected to an ultrasound recording interface plugged into a computer equipped with Avisoft-SASLab Pro 5.2.09 recording software, then analyzed using SASLab Pro (Avisoft Bioacoustic). Spectrograms were generated for each call detected (frequency resolution: FFT-length: 512; frame size: 100%; overlap: 50%). The parameters used for the automatic quantification of the ultrasonic vocalizations were: cut-off frequency of 30 kHz, element separation based on an automatic single threshold with a hold time of 15 ms. The percentage of animals emitting vocalizations was determined.

171 **2.2.4. Mating**

172 Each male mouse was tested in its home cage for 10 h after introduction of the receptive female. The  
173 latency to the first intromission or ejaculation (time from the female introduction into the cage until  
174 the behavioral event), the mating duration (time from the first mount until ejaculation), the frequencies  
175 of mounts, intromissions and thrusts were scored.

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177 **2.2.5. Locomotor activity**

178 Activity was analyzed in a computed circular corridor. Briefly, the male subject was introduced into a  
179 circular corridor made up of two concentric cylinders crossed by four diametrically opposite infrared  
180 beams (Imetronic). Locomotor activity was counted when animals interrupted two successive beams  
181 and had thus traveled a quarter of the circular corridor. Spontaneous activity was recorded for 140 min  
182 and was expressed as cumulative activity every 20 min or cumulative activity over the whole 140 min  
183 test.

184

185 **2.2.6. Anxiety-related behavior**

186 Males were placed in the closed arms of the O-maze and were allowed to explore the maze freely for 9  
187 min. The latency time before entry into the open arms, the number of entries and the time spent in the  
188 open arms were analyzed. A mouse was considered to be in an open arm once all its 4 paws had  
189 entered the arm. The light intensity was 60 lux.

190

191 **2.3. Immunohistochemistry**

192 Animals were euthanized and transcardially perfused with a solution of 4% paraformaldehyde (PFA)  
193 in phosphate buffer. Brains were post-fixed overnight in 4% PFA, cryoprotected in sucrose and stored  
194 until analyses. Brains were sliced into coronal sections of 30  $\mu$ m and immunolabeling processed as  
195 performed previously (Capela et al., 2018; Dombret et al., 2017).

196 Kisspeptin immunolabeling was processed with anti-kisspeptin AC053, and sections were  
197 counterstained with Hoechst. The number of labeled cell soma and fiber density in the anteroventral  
198 periventricular (AVPV) and periventricular nuclei (PeN) of RP3V, and the arcuate nucleus were

199 quantified. Images for fiber were acquired with an LSM 700 confocal microscope (Zeiss) under x40  
200 magnification. Quantifications were performed on three sections sampled at one anteroposterior level  
201 of RP3V corresponding to the AVPV (plate 29 of Paxinos and Franklin atlas) and at two levels  
202 corresponding to the PeN (plates 30 and 31-32), and on three sections of the arcuate nucleus sampled  
203 at the levels of the anterior, median and caudal arcuate nuclei (plates 43, 47 and 50).

204 AR- and ER $\alpha$ -immunolabeling were processed as previously described (Capela et al., 2018; Dombret  
205 et al., 2017). Images were acquired with a Nikon Eclipse 80i light microscope under x10  
206 magnification. The number of labeled cells per section were counted in anatomically matched  
207 sections. Counting surfaces for medial amygdala (plate 45 of Paxinos and Franklin atlas), bed nucleus  
208 of stria terminalis (plate 33), and medial preoptic nucleus (plate 30) were respectively 0.07 mm<sup>2</sup>, 0.055  
209 mm<sup>2</sup> and 0.31mm<sup>2</sup>.

210

#### 211 **2.4. Urogenital tract weight and hormone measurements**

212 Animals were euthanized to collect their blood and to weigh testes and seminal vesicles. Serum was  
213 prepared as previously described (Raskin et al., 2009), and circulating levels of testosterone were  
214 measured by RIA using <sup>3</sup>H-testosterone at the hormonal assay platform of the laboratory of behavioral  
215 and reproductive physiology (UMR INRA/ CNRS / Université de Tours). The mean intra-assay  
216 variation coefficient was 7%, and the assay sensitivity was 125 pg/ml.

217

#### 218 **2.5. Statistical analyses**

219 The percentage of animals emitting ultrasonic vocalizations were compared by chi square Test. The  
220 other data were expressed as the mean  $\pm$  S.E.M. Two-way ANOVA was used to analyze the main  
221 effects of treatment and stimulus on olfactory preference (number of entries into the arms), and of  
222 treatment and time on locomotor activity. One-way ANOVA was used to analyze the effects of  
223 exposure on the remaining data. Tukey post-hoc tests were used to determine group differences. P  
224 values of less than 0.05 were considered to be significant.

225

## 226 **3. Results**

### 227 **3.1. Effects of adult co-exposure to DEHP and NP on sexual behavior**

228 In the presence of a sexually receptive female, the male mouse exhibits olfactory investigation of the  
229 female genitals. The pheromonal cues emitted by the female are detected by the olfactory bulb, which  
230 transmits signals to the chemosensory areas involved in the activation of male sexual behavior. Male  
231 mice exposed to the vehicle or DEHP/NP mixture at 0.5 and 5  $\mu\text{g}/\text{kg}/\text{day}$  were therefore analyzed for  
232 their olfactory preference in a behavioral test using anesthetized gonadally intact males versus  
233 sexually receptive females. Figure 1A shows that there was no effect of DEHP/NP exposure on the  
234 time spent in total chemo-investigation ( $p = 0.36$ ). Two-way ANOVA showed no effect of exposure to  
235 DEHP/NP ( $F_{(2, 21)} = 0.50$ ,  $p = 0.61$ ) or stimulus ( $F_{(1, 21)} = 2.39$ ,  $p = 0.14$ ) on the number of entries into  
236 the Y maze arms (Fig. 1B). In contrast, an effect of DEHP/NP exposure on the ability of mice to  
237 discriminate between male and female stimuli was observed ( $p = 0.0001$ ; Fig. 1C). Indeed, male  
238 groups exposed to DEHP/NP at 0.5  $\mu\text{g}/\text{kg}/\text{day}$  showed no preference for females over males, while  
239 such a preference was observed for the vehicle and DEHP/NP-5 groups.

240 Olfactory stimulation activates the emission of ultrasonic vocalization by males (Bean, 1982; Dizinno  
241 and Whitney, 1977; Nyby et al., 1977), which seem to play a key role in female attraction and may  
242 facilitate copulation (Pomerantz et al., 1983). Male mice mainly vocalized at a frequency of 40-110  
243 kHz. The percentage of vocalizing animals differed between the three treatment groups, since only  
244 25% of the male group exposed to a DEHP/NP mixture at 0.5  $\mu\text{g}/\text{kg}/\text{day}$  emitted ultrasonic  
245 vocalizations in the presence of sexually receptive females ( $p = 0.049$ ). In contrast more than 60% of  
246 males did so in the two other groups (Fig. 1D).

247 In mating tests, one-way ANOVA showed no significant effect of treatment on the latency to initiate  
248 the first mount and first intromission, or time to reaching ejaculation, despite a tendency for these  
249 events to be increased in the DEHP/NP-0.5 group (Fig. 1E). Mating duration was also unaffected by  
250 exposure to DEHP/NP (Figure 1F). Quantification of the frequency of behavioral events showed an  
251 effect of exposure on the number of mounts ( $p = 0.02$ ) and thrusts ( $p = 0.03$ ), with males of the

252 DEHP/NP-0.5 group exhibiting less mounts and thrusts than vehicle-exposed mice (Figure 1G). No  
253 significant effects on the number of intromissions were observed.

254

### 255 **3.2. General behavioral analysis of males exposed or not to the DEHP/NP mixture**

256 General behavior such as locomotor activity or anxiety-state levels were assessed. Two-way ANOVA  
257 showed an effect of time ( $F_{(6, 114)} = 166.4$ ,  $p < 0.0001$ ) and exposure ( $F_{(2, 19)} = 3.661$ ,  $p = 0.04$ ) on  
258 locomotor activity assessed over 140 min (Fig. 2A). Post hoc analyses showed a higher activity for the  
259 DEHP/NP-0.5 group by comparison to males exposed to DEHP/NP-5 at 80 and 100 min of the test. In  
260 agreement with this, total activity showed an effect of exposure ( $p = 0.04$ ), with an increased activity  
261 of males treated with 0.5  $\mu\text{g}/\text{kg}/\text{day}$  of the DEHP/NP mixture (Fig. 2A). In the O-maze test, no effect  
262 of exposure on the latency to enter in the open arms was observed ( $p = 0.33$ ), but an effect of exposure  
263 was found on the number of entries ( $p = 0.02$ ) and on the time spent in the open arms ( $p = 0.03$ ), as  
264 shown in Fig. 2B. Post hoc analyses showed that the DEHP/NP mixture at 5  $\mu\text{g}/\text{kg}/\text{day}$  induced an  
265 anxiolytic behavior as evidenced by the higher number of entries ( $p < 0.05$  versus the vehicle group)  
266 and increased time spent in the open arms ( $p < 0.05$  versus the vehicle group), while no effect was  
267 seen at the lower dose of 0.5  $\mu\text{g}/\text{kg}/\text{day}$ .

268 Overall, behavioral data show an effect of exposure to the DEHP/NP mixture on male sexual behavior,  
269 including olfactory preference, production of ultrasonic vocalizations, and mating. Effects are seen in  
270 particular at the lower dose of pollutants of 0.5  $\mu\text{g}/\text{kg}/\text{day}$ . The sexual alterations cannot be attributed  
271 to changes in general behavior patterns, since males of this group exhibited a higher activity and  
272 showed no anxiety-like behavior.

273

### 274 **3.3. Adult co-exposure to a DEHP and NP mixture did not affect the gonadotropic axis**

275 Male behaviors are tightly controlled by testosterone. Therefore, DEHP/NP treatment could indirectly  
276 induce behavioral modifications through changes in the gonadotropic axis. At the hypothalamic level,  
277 we analyzed kisspeptin immunoreactivity in two subdivisions of RP3V (AVPV and PeN subregions)  
278 and in the arcuate nucleus. Data illustrated in Figure 3A show the presence of few neurons in the

279 AVPV and PeN subdivisions of RP3V, as is widely known for male mice (Kauffman et al., 2007).  
280 One-way ANOVA showed no effect of exposure on kisspeptin immunoreactivity in these  
281 hypothalamic areas (Fig. 3A). No differences in fiber density were seen in the arcuate nucleus (Fig.  
282 3B). In terms of hormones, we measured circulating levels of testosterone and weights of androgeno-  
283 dependent tissues. No effects on circulating levels of testosterone were observed (Fig. 3C). In  
284 agreement with these data, the weight of seminal vesicles and testis were comparable between the  
285 three treatment groups (Fig. 3D-E). These data show that the DEHP/NP mixtures did not affect the  
286 gonadotropic axis at the doses studied.

287

### 288 **3.4. Quantification of AR and ER $\alpha$ immunoreactivity in the neural circuitry underlying sexual** 289 **behavior**

290 Our previous studies showed that chronic exposure to DEHP alone or NP alone altered the number of  
291 neurons expressing AR or ER $\alpha$  in the neural circuitry that underlies male sexual behavior (Capela et  
292 al., 2018; Dombret et al., 2017). These two receptors play complementary roles in the expression of  
293 male behavior (Naulé et al., 2016; Ogawa et al., 1997; Ogawa et al., 1998; Raskin et al., 2009;  
294 Wersinger et al., 1997). Immunohistochemical studies show AR and ER $\alpha$  nuclear staining in the  
295 medial amygdala, bed nucleus of stria terminalis, and medial preoptic nuclei (Fig. 4A; 5A). The  
296 quantification of AR and ER $\alpha$ -expressing neurons did not show any significant difference between the  
297 three treatment groups (Fig. 4B; 5B).

298 Taken together, our data show that exposure to the DEHP/NP mixture at the doses tested did not affect  
299 the expression of neural AR or ER $\alpha$  in the neural circuitry that underlies male sexual behavior.

300

### 301 **3.5. Effects of adult exposure to DEHP/NP mixture compared to DEHP and NP alone**

302 Table 1 summarizes the effects of the DEHP/NP mixture compared to each compound alone. Several  
303 observations can be drawn from such a comparison. Firstly, when males were exposed to each  
304 molecule alone, sexual behavior (emission of ultrasonic vocalizations, latency to intromission and  
305 ejaculation) or anxiety-state levels were affected at the dose of 5  $\mu$ g/kg/day, while no effects were

306 observed at a 10-fold lower dose (0.5  $\mu\text{g}/\text{kg}/\text{day}$ ). In the present study, combined exposure induced  
307 sexual alterations at the dose of 0.5  $\mu\text{g}/\text{kg}/\text{day}$ , whereas no behavioral effects were observed at 5  
308  $\mu\text{g}/\text{kg}/\text{day}$ . Secondly, olfactory preferences and locomotor activity were not affected by treatment with  
309 each compound alone, whatever the dose used, while the mixture induced effects at the lower dose of  
310 0.5  $\mu\text{g}/\text{kg}/\text{day}$ . Thirdly, while the behavioral effects induced by each compound alone at 5  $\mu\text{g}/\text{kg}/\text{day}$   
311 were associated with changes in the expression level of AR and ER $\alpha$  in the neural circuitry that  
312 underlies sexual behavior, the mixture did not induce any significant modification. Finally, for both  
313 types of exposure (to a single molecule or two molecules), kisspeptin-immunoreactivity was unaltered  
314 in the hypothalamic nuclei involved in the regulation of the gonadotropic axis. These data were  
315 confirmed by unchanged hormonal levels and weights of androgeno-dependent tissues.

316



#### 317 **4. Discussion**

318 The present study shows that co-exposure of adult male mice to a DEHP/NP mixture induced  
319 behavioral alterations, mainly at the lowest dose tested of 0.5  $\mu\text{g}/\text{kg}/\text{day}$ . This dose did not trigger any  
320 changes when each compound was tested separately. Moreover, the behavioral modifications induced  
321 by each compound alone, seen in previous studies at the dose of 5  $\mu\text{g}/\text{kg}/\text{day}$ , were no longer  
322 observable with the mixture of the two products.

323

324 Male mice exposed to the DEHP/NP mixture at a dose of 0.5  $\mu\text{g}/\text{kg}/\text{day}$  did not exhibit a  
325 preference for receptive females. Such alterations can probably explain the lower number of male  
326 mice in the treatment group emitting ultrasonic vocalizations, since this behavior is induced by  
327 olfactory stimulation following female anogenital investigation. Despite an altered olfactory  
328 preference, male mice exposed to the DEHP/NP mixture at 0.5  $\mu\text{g}/\text{kg}/\text{day}$  initiated mating. The  
329 latencies to initiate the first mount, and intromission, and to reach ejaculation, were not statistically  
330 significant despite an increased tendency of these events to be increased in this exposed group. In  
331 contrast, the frequency of behavior was significantly affected, as evidenced by a lower total number of  
332 mounts and thrusts. These behavioral alterations could not be explained by changes in locomotor  
333 activity or anxiety-state levels. Indeed, male mice exposed to a DEHP/NP mixture at 0.5  $\mu\text{g}/\text{kg}/\text{day}$   
334 showed an increased activity and normal anxiety-state level. Altogether, our data point out a sexual  
335 alteration induced by chronic exposure of adult male mice to a DEHP/NP mixture at 0.5  $\mu\text{g}/\text{kg}/\text{day}$ .

336 Behavioral alterations induced by the DEHP/NP mixture may be caused by an endocrine disrupting  
337 mode of action involving i) changes in circulating levels of gonadal testosterone, ii) interference with  
338 hormone-receptor binding, or iii) modifications in the expression levels of sex steroid receptors in  
339 neural areas that underlie male behavior. No changes were observed in testosterone levels, as was  
340 confirmed by the unaltered weight of androgeno-dependent tissues. In agreement with this, analysis of  
341 the number of kisspeptin-immunoreactive cells in the two hypothalamic areas involved in the  
342 regulation of the gonadotropic axis revealed no differences between the three treatment groups. In  
343 addition, the number of AR- and ER $\alpha$ -immunoreactive cells in the neural circuitry that underlies

344 sexual behavior was comparable between the three treatment groups. Therefore, one possibility could  
345 be that chronic exposure to the DEHP/NP mixture at 0.5 µg/kg/day interfered with hormone-receptor  
346 binding, although this hypothesis remains difficult to demonstrate clearly in vivo. Alternatively, the  
347 behavioral changes could also be triggered by mechanisms that are not part of an endocrine disrupting  
348 mode of action.

349

350 A common feature of exposure to one (DEHP or NP) or two (DEHP and NP) molecules is the lack  
351 of effects on the gonadotropic axis. Indeed, no changes were seen in circulating levels of testosterone  
352 or kisspeptin in the preoptic area or arcuate nucleus, whatever the dose of pollutants used (Capela et  
353 al., 2018; Dombret et al., 2017; present study). We have previously discussed the fact that the  
354 hypothalamus-pituitary-gonad axis seems less vulnerable in adults to exposure to low doses of DEHP  
355 alone (Dombret et al., 2017) or NP alone (Capela et al., 2018). The present data show that exposure to  
356 low doses of the DEHP/NP mixture does not affect this axis.

357 At the behavioral level, the majority of changes induced by exposure to the DEHP/NP mixture were  
358 observed at a dose of 0.5 µg/kg/day. When used separately, neither DEHP nor NP could affect male  
359 behavior at this dose. It is possible that a synergistic effect occurred in the neural circuitry that  
360 underlies sexual behavior when the two substances were combined. Another interesting finding is that  
361 exposure to the DEHP/NP mixture at 5 µg/kg/day did not induce any sexual alteration. DEHP and NP  
362 have been respectively described for their anti-androgenic and estrogenic activities. When male mice  
363 were exposed to each compound alone, the observed effects were not comparable. Firstly, exposure to  
364 DEHP lengthened the motivational phase by affecting the emission of ultrasonic vocalizations and  
365 male attractiveness (Dombret et al., 2017), while NP increased the emission of ultrasonic vocalizations  
366 and the time from the first mount to ejaculation. There was a significant increase in the number of  
367 mounts, intromissions and thrusts (Capela et al., 2018). Secondly, DEHP-induced effects could mainly  
368 be attributed to an anti-androgenic activity through down-regulation of the neural AR without any  
369 effect on ER $\alpha$  expression (Dombret et al., 2017), whereas NP exposure brought to mind the effects  
370 reported for estradiol administration to adult rodents (Retana-Márquez et al., 2016). It was associated

371 with changes in the expression levels of both receptors in the neural circuitry underlying sexual  
372 behavior (Capela et al., 2018). Whether anti-androgenic or estrogenic, each compound alone was able  
373 to induce sexual alterations, suggesting that a physiological balance between both signaling pathways  
374 is necessary to elicit normal behavior. Indeed, the AR and ER $\alpha$  signaling pathways play a  
375 complementary role in the activation of male sexual behavior. When they are combined, it is possible  
376 that these anti-androgenic and estrogenic substances exert antagonistic effects, which may explain the  
377 absence of effects on male behavior. A comparable observation was reported in pre-pubertal male rats,  
378 where exposure to a mixture of DBP and NP exerted antagonistic effects on LH and FSH levels in  
379 comparison to each compound alone (Hu et al., 2014b).

380

## 381 **5. Conclusion**

382 The present work shows that exposure of adult male mice to a mixture of DEHP and NP affects  
383 their olfactory preference, emission of ultrasonic vocalizations, and frequency of behavioral events  
384 during mating. The induced changes in sexual behavior, together with increased locomotor activity,  
385 were observed at the lowest dose tested (0.5  $\mu\text{g}/\text{kg}/\text{day}$ ). This did not trigger changes when male mice  
386 were exposed to each compound separately, suggesting a synergistic effect of co-exposure to the two  
387 compounds. The data obtained could be extremely relevant for several vertebrate species that use  
388 olfactory cues and ultrasonic vocalization to detect and attract their female partner. A study in humans  
389 reported an association between the levels of urine DEHP and NP metabolites and hypospadias in  
390 boys (Choi et al., 2012). This indicates that combined exposure to these molecules can trigger adverse  
391 effects. Whether such exposure also induces sexual or erectile deficiencies during adulthood remains  
392 to be investigated. The results also show that behavioral effects, which were reported for each  
393 molecule when used alone at the dose of 5  $\mu\text{g}/\text{kg}/\text{day}$  (Capela et al., 2018; Dombret et al., 2017), were  
394 no longer observable upon co-exposure, suggesting antagonistic effects of the two molecules. Overall,  
395 despite the use of only two molecules, the present data highlight and confirm the need to assess  
396 environmental endocrine disruptors in the context of combined exposure.

397

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403

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482

483 **Figure legends**

484 **Figure 1. Effects of adult exposure to a DEHP/NP mixture on male sexual behavior.** **A.** Total time  
485 spent in the chemo-investigation of male and sexually receptive female stimuli by males exposed to  
486 the vehicle (Veh) or DEHP/NP mixture (0.5 or 5 µg/kg/day). **B.** Number of entries into the male or  
487 female arm of the Y maze. **C.** Discrimination index expressed as time spent investigating the female  
488 minus time spent investigating the male divided by the total time of chemo-investigation. Data are  
489 expressed as the means ± S.E.M. of 9-10 males per treatment group. One-way ANOVA (<sup>a</sup>p < 0.001)  
490 demonstrated an effect of exposure; post hoc analyses showed differences (p < 0.001) between males  
491 exposed to the mixture at 0.5 µg/kg/day and the other two groups. **D.** Percentage of males emitting  
492 ultrasonic vocalizations in the presence of a sexually receptive female. Chi square test analysis showed  
493 a statistically significant difference (\*p < 0.05). **E-F.** Latency to the first mount, intromission, and to  
494 ejaculation (**E**) and duration of mating expressed as the time between the first mount and ejaculation  
495 (**F**) for males exposed to the vehicle (Veh) or a DEHP/NP mixture at 0.5 or 5 µg/kg/day. **G.** Number  
496 of mounts (left), intromissions (middle) and thrusts (right) exhibited by males. One-way ANOVA (a)  
497 showed an effect of exposure; \*p < 0.05 versus the vehicle group.

498

499 **Figure 2. Anxiety and locomotor activity were affected by exposure to a DEHP/NP mixture.** **A.**  
500 **Left.** Spontaneous activity by males exposed to the vehicle (Veh) or to DEHP/NP at 0.5 or 5  
501 µg/kg/day measured for 140 min. \*p < 0.05 or \*\*p < 0.01, post hoc analyses showing a higher activity  
502 for the DEHP/NP-0.5 group by comparison to males exposed to DEHP/NP-5 at 80 and 100 min of the  
503 test. **Right.** Cumulative activity recorded for the three treatment groups during the 140 min of the test.  
504 Data are expressed as the means ± S.E.M. of 9-10 males per treatment group; <sup>a</sup>p < 0.05 general effect  
505 of exposure. **B.** Anxiety-state level measured in the zero maze paradigm. Latency to the first entry into  
506 the open arm (Left), the number of entries into the open arm (middle) and time spent in the open arms  
507 (right) were analyzed. Data are expressed as the means ± S.E.M. of 9-10 males per treatment group.  
508 One-way ANOVA (a) showed an effect of exposure to DEHP/NP. \*Post hoc analyses showed  
509 significant differences between DEHP/NP-5 and vehicle groups.



510

511 **Figure 3. Kisspeptin immunoreactivity and hormonal levels are unchanged following chronic**  
512 **exposure to a DEHP/NP mixture.** Mice were exposed to the vehicle or DEHP/NP mixture at 0.5 or 5  
513  $\mu\text{g}/\text{kg}/\text{day}$ . **A. Upper panel:** representative immunolabeling in the periventricular nuclei is shown  
514 (left), with increased magnification of a kisspeptin-immunoreactive cell body (right). **Lower panel:**  
515 quantification of the number of kisspeptin-immunoreactive neurons and fiber density in the  
516 anteroventral periventricular (AVPV) and periventricular (PeN) nuclei. **B.** Representative  
517 immunolabeling in the arcuate nucleus (upper panel) and quantification of fiber density (lower panel).  
518 Data are expressed as the means  $\pm$  S.E.M. of 6 males per treatment group. Scale bar = 20  $\mu\text{m}$  (**A-right**  
519 panel) and 50  $\mu\text{m}$  (**A-left panel and B**). **C-E.** Circulating levels of testosterone (**C**) and weight of  
520 seminal vesicles (SV; **D**) and testes (**E**) are expressed as a percentage of body weight (bw). Data are  
521 expressed as the means  $\pm$  S.E.M. of 9-10 males per treatment group.

522

523 **Figure 4. The number of androgen receptor (AR)-immunoreactive (ir) cells was unchanged upon**  
524 **exposure to a DEHP/NP mixture.** **A.** Representative AR-ir cells in the posterodorsal medial  
525 amygdala (MeA), bed nucleus of stria terminalis (BNST) and medial preoptic nucleus (MPN) of males  
526 exposed to the vehicle (Veh) or a DEHP/NP mixture at 0.5 or 5  $\mu\text{g}/\text{kg}/\text{day}$ . **B.** Quantitative analyses of  
527 the number of AR-ir neurons in chemosensory areas. Data are expressed as the means  $\pm$  S.E.M. of 6  
528 males per treatment group. Scale bar, 100  $\mu\text{m}$ .

529

530 **Figure 5. The number of  $\alpha$  estrogen receptor (ER $\alpha$ )-immunoreactive (ir) cells was unchanged**  
531 **upon exposure to the DEHP/NP mixture.** **A.** Representative ER $\alpha$ -ir cells in the posterodorsal medial  
532 amygdala (MeA), bed nucleus of stria terminalis (BNST) and medial preoptic nucleus (MPN) of males  
533 exposed to the vehicle (Veh) or a DEHP/NP mixture at 0.5 or 5  $\mu\text{g}/\text{kg}/\text{day}$ . **B.** Quantitative analyses of  
534 the number of ER $\alpha$ -ir neurons in chemosensory areas. Data are expressed as the means  $\pm$  S.E.M. of 6  
535 males per treatment group. Scale bar, 100  $\mu\text{m}$ .

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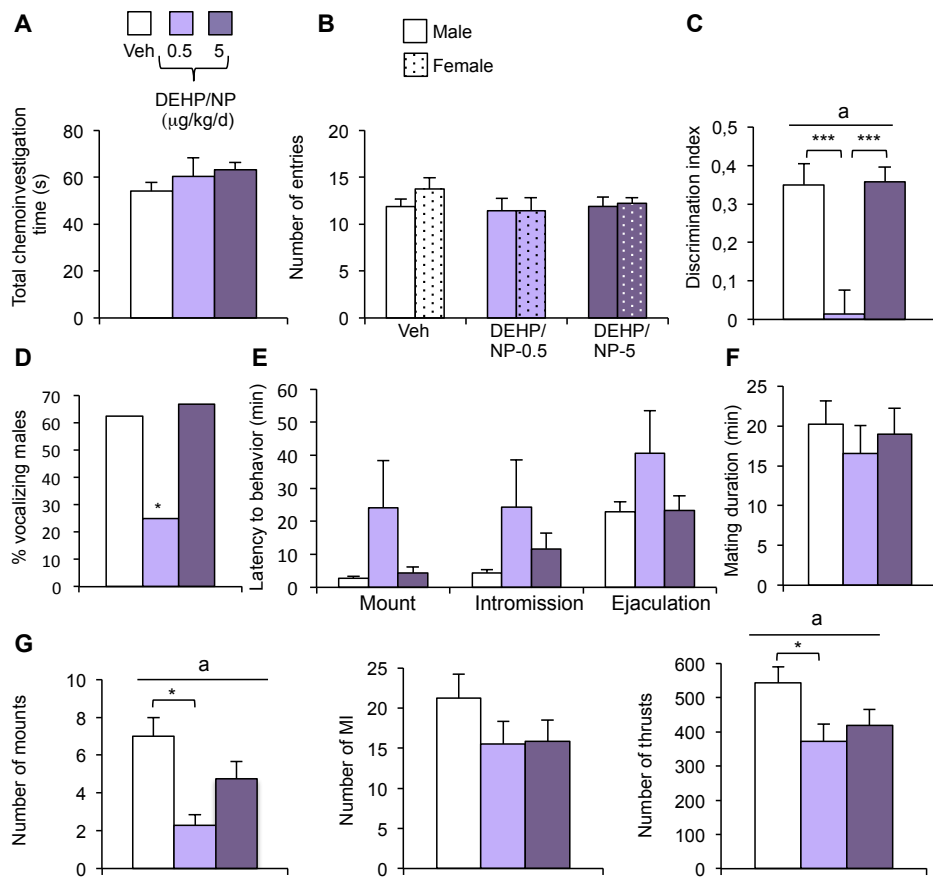
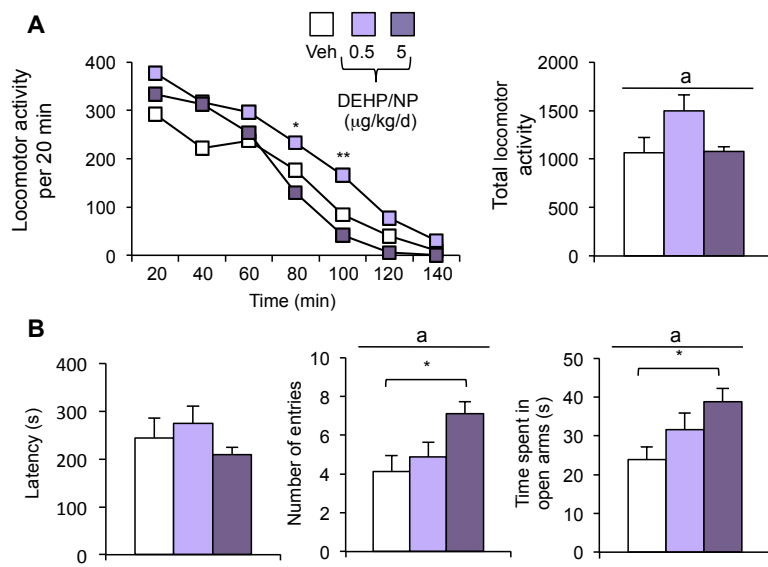


Figure 1

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538



**Figure 2**

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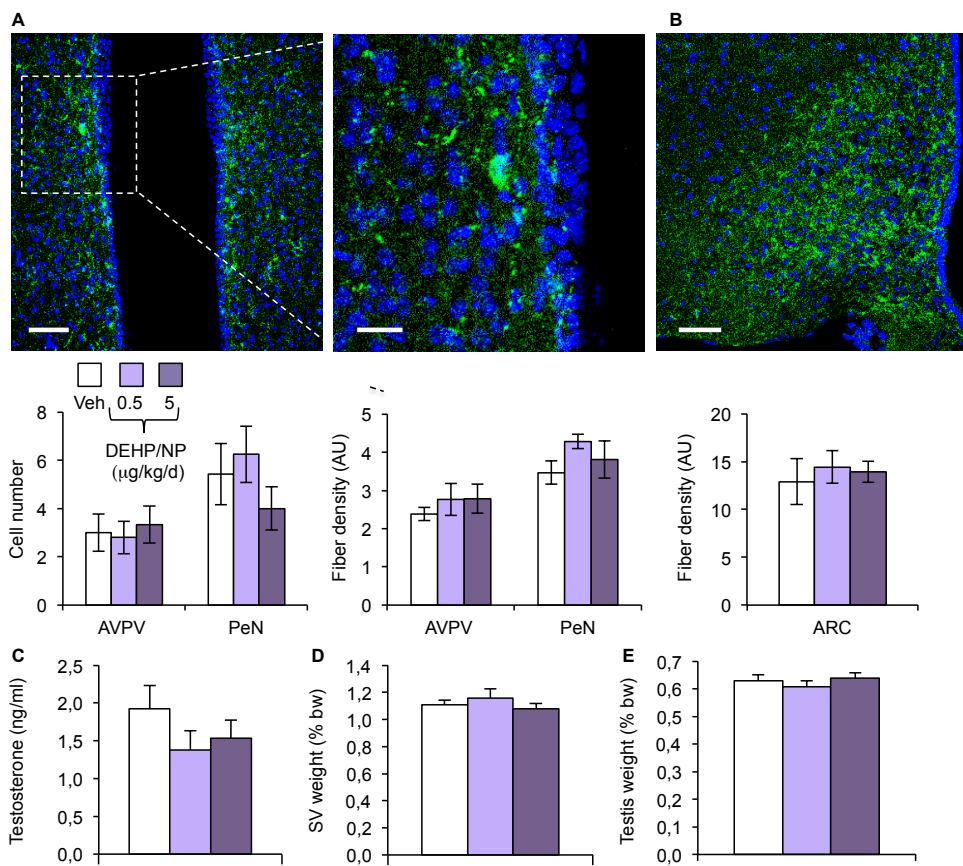
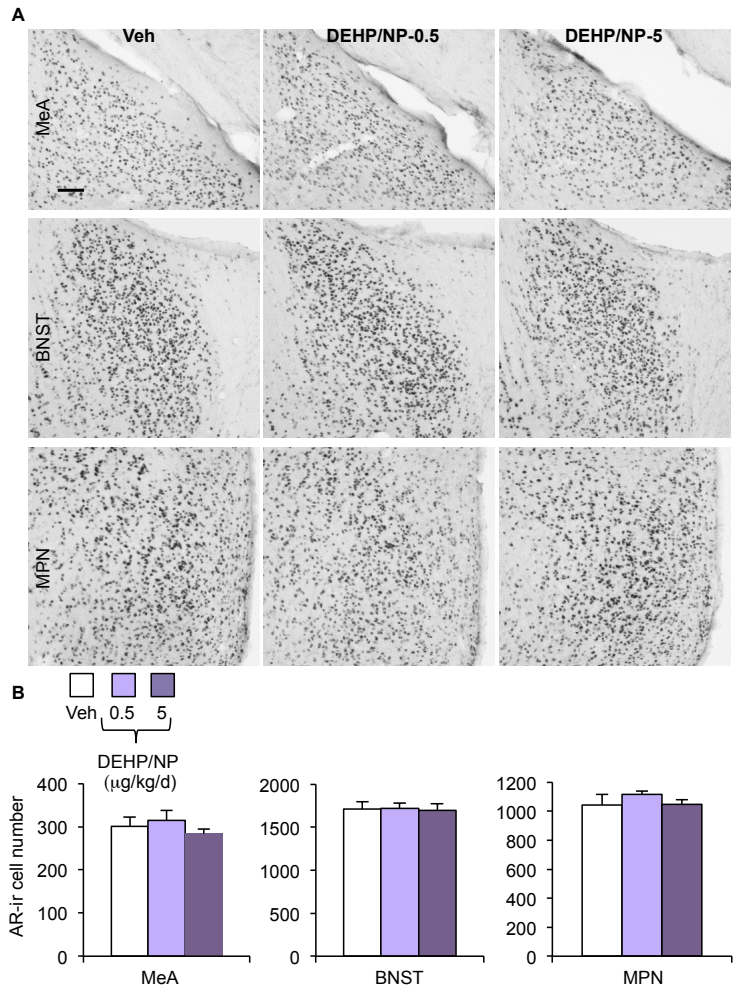


Figure 3

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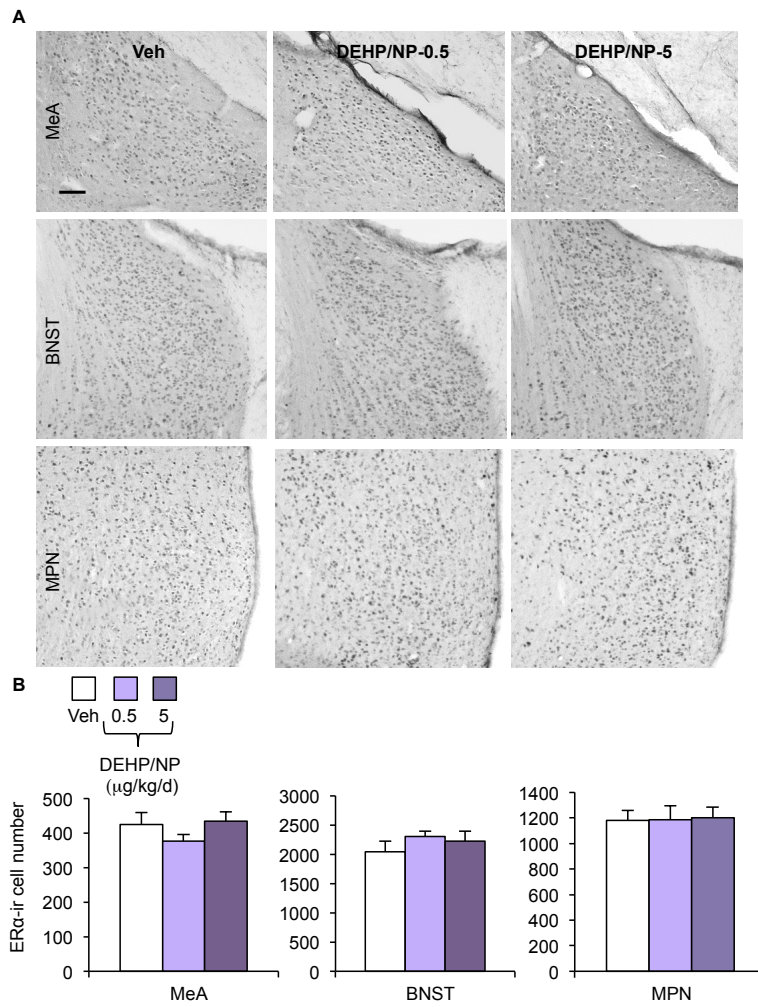
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**Figure 4**

543

544



**Figure 5**

545

546

Molecules	DEHP (Dombret et al., 2017)		NP (Capela et al., 2018)		DEHP/NP mixture (present study)	
	0.5 µg/kg/day	5 µg/kg/day	0.5 µg/kg/day	5 µg/kg/day	0.5 µg/kg/day	5 µg/kg/day
<b>Behavioral effects</b>	<b>Sexual behavior</b>					
Olfactory preference	Unaffected	Unaffected	Unaffected	Unaffected	No preference	Unaffected
Ultrasonic vocalizations	Unaffected	Unaffected number and duration, altered ratio of USVs syllables	Unaffected	Increased number and duration	Few males vocalized	Unaffected
Latency to intromission, and ejaculation	Unaffected	Increased latency to intromission and ejaculation	Unaffected	Increased latency to ejaculation	No significant effect	Unaffected
Number of mounts, intromissions and thrusts	Unaffected	Unaffected	Unaffected	Increased number of mounts, intromissions and thrusts	Reduced number of mounts and thrusts	Unaffected
	<b>General behaviors</b>					
Locomotor activity	Unaffected	Unaffected	Unaffected	Unaffected	Increased	Unaffected
Anxiety-state level	Unaffected	Unaffected	Unaffected	Increased	Unaffected	Reduced
<b>Chemosensory areas</b>	<b>Number of androgen receptor (AR)- and estrogen receptor (ER)α-immunoreactive cells</b>					
Medial amygdala	Unaffected	Reduced for AR	Unaffected	Reduced for AR Increased for ERα	Unaffected	Unaffected
Bed nucleus of stria terminalis	Unaffected	Reduced for AR	Unaffected	Increased for AR	Unaffected	Unaffected
Medial preoptic nucleus	Unaffected	Reduced for AR	Unaffected	Reduced for AR	Unaffected	Unaffected
	<b>Number of kisspeptin-immunoreactive cells</b>					
Medial preoptic nucleus	Unaffected	Unaffected	Unaffected	Unaffected	Unaffected	Unaffected
Arcuate nucleus	Unaffected	Unaffected	Unaffected	Unaffected	Unaffected	Unaffected

547

548

549 Table 1. Summary of neuroendocrine and behavioral effects induced by adult exposure to DEHP

550 alone, NP alone or combined exposure to both molecules in male mice.