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

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Title

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Running title

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

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Abstract

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

Highlights

- Adult co-exposure to DEHP/NP alters male sexual behavior.
- DEHP/NP mixture increases locomotor activity and reduces anxiety-state level.
- The lower dose of DEHP/NP mixture triggers the majority of behavioral effects.
- No effects of DEHP/NP mixture on the gonadotropic axis.
- Unaltered number of AR- and ERα-immunoreactive cells in exposed males.

Keywords

Nervous system

Endocrine disruptor

Phthalates

Nonylphenol

Behavior

Reproduction

1. Introduction

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

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

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

The present study was undertaken to characterize the effects of chronic exposure of male mice to low doses of the two molecules together. The same behavioral and neuroendocrine endpoints previously tested for each compound alone were analyzed. To this end, male mice were treated orally with the vehicle alone or with DEHP and NP (5 and 0.5 μg/kg/day) for four weeks. Courtship behavior of male mice was analyzed by testing their olfactory preference and emission of courtship vocalization in the presence of receptive females. Latency and frequency of copulatory behavioral events were also quantified. Furthermore, we measured locomotor activity and the anxiety-state level, which, if altered, could interfere with the expression of sexual behavior. Testosterone levels and weight of androgen-sensitive tissues of the genital tract were compared between the three groups. Kisspeptin-immunoreactive neurons were quantified in the rostral preoptic periventricular nucleus (RP3V) and in 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

2. Materials and Methods

2.1. Animals and treatment

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

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

2.2. Behavioral tests

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

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 μg of estradiol-benzoate (Sigma-Aldrich) in 30 μl 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 μl 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.

2.2.4. Mating

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

2.2.5. Locomotor activity

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

2.2.6. Anxiety-related behavior

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

2.3. Immunohistochemistry

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

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

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

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

2.4. Urogenital tract weight and hormone measurements

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

2.5. Statistical analyses

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

3. Results

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

In the presence of a sexually receptive female, the male mouse exhibits olfactory investigation of the female genitals. The pheromonal cues emitted by the female are detected by the olfactory bulb, which transmits signals to the chemosensory areas involved in the activation of male sexual behavior. Male mice exposed to the vehicle or DEHP/NP mixture at 0.5 and 5 μg/kg/day were therefore analyzed for their olfactory preference in a behavioral test using anesthetized gonadally intact males versus sexually receptive females. Figure 1A shows that there was no effect of DEHP/NP exposure on the time spent in total chemo-investigation ($p = 0.36$). Two-way ANOVA showed no effect of exposure to 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 the Y maze arms (Fig. 1B). In contrast, an effect of DEHP/NP exposure on the ability of mice to discriminate between male and female stimuli was observed ($p = 0.0001$; Fig. 1C). Indeed, male groups exposed to DEHP/NP at 0.5 μg/kg/day showed no preference for females over males, while such a preference was observed for the vehicle and DEHP/NP-5 groups.

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

In mating tests, one-way ANOVA showed no significant effect of treatment on the latency to initiate the first mount and first intromission, or time to reaching ejaculation, despite a tendency for these events to be increased in the DEHP/NP-0.5 group (Fig. 1E). Mating duration was also unaffected by exposure to DEHP/NP (Figure 1F). Quantification of the frequency of behavioral events showed an 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 μg/kg/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 μg/kg/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 μg/kg/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 μg/kg/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

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

3.4. Quantification of AR and ERα immunoreactivity in the neural circuitry underlying sexual behavior

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

5. Conclusion

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

Acknowledgements

This work was supported by the "Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail" (Anses; Project n° 2012-2-077). We thank the INRA hormonal assay platform for testosterone assays and the UPMC and INRA (UEPAO) platforms for taking care of the animals.

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482

Figure legends

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

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

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

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

Figure 5. The number of α estrogen receptor (ERα)-immunoreactive (ir) cells was unchanged upon exposure to the DEHP/NP mixture. **A**. Representative ERα-ir cells in the posterodorsal medial amygdala (MeA), bed nucleus of stria terminalis (BNST) and medial preoptic nucleus (MPN) of males exposed to the vehicle (Veh) or a DEHP/NP mixture at 0.5 or 5 μg/kg/day. **B**. Quantitative analyses of the number of ERα-ir neurons in chemosensory areas. Data are expressed as the means ± S.E.M. of 6 males per treatment group. Scale bar, 100 μm.

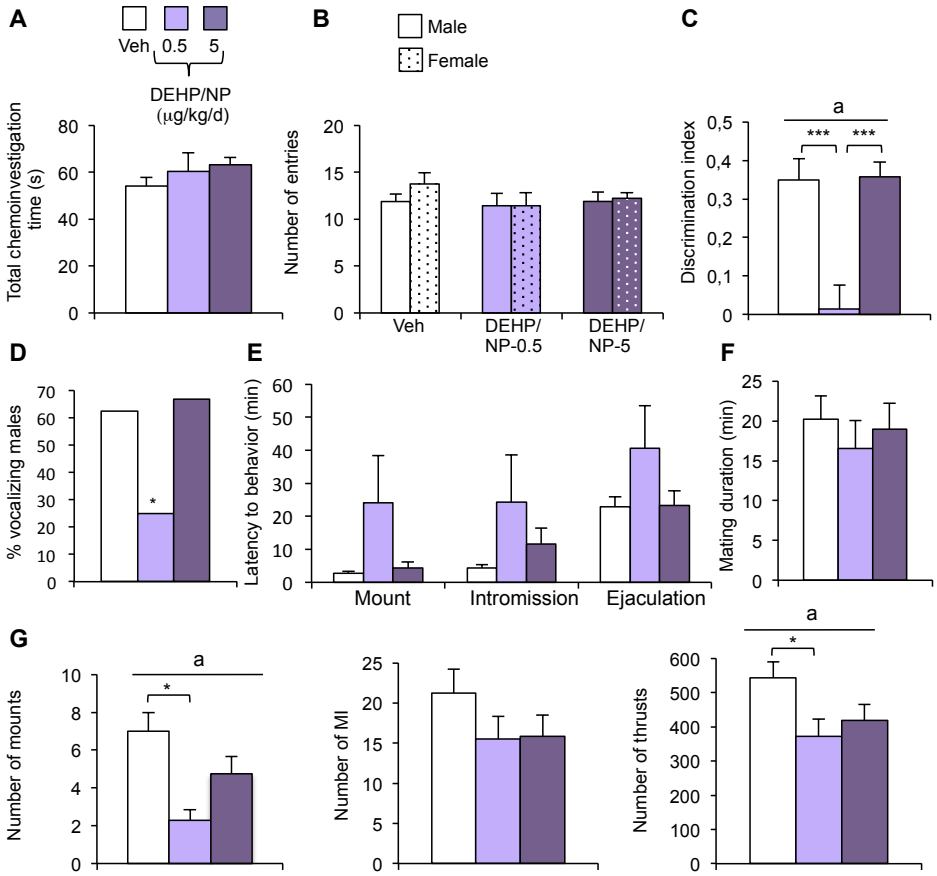
A
Veh
0.5
5
DEHP/NP
(µg/kg/d)
Total chemoinvestigation time (s)
B
Male
Female
Number of entries
Veh
DEHP/ NP-0.5
DEHP/ NP-5
C
a
***
***
Discrimination index
D
% vocalizing males
E
Latency to behavior (min)
Mount
Intromission
Ejaculation
F
Mating duration (min)
G
a
Number of mounts
Number of MI
a
Number of thrusts

Figure 1

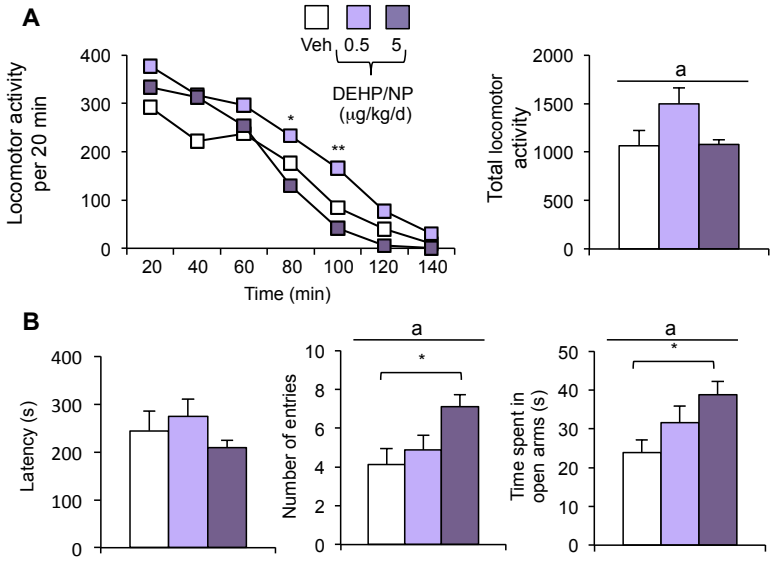

Figure 2

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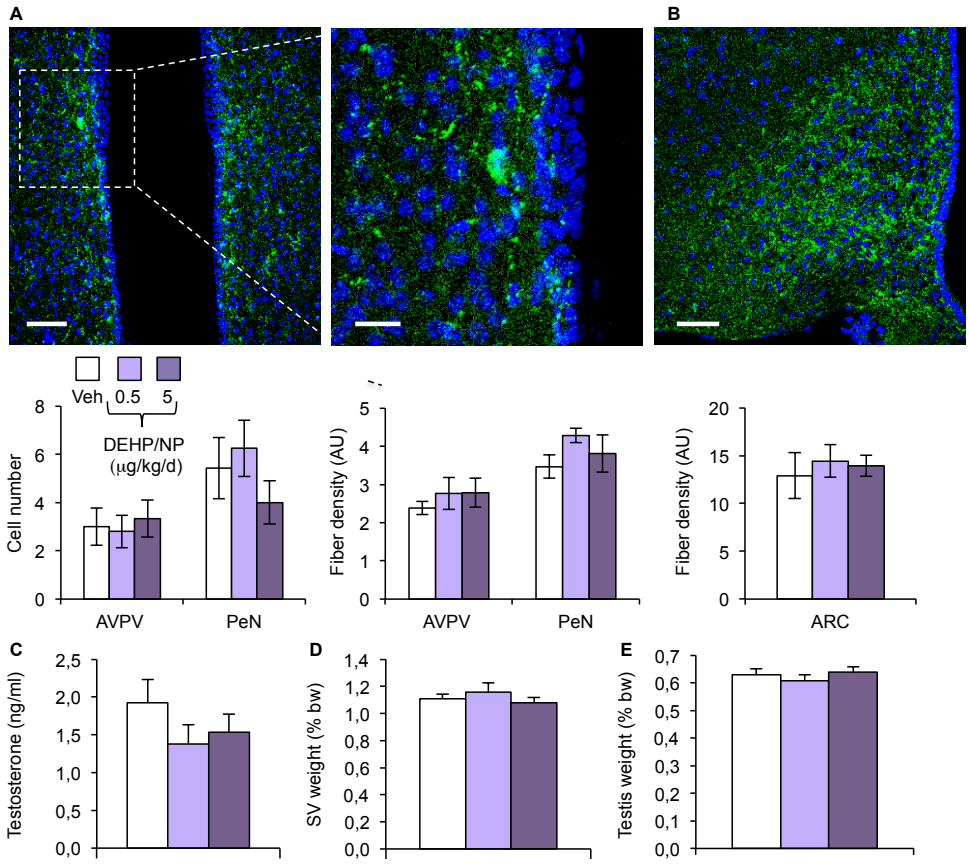
A
B
Veh
0.5
5
DEHP/NP
(µg/kg/d)
Cell number
AVPV
PeN
Fiber density (AU)
AVPV
PeN
Fiber density (AU)
ARC
C
Testosterone (ng/ml)
D
SV weight (% bw)
E
Testis weight (% bw)

Figure 3

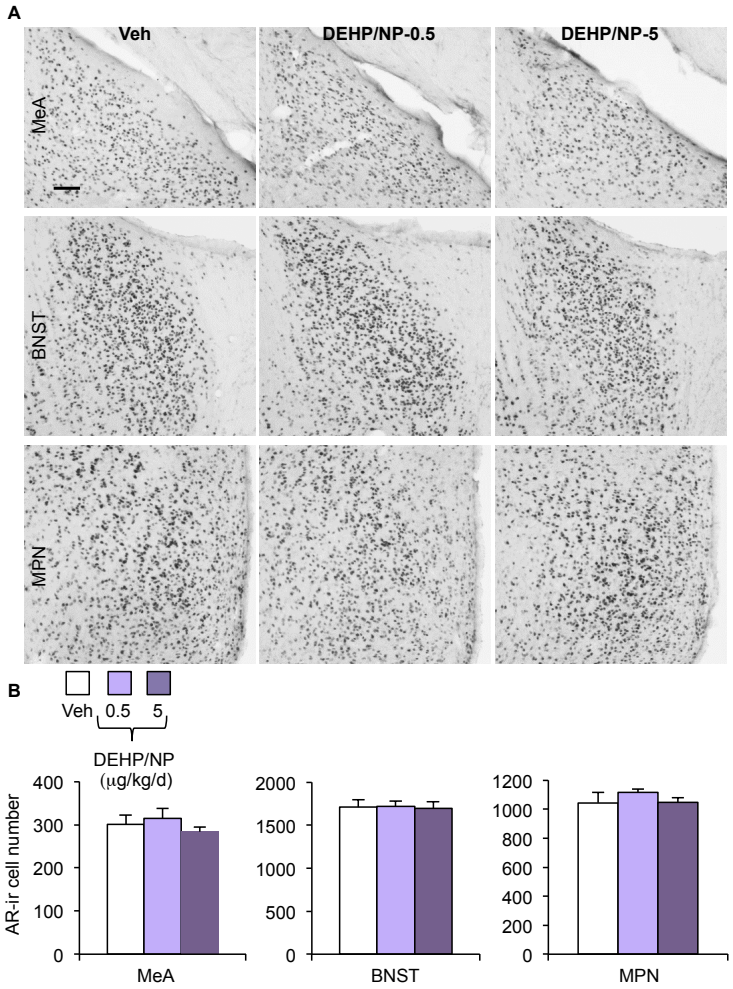

Figure 4

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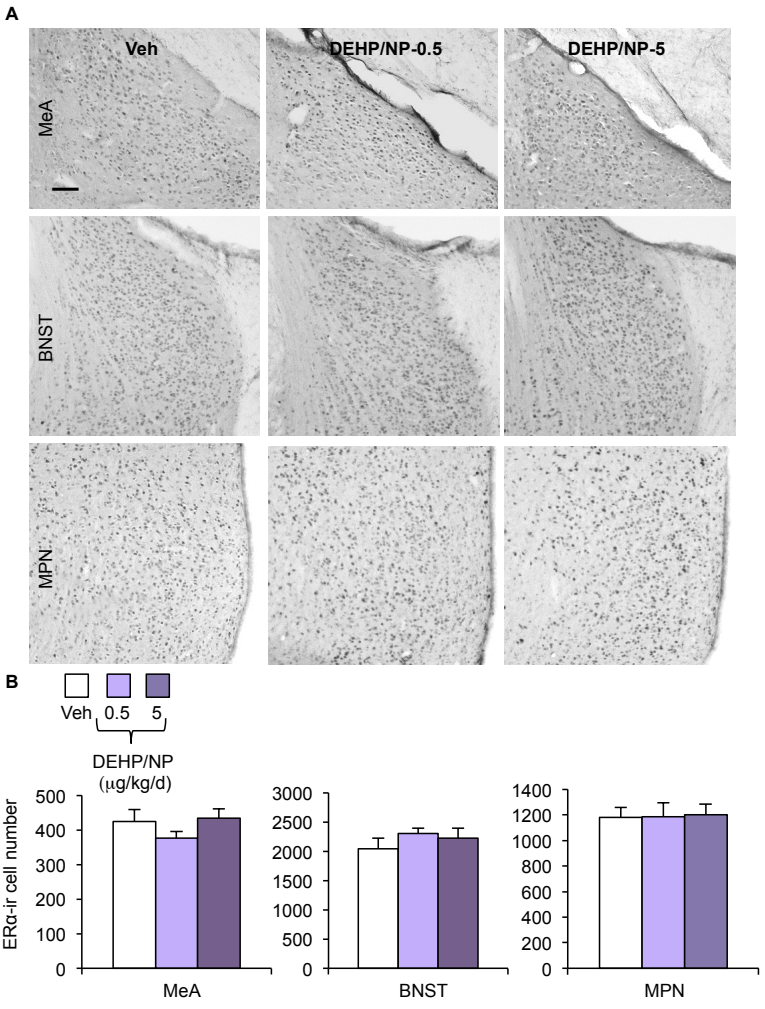

Figure 5

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Molecules	**DEHP (Dombret et al., 2017)**		**NP (Capela et al., 2018)**		**DEHP/NP mixture (present study)**	
Doses	**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**
Behavioral effects	**Sexual behavior**					
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
	General behaviors					
Locomotor activity	Unaffected	Unaffected	Unaffected	Unaffected	Increased	Unaffected
Anxiety-state level	Unaffected	Unaffected	Unaffected	Increased	Unaffected	Reduced
Chemosensory areas	**Number of androgen receptor (AR)- and estrogen receptor (ER)α–immunoreactive cells**					
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
Number of kisspeptin-immunoreactive cells						
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.