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Environmental concentration of nonylphenol alters the development of urogenital and visceral organs in avian model.

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ABSTRACT

Nonylphenol (NP) is an endocrine disruptor with harmful effects including feminization and carcinogenesis on various organisms. This substance is a degradation product of nonylphenol ethoxylates (NPEO) that is used in several industrial and agricultural processes. In this paper, we examined the assessment of NP exposure on chick embryo development, using a concentration consistent with the environmental concentrations of NP.

With this aim, NP (between 0.1 and 50 µg/egg) was injected into the yolk of egg through a small needle hole in the shell.

We report the effect of NP on chick reproductive system development although the effect we observed is lower than those observed by exposition to other endocrine disruptors. However, histological analysis highlighted a decrease of intraluminal seminiferous surface area in 64.12% of case ($P = 0.0086$) and an heterogeneous organization of the renal tubules when 10µg/egg were injected. Moreover, an impairment of liver development with an abnormal bile spillage was observed when higher concentration of NP was injected (50µg/egg).

KEY WORDS:

Nonylphenol, chick embryo, estrogenic effect, environment.

INTRODUCTION

Endocrine disrupting chemicals (EDC) are a wide variety of environmental exogenous chemicals, including synthetic substances and naturally occurring hormones, which impair the normal function of the endocrine system of both wildlife and humans (WHO/IPCS, 2002). Among them, nonylphenol (NP), a degradation product of nonylphenol ethoxylate (used as nonionic surfactants in a variety of industrial and agricultural processes and as cleaning agents (Tolls et al., 1994)), has been identified in various environmental compartments in concentrations ranging from ng to hundreds of ng/L in surface water (Tao et al., 2011; Ra et al., 2010; Micić and Hofmann, 2009), ng to hundreds of ng/g in sediment (Gong et al., 2011; Mayer et al., 2007) and mg to hundreds of mg/kg in sewage sludge (González et al., 2010; Micić and Hofmann, 2009; Brix et al., 2010). Such concentrations have been shown to exhibit an (eco) toxicity toward a wide range of species. Many studies have focused on the endocrine disrupting effects of NP on the developing reproductive system of environmental aquatic organisms of reference (fish, crustaceans, algae), in rat and in mouse models. The effect on animal reproduction (Yang et al., 2006; Zha et al., 2008; Wu et al., 2010), and sexual

development (Chen et al., 2009; Hirano et al., 2009; Jobling et al., 1996), induction of vitellogenin (Cionna et al., 2006; DelGiudice et al., 2012) or other markers (Petridis et al., 2009; Shelley et al., 2012) have been particularly investigated showing in some studies the weaker estrogenic effect of NP compared to estrogens (Schoenfuss et al., 2008; Yang et al., 2006). NP has also been demonstrated to have the potential to enhance carcinogenesis in vivo (Seike et al., 2003; Fukamachi et al., 2004). Because of their ecotoxicological properties and their presence in the environment, Europe has begun to take measures with regard to NP and its ethoxylates. They were included in several lists of compounds under particular surveillance such as the OSPAR Convention (2000) and the Water Framework Directive on water (European Union, 2000). In the United States, the EPA published a guide to water quality recommending concentrations of nonylphenol $< 6.6\mu\text{g/L}$ in surface water, and < 1.7 for seawater (Brooke et al., 2005). Today, NP and NP ethoxylates are even on SIN (Substitute It Now!), list of dangerous chemical products established by the International Chemical Secretariat (ChemSec) working for an environment without toxicity.

In this paper we assessed the endocrine effect of NP by using an avian model. We selected this model based on the recent OECD guideline (“testing of chemicals, Section 2, effects on biotic systems” (OECD 223, 2010)), which recommends using a bird model to assess acute and oral toxicity of substances. In practice, the suggested species are the Northern Bobwhite quail (*Colinus virginianus*), the Japanese quail (*Coturnix japonica*) and sometime the mallard duck (*Anas platyrhynchos*), feral pigeon (*Columba livia*) zebra finch (*Poephila guttata*) and budgerigar (*Melopsittacus undulates*) which are fed by a diet containing the test substance at different concentrations for a period of less than 20 weeks. The guideline considers adults and first generation, and the mortality of adults, eggs breeding, cracked eggs, thickness of the shell, viability, hatchability and the effects on young birds during the study are observed as the endpoints (OECD 223, 2010). In addition, some investigators have used avian models in other experimental designs for studies of various environmental pollutants (Heinz et al., 2011, 2012; Manning et al., 2012; Grasman et al., 2013).

However, compared to the aquatic models, few studies on the assessment of endocrine disruptor effects have been conducted using avian models. Table 1 presents the dozen available studies, most of them based on observations of the sexual organs in Japanese quail. Among the molecules studied, estrogenic compounds (estradiol, ethynylestradiol, diethylstilbestrol) were investigated in majority, as well as other endocrine disrupting

substances such as dioxins, methylmercury and pesticides substances. NP was studied in three studies involving avian model, all using the quail embryo and comparing its impacts with those of estradiol. Though NP concentrations were higher than the environmental exposure, each study showed a lesser effect than estradiol.

In this study, we chose the chick embryo as avian model because it is a good model for rapid organogenesis studies. In addition we already used the model in a previous study dealing with the effect of strong estrogenic compounds (Biau et al., 2007).

The goal of the study was to investigate the toxic effect of NP at environmental concentrations on chick embryonic development. For this purpose, we explored the development of the chicken urogenital sphere after in-ovo nanoinjections of NP and the effects were compared to those obtained with estrogens. Interestingly, we also observed an effect of NP on visceral organs development, indicating that NP has other effects in addition to being an endocrine disruptor.

MATERIAL AND METHODS

Chick embryos

Timed-fertilized white Leghorn chick eggs were obtained from a commercial supplier (Haas Farm, France) and incubated at 38 °C in a constant humidified incubator (Coudelou, France) until they reach the expected level of embryonic development (14 to 18 days), determined according to the table of development established by Hamburger and Hamilton, (1951).

NP injection

Two different sources of NP (CAS: 104-40-5) from two companies: nonylphenol pestanal 98%GC (from Sigma, reference number 46405) and 4-n-Nonylphenol, 98+% (from Alfa Aesar, reference number ALFAA15609.03) were used during the study. Chick embryos were injected at 4 days of embryonic development (or E4) when the gonads are still undifferentiated (Biau et al., 2007). NP solutions of different concentrations were prepared in phosphate-buffered saline (PBS) (137mM NaCl (Merck), 2.7 mM KCl (Merck), 4.3 mM Na₂HPO₄/2H₂O (Merck), 1.4 mM KH₂PO₄ (Merck), pH 7.4). For the tests, 20 µl of solution were injected into the yolk of each egg through a small needle hole in the shell as previously described (Biau et al., 2007) for a final concentration of 0.1, 1, 10 or 50 µg/egg. Control embryos were injected with an equal volume of PBS. The embryos were then re-incubated at 38°C in order to continue their development until the expected embryonic development stage

was reached depending on the organ studied. 117 embryos (table 2) were analyzed during this study after NP injection and compared to 24 controls. We did not observe an increase in embryo mortality when compared to controls. In addition, we analyzed the impact of 50 µg of NP on eggs and observed no significant impact on mortality (44 survivals for 54 injected, 81.5%) (Table 3).

Embryos dissection

Embryos were sacrificed at day 14 (E14) or 18 (E18) of their embryonic life and then dissected in PBS. The shape and appearance of the gonads as well as the state of the Mullerian ducts were observed. Dissections of the genital tract and embryonic kidney (mesonephros) were carried out under binocular microscope (Nikon SMZ1000) and all photographed using a camera (Nikon DXM1200). Tissues were then fixed in a 4% paraformaldehyde (Euromedex) solution in PBS. Samples of digestive tract of all embryos (controls and injected) were systematically collected and stored at -80°C to determine their genetic sex by molecular sexing.

DNA extraction and determination of sexual genotype

All embryos were typed for their sexual ZZ (male) or ZW (female) genotype, using the method of Fridolfsson and Ellegren, (1999) based on PCR with a single set of primers. DNA extraction was performed from a sample of digestive tract as previously described (Biau et al., 2007). Tissues were homogenized in 500 µl of a lysis solution (100mM Tris-HCl pH 8.5, 5 mM EDTA pH 8, 0.2 % SDS, 200 mM NaCl, 100 µg/ml Proteinase K). The whole mixture was incubated 12h at 55°C and then precipitated with an equal volume of isopropanol. Finally the pellet of DNA was re-suspended in Tris-EDTA buffer.

PCR reactions were performed using the primers 2550F “5-GTT ACT GAT TCG TCT ACG AGA-3” and 2718R “5-ATT GAA ATG ATC CAG TGC TTG-3”. Amplifications were performed by using a MJ Mini ThermoCycler (BioRad). Thermal cycling conditions consisted of DNA Polymerase activation at 94 °C for 2 min, 9 cycles of denaturation at 94 °C for 30 s, annealing at 50°C for 30 s (decrease of 1°C at each cycle), and elongation at 72 °C for 1min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 42°C for 30 s and elongation at 72 °C for 1min, and 1 cycle at 72°C for 5min. PCR products were separated on 2% agarose gels and photographed. All the females had a 450-bp CHD1-W female-specific fragment. The embryos of both sexes had a 600-bp CHD1-Z specific fragment.

Immunohistochemistry

Various dissected embryonic chicken structures were fixed in 4% paraformaldehyde in PBS. Fixed chick tissues were embedded in paraffin. Sections (3 to 5 μm) were performed on Superfrost Plus slides (Fisher). Immunohistochemical studies were performed using standard techniques (Moniot et al., 2004; LeGuen et al., 2009). Endogenous peroxidase was blocked in 1.5% hydrogen peroxide for 30–60 min. An antigen unmasking step was performed in 0.1 M sodium citrate solution by boiling the samples for 10 min. The sections were briefly washed in PBS and a blocking step was performed in 10% chick serum by a 30 min incubation at room temperature. Primary antibody solutions were made in PBS with 1% serum plus 0.05 % triton (Triton X-100). The antibodies were incubated in a humidity chamber at 4°C overnight as previously described (Moniot et al., 2004; Le Guen et al., 2009; Notarnicola et al., 2012). The following primary antibodies were used: a mouse anti α -SMA (1:200, Sigma), a mouse monoclonal anti-Desmin (1:500, Euromedex), and a rabbit anti-SOX9 (1:200, Chemicon). The sections were washed in PBS and incubated respectively with biotinylated goat anti-mouse IgM, biotinylated goat anti-mouse IgG, and biotinylated goat anti-rabbit IgG immunoglobulins (1:800 AbCys, Paris) for 1 hr at room temperature. Sections were washed several times in PBS at room temperature, followed by an incubation in ABC reagent according to the manufacturer's instructions for 20 min (AbCys, Paris). Sections were washed again and then incubated in hydrogen-peroxide/3,3'-diaminobenzidine hydrochloride (DAB, Sigma) up to 20 min. Samples were then washed several times in PBS, mounted with Entellan (Sigma) and observed by microscopy (Zeiss Axiophot). For each tissue, immunohistochemistry control experiments were performed by excluding the primary antibody and continuing until the absence of signals (data not shown).

Statistical analyses

To analyze the impact of 10 $\mu\text{g}/\text{egg}$ of NP on testes, the surface of intraluminal seminiferous tubules of the controls was monitored as well as NP treated testes. Data were analyzed by using the Student's t test (after verification of the normal distribution of the data) and results were considered statistically significant when P value < 0.01.

Histological analyses

Tissues were colored with hematoxylin (nucleus dye) and eosin (cytoplasm dye) as previously described (Le Guen et al., 2009). Sections were dehydrated in ethanol, followed by xylene

treatment, mounted with Entellan New (Electron Microscopy Sciences) and observed with a Zeiss Axiophot microscope and photographed with a Nikon DXM1200.

Results

Reprotoxic effect of NP on urogenital tract development

We first examined the reprotoxic effect of NP on urogenital tract development. The experimental procedure, using the chick embryo as a model system, is detailed in the material and methods section. The doses used in this study ranked from 0.1 to 50 μg per egg, doses close to the ones used in the previously published studies to investigate the potential reprotoxic effects of this class of compound (Table 1). In addition, such concentrations are representative of an environmental exposure. Indeed, this range of concentration are those often founded in surface water (Brix et al., 2010; MM González et al., 2010; Tao et al., 2011), in food like eggs or milk (Shao et al., 2007) and in plastic packaging (Loyo-Rosales et al., 2004). At all these doses of NP tested, we did not observe neither an impact on survival rate (Table 3) nor a drastic morphological defect in the urogenital system. Indeed, the size of the gonads was conserved as well as the persistence of the different ducts (Figure 1 and data not shown). Although the size of the gonads in females seems to increase in certain cases (n=9 for 57 female NP-injected samples, Figure 1B), no sexual reversion could be observed for the 117-NP injected embryos that we analyzed.

Reprotoxic effect of nonylphenol in testis organization

We next examined the reprotoxic effect of NP on testis and ovary organization using histological staining and immunohistochemistry analysis. Sections of ovaries and testes dissected from control embryos and embryos injected with 10 $\mu\text{g}/\text{egg}$ of NP (Figure 1) were analyzed by hematoxylin and eosin staining. These classical histological stains demonstrated in both case the presence of regular ovary organization (data not shown). In contrast, we observed the presence of dense seminiferous tubules in testes in NP-injected samples compare to control testes (Figure 2A). Immunohistochemistry analyses using specific antibodies directed against markers of mesenchymal (Desmin) and smooth muscle (αSMA for alpha Smooth Muscle Actin cells) that labeled mesenchymal cells out of the seminiferous tubules showed that NP treatment did not affect the presence and the organization of these cell types in the testes of treated embryos. Interestingly, analysis of adjacent sections using an anti-SOX9

antibody that specifically stained Sertoli cells present inside seminiferous tubules revealed a higher expression of SOX9 in testes injected with NP compared to its normal expression in control testes. This result prompted us to evaluate the seminiferous tubule area compared to the total testis area and we observed no significant difference between NP and control conditions suggesting that NP treatment did not induce any change of the seminiferous tubules in the global organization of the resulting testes. However, we observed a statistical decrease of almost 64.12% on 5 NP-testes analyzed (P value=0.0086) of the intra-lumen of the seminiferous tubule of the NP treated testes (10 μ g/egg) compared to control testes (Figure 3). These results suggested an impairment of the NP testes and could suggest a functional alteration of testes under NP treatment. Furthermore, the same immunohistochemistry analysis of the impact of the NP treatment on ovarian development was performed and showed little difference on ovarian organization (data not shown). Finally, we also tested the impact of injection of 50 μ g NP per embryo (Figure 2B). We observed no impact on mesenchyme or smooth muscle presence and organization, but confirmed the impact of NP on the decrease of intraluminal seminiferous surface area (Figure 2B).

Effect of nonylphenol on the development of visceral organs

In addition to the impact of NP on seminiferous tubule development, the impact of high dose of NP compounds (10 and 50 μ g/egg) was analyzed in visceral derivate organs such as the kidney, liver and gastrointestinal tract. After injection of 10 μ g of NP (Figure 4, bottom panel), we confirmed with histological tests the alteration of the seminiferous tubules in the NP-testes and observed a heterogeneous organization of the renal tubules (Figure 4, black arrows) compared to the untreated control kidneys (Figure 4, upper panel).

We also observed the impact of 50 μ g of NP on visceral organs development and specifically observed at this dose that the development of the liver was impaired (Figure 5). In the left panel showing the control liver, the liver shows in the form of two lobes in the abdominal cavity. After injection of 50 μ g NP (right panel), the resulting liver showed an altered phenotype (green) suggesting an abnormal bile spillage at this point (black arrows).

Discussion

NP is considered to be an endocrine disruptor. Its mode of action as classically described consists of binding the estrogen receptors, thus blocking or modifying the functions of

endogenous estrogens at different stages of reproduction and development (Hong et al., 2004).

Some authors have discussed this action arguing that the estrogenic potential of NP is largely weak compared to natural, synthetic estrogenic compounds and other xenoestrogens (CEPAD, 2007). Indeed, the estrogenic potential of nonylphenol is 100,000 (10^5) to 120,000 times lower than those of model estrogens (estradiol, EE2), or even ten times stronger than those of phthalates (Table 4).

Our study confirmed the weaker estrogenic effect of NP compared to compounds with stronger estrogenic potential, at least for the macroscopic observations. Regarding a previous study (Biau et al., 2007) where injection of ethynyl estradiol, estrone and estriol (1 μ g/eggs injected) resulted in the modification of the right gonad size for the first one and a statistically reliable sexual reversion for the two others, injection of NP, at higher doses (10 to 50 times) did not produce similar macroscopic damage in our study. Razia et al. (2006) also observed such differences between NP and estradiol on the development of an ovotestis in Japanese quail embryos. In spite of a concentration 100 to 1000 times higher (1-100 ng/g egg for E2 vs 1-100 μ g/g egg for NP) NP did not induce the development of an ovotestis contrarily to E2. On another hand, Oshima et al. (2012) showed a gonad feminization in a dose independent way (concentration of exposure varied from 0.2 to 200 μ g/L) but by using particular species of quail (obtained by criss crossing).

Then, although the doses administered were higher than those for E2, NP is still much weaker but histological changes were observed. In particular, denser seminiferous tubules and over expression of some genes have been observed.

These outcomes are broadly in agreement with studies on aquatic organisms, especially fish for which sexual behavior is one of the main parameters investigated. Indeed, compared to natural or synthetic estrogens, the effects due to NP exposure are weakly macroscopic (gonad morphology, sex reversion) but more strongly cellular or molecular. Zha et al. (2008) showed, for example, the non-effect of 4 ng/L of NP exposure on fertility and reproduction in several fish species (Zebrafish, medaka, fathead minnow whatever their sensitivity), and other authors illustrate the impairment of reproductive power by the expression of specific proteins (especially vitellogenin) in males (Yang et al., 2006; Schoenfuss et al., 2008).

The effect of NP on aquatic organisms has also been demonstrated by using other sexual parameters of interest (also called "endpoints") such as (i) the reduction of egg production and fertility, the increase of hepatic vitellogenin, the hatchability and time of hatching in medaka (Ishibashi et al., 2006), and (ii) the production and quality of sperm in trout (Lahnsteiner et al., 2005) (exposure concentrations varied from 100 ng/L to 100 µg/L and exposure from 30 to 60 days in these studies).

Finally, NP exposure of aquatic and terrestrial organisms can result in various other mechanisms. Recently, Palermo et al. (2012) suggested that NP modulates the hypothalamic–pituitary–interrenal axis (HPI) in the sole (*Solea solea*), inducing a cortisol-mediated stress response via a non-estrogen receptor (ER)-mediated mechanism. Several authors (Bhattacharya et al., 2008; Zha et al., 2008) showed that NP (at concentrations from 37 to 150 µg/L) caused a modification of the structure and function of the kidney and other organs such as the liver and gills. The reactivity of visceral organs has also been demonstrated in our study: the organization of the urinary tubules and seminiferous tubules of the kidney was found altered. Such impairment was previously observed on chick embryos in the presence of estrogen models as shown in figure 6 (Biau, 2007). In addition, the liver sensitivity highlighted in our study deserves to be addressed. Indeed, a failure of hepatic morphogenesis during NP injection at 50 µg was observed. These results tend to corroborate results published recently on the effects of NP on the hepatic system of rats presented in Table 5 and showing that although presenting a weak estrogenic potential, NP causes liver damage that may be more or less severe.

Conclusion

This study showed that NP has a reprotoxic effect apparently lower than those of other endocrine disruptors: the effects it causes on organisms is weak at the morphological level but can be significant at the functional level; these effects can be highlighted by the use of new relevant "endpoints". For that, the use of the chick embryo can represent an alternative. Indeed, though the phenotypes of male and female embryos were unaffected by the injected concentrations, the histological study showed a narrowing of the seminiferous tubules. Therefore, the reproductive function is affected. It would therefore be interesting to further examine these observations and to quantify more robustly if there is a dose response relationship for instance. Moreover, our study showed that the whole urogenital sphere was

affected; kidney lesions were observed. Finally, the use of the chick embryo made it possible to visualize effects on other organs, showing that the mode of action of NP may not be limited to the endocrine system, but can also target other visceral organs.

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1 **Table 1** : Avian model and endocrine disruptors studies

Avian model	Tested compound	Conditions	Observations
Japanese quail embryos (Berg et al., 1999)	Diethylstilbestrol (DES) Ethinylestradiol (EE2)	Injection into the yolks at day 3 of incubation. Doses range between 0.7 and 170 ng/g egg. Dissection at day 15 (2 days before hatching)	EE2: at 2ng/egg, all male embryos became feminized, containing ovary-like tissue in the left testis. Persistent Mullerian ducts oviducts in male embryos, and malformations of the Mullerian ducts in females DES: approximately one-third to one-tenth as potent as EE2. Morphological changes were dose-dependent,
Japanese quail (Halldin, 2005)	DDT	Injection into the yolk at day 3 of incubation. Study of the behavior from 7 weeks of age	Alteration of the development of the neural system and demasculinization of male quail. Morphological changes of the oviduct and reduction of egg laying in females.
Japanese quail embryos (Ottinger et al., 2005)	Estradiol DDE Methoxychlor Vinclozolin	Injection at day 11 of embryonic development. Exposure of adult via diet.	Embryonic injection: demasculinization of male sexual behavior, alteration of hypothalamic neurotransmitters and reduction of hen day production with estradiol injection; impairment of male sexual behavior in adult quail with methoxychlor or vinclozolin; impairment of reproductive and immune related end points with DDE. Adult methoxychlor diet exposure: reproductive consequences observable at both lower and higher doses in offspring F1 and F2 generations
Japanese quail embryos (Razia et	17 β -estradiol p-nonylphenol	Injection into the yolk at day 3 of incubation. Doses range between 1 and 100 ng/g egg. Dissection at day 15	Estradiol: disappearance of lymphoid cells; decrease of area of thyroid follicles; increase of follicular appearance of the thymus; and development of an ovotestis in male embryos. NP : no development of an ovotestis in male embryos; increase of the

al., 2006)			disappearance of lymphoid cells and increase of the follicle-like structure in the thymus in male embryos
Japanese quail embryos (Ottinger et al., 2009)	Estradiol, atrazine, Methoxychlor, PCB 126, Vinclozolin,	Injection between day 0 and 4 into the egg, and measurement of hypothalamic gonadotropin releasing hormone-I (GnRH-I) in hatchlings and adults.	Vinclozolin and PCB126: depression of the concentration of embryonic GnRH-I peptide; Methoxychlor: inconsistent stimulatory effect. Estradiol, atrazine: no significant effects on GnRH-I in adults
Japanese quail embryos (Oshima et al., 2012)	Bisphenol A Nonylphenol	Injection into the egg white just before incubation. Concentration ranges between 20 à 200000ng/egg. Dissection and observation at day 16.	Ovotestis (developed in the left testis) significantly higher than that in the control group, but no dose-dependent
Quail embryos in vitro culture (Nishijima et al., 2003)	17 β -Estradiol, Nonylphénol	Transfer the content of quail eggshells of 2.5 d culture into to chicken eggshells. Addition of extra albumen supplemented calcium lactate was added. Dissection after 10d more culture.	17 β -Estradiol: Feminization of the gonads of male embryos; increase in the size of the left gonad and female-specific aromatase expression. NP: reduction of the viability and body weight of embryos. Feminization of the gonads of male embryos.
White ibises (Jayasena et al., 2011)	Methylmercury	Exposure of captive white ibises for 3.5 years to dietary methylmercury at 0.05, 0.1 and 0.3 ppm wet weight in diet + measurement of fecal concentrations of estradiol and testosterone metabolites in two	Changes in reproductive behavior, reduced reproductive success and altered mate choice in males Decrease of estradiol and testosterone concentrations in females

		consecutive breeding seasons.	
Female starlings (Van den Steen et al., 2009)	PBDE	Subcutaneous implants of 150 µg + measurement of levels and profiles of PBDEs together with reproductive effects	Higher egg weight and volume in the expose population A general negative effect on reproductive performance
Female starlings (Van den Steen et al., 2010)	PBDE	Subcutaneous implants of 1740 µg/kg body weight) + measurement of hormone level in the plasma.	Lower level of estradiol and thyroid hormone in the exposed group
Japanese quail and chicken (Brunström et al., 2009)	Estrogen	Injection into the yolk at day 3 of incubation. Dissection at day 16 and week 3 and 9. Measurement of influence of ER α and ER β	Abundant expression of ER α mRNA in gonads and Müllerian ducts of early Japanese quail embryos Detection of ER α and ER β transcripts in early embryo brains of Japanese quail No effect of an ER α agonist propyl on the male copulatory behavior
Chicken embryos (Bruggeman et al., 2003)	Dioxin (TCDD)	Injection of 0, 8, 20 or 50 ng/egg at day 4, 8 or 12 of incubation and exam at hatch of weigh, sex and morphology. Additional chicken of 6 weeks of age studied (body and organ masses, plasma concentrations of thyroid hormones, triglycerides and glucose)	High toxicity for 4-day-old chicken embryos with 50 ng. Less toxicity for chicken embryos of 8- and especially 12-days old. Depression of post-hatch body mass gain for injected at day 4 or day 8 with 20 and 50 ng, respectively. No changes in plasma triglycerides or glucose concentrations during postnatal life No changes of organ masses of 6-week-old chickens

Chicken embryos (Biau et al., 2007)	Estone, Estriol, Ethynylestradiol	Injection between 20 and 600ng/egg at day 3 of incubation. Dissection at day 18 and observation of morphological phenotype	Estriol and estrone: persistence of Müllerian ducts in 50% of male embryos and hypertrophic oviducts in 71% of females. Kidney dysfunction with estrone Ethynylestradiol: weak morphological effect
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Table 2 : Summary of chick embryo analyzed after NP injection

Control N=24	Male = 11
	Female = 13
0.1 µg of NP/egg N=20	Male = 11
	Female = 9
1 µg of NP/egg N=23	Male = 10
	Female = 13
10 µg of NP/egg N=64	Male = 35
	Female = 29
50 µg of NP/egg N=10	Male = 4
	Female = 6

Table 3 : Summary of chick embryo survival after NP injection

	Number of injected eggs	Survival	Percentage
Control	28	24	85.7%
Total NP doses from 0.1 to 50 µg/egg	181	151	83.4%
0.1 µg of NP/egg	23	20	86.9%
1 µg of NP/egg	28	23	82.1%
10 µg of NP/egg	76	64	84.2%
50 µg of NP/egg	54	44	81.4%

Table 4 : Estrogenic potential of some EDC compounds

Substances	Estrogenic potential	Reference
Estradiol	1	
Ethynylestradiol (EE2)	1.2	(Legler et al., 2002)
PCB: delor 103	4.10^{-4}	(Svobodová et al., 2009)
Bisphenol A	1.10^{-4}	(Bergeron et al., 1999)
Benzyl Parabenes	1.10^{-4}	(Routledge et al., 1998)
Genistein	$6.0.10^{-5}$	(Legler et al., 1999)
Nonylphenols	1.10^{-5}	(White et al., 1994)
Pesticides DDT	$9.1.10^{-6}$	(Legler et al., 1999)
Butylbenzyl Phthalates	1.10^{-6}	(Harries et al., 1997)

Table 5: Hepatic response of animals exposed to NP

Animal	Exposure conditions	Effect described
Adult male rat (Jubendradass et al., 2012)	Oral gavage at the doses of 15,150 and 1500 $\mu\text{g}/\text{kg}$ body weight per day for 45 days.	NP induces apoptosis in liver involving both mitochondria-dependent pathways and Fas–Fas-L Apoptosis-related proteins increased leading to hepatic damage in rats
Adult male rat (Jubendradass et al., 2012)	Oral gavage at the doses of 15,150 and 1500 mg/kg body weight per day for 45 days.	NP down regulates insulin signalling in liver, which could be due to ROS production and oxidative damage
Adult male rat (Jubendradass et al., 2011)	Oral gavage at the doses of 15,150 and 1500 mg/kg body weight per day for 45 days.	NP causes oxidative stress in pancreas and impairs liver glucose homeostasis
Male rat (Korkmaz et al., 2010)	Oral administration of 25 $\text{mg}/\text{kg}/\text{jour}$ three times a week for 50 days	NP causes oxidative damage by disturbing the balance between Reactive oxygen species and antioxidant defences system in liver.
Chalcalburnus tarichi (fish) (Kaptaner and Unal, 2011)	Exposure to 10, 60, 200 $\mu\text{g}/\text{L}$ for 32 days under semi-static daily renewal conditions	Exposure to the highest concentrations (60 - 200 $\mu\text{g}/\text{L}$) caused a hepatotoxicity causally related to a significant increases in the extent of apoptosis in liver
Juvenile Atlantic cod (Olsvik et al., 2009)	Exposure to 30 $\mu\text{g}/\text{L}$ for 3 weeks	NP exposure produced a significant reduction of five cytochrome P450s genes an increase (4.6-fold) of GST (Phase II conjugating enzymes) in liver

Figure legends

Figure 1: Impact of NP on the morphology of developing urogenital structures. A/ Male E14 gonads were analyzed in situ or after dissection under a dose of 10 µg/eggs. Black arrows indicate testis. B/ Female E14 gonads were analyzed in situ or after dissection under a dose of 10 µg/eggs. Black arrows indicate ovary. Note the normal ovary disymmetry with the presence of left functional ovary.

Figure 2: Impact of NP on testis organization. A/ Male E14 control and 10 µg/egg injected NP testes were analyzed by histological and immunohistochemistry respectively in the upper and below panels. Panels represent the common observation obtained after NP treatment. Scale bars, 100 µm. Histological staining indicate the tissue composition of testes with dense seminiferous tubules and indicate normal organization of NP treated testes. αSMA and Desmin immunodections allow the detection of peritubular myoid cells surrounding seminiferous tubules and indicate normal composition of these cell types in BO treated testes. SOX9 immunodetection indicates the presence of Sertoli cells into seminiferous tubules of control and treated testes. The amount of reduction (64%) of decrease in seminiferous area in NP treated testes. Black arrows indicate seminiferous tubules. B/ Impact of 50 µg/egg of NP on testis organization. Panels represent the common observation obtained after NP treatment. Histological, αSMA and Desmin stainings revealed normal organization of NP treated testis. As observed in A, we observed that SOX9 immunostaining reveals similar decrease of intraluminal seminiferous area. Black arrows indicate seminiferous tubules. Scale bars, 100 µm.

Figure 3: Quantification of intra-lumen seminiferous tubes after 10 µg/egg of NP treatment. Statistical decrease of almost 64 % on 5 NP-testes analyzed (P value=0.0086) of the intra-lumen of the seminiferous tubule compare to control testes.

Figure 4: Kidney organization after 10 µg/egg of NP injection. Hematoxylin and eosin (H&E)-stained transverse sections of Control and NP injected testis-mesonephric tubules. Histological stainings indicate the alteration of mesonephric tubules in NP injected embryos.

Black arrowheads and arrows indicate respectively seminiferous and mesonephric tubules. Scale bars are 500 μm and 100 μm for magnified views.

Figure 5: Impact of 50 $\mu\text{g}/\text{egg}$ of NP on Liver development at 14 days. Under 50 μg of NP, we observed the presence of green bile into liver lobes. Black arrows indicate aberrant liver lobes.

Figure 6: Histology of chick embryo kidneys after injection of estrone and estriol. T.U: Urinary tubule. (Bars: 200 μm) (Sandrine Biau, 2007).





