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EFFECTS OF IN UTERO AND LACTATIONAL EXPOSURE TO DI(2-ETHYL-HEXYL) PHTHALATE (DEHP) AND POLYCHLORINATED BIPHENYLS (PCBs) IN MICE: REPRODUCTIVE TOXICITY AND MULTIGENERATIONAL TRANSMISSION

Doctoral Thesis

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ABSTRACT

Several studies indicate that *in utero* and peri-natal exposure to some classes of endocrine disruptors induces adverse reproductive effects, but it remains unclear whether such effects may be transmitted to subsequent generations. The present study examined the effects in mice of exposure to di(2-ethyl-hexyl) phthalate (DEHP) or to polychlorinated biphenyls (PCBs) throughout pregnancy and lactation on reproductive health in male and female offspring, at adult age, over three generations.

Groups of two to three dams were exposed to increasing doses of contaminants with the diet from gestational day 0.5 until the end of lactation. The doses employed were within the range of environmental exposure levels in humans (DEHP: 0, 0.05, 5, and 500 mg/kg/day; PCBs 101+118: 0, 1, 10, 100 μg/kg/day).

In DEHP experiments, treatment of pregnant F0 dams with the 500 mg dose caused complete pregnancy failure, while a slight reduction in litter size in the 5 mg was observed. Male and female F1 offspring born from dams treated with 5 and 0.05 DEHP doses showed, once they reache adult age, significant morphological and functional alterations of the reproductive system. Specifically: i) lower body weight; ii) altered gonad weight (i.e.: lighter testis and heavier ovary) and morphology; iii) reduced germ cells quality; iv) low expression of steroidogenesis and gonadotropin-receptor genes in the gonads; and v) up-regulated gonadotropin subunits gene expression in the pituitary.

DEHP exposure altered male offspring morphological and reproductive indices only in the first generation. Conversely, F2 and F3 female offspring exhibited altered gonadal weight and morphology, concomitantly with poor embryo quality, similarly to what observd in F1, thus showing a transgenerational transmission of reproductive adverse effects. Interestingly, also disregulation of selected ovarian and embryonic genes was maintained up to the third generation.

In PCBs experiments, treatment did not affect F0 dams' reproductive outcome. Nevertheless, whole-body PCB burden increased in a dose-dependent manner confirming the effectiveness of the treatment. Furthermore, concentrations at all doses investigated were greater in the offspring than in the dams, confirming that the progeny were exposed as a result of maternal exposure.

Pre- and peri-natal exposure to PCBs resulted in male and female offspring showing significant reproductive abnormalities, at adult age. Specifically, compared to controls, they showed reductions in: i) testis weight and seminiferous tubule diameter; ii) sperm viability and developmental capacity; iii) ovary weight; iv) oocyte developmental capacity. Furthermore, F1 ovaries showed a dose-dependent increase in follicular atresia, associated with down-regulation of *cyp19a1* and *pten* mRNA levels.

PCBs adverse reproductive effects in females were limited to F1 generation. In contrast, male offspring exhibited reduced sperm viability and altered seminiferous tubule distribution up to the third generation. These results evidence that maternal exposure to PCBs can affects reproductive health in multiple generations.

In conclusion, our data indicate that exposure to the endocrine disruptors DEHP or PCBs, at the time of gonadal sex determination, perturbed significantly the reproductive indices of male and female adult offspring. Furthermore, some of the reproductive deficiencies observed upon direct exposure have been observed up to the third generation. These findings have significant implications for reproductive health and fertility of animals and humans.

Ai miei cari,
per esser stati un costante sostegno durante questo entusiasmante percorso.

Alla mia dolce metà,
per aver camminato al mio fianco dall'inizio alla fine.

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INTRODUCTION

Chapter 1 – Endocrine disruptors

1.1 *What are the endocrine disruptors?*

Recently, there has been concern among the scientific community, policy makers and general public regarding the potential reproductive and health hazards of a range of environmental chemicals known as 'endocrine disruptors'. An endocrine disruptor (ED) is a natural or synthesized compound able to interfere with the normal functioning of the endocrine system and, consequently, to induce adverse health effects in an intact organism, or its progeny. Like hormones, small amounts of these chemicals (parts per trillion) are believed to affect the endocrine system of animals and humans.

A wide range of substances, diverse as regards the use, chemical structure and mechanism of action, are thought to cause endocrine disruption, including: persistent organic pollutants (POPs) such as dioxins, DDT and other pesticides, polybrominated diphenyl ethers (PBDE) and polychlorinated biphenyls (PCBs); fungicides used in plant or animal food production (azole or dicaroximide); heavy metals (arsenic, cadmium, lead, mercury); plasticizers such as phthalates and bisphenol A (BPA) (Hotchkiss *et al.* 2008; Martino-Andrade and Chahoud 2010).

The homeostasis of sex steroids and thyroid hormones are the main targets of endocrine disruption and, therefore, reproductive health, considered as a continuum from gamete production and fertilization right through intrauterine and post-natal development of progeny, is recognized as being especially vulnerable to endocrine disruption

To date, different endocrine-disrupting mechanisms have been identified: (a) mimic the effect of endogenous hormones (McLachlan 1993); (b) antagonize the effect of endogenous hormones (McLachlan 1993); (c) interfere with the synthesis and metabolism

of endogenous hormones (Bradlow *et al.* 1995); (d) modify the synthesis of hormone receptors or the hormone transport. Some examples of EDs action are given in Table 1.

Table 1 Some examples of chemicals exerting endocrine disrupting activity grouped according to their proposed mechanism of action.

Proposed mechanism of action	**Chemicals**
Estrogen-receptor mediated	DDT, Bisphenol-A, Methoxychlor
Anti-estrogenic	Dioxin, endosulphan
Anti-androgenic	DDE, Vinclozolin, phthalates
Modulation of circulating steroid levels	Fungicides, endosulphan, dioxin, PCBs
Anti-thyroid hormone action	Phthalates, herbicide, PCBs

(Depledge *et al.* 1999) - modified)

1.2 *Sources of exposure*

The sources of exposure to EDs are diverse and vary widely around the world. Humans and animals exposure may result from the involuntary ingestion of contaminated food and water, breathing of contaminated air or absorption through the skin. In humans, by far the largest exposure to EDs occurs through the ingestion of contaminated food.

Some EDs were designed to have long half-lives; this was beneficial for their industrial use, but it has turned out to be detrimental to wildlife and humans. These substances do not decay easily, they may not be metabolized, or they may be metabolized or broken down into more toxic compounds than the parent molecule. Therefore, even substances that were banned decades ago may remain in high levels in the environment, and they can be detected as part of the body burden of virtually every tested individual, animal or human (Calafat and Needham 2008; Porte *et al.* 2006) . Furthermore, endocrine disruptors such as

PCBs, phthalates, pesticides and dioxins, can be transported by air or water currents and contaminate sites distant from the release point (Chiu *et al.* 2004; Loganathan BG and K 1994). Finally, EDs sharing lipophilic characteristics may undergo bioaccumulation and biomagnification, putting species at the top of the food chain at particular risk.

In humans, vulnerability of different groups in the population will be affected by lifestyle factors (e.g., subsistence hunting and fishing and avid sportsmen who consume fish and wildlife), genetic factors (e.g., metabolic differences that can determine sensitivity), special dietary habits, and age (e.g., the types and rates of food consumption in children).

It is nothworthy to notice that distinct EDs-related effects can occur at different life stages. Indeed, exposure of an adult to an EDs may have very different consequences from exposure to a developing fetus or infant whose growth and development are highly controlled by the endocrine system (Bern 1992). Therefore, a growing concern has been raised by the scientific community, concerning the exposure to endocrine disruptors in the womb or early in life. In fact, many of ED contaminants can pass through the placental barrier and/or to the breast milk and the potential exists also for *in utero* exposure of the developing organism, as well as exposure of neonates during critical developmental periods. Suckling infants, for example, may be exposed to contaminants during sensitive developmental periods at levels 10-40 times higher than levels the general population is exposed to (WHO 1989). The basis for this concern is that development is coordinated by hormonal signals controlling cell proliferation, differentiation, and organ development and disruption of these signals can lead to irreversible changes in organ function creating the greatest potential for adverse health effects. Therefore, in the last years, the field of endocrine disruption has embraced the terminology “the fetal basis of adult disease” (Newbold 2011) to describe observations that the environment of a developing organism, which includes both the maternal and the external environment, interacts with the

individual's genes to determine the propensity of that individual to develop a disease or dysfunction later in life.

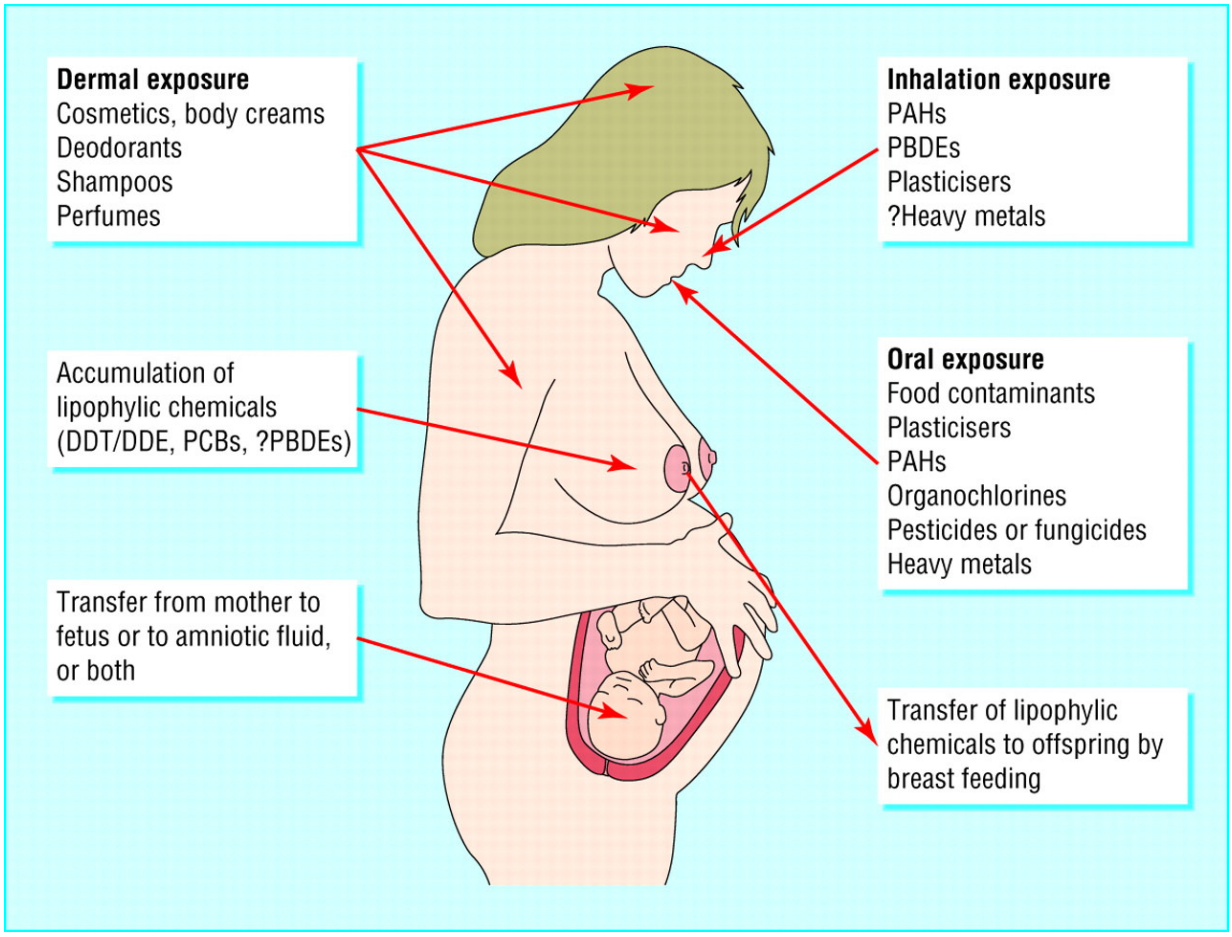

Figure 1 Routes of human exposure to some common environmental chemicals.
DDE=1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT=dichlorodiphenyltrichloroethane
PAHs=polycyclic aromatic hydrocarbons
PCBs=polychlorinated biphenyls
Reproduced from [How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? Richard M Sharpe, D Stewart Irvine; 328:447–51, 2004] with permission from BMJ Publishing Group Ltd.

1.3 *EDs and health effects*

In general, health effects associated with EDs include a range of reproductive problems (reduced fertility, male and female reproductive tract abnormalities, skewed male/female sex ratios, abortion and miscarriage, menstrual problems), brain and behaviour problems, impaired thyroid and immune functions, and various cancers.

There is increasing evidence that the central neuroendocrine systems are target of endocrine-disrupting chemicals. Most studies have focused on the Hypothalamic-Pituitary-Gonadal (HPG) axis, however, there is a body of literature showing that also the Hypothalamic-Pituitary-Thyroid (HPT) axis, is highly susceptible to endocrine disruption. Finally, relatively few studies have investigated links between endocrine disruptors and stress [Hypothalamic-Pituitary-Adrenal (HPA) axis] (for review see: (Gore 2010).

EDs exposure has been linked to disruption of a variety of immune maturational events resulting in a wide range of adverse outcomes in the form of immune dysfunction including increased risk of infectious disease (Dallaire *et al.* 2006; Heilmann *et al.* 2006), cancer (Dietert 2009), allergic diseases (Dietert and Zelikoff 2008), autoimmunity (Mustafa *et al.* 2011), and neuroinflammatory-associated conditions (Dietert and Dietert 2008a, b; Hertz-Picciotto *et al.* 2008).

Although endocrine disruptors have adverse effects on different hormone-dependent functions as described above, studies have focused mainly on development and reproduction.

Maintenance of species is dependent on an integrated interplay of the entire endocrine system. Reproduction ceases when life-supporting hormonal systems are disturbed, but "performance" supporting hormones are also needed to maintain normal reproduction.

Of major importance for reproduction is normal functioning of the hypothalamic-pituitary-gonadal axis, as well as the proper functioning of other accessory organs

regulated by gonadal steroids in both female and males. Most EDs that disturb reproductive health act on steroidal signalling, interfering with the proper action of estrogens or androgens, inhibiting the synthesis of sex steroids or interfering with their metabolism (Fig 2). Most EDs with adverse effects on reproduction bind either to the estrogen receptors (ERs) or to the androgen receptor (AR) and, by doing so, may either stimulate or inhibit the transcriptional or post-transcriptional mechanisms; moreover, EDs can modify the existing signal transduction by steroid hormones acting on either ion channels or 2nd messengers (Massaad *et al.* 2002). By means of these mechanisms, they may interfere with fundamental sex steroid effects on the brain, the pituitary gland, the gonads and the accessory sex organs, such as the uterus and mammary gland in females and the prostate and seminal vesicles in males.

Early evidence leading to a widespread awareness of the impact of environmental chemicals on reproduction and development was observed in wildlife. A suite of reproductive and congenital defects was identified that were attributed to high concentrations of organochlorine pesticides and industrial chemicals in birds, reptiles, and mammals across the world. Specifically, much interest has been directed towards species living in an aquatic environment or associated with the aquatic food chain. In fact, a variety of EDs, including chlorinated hydrocarbons and heavy metals, are discharged into rivers and estuaries and therefore accumulate into fresh and marine waters. Reproductive and developmental effects in a number of species have been reviewed by Vos et al. (Vos *et al.* 2000). Examples include:

- Masculinization in female marine snails, caused by tributylin (Horiguchi 2006; Iguchi *et al.* 2008).
- Eggshell thinning in birds, caused by dichlorodiphenyldichloroethylene (DDE).
- Effects on reproductive organs in a variety of fish species caused, for example, by effluents from water treatment plants.

- Distorted sex organ development and function in alligators, caused by dichlorodiphenyltrichloroethane (DDT).
- Reduced fecundity, decrease survival of juveniles, and depress sperm quality (count, viability, motility, and percentage of abnormalities in a variety of marine species, caused by phthalate (Younglai *et al.* 2007).
- Impaired reproduction and immune function in Baltic grey and ringed seals, as well as in harbour seals in the Wadden Sea, firmly linked to PCBs.

These effects, although at first rather subtle, could over the course of a breeding season, or several seasons, result in reduced reproductive success and eventual population decline.

In humans, reproductive and fertility problems appear to be on the rise. Despite many questions are still controversial due the difficulty of predicting the exact relationship between exposure and effect, there are a variety of individual pieces of evidence that, altogether, suggest that exposure to EDs may be responsible for the increased occurrence of reproductive abnormalities and infertility in both male and females. The current concerns about the effects of EDs on humans are largely based on a series of observations which, when considered together, implicate these chemicals in a process that leads to deleterious effects in reproductive tract development and function. Unexplained increases in testicular prostate cancer, genital deformities in males, and breast cancer, endometriosis and earlier onset of puberty in females in recent decades have raised the concern about the role of endocrine disruptors in these health trends (Colborn *et al.* 1993).

A number of adverse trends in male reproductive health have been observed in many developed countries including poor semen quality, low sperm count, low ejaculate volume, high number of morphologically abnormal sperms and low number of motile sperms as well as testicular cancer, reproductive organ malformations (for example, undescended testes, small penis size and hypospadias), prostate diseases and other abnormalities of male reproductive tissues (reviewed by: (Olea and Fernandez 2007). The relevance of

environmental factors in the development of these problems is emphasised by the striking geographical variations reported between different countries, as well as the temporal changes in incidence rate that have occurred over the last 50 years, which cannot be accounted for by random fluctuations in prevalence rate, nor by differences in study design or by method of effect ascertainment.

The major limiting factor in drawing any conclusions about female reproductive system effects and EDs is the scarceness of actual exposure data. In fact, contrary to male fertility, data on EDs effects on female reproductive health are sparse both in the human and experimental literature. Despite these drawbacks, exposure to environmental chemicals recently has been proposed to contribute to several gynecological pathologies, especially when exposures occur during critical periods of development (Caserta *et al.* 2008). An analysis of female reproductive outcomes reveals that conception rates have declined by 44% since 1960 in developed countries (Hamilton and Ventura 2006; Jensen *et al.* 2008). In addition, hormone-related diseases such as disorders of pubertal development, polycystic ovary syndrome (PCOS), endometriosis, and uterine fibroids are common, although few data on global or population-based trends are available. The combination of reduced conception rates and increasing occurrences of female reproductive organ diseases raises concern that environmental factors may be having a negative impact on female reproductive health.

The time of development when exposure takes place may be also critical to define the relationships of EDs for female reproductive disorders. The perinatal period and the period between age at menarche and age at first full-term pregnancy may be particularly important for reproductive abnormalities development and latency. The case of DES is the most well-known example, with young adult offspring exposed in utero to this potent drug having a higher rate of reproductive tract abnormalities in both sexes as well as of the rare clear-cell vaginal adenocarcinoma in female offspring (Swan 2000). In conclusion, the

currently available human data are inadequate to support a conclusion about whether the female reproductive system is adversely affected by exposure to EDs; however, the weight of the evidence is adequate to address further studies.

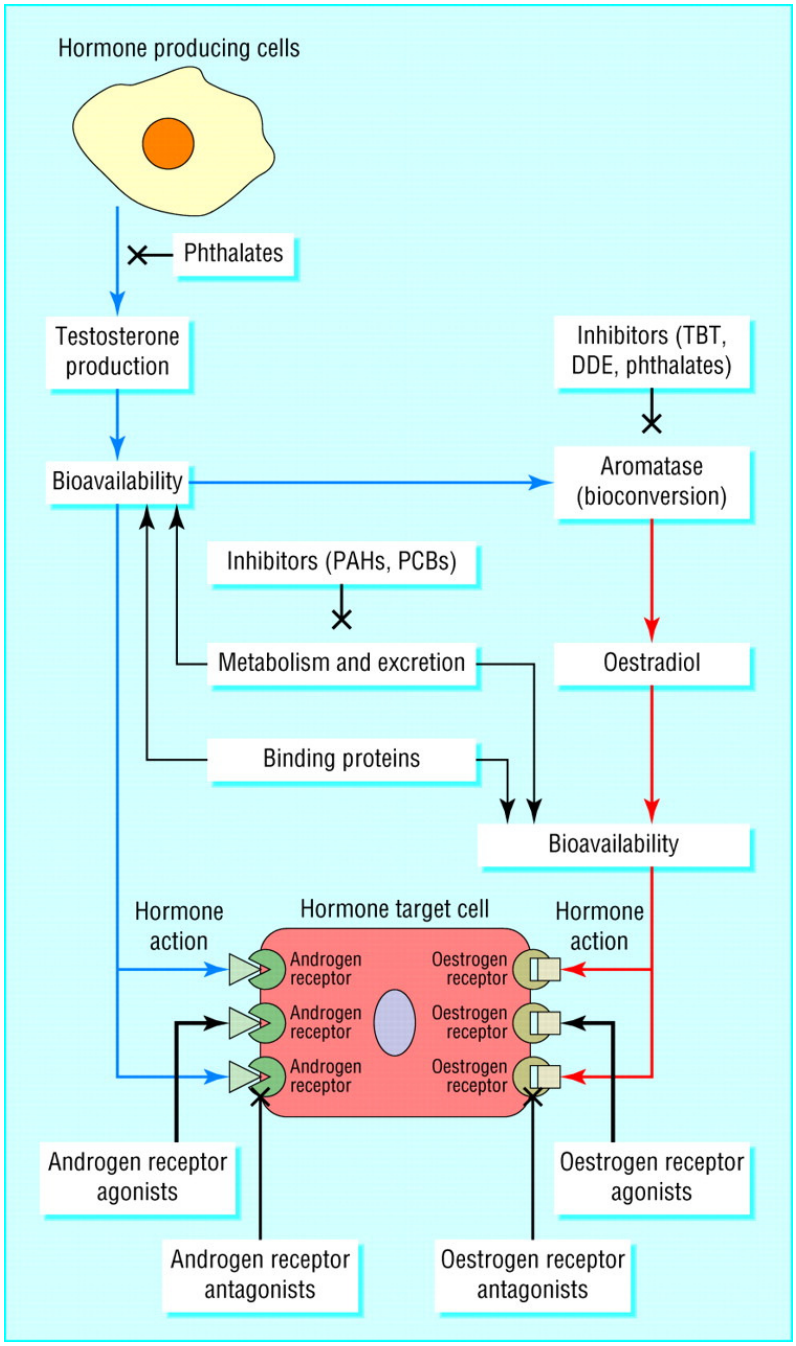

Figure 2 Potential pathways of endocrine disruption by environmental chemicals. Reproduced from [How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? Richard M Sharpe, D Stewart Irvine; 328:447–51, 2004] with permission from BMJ Publishing Group Ltd.

Chapter 2 – Phthalates

2.1 General background

Phthalates (phthalic acid esters) are plasticizers that are added to polymers, especially PVC, to impart softness and flexibility. They are widely used in the manufacture of a broad range of consumer goods such as medical devices, clothing, packaging, food containers, personal-care products and children's toys (Kavlock *et al.* 2002). The most common used phthalate is the Di(2-ethylhexyl) phthalate (DEHP – Fig 3) with a production of approximately one to four million tons per year, which makes DEHP one of the most widespread environmental contaminant worldwide (Akingbemi *et al.* 2001; McKee *et al.* 2004). Phthalates does not form strong linkages with the polymer at the molecular level. Therefore, they can diffuse throughout the matrix and leach into the environment (Bosnir *et al.* 2003; Petersen and Breindahl 2000). As a result, the general population is widely and continuously exposed to phthalates via ingestion, inhalation or dermal absorption. Therefore, since phthalates exert endocrine disrupting activity, they pose significant public health concerns (Silva *et al.* 2004; Wittassek and Angerer 2008).

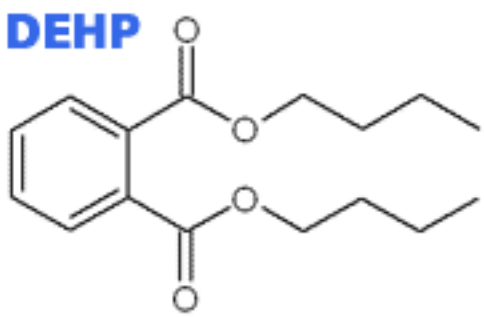

Figure 3 Chemical structure of the di(2-ethylhexyl) phthalate

2.2 *Effects on reproduction*

The reproductive system is particularly susceptible to the endocrine disrupting activity of phthalates. In rats, these effects include reduction in fertility (Agarwal *et al.* 1989), litter size/viability (Agarwal *et al.* 1985a; Tyl *et al.* 1988), sperm density and motility (Agarwal *et al.* 1985b), and biochemical and morphological alterations of male and female gonads (Agarwal *et al.* 1989). Furthermore, phthalates are able to cross the placental barrier and, also, to pass into breast milk, causing a significant risk of damages for the developing fetus and newborn due to inappropriate modulation in hormonal levels which can result in permanent structural and functional changes (Dostal *et al.* 1987; Latini *et al.* 2003). Worryingly, many of the reproductive abnormalities derived from developmental exposure only become apparent after puberty (long-latency effect), which strongly hinders the development of a cause and effect relationship.

In rats recent studies demonstrated that pre- and peri-natal exposure to DEHP induces of reproductive tract abnormalities in male offspring such as hypospadias, undescended testis, underdeveloped epididymis and seminiferous tubules, and reduces daily sperm production (Barlow and Foster 2003; Li *et al.* 2000; Swan *et al.* 2005). Morphological and functional alterations of the reproductive system observed in animal models, strongly suggest phthalate-mediated alteration in steroid hormone-dependent processes in both the male and the female. Reduced anogenital distance and impaired testicular descent, which is consistent with the disruption of androgen-dependent development, have been observed in boys of mothers showing elevated phthalate exposure during pregnancy (Swan *et al.* 2005). Furthermore, in male rats, in utero exposure to DEHP inhibits fetal testosterone synthesis (Mylchreest *et al.* 2002). Finally, it has been observed that phthalates exposure may disrupt estrogen biosynthesis pathways through interference with the expression of aromatizing enzymes (e.g. CYP19a1) in both female and male gonads (Andrade *et al.* 2006; Davis *et*

al. 1994; Kim *et al.* 2003; Lovekamp and Davis 2001; Lovekamp-Swan and Davis 2003; Noda *et al.* 2007). Effects of pre- and peri-natal exposure to DEHP on the female have been less well studied than for the male (Lovekamp-Swan and Davis 2003). The limited number of studies have shown effects such as delayed onset of puberty (Grande *et al.* 2006) and disturbed post-pubertal reproductive functions (Ma *et al.* 2006) following exposure of pre-pubertal rats to DEHP by inhalation.

Chapter 3 – Polychlorinated biphenyls (PCBs)

3.1 General background

Polychlorinated biphenyls (PCBs) are a group of chemical compounds consisting of more than 200 possible congeners that differ in the number and position of the chlorine atoms on two basic benzene rings.

PCBs have been produced for industrial purposes for a long time and on a large scale. Even though the commercial production of PCBs was banned at the end of the 1970s, they continue to be widely persistent in the environment due to their high chemical stability lipophilicity, and persistence (the log*K*ow values range from 4.6 to 8.4), and therefore they are rapidly adsorbed in sediments and other particulate matter (Birkett 2003).

PCBs tend to bio-accumulate progressively in the food chains in proportion to the trophic level (Agency for Toxic Substances and Disease Registry (ATSDR) 1997) and can be detected in plasma, tissue samples and in the breast milk of a wide variety of species including humans (AMAP 2009; McFarland and Clarke 1989). Their occurrence has been linked to adverse health effects in humans and wildlife such as endocrine disruption and reproductive toxicity (Colborn *et al.* 1993; Lie *et al.* 2004; Ropstad *et al.* 2006; Safe 2004). Indeed, PCBs have been classified by the International Agency for Research on Cancer (IARC) as potentially carcinogenic to humans and may have non-carcinogenic health effects, particularly as endocrine disruptors (IARC 1998).

Toxic effects of a PCB congener are structure-dependent and are related to the degree of chlorination and pattern of chlorine substitution (Fig 4). In fact, depending on the chlorination of the ortho positions of the molecule, PCBs can be divided into non-ortho substituted (coplanar) and ortho-substituted (non-coplanar) groups. Non-ortho PCBs act through the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor

mediating toxicity of several structurally related pollutants (Ema *et al.* 1994), exerting dioxin-like activity. Ortho PCBs do not bind to the AhR, but are involved in alteration of relevant signal transduction systems, exerting neurotoxicity, endocrine disruption, and reproductive toxicity in a wide variety of species, including humans (Fischer *et al.* 1998; Selgrade 2007).

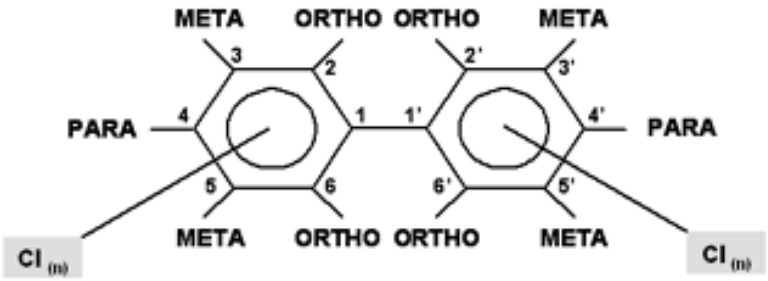

Figure 4 PCB molecule with Cl-binding positions

3.2 *Effects on reproduction*

It has been suggested that chemicals such as PCBs in the environment can mimic natural hormones when internalised and that this endocrine disruption can lead to infertility, certain types of cancer, hermaphroditism, reduced testosterone levels, other hormone-related disorders, a shortened menstrual cycle and other non-specific effects on the female reproduction (Mendola *et al.* 1997). PCBs and their metabolites can exhibit agonistic and antagonistic behaviours in oestrogenic systems, although the exact mechanism by which hydroxylated PCBs produce their effects has not been established (Kester *et al.* 2000; Li and Hansen 1996).

Since PCBs accumulate in adipose tissue and milk (Safe 1990) and can easily cross the placenta (ATSDR 2004; Eyster *et al.* 1983; Guvenius *et al.* 2003; Jacobson *et al.* 1984),

mammalian offspring is likely to be exposed to high concentrations of these pollutants during prenatal development and breast-feeding (Colciago *et al.* 2006; Kaya *et al.* 2002). Due to the heightened sensitivity of the fetal life stage, the fetus is extremely vulnerable to environmental challenges which can result in permanent structural and functional changes (Dostal *et al.* 1987; Latini *et al.* 2003). Many of the reproductive effects resulting from early exposure, only become apparent later in life, and this is a strong obstacle to identify cause and effect relationship.

In spite of this limitation, different studies correlated maternal PCB exposure with onset of reproductive disorders at adult age. In males, *in utero* and lactational exposure to PCBs has been shown to reduce ano-genital distance, alter testosterone homeostasis, induce significant changes in reproductive organ weights and decreases in sperm number and motility in different species, including humans (Hauser *et al.* 2003; Hauser *et al.* 2005; Hsu *et al.* 2007; Kuriyama and Chahoud 2004; Richthoff *et al.* 2003). Conversely, data on the effects of maternal exposure to PCBs on female progeny is still limited. Sager and Girard (1994) reported delays in puberty, impaired estrous cyclicity and impaired fertility in the rat. More recently, two different studies reported that in-utero and lactational exposure to PCBs altered ovarian follicle dynamics, preferentially targeting pre-antral follicles [rat: (Baldridge *et al.* 2003); sheep: (Kraugerud *et al.* 2011)].

Chapter 4 - Transgenerational exposure to EDs

Recent observations indicated that exposure to environmental endocrine disrupting chemicals at the time of gonadal sex determination not only may directly affect the reproductive health of the exposed individual, but it can also induce adverse effects on subsequent generations that may differ from those associated with primary exposure (Anway *et al.* 2005; Fernie *et al.* 2003; Shipp *et al.* 1998).

Exposure to environmental compounds during early development has important consequences on adult stages (Danzo 1998; McLachlan 2001; Skinner *et al.* 2010). One of the periods most sensitive to endocrine disruptor exposure is embryonic gonadal sex determination, when the germ line undergoes epigenetic programming and DNA re-methylation (Anway *et al.* 2005; Guerrero-Bosagna and Skinner 2009; Skinner *et al.* 2010). Prenatal and early postnatal exposure to EDs are likely more critical in disease etiology later in life than the adult exposure, which are more resistant to epigenetic change due to increased level of cellular and tissue differentiation versus the sensitivity of epigenetic systems during early stages of development. When exposure to endocrine disruptors occurs during this sensitive period, the effects can be transgenerationally transmitted (Anway *et al.* 2005; Skinner *et al.* 2010).

The precise mechanisms through which endocrine disrupting effects transmit to subsequent generations are not well understood but emerging evidence suggests that this transgenerational transmission may be based on epigenetic modifications (Anway *et al.* 2005). Epigenetic inheritance involves changes in gene expression patterns that in spite of not altering DNA sequence can produce important and permanent changes in the phenotype. Such effects include histone and chromatin structure modifications, non-coding RNA and DNA methylation and hydroxymethylation. In most cases, methylation of gene

promoter regions abrogates gene transcription while acetylation of the histone tail enhances it. The majority of environmental toxicants do not have the capacity to act directly on DNA sequence or promote mutations. Conversely they may have the potential to alter the epigenome. In the event an environmental toxicant modifies the epigenome of a somatic cell, this may promote disease in the individual exposed, but not be transmitted to the next generation. In contrast, in the event an environmental factor modifies the epigenome of the germ line permanently, the transmission of this altered genome could promote a transgenerational inheritance of corresponding phenotypes to subsequent generations and progeny (Giusti *et al.* 1995; Gore 2008; Jirtle and Skinner 2007; Skinner and Guerrero-Bosagna 2009).

Transgenerational epigenetic actions of EDs were first reported in the rat following exposure to the antiandrogenic compound vinclozolin, a fungicide commonly used in agriculture (Wong *et al.* 1995), and the oestrogenic compound methoxichlor, during gonadal sex determination (Anway *et al.* 2005; Anway and Skinner 2006) in rats. It has been observed that embryonic exposure of rats to the vinclozolin induced in adult male offspring a number of disease states or tissue abnormalities including prostate disease, kidney disease, immune system and blood abnormalities, testis abnormalities, and tumor development. Interestingly, these defects were found to be transgenerational up to the fourth generation likely due to a permanent altered DNA methylation of the male germ-line (Anway *et al.* 2005; Anway *et al.* 2006a; Anway *et al.* 2006b). In contrast, in the females, only milder pathologies with regard to reproductive development (e.g., uterine bleeding and mammary-gland tumours) have recently been reported in F2 and F3 offspring when both parents were exposed to vinclozolin (Nilsson *et al.* 2008). The reasons for this lower penetrance in females may be related to the timing of the exposure and to the fact that establishment of genomic imprinting varies between males and females. In fact, to be

most effective, the treatment for females should include in utero as well as early postnatal life, when the epigenetic reprogramming of female germ cells takes place.

Recently, other EDs were also reported to cause transgenerational epigenetic effects. For example, perinatal exposure to Bisphenol A (BPA) can induce gene expression alterations in testicular steroid receptor co-regulators up to the third generation (Salian *et al.* 2009). More recently, transgenerational epigenetic effects of phthalates in the gonad and dioxins in the uterus were also reported (Kim 2009; Osteen 2009). Finally, one study in rats has reported intergenerational effects (up to the second generation) of maternal exposure to PCB in mammals (Steinberg *et al.* 2008).

AIM OF THE STUDY

Aim of the present study was to evaluate, in mice given DEHP or PCBs 101+118 throughout pregnancy and lactation, the effects on the reproductive function in offspring once they reach adult age, and to investigate the transmission of the observed effects through subsequent generations, along the female lineage.

To address this objective, F0 dams were exposed to DEHP or PCBs from conception to weaning and examined the effects on reproductive performance in male and female offspring of F1, F2 and F3 generation. Determination of reproductive and developmental endpoints, such as ano-genital distance, physical and functional development and gonadal morphology were included.

Dams were exposed until the end of lactation in order to cover the complete time window of reproductive system development in the mouse, which occurs largely in post-natal period, while in other mammals, including human, reproductive organs' development is completed *in utero* (Fig 5).

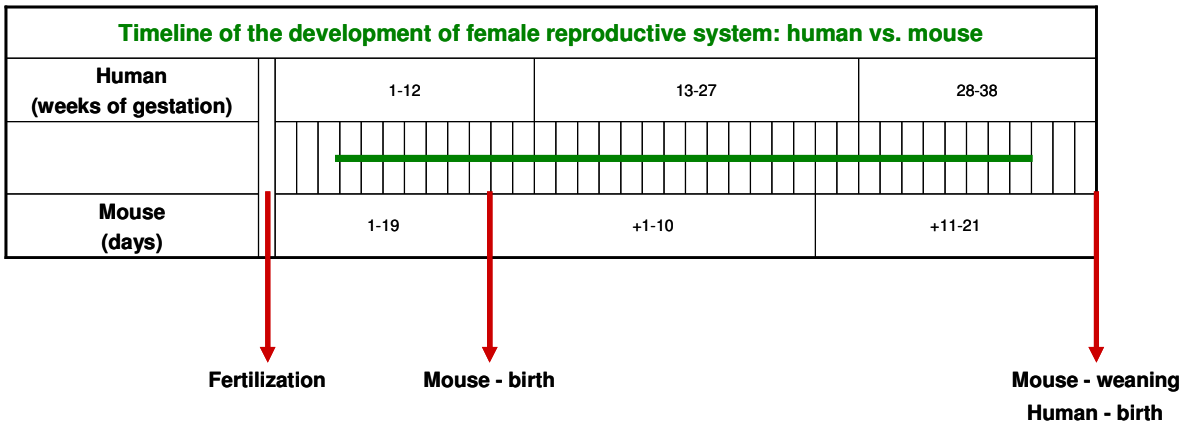

Figure 5 Comparison of the developmental stages between human and mouse female reproductive system.

EDs analyzed in the present study have been chosen for their ubiquitous distribution in the environment and for the health risk related to human exposure. Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used phthalate with a production of one to four million tons per year, which makes it one of the most widespread environmental contaminants worldwide (Akingbemi *et al.* 2001; McKee *et al.* 2004). As a result, the general population is widely and continuously exposed to this compound which, therefore, pose significant public health concerns on account of its endocrine disrupting activity (Silva *et al.* 2004; Wittassek and Angerer 2008). The mixture of the two PCBs congeners, was selected because: i) they represent a high proportion of the total PCB burden in biological samples (both PCB 101 and 118 are part of the so called ICES 7 and ii) represent approximately 65% of the total PCBs in biological samples (Bernhoft *et al.* 1997; Cerna *et al.* 2008; Connor *et al.* 1997; ICES 1992; Shen *et al.* 2009). The proportions of individual congeners vary between matrices but, on average, PCB 101 and 118 contribute in a ratio of 1:1 to the sum of the seven indicators (VKM 2008). It is also noteworthy to notice that PCB 101 is a di-ortho substituted PCB and, therefore, acts as typical non-coplanar PCB, whereas PCB 118, being a mono-ortho substituted PCB, is able to assume a partial coplanar conformation and therefore may share some effects with non-ortho PCBs (Fig 6).

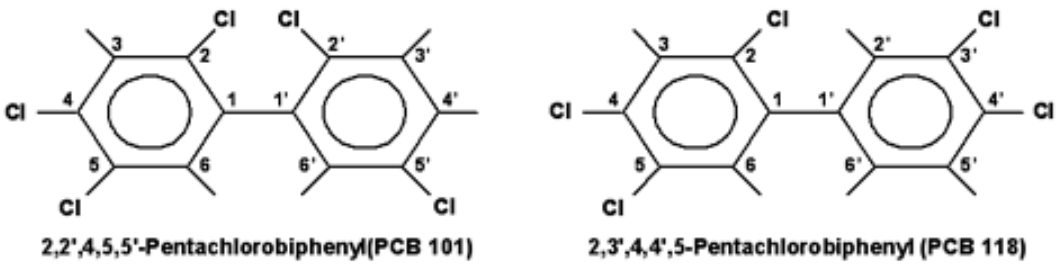

Figure 6 Structural formula of PCB 101 (2,2',4,5,5'-Pentachlorobiphenyl – CAS # 37680-73-2) and PCB 118 (2,3',4,4',5-Pentachlorobiphenyl – CAS # 31508-00-6)

Finally, previous studies in an ovine model, have shown that DEHP and both PCBs 101 and 118 preferentially accumulate in the offspring compared to their dams (Rhind *et al.* 2009; Rhind *et al.* 2010), and therefore their effects may be particularly important during critical developmental stages.

Administration via food and dosages for both DEHP and PCBs were chosen for their relevance to human exposure. The dose range was selected to overlap PCB concentrations reported in women in industrialized countries (Kavlock *et al.* 2002); (WHO 1996). See details given in Material and Methods.

***Trangenerational approach**:* A substantial distinction has to be made between intergenerational transmission involving direct exposure to the environmental factor, and transgenerational effects involving germ-line transmission without direct exposure of the affected generation (Skinner 2008). Therefore, for the transgenerational inheritance of environmental effects, these changes must be maintained in at least the F3 generation when an embryonic exposure is involved. In fact, exposure of the F0 gestating female not only exposes developing F1 but also F2 generation germ-line present in the F1 fetus which imply that effects observed can be defined as "multigenerational". F3 animals are the first which did not experience any direct exposure therefore observed effects are "transgenerational" (Fig 7).

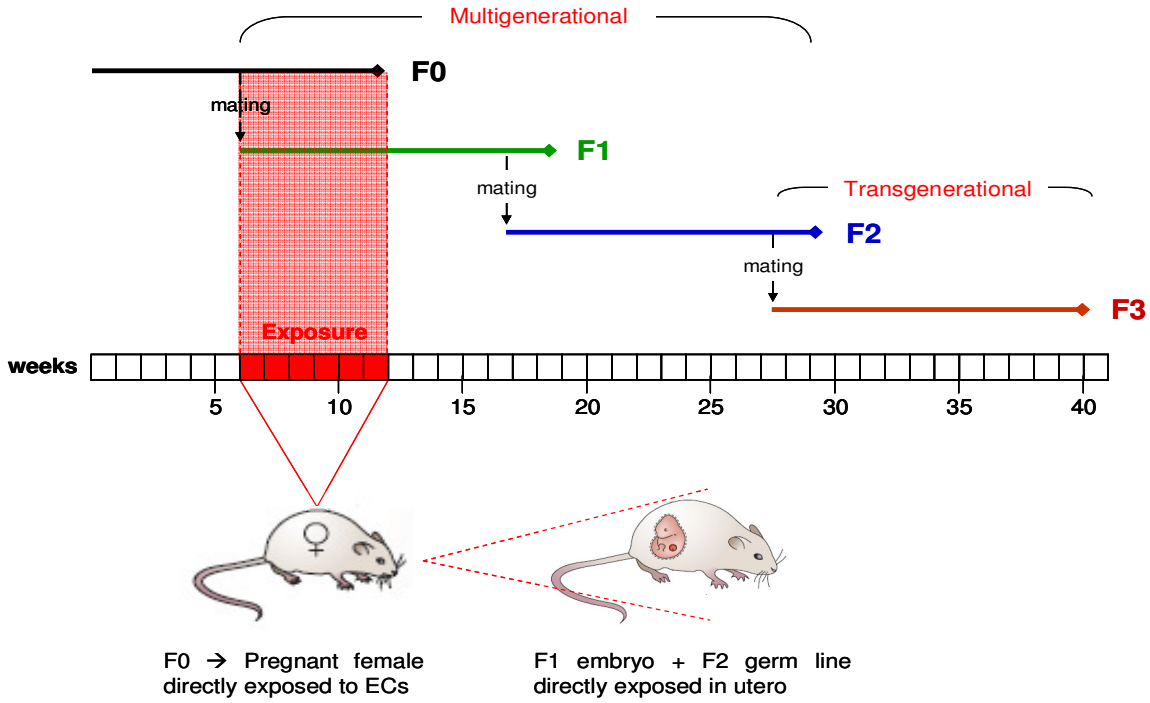

Figure 7 Schematic of the experimental approach with focus on multigenerational and transgenerational phenomena and direct exposure of the F0 mother, F1 embryo, and F2 germ-line. The F3 generation is the first without direct exposure.

MATERIALS AND METHODS

Animals

The CD-1 mouse strain was selected due to its large litter size, high fecundity and robustness, all of which are useful traits in reproductive studies and embryology.

Virgin female, five-weeks old, CD-1 mice were purchased from Charles River (Calco, Italy) and allow acclimatize for 2 weeks. Animals were maintained in the animal facilities of the Dept. of Animal Pathology and Health, Faculty of Veterinary Medicine, University of Milan, under controlled conditions (23 ± 1 °C, 12 h light/dark cycle). Standard pellet food (4RF21, Charles River) and tap water were available *ad libitum*. Groups of two to three female mice were mated with one male overnight and the day of vaginal plug was considered as day 0.5 of gestation (day post coitum - dpc +0.5). The pregnant females were randomly assigned among the treatment groups and housed individually in type II cages with stainless steel covers and hard wood shavings as bedding. Care and experimental procedures with mice were in accordance with National Regulations and were approved by the University of Milano's ethical committee.

Dose and treatments

DEHP (Sigma-Aldrich, Hamburg, Germany) or PCBs 101+118 mixture [LGC Standards GmbH (Wesel, Germany) and certified 99.8% pure] were diluted in commercial sunflower oil and sent to a manufacturer of special and customized animal diets (Altromin, Lage, Germany), to prepare both PCB and vehicle-treated chow. The amount of ED added to the chow in order to obtain the desired doses was calculated on the basis of the mean daily food intake of mice. This was assessed in a preliminary study, under the same

physiological conditions, and confirmed by literature (Johnson *et al.* 2001). Each batch of diet was tested before use in an accredited laboratory (SGS laboratory GmBH, Hamburg, Germany). Pregnant mice were given diets formulated to contain known amounts of ED or vehicle-containing diet from dpc 0 throughout lactation until weaning (post-natal day 21 – PND 21). Two to three pregnant mice were randomly assigned among the groups, and the experiment replicated at least three times (total 7-10 dams/treatment).

DEHP: The amount of DEHP added to the chow in order to obtain the desired mg/kg/day doses (0, 0.05, 5 and 500 mg DEHP/kg/day) was calculated based on the mean daily food intake of CD1 mice as above described. Therefore, the chow was dosed by the concentrations of 0.2857, 28.57 and 2857.0 mg/kg/food in order to ensure a mean daily intake of 0.05, 5 and 500 mg/kg/day, respectively, for the three experimental groups. The dose range was selected considering as reference value an amount close to the estimated daily intake of the general population (0.058 mg/kg/day) as reported by Kavlok et al. (Kavlock *et al.* 2002). Because of the scant data on mice the highest dose was based on data reported for rats. Therefore, the two highest doses were calculated by applying a factor of 100 so that the largest (500 mg/kg/day) was known to induce reproductive adverse effects in rat offspring without causing overt maternal toxicity (Moore *et al.* 2001).

PCBs: The two congeners, in a proportion of 1:1, were diluted in commercial sunflower oil and sent to a manufacturer of special and customized animal diets (Altromin, Lage, Germany) to prepare both PCB and vehicle treated chow. The amount of PCB mixture added to the chow in order to obtain the desired doses (0, 1, 10 and 100 μg PCB/kg/day) was calculated on the basis of the mean daily food intake of mice. The dose range was selected to overlap PCB concentrations reported in the breast milk of women in industrialized countries (19 ng/g/fat of PCB 118) (WHO 1996). The average daily PCB intake of the dams has been calculated based on the procedures proposed by US EPA (US-

EPA 2000). This approach assumes that the concentration in breast milk fat is the same as in maternal fat; the estimated intake of PCBs is calculated as follows: Daily maternal intake = (C_{milk} x maternal fat content). Considering that approximately 20% of the body weight of an adult female mouse is composed of adipose tissue (Kuriyama and Chahoud 2004), such a concentration is achieved by a daily oral dose of 3.8 µg/kg bodyweight. Therefore, the range of concentrations chosen for the present study (1 to 100 µg/kg/day) represented doses estimated to represent between 0.3 and 30x the mean concentration to which human infants are exposed.

F0 reproductive outcome

Dams and lactating offspring were examined daily for clinical signs of toxicity and during treatment dam body weights were recorded twice weekly. At PND 21 dams were sacrificed by CO_2 inhalation for collection of organs. Variables including litter size, sex ratio, pup weight, and the number of viable pups were also assessed. The liver, ovaries and uterus were removed, weighed and snap frozen in liquid nitrogen for later analyses.

To determine DEHP or PCBs tissue content, the digestive tract, skin and head were removed from treated animals and the remaining whole-animal body, including liver and fat, was wrapped in aluminium foil, freeze-dried and stored at -20 °C until it was analysed.

To evaluate post-implantation losses, an additional group of 15 dams/treatment was exposed to treated diet from dpc +0.5 and sacrificed at specific timepoints during pregnancy (dpc +9.5, +10.5, +11.5, +13.5, and +15.5). Fetuses and placenta morphology were macroscopically compared between groups.

F1 offspring data

At PND 21 all pups were sexed and body weight was recorded. At least two offspring per litter were sacrificed. The digestive tract, skin and head were removed and the remaining whole-animal body was wrapped in aluminium foil, freeze-dried and stored at -20 °C until analysis for PCBs tissue content.

Remaining male and female pups from each litter were housed in separated groups for further 3 or 9 weeks (PND 42 or PND 84), according to the experimental design. Standard pellet food (Charles River 4RF21) and tap water were available *ad libitum*.

At sexual maturity, at least three animals of each sex per litter were randomly selected for measurement of body weight, and ano-genital distance (AGD), and for autopsy. Animals were sacrificed by CO_2 inhalation followed by cervical dislocation and AGD (defined as the distance between the centre of the anus and the basis of the genital bud) was measured using a manual caliper by a single investigator. The animals were carefully handled to avoid variation in the measurement due to stretching of the perineal region. AGD data were analysed by the calculated AGD index, defined as AGD divided by the cube root of the body weight (Gallavan *et al.* 1999). In males, external genitalia were examined for malformations and testicular position was recorded after opening the abdominal cavity. Pituitaries and reproductive organs in both genders were removed, weighed and the mean weight was used in subsequent analyses. All organs that had a significant correlation with body weight were adjusted for body weight. Thereafter, collected organs were snap frozen in liquid nitrogen, or formalin- or bouin-fixed for later analyses.

Intergenerational study

To study the transmission over multiple generations of DEHP and PCBs effects, at least seven F1 females from different litters were randomly selected and subsequently mated with CD-1 non-exposed males of proven fertility, in order to obtain F2 offspring. F2 offspring were analyzed as described for F1 and at the same ages. Furthermore, at least seven F2 females from different litters were randomly chosen and mated as described for F1. The experiment ended when F3 offspring reached adult age (PND 84).

Sperm collection and dead/live ratio

Sperm were obtained from the cauda epididymis of adult male offspring. Both cauda were dissected out from the body and transferred into 500 µl of Whittingham medium previously equilibrated (37°C at 5% CO2 in air). Sperm were passively released into the culture medium by puncturing 3-4 times the cauda with a 27G needle. A part of the samples were diluted (1:100) with water and used for sperm count in a Neubauer chamber. A part of the samples were diluted (1:20) with 0.9% NaCl, and were stained by modified Kovács-Foote method (Kovacs and Foote 1992). Briefly, one drop diluted samples were mixed on a microscope slide with one drop of isoosmotic 0.2% trypan blue (Sigma T-8154) and smeared with the edge of another slide. After vertical air-drying the slides were fixed for two minutes with fixative solution [86 ml of 1 N HCL plus 14 ml of 37% formaldehyde solution and 0.2 g neutral red (Fluka, 72210)] and then rinsed with tap and distilled water. Finally, the slides were dried in air, covered with Eukitt (Fluka, 03989) and coverslip. Stained smears were evaluated by light microscopy at 400x magnification. The status of the head and tail of at least 100 spermatozoa was classified in each smear. Sperm

with white or light pink heads (intact plasma membrane) were classified as alive, conversely, sperm with black to dark-purple heads (damaged membrane) were classified as dead.

In vitro fertilization and embryo culture

Females were superovulated by intraperitoneal (i.p.) injection of 3.5 IU Folligon (PMSG, Intervet International, Netherlands), followed 48 h later by an i.p. injection of 5 IU Chorulon (hCG, Intervet).

Spermatozoa were collected as described above and capacitated for 60 min in Whittingham medium (37°C at 5% CO2 in air). Fourteen hours post-hCG, cumulus oocyte complexes were recovered from oviducts in M2 medium (Sigma-Aldrich). After rinsing in Whittingham medium, cumulus-oocyte complexes were inseminated with $2*10^6$ capacitated spermatozoa. Putative fertilized eggs (6 h post-insemination) were then transferred to 250 µl drops of M16 medium (Sigma-Aldrich) covered with paraffin oil and incubated at 37°C at 5% CO2 in air for further 96 h. Cleavage and blastocyst rate were assessed at 24 h and 96 h post-insemination, respectively.

Histological analysis

Ovary: Ovaries collected from F1, F2 and F3 female offspring were fixed 1 in 10% neutral buffered formalin and subsequently embedded in paraffin. Specimens were serially sectioned (8 µm), mounted on glass slides and stained with hematoxylin and eosin (HE) according to standard procedures. Follicle counts were performed as described by Tomic et al. (Tomic *et al.* 2002). Briefly, a stratified sample consisting of ten sections was used to estimate the number of different follicles class per ovary. The selected sections from each

ovary were randomized and follicles were counted on the entire section. Only follicles with a visible nucleolus in the oocyte were counted to avoid counting follicles twice. The number of follicles in the marked sections was then multiplied by 10 (because every 10^{th} section was analyzed) and subsequently by 8 (accounting for section thickness) to obtain an estimate of the total number in each ovary. Follicles were categorized according to Flaws et al. (Flaws *et al.* 2001). They were classified as i) primordial when they contained an intact oocyte surrounded by a one-layer ring of fusiform granulosa cells, as ii) primary if they consisted of an oocyte and a single layer of cuboidal granulosa cells, iii) secondary if they contained an oocyte and more than one layer of granulosa cells, iv) tertiary if they contained more than one layer of granulosa cells and an antral space, and v) end-stage atretic follicles if containing zona pellucida remnants. All sections were evaluated "blind", without knowledge of the treatment group of the animals.

Testis: Testes collected from F1, F2 and F3 animals were fixed in Bouins fixative, dehydrated and embedded in paraffin. Then, 5-µm serial microscopic sections were prepared and at least 6 slides from each testis were stained with hematoxylin and eosin for histological assessment. In cross sections of randomly selected tubular profiles that were round in shape, the diameters of the tubules and epithelium thickness were measured using light microscopy, accordingly to the methods of Koruji et al. (Koruji *et al.* 2008). Furthermore, the percentages of each of 3 types of tubules were determined: i) normal seminiferous tubule with sperm (type I), ii) normal seminiferous tubules without sperm (type II) and iii) depleted seminiferous tubule (type III). Each parameter measurement was based on examination of at least 45 fields in histological sections from at least 6 testes per group.

RNA Isolation and Reverse-Transcription PCR

Total RNA was isolated from one testis or ovary of all necroscopized animals using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was checked for integrity and DNA contamination using an ultraviolet (UV) spectrophotometer and 1.3% agarose gel electrophoresis. 1 µg of total RNA extracted from each sample was used to synthesize the cDNA using a SuperScript kit (Invitrogen). The RT reaction was carried out at 42 ºC for 1 h, and terminated by heating at 94ºC for 2 min.

Polyadenylated [poly(A)+] RNA from pituitaries and pre-implantation blastocysts was extracted using Dynabeads mRNA DIRECT kit (Deutsche Dynal, Hamburg, Germany). Briefly, single pituitaries were lysed for 10 min at room temperature in 200 µl lysis buffer [100mmol Tris-HCl (pH 8.0), 500 mmol LiCl, 10 mmol EDTA, 1% (wt/vol) sodium dodecyl sulfate, and 5 mmol dithiothreitol] while a pool of six blastocysts were lysed using 50 µl of lysis buffer. After lysis, 10 µl prewashed dynabeads-oligo (deoxythymidine) were pipetted into the tube, and binding of poly(A)+ RNAs to oligo(deoxythymidine) was allowed for 5 min at room temperature. The beads were then separated with a Dynal MPC-E magnetic separator and washed twice with 50 µl washing buffer A [10 mmol Tris-HCl (ph 8.0), 0.15 mmol LiCl, 1 mmol EDTA, and 0.1% (wt/vol) sodium dodecyl sulfate] and three times with 50 µl washing buffer B [10 mmol Tris-HCl (ph8.0), 0.15mm LiCl, and 1 mmol EDTA]. Poly(A)+ RNAs were then eluted from the beads by incubation in 11 µl diethylpyrocarbonate-treated sterile water at 65 °C for 2 min. Aliquots were immediately used for RT using the PCR Core Kit (Perkin Elmer, Wellelsey, MA), using 2.5 µmol random hexamers to obtain the widest array of cDNAs. The RT reaction was carried out in a final volume of 20 µl at 25 C for 10 min and 42 C for 1h, followed by a denaturation step at 99 C for 5 min and immediate cooling on ice.

Table 2 lists the primers and PCR conditions for the genes analyzed..In gonads, analyzed transcripts were chosen in the following categories: a) Genes related to response to pituitary gonadotropins (FSH-R, LH-R); b) Genes related to oocyte quality and follicle health (GDF9, BMP15, pTEN); c) Genes related to steroidogenesis (StAR, Cyp11, Cyp17, Cyp19) and estrogen signalling (ER and PgR). In blastocysts specific mRNAs were selected for their role in the embryonic development and trophoblast differentiation and implantation. Finally, in pituitaries, the expression profile of mRNAs for pituitary hormones related to ovarian function (*fsh, lh* and *gh*) was investigated.

For each set of primers, the optimal cycle number at which the transcript was amplified exponentially was established running a linear cycle series and the number of PCR cycles was kept within this range. Approximately 1 µl cDNA per sample was used for amplification. The cDNA fragments were generated by initial denaturation at 94°C for 3 min. The PCR products were separated by electrophoresis on a 1.3 % agarose gel and detected under UV light. To normalize signals from different RNA samples, GAPDH transcripts were co-amplified as an internal standard. Quantitative expression was analyzed with Quantity One software using the Volume analysis Report of Quantity One software (Bio-Rad, Hercules, CA, USA).

Table 2 Primers list and PCR conditions for gene expression analysis

Gene	Accession Nr	Primers	Annealing T°	Product size
lhr	NM013582	F: TCTACCTGCTGCTCATTGCCTC R: AAGGCAGCTGAGATGGCAAAG	57	553
fshr	NM013523	F: ATGTGTAACCTCGCCTTTGCTG R: AACATACAGCTGCGACAAAGGG	57	393
star	BC082283	F: GAAGGAAAGCCAGCAGGAGAAC R: CTGCGATAGGACCTGGTTGATG	58	496
cyp11	BC068264	F: CAACCTTTCCTGAGCCCTACG R: AGGACGATTCGGTCTTTCTTCC	57	404
cyp17a1	AY594330	F: ACGGTGGGAGACATCTTTGGG R: CCTTCGGGATGGCAAACTCTC	57	283
cyp19a1	NM007810	F: CCTCTGGATACTCTGCGACGAG R: CGAATGGTGGAAGTTTGTGTGG	56	508
lhbeta	NM008497	F: CATCACCTTCACCACCAGCATC R: GAGGTCACAGGCCATTGGTTG	60	259
fshbeta	NM008045	F: CTGCCATAGCTGTGAATTGACC R: CACAGCCAGGCAATCTTACG	55	203
gh	NM008117	F: TCGAGCGTGCCTACATTCCC R: AGCGGCGACACTTCATGACC	59	456
pgr	NM008829	F: GATGAGCCTGATGGTGTTTGGC R: GGGCAACTGGGCAGCAATAAC	57	490
gapdh	NM008084	F: TCACCATCTTCCAGGAGCG R: CTGCTTCACCACCTTCTTGA	57	572
gdf-9	NM008110	F: GGCACTTCCCAGCAACTTCC R: GGTGACTTCTGCTGGGTTTGG	58	326
bmp15	NM009757	F: GCAAGGAGATGAAGCAATGGC R: GAAGCGGAGGCGAAGAACAC	57	458
pten	NM_008960	F: TGGAAAGGGACGGACTGGTG R: CCGCCACTGAACATTGGAATAG	55	249
nanog	NM028016	F: TGCCAGGAAGCAGAAGATG R: TTATGGAGCGGAGCAGCATTC	57	471
oct4	NM013633	F: ATCACTCACATCGCCAATCAGC R: CAGAGCAGTGACGGGAACAGAG	58	279
cdx2	NM007673	F: GAAACCTGTGCGAGTGGATGC R: TGCTGCTGCTGCTTCTTCTTG	58	265
eomes	NM001164789	F: GTGGAAGTGACAGAGGACGGTG R: GAGGCAAAGTGTTGACAAAGGG	57	250
lif	NM008501	F: TGGCAACGGGACAGAGAAGAC R: ACGGTACTTGTTGCACAGACGG	58	202
gata3	NM008091	F: CTGGAGGAGGAACGCTAATGG R: TGTGGCTGGAGTGGCTGAAG	57	263

Electrophoresis and immunoblot analysis

Protein from ovaries and testis were extracted using RIPA buffer additioned with proteinase and phosphatase cocktail (cat # P 2714 and cat # P5726, respectively). The lysates were mixed 1:1 with 2X Laemmli sample buffer and heated to 90 °C for 5 minutes, then centrifuged at 13,000 rpm for 2 min. Immunoblot analysis was then performed as described previously (Pocar *et al.* 2004). Cyp191a1 was detected using a goat polyclonal anti-Cyp19 antibody (SC-1425; Santa Cruz Biotech., USA). The secondary antibody used for the detection of Cyp191a1-primary antibody complex was horseradish peroxidase-conjugated bovine anti-goat IgG (SC-2378; Santa Cruz Biotech., USA). Proteins on the membranes were visualized using the WestPico ECL detection system (Pierce Chemical Co., IL, USA). After the initial analysis, the membranes were washed in a stripping buffer (2% SDS, 100 mM beta-mercaptoethanol, 50 mM Tris, pH 6.8) to remove bound antibodies and were re-probed with a monoclonal anti-β-actin antibody (cat # A1978). The secondary antibody used for the detection of β-actin-primary antibody complex was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce Chemical Co.). Protein content was analyzed in each blot (from three different experiments) using the Volume analysis Report of Quantity One software (Bio-Rad).

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software 5.03, San Diego CA). The mean number of pups per litter was calculated from at least six different mating pairs per treatment group, and mean body and organ weights were calculated for at least 6 different litters per treatment group. Differences between the means for litter size, AGD, organ weight, semen parameters and gene expression were tested by D'Agostino and Pearson normality test in order to confirm Gaussian distribution and then examined by

one-way ANOVA, with statistical significance at P ≤0.05. The mean numbers of follicles per ovary were calculated using ovaries from at least five different animals. Seminiferous tubule morphology was assessed using testes from at least six different animals. Differences between the means were examined by one-way ANOVA, with statistical significance assigned at $P \leq 0.05$. When ANOVA gave a significant P value, the Newmann-Keuls' test was used in the *post hoc* analysis.

Data *for in vitro* embryo culture were analyzed by binary logistic regression. Controls were taken as the reference group. Experiments were replicated at least three times, and each replicate was fitted as a factor. The log likelihood ratio statistic was used to detect between-treatment differences and significance was set at P<0.05.

Effects of in utero and lactational exposure to DEHP on reproductive health of F1 to F3 adult offspring

DEHP tissue accumulation

Concentrations of DEHP in tissues from dams and F1 weaned offspring were analyzed at The James Hutton Institute, Aberdeen, Scotland (UK), according to Pocar et al. (Pocar *et al.* 2011).

Data indicate that, an approximately 9 fold higher amount of DEHP was found in tissues from dams exposed to 500 mg DEHP/kg/day dose. In the 0.05 and the 5 mg group, despite a tendency in higher values compared to controls, differences in tissue concentration did not reach statistical significance. In weaned offspring, at PND 21, no statistical differences between exposure groups or compared with dams, were observed (Fig 8).

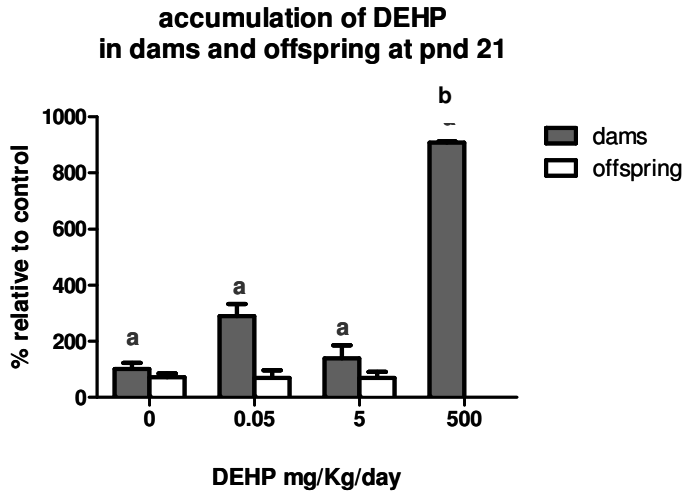

Figure 8 DEHP accumulation analysis in tissues from dams (F0 - grey bars) and F1 weaned offspring (PND 21 – white bars). Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p<0.05$).

DEHP disturbs maternal reproductive outcome

Reproductive outcome of F0 dams are shown in Table 3. Exposure to 500 mg/Kg/day resulted in dramatic increase in post-implantation losses, with only one out of 10 females able to deliver. Necroscopy of dams at specific timepoints during pregnancy indicated that embryonic vesicles appear to be macroscopically normal until dpc +9.5. Between dpc +10.5 and +11.5 resorption starts and fetuses and fetal envelopes rapidly degenerate. At dpc +15.5 only hemorragic remnants can be seen in the uterine cavity (Fig 9).

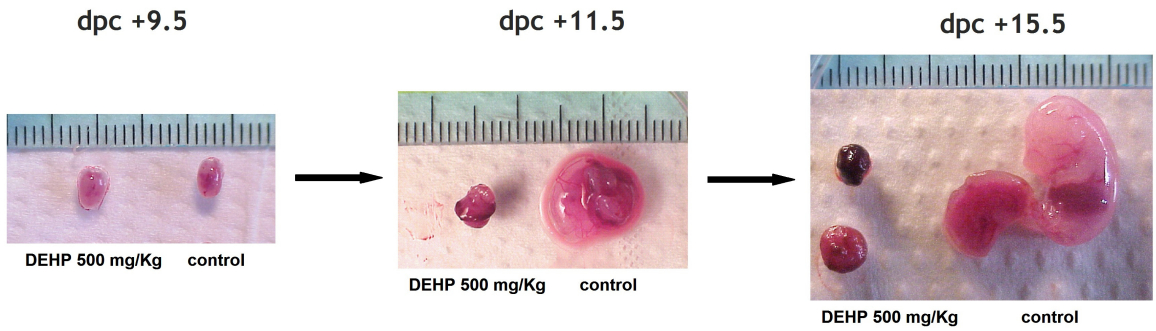

Figure 9 Morphological abnormalities of 500 mg DEHP/Kg/day treated foetuses and extraembryonic tissues taken at dpc + 9.5, 11.5, and 15.5 compared with no exposure to DEHP at the same timepoint.

No signs of maternal toxicity were observed and at dpc +19.5 (time of predicted delivery), and uterus recovered almost completely. Sometimes implantation sites were still evident. In the 5 mg/Kg/day group a slightly reduced mean litter size was observed compared to control. However, variability in litter size between dams meant that the percentage of post-implantation losses did not correlate significantly with the observed reduction in mean litter size. No differences in mean litter size and post-implantation losses were observed in the 0.05 mg/Kg/day compared to control. Offspring sex ratio and

viability index were unaffected by treatments and there were no adverse clinical findings in the new-born pups. DEHP treated dams showed a dose-dependent increase in mean liver weight compared to control. DEHP treatment did not affect ovary and uterus weight of dams.

Table 3 Reproductive outcome and organ weights of dams treated with DEHP through pregnancy and lactation

	DEHP mg/Kg/day			
	0	**0.05**	**5**	**500**
Nr. Of dams	10	7	7	10
Pregnancy at term (%)	10/10 (100) [a]	6/7 (85.7) [a]	7/7 (100) [a]	1/10 (10) [b]
Abortion/miscarriage	0/10 [a]	1/7 [a]	0/7 [a]	9/10 [b]
Litter size	12.9 ± 0.7	15.0 ± 1.5	10.6 ± 1.5	-[1]
Viability index (%)	98.5 ± 1.5	98.3 ± 1.4	100.0 ± 0.0	-
Liver weight (% on BW)	8.5 ± 0.2 [a]	9.4 ± 1.2 [ab]	10.0 ± 0.8 [b]	-
Ovary weight (% on BW)	0.019 ± 0.02	0.018 ± 0.02	0.021 ± 0.02	-
Uterus weight (% on BW)	0.48 ± 0.05	0.53 ± 0.07	0.55 ± 0.09	-

Note: Values are means ± SE
a, b: different superscripts indicate statistical differences for $P \leq 0.05$
[1] From a total of 10 pregnancies only one reached term and one single male pup was born, 500 mg/Kg/day group was excluded from the analysis of the parameters reported in this table.
Viability index: (number of pups at weaning/number of pups alive at PND 2) * 100

DEHP affects morphological and reproductive indices in offspring

No adverse effects on clinical findings were observed in pups over the three generations analyzed neither before nor after weaning in any of the DEHP-treated groups compared to control.

Litter data of F1, F2 and F3 generations at weaning (PND 21) are shown in Table 4. Pre- and perinatal treatment with 0.05 and 5 mg/Kg/day DEHP significantly decreased body weight of both female and male mice either at PND 21. At weaning, both male and female DEHP treated offspring were 20-25% lighter than control animals, being the males in the 5 mg/Kg/day the most affected group. In F2 and F3 male and female offspring no statistical differences were observed for any of the parameters analyzed.

Table 4 Litter data of F1, F2 and F3 generations at weaning (PND 21)

		DEHP mg/Kg/day		
		0	**0.05**	**5**
F1 generation				
No. of animals		66	51	55
Sex ratio (females : males %)		43.3 : 56.7	48.7 : 51.3	50.9 : 49.1
Body weight (g)	*males*	9.9 ± 0.3^{a}	8.1 ± 0.3^{b}	7.5 ± 0.4^{c}
	females	9.5 ± 0.5^{a}	7.2 ± 0.6^{b}	7.8 ± 0.2^{b}
F2 generation				
No. of animals		50	48	41
Sex ratio (females : males %)		54.8 : 45.2	43.3 : 56.7	55.0 : 44.0
Body weight (g)	*males*	12.8 ± 0.6	11.8 ± 0.6	13.1 ± 0.5
	females	11.9 ± 0.5	11.7 ± 0.3	11.9 ± 0.5
F3 generation				
No. of animals		41	41	45
Sex ratio (females : males %)		45.2 : 54.8	56.7 : 43.3	55.0 : 45.0
Body weight (g)	*males*	12.78 ± 0.6	12.6 ± 0.9	14.3 ± 0.5
	females	13.0 ± 0.4	12.9 ± 0.5	14.4 ± 0.7

Note: Values are means ± SE
a, b, c: different superscripts indicate statistical differences for $P \leq 0.05$

Morphological indices of male and female adult offspring are shown in Table 5. In F1, the decrease in body weight observed at weaning persisted up to 6 weeks of age, when treated offspring still weighted between 6% and 14% less than control animals of the same gender. Abdominal fat weight was significantly reduced in females, showing the 0.05 mg/Kg/day and the 5 mg/Kg/day group 41% and 30% reduction, respectively, compared to controls. No significant differences were seen in male adiposity.

Liver weight was unaffected by DEHP treatments in any of the doses or gender analyzed.

In F2 and F3 offspring there were no statistical differences in any parameter investigated for both genders.

Reproductive indices for both male and female adult offspring (PND 42) are shown in Table 6. In F1 female offspring, ovarian weight was significantly higher in both the 0.05 and the 5 mg/Kg/day group compared to controls. Ovarian weight was increased of about 45% and 35% in the 0.05 mg/Kg/day and 5 mg/Kg/day, respectively. In contrast, in both F2 and F3 females, a significantly reduced ovarian weight was observed in the 0.05 mg/Kg/day group at adult age. Furthermore, in the F3, the reduced weight of the ovaries was observed also in the 5 mg/Kg/day group. Uterus weights were unaffected by treatment in any of the group investigated in the F1 and F3 generation. However, in the F2 females, relative uterus weight in the 0.05 mg/Kg/day group was significantly reduced.

In F1 male offspring, DEHP doses significantly decreased testes and seminal vesicles weight. Seminal vesicles in both dosage groups were about 20-25% lighter while testes from the 0.05 mg/Kg/day group show approximately a 13 % decreased weight.

AGD index were unaffected by DEHP treatments in any of the doses or gender analyzed.

In F2 and F3 male offspring there were no statistical differences in any of the parameter investigated.

Table 5 Morphological indices in male and female adult offspring (PND 42) in F1, F2 and F3 generations

	DEHP mg/Kg/day		
	0	**0.05**	**5**
F1 generation			
Nr of animals	66	51	55
		females	
Body Weight (g)	30.5 ± 0.5^{a}	28.4 ± 0.6^{b}	29.1 ± 0.9^{ab}
Liver weight (% on BW)	4.9 ± 0.2	5.1 ± 0.1	5.1 ± 0.2
Abdominal fat weight (% on BW)	2.3 ± 0.2^{a}	1.4 ± 0.3^{c}	1.7 ± 0.1^{b}
		males	
Body Weight (g)	32.9 ± 0.4^{a}	28.2 ± 0.8^{b}	30.0 ± 0.7^{b}
Liver weight (% on BW)	6.3 ± 0.2	6.0 ± 0.1	6.5 ± 0.1
Abdominal fat weight (% on BW)	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
F2 generation			
Nr of animals	50	48	41
		females	
Body Weight (g)	34.6 ± 1.2	37.8 ± 1.9	35.4 ± 1.4
Liver weight (% on BW)	4.5 ± 0.2	4.5 ± 0.6	4.4 ± 0.1
Abdominal fat weight (% on BW)	2.1 ± 0.3	2.8 ± 0.5	2.9 ± 0.5
		males	
Body Weight (g)	33.6 ± 0.8	33.9 ± 0.6	34.75 ± 0.7
Liver weight (% on BW)	5.2 ± 0.1	5.3 ± 0.2	5.1 ± 0.2
Abdominal fat weight (% on BW)	1.8 ± 0.1	1.9 ± 0.1	1.4 ± 0.1
F3 generation			
Nr of animals	41	41	45
		females	
Body Weight (g)	29.9 ± 0.8	31.09 ± 1.2	32.15 ± 0.8
Liver weight (% on BW)	4.8 ± 0.1	5.1 ± 0.1	4.7 ± 0.2
Abdominal fat weight (% on BW)	1.6 ± 0.2	1.7 ± 0.2	1.9 ± 0.2
		males	
Body Weight (g)	36.6 ± 0.6	37.6 ± 1.0	38.4 ± 0.7
Liver weight (% on BW)	5.2 ± 0.1	5.3 ± 0.2	5.1 ± 0.2
Abdominal fat weight (% on BW)	1.6 ± 0.2	1.7 ± 0.2	1.9 ± 0.2

Note: Values are means ± SE

a, b, c: different superscripts indicate statistical differences for $P \leq 0.05$

Table 6 Reproductive indices in male and female adult offspring (PND 42) in F1, F2 and F3 generations

	DEHP mg/Kg/day		
	0	**0.05**	**5**
F1 generation			
No. of animals examined	33	27	27
		females	
AGD [cm/BW^(1/3)]	0.23 ± 0.004	0.23 ± 0.005	0.22 ± 0.007
Ovary weight (g)	0.0063 ± 0.0002[a]	0.0083 ± 0.0002[c]	0.0071 ± 0.0002[b]
Uterus weight (g)	0.16 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
		males	
AGD [cm/BW^(1/3)]	0.53 ± 0.004	0.52 ± 0.005	0.52 ± 0.007
Testis weight (g)	0.086 ± 0.002[a]	0.075 ± 0.003[b]	0.089 ± 0.003[a]
Seminal vesicles weight (g)	0.179 ± 0.007[a]	0,143 ± 0.008[b]	0.132 ± 0.008[b]
F2 generation			
No. of animals examined	33	27	27
		females	
AGD [cm/BW^(1/3)]	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01
Ovary weight (g)	0.0102 ± 0.001 [a]	0.0078 ± 0.001 [b]	0.0111 ± 0.001 [a]
Uterus weight (g)	0.18 ± 0.03 [a]	0.12 ± 0.01 [b]	0.15 ± 0.03 [a]
		males	
AGD [cm/BW^(1/3)]	0.52 ± 0.02	0.52 ± 0.01	0.52 ± 0.02
Testis weight (g)	0.101 ± 0.002	0.096 ± 0.003	0.100 ± 0.002
Seminal vesicles weight (g)	0.233 ± 0.014	0.256 ± 0.019	0.247 ± 0.013
F3 generation			
No. of animals examined	33	27	27
		females	
AGD [cm/BW^(1/3)]	0.24 ± 0.01	0.23 ± 0.01	0.24 ± 0.01
Ovary weight (g)	0.0125 ± 0.001 [a]	0.0099 ± 0.001 [b]	0.0102 ± 0.001 [b]
Uterus weight (g)	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.02
		males	
AGD [cm/BW^(1/3)]	0.55 ± 0.01	0.52 ± 0.01	0.52 ± 0.01
Testis weight (g)	0.11 ± 0.03	0.12 ± 0.01	0.11 ± 0.03
Seminal vesicles weight (g)	0.30 ± 0.02	0.30 ± 0.01	0.31± 0.03

Note: Values are means ± SE
a, b, c: different superscripts indicate statistical differences for $P \leq 0.05$

Histological analysis of male and female gonads of F1, F2 and F3 generations

Results of the histopathological analysis of ovaries from F1, F2 and F3 adult offspring are shown in Fig 10. In ovaries from all generations investigated a significant decrease in the percent number of primordial follicles was observed in both the 0.05 and the 5 mg/Kg/day group. The percent number of growing pre-antral follicles (primary and secondary follicles) were significantly increased in both the 0.05 and the 5 mg/Kg/day group in F1 females, whereas only in the 0.05 mg/Kg/group in both the F2 and the F3 generation. Furthermore, in ovaries from the 0.05 mg/Kg/day a significant larger number of atretic follicles was observed in the F1 generation, whereas a decrease in percent number of large antral follicles compared to control was present in the F2. There was no statistically significant difference between the treatments or generation investigated in the total number of follicles.

In males, no histologically observable alterations were observed in testes and accessory glands morphology irrespective of treatment or generation investigated.

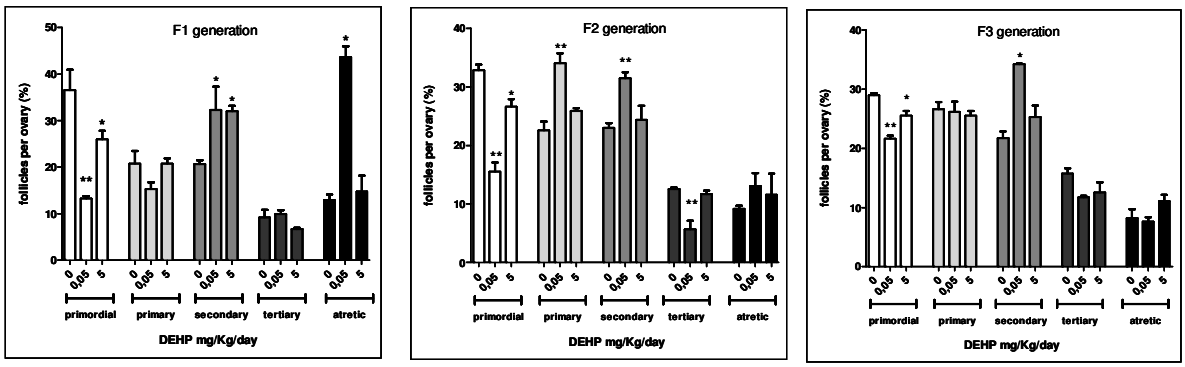

Figure 10: Histological analysis of F1-F2 and F3 ovaries. Percentages of follicles in each follicular stage in ovaries of the F1, F2 and F3 offspring. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p<0.0001$).

Gamete quality in both male and female offspring is affected by DEHP

Semen characteristics and in vitro developmental capacity in adult male offspring: Fig 11 shows semen characteristics of 6 week old offspring that were exposed to DEHP in utero and during lactation. Sperm concentration and viability, intended as membrane integrity, were significantly decreased following exposure to DEHP at both 0.05 and 5 mg/Kg/day doses as compared with vehicle. Semen recovered from treated animals was about 50% less concentrated than those from controls (DEHP 0: 5.9 * 10^6 ml^{-1} ± 0.8; DEHP 0.05: 2.8 * 10^6 ml^{-1} ± 0.2; DEHP 5: 2.9 * 10^6 ml^{-1} ± 0.4), and nearly 20% less viable (viable sperm % - DEHP 0: 71.3 ± 2.2; DEHP 0.05: 56.7 ± 5.3; DEHP 5: 57.1 ± 3.5).

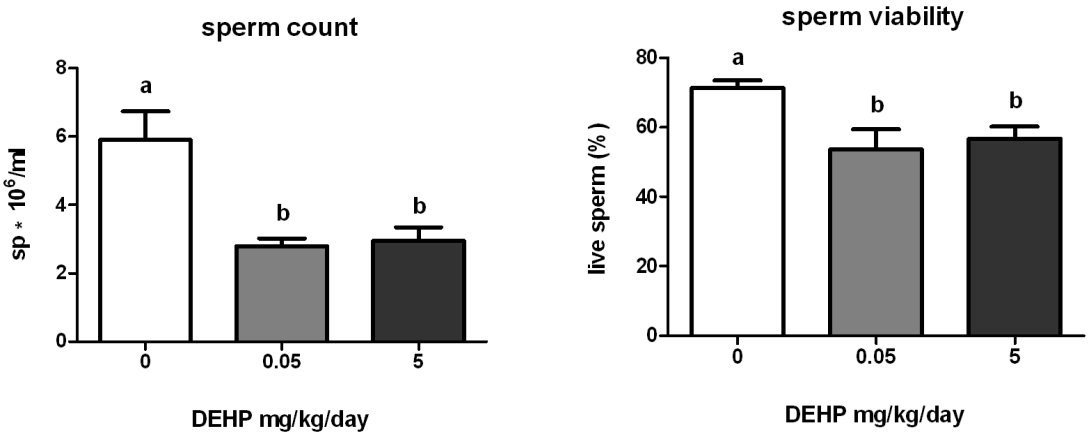

Figure 11: Counts and viability of epididymal caudal sperm from in utero and lactational DEHP treated animals. Different superscripts denote significant differences between columns ($p<0.05$).

DEHP exposure compromised sperm developmental capacity but not its fertilization capacity. In tests using oocytes (a total of 404) from untreated females and *in vitro* fertilization protocols, the sperm from both the 0.05 and the 5 mg/kg/day groups resulted in zygotes with the same ability to complete first mitotic division, but with a significantly reduced capacity to reach the blastocyst stage, compared to controls ($P \leq 0.05$; Table 7).

Table 7 Effect of pre- and perinatal DEHP exposure on developmental capacity of gametes from adult male offspring

	DEHP mg/kg/day		
	0	**0.05**	**5**
F1 Males			
No. of oocytes	162	138	140
Cleavage rate (%)	65.2 ± 9.3	65.0 ± 10.2	47.4 ± 18.3
Blastocyst rate (%)	43.9 ± 11.5 [a]	13.50 ± 6.5 [b]	4.4 ± 0.8 [b]

Note: Values are means ± SE
a, b: different superscripts indicate statistical differences for $P \leq 0.05$

In vitro oocyte developmental competence in adult female offspring from F1 to F3 generation: Data of in-vitro embryo production from oocytes derived from F1, F2 and F3 adult offspring are shown in Table 8. Developmental competence of female gametes was tested on a total of 909 in vivo matured oocytes. The oocytes recovered from the 0.05 mg/Kg/day group from all generations analyzed, when fertilized with gametes from untreated animals, produced embryos with a significantly reduced competence to complete the first mitotic division and to subsequently reach the blastocyst stage ($P<0.001$; Table 8). There was no significant difference in the total number of oocytes collected per animal.

Table 8 Effect of DEHP exposure on in vitro embryo production from oocytes derived from F1, F2 and F3 offspring

	DEHP mg/Kg/day		
	0	**0.05**	**5**
F1 females			
Oocytes/animal	35.20 ± 3.1	36.80 ± 2.3	32.80 ± 5.1
Cleavage rate (%)	59.63 ± 4.5 [a]	34.48 ± 4.2 [b]	63.61 ± 4.6 [a]
Blastocyst rate (%)	42.46 ± 5.6 [a]	9.03 ± 2.4 [b]	48.17 ± 3.6 [a]
F2 females			
Oocytes/animal	30.71 ± 3.3	27.00 ± 3.4	30.40 ± 4.6
Cleavage rate (%)	80.88 ± 6.9 [a]	55.46 ± 2.1 [b]	96.77 ± 1.9 [a]
Blastocyst rate (%)	74.46 ± 7.0 [a]	39.37 ± 3.1 [b]	79.92 ± 13.7 [a]
F3 females			
Oocytes/animal	26.00 ± 1.9	28.00 ± 3.9	28.00 ± 2.3
Cleavage rate (%)	82.09 ± 4.1 [a]	69.62 ± 1.9 [b]	84.38 ± 3.5 [a]
Blastocyst rate (%)	79.78 ± 4.5 [a]	26.91 ± 7.7 [b]	57.61 ± 10.6 [a]

Note: Values are means ± SE
a, b: different superscripts indicate statistical differences for $P \leq 0.05$

DEHP-induced alterations in gene expression profiles of adult offspring gonads and pituitaries

Expression profile of selected transcripts involved in the pituitary-gonadal crosstalk in adult male and female offspring: In F1, a significant down-regulation in *cyp19a1* transcript occurs in both ovaries (0.05 and 5 mg/Kg/day – Fig 12a) and testes (5 mg/Kg/day group – Fig 12b). Furthermore, in ovaries, the 5 mg/Kg/day dose caused a decrease in the expression level of *cyp17a1* transcript (Fig 12a). Gene expression for *cyp19a1* was confirmed by immunoblot analysis (Fig 12c). Transcript for progesterone receptor (*pgr*) was significantly down-regulated in both testes (5 mg/Kg/day) and ovaries (0.05 and 5 mg/Kg/day) (Fig 12d).

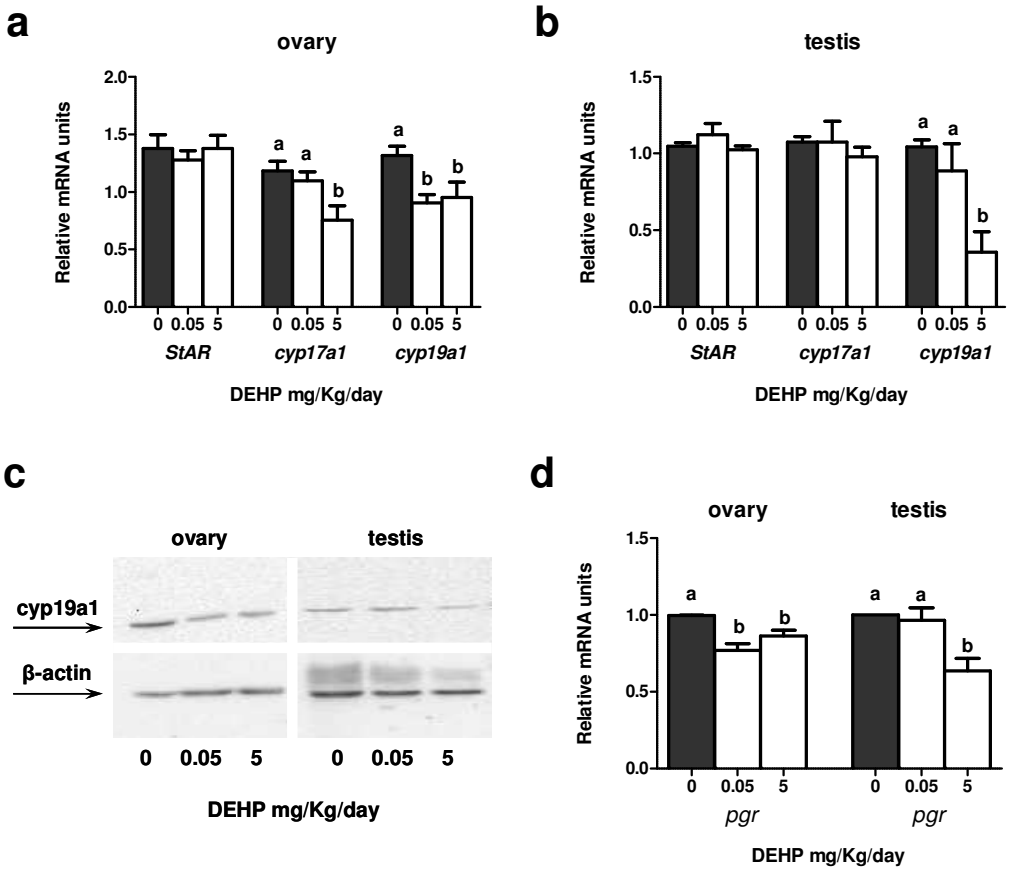

Figure 12: Effect of DEHP on the mRNA levels of target genes in the gonads of female and male mice offspring at PND 42 (a, b, d). Quantitative analysis of *StAR, cyp17a1, cyp19a1,* and *pgr* mRNA levels in the gonads exposed to DEHP during pregnancy throughout lactation. Each column represents the mean ± SE of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in Materials and Methods. Different superscript denote significant differences between columns ($p \leq 0.05$). (c) Representative immunoblot analysis of cyp19a1 and β-actin protein from total ovary and testis lysate of adult female and male mice offspring at PND 42.

In both ovaries and testes, the mRNA levels for gonadotropins' receptors, *fshr* and *lhr*, were significantly down-regulated in DEHP treated animals at all doses investigated (Fig 13a).

In the female pituitaries, a dose dependent increase on the expression of *lhβ* mRNA was observed in DEHP treated animals, whereas the expression of *fshβ* mRNA did not differ between groups (Fig 13b). The *lhβ* mRNA expression in the control animals was 2 and 3.5 fold lower compared to the 0.05 mg/Kg/day and 5 mg/Kg/day doses, respectively.

In males, the pituitary expression of both *lhβ* and *fshβ* mRNA was significantly up-regulated only in the 5 mg/Kg/day group (1.3 fold and 1.5 fold the control levels, respectively).

There were no significant effects of DEHP exposure on the expression profile of transcripts involved in the regulation of the pituitary-gonadal axis in both F2 and F3 generations.

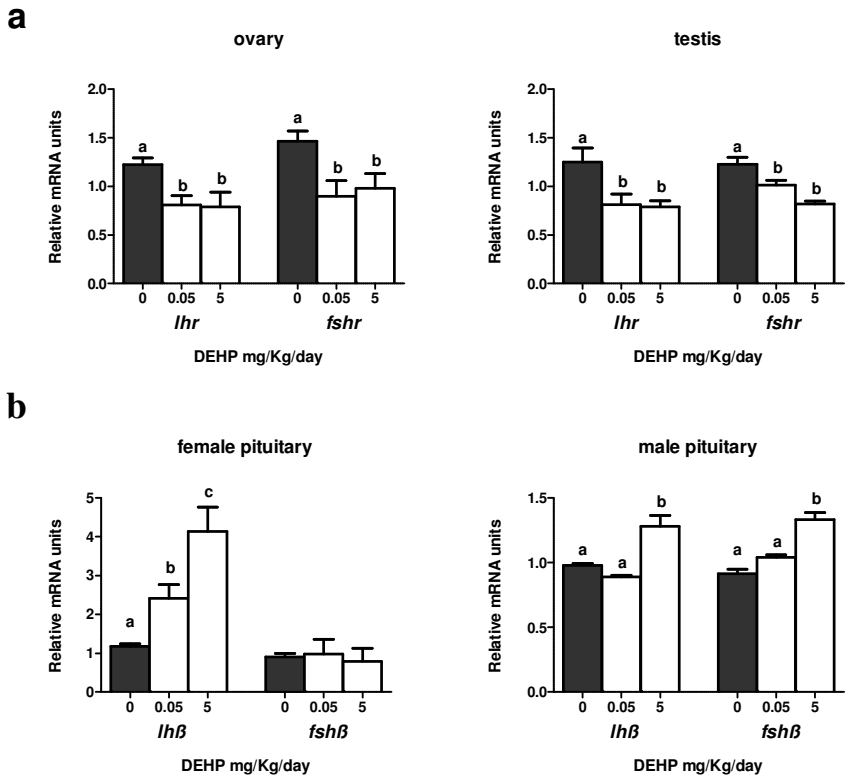

Figure 13: Effect of DEHP on the mRNA levels of target genes in the pituitaries and gonads of female and male mice offspring at PND 42 (a) Quantitative analysis of *lhr* and *fshr* mRNA levels in the gonads. (b) Quantitative analysis of *lhβ* and *fshβ* mRNA levels in the pituitaries. Each column represents the mean ± SE of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in Materials and Methods. Different superscript denote significant differences between columns ($p<0.05$).

Expression of genes related to oocyte and follicular development in the ovaries: in F1 ovaries a significant up-regulation of the transcript level for *gdf9* occurred in the 5 mg DEHP/Kg/day group related to control (Fig 14). Interestingly, up-regulation of *gdf-9* transcript is maintained up to the third generation. In F2 and F3 ovaries, *gdf-9* mRNA is up-regulated in both 0.05 and 5 mg DEHP/Kg/day group (Fig 14a). No differences were observed in level expression for *bmp15* and *pten* transcripts in any of doses and generations investigated (fig 14b).

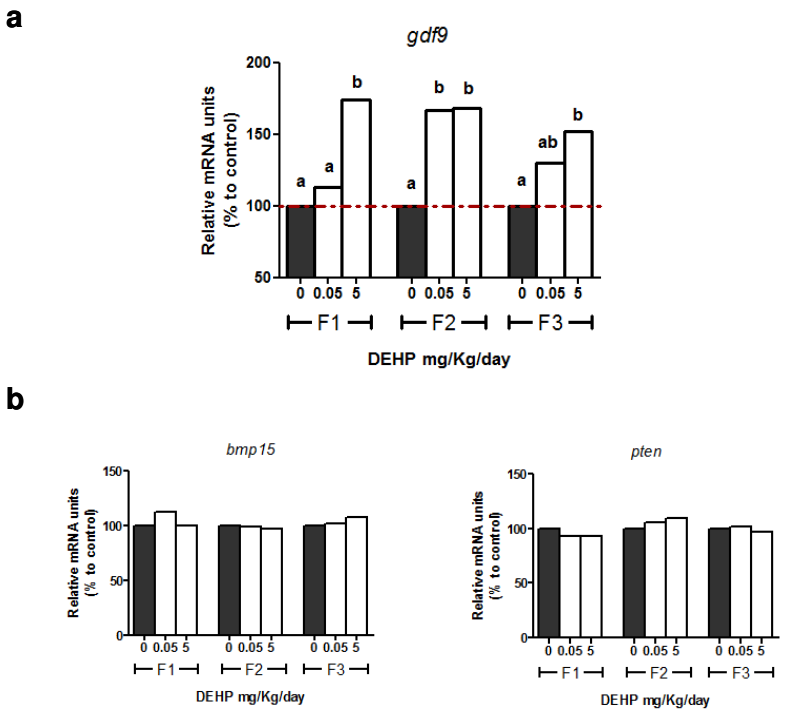

Figure 14: Expression profile of genes related to oocyte and follicular quality and development in adult ovaries from F1 to F3 adult animals. (a) Quantitative analysis of *gdf9* mRNA levels in the gonads from female adult offspring. (b) Quantitative analysis of *bmp15* and *pTEN* mRNA levels in the ovaries.

DEHP-induced alterations in gene expression profile of pre-implantation blastocysts throughout all generations.

Significant alterations in the expression profile of key genes involved in embryonic development, and trophoblast differentiation and implantation occurred in all generations investigated (Fig 15). In both treated groups (0.05 and 5 mg/kg/day) an up-regulation of transcript levels for *Oct-4* was observed in F1 and F2 generation; while transcript levels of Nanog were down-regulated. Furthermore, in the same generations, the expression profile of *Gata3* was up-regulated in both treated groups. Interstingly, the transcript levels for other genes related to trophoblast development, differentiation and implantation, while not having shown differences in the F1 generation, were disregulated in the F2 and F3 generation. In particular, the expression of *cdx2* and *eomes* transcripts were up-regulated in F2 and F3 generations in both 0.05 and 5 mg/kg/day treated groups, whereas *Lif* transcript level was up-regulated in the 0.05 mg/kg/day in the F2 and in both treated groups in the third generation (figure 15).

Maternal and reproductive outcome data in F1 and F2

There were no significant differences in the reproductive outcome of F1 and F2 animals. In both F1 and F2 parental animals, all mated females from each exposure group become pregnant. Gestation length was unaffected by treatment. There was no significant difference in the number of pups delivered, mean weaning weight, viability index and sex ratio between control and DEHP treated females.

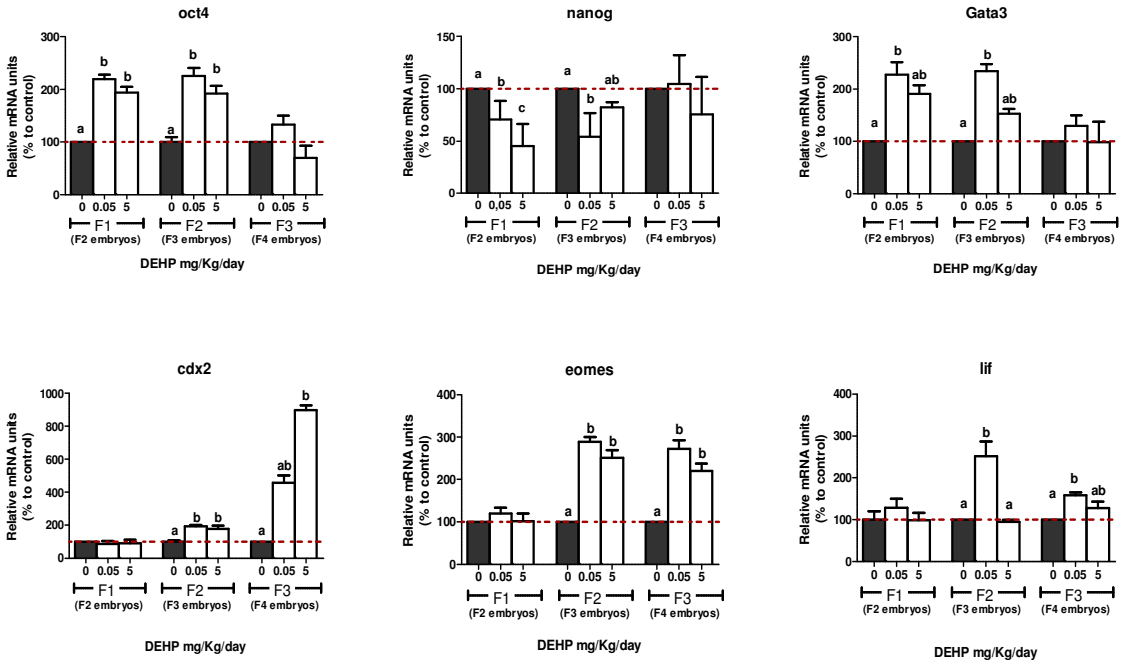

Figure 15 Quantitative analysis on the mRNA levels of key genes involved in pre-implantation blastocysts over three generations (F1 to F3) upon exposure of F0 dams to DEHP during pregnancy and lactation.

Effects of in utero and lactational exposure to PCBs 101+118 on reproductive health of F1 to F3 adult offspring

PCB congener concentration in F0 and F1 body tissues

Determination of the 101 and 118 PCB abundance in tissues from dams and weaned offspring were analyzed at The James Hutton Institute, Aberdeen, (Scotland; UK), according to the method previously described (Pocar *et al.* 2011).

Total PCB concentrations in tissues of dams (F0) and of their offspring (F1) at weaning (PND 21) increased in a dose-dependent manner. Offspring accumulated about 1.4 – 1.9 folds more PCB per unit of weight than their mothers (Table 9).

The ratio of the PCB congeners 101 and 118 in the body of both dams and offspring in each of the 10 µg/kg/ and 100 µg/kg/day groups was about 1:2 to 1:3, despite being present in the diet in a proportion of 1:1.

Table 9: PCB 101 and 118 tissue accumulation in both dams (F0) and offspring (F1) exposed from dpc 0.5 to PND 21.

	PCBs 101+118 µg/kg/day			
	0	**1**	**10**	**100**
Total PCBs dams (µg/kg)	0.9 ± 0.2^{a}	4.6 ± 0.4^{b}	34.42 ± 3.6^{c}	412.1 ± 24.6^{d}
Total PCBs offspring (µg/kg)	1.7 ± 0.2^{a}	$7.7 \pm 0.3^{b*}$	$66.3 \pm 2.0^{c*}$	$561.2 \pm 21.4^{d*}$
PCB 101:118 dams (%)	60:40	33:67	27:63	28:72
PCB 101:118 offspring (%)	47:53	44:56	31:69	27:73

a, b, c, d: different superscripts within a row indicate statistical differences for $P \leq 0.05$
*: indicates statistical difference between columns for $P \leq 0.05$

F0 reproductive outcome

There were no signs of toxicity in dams exposed to PCB. Necroscopy revealed no significant change in mean liver weight, visceral fat content or reproductive organ weights in any of the treatment group, relative to the control. Furthermore, treatment with PCB did not affect duration of gestation, litter size, sex ratio and viability index in any of the treated group, relative to control (Table 10).

Table 10 Reproductive outcome of F0 dams treated with PCB throughout pregnancy and lactation

	PCBs 101+118 µg/kg/day			
	0	**1**	**10**	**100**
Nr. of dams	10	9	10	10
Pregnancy at term (%)	100	100	100	100
Litter size	13.5±0.4	13.0±0.4	14.4±0.5	13.3±0.4
Sex ratio (females:males %)	48:52	49:51	52:48	54:46
Viability index[1]	97.1±2.0	98.7±1.3	97.3±1.6	98.1±1.9

[1]Viability index: (number of pups at weaning/number of pups alive at PND 2) *x* 100

Morphological and reproductive indices in F1, F2 and F3 male and female offspring

No adverse effects on clinical findings were observed in pups over the three generations analyzed neither before nor after weaning in any of the PCB-treated groups compared to control. Furthermore, no differences in body weight (both at weaning and at adult age), visceral fat content and liver weight were observed in treated male and female offspring irrespective of treatment or generation investigated.

Reproductive indices for both male and female adult offspring are shown in Table 11. In F1 offspring a significant reduction in testis and ovary weight was observed with all the

PCBs doses compared to controls ($P \leq 0.001$). Furthermore, at 100 μg/kg/day PCB a reduction in AGD index was observed ($P \leq 0.0001$).

F2 male offspring of all three PCB treatment groups, as for F1 animals, had significantly lighter testis compared to controls ($P \leq 0.0001$). Conversely, in F2 females, the mean ovary weight was unaffected, although the 100 μg/kg/day dose was associated with a non-significant reduction in mean ovarian weight. In F3 animals, there were no differences in reproductive indices between treated and control groups irrespective of gender.

Table 11 Reproductive indices in male and female adult F1, F2 and F3 offspring

	PCB µg/kg/day			
	0	1	10	100
F1 generation				
No. of animals	104	92	75	105
		males		
AGD [cm/BW^(1/3)]	0.517±0.009[a]	0.509±0.009[a]	0.514±0.005[a]	0.478±0.004[b]
Testis (g)	0.107±0.002[a]	0.093±0.003[b]	0.087±0.003[b]	0.091±0.001[b]
Seminal vesicles (g)	0.182±0.006	0.169±0.009	0.193±0.003	0.192±0.005
		females		
AGD [cm/BW^(1/3)]	0.249±0.004	0.246±0.004	0.254±0.005	0.247±0.009
Ovary (g)	0.0086±0.0008[a]	0.0070±0.0005[b]	0.0067±0.0004[b]	0.0065±0.0004[b]
Uterus (g)	0.134±0.014	0.106±0.006	0.119±0.008	0.128±0.003
F2 generation				
No. of animals	74	52	57	37
		males		
AGD [cm/BW^(1/3)]	0.537±0.007	0.528±0.006	0.533±0.008	0.523±0.004
Testis (g)	0.113±0.001[a]	0.100±0.003[b]	0.100±0.001[b]	0.101±0.004[b]
Seminal vesicles (g)	0.179±0.005	0.183±0.007	0.153±0.001	0.162±0.009
		females		
AGD [cm/BW^(1/3)]	0.268± 0.005	0.277±0.006	0.266±0.003	0.261±0.006
Ovary (g)	0.0082±0.0002	0.0081±0.0004	0.0080±0.0004	0.0071±0.0002
Uterus (g)	0.143±0.007	0.135±0.019	0.124±0.005	0.118±0.008
F3 generation				
No. of animals	57	62	41	61
		males		
AGD [cm/BW^(1/3)]	0.548±0.005	0.537±0.006	0.558±0.008	0.536±0.007
Testis (g)	0.102±0.002	0.101±0.002	0.101±0.001	0.097±0.001
Seminal vesicles (g)	0.147±0.011	0.168±0.016	0.164±0.001	0.146±0.009
		females		
AGD [cm/BW^(1/3)]	0.263±0.007	0.267±0.005	0.251±0.005	0.266±0.005
Ovary (g)	0.0085±0.0006	0.0082±0.0010	0.0078±0.0004	0.0079±0.0006
Uterus (g)	0.124±0.005	0.125±0.014	0.123±0.011	0.141±0.010

Note: Values are means ± SE
a, b, c: different superscripts indicate statistical differences within rows for $P \leq 0.05$

Histological analysis of male and female gonads in F1, F2 and F3 generations

The distribution of different types of seminiferous tubules was measured in the testes of males from all three generations (Fig 16). *In utero* and lactational exposure to PCB at 10 and 100 ug/kg/day significantly increased type II tubules at the expenses of type I tubules in male offspring in all generations. Incidence of depleted (type III) tubules was unaffected by treatment at any dose or generation investigated.

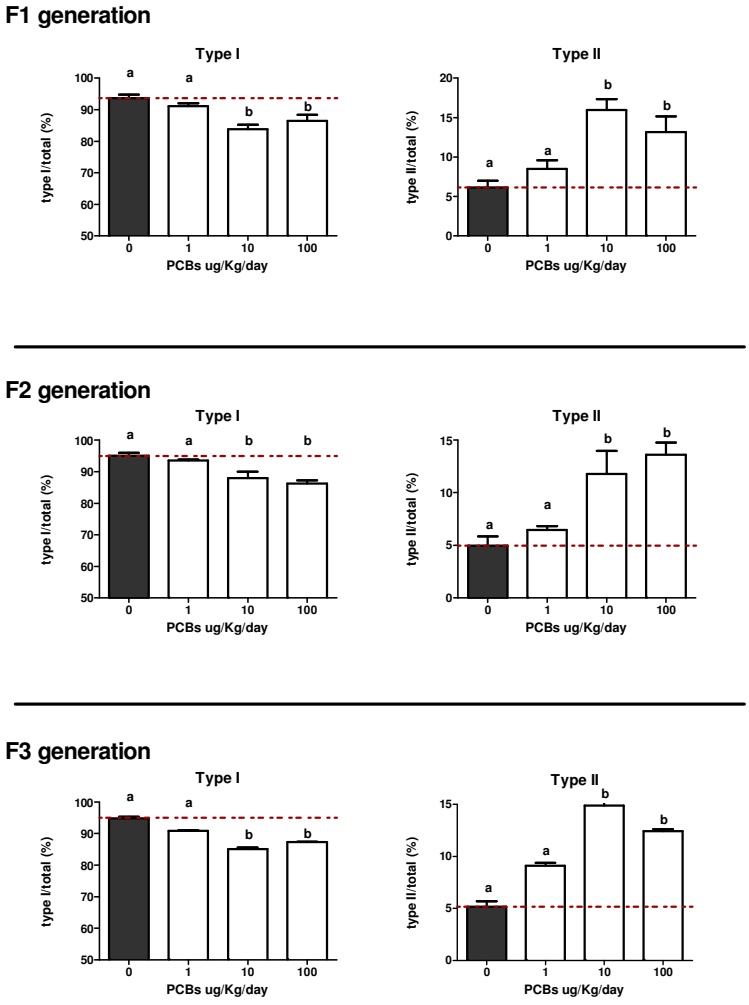

Figure 16: Seminiferous tubules distribution in testes of the F1, F2 and F3 offspring. Type I: normal seminiferous tubule with sperm; Type II: normal seminiferous tubules without sperm. Each column represents the mean ± SE of at least three separate experiments. Horizontal dashed lines represent mean control levels. Different superscripts denote significant differences between columns ($p<0.05$).

Table 12 shows tubule diameter and epithelium height in testis from F1, F2 and F3 generations. In F1 tubule diameter was significantly decreased by all PCB doses compared to controls, without changes of epithelium height. Similar changes of tubules morphometrical indices were observed in F2 testes, whereas no differences were observed between treated and control groups in the F3 generation.

Table 12 Tubule diameters and epithelium height in testes from F1, F2 and F3 adult male offspring

	PCBs 101+118 µg/kg/day			
	0	**1**	**10**	**100**
F1 generation				
Number of fields	79	58	79	66
Tubule diameter (µm)	256.7 ± 2.2 ^{a}	234.9 ± 3.1 ^{b}	242.8 ± 1.9 ^{b}	244.8 ± 2.9 ^{b}
Epithelium height (µm)	67.20 ± 0.79	63.85 ± 1.18	67.42 ± 0.96	66.17 ± 1.37
F2 generation				
Number of fields	45	48	45	46
Tubule diameter (µm)	264.2 ± 5.8 ^{a}	231.7 ± 3.1 ^{b}	230.4 ± 4.3 ^{b}	236.0 ± 4.0 ^{b}
Epithelium height (µm)	72.40 ± 1.27	66.44 ± 1.31	67.89 ± 2.00	66.73 ± 1.95
F3 generation				
Number of fields	47	51	46	48
Tubule diameter (µm)	251.3 ± 3.9	244.7 ± 2.7	245.1 ± 3.1	238.0 ± 2.6
Epithelium height (µm)	65.46 ± 1.81	64.57 ± 1.37	66.35 ± 1.30	64.58 ± 1.65

Note: Values are means ± SE
a, b: different superscripts indicate statistical differences for $P \leq 0.05$

In ovaries, exposure to PCBs during pregnancy and lactation did not affected percent distribution of pre-antral or antral follicles in female offspring in any of the generation investigated. Conversely, pre- and peri-natal exposure to PCBs significantly induced follicular atresia only in F1 adult offspring. In fact, ovaries of F1 generation of the three PCB groups showed a dose-dependent increase of atretic follicles compared to controls (Fig 17).

No differences in follicular distribution or in the incidence of atretic follicles were observed in F2 and F3 generations, irrespective of treatment.

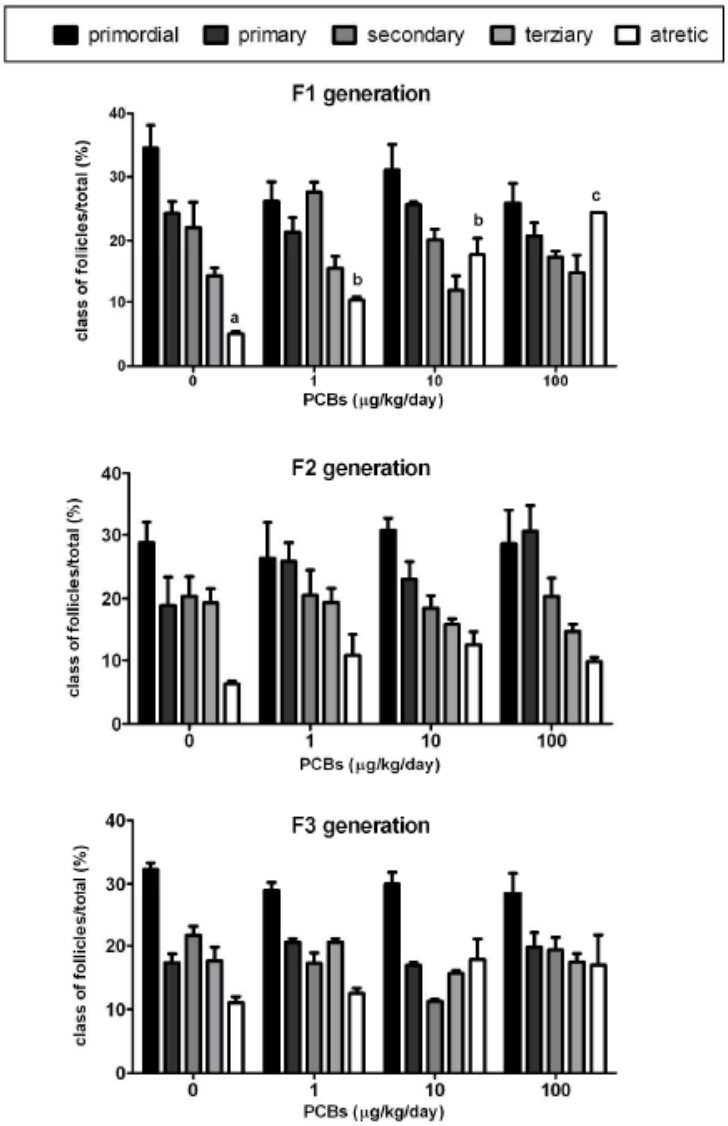

Figure 17 Percentages of follicles in each follicular stage in ovaries of the F1, F2 and F3 offspring. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p<0.0001$).

Gamete quality in F1, F2 and F3 male and female offspring

Semen characteristics and in vitro developmental capacity in adult male offspring: Figure 18 shows sperm concentration and viability of adult mice offspring of F1, F2 and F3 generations. PCB exposure of pregnant and lactating F0 dams significantly depressed the sperm viability of adult offspring up to the third generation. In general, sperm of F1, F2 and F3 treated groups was about 20-30% less viable than in controls of the same generation ($P \leq 0.0001$). There was no effect of PCB doses. Sperm concentration was unaffected by PCB exposure in any generation.

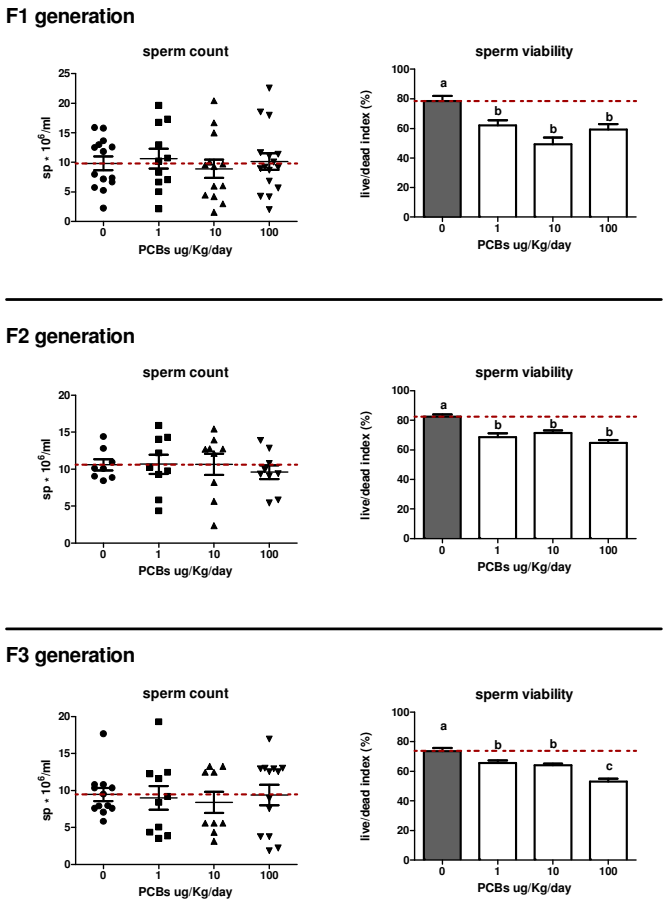

Figure 18: Counts and viability of epididymal caudal sperm from offspring of the F1, F2 and F3 generations. Horizontal dashed lines represent mean control levels. Different superscripts denote significant differences between columns ($p<0.0001$).

The fertility and developmental ability of sperm of F1 offspring were also analyzed. PCB exposure compromised sperm developmental capacity but not its fertilization capacity. In fact, in *in vitro* fertilization protocols using oocytes from untreated mice, the sperm from the 10 and the 100 μg/kg/day groups resulted in zygotes with the same ability to complete first mitotic division, but with a significantly reduced capacity to reach the blastocyst stage, compared to controls ($P \leq 0.05$; Fig 19).

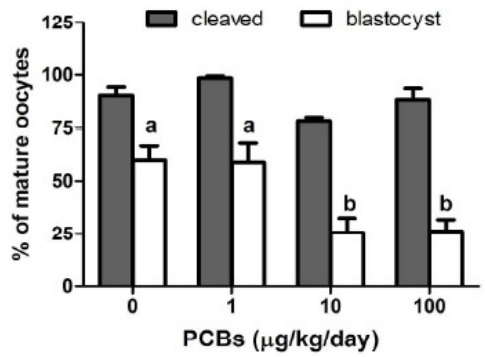

Figure 19 Fertility and developmental ability of epididymal caudal sperm from mice treated in utero and during lactation with PCBs 101 and 118. Embryo cleavage (grey bars) and blastocyst development (white bars) following in vitro fertilization of untreated oocytes. Each column represents the mean ± SE of at least three separate experiments. Different superscripts denote significant differences between columns ($p<0.05$).

In vitro oocyte developmental competence in adult female offspring from F1, F2 and F3 generations: Table 13 shows oocyte developmental competence of adult mice offspring of F1, F2 and F3 generations. In F1, the ability of oocytes to reach blastocyst stage was significantly reduced in the 100 μg/kg/day group compared to control, the 10 μg/kg/day dose showing intermediate values. No further differences were observed in developmental competence of F2 and F3 derived oocytes. PCB did not affect the number of ovulated mature oocytes per animal irrespective of the doses and generation investigated.

Table 13 Effects of exposure to PCBs 101+118 on in-vitro embryo production from oocytes derived from F1, F2 and F3 offspring

	PCBs µg/Kg/day			
	0	**1**	**10**	**100**
F1 generation				
Cleavage rate (%)	79.2 ± 7.0	74.1 ± 7.0	72.3 ± 6.0	69.8 ± 6.1
Blastocyst rate (%)	66.9 ± 4.4 ^{a}	64.0 ± 5.0 ^{a}	56.0 ± 3.1 ^{ab}	35.3 ± 3.0 ^{b}
F2 generation				
Cleavage rate (%)	82.6 ± 6.4	79.9 ± 11.2	73.6 ± 6.6	80.8 ± 6.2
Blastocyst rate (%)	58.4 ± 6.6	50.8 ± 7.3	51.6 ± 9.1	54.2 ± 10.7
F3 generation				
Cleavage rate (%)	78.2 ± 6.1	82.0 ± 4.8	89.7 ± 2.2	85.7 ± 3.8
Blastocyst rate (%)	59.0 ± 4.7	55.4 ± 3.9	51.2 ± 11.2	56.2 ± 9.3

Note: Values are means ± SE
a, b: different superscripts indicate statistical differences for $P \leq 0.05$

Reproductive outcome of female mice of the F1 and F2 generations

Table 14 shows reproductive outcome of F1 and F2 female mice, mated at sexual maturity with control unexposed males. All females of both generations become pregnant and had regular gestational duration. Nevertheless, F1 dams of all three PCB treated groups gave birth to significantly smaller litters, of about two pups less than controls.

Table 14 Reproductive outcome of female offspring of the F1 and F2 generations

	PCBs 101+118 µg/kg/day			
	0	**1**	**10**	**100**
F1 generation				
Nr. of dams	8	7	7	8
Pregnancy at term (%)	100	100	100	100
Litter size	14.71 ± 0.64 ^{a}	12.60 ± 0.24 ^{b}	12.20 ± 0.49 ^{b}	11.67 ± 0.67 ^{b}
Sex ratio (females:males %)	49:51	50:50	53:47	57:43
Viability index[1]	92.06 ± 4.88	96.08 ± 3.92	91.96 ± 5.45	91.38 ± 5.27
F2 generation				
Nr. of dams	7	7	7	8
Pregnancy at term (%)	100	100	100	100
Litter size	13.57 ± 0.87	13.43 ± 0.69	14.83 ± 0.70	13.17 ± 0.31
Sex ratio (females:males %)	45:55	49:51	49:51	50:50
Viability index[1]	95.99 ± 1.64	98.75 ± 1.25	97.92 ± 2.08	98.90 ± 1.10

[1] Viability index: (number of pups at weaning/number of pups alive at PND 2) *x* 100.

PCBs-induced alterations in gene expression profiles of adult offspring ovaries and pituitaries from F1 to F3 generation.

Expression profile of selected transcripts involved in the pituitary-gonadal crosstalk in adult female offspring: in F1 animals, a dose-dependent down-regulation in *cyp19a1* occurs in ovaries at all doses investigated, whereas no other steroidogenesis-related transcripts (*Cyp11, Cyp17a1* and *StAR*) resulted affected by PCB exposure in F1 (Fig 20a). No differences in expression levels of genes related to steroidogenesis, estrogen-signalling nor for gonadotropins receptors were observed in subsequent generations investigated. Furthermore, in PCB-treated animals, no significant alteration in the expression profile of pituitary hormone transcripts were observed independently from treatment in any of the generation investigated (Fig 20b).

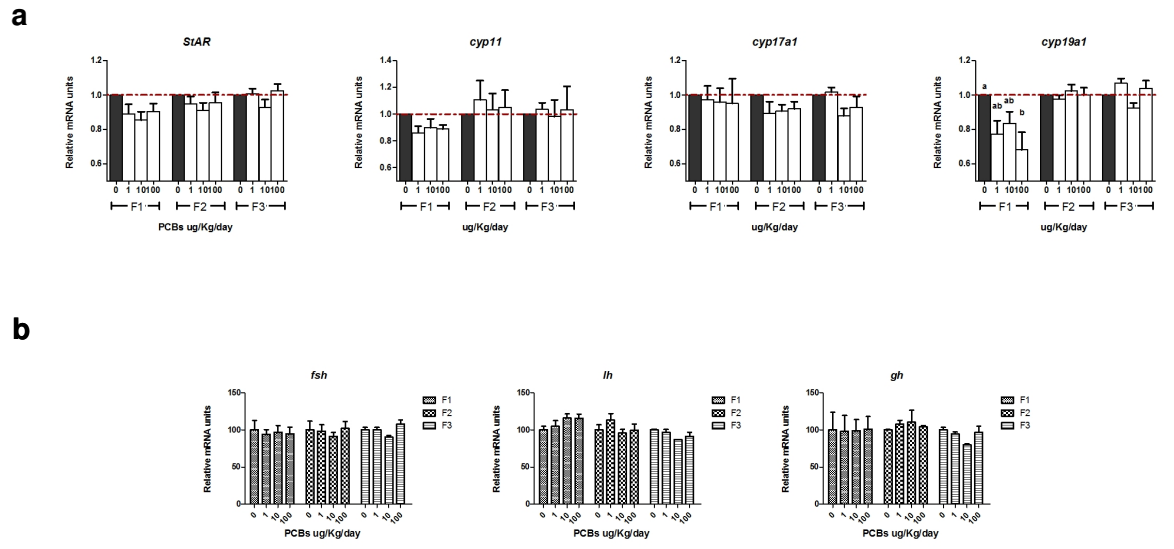

Figure 20 Effects of PCB mixture during pregnancy and lactation on the expression profile of genes related to steroidogenesis in the ovaries and of transcripts for pituitary hormones in adult F1 to F3 animals. (a) Quantitative analysis of c*yp11*, *cyp17a1*, *cyp19a1* and *star* mRNA levels in female gonads. Horizontal dashed lines represent mean control levels. (b) Effects of PCB mixture on the mRNA levels of selected hormones (*lh, fsh* and *gh*) in the pituitaries from F1 to F3 adult females. Each column represents the mean ± SE of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in Materials and Methods. Different superscripts denote significant differences between columns ($p<0.05$)

Expression of genes related to oocyte and follicular development in the ovaries: Analysis of ovarian transcripts related to folliculogenesis and oocyte quality revealed that transcript levels of *pten* were significantly down-regulated in all treated groups in ovaries from both F1 and F2 females mice at adult age, whereas there were no alterations in the expression profile of *pten* in the third generation. No differences were observed in level expression of *bmp15* and *gdf9* transcripts in any of the generation investigated (Fig 21).

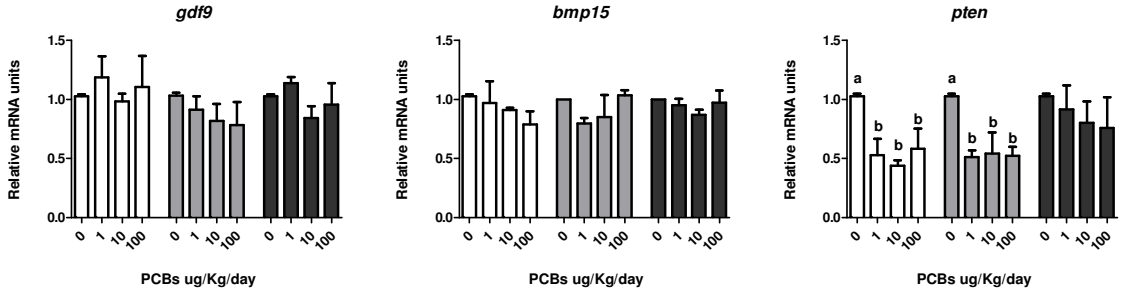

Figure 21: Effects of PCB mixture during pregnancy and lactation on the expression profile of genes related to oocyte and follicular development. Quantitative analysis of *gdf9*, *Bmp15*, and *pTEN* mRNA levels in ovaries. Each column represents the mean ± SE of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in Materials and Methods. Different superscripts denote significant differences between columns ($p<0.05$).

DEHP - DISCUSSION

To our knowledge this is the first comprehensive multigenerational study that evaluate the reproductive effects of pre- and perinatal exposure to doses of DEHP in the range of the estimated exposure of the general human population (Blount *et al.* 2000; Kavlock *et al.* 2002; Koch *et al.* 2003; Koch *et al.* 2006; Silva *et al.* 2004) on male and female offspring at adult age up to the third generation.

In the present study we elicit to expose dams during the entire period of pregnancy and lactation, in order to cover the complete time window of reproductive system development in the mouse. In fact, development of gonads and reproductive tract in rodents occurs largely in post-natal period, while in other mammals, including human, reproductive organs' development is completed in utero.

Upon exposure, no significant clinical signs of toxicity were observed in F0 dams. The increase in liver weight observed in treated animals, was considered a general biological reaction to exogenous xenobiotica, and assumed as irrelevant because not associated with histopathological changes and not consistent among generations.

One dramatic acute consequence of DEHP treatment on pregnant dams was observed in our investigation: the complete pregnancy failure with the highest dose (500 mg/kg/day). These data are in agreement with recent data reported by Gray et al. (Gray *et al.* 2006) reporting midgestation abortions in rats upon exposure to high doses (500 and 1000 mg/Kg/day) of phthalates. Furthermore, limited studies in human populations suggest an association between phthalate exposure and adverse reproductive health outcomes. Chronic occupational exposure to high levels of phthalates was associated with low pregnancy rates and high rates of miscarriage in female factory workers (Aldyreva *et al.* 1975). In the present study, examination of conceptuses revealed that, at dcp +10.5, 500 mg/Kg/day treated embryos and their extraembryonic envelopes start to degenerate and

embryos from all treated dams became non-viable before dpc +11.5. Vascular development in the postimplantation mouse embryo and placentation essentially begin at dpc +6.5, resulting in a fully functional embryonic circulation by dpc +12.5 (Coffin and Poole 1991). Furthermore, DEHP is able to activate the peroxisome proliferator-activated receptors (PPARs), a family of transcription factors which has been recently implicated in the inhibition of proliferation and differentiation of endothelial cell in vitro and in impaired in vivo neovascularization (Bishop-Bailey and Hla 1999; Murata *et al.* 2000; Panigrahy *et al.* 2002; Xin *et al.* 1999). Based on these data, it is possible to hypothesize that DEHP exposure in pregnant mice may affect placental vascularization through activation of PPARs leading to the total pregnancy failure at high doses (500 mg/Kg/day). Likewise, a similar mechanism could be responsible for the reduced litter size observed at lower doses (5 mg/Kg/day), despite necroscopy at specific timepoints was not able to reveal significant increase in post implantation losses.

The results of our investigation indicate that direct exposure to DEHP during pre- and perinatal development induces in F1 male and female offspring: i) lower body weight; ii) altered gonadal weight (i.e.: lighter testis and heavier ovary); iii) altered ovarian morphology; iv) reduced germ cells quality; v) low expression of steroidogenesis and gonadotropin-receptor genes in the gonads; and vi) up-regulated gonadotropin subunits gene expression in the pituitary.

Reduced body weight and abdominal fat content was observed in F1 offspring, at all doses investigated, both at weaning and adult age. No further alteration in body weight was observed in subsequent generations, pointing to an effect of direct mother exposure. The reduced fat content observed in F1 offspring suggests that lypodystrophy is a component of the pleiotropic response to phthalate exposure. In fact, it has been observed that a variety of peroxisome proliferators, including DEHP, induce adipose tissue atrophy in mice, and

that this effect is mediated by PPARs' activation (Xie *et al.* 2002). The reduced body weight of the F1 offspring that were exposed to DEHP during pre- and perinatal period are in accordance with previously published results. For example, mouse foetuses exposed to DEHP during gestation from conception through dpc 17 showed significantly reduced body weight (NTP 1984). As well, rats exposed to di-n-butyl phthalate (DBP) during pregnancy, showed decreased birth weight and reduced weight gain (Marsman 1995). Furthermore, in humans, in last decades it has been strongly suggested that, beside malnutrition and socio-economical factors, environmental pollution, may play a role in the development of low birth weight. Low birth weight is an important risk factor in development and worldwide data indicate that infants showing low birth weight are at greater risk of early mortality or long-term diseases. Interestingly, a recent nested case-control study in human population positively correlates higher phthalate levels in umbilical vein blood serum in low birth weight cases compared to normal weight newborns (Zhang *et al.* 2009). Furthermore, for both DBP and DEHP, association between reduced weight and exposure level appear to be dose-dependent (Zhang *et al.* 2009).

As regard as the significant weight alteration of both testis and ovary in adult offspring after exposure to DEHP in utero and during lactation, results are in agreement with literature data describing changes in gonad weight when DEHP was administered through pathways and doses similar to the one used in this work (Akingbemi *et al.* 2001; Arcadi *et al.* 1998; Borch *et al.* 2006; Howdeshell *et al.* 2007; Stroheker *et al.* 2005; Wilson *et al.* 2004). In addition, F1 ovaries from treated females, demonstrated an altered morphology with a reduced proportion of primordial follicles at any of the dosages investigated, and a reciprocal increase in the proportion of follicles that had started to grow. Traditionally, qualitative measures of female reproductive capability, such as fertility and abnormal clinical symptoms, have been used to assess toxicity. Recently, however, differential

follicle count has been found to be a sensitive and a quantifiable endpoint of ovarian injury (Bolon *et al.* 1997; Yu *et al.* 1999), thus counting preantral follicular populations can provide a direct estimate of the ovarian functional reserve after exposure to reproductive toxicants (Bolon *et al.* 1997). In addition, the total number of follicles counted from serially sectioned ovaries does not differ from the number obtained when random sections are used (Bucci *et al.* 1997; Smith *et al.* 1991). Thus, sampling populations of preantral follicles provides a good indication of ovarian injury and may be a more sensitive endpoint of female reproductive toxicity than traditional methods of assessment. The depletion of the primordial follicular pool in DEHP treated mice during adult life observed in the present study may be caused by two different mechanisms: (1) a disturbance in the process of follicular formation and the establishment of the primordial follicle pool due to DEHP exposure. The disruption of primordial germ cell migration and granulosa cell differentiation have been suggested as mechanisms of toxicity for a variety of xenobiotica (MacKenzie and Angevine 1981; Tam and Snow 1981; Wide 1985); (2) depletion can be due to an accelerated rate of follicle recruitment from the resting pool. Our data support more this second hypothesis, since the decline in the proportion of primordial follicles is also characterized by higher percent number of growing follicles in ovaries from DEHP treated animals, suggesting that increased follicular recruitment contributes, at least, in part to this decline. These data nicely correlate with the observed up-regulation of *gd9* transcript levels identified in treated ovaries. Indeed, recent studies have shown that the administration of GDF-9 to neonatal rats increases the number of primary and preantral follicles and decreases the complement of primordial follicles, collectively suggesting that GDF-9 promotes initial recruitment (Vitt *et al.* 2000). Of particular interests is the fact that similar ovarian phenotype, together with dysregulation of *gdf9* expression levels, were observed in all generations investigated.

With respect to the reduced ovarian reserve, it can be, therefore, postulated that accelerated follicular recruitment would rapidly exhaust the primordial pool leading to early reproductive failure. In humans, premature ovarian failure (POF) is clinically defined as the spontaneous and irreversible cessation of menses due to ovarian failure prior to the age of 40 years (Gosden *et al.* 2007). Population based studies estimate that 1% of women experience POF (Coulam *et al.* 1986; Cramer and Xu 1996; Luborsky *et al.* 2003). The known causes of POF are heterogeneous; they include genetic factors, ovarian autoimmunity, infection (mumps), treatment with anticancer drugs, and chemical exposures (Goswami and Conway 2005; Sharara *et al.* 1998). Regarding the latter, it has been recently reported that hairdresser, a major occupational group of female workers of reproductive age who sustain continuous exposure to chemicals, including phthalates, during their reproductive lifespan, are at increased risk of POF compared with women of reproductive age in other occupations (Gallicchio *et al.* 2009).

The ovarian phenotype observed in the present study in DEHP treated mice, characterized by primordial pool failure, accelerated growth of preantral follicle and increased atresia, corresponds to the histological appearance of FSH-R insufficient mice. Despite the exact cause-and-effect relationships between endocrine and follicular changes and their impact on ovarian aging are still unclear, an age-related reduction in binding of FSH to its receptors was demonstrated in FSH-R heterozygous and wild-type mice (Danilovich *et al.* 2002) and premature ovarian failure has been shown in haploinsufficient FSH-R mice, suggesting that a full complement of functional FSH-R signaling is required to maintain follicle numbers and to protect the ovary from atresia (Danilovich and Sairam 2002). Although we did not measure circulating hormones, in the present study a disregulation of gonadotropin signalling is strongly suggested by alteration in the expression levels of both gonadotropins and gonadotropins' receptors in the pituitary and in the ovary, respectively. Therefore, it is possible to hypothesize that DEHP, affecting

FSH-R signalling in treated mice, can induce premature ovarian failure. In the present study we did not observed reduced fertility in *in utero* treated mice and subsequent generations. However, mating was performed at early age (8 weeks), where the reproductive activity in mice did not decline up to the 8-12 month of age, depending on strain. It is therefore likely that follicle number present in the ovary at the investigated age is sufficient to support pubertal development and fertility, and ovarian failure due to follicle depletion would be visible at later age. Furtermore, altered receptor-hormone interactions in the ovarian aging have been reported in older women (Kim 1995) and cattle (Lerner *et al.* 1986), before fertility decline.

The novelty of the present investigation is that the morphological changes were related to the functionality of the ovaries. To our knowledge this is the first study showing that in vivo pre- and perinatal exposure to DEHP concomitantly causes morphological alterations and impairs oocyte developmental competence, reducing the ability of oocytes to complete first mitotic division, after *in vitro* fertilization, and to reach the blastocyst stage. In addition, blastocysts derived from fertilized oocytes of treated animals evidenced a disregulation of the expression profile for key genes involved in early embryonic differentiation and development (*Oct-4, Nanog and Gata3*) in both F1 and F2. Interestingly, in F2 and F3 generation a further up-regulation of trophoblast differentiation markers, cdx2 and eomes, was observed. Recent studies underlined the critical role of Nanog and Oct-4 in the pre-implantation development of fertilized oocytes: Nanog prevents differentiation of Embryonic Stem Cells (ESC) into extraembryonic endoderm and actively maintains pluripotency, while Oct-4 has the primary function to prevent trophoectoderm differentiation of Inner Cell Mass (ICM) and ESC (Mitsui *et al.* 2003). The same authors further demonstrated that during preimplantation development, Nanog and Oct-4 are differentially expressed at morula or blastocyst stage; at morula stage Nanog expression level is low and Oct-4 is expressed in ICM, while, at blastocyst stage, cells that

express Nanog remain pluripotent and the other cells differentiate into primitive endoderm, so Nanog estabilished the destiny of the ESC in the embryo and its down-regulation orchestrate the differentiation of murine and human ESC into extra-embryonic lineages (Hough *et al.* 2006; Hyslop *et al.* 2005). The complementary overexpression of Oct-4 is directly related to Nanog levels because pluripotency factors form an interconnecting autoregulation loop to maintain ES identity so, if Oct-4 levels rise above a steady level, Nanog expression would be repressed (Pan and Thomson 2007).

With regard to trophoectoderm development, before formation of the blastocyst, the complementary expression patterns and reciprocal repression of the transcription factors Cdx2 (in the outermost cells) and Oct-4 (in the innermost ones) defines the segregation of TE and ICM (Niwa *et al.* 2005). Cdx2 then activates Eomes expression in the TE, both transcription factors being essential for the specification and maintenance of the TE-derived trophoblast lineage. Furthermore, Gata3 is expressed only in the trophoblast lineage and is both necessary and sufficient to promote trophoblast maturation regulating the process of morula to blastocyst transformation (Ralston *et al.* 2010). Recently, it has been observed that a tight interconnection exists in the expression of genes involved in early trophoblast differentiation. Tead4 is an early transcription factor required for specification and development of the trophectoderm lineage, which includes expression of both *gata3* and *cdx2* genes. In addition, Home et al (Home *et al.* 2009) demonstrated that *gata3* directly regulates *cdx2* transcription in a positive fashion, suggesting that *gata3* maintains higher levels of *cdx2* transcription in outer cells after initial induction of *cdx2* expression by a Tead4-dependent mechanism. Based on these observation, the overexpression of *oct4* concomitantly with a down-regulation of *nanog* transcript levels in treated embryos observed in the present study indicate an altered profile of pluripotency factors where down-regulation of *nanog* may indicate altered pattern of extra-embryonic

lineages differentiation. This hypothesis is further supported by the up-regulation of the trophoectoderm differentiation markers *gata3*, *cdx2* and *eomes*, and might explain the reduced oocyte developmental competence observed in the present study.

The mechanisms underlying phthalates' influence on pre-implantation development is not yet fully understood. Recent *in vitro* studies showed impairment of meiotic maturation and embryo development in oocytes directly exposed during culture to either DEHP or MEHP (Dalman *et al.* 2008; Mlynarcikova *et al.* 2009), thus supporting our observations upon *in vivo* treatment. Oocyte developmental competence is acquired during folliculogenesis as oocyte grow and during oocyte maturation (cytoplasmatic and nuclear). During follicle growth, FSH signal transduction (via cAMP) in granulosa cells drive the orderly production of aromatizable androgens, estrogens and progesterone that is essential for the maturation of healthy preovulatory follicles (Skinner 2005).

Recent data suggest that phthalates may induce disruption of ovarian estrogen biosynthesis pathways through a PPAR-mediated way (Lovekamp-Swan and Davis 2003), and that lower estradiol secretion from granulosa cells may be responsible for impaired oocyte quality (Eimani *et al.* 2005). There is evidence that in vitro exposure to phthalates suppresses *cyp19a1* transcript levels and decreases E2 production in rat (Lovekamp and Davis 2001) and human (Reinsberg *et al.* 2009) granulosa cells, and that both DEHP and MEHP reduce E2 production and *cyp19a1* transcript levels, leading to inhibition of growth of cultured whole antral follicles from mice (Gupta *et al.*;, 2010). In agreement with these observations, the results of the present study may suggest that adverse effects observed in oocyte developmental competence may be related to dysregulated steroid synthesis. In fact, in F1 female offspring, concomitantly with reduced oocyte competence, we observed a significant down-regulation of both *Cyp17a1* and C*yp19a1* genes expression in ovaries, suggesting a persistent altered estrogen synthesis pathway in adult mice maternally

exposed to DEHP. This hypothesis is further supported by the significant reduced expression of the *pr* gene, a known target gene of estrogen receptor. It is therefore possible to speculate that DEHP-mediated PPARs activation could interfere with estrogen biosynthesis in ovaries form maternally exposed mice and may explain suboptimal ability of the oocyte to accomplish its reproductive purpose, observed in the present study.

Interestingly, the cause-effect relationship between DEHP-induced altered estradiol synthesis and low reproductive performance of F1 female mice observed here, may also apply to male offspring from the same litter. In fact, in testes of DEHP treated F1 male offspring we observed a decrease of both *cyp19a1* and *pgr* expression associated to a reduction of sperm count and sperm viability. It is therefore possible to speculate that also male mice exposed to phthalates pre- and perinatally have long-lasting altered estrogen biosynthesis in the testis, which, in turn, results in disturbances of sperm count and viability at adult age. This conclusion is supported by recent studies showing that in men impaired aromatase activity due to defective *cyp19a1* is related to decreased sperm concentration and motility (Lazaros *et al.* 2011) and to disturbance of acrosome formation (Pentikainen *et al.* 2000; Robertson *et al.* 1999), as well by the evidence of a strong inverse associations between estradiol levels and sperm DNA damage (Meeker *et al.* 2009). Furthermore, studies have demonstrated that in vivo exposure to DEHP and/or MEHP suppress aromatase in the brain and testes of in vivo treated young male rats (Andrade *et al.* 2006; Davis *et al.* 1994; Kim *et al.* 2003; Lovekamp and Davis 2001; Noda *et al.* 2007). It has also been suggested that estrogen deficiency or insensitivity in man might result in the accumulation of fluid in efferent ductules and subsequent atrophy of the testis (Hess *et al.* 1997). These data are in agreement with our findings reporting decreased testis weight in exposed males, further supporting a disregulation in estradiol synthesis.

Although we did not measure circulating steroid hormone concentrations, decreased gamete quality from exposed animals, together with down-regulation *cyp19a1* and *pgr* expression observed in adult offspring of both gender, strongly suggest low serum estrogen levels. This is likely to have effects on the hypothalamus-pituitary-gonadal axis negative feedback mechanism. We therefore finally suggest that the reproductive health worsening of male and female mice exposed in utero and during lactation to DEHP, can overall be caused by a long-lasting damage of the entire pituitary-gonadal axis. This assumption is supported by our data showing up-regulated expression levels of gonadotropins' β subunits in pituitaries of both male and female treated offspring, likely reflecting attenuated negative feedback by estradiol on the pituitary. According to these observations, in adult rats it has been recently reported that direct exposure to DEHP, enhanced the capacity of pituitary cells to secrete LH, thereby resulting in elevated serum levels of LH (Akingbemi *et al.* 2001; Svechnikova *et al.* 2007). Taken together, these observations may suggest that overexpression of *lh* and *fsh* transcripts observed in the present study in the pituitaries of both males and females animals may lead to increased gonadotropin serum levels. This hypothesis is further supported by the down-regulation of *fshr* and *lhr* mRNA observed in testes and ovaries of DEHP treated animals. In fact, like other polypeptide hormone receptors, gonadotropins receptors undergo down-regulation in response to ligand (Hoffman *et al.* 1991; LaPolt *et al.* 1990; Lu *et al.* 1993; Peegel *et al.* 1994). Beside the rapid loss of receptors through internalization, it has been demonstrated that elevated levels of both FSH and LH/hCG caused rapid receptor mRNA loss (Kishi *et al.* 1997; Maguire *et al.* 1997; Murphy and Dobias 1999), similarly to what observed in the present study.

In conclusion, our findings suggest that in maternally exposed male and female mice, DEHP acts on multiple pathways involved in maintaining steroid homeostasis, causing

significant alterations in gonadal morphology and functionality in both genders at adult age.

Further studies will be necessary to better understand how genes associated with regulation of pituitary-gonadal axis are regulated after pre- and perinatal DEHP exposure. The DEHP doses we employed were within the range of real environmental exposure levels in humans. Therefore, our observations of the inhibitory effect of DEHP on estrogen production and, in turn, on reproductive performance, represent reason of concern. In fact, despite mouse data must be assessed very carefully before being extrapolated to the human fetus, the pathways leading to ovarian hormone production are similar in rodents and humans and phthalates can cross the placenta in both species, it is, therefore, reasonable to assume that human fetal DEHP exposure during critical points in development would also act on pituitary-gonadal axis. Moreover, fetal gonad development in rodents appears to be primarily a pituitary-independent phase and gonadotropin dependent development does not occur until after birth. In contrast, in humans, ovarian and testicular development occurs prenatally and are largely dependent on pituitary gonadotropins in later gestation (Fowler *et al.* 2011; O'Shaughnessy and Fowler 2011), which may, in turn, make DEHP effects potentially more marked in human than in the mouse.

Of particular interest is the fact that the present study indicates that DEHP administration to pregnant female mice induces alterations in the reproductive health of female offspring also in subsequent generations. Specifically, effects on ovarian follicular distribution and in embryonic development were observed up to the third generation, pointing to a transgenerational effect of DEHP in female offspring.

Recent observations indicated that exposure to environmental chemicals at the time of gonadal development not only directly affects the reproductive health of the exposed individual, but can also induce effects in subsequent generations that may differ from those

associated with the primary exposure (Anway *et al.* 2005; Fernie *et al.* 2003; Shipp *et al.* 1998). The mechanisms of transmission along multiple generations are still to be clarified. In this matter a substantial distinction has to be made between intergenerational transmission involving direct exposure to the environmental factor, and transgenerational effects involving germ-line transmission without direct exposure of the affected generation (Skinner 2008). In fact, upon exposure to non-persistent chemicals, including phthalates, exposure of the F0 gestating female also exposes the F2 germ-line present in the F1 developing offspring, whereas F3 animals did not experience any direct exposure, indicating true transgenerational transmission.

The critical target cell for transgenerational phenotypes and toxicology is the germ line. Recent studies have been shown that exposure to endocrine disruptors, such as the anti-androgen vinclozolin, during embryonic gonadal sex determination induces adult onset disease for multiple generations up to the F4 (Anway *et al.* 2006a; Anway *et al.* 2006b), and it has been postulated that vinclozolin-induced transgenerational phenotype resulted from an epigenetic change in the male germ-line (Anway *et al.* 2006a). The fact that adverse, DEHP-mediated, reproductive effects in the male were highly consistent, between individuals and amongst litters, suggests that genetic DNA sequence mutations are not the most likely cause (Barber *et al.* 2002; Dong *et al.* 2004). In contrast, epigenetic variations involving the germ-line could result in the high frequency observed (Anway *et al.* 2005). Further analyses are necessary to identify epigenetic modifications which might explain transgenerational transmission of DEHP adverse effects.

One of the main questions involving phthalates, is whether the level of human exposure is sufficient to adversely affect female reproductive health. Recently, it has been reported that treatment of rat dams with active phthalates may result in non linear, mainly U-shaped, dose response curves effects in the offspring (Andrade *et al.* 2006; Lahousse *et al.* 2006; Lehmann *et al.* 2004). This is in agreement with results reported in the present study.

Specifically, adverse effects in dams were mainly observed in the highest doses investigated (500 mg/Kg/day). However, in the offspring, major adverse effects were observed in the lowest investigated dose (0.05 mg/Kg/day), suggesting non-monotonic response curves and low-dose effects. It is noteworthy to observe that the current no observed adverse effect level (NOAEL) adopted by the European Food Safety Authority for DEHP is 5 mg/Kg/day, based on a multigenerational study using alterations in male reproductive organs as endpoint (EFSA 2005). However, it is also important to note that the significance of low-dose changes, as observed in the present study, is still largely unknown and, due to difficulties in animal to human extrapolation, cannot be taken as clear evidence for concern about human health. To address this question, further analysis in animal models are necessary in order to better understand the cellular and molecular mechanisms at the basis of the effects observed and their relevance for human health.

PCBs DISCUSSION

The present study shows that pre- and peri-natal exposure of mice to a mix of PCBs 101 and 118, at doses designed to simulate human exposure, induced permanent morphological and functional reproductive alterations in both male and female adult offspring. Furthermore, the results indicate that reproductive deficiencies may be transmitted intergenerationally.

PCBs tissue concentration increased with the dose administered in a broadly linear manner, suggesting that the desired differences in treatment were actually achieved. Lower burdens of PCB 101 compared to PCB 118 could be explained by differences in clearance and redistribution rates; these processes are more rapid for PCB 101 than for PCB 118 (24h vs. one week) (Oberg *et al.* 2002). PCBs concentration measured in dams were in the range of those found in adipose tissue in the human population (up to 134 μ g/kg) (Kiviranta *et al.* 2005). In fact, multiplication by a factor of 5 of the whole-body burden measured in the present study (mouse body fat content being approximately 20% of the total body mass), indicates that the concentrations in dams treated with the low and medium doses overlap PCB human levels.

The finding of preferential accumulation of PCBs in the offspring, compared to their dams, confirms previous studies in sheep exposed to contaminated pastures (Rhind *et al.* 2009; Rhind *et al.* 2010), and indicates that the most vulnerable developmental stages are subject to the greatest PCB burden following maternal exposure, an observation of particular concern.

In the present study, a significant reduction in ovarian weight and an increased follicular atresia was observed in PCB-exposed F1 female offspring. These data are in agreement with studies indicating that reduction in mammalian ovary weight may be related to morphological changes, such as a reduction in the number of antral follicle and/or increase in atretic follicle number (Rao and Kaliwal 2002; Shirota *et al.* 2006). The observation of increased atresia without effects on total follicle count or on the proportion

of follicles of each class is singular. However, it has been suggested that maternal exposure to coplanar and non coplanar PCBs may exert opposite effects on follicular dynamics. Baldridge et al. (Baldridge *et al.* 2003) reported that maternal exposure to a mixture of non-coplanar PCBs reduced preantral and antral follicle numbers in rat ovaries while concurrently causing an increase in atresia. In contrast, *in utero* exposure to coplanar PCBs has been demonstrated to increase follicle number in different species (Kraugerud *et al.* 2011; Ronnback and de Rooij 1994). Considering that the mono–ortho PCB 118 may share some effects with coplanar PCBs whereas the di–ortho PCB 101 is a typical non-coplanar congener, it is postulated that the interaction of the two congeners could have a stimulatory effect on follicle count that would be masked by increased rate of follicular loss through atresia. This hypothesis is further supported by the observation that, despite increased atresia, no differences in mean numbers of ovulated oocytes were observed in any of the treatment groups. It is interesting to notice that in F1, ovarian phenotype was accompanied by a significant down-regulation of *pten* expression. Recent data indicate that, although Pten deletion activates the PI3K-Akt pathway in both oocytes and granulosa cells, the resulting outcome is different. In fact, PTEN-deficiency in oocytes leads to premature activation of the entire pool of primodial follicles and drastically reduces the ovarian follicle reserve, thereby rapidly advancing infertility (Daikoku and Dey 2008; Reddy *et al.* 2008). In contrast, granulosa cells lacking *Pten* induces induced ovulation and enhanced fertility (Fan *et al.* 2008) providing evidence that *pten* is expressed in a cell-specific manner in the ovary. It is therefore possible to hypothesize that the exposure to the PCB mixture employed in the present study might act on follicular dynamics differentially acting on PTEN signaling pathway in a cell-specific manner and further analyses are required to clarify the molecular mechanism underlying this phenomenon.

In view of the observed patterns of follicle populations in F1 treated animals, no alteration in fertility rate might be expected but in fact a significant reduction in mean litter

size was observed in PCB-treated F1 parental animals. Animal and human studies have shown that PCBs cause implantation failure, resorption and reduced litter size (Jonsson *et al.* 1975; Seiler *et al.* 1994). Reduced litter size is also consistent with impaired developmental competence of oocytes derived from F1 treated females observed in the present investigation.

Several studies have shown that exposure to PCBs reduces oocyte maturation, fertilization and blastocyst development (Campagna *et al.* 2002; Kholkute and Dukelow 1997; Kholkute *et al.* 1994a, b; Krogenaes *et al.* 1998; Kuchenhoff *et al.* 1999; Lindenau and Fischer 1996; Pocar *et al.* 2001). Studies in women showed preferential accumulation of PCB 118 in the follicular fluid (Pauwels *et al.* 1999), which may explain the adverse effects on oocyte developmental competence observed in the current study.

Despite, in the present study, intergenerational adverse effects of PCBs were not detected in females of F2 and F3 generations at both morphological and functional level, it is noteworthy to notice that, similarly to what observed in F1, *pten* down-regulation was evidenced also in F2 ovaries. It is therefore possible to hypothesize that subtle adverse effects of pre- and peri-natal exposure to PCBs could be transferred to subsequent generations also in females and deserve further investigations.

Effects of PCBs were not confined to the female offspring. In adult F1 male offspring reduced mean testis weight, and reduced sperm quality and developmental capacity were observed, suggesting an anti-androgenic effect attributable to maternal exposure to PCBs. This hypothesis is consistent with the reduced AGD observed in the highest dose group. In agreement with present data, rats and guinea pigs prenatally exposed to PCBs showed reduced testis weight and limited reproductive capability (Brouwer *et al.* 1995; Kuriyama and Chahoud 2004; Lundkvist 1990).

Histological analysis of PCB-treated animals has also been shown to be associated with a significant increase in sperm depleted seminiferous tubules and reduced diameter of

seminiferous tubules, findings that may indicate spermatogenic cell loss and tubules disorganization (Creemers *et al.* 2002). Thus, while no significant alteration in epididymal sperm count was observed, the possibility of a sub-clinical reduction in sperm production cannot be ruled out.

Our data are consistent with cohort studies indicating that men exposed to PCBs (directly or *in utero*) had a higher percentage of oligospermia, abnormal sperm morphology and reduced sperm penetration capacity (Guo *et al.* 2000; Hsu *et al.* 2003).

In rodents, sperm production can be reduced by up to 90% without compromising fertility (Aafjes *et al.* 1980; Faqi *et al.* 1997), whereas in men, relatively small changes in sperm concentration and quality may have severe consequences because the sperm count is near the critical lower threshold for fertility (Zenick and Clegg 1989).

Of particular concern is the observation that PCB effects on seminiferous tubules distribution and of sperm viability were detected in later generations, up to the F3, an observations consistent with other physiological systems and environmental toxicants (Anway *et al.* 2005; Wolf *et al.* 1999). To our knowledge, this is the first study reporting long-lasting reproductive effects of PCBs spanning three generations in mammals. Nevertheless other environmental chemicals have been shown to induce reproductive abnormalities for multiple generations (Anway *et al.* 2006b). Different mechanisms have been postulated to explain multigenerational reproductive adverse phenotype, including epigenetic changes in the germ-line (Anway *et al.* 2006a).

Specifically, imprinting defects of paternally- or maternally-methylated genes have been associated with altered spermatogenesis (Marques *et al.* 2010; Roeleveld and Bretveld 2008) and have been observed in oligospermic or azoospermic patients.

The mechanisms underlying transmission of PCBs effects observed in the present study could involve intergenerational and/or transgenerational transmission. In fact, considering the congeners half-lives (between 54 and 124 days (Oberg *et al.* 2002), PCB burden in F1

females at time of mating could still be significant. Therefore remaining chemicals could represent a source of direct exposure for both F2 and F3 generations; i.e. intergenerational transmission may be the result of transfer of maternal PCB burdens to the fetuses, in conjunction with preferential accumulation by them. However, since PCB-induced effects were present up to the F3, which is the first on that can exhibit true transgenerational effects (Skinner 2008), transmission to subsequent generations of permanent molecular changes induced in the F1 cannot be ruled out. The fact that adverse, PCB-mediated, reproductive effects in the male were highly consistent, between individuals and amongst litters, suggests that genetic DNA sequence mutations are not the most likely cause (Barber *et al.* 2002; Dong *et al.* 2004). In contrast, epigenetic variations involving the germ-line could result in the high frequency observed (Anway *et al.* 2005). Analyses are required to identify epigenetic patterns which might explain intergenerational transmission of PCB effects.

In the present study no obvious dose-response relationships were found for any of the endpoints analyzed. This may reflect the multiple mechanisms of action of the PCB mixture and the different, sometimes opposite, actions of the congeners. Furthermore, the induction of enzyme activity at high doses may alter toxicokinetics and this, in turn, may change the effective dose at the site of action resulting in non linear dose-response (Kupfer 1987; Li and Hansen 1996).

In conclusion, our study demonstrates that *in utero* and lactational exposure to a congener mixture of PCBs 101 and 118 has multiple effects on the reproductive system in adult offspring of both sexes. In F1, several effects including reductions in gonadal weight, impaired gamete quality and reduced developmental competence were similar in both male and female offspring which may suggest a common mechanism of action in both sexes. The observation that reproductive abnormalities in males occurred in subsequent generations, up to F3, suggests vertical transmission of PCB-mediated adverse effects.

Finally, it needs to be emphasized that the adverse effects have been observed in a dose range relevant to human exposure and that preferential accumulation of PCBs was observed in the offspring, pointing to the progeny as a target of maternal exposure to PCB.

CONCLUSIONS

In the present study, we focused the attention on the possible effects of *in utero* and lactational exposure to two different endocrine disruptors, the plasticizer di(2-ethyl-hexyl) phthalate (DEHP) and a mixture of the two polychlorinated biphenyls congeners PCB 101 and PCB 118, on the reproductive health of adult offspring of both genders in the mouse. Furthermore, possible permanent effects over multiple generations were investigated up to the third generation along the female lineage without further exposure.

Independently of the chemical analyzed, our study demonstrates that pre and perinatal exposure to the selected EDs has multiple effects on the reproductive system in offspring at adult age of both sexes. Specifically, both DEHP and PCBs treated F1 offspring show, at sexual maturity, altered gonad weight and morphology and reduced gamete quality and developmental competence. However, the molecular mechanisms underlying the observed adverse effects depend on the contaminant investigated. In particular, DEHP alters estrogen biosynthesis pathways in both male and female gonads leading to imbalance of pituitary-gonadal cross-talk. In contrast, no molecular alterations in the same endocrine signaling were observed upon exposure to PCBs. To date, no obvious mechanism of action has been identified at molecular level which can clarify the complexity of the adverse phenotype observed in PCB-exposed F1 animals. This can likely be explained by the different, sometimes opposite, mechanism of action exerted by the two congeners investigated, a typical ortho-substituted PCB (PCB 101) and a mono-ortho substituted PCB (PCB 118) which may share some characteristic with dioxin-like PCBs.

Of particular concern is the observation that both EDs classes, besides affecting reproductive health in *in utero* and lactationally exposed offspring, may induce reproductive effects also in later generations, an observation coherent with other physiological system and environmental toxicants.

Independently of the mechanism underlying the inheritance of adverse effects along several generations, the observations that an endocrine disruptor can cause a multigenerational effect on male and female reproduction have a significant impact on our understanding of the potential hazards of these compounds in mammals. Elucidation of the mechanism involved in multigenerational endocrine disruptor actions will undoubtedly provide insights into diagnostics and therapeutics for environmental exposures, risk assessment and adult-onset disease.

Finally, it needs to be emphasized that the adverse effects have been observed in a dose range relevant to human exposure. Data from *in vivo* animal models may be difficult to extrapolate to humans for several reasons, including species differences in ontogeny of reproductive system and functions, differences in metabolism of sex steroids, and variable body burdens. Furthermore, human exposure to EDs is complex, being individuals and populations exposed to environmental compounds as mixtures, rather than individual chemicals, thus the impact on human populations remains to be elucidated in further toxicology studies. Despite these limitations, considering the substantial conservation of endocrine and reproductive processes across species, the results of the present study give great reason for concern and make it clear that more investigations in this field are of a high priority.

REFERENCES

Aafjes, J. H., Vels, J. M., and Schenck, E. (1980). Fertility of rats with artificial oligozoospermia. *J Reprod Fertil* **58**, 345-51.

Agarwal, D. K., Lawrence, W. H., and Autian, J. (1985a). Antifertility and mutagenic effects in mice from parenteral administration of di-2-ethylhexyl phthalate (DEHP). *J Toxicol Environ Health* **16**, 71-84.

Agarwal, D. K., Lawrence, W. H., Turner, J. E., and Autian, J. (1989). Effects of parenteral di-(2-ethylhexyl)phthalate (DEHP) on gonadal biochemistry, pathology, and reproductive performance of mice. *J Toxicol Environ Health* **26**, 39-59.

Agarwal, D. K., Maronpot, R. R., Lamb, J. C. t., and Kluwe, W. M. (1985b). Adverse effects of butyl benzyl phthalate on the reproductive and hematopoietic systems of male rats. *Toxicology* **35**, 189-206.

Akingbemi, B. T., Youker, R. T., Sottas, C. M., Ge, R., Katz, E., Klinefelter, G. R., Zirkin, B. R., and Hardy, M. P. (2001). Modulation of rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. *Biol Reprod* **65**, 1252-9.

Aldyreva, M. V., Klimova, T. S., Iziumova, A. S., and Timofeevskaia, L. A. (1975). [The effect of phthalate plasticizers on the generative function]. *Gig Tr Prof Zabol*, 25-9.

AMAP (2009). AMAP Assessment 2009: Human Health in the Arctic., p. xiv+256. Arctic Monitoring and Assessment Programme (AMAP), Oslo, Norway.

Andrade, A. J., Grande, S. W., Talsness, C. E., Grote, K., and Chahoud, I. (2006). A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity. *Toxicology* **227**, 185-92.

Anway, M. D., Cupp, A. S., Uzumcu, M., and Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308**, 1466-9.

Anway, M. D., Leathers, C., and Skinner, M. K. (2006a). Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. *Endocrinology* **147**, 5515-23.

Anway, M. D., Memon, M. A., Uzumcu, M., and Skinner, M. K. (2006b). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J Androl* **27**, 868-79.

Anway, M. D., and Skinner, M. K. (2006). Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology* **147**, S43-9.

Arcadi, F. A., Costa, C., Imperatore, C., Marchese, A., Rapisarda, A., Salemi, M., Trimarchi, G. R., and Costa, G. (1998). Oral toxicity of bis(2-ethylhexyl) phthalate during pregnancy and suckling in the Long-Evans rat. *Food Chem Toxicol* **36**, 963-70.

ATSDR (2004). Annual Report. Agency for Toxic Substances and Disease Registry, Atlanta.

Baldridge, M. G., Stahl, R. L., Gerstenberger, S. L., Tripoli, V., and Hutz, R. J. (2003). Modulation of ovarian follicle maturation in Long-Evans rats exposed to polychlorinated biphenyls (PCBs) in-utero and lactationally. *Reprod Toxicol* **17**, 567-73.

Barber, R., Plumb, M. A., Boulton, E., Roux, I., and Dubrova, Y. E. (2002). Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice. *Proc Natl Acad Sci U S A* **99**, 6877-82.

Barlow, N. J., and Foster, P. M. (2003). Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to Di(n-butyl) phthalate. *Toxicol Pathol* **31**, 397-410.

Bern, H. A. (1992). The development of the role of hormones in development--a double remembrance. *Endocrinology* **131**, 2037-8.

Bernhoft, A., Wiig, O., and Skaare, J. U. (1997). Organochlorines in polar bears (Ursus maritimus) at Svalbard. *Environ Pollut* **95**, 159-75.

Birkett, J. W. (2003). *Scope of the problem*. Lewis Publishers, London.

Bishop-Bailey, D., and Hla, T. (1999). Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-Delta12, 14-prostaglandin J2. *J Biol Chem* **274**, 17042-8.

Blount, B. C., Silva, M. J., Caudill, S. P., Needham, L. L., Pirkle, J. L., Sampson, E. J., Lucier, G. W., Jackson, R. J., and Brock, J. W. (2000). Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* **108**, 979-82.

Bolon, B., Bucci, T. J., Warbritton, A. R., Chen, J. J., Mattison, D. R., and Heindel, J. J. (1997). Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. *Fundam Appl Toxicol* **39**, 1-10.

Borch, J., Axelstad, M., Vinggaard, A. M., and Dalgaard, M. (2006). Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis. *Toxicol Lett* **163**, 183-90.

Bosnir, J., Puntaric, D., Skes, I., Klaric, M., Simic, S., and Zoric, I. (2003). Migration of phthalates from plastic products to model solutions. *Coll Antropol* **27 Suppl 1**, 23-30.

Bradlow, H. L., Davis, D. L., Lin, G., Sepkovic, D., and Tiwari, R. (1995). Effects of pesticides on the ratio of 16 alpha/2-hydroxyestrone: a biologic marker of breast cancer risk. *Environ Health Perspect* **103 Suppl 7**, 147-50.

Brouwer, A., Ahlborg, U. G., Van den Berg, M., Birnbaum, L. S., Boersma, E. R., Bosveld, B., Denison, M. S., Gray, L. E., Hagmar, L., Holene, E., and et al. (1995). Functional aspects of developmental toxicity of polyhalogenated aromatic hydrocarbons in experimental animals and human infants. *Eur J Pharmacol* **293**, 1-40.

Bucci, T. J., Bolon, B., Warbritton, A. R., Chen, J. J., and Heindel, J. J. (1997). Influence of sampling on the reproducibility of ovarian follicle counts in mouse toxicity studies. *Reprod Toxicol* **11**, 689-96.

Calafat, A. M., and Needham, L. L. (2008). Factors affecting the evaluation of biomonitoring data for human exposure assessment. *Int J Androl* **31**, 139-43.

Campagna, C., Guillemette, C., Paradis, R., Sirard, M. A., Ayotte, P., and Bailey, J. L. (2002). An environmentally relevant organochlorine mixture impairs sperm function and embryo development in the porcine model. *Biol Reprod* **67**, 80-7.

Caserta, D., Maranghi, L., Mantovani, A., Marci, R., Maranghi, F., and Moscarini, M. (2008). Impact of endocrine disruptor chemicals in gynaecology. *Hum Reprod Update* **14**, 59-72.

Cerna, M., Maly, M., Grabic, R., Batariova, A., Smid, J., and Benes, B. (2008). Serum concentrations of indicator PCB congeners in the Czech adult population. *Chemosphere* **72**, 1124-31.

Chiu, A., Beaubier, J., Chiu, J., Chan, L., and Gerstenberger, S. (2004). Epidemiologic studies of PCB congener profiles in North American fish consuming populations. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **22**, 13-36.

Coffin, J. D., and Poole, T. J. (1991). Endothelial cell origin and migration in embryonic heart and cranial blood vessel development. *Anat Rec* **231**, 383-95.

Colborn, T., vom Saal, F. S., and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* **101**, 378-84.

Colciago, A., Negri-Cesi, P., Pravettoni, A., Mornati, O., Casati, L., and Celotti, F. (2006). Prenatal Aroclor 1254 exposure and brain sexual differentiation: effect on the expression of testosterone metabolizing enzymes and androgen receptors in the hypothalamus of male and female rats. *Reprod Toxicol* **22**, 738-45.

Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balaguer, P. (1997). Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. *Toxicol Appl Pharmacol* **145**, 111-23.

Coulam, C. B., Adamson, S. C., and Annegers, J. F. (1986). Incidence of premature ovarian failure. *Obstet Gynecol* **67**, 604-6.

Cramer, D. W., and Xu, H. (1996). Predicting age at menopause. *Maturitas* **23**, 319-26.

Creemers, L. B., Meng, X., den Ouden, K., van Pelt, A. M., Izadyar, F., Santoro, M., Sariola, H., and de Rooij, D. G. (2002). Transplantation of germ cells from glial cell line-derived neurotrophic factor-overexpressing mice to host testes depleted of endogenous spermatogenesis by fractionated irradiation. *Biol Reprod* **66**, 1579-84.

Daikoku, T., and Dey, S. K. (2008). Two faces of PTEN. *Nat Med* **14**, 1192-3.

Dallaire, F., Dewailly, E., Vezina, C., Muckle, G., Weber, J. P., Bruneau, S., and Ayotte, P. (2006). Effect of prenatal exposure to polychlorinated biphenyls on incidence of acute respiratory infections in preschool Inuit children. *Environ Health Perspect* **114**, 1301-5.

Dalman, A., Eimani, H., Sepehri, H., Ashtiani, S. K., Valojerdi, M. R., Eftekhari-Yazdi, P., and Shahverdi, A. (2008). Effect of mono-(2-ethylhexyl) phthalate (MEHP) on resumption of meiosis, in vitro maturation and embryo development of immature mouse oocytes. *Biofactors* **33**, 149-55.

Danilovich, N., Javeshghani, D., Xing, W., and Sairam, M. R. (2002). Endocrine alterations and signaling changes associated with declining ovarian function and advanced biological aging in follicle-stimulating hormone receptor haploinsufficient mice. *Biol Reprod* **67**, 370-8.

Danilovich, N., and Sairam, M. R. (2002). Haploinsufficiency of the follicle-stimulating hormone receptor accelerates oocyte loss inducing early reproductive senescence and biological aging in mice. *Biol Reprod* **67**, 361-9.

Danzo, B. J. (1998). The effects of environmental hormones on reproduction. *Cell Mol Life Sci* **54**, 1249-64.

Davis, B. J., Maronpot, R. R., and Heindel, J. J. (1994). Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol* **128**, 216-23.

Depledge, M., Galloway, T., and Billinghurst, Z. (1999). Issues in Environmental Science and Technology. In Endocrine Disrupting Chemicals (R. H. a. R. Harrison, ed., Vol. 12. The Royal Society of Chemistry, Thomas Graham House, Cambridge.

Dietert, R. R. (2009). Developmental immunotoxicity (DIT), postnatal immune dysfunction and childhood leukemia. *Blood Cells Mol Dis* **42**, 108-12.

Dietert, R. R., and Dietert, J. M. (2008a). Possible role for early-life immune insult including developmental immunotoxicity in chronic fatigue syndrome (CFS) or myalgic encephalomyelitis (ME). *Toxicology* **247**, 61-72.

Dietert, R. R., and Dietert, J. M. (2008b). Potential for early-life immune insult including developmental immunotoxicity in autism and autism spectrum disorders: focus on critical windows of immune vulnerability. *J Toxicol Environ Health B Crit Rev* **11**, 660-80.

Dietert, R. R., and Zelikoff, J. T. (2008). Early-life environment, developmental immunotoxicology, and the risk of pediatric allergic disease including asthma. *Birth Defects Res B Dev Reprod Toxicol* **83**, 547-60.

Dong, H., Bonala, R. R., Suzuki, N., Johnson, F., Grollman, A. P., and Shibutani, S. (2004). Mutagenic potential of benzo[a]pyrene-derived DNA adducts positioned in codon 273 of the human P53 gene. *Biochemistry* **43**, 15922-8.

Dostal, L. A., Weaver, R. P., and Schwetz, B. A. (1987). Transfer of di(2-ethylhexyl) phthalate through rat milk and effects on milk composition and the mammary gland. *Toxicol Appl Pharmacol* **91**, 315-25.

EFSA (2005). Statement of the Scientific Panel on Food Additives and Materials in Contact with food on a request from the Commission on the possibility of allocating a

group TDI for BBP, DBP, DEHP, DINP, and DIDP. European Food Safety Authority, Parma, Italy.

Eimani, H., Dalman, A., Sepehri, H., Kazemi, S., Hassani, F., Baharvand, H., and Shahverdi, A. (2005). Effect of DEHP (di(2-ethylhexyl) phthalate) on resumtion of meiosis and in vitro maturation of mouse oocytes and development of resulting embryos. *Yakhteh Medical Journal* **7**, 56-61.

Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., and Fujii-Kuriyama, Y. (1994). Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. *J Biol Chem* **269**, 27337-43.

Eyster, J. T., Humphrey, H. E., and Kimbrough, R. D. (1983). Partitioning of polybrominated biphenyls (PBBs) in serum, adipose tissue, breast milk, placenta, cord blood, biliary fluid, and feces. *Arch Environ Health* **38**, 47-53.

Fan, H. Y., Liu, Z., Cahill, N., and Richards, J. S. (2008). Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. *Mol Endocrinol* **22**, 2128-40.

Faqi, A. S., Klug, A., Merker, H. J., and Chahoud, I. (1997). Ganciclovir induces reproductive hazards in male rats after short-term exposure. *Hum Exp Toxicol* **16**, 505-11.

Fernie, K., Bortolotti, G., Drouillard, K., Smits, J., and Marchant, T. (2003). Developmental toxicity of in ovo exposure to polychlorinated biphenyls: II. Effects of maternal or paternal exposure on second-generation nestling american kestrels. *Environ Toxicol Chem* **22**, 2688-94.

Fischer, L. J., Seegal, R. F., Ganey, P. E., Pessah, I. N., and Kodavanti, P. R. (1998). Symposium overview: toxicity of non-coplanar PCBs. *Toxicol Sci* **41**, 49-61.

Flaws, J. A., Langenberg, P., Babus, J. K., Hirshfield, A. N., and Sharara, F. I. (2001). Ovarian volume and antral follicle counts as indicators of menopausal status. *Menopause* **8**, 175-80.

Fowler, P. A., Anderson, R. A., Saunders, P. T., Kinnell, H., Mason, J. I., Evans, D. B., Bhattacharya, S., Flannigan, S., Franks, S., Monteiro, A., and O'Shaughnessy, P. J. (2011). Development of Steroid Signaling Pathways during Primordial Follicle Formation in the Human Fetal Ovary. *J Clin Endocrinol Metab* **96**, 1754-62.

Gallavan, R. H., Jr., Holson, J. F., Stump, D. G., Knapp, J. F., and Reynolds, V. L. (1999). Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. *Reprod Toxicol* **13**, 383-90.

Gallicchio, L., Miller, S., Greene, T., Zacur, H., and Flaws, J. A. (2009). Premature ovarian failure among hairdressers. *Hum Reprod* **24**, 2636-41.

Giusti, R. M., Iwamoto, K., and Hatch, E. E. (1995). Diethylstilbestrol revisited: a review of the long-term health effects. *Ann Intern Med* **122**, 778-88.

Gore, A. C. (2008). Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Front Neuroendocrinol* **29**, 358-74.

Gore, A. C. (2010). Neuroendocrine targets of endocrine disruptors. *Hormones (Athens)* **9**, 16-27.

Gosden, R. G., Treloar, S. A., Martin, N. G., Cherkas, L. F., Spector, T. D., Faddy, M. J., and Silber, S. J. (2007). Prevalence of premature ovarian failure in monozygotic and dizygotic twins. *Hum Reprod* **22**, 610-5.

Goswami, D., and Conway, G. S. (2005). Premature ovarian failure. *Hum Reprod Update* **11**, 391-410.

Grande, S. W., Andrade, A. J., Talsness, C. E., Grote, K., and Chahoud, I. (2006). A dose-response study following in utero and lactational exposure to di(2-ethylhexyl)phthalate: effects on female rat reproductive development. *Toxicol Sci* **91**, 247-54.

Gray, L. E., Jr., Laskey, J., and Ostby, J. (2006). Chronic di-n-butyl phthalate exposure in rats reduces fertility and alters ovarian function during pregnancy in female Long Evans hooded rats. *Toxicol Sci* **93**, 189-95.

Guerrero-Bosagna, C. M., and Skinner, M. K. (2009). Epigenetic transgenerational effects of endocrine disruptors on male reproduction. *Semin Reprod Med* **27**, 403-8.

Guo, Y. L., Hsu, P. C., Hsu, C. C., and Lambert, G. H. (2000). Semen quality after prenatal exposure to polychlorinated biphenyls and dibenzofurans. *Lancet* **356**, 1240-1.

Gupta, R. K., Singh, J. M., Leslie, T. C., Meachum, S., Flaws, J. A., and Yao, H. H. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. *Toxicol Appl Pharmacol* **242**, 224-30.

Gupta, R. K., Singh, J. M., Leslie, T. C., Meachum, S., Flaws, J. A., and Yao, H. H. (2010). Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. *Toxicol Appl Pharmacol* **242**, 224-30.

Guvenius, D. M., Aronsson, A., Ekman-Ordeberg, G., Bergman, A., and Noren, K. (2003). Human prenatal and postnatal exposure to polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorobiphenylols, and pentachlorophenol. *Environ Health Perspect* **111**, 1235-41.

Hamilton, B. E., and Ventura, S. J. (2006). Fertility and abortion rates in the United States, 1960-2002. *Int J Androl* **29**, 34-45.

Hauser, R., Chen, Z., Pothier, L., Ryan, L., and Altshul, L. (2003). The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. *Environ Health Perspect* **111**, 1505-11.

Hauser, R., Williams, P., Altshul, L., and Calafat, A. M. (2005). Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. *Environ Health Perspect* **113**, 425-30.

Heilmann, C., Grandjean, P., Weihe, P., Nielsen, F., and Budtz-Jorgensen, E. (2006). Reduced antibody responses to vaccinations in children exposed to polychlorinated biphenyls. *PLoS Med* **3**, e311.

Hertz-Picciotto, I., Park, H., Dostal, M., Kocan, A., Trnovec, T., and Sram, R. (2008). Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development. *Basic Clin Pharmacol Toxicol.* **102**, 146-154.

Hess, R. A., Bunick, D., Lee, K. H., Bahr, J., Taylor, J. A., Korach, K. S., and Lubahn, D. B. (1997). A role for oestrogens in the male reproductive system. *Nature* **390**, 509-12.

Hoffman, Y. M., Peegel, H., Sprock, M. J., Zhang, Q. Y., and Menon, K. M. (1991). Evidence that human chorionic gonadotropin/luteinizing hormone receptor down-regulation involves decreased levels of receptor messenger ribonucleic acid. *Endocrinology* **128**, 388-93.

Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M., and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. *J Biol Chem* **284**, 28729-37.

Horiguchi, T. (2006). Masculinization of female gastropod mollusks induced by organotin compounds, focusing on mechanism of actions of tributyltin and triphenyltin for development of imposex. *Environ Sci* **13**, 77-87.

Hotchkiss, A. K., Rider, C. V., Blystone, C. R., Wilson, V. S., Hartig, P. C., Ankley, G. T., Foster, P. M., Gray, C. L., and Gray, L. E. (2008). Fifteen years after "Wingspread"--environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol Sci* **105**, 235-59.

Hough, S. R., Clements, I., Welch, P. J., and Wiederholt, K. A. (2006). Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. *Stem Cells* **24**, 1467-75.

Howdeshell, K. L., Furr, J., Lambright, C. R., Rider, C. V., Wilson, V. S., and Gray, L. E., Jr. (2007). Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. *Toxicol Sci* **99**, 190-202.

Hsu, P. C., Huang, W., Yao, W. J., Wu, M. H., Guo, Y. L., and Lambert, G. H. (2003). Sperm changes in men exposed to polychlorinated biphenyls and dibenzofurans. *Jama* **289**, 2943-4.

Hsu, P. C., Pan, M. H., Li, L. A., Chen, C. J., Tsai, S. S., and Guo, Y. L. (2007). Exposure in utero to 2,2',3,3',4,6'-hexachlorobiphenyl (PCB 132) impairs sperm function and alters testicular apoptosis-related gene expression in rat offspring. *Toxicol Appl Pharmacol* **221**, 68-75.

Hyslop, L., Stojkovic, M., Armstrong, L., Walter, T., Stojkovic, P., Przyborski, S., Herbert, M., Murdoch, A., Strachan, T., and Lako, M. (2005). Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. *Stem Cells* **23**, 1035-43.

IARC (1998). Evaluation of Carcinogenic Risks to Humans (I. Monographs, ed.), pp. 369-373, Supplement 7.

ICES (1992). A compilation of standards and guidance values for contaminants in fish, crustaceans and molluscs for the assessment of possible hazards to human health. Document JMG 17/3/10E. International Council for the Exploration of the Seas.

Iguchi, T., Watanabe, H., Ohta, Y., and Blumberg, B. (2008). Developmental effects: oestrogen-induced vaginal changes and organotin-induced adipogenesis. *Int J Androl* **31**, 263-8.

Jacobson, J. L., Fein, G. G., Jacobson, S. W., Schwartz, P. M., and Dowler, J. K. (1984). The transfer of polychlorinated biphenyls (PCBs) and polybrominated biphenyls

(PBBs) across the human placenta and into maternal milk. *Am J Public Health* **74**, 378-9.

Jensen, T. K., Sobotka, T., Hansen, M. A., Pedersen, A. T., Lutz, W., and Skakkebaek, N. E. (2008). Declining trends in conception rates in recent birth cohorts of native Danish women: a possible role of deteriorating male reproductive health. *Int J Androl* **31**, 81-92.

Jirtle, R. L., and Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nat Rev Genet* **8**, 253-62.

Johnson, M. S., Thomson, S. C., and Speakman, J. R. (2001). Limits to sustained energy intake. I. Lactation in the laboratory mouse Mus musculus. *J Exp Biol* **204**, 1925-35.

Jonsson, H. T., Jr., Keil, J. E., Gaddy, R. G., Loadholt, C. B., Hennigar, G. R., and Walker, E. M., Jr. (1975). Prolonged ingestion of commercial DDT and PCB; effects on progesterone levels and reproduction in the mature female rat. *Arch Environ Contam Toxicol* **3**, 479-90.

Kavlock, R., Boekelheide, K., Chapin, R., Cunningham, M., Faustman, E., Foster, P., Golub, M., Henderson, R., Hinberg, I., Little, R., Seed, J., Shea, K., Tabacova, S., Tyl, R., Williams, P., and Zacharewski, T. (2002). NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reprod Toxicol* **16**, 529-653.

Kaya, H., Hany, J., Fastabend, A., Roth-Harer, A., Winneke, G., and Lilienthal, H. (2002). Effects of maternal exposure to a reconstituted mixture of polychlorinated biphenyls on sex-dependent behaviors and steroid hormone concentrations in rats: dose-response relationship. *Toxicol Appl Pharmacol* **178**, 71-81.

Kester, M. H., Bulduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C. N., Coughtrie, M. W., Bergman, A., Safe, S. H., Kuiper, G. G., Schuur, A. G., Brouwer, A., and Visser, T. J. (2000). Potent inhibition of estrogen sulfotransferase by hydroxylated PCB

metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* **141**, 1897-900.

Kholkute, S. D., and Dukelow, W. R. (1997). Effects of polychlorinated biphenyl (PCB) mixtures on in vitro fertilization in the mouse. *Bull Environ Contam Toxicol* **59**, 531-6.

Kholkute, S. D., Rodriguez, J., and Dukelow, W. R. (1994a). Effects of polychlorinated biphenyls (PCBs) on in vitro fertilization in the mouse. *Reprod Toxicol* **8**, 69-73.

Kholkute, S. D., Rodriguez, J., and Dukelow, W. R. (1994b). Reproductive toxicity of Aroclor-1254: effects on oocyte, spermatozoa, in vitro fertilization, and embryo development in the mouse. *Reprod Toxicol* **8**, 487-93.

Kim, H. S., Saito, K., Ishizuka, M., Kazusaka, A., and Fujita, S. (2003). Short period exposure to di-(2-ethylhexyl) phthalate regulates testosterone metabolism in testis of prepubertal rats. *Arch Toxicol* **77**, 446-51.

Kim, K. (2009). Transgenerational changes after embryonic exposure to plasticizer phthalates. In PPTOXII: Role of Environmental Stressors in the Developmental Origins of Disease, Vol. 45, Miami, Florida.

Kim, S. H. (1995). Female aging and superovulation induction for IVF. *J Obstet Gynaecol (Tokyo 1995)* **21**, 75-82.

Kishi, H., Minegishi, T., Tano, M., Abe, Y., Ibuki, Y., and Miyamoto, K. (1997). Down-regulation of LH/hCG receptor in rat cultured granulosa cells. *FEBS Lett* **402**, 198-202.

Kiviranta, H., Tuomisto, J. T., Tuomisto, J., Tukiainen, E., and Vartiainen, T. (2005). Polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in the general population in Finland. *Chemosphere* **60**, 854-69.

Koch, H. M., Drexler, H., and Angerer, J. (2003). An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. *Int J Hyg Environ Health* **206**, 77-83.

Koch, H. M., Preuss, R., and Angerer, J. (2006). Di(2-ethylhexyl)phthalate (DEHP): human metabolism and internal exposure-- an update and latest results. *Int J Androl* **29**, 155-65; discussion 181-5.

Koruji, M., Movahedin, M., Mowla, S. J., Gourabi, H., and Arfaee, A. J. (2008). The morphological changes of adult mouse testes after 60Co gamma-Radiation. *Iran Biomed J* **12**, 35-42.

Kovacs, A., and Foote, R. H. (1992). Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotech Histochem* **67**, 119-24.

Kraugerud, M., Aleksandersen, M., Nyengaard, J. R., Ostby, G. C., Gutleb, A. C., Dahl, E., Berg, V., Farstad, W., Schweder, T., Skaare, J. U., and Ropstad, E. (2011). In utero and lactational exposure to PCB 118 and PCB 153 alter ovarian follicular dynamics and GnRH-induced luteinizing hormone secretion in female lambs. *Environ Toxicol*.

Krogenaes, A. K., Nafstad, I., Skare, J. U., Farstad, W., and Hafne, A. L. (1998). In vitro reproductive toxicity of polychlorinated biphenyl congeners 153 and 126. *Reprod Toxicol* **12**, 575-80.

Kuchenhoff, A., Eckard, R., Buff, K., and Fischer, B. (1999). Stage-specific effects of defined mixtures of polychlorinated biphenyls on in vitro development of rabbit preimplantation embryos. *Mol Reprod Dev* **54**, 126-34.

Kupfer, D. (1987). Critical evaluation of methods for detection and assessment of estrogenic compounds in mammals: strengths and limitations for application to risk assessment. *Reprod Toxicol* **1**, 147-53.

Kuriyama, S. N., and Chahoud, I. (2004). In utero exposure to low-dose 2,3',4,4',5-pentachlorobiphenyl (PCB 118) impairs male fertility and alters neurobehavior in rat offspring. *Toxicology* **202**, 185-97.

Lahousse, S. A., Wallace, D. G., Liu, D., Gaido, K. W., and Johnson, K. J. (2006). Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl)

phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. *Toxicol Sci* **93**, 369-81.

LaPolt, P. S., Oikawa, M., Jia, X. C., Dargan, C., and Hsueh, A. J. (1990). Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and luteinization. *Endocrinology* **126**, 3277-9.

Latini, G., De Felice, C., Presta, G., Del Vecchio, A., Paris, I., Ruggieri, F., and Mazzeo, P. (2003). In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. *Environ Health Perspect* **111**, 1783-5.

Lazaros, L., Xita, N., Kaponis, A., Hatzi, E., Plachouras, N., Sofikitis, N., Zikopoulos, K., and Georgiou, I. (2011). The association of aromatase (CYP19) gene variants with sperm concentration and motility. *Asian J Androl* **13**, 292-7.

Lehmann, K. P., Phillips, S., Sar, M., Foster, P. M., and Gaido, K. W. (2004). Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. *Toxicol Sci* **81**, 60-8.

Lerner, S. P., Thayne, W. V., Baker, R. D., Henschen, T., Meredith, S., Inskeep, E. K., Dailey, R. A., Lewis, P. E., and Butcher, R. L. (1986). Age, dose of FSH and other factors affecting superovulation in Holstein cows. *J Anim Sci* **63**, 176-83.

Li, L. H., Jester, W. F., Jr., Laslett, A. L., and Orth, J. M. (2000). A single dose of Di-(2-ethylhexyl) phthalate in neonatal rats alters gonocytes, reduces sertoli cell proliferation, and decreases cyclin D2 expression. *Toxicol Appl Pharmacol* **166**, 222-9.

Li, M. H., and Hansen, L. G. (1996). Enzyme induction and acute endocrine effects in prepubertal female rats receiving environmental PCB/PCDF/PCDD mixtures. *Environ Health Perspect* **104**, 712-22.

Lie, E., Larsen, H. J., Larsen, S., Johansen, G. M., Derocher, A. E., Lunn, N. J., Norstrom, R. J., Wiig, O., and Skaare, J. U. (2004). Does high organochlorine (OC) exposure

impair the resistance to infection in polar bears (Ursus maritimus)? Part I: Effect of OCs on the humoral immunity. *J Toxicol Environ Health A* **67**, 555-82.

Lindenau, A., and Fischer, B. (1996). Embryotoxicity of polychlorinated biphenyls (PCBS) for preimplantation embryos. *Reprod Toxicol* **10**, 227-30.

Loganathan BG, and K, K. (1994). Global Organochlorine Contamination trends: an overview. *Ambio* **23**, 187-191.

Lovekamp, T. N., and Davis, B. J. (2001). Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. *Toxicol Appl Pharmacol* **172**, 217-24.

Lovekamp-Swan, T., and Davis, B. J. (2003). Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ Health Perspect* **111**, 139-45.

Lu, D. L., Peegel, H., Mosier, S. M., and Menon, K. M. (1993). Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced down-regulation occurs post transcriptionally. *Endocrinology* **132**, 235-40.

Luborsky, J. L., Meyer, P., Sowers, M. F., Gold, E. B., and Santoro, N. (2003). Premature menopause in a multi-ethnic population study of the menopause transition. *Hum Reprod* **18**, 199-206.

Lundkvist, U. (1990). Clinical and reproductive effects of Clophen A50 (PCB) administered during gestation on pregnant guinea pigs and their offspring. *Toxicology* **61**, 249-57.

Ma, M., Kondo, T., Ban, S., Umemura, T., Kurahashi, N., Takeda, M., and Kishi, R. (2006). Exposure of prepubertal female rats to inhaled di(2-ethylhexyl)phthalate affects the onset of puberty and postpubertal reproductive functions. *Toxicol Sci* **93**, 164-71.

MacKenzie, K. M., and Angevine, D. M. (1981). Infertility in mice exposed in utero to benzo(a)pyrene. *Biol Reprod* **24**, 183-91.

Maguire, S. M., Tribley, W. A., and Griswold, M. D. (1997). Follicle-stimulating hormone (FSH) regulates the expression of FSH receptor messenger ribonucleic acid in cultured Sertoli cells and in hypophysectomized rat testis. *Biol Reprod* **56**, 1106-11.

Marques, C. J., Francisco, T., Sousa, S., Carvalho, F., Barros, A., and Sousa, M. (2010). Methylation defects of imprinted genes in human testicular spermatozoa. *Fertil Steril* **94**, 585-94.

Marsman, D. (1995). NTP technical report on the toxicity studies of Dibutyl Phthalate (CAS No. 84-74-2) Administered in Feed to F344/N Rats and B6C3F1 Mice. *Toxic Rep Ser* **30**, 1-G5.

Martino-Andrade, A. J., and Chahoud, I. (2010). Reproductive toxicity of phthalate esters. *Mol Nutr Food Res* **54**, 148-57.

Massaad, C., Entezami, F., Massade, L., Benahmed, M., Olivennes, F., Barouki, R., and Hamamah, S. (2002). How can chemical compounds alter human fertility? *Eur J Obstet Gynecol Reprod Biol* **100**, 127-37.

McFarland, V. A., and Clarke, J. U. (1989). Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. *Environ Health Perspect* **81**, 225-39.

McKee, R. H., Butala, J. H., David, R. M., and Gans, G. (2004). NTP center for the evaluation of risks to human reproduction reports on phthalates: addressing the data gaps. *Reprod Toxicol* **18**, 1-22.

McLachlan, J. A. (2001). Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocr Rev* **22**, 319-41.

McLachlan, M. S. (1993). Digestive tract absorption of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in a nursing infant. *Toxicol Appl Pharmacol* **123**, 68-72.

Meeker, J. D., Calafat, A. M., and Hauser, R. (2009). Urinary metabolites of di(2-ethylhexyl) phthalate are associated with decreased steroid hormone levels in adult men. *J Androl* **30**, 287-97.

Mendola, P., Buck, G. M., Sever, L. E., Zielezny, M., and Vena, J. E. (1997). Consumption of PCB-contaminated freshwater fish and shortened menstrual cycle length. *Am J Epidemiol* **146**, 955-60.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631-42.

Mlynarcikova, A., Nagyova, E., Fickova, M., and Scsukova, S. (2009). Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes. *Toxicol In Vitro* **23**, 371-7.

Moore, R. W., Rudy, T. A., Lin, T. M., Ko, K., and Peterson, R. E. (2001). Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. *Environ Health Perspect* **109**, 229-37.

Murata, T., He, S., Hangai, M., Ishibashi, T., Xi, X. P., Kim, S., Hsueh, W. A., Ryan, S. J., Law, R. E., and Hinton, D. R. (2000). Peroxisome proliferator-activated receptor-gamma ligands inhibit choroidal neovascularization. *Invest Ophthalmol Vis Sci* **41**, 2309-17.

Murphy, B. D., and Dobias, M. (1999). Homologous and heterologous ligands downregulate follicle-stimulating hormone receptor mRNA in porcine granulosa cells. *Mol Reprod Dev* **53**, 198-207.

Mustafa, A., Holladay, S. D., Witonsky, S., Sponenberg, D. P., Karpuzoglu, E., and Gogal, R. M., Jr. (2011). A single mid-gestation exposure to TCDD yields a postnatal

autoimmune signature, differing by sex, in early geriatric C57BL/6 mice. *Toxicology* **290**, 157-69.

Mylchreest, E., Sar, M., Wallace, D. G., and Foster, P. M. (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reprod Toxicol* **16**, 19-28.

Newbold, R. R. (2011). Developmental exposure to endocrine-disrupting chemicals programs for reproductive tract alterations and obesity later in life. *Am J Clin Nutr* **94**, 1939S-42S.

Nilsson, E. E., Anway, M. D., Stanfield, J., and Skinner, M. K. (2008). Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. *Reproduction* **135**, 713-21.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* **123**, 917-29.

Noda, M., Ohno, S., and Nakajin, S. (2007). Mono-(2-ethylhexyl) phthalate (MEHP) induces nuclear receptor 4A subfamily in NCI-H295R cells: a possible mechanism of aromatase suppression by MEHP. *Mol Cell Endocrinol* **274**, 8-18.

NTP (1984). Di(2-ethylhexyl)phthalate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. Final Report. In NTP-84-079. National Toxicology Program, Research Triangle Park, NC.

Oberg, M., Sjodin, A., Casabona, H., Nordgren, I., Klasson-Wehler, E., and Hakansson, H. (2002). Tissue distribution and half-lives of individual polychlorinated biphenyls and serum levels of 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl in the rat. *Toxicol Sci* **70**, 171-82.

Olea, N., and Fernandez, M. F. (2007). Chemicals in the environment and human male fertility. *Occup Environ Med* **64**, 430-1.

O'Shaughnessy, P. J., and Fowler, P. A. (2011). Endocrinology of the mammalian fetal testis. *Reproduction* **141**, 37-46.

Osteen, K. (2009). Transgenerational effect of dioxin on the endometriosis phenotype. In PPTOXII: Role of Environmental Stressors in the Developmental Origins of Disease., Vol. 43-44, Miami, Florida.

Pan, G., and Thomson, J. A. (2007). Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res* **17**, 42-9.

Panigrahy, D., Singer, S., Shen, L. Q., Butterfield, C. E., Freedman, D. A., Chen, E. J., Moses, M. A., Kilroy, S., Duensing, S., Fletcher, C., Fletcher, J. A., Hlatky, L., Hahnfeldt, P., Folkman, J., and Kaipainen, A. (2002). PPARgamma ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *J Clin Invest* **110**, 923-32.

Pauwels, A., Covaci, A., Delbeke, L., Punjabi, U., and Schepens, P. J. (1999). The relation between levels of selected PCB congeners in human serum and follicular fluid. *Chemosphere* **39**, 2433-41.

Peegel, H., Randolph, J., Jr., Midgley, A. R., and Menon, K. M. (1994). In situ hybridization of luteinizing hormone/human chorionic gonadotropin receptor messenger ribonucleic acid during hormone-induced down-regulation and the subsequent recovery in rat corpus luteum. *Endocrinology* **135**, 1044-51.

Pentikainen, V., Erkkila, K., Suomalainen, L., Parvinen, M., and Dunkel, L. (2000). Estradiol acts as a germ cell survival factor in the human testis in vitro. *J Clin Endocrinol Metab* **85**, 2057-67.

Petersen, J. H., and Breindahl, T. (2000). Plasticizers in total diet samples, baby food and infant formulae. *Food Addit Contam* **17**, 133-41.

Pocar, P., Augustin, R., and Fischer, B. (2004). Constitutive expression of CYP1A1 in bovine cumulus oocyte-complexes in vitro: mechanisms and biological implications. *Endocrinology* **145**, 1594-601.

Pocar, P., Fiandanese, N., Secchi, C., Berrini, A., Fischer, B., Schmidt, J. S., Schaedlich, K., Rhind, S. M., Zhang, Z., and Borromeo, V. (2011). Effects of Polychlorinated Biphenyls In Cd-1 Mice: Reproductive Toxicity And Intergenerational Transmission. *Toxicol Sci*, Ahead of print.

Pocar, P., Perazzoli, F., Luciano, A. M., and Gandolfi, F. (2001). In vitro reproductive toxicity of polychlorinated biphenyls: effects on oocyte maturation and developmental competence in cattle. *Mol Reprod Dev* **58**, 411-6.

Porte, C., Janer, G., Lorusso, L. C., Ortiz-Zarragoitia, M., Cajaraville, M. P., Fossi, M. C., and Canesi, L. (2006). Endocrine disruptors in marine organisms: approaches and perspectives. *Comp Biochem Physiol C Toxicol Pharmacol* **143**, 303-15.

Ralston, A., Cox, B. J., Nishioka, N., Sasaki, H., Chea, E., Rugg-Gunn, P., Guo, G., Robson, P., Draper, J. S., and Rossant, J. (2010). Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development* **137**, 395-403.

Rao, R. P., and Kaliwal, B. B. (2002). Monocrotophos induced dysfunction on estrous cycle and follicular development in mice. *Ind Health* **40**, 237-44.

Reddy, P., Liu, L., Adhikari, D., Jagarlamudi, K., Rajareddy, S., Shen, Y., Du, C., Tang, W., Hamalainen, T., Peng, S. L., Lan, Z. J., Cooney, A. J., Huhtaniemi, I., and Liu, K. (2008). Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science* **319**, 611-3.

Reinsberg, J., Wegener-Toper, P., van der Ven, K., van der Ven, H., and Klingmueller, D. (2009). Effect of mono-(2-ethylhexyl) phthalate on steroid production of human granulosa cells. *Toxicol Appl Pharmacol* **239**, 116-23.

Rhind, S. M., Kyle, C. E., Mackie, C., and McDonald, L. (2009). Accumulation of endocrine disrupting compounds in sheep fetal and maternal liver tissue following exposure to pastures treated with sewage sludge. *J Environ Monit* **11**, 1469-76.

Rhind, S. M., Kyle, C. E., Mackie, C., McDonald, L., Zhang, Z., Duff, E. I., Bellingham, M., Amezaga, M. R., Mandon-Pepin, B., Loup, B., Cotinot, C., Evans, N. P., Sharpe, R. M., and Fowler, P. A. (2010). Maternal and fetal tissue accumulation of selected endocrine disrupting compounds (EDCs) following exposure to sewage sludge-treated pastures before or after conception. *J Environ Monit* **12**, 1582-93.

Richthoff, J., Rylander, L., Jonsson, B. A., Akesson, H., Hagmar, L., Nilsson-Ehle, P., Stridsberg, M., and Giwercman, A. (2003). Serum levels of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) in relation to markers of reproductive function in young males from the general Swedish population. *Environ Health Perspect* **111**, 409-13.

Robertson, K. M., O'Donnell, L., Jones, M. E., Meachem, S. J., Boon, W. C., Fisher, C. R., Graves, K. H., McLachlan, R. I., and Simpson, E. R. (1999). Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* **96**, 7986-91.

Roeleveld, N., and Bretveld, R. (2008). The impact of pesticides on male fertility. *Curr Opin Obstet Gynecol* **20**, 229-33.

Ronnback, C., and de Rooij, D. G. (1994). Effects of 3,3',4,4'-tetrachlorobiphenyl on foetal germ cells in two mouse strains after repeated treatment of the dams during and after pregnancy. *Pharmacol Toxicol* **74**, 287-93.

Ropstad, E., Oskam, I. C., Lyche, J. L., Larsen, H. J., Lie, E., Haave, M., Dahl, E., Wiger, R., and Skaare, J. U. (2006). Endocrine disruption induced by organochlorines (OCs): field studies and experimental models. *J Toxicol Environ Health A* **69**, 53-76.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic

considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev Toxicol* **21**, 51-88.

Safe, S. (2004). Endocrine disruptors and human health: is there a problem. *Toxicology* **205**, 3-10.

Salian, S., Doshi, T., and Vanage, G. (2009). Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. *Life Sci* **85**, 742-52.

Seiler, P., Fischer, B., Lindenau, A., and Beier, H. M. (1994). Effects of persistent chlorinated hydrocarbons on fertility and embryonic development in the rabbit. *Hum Reprod* **9**, 1920-6.

Selgrade, M. K. (2007). Immunotoxicity: the risk is real. *Toxicol Sci* **100**, 328-32.

Sharara, F. I., Seifer, D. B., and Flaws, J. A. (1998). Environmental toxicants and female reproduction. *Fertil Steril* **70**, 613-22.

Shen, H., Yu, C., Ying, Y., Zhao, Y., Wu, Y., Han, J., and Xu, Q. (2009). Levels and congener profiles of PCDD/Fs, PCBs and PBDEs in seafood from China. *Chemosphere* **77**, 1206-11.

Shipp, E. B., Restum, J. C., Bursian, S. J., Aulerich, R. J., and Helferich, W. G. (1998). Multigenerational study of the effects of consumption of PCB-contaminated carp from Saginaw Bay, Lake Huron, on mink. 3. Estrogen receptor and progesterone receptor concentrations, and potential correlation with dietary PCB consumption. *J Toxicol Environ Health A* **54**, 403-20.

Shirota, M., Mukai, M., Sakurada, Y., Doyama, A., Inoue, K., Haishima, A., Akahori, F., and Shirota, K. (2006). Effects of vertically transferred 3,3',4,4',5-pentachlorobiphenyl (PCB-126) on the reproductive development of female rats. *J Reprod Dev* **52**, 751-61.

Silva, M. J., Barr, D. B., Reidy, J. A., Malek, N. A., Hodge, C. C., Caudill, S. P., Brock, J. W., Needham, L. L., and Calafat, A. M. (2004). Urinary levels of seven phthalate

metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999-2000. *Environ Health Perspect* **112**, 331-8.

Skinner, M. K. (2005). Regulation of primordial follicle assembly and development. *Hum Reprod Update* **11**, 461-71.

Skinner, M. K. (2008). What is an epigenetic transgenerational phenotype? F3 or F2. *Reprod Toxicol* **25**, 2-6.

Skinner, M. K., and Guerrero-Bosagna, C. (2009). Environmental signals and transgenerational epigenetics. *Epigenomics* **1**, 111-117.

Skinner, M. K., Manikkam, M., and Guerrero-Bosagna, C. (2010). Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends Endocrinol Metab* **21**, 214-22.

Smith, B. J., Plowchalk, D. R., Sipes, I. G., and Mattison, D. R. (1991). Comparison of random and serial sections in assessment of ovarian toxicity. *Reprod Toxicol* **5**, 379-83.

Steinberg, R. M., Walker, D. M., Juenger, T. E., Woller, M. J., and Gore, A. C. (2008). Effects of perinatal polychlorinated biphenyls on adult female rat reproduction: development, reproductive physiology, and second generational effects. *Biol Reprod* **78**, 1091-101.

Stroheker, T., Cabaton, N., Nourdin, G., Regnier, J. F., Lhuguenot, J. C., and Chagnon, M. C. (2005). Evaluation of anti-androgenic activity of di-(2-ethylhexyl)phthalate. *Toxicology* **208**, 115-21.

Svechnikova, I., Svechnikov, K., and Soder, O. (2007). The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats. *J Endocrinol* **194**, 603-9.

Swan, S. H. (2000). Intrauterine exposure to diethylstilbestrol: long-term effects in humans. *Apmis* **108**, 793-804.

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., Mao, C. S., Redmon, J. B., Ternand, C. L., Sullivan, S., and Teague, J. L. (2005). Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* **113**, 1056-61.

Tam, P. P., and Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *J Embryol Exp Morphol* **64**, 133-47.

Tomic, D., Brodie, S. G., Deng, C., Hickey, R. J., Babus, J. K., Malkas, L. H., and Flaws, J. A. (2002). Smad 3 may regulate follicular growth in the mouse ovary. *Biol Reprod* **66**, 917-23.

Tyl, R. W., Price, C. J., Marr, M. C., and Kimmel, C. A. (1988). Developmental toxicity evaluation of dietary di(2-ethylhexyl)phthalate in Fischer 344 rats and CD-1 mice. *Fundam Appl Toxicol* **10**, 395-412.

Vitt, U. A., McGee, E. A., Hayashi, M., and Hsueh, A. J. (2000). In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology* **141**, 3814-20.

VKM (2008). Opinion of the Panel on Contaminants of the Norwegian Scientific Committee for Food Safety - Risk assessment of non dioxin-like PCBs in Norwegian food (N. S. C. f. F. Safety, ed., Oslo, Norway.

Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambre, C., Tarazona, J. V., Brandt, I., and Vethaak, A. D. (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Crit Rev Toxicol* **30**, 71-133.

WHO (1989). Levels of PCBs, PCDDs, and PCDFs in Breast Milk. World Health Organization Regional Office for Europe.

WHO (1996). Levels of PCBs, PCDDs and PCDFs in Human Milk: Second Round of WHO-Coordinated Exposure Study. In Environmental Health in Europe No 3. World

Health Organization European Centre for Environment and Health., Bilthoven, Netherlands.

Wide, M. (1985). Lead exposure on critical days of fetal life affects fertility in the female mouse. *Teratology* **32**, 375-80.

Wilson, V. S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., and Gray, L. E., Jr. (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol Lett* **146**, 207-15.

Wittassek, M., and Angerer, J. (2008). Phthalates: metabolism and exposure. *Int J Androl* **31**, 131-8.

Wolf, C. J., Ostby, J. S., and Gray, L. E., Jr. (1999). Gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female hamster offspring. *Toxicol Sci* **51**, 259-64.

Wong, C., Kelce, W. R., Sar, M., and Wilson, E. M. (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem* **270**, 19998-20003.

Xie, Y., Yang, Q., Nelson, B. D., and DePierre, J. W. (2002). Characterization of the adipose tissue atrophy induced by peroxisome proliferators in mice. *Lipids* **37**, 139-46.

Xin, X., Yang, S., Kowalski, J., and Gerritsen, M. E. (1999). Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. *J Biol Chem* **274**, 9116-21.

Younglai, E. V., Wu, Y. J., and Foster, W. G. (2007). Reproductive toxicology of environmental toxicants: emerging issues and concerns. *Curr Pharm Des* **13**, 3005-19.

Yu, X., Kamijima, M., Ichihara, G., Li, W., Kitoh, J., Xie, Z., Shibata, E., Hisanaga, N., and Takeuchi, Y. (1999). 2-Bromopropane causes ovarian dysfunction by damaging primordial follicles and their oocytes in female rats. *Toxicol Appl Pharmacol* **159**, 185-93.

Zenick, H., and Clegg, E. D. (1989). Assessment of male reproductive toxicity: a risk assessment approach. In Principles and Methods of Toxicology (E. W. Hayes, ed., p. 275–309. Raven Press, New York.

Zhang, Y., Lin, L., Cao, Y., Chen, B., Zheng, L., and Ge, R. S. (2009). Phthalate levels and low birth weight: a nested case-control study of Chinese newborns. *J Pediatr* **155**, 500-4.

APPENDIX

The present PhD thesis is based on the work contained in the following papers and presentations:

Publications

I. **Effects of polychlorinated biphenyls in CD-1 mice: reproductive toxicity and intergenerational transmission.** Paola Pocar, Nadia Fiandanese, Camillo Secchi, Anna Berrini, Bernd Fischer, Juliane-Susanne Schmidt, Kristina Schaedlich, Stewart M. Rhind, Zulin Zhang, and Vitaliano Borromeo. Toxicological Science 2011. *Ahead of print* December 7, 2011 - doi: 10.1093/toxsci/kfr327.

II. **Exposure to di(2-ethyl-hexil) phthalate (DEHP) in utero and during lactation causes long-term pituitary-gonadal axis disruption in mouse male and female offspring.** Pocar P, Fiandanese N, Secchi C, Berrini A, Fischer B, Schmidt JS, Hart K and Borromeo V. Endocrinology 2011. *Ahead of print* December 6, 2011 - doi: 10.1210/en.2011-1450.

III. **Di(2-ethylhexyl) Phthalate (DEHP) Impairs Female Fertility and Promote Adipogenesis in C3H/N Mice.** Juliane-Susanne Schmidt, Kristina Schaedlich, Nadia Fiandanese, Paola Pocar and Bernd Fischer 2011. *Submitted to Environ Health Perspect.,* Final Revision.

Posters and oral presentations

IV. **In utero and lactational exposure to di(2-di-ethyl-hexil) phthalate (DEHP) disturbs pituitary-gonadal axis development in mice .** Fiandanese N, Borromeo V, Secchi C, Berrini A and Pocar P. 2011 26-29 April - 6th Copenhagen Workshop on Endocrine Disrupters, Copenhagen, Denmark.

V. **In-utero and lactational exposure to PCB 101 and 118 results in transgenerational disturbance of reproductive development.** Pocar P, Fiandanese N, Borromeo V, Berrini A, Rhind SM, Zhang ZL, Fischer B, Schmidt JS, Schädlich K, and Secchi C. 6th Copenhagen Workshop on Endocrine Disrupters, 26-29 April 2011, Denmark.

VI. **Effects of di(2-ethylhexil) phthalate (DEHP) on hypophisial-gonadal axis in male mice following in utero and lactational exposure.** Fiandanese N, Borromeo V, Secchi C, Berrini A and Pocar P. Gordon Research Conference: Environmental Endocrine Disrupters, 30 May-4 June 2010, Les Diablerets, Switzerland.

VII. **Effects of di(2-ethylhexyl) phthalate (DEHP) exposure during pregnancy and lactation on reproductive health of female mouse offspring: a transgenerational study over three generation.** Pocar P, Fiandanese N, Borromeo V, Berrini A, Fischer B, Cotinot C, Rhind SM, Sinclair K, Lea RG, Fowler PA and Secchi C. Gordon Research Conference: Environmental Endocrine Disrupters, 30 May-4 June 2010, Les Diablerets, Switzerland.

Other papers and presentations not included in this thesis:

Publications

I. **Effects of leptin on in vitro maturation, fertilization and embryonic cleavage after ICSI and early developmental expression of leptin (Ob) and leptin receptor (ObR) proteins in the horse.** Lange Consiglio A, Dell'Aquila ME, Fiandanese N, Ambruosi B, Cho YS, Bosi G, Arrighi S, Lacalandra GM, Cremonesi F. Reprod Biol Endocrinol. 2009 Oct 16;7:113.

II. **The extracellular calcium-sensing receptor is expressed in the cumulus-oocyte complex in mammals and modulates oocyte meiotic maturation.** De Santis T, Casavola V, Reshkin SJ, Guerra L, Ambruosi B, Fiandanese N, Dalbies-Tran R, Goudet G, Dell'Aquila ME. Reproduction. 2009 Sep;138(3):439-52. Epub 2009 Jun.

Posters and oral presentations

III. **Exposure to di(2-ethylhexyl)phthalate (DEHP) stimulates adipogenesis in female C3H/N mice and their offspring.** Schmidt JS, Schaedlich K, Fiandanese N, Pocar P and Fischer B. 6th Copenhagen Workshop on Endocrine Disrupters, 26-29 April 2011, Denmark.

IV. **Effects of the di(2-ethylhexyl) phthalate (DEHP) on in-vitro oocyte maturation in the mouse.** Pocar P, Borromeo V, Fiandanese N & Secchi C. 5th Copenhagen Workshop on Endocrine Disrupters – Copenhagen 20-22 May 2009.

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