# Endocrine disruption in the female following *in utero* exposure to the plasticizer di(2-ethylhexyl) phthalate

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science in Biochemistry

by

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## Abstract

Di-2-ethylhexyl phthalate (DEHP) is an endocrine disruptor used as a plasticizer in industry as an additive to polyvinyl chloride commodities which include consumer and medical products. Developmental delay induced by exposure to chemicals such as DEHP has been a cause for concern. In our work, we use a model in which pregnant dams are gavaged with DEHP from gestational day 14 until birth. We have previously provided evidence that demonstrates the adverse effects of DEHP on the male endocrine system following *in utero* exposure; however, knowledge of analogous effects of DEHP in the female is lagging in comparison. In the present study, we set out to characterize the anti-estrogenic effects of DEHP on the estrous cycle of female offspring exposed to 1 - 300 mg DEHP/kg/day. *In utero* exposure to DEHP resulted in a perturbation of the ovarian steroids profiles as well as an underdevelopment of ovarian follicles. In the ovaries we reported a decrease in the expression of *Cyp17a1,* a gene that encodes for the enzyme necessary for androgen formation. Furthermore, pituitary-derived FSH serum levels were significantly increased pre and post ovulation starting at the lowest dose. The feedback mechanism in the hypothalamic-pituitary-gonadal axis was presumably not affected since pituitary and hypothalamus expression of genes involved in the regulation of the axis was not altered. Anti-Müllerian hormone gene expression was significantly decreased in the ovary. Mating studies showed a decrease in first cycle pregnancy. The data presented herein suggest that *in utero* exposure to DEHP likely targets ovarian follicular development.

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# List of abbreviations

| | |
|---|---|
| 3βHSD | 3beta-hydroxysteroid dehydrogenase/delta(5)-delta(4) isomerase |
| 17βHSD | Hydroxysteroid **(17-**Beta**)** Dehydrogenase |
| AMH | Anti-Müllerian hormone |
| ATAD3 | ATPase family AAA Domain-containing protein 3 |
| CGA | Glycoprotein hormones, alpha polypeptide |
| CL | Corpus luteum |
| CYP11A1 | Cholesterol side-chain cleavage enzyme |
| CYP17A1 | 17α-hydroxylase/17, 20 lyase/17,20 desmolase |
| CYP19A1 | Cytochrome P450, family 19, subfamily A, aromatase |
| DEHP | Di-(2-ethylhexyl) phthalate |
| E1 | Estrone |
| E2 | Estradiol, 17β-estradiol |
| ESR1 | Estrogen receptor alpha |
| ESR2 | Estrogen receptor beta |
| FSH | Follicle stimulating hormone |
| FSHb | Follicle stimulation hormone beta subunit |
| FSHR | Follicle stimulating hormone receptor |
| GD | Gestational day |
| GnRH | Gonadotropin releasing hormone |
| GNRHR | Gonadotropin releasing hormone receptor |
| hCG | Human chorionic gonadotropin |
| HED | Human equivalent dose |

| | |
|---|---|
| HMGCR | 3-hydroxy-3-methylglutaryl-CoA reductase |
| HMGCS1 | 3-hydroxy-3-methylglutaryl-CoA synthase 1 |
| HPG | Hypothalamic-pituitary-gonadal axis |
| IMM | Inner mitochondrial membrane |
| KISS1 | Kisspeptin-1 |
| KISS1R | Kisspeptin-1 receptor |
| LDLR | Low density lipoprotein receptor |
| LH | Luteinizing hormone |
| LHb | Luteinizing hormone beta subunit |
| LHCGR | Luteinizing Hormone/Choriogonadotropin Receptor |
| MEHP | Mono(2-ethylhexyl) phthalate |
| OMM | Outer mitochondrial membrane |
| P4 | Progesterone |
| PCOS | Polycystic ovarian syndrome |
| PGR | Progesterone receptor |
| PND | Postnatal day |
| PPAR | Peroxisome proliferator activated receptor |
| PVC | Polyvinyl chloride |
| Q-PCR | Quantitative polymerase chain reaction |
| SCARB1 | Scavenger Receptor Class B, Member 1 |
| StAR | Steroidogenic Acute Regulatory Protein |
| TSPO | Translocator Protein |
| VDAC | Voltage-Dependent Anion Channel |

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# Introduction

## The female reproduction system

The reproductive system in the female comprises of the ovaries, reproductive tract and the mammary glands. Although the female gonads have a gametogenic function, they are also responsible for an endocrine function that is intertwined in a hypothalamic-pituitary-ovarian axis, or hypothalamic-pituitary-gonadal axis (HPG). In fact, the integrity of the female reproductive tract relies heavily on ovarian steroid hormones. Moreover, the hormonal interactions between the brain and the gonads are critical in sustaining the tightly regulated mechanisms that set the stage for female reproduction.

### *The hypothalamic-pituitary-ovarian axis*

The hypothalamus consists of 0.3% of the adult brain (1). Notwithstanding its small size, the hypothalamus is responsible for a variety of biological processes including metabolism, endocrine regulation and reproduction, temperature, and electrolyte balance (2-5). Gonadotropin releasing hormone, also known as GnRH, is a decapeptide secreted by GnRH neurons in the hypothalamus (6) and is a key regulator of mammalian fertility. In humans, three types of GnRH peptides have been detected, GnRH-I, GnRH-II and GnRH-III, with GnRH-I being the characteristic stimulus of the pituitary gland, regulating the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) through its receptor, GnRHR (7-9). GnRH-I is secreted in a pulsatile manner; this oscillation is necessary for its function (10). Its continuous secretion has been shown to suppress gonadotropin secretion and gene transcription (11;12). The variation in magnitude and frequency of GnRH pulses mediate the shift between the positive and negative feedback in the axis and is the basis of the hypothalamus' role in neuroendocrine function.

The hypothalamus and pituitary gland work synchronously to regulate LH and FSH secretions. The pituitary can be divided in to two lobes: anterior and posterior. Gonadotropes are neurons that reside in the anterior lobe of the pituitary and upon GnRH stimulation, these cells produce and secrete FSH and LH. The anterior pituitary also locally secretes activin, follistatin and inhibin (13). FSH and LH are glycoproteins consisting of an alpha and beta subunit. The alpha subunit is a commonality between the gonadotropins while the beta subunit dictates hormone specificity. The gonadotropes rely on GnRH stimulation and activin for FSH beta subunit synthesis (14;15). Levels of FSH increase at the beginning of the cycle in order to stimulate the maturation of a cohort of antral follicles in the ovary, and the midcycle LH surge induces the ovulation and luteinisation of the dominant follicle(s). Early studies provide evidence that the differential secretion of the gonadotropins is thought to be caused by changes in the frequency of GnRH stimulation; low frequency favors FSH secretion and high frequency favors LH secretion (16). The ovarian steroid hormones also play a bimodal role on gonadotropin secretion at the mRNA level by simultaneously inhibiting FSH transcription and inducing that of LH.

Ovarian steroidogenesis is compartmentalized within each follicle in the ovary (17). Distinctive follicular microenvironments result in the presence of follicles at various stages of development in the ovary throughout the cycle. Ovarian steroid hormones estradiol (E2) and progesterone (P4) are both critical regulators of the feedback mechanisms that modulate the periodicity of the cycle. Ovarian P4 can diffuse across the blood-brain barrier and mediate GnRH secretions through the action of its P4 receptor (PGR). The transcription of PGR in the brain is activated via its transcription factor, E2-bound estrogen receptor alpha (ERα), which acts by binding to the PGR promoter region (18).

The ovaries also produce FSH-regulating proteins, inhibin and activin, whose functions are to inhibit and stimulate pituitary FSH secretion, respectively. Both proteins are secreted during the follicular and luteal phase of the cycle. Figure 1 shows a schematic representation of the hypothalamic-pituitary-ovarian axis.

*Follicle development throughout the ovarian cycle*

In humans, the foetal ovary establishes the finite pool of primordial follicles and the recruited growing follicle population before birth. In the rodent, although by GD14.5 oocytes have already formed loose ovigerous cord structures with pre-granulosa somatic cells (19-21), follicle formation and recruitment only occurs after birth. That said, novel evidence suggests the presence of follicles at GD 17.5 (22). Both human and rodent ovaries will continue to recruit follicles for subsequent growth and maturation until their respective primordial follicle population is depleted (23;24).

The follicle is the multifaceted primary component of the ovary; it is the site of gamete growth and steroidogenesis. Development and regression of ovarian follicles follow a reoccurring cyclical mechanism that continuously spans the female reproductive life. Autocrine, paracrine, as well as endocrine signals are responsible for the progression of follicular maturation throughout the growth phases. Oogonia that have entered the first stage of meiosis form primary oocytes (25). At the earliest stage, primary oocytes form cell-oocyte gap junctions with the surrounding somatic cells which will further differentiate into mural and cumulus granulosa cells (26). These primordial follicles represent the finite pool of ovarian follicles (27) which, in women, degenerate from 7 million to approximately 300 000 at reproductive maturity, with about 400 ever reaching ovulation. Granulosa cells will remain closely coupled with the oocyte throughout follicular maturation. At this point, the primary oocytes advance through meiosis I

and are arrested in the diplotene stage of prophase I due to the lack of necessary proteins required to support meiosis progression (25). They are, therefore, meiotically incompetent.

Primordial follicles progress into the primary stage which is characterized by a single surrounding layer of squared granulosa cells. Subsequent granulosa cell proliferation gives rise to a multi-granulosa-layer secondary follicle, which secretes paracrine factors in order to recruit and induce differentiation of stromal cells in to an outermost layer of flattened theca cells (28). Although at this point the granulosa cells express the FSH receptor, they do not produce steroid hormones and function in a gonadotropin-independent manner, relying primarily on factors secreted by the oocyte (29). Once maturing follicles develop multiple granulosa cell layers, fluid accumulates in the antrum of the follicle, hence the name antral follicles. Granulosa cells are divided in to two distinct categories: mural granulosa cells, those that continue to border the follicle, and cumulus granulosa cells, those that cradle the oocyte (30). From the early stages, fluid-filled antral follicles are highly dependent on FSH for growth and sustainability and it is at this point that the mural granulosa cells become highly steroidogenic. Oocytes residing in antral follicles secrete enough meiosis-associated proteins to complete meiosis I, however, the process is arrested until the LH surge that precedes ovulation (31).

During the follicular phase of the cycle, large antral follicles are selected to undergo further development in a gonadotropin-dependent manner. These recruited follicles are subject to atresia by a highly regulated process until the biological species-depend quota is reached (32). The dominant follicle expresses higher levels of gonadotropin receptors and as a result, its steroidogenesis is increased and it will be the only follicle that will reach ovulation. Ovulation of the dominant follicle occurs following the periodic midcycle LH surge (33). In rat granulosa cells, it has been shown that a cellular response to LH is dependent on a high density of receptors

(34). The rise in E2 and inhibin impedes the secretion of FSH and the remaining mature antral follicles expressing lower levels of the receptors will no longer be stimulated (35). This induces a decrease in the granulosa cell aromatase expression and, consequently, the androgen-estrogen ratio rises. An androgenic follicular state induces oocyte degeneration and cellular apoptosis (36), thus, the remaining antral follicles are forced in to an atretic state.

Following ovulation, the remaining granulosa and theca cells of the ovulated follicle differentiate in to luteal cells to ultimately form a highly steroidogenic gland termed the corpus luteum (CL). This characterizes the luteal phase of the cycle. The high levels of P4 secreted by the CL inhibit hypothalamic GnRH pulses (37;38), a phenomenon that is necessary in maintaining pregnancy by preventing subsequent ovulation. P4 also prepares the lining of the uterus for implantation (39). While there is no successive surge in LH, the CL relies on basal levels of LH for sustainability (40). Fertilization of the oocyte occurs in the oviducts, or fallopian tubes, and implantation occurs in the uterus. In the rat, coital stimulation, independently of implantation, induces the pituitary secretion of prolactin, a protein necessary in maintaining the CL throughout pregnancy (41). In humans, following implantation, the placenta will begin to secrete hCG in order to sustain the CL through stimulation of the desensitized LH receptors (42). If implantation does not occur, the CL breaks down and regresses, P4 levels fall, and the hypothalamic-pituitary axis is released from the hormonal suppression of the CL and the cycle resumes in a timely fashion throughout the entirety of the female reproductive life. Figure 2 depicts the various stages in follicular maturation, hormonal stimulation and synthesis.

The process of FSH recruitment of large antral follicles until CL formation occurs every one-month menstrual cycle in humans, and every four day estrous cycle in rats. Rodents reabsorb the endometrium, like many other small mammals, and do not menstruate. The estrous cycle can be

divided in to four stages: proestrus, estrus, metestrus and diestrus, with follicle selection occurring at metestrus and ovulation occurring between proestrus and estrus (43). The hormonal fluctuations of the rat's four to five day estrous cycle have been well characterized. It has been reported that FSH, E2, LH and P4 peak within the window of proestrus, respectively (44).

*Steroidogenesis in the female gonads*

Gonadal steroid hormones consisting of androgens, progestins and estrogens are, systemically, the most abundant of all steroid hormones (45). For all steroid hormones, cholesterol is the primary building block. Bioavailability of cholesterol in steroidogenic tissue can be achieved through the *de novo* synthesis pathway or the uptake of lipoprotein-bound cholesterol acquired from dietary sources (46). The transport of cholesterol from the OMM to the IMM is the rate limiting step in the synthesis of all steroids (47;48). This process is enabled by hormone-induced StAR, a protein whose function is to activate cholesterol movement from the outer to the inner mitochondrial membrane, and a complex of mitochondrial proteins, allowing for the transfer and targeting of cholesterol to CYP11A1 in the matrix side of the inner mitochondrial membrane (49), where it is converted to pregnenolone. These mitochondrial proteins include the translocator protein, TSPO, voltage dependent anion channel VDAC and AAA+ ATPase domain ATAD3. Pregnenolone is then subject to further modification by 3βHSD, present both in mitochondria and the endoplasmic reticulum, yielding progesterone. Steroid hormone biosynthesis is a result of cellular-specific enzymatic expression that occurs after this step (50). For instance, in the testes, Leydig cells express CYP17A1 which catalyzes the formation of testosterone. In the ovaries, theca cells express this androgen-forming enzyme. Although analogous across gender in their ability to produce androgens, theca cells differ from

Leydig cells in their lower expression of 17βHSD. This results in androstenedione being the major product of theca cells, unlike testosterone in Leydig cells.

Steroidogenesis in the ovary occurs at the level of mature antral follicles and necessitates both theca and granulosa cells. As previously mentioned, theca cells are the site of androgen formation in the ovary. Theca cells express LH receptors to allow basal levels of LH to modulate the expression of enzymes involved in steroidogenesis (see above), including LDL receptors. Theca cells express CYP17A1; its 17 alpha-hydroxylase/C17,20 lyase enzymatic system results in the biosynthesis of androgens (androstenedione and testosterone), which can diffuse to the blood vessels surrounding the follicle or to the mural granulosa cells. These cells are functionally dissimilar to theca cells. This can be explained by the difference in gene expression profiles. For instance, granulosa cells express FSH receptors, which, upon stimulation, trigger the expression of LH receptors and CYP19A1 (aromatase). This enzyme is responsible for the conversion of androstenedione to estrone (E1) and testosterone to E2. In granulosa cells, elevated expression of 17βHSD isoforms forces the enzymatic reaction towards the production of the more potent estrogen, E2. This interaction between the mechanistically distinct granulosa and theca cells is what is known as the two-cell model of ovarian steroidogenesis, see figure 3.

**Di-(2-ethylhexyl) phthalate**

*Phthalates*

Phthalate esters are widely used industrial plasticizers that are most commonly incorporated in to polyvinyl chloride products to increase the flexibility and the transparency of the plastic. Plasticized PVC is the primary material used in the fabrication of various industrial and consumer products including food wrappings, personal care and household products, toys,

waxes, medical devices, insecticides and building materials (51;52). Ironically, patients in intensive care are in an exceptionally high risk group with regards to phthalate exposure due to the continuous contact with medical tubing and devices. The high levels of phthalates in medical devices were also deemed a recent cause for concern in children (53;54). Many phthalates are continuously being mass produced and incorporated in to ubiquitous plastic commodities.

Di-(2-ethylhexyl) phthalate, also known as DEHP, is the most commonly used phthalate plasticizer in industry, with more than three million metric tons being produced each year (55). This phthalate is not covalently bound to the polymer structure of PVC and, as a result, can freely leech out and contaminate the surrounding environment. Direct human exposure can occur via dermal contact (56), inhalation (56), oral ingestion (57) and intravenous injection (53). Phthalates have also been detected in amniotic fluid (58), umbilical cord blood (59), breast milk (60) and baby formula (61) suggesting that prenatal and perinatal exposure to these phthalates allows their potential toxicity to target development.

*Pharmacokinetics of DEHP*

DEHP is a high molecular weight organic compound, with the molecular formula $C_6H_4(C_8H_{17}COO)_2$. It is composed of an aromatic benzene ring ortho-substitued by two symmetrical ester groups containing branched hydrocarbon chains. Upon ingestion, DEHP is hydrolyzed by non-specific blood bound esterases, liver esterases and pancreatic lipases (62-64). Studies done in Sprague-Dawley rats have shown that DEHP has a half-life of 13.49 hours (65). Degradation of this phthalate yields a variety of 15 metabolites, including mono(2-ethylhexyl) phthalate, or MEHP (66;67). The formation of this particular metabolite is achieved through one deesterification reaction of the parental compound DEHP. In vitro studies have shown that MEHP is approximately ten times more potent in terms of toxicity than DEHP and is thought to

be the primary acting metabolite of DEHP (68;69). DEHP and its metabolites do not accumulate as they are rapidly excreted from the system (70;71) so the presence of conjugated MEHP in urine samples is considered a reliable biomarker in estimating single acute exposure to DEHP (53;71-73). The majority of human studies correlating abnormal phenotypes to DEHP exposure implicate single urine sample testing. The average human daily exposure to phthalates is estimated to be between 1.7 and 52.1 μg/kg/day (70;74;75). In our studies, we administer quantities that correspond to human equivalent doses (HED). This is based on a formula that suggestively converts substance exposure across species by taking in to account many relevant features including body surface area and metabolism, which are thought to be more reliable conversion factors compared to body mass (76). Based on these calculations, human exposure corresponds to 0.18 to 62.5 mg DEHP/kg/day rat exposures. Our methods comprise DEHP doses ranging from 1 to 300 mg DEHP/kg/day, thus spanning the window of environmentally relevant doses for humans.

*Acute exposure to DEHP affects the female reproductive system*

Studies of the adverse effect of phthalates on the female reproductive system are limited, and related research notably lags behind that of the male. Human population studies that employed single urinary measurements of phthalate metabolites suggest a link between reproductive abnormalities and environmental exposure. For instance, an increase in miscarriages and a decrease in pregnancy rate was associated with MEHP urinary levels in female factory workers (77). Women living in proximity to plastic manufacturers had urinary phthalate metabolite levels that correlated to an increase incidence of pregnancy complications including toxemia, anemia and preeclampsia; human exposure to DEHP has also been linked to shortened gestational periods (59;78) and endometriosis (79). Premature breast development was

correlated to urinary levels of phthalates in a cohort of young Puerto Rican girls, although disputed for lack of substantial proestrogenic evidence (80;81). It has been proposed that multiple urinary quantifications of MEHP would enhance the acute bioexposure associations between phthalate exposure and abnormal findings (45). Urinary measurements are limited in extrapolating valid bioexposure data since the values are more likely biased towards dietary intake rather than overall environmental exposure. In fact, more recent studies implicated serum measurements of metabolites in order to better assess phthalate exposure.

Animal models have been used to study the mechanism of action of phthalates on the reproductive system. DEHP is the most potent of the phthalates in terms of reproductive toxicity (82) and has been shown to exert an effect on both sexes. Initial studies in the male rodent reported decreased testes weight, reduced sperm production, (83) and infertility (84). More recent work has reported that DEHP particularly targets gene expression of enzymes involved in steroidogenesis in the testes and adrenal glands (85;86). It is, therefore, more than likely that DEHP exerts parallel effects on the female endocrine system, although research in this area is notably lagging in comparison to the male data.

The endocrine disrupting effects of DEHP are generally less pronounced in the females compared to the males (87). Nonetheless, acute exposure to DEHP at exceedingly high doses has been shown to impede the female reproduction system. It has been reported that doses as high as 2 g DEHP/kg/day induces anovulation and prolongation of the estrous cycle in regularly cycling rats by eliminating the preovulatory peak of E2 and consequently LH (88). Cyst-like formations in the ovaries and a decrease in the size of granulosa cells have also been reported (88). In fact, DEHP is the only phthalate that has been shown to affect E2 levels by directly targeting

granulosa cells by decreasing aromatase expression (89) in a peroxisome proliferator-activated receptor (PPAR) mediated mechanism (90;91).

Multiple treatment regimens have been used in various studies, making the results difficult to compare. Still, certain findings have been reoccurring. In several studies, acute short-term exposure to DEHP results in reduced E2 and aromatase expression levels, while prolonged treatment has been shown to result in an increase in the atretic follicle count (91-95). One study showed that prolonged 60 day exposure to 300 and 600 mg DEHP/kg/day resulted in a decrease in the number of ovarian follicles and an increase in granulosa cell apoptosis (91). A very high dose of 3 g DEHP/kg/day was shown to decrease pregnancy rates (96). Regarding the HPG axis, LH production was notably increased in a primary pituitary culture from female rats exposed to 500 mg DEHP/kg/day when stimulated with GnRH (92). However, in sheep treated with 50 mg DEHP/kg three times a week, basal LH levels were decreased compared to the control group and the LH pulsatile secretions were undetectable in the DEHP-treated animals (97).

*Maternal exposure to DEHP*

Acute exposure is not the only mode through which toxins can exert an effect on an organism. In fact, the foetal origin of adult disease hypothesis is based on the notion that contact with a chemical or toxin during embryogenesis and development will result in long term physiological effects in the offspring (98). With regards to the ovary, gestational exposure to androgens in rhesus monkeys (99), sheep (100) and rats (101) resulted in polycystic ovarian syndrome (PCOS) characteristically similar to the human disease, while acute androgen exposure failed to yield the diseased state (102). It is likely that exposure to a toxin in the adult compared to the foetus amounts to different health outcomes. It has also been suggested that ovarian cancer stems from foetal exposure to maternal gonadotropins (103;104).

Phthalates have been shown to modulate changes in maternal hormone levels and function during gestation (105). In the rodent, changes in hormonal serum levels during this developmental period can induce birth defects (106). Studies have also shown that prenatal exposure to DEHP was negatively correlated to free testosterone levels in cord blood (107) and although there is evidence presenting a negative correlation between E2 and DEHP exposure (88), the studies have yet to be conducted in pregnant women. In humans, phthalate exposure during development has been linked to abnormal phenotypes in children including reduced anogenital distance (108) and abnormal sex specific behavior (109). During pregnancy, maternal hormone levels can be representative of foetal production (110). Although it remains to be elucidated, aberrations in those levels suggest that DEHP may exert its effect by modulating sex steroid synthesis and mechanistic function of the foetus during development which may lead to health outcomes later in life. Although it is unknown whether phthalates act indirectly or directly on female foetal steroids, there is evidence suggesting that DEHP exercises long term effects by altering the epigenome.

*Epigenetic effects of DEHP*

*In utero* and prepubertal DEHP exposure, in particular during embryogenesis, has shown to propel long term lasting effects on the endocrine system (111). One theory explaining the relation between diseased states in adulthood to gestational exposure to toxins suggests a mechanistic reprogramming induced by epigenetic changes (112-114). The epigenome consists of hereditary surface modifications of the genome, including methylation and acetylation, which alter gene expression without modifying the genome. A stressor that modifies the epigenome can establish a diseased state. In fact epigenetic therapy is a novel treatment implicated in reprogramming epigenetic abnormalities found in certain cancers (115).

There is some evidence that suggests that DEHP alters DNA methylation. In mice, foetal and perinatal exposure to 500 mg DEHP/kg/day was reported to increase global gene methylation in the testes (116;117). Work from this laboratory has also shown differential methylation in the promoter region of the mineralcorticoid receptor which affects gene expression in the testes of the adult rat following *in utero* exposure to DEHP (118). In animal models, short term administration of DEHP during development mediates physiological abnormalities much later in life, long after the window of exposure. Thus, epigenetic modification during foetal life is a plausible explanation for the long term adverse effects of DEHP, although this has yet to be extensively explored in the female.

In the female rodent, *in utero* exposure to DEHP has been shown to have a detrimental impact on the reproductive system. The oocyte and precursor granulosa and theca cells that are present in the fetus during the window of exposure will evolve in to the mature functional units of the ovary throughout the entire reproductive lifespan. CD-1 female mice that had been exposed to 0.05 mg DEHP/kg/day in the chow from GD0.5 until the end of lactation, as adults (PND41), had irregular gene expression profiles of the steroidogenic pathway and the HPG axis feedback loop. Decreases were reported in the ovarian expression of *cyp17a1*, *cyp19a1*, and the P4, LH and FSH receptors (*pgr, lhcgr, fshr*) as well as in the number of oocytes (119). In other studies, exposure to at least 15 mg DEHP/kg/day from GD6 until PND21 showed delays in vaginal openings, while higher doses (135, 405 mg DEHP/kg/day) delayed the onset of the first estrus and increased the atretic follicle count (120;121). Furthermore, absence of a vaginal orifice was reported in the offspring of Wistar rats that were gavaged from GD12 until GD17 at doses of 750 and 100 mg DEHP/kg/day (122). Interestingly, metabolites of DEHP also induced similar detrimental effects. Gestational exposure to 500 and 1000 mg MEHP/kg/day in C57/B16

mice resulted in increased levels of E2 and FSH and delayed onset of estrus. At one year of age, mammary gland hyperplasia was observed in the 1000 mg MEHP/kg/day dose (123).

In our model, timed-pregnant Sprague-Dawley rats are gavaged with doses ranging between 1 and 300 mg DEHP/kg/day from GD14 until birth. We have previously reported reduced levels of E2 and increased levels of aldosterone in the adult female rat aged 60 days (PND60) (85). The long-term effect of *in utero* exposure to environmentally relevant DEHP doses on the female reproductive system requires further exploration. The magnitude of the DEHP-induced stress on the system is unclear and the mechanism by which it acts needs to be elucidated.

## Figures 1 to 3

**Figure 1. The sequence of events governing the hypothalamic-pituitary-ovarian axis**

1. GnRH secretion from the hypothalamus and stimulation of the pituitary gland, 2. Pituitary secretion of FSH, and stimulation of the ovaries, 3. Ovarian synthesis and secretion of E2 and inhibin resulting in feedback to the pituitary inhibiting FSH secretion, 4. Induction of LH secretion from the pituitary, 5. Ovarian secretion of P4, E2 and inhibin resulting in the feedback inhibition of the pituitary and hypothalamus.

**Figure 1. The sequence of events governing the hypothalamic-pituitary-ovarian axis**

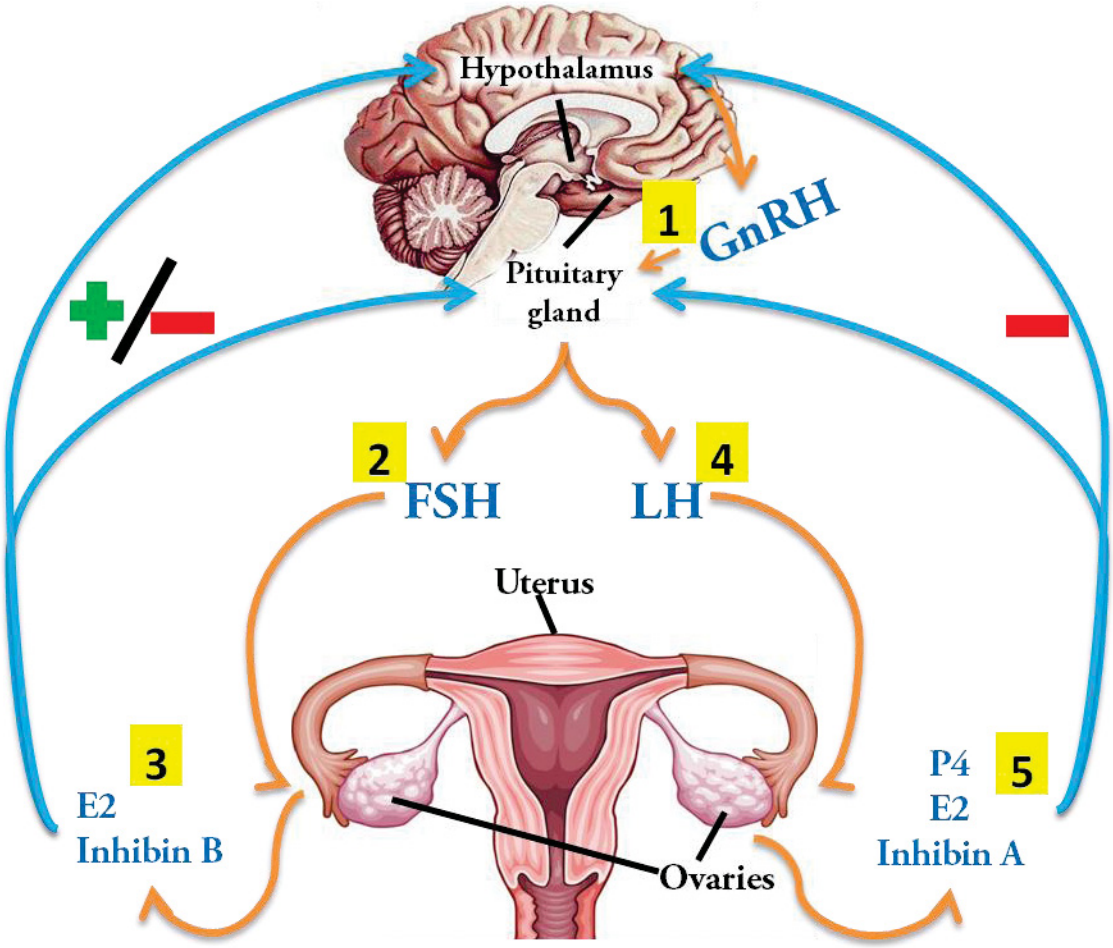


**Figure 2. The different phases of follicular growth and maturation**

AMH is secreted by the growing cohort of follicles and FSH stimulates follicles in the antral stage. E2 and inhibin-secreting mature antral follicles that are not selected for ovulation undergo atresia. LH induces ovulation. The ovulated oocyte travels to the oviducts for fertilization and then to the uterus for implantation. The remaining cells differentiate in to the corpus luteum. The CL secretes P4, and to a lesser extent, E2 and inhibin.

**Figure 2. The different phases of follicular growth and maturation**

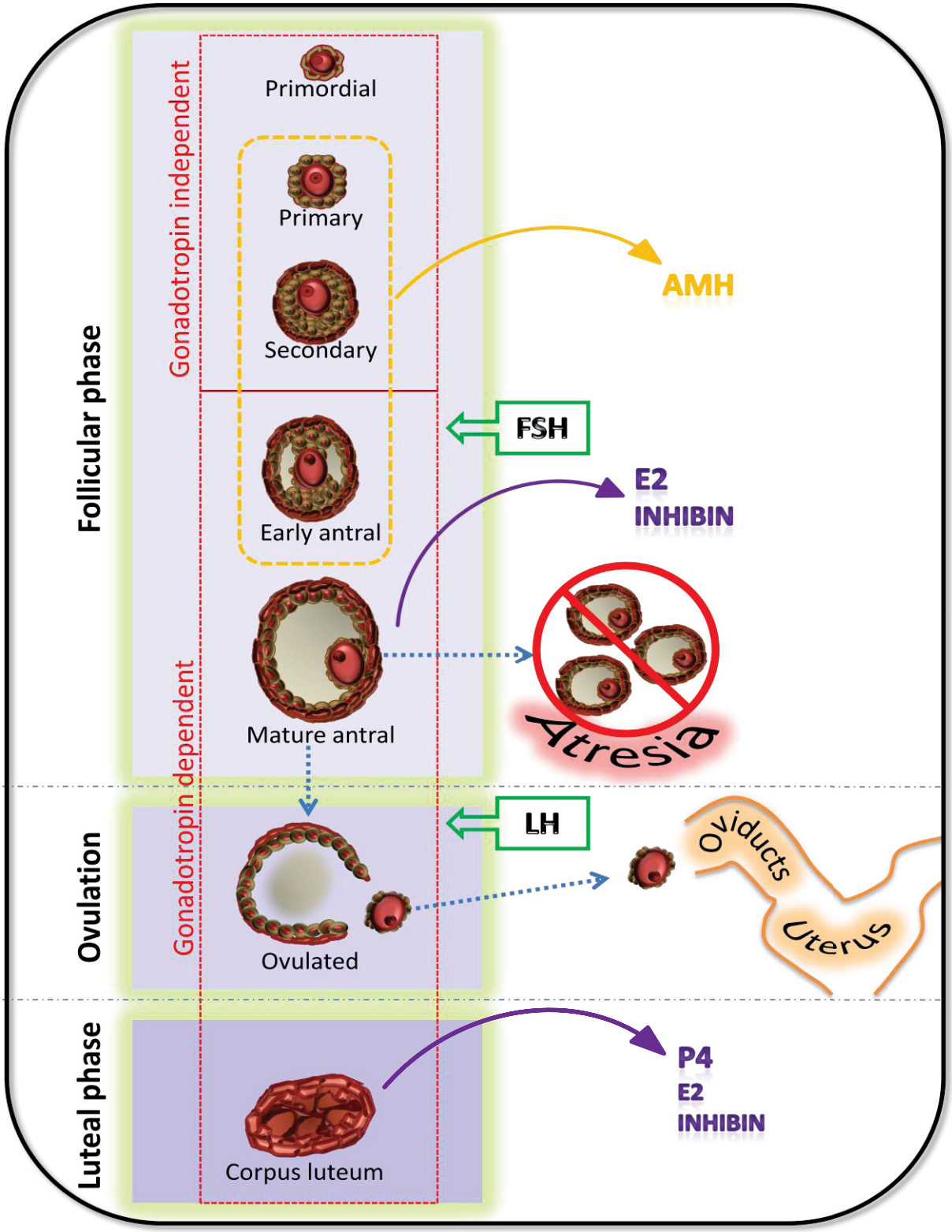
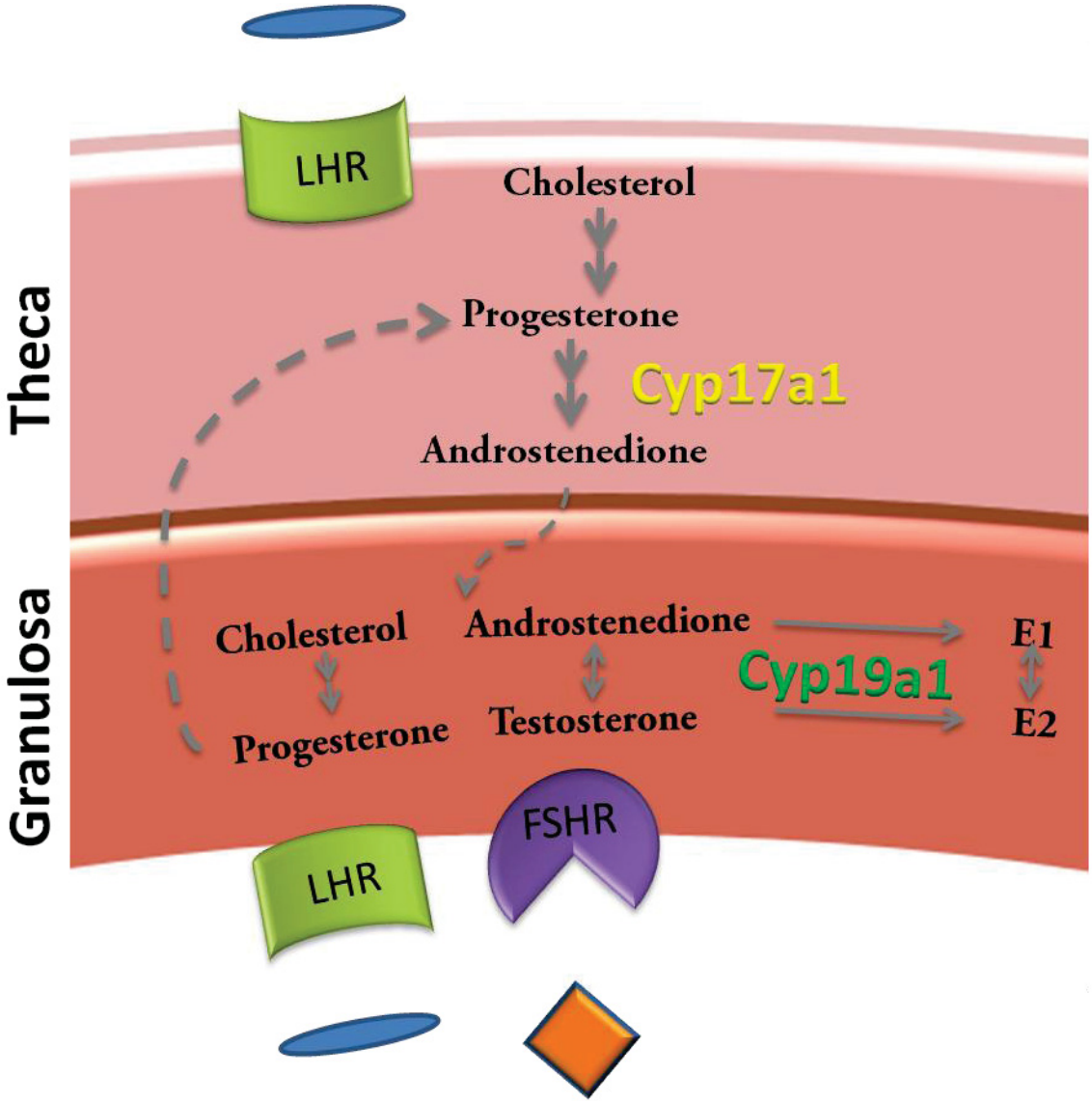


**Figure 3. The two-cell two-gonadotropin model**

FSHR and LHR-expressing granulosa cells convert cholesterol to progesterone that is then diffused across the basal lamina to the LHR-expressing theca cells. Theca cells express *Cyp17a1* and can synthesize androstenedione which diffuses back across the basal lamina to the granulosa cells. Androgens undergo aromatization in the *Cyp19a1*-expressing granulosa cells, and yield the estrogens, E1 and E2.

**Figure 3. The two-cell two-gonadotropin model**



# *In utero* exposure to the endocrine disruptor di(2-ethylhexyl) phthalate targets ovarian follicular development and steroidogenic function in the adult female rat

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**Abstract**

Di-2-ethylhexyl phthalate (DEHP) is an endocrine disruptor used in industry as an additive to polyvinyl chloride-based consumer, and medical products. We use a model in which pregnant dams are gavaged with various concentrations of DEHP from gestational day 14 until birth. We previously reported that *in utero* DEHP exposure results in decreased testosterone levels in the male offspring starting at the 100 mg DEHP/kg/day dose and reduced estrogen levels in the female offspring exposed to 300 mg DEHP/kg/day. In the present study, we set out to characterize the anti-estrogenic effects of DEHP in the female offspring exposed to 1, 20, 50 or 300 mg DEHP/kg/day. *In utero* exposure to DEHP resulted in reduced estrogen levels at proestrus in the 300 mg DEHP/kg/day dose. Serum estrogen levels averaged across the cycle were not significantly affected by DEHP. Expression of *Cyp17a1* in the ovary was significantly decreased in the 300 mg DEHP/kg/day dose and correlated with a decrease in the theca cell layer thickness starting at the 50 mg DEHP/kg/day dose. Granulosa cell layer thickness was not significantly reduced. FSH levels were significantly increased at proestrus, estrus, and when averaged across the cycle starting at the lowest dose, 1 mg DEHP/kg/day. Pituitary and hypothalamus expression of genes involved in the regulation of LH and FSH formation were not affected. Anti-Müllerian hormone gene expression levels were significantly decreased in the ovaries. Mating studies showed a decrease in first cycle pregnancy. The data presented herein suggest that *in utero* exposure to DEHP targets follicular development in the ovary and the steroid surges in the estrous cycle.

## Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly introduced to polyvinyl chloride (PVC) products to increase their flexibility and transparency (52). DEHP is ubiquitously found in the environment because of its large-scale use in industrial, consumer, and medical products (124). Exposure to DEHP occurs mainly through oral ingestion (57), dermal contact (56), and inhalation (125). Once ingested, lipases generate the active metabolite MEHP which is ten-times more potent than the parent compound (68;69). DEHP and its metabolites were found in several bodily fluids (126) including amniotic fluid (127), umbilical cord blood (59), and breast milk (128). In addition, baby formulas were found to be contaminated with phthalates (61;129), raising concerns for fetal and neonatal development. Human exposure to DEHP is in the range of 1.7 - 52.1 μg/kg/day (70;75) and it has been proposed that newborns and infants are at greater risk due to their low surface area and increased contact with DEHP contaminated products (51;52). DEHP has a short-half life (66) and its metabolites can be readily detected in urine (62-64). Urine levels of DEHP and its metabolites reflect recent contact and are frequently used to estimate phthalate exposure (53;130).

DEHP is an endocrine disruptor and reports correlating DEHP levels with negative endocrine effects in females are increasing (45). In humans, studies have shown an association between high levels of DEHP and endometriosis (79), decreased gestational times (78), and increased rate of early miscarriages (131). DEHP was also reported to be associated with premature thelarche in Puerto Rican girls (80). In addition, occupational exposure to DEHP has been associated with hypoestrogenism and anovulation in middle aged women (132). In rodents, numerous studies have been conducted to elucidate the endocrine disruption of phthalates on the male but female research is lagging (45). Exposures to DEHP or its bioactive metabolite mono(2-ethylhexyl)

phthalate (MEHP) have been found to target steroidogenesis, follicle development, and have resulted in decreased fertility (133). Davis et al. (88;134), initially showed that high doses of DEHP (2gr/kg/day) targeted the ovary resulting in decreased E2 levels, absence of the LH surge, anovulation, and prolonged estrous cycle. Effects in estrogen-dependent tissues were reported in rats exposed to MEHP where there was delayed estrous, shortening of reproductive life, and mammary hyperplasia (123). In addition, *in utero* and lactational exposures with high doses of DEHP were shown to delay puberty in the female offspring (121). *In vitro* models mainly focused on ovarian follicle cultures because the granulosa and thecal cell layers are retained. Using these models, studies have shown MEHP to target the follicular development and induce apoptosis of granulosa cells (93;135).

Until now, most of the research has focused on acute or prolonged exposures to DEHP and there is little known on the long-term effects of *in utero* exposure to DEHP in the female offspring (136). The various dose regiments and time points used in DEHP studies make the data difficult to compare but, in general, earlier exposures are associated with long-lasting effects (45). We used an animal model where pregnant dams are gavaged with DEHP from gestational day (GD) 14 until birth to study the short and long-term effects of DEHP (85;86;118;137;138). We previously reported that DEHP reduced circulating levels of testosterone and aldosterone in the male offspring (85;86;118) and decreased estrogen levels in the female offspring exposed to 300 mg DEHP/kg/day at PND60 (85).

Herein we characterize the long-term effects of *in utero* exposure to DEHP on the reproductive function of the F1 females. We report that DEHP induces long-term endocrine disrupting effects in steroidogenesis and follicular development.

## Methods

*DEHP Treatment and Animal Care*

Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories and gavaged daily with corn oil or with 1, 20, 50 or 300 mg DEHP/kg/day (Sigma-Aldrich) from GD14 until postnatal day 0. Pregnant dams were weighed every two days and the doses were adjusted accordingly. Estrous cycle stage was determined by crystal violet staining of daily vaginal lavage (139). Female offspring of all treatment groups were euthanized between PND60 and PND68 at the proestrus, estrus, metetrus, or diestrus stage. Ovaries were collected and snap frozen in liquid nitrogen or fixed in 4% formaldehyde. Animals were handled according to protocols approved by the McGill University Animal Care and Use Committees.

*Serum Measurements*

Blood was collected by percutaneous cardiac puncture from all treatment groups at each estrous cycle stage and the serum was separated and stored until further use. Enzyme-linked immunosorbent assays (ELISAs) from USCN Life Science Inc were used to quantify circulating levels of estradiol (E2; Cat# E90461Ge), luteinizing hormone (LH; Cat# E90441Ra), progesterone (P4; Cat# E90459Ge), follicle stimulating hormone (FSH; Cat# E90830Ra), inhibin B (inhb; Cat# CEA760Ra), inhibin A (inha; Cat# SEA395Ra), activin A (acta; Cat# CEA001Ra) and anti-Müllerian hormone (AMH; Cat# SEA228Ra).

*RNA extraction*

Tissue extraction to measure gene expression using Q-PCR was performed as previously described (85). Briefly, total RNA was extracted from snap frozen ovaries using RNAeasy kit (Qiagen). The RNA was then converted to cDNA using QuantiTect Reverse Transcription kit (Qiagen).

*Quantitative Real-Time PCR Analysis*

GAPDH was used as an endogenous control to normalize the gene targets obtained from one female offspring from each of three to four litters per treatment, with each sample processed in duplicates. The delta Ct method was used to express the results relative to the reference gene. Supplemental Table T1 contains a list of the TaqMan probes used with the TaqMan gene expression master mix (Invitrogen). Q-PCR measurements were carried out in a LightCycler 480 (Roche Diagnostics).

*Morphometry, histology, hematoxylin & eosin and Oil Red O staining*

Ovaries fixed in 4% paraformaldehyde were embedded in paraffin and sectioned (4 μm). Slides were stained with hematoxylin & eosin. Two sections per ovary spaced 700 microns apart were assessed with two preovulatory follicles per section being selected in order to measure theca and granulosa cell layers in three different areas. The quantification was done using PhotoShop CS5. For Oil Red O staining, samples were shipped to Cytochem (Montreal, QC). Tissues were embedded in cold polymer Optimal Cutting Medium (OCT, Tissue-Tek) and frozen in isopentane at -40°C, and then cut into 8 μm sections with Hacker/Bright OTF cryostat and the sections thaw-mounted into arrays on glass microscopy slides. Tissue sections were fixed in 4% formaldehyde in phosphate buffer 0.1 M pH=7.4, for 60 minutes and washed for 2 minutes in PBS. Sections were then gently immersed 2 x 1 second in 60% isopropanol and after dipping were transferred into freshly filtrated Oil Red O solution for 20 minutes. After Oil Red O staining, sections were washed 2 minutes in PBS and stained in freshly filtrated hematoxylin (H) for 40 seconds, washed in distilled water by 4 x 1-second dipping and closed in water mounting medium.

*Reproductive performance*

Three-month old females from all treatment groups were mated with three-month old normal males of known fertility for a minimum of three estrous cycles and their reproductive performance was assessed by rate of pregnancy, litter size, birth weight and anogenital distance. Assuming a 4 to 5 day long cycle, first cycle pregnancy was estimated to be between day 21 and 26, from initial encounter with a male.

*Microarray*

cDNA samples, converted from mRNA, were hybridized on Affymetrix GeneChip Rat Genome 230 2.0 arrays. Initial analysis was executed using the Affymetrix Expression Console and PLIER algorithm. Quality control procedures were performed in order to verify expression values, global average values and standard deviations, distributions of expression values, correlations, MVA plots and include principal component analysis (PCA). Normalization was completed using the Affymetrix Expression Console during the primary analysis of the CEL files using the quantile normalization option. The data were then further normalized to 100% of the mean value. Analysis of the differential expression was carried out using the Cyber-T software based on Bayesian approach complemented with the nonparametric method of consecutive sampling (140-144).

*Statistical Analyses*

Statistical analysis of the data using one-way analysis of variance followed by Dunnett's post-test was performed with Prism v4.02 (GraphPad, Inc.). For all experiments, the experimental unit was the pregnant dam, and the responses of female offspring from at least three dams (n = 3 pregnant dams) for each treatment was examined independently.

## Results

*In utero exposure to DEHP disrupts the preovulatory E2 surge in the estrous cycle*

To establish the effect of *in utero* exposure to DEHP on the estrous cycle of the adult offspring, pregnant Sprague-Dawley rats were gavaged from GD14 until birth with corn oil, or with 1, 20, 50, or 300 mg DEHP/kg/day. Female offspring were euthanized between PND60 and PND68 and serum samples were collected. At least 3 to 4 rats were euthanized per estrous cycle stage. Serum E2 levels were decreased at proestrus. E2 levels of female offspring exposed to 300 mg DEHP/kg/day were significantly decreased as shown by Bonferroni's post-test (Fig. 4A). The preovulatory E2 surge at proestrus was reduced in the offspring exposed to 1 and 50 mg DEHP/kg/day although not significant. The 50 and 300 mg DEHP/kg/day doses showed increases in E2 levels at estrus and metestrus but the effects were not statistically significant. Circulating E2 levels averaged across the cycle did not change significantly (Figure 4B). Circulating levels of P4 showed changes that varied according to the estrous cycle but they were not significant by ANOVA (Figure 4A and 4C).

*In utero exposure to DEHP reduces ovarian expression of Cyp17a1*

To determine the effect of DEHP in expression of encoding for proteins and enzymes involved in the biosynthesis of E2, we collected ovaries from female offspring which were *in utero* exposed to 1 – 300 mg DEHP/kg/day. Females were euthanized when staged at proestrus between PND60 and PND68. Q-PCR analysis showed the mRNA levels of *Star*, *Tspo, Cyp11a1, Hsd3b1,* and *Hsd17b3* were not affected by DEHP (supplemental Figure S1). *Cyp17a1* mRNA levels were decreased at 300 mg DEHP/kg/day at proestrus (Figure 5A) and was the only affected protein or enzyme involved in the biosynthesis of E2. *Cyp19a1* mRNA levels show

dose-dependent changes that correlate with circulating E2 levels but where not significant (Figure 5B). DEHP did not affect the expression of genes involved in the import or *de novo* synthesis of cholesterol at proestrus (supplemental Figure S1).

*In utero exposure to DEHP decreases theca cell layer thickness*

To determine the effect of DEHP exposure in granulosa and theca cell development at proestrus, ovaries from PND60 to PND68 offspring exposed *in utero* to 1 – 300 mg DEHP/kg/day were sectioned and the granulosa and thecal layer thickness were quantified. The thecal cell layer was significantly reduced at proestrus at the 50 and 300 mg DEHP/kg/day doses (ANOVA P = 0.0291) and the 50 DEHP/kg/day dose was significant by Dunnett's post-test (Figure 6A and 6C). The granulosa cell layer showed a decreasing trend across doses (Figure 6A and 6C), although not significant. Furthermore, Oil Red O staining showed that DEHP did not affect lipid droplet accumulation in the thecal layer of the ovarian follicles, although the staining appears to be slightly more intense in the treated groups. Oil Red O staining corroborates with the observed reduced thickness (Figure 6B).

To determine whether DEHP targeted the gonadotropin receptors in the ovary we quantified the mRNA levels of follicle-stimulating hormone receptor (*Fshr*) and luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) and found no significant changes (Figure 6D and 6E).

*In utero exposure to DEHP increases serum levels of FSH*

To determine the effects of DEHP exposure on the HPG axis, we collected serum from female offspring exposed *in utero* to 1 – 300 mg DEHP/kg/day and quantified circulating levels

of FSH, LH, inhibin B, inhibin A and activin A. The offspring were euthanized between PND60 and PND68 and at least 3 to 4 rats were euthanized per estrous cycle stage. FSH levels were significantly increased at proestrus in the 1, 50, and 300 mg DEHP/kg/day doses and at estrus in the 50 and 300 mg DEHP/kg/day doses (Figure 7A). FSH serum levels averaged across the estrous cycle stages were significantly increased (Figure 7B; ANOVA P = 0.0023). Circulating levels of LH were not affected per estrous cycle stage or overall in the *in utero* exposed offspring (Figure 7C and 7D). Circulating serum levels of inhibin B, inhibin A and activin A were also not significantly affected by DEHP (Figure 7E-G).

We quantified the mRNA levels of pituitary and hypothalamic genes involved in the regulation of FSH and LH serum levels in the 20 and 300 mg DEHP/kg/day doses. We found no significant changes in the pituitary genes, *Esr1*, *Esr2*, *Pgr, Gnrhr*, *Cga*, *Fshb*, *Lhb* or in the hypothalamus genes, *Esr1*, *Esr2*, *Pgr, Gnrh1, Kiss1, Kiss1r* (supplementary Figure S2).

*In utero exposure to DEHP decreases anti-Müllerian hormone gene expression*

To evaluate the ovarian oocyte reserve, we quantified gene expression levels of *Amh* in the ovaries of PND60 to PND68 offspring exposed *in utero* to 1 – 300 mg DEHP/kg/day, staged at proestrus. *Amh* RNA levels were significantly decreased at the 50 and 300 mg DEHP/kg/day doses (Figure 8A; ANOVA P = 0.0362). Serum levels of AMH were measured at proestrus (Figure 8B) and were not significant. We also quantified the number of preovulatory mature follicles. Ovaries from offspring exposed to 1 - 300 mg DEHP/kg/day were collected at proestrus, sectioned, and stained with hematoxylin and eosin. Although not significant by ANOVA, the mature follicle count demonstrated a decreasing trend (Figure 8C).

*In utero exposure to DEHP decreases first cycle pregnancy rate*

Reproductive performance in the female was assessed following *in utero* exposure to 20 and 300 mg DEHP/kg/day by breeding PND90 female offspring (F1) with PND90 non-treated males of known fertility. *In utero* exposure to DEHP did not affect the overall pregnancy rate (Figure 9A), however the first cycle pregnancies for the 20 and 300 mg DEHP/kg/day were decreased to 42% compared to 75% in the controls (Figure 9B). Litter size, male to female ratio, pup weight as well as anogenital distance were not affected (Figure 9 C-H).

*In utero exposure to DEHP induces similar genetic alterations across treatment groups*

In order to assess global transcript changes, microarray data of the ovaries was acquired from PND60 to PND68 offspring exposed *in utero* to 20 and 300 mg DEHP/kg/day, staged at proestrus. The microarray data was compiled in a Venn diagram. The treatment groups had 247 common genes that were targeted by DEHP, with the 20 mg DEHP/kg/day dose exhibiting more gene expression changes compared to the 300 mg DEHP/kg/day dose (Figure 10).

**Discussion**

This work originated from our previous observation that E2 levels were decreased in the PND60 female offspring *in utero* exposed to 300 mg DEHP/kg/day (85). In the male, *in utero* exposure to DEHP results in decreased circulating testosterone and aldosterone levels in the adult offspring (85;86;118;138). Herein, we address the mechanisms behind the endocrine disrupting effects of DEHP in ovarian steroidogenesis and function. The window of DEHP exposure was originally chosen to target testosterone production of fetal-type Leydig cells responsible for primary male sexual development (145-147). In the female, this window of

exposure corresponds to the period when pre-granulosa cells gather around ovules to form chordae (148). MEHP is an activator of the PPARα and PPARγ nuclear receptors (149). These receptors were shown to be a critical mediators of the decrease in E2 and *Cyp19a1* levels of granulosa cells exposed to MEHP (89). In our model, the finding that steroidogenesis was affected in both sexes suggests that the PPAR pathway was critical for the correct development of the progenitor cells of the adult steroidogenic cells. Since DEHP and its metabolites are rapidly cleared from the circulation, we hypothesised that an epigenetic mechanism targeting the PPAR pathway is partially responsible for mediating the effects of DEHP in the adult steroidogenic cells of both sexes.

We began our study by characterizing the steroidogenic profile across the estrous cycle in the female offspring. The data show a decrease in the preovulatory sex steroid surge. E2 levels peak during proestrus and set off a series of events that culminate with ovulation. This event is followed by formation of the luteal body responsible for producing P4. We observed high P4 levels at 20 and 50 mg DEHP/kg/day at proestrus that may also interfere with ovulation, however, LH levels were not significantly altered across the cycle at any of the doses. Interestingly, DEHP treatment induced hormonal fluctuations in a non-monotonic manner; alterations were seen at the lower doses that were not amplified at the higher doses. A possible explanation is that DEHP targets an array of mechanisms related to steroidogenesis in the ovary resulting in the absence of a distinct dose-response trend. In fact, while the microarray data comparison between the 20 and 300 mg DEHP/kg/day showed 247 common targeted genes, the lower dose demonstrated more gene expression changes with 692 additional altered genes compared to 21 in the higher dose group. DEHP has been associated with antiestrogenic effects in females (88;134), and indeed, our data supports these observations at proestrus. Conversely,

averaged levels of E2 and P4 across the cycle were not significantly affected by DEHP. These findings suggest that although *in utero* exposure to DEHP may not affect basal steroid levels, it is likely that it specifically disrupts the female sex steroid surge at proestrus that is critical for ovulation. The alteration in the steroid profile could possibly impact estrogen-dependent tissues such as the endometrium and mammary gland, although this has yet to be further explored. The results suggest that DEHP propelled a long-term perturbation of the mechanisms governing preovulatory steroidogenesis.

We chose to study the proestrus stage because it precedes ovulation and exhibits the highest levels of E2. We measured the mRNA levels of proteins and enzymes involved in the biosynthesis of E2 and P4. The results show that *Cyp17a1* mRNA levels were significantly decreased by DEHP at proestrus. *Cyp17a1* is expressed in theca cells and catalyzes the conversion of 17α-hydroxyprogesterone to androstenedione, which diffuses to the granulosa cell layer to finalize estrogen biosynthesis. Interestingly, *Cyp19a1* levels were not significantly changed, however, they closely correlated with the fluctuations in E2 levels at proestrus. Morphometric analysis indicated that the development of the thecal cell layer of mature follicles starting at the 50 mg DEHP/kg/day dose was decreased. We also observed decreases in granulosa cell layer although not significant. Our findings are in agreement with previous reports showing decreases of *Cyp19a1* mRNA and E2 levels in human granulosa cells (150) and growth inhibition of mouse antral follicles after treatment with MEHP (69). Moreover, high levels of DEHP *in vivo* treatment for 16 weeks was also shown to arrest growth and apoptosis of granulosa cells in mice (151). A decrease in cell volume of granulosa cells was also reported in rats treated with DEHP (88). Cell proliferation in the granulosa and theca layers is, potentially, a common short and long-term target of DEHP.

In our previous findings, *in utero* exposure to DEHP affected cholesterol import in the adrenal gland of the male offspring resulting in lipid droplet accumulation in the aldosterone-producing cells (85). Similar findings were observed in ovarian granulosa cells from 20 day old rats exposed for 10 days to 500 mg DEHP/kg/day (92). We quantified the expression of genes involved in cholesterol import and *de novo* cholesterol synthesis and found them not to be affected by *in utero* exposure to DEHP in the proestrus ovary at PND60. In addition, Oil Red O staining indicated no significant change in lipid droplet accumulation in the thecal layer, although the staining appears to be slightly more intense in the treated groups. It is possible that this observation is a consequence of the same amount of droplets congregating in the reduced-in-size thecal cell layer area. It remains to be elucidated if cholesterol import could account for the decrease in E2 at the proestrus stage in the female offspring exposed *in utero* to DEHP.

To assess the integrity of the HPG axis signalling pathway, we explored the expression of the gonadotropin receptors in the ovary. *Fshr* and *Lhcgr* mRNA levels in the ovaries of DEHP exposed animals were similar to those of control animals. Since there was a decrease in the granulosa/theca thickness and because the mRNA levels of the receptors were unchanged, we can speculate that perhaps the volume of the cells might be decreased. The latter coincides with previous findings that showed that acute exposure to DEHP decreased granulosa cell size (88), as previously mentioned. We then quantified FSH and LH serum levels and found a significant increase in FSH levels at the proestrus and estrus stages while LH levels were unaffected by DEHP. These increases in FSH significantly affected the average levels of FSH across the cycle. We measured the expression of pituitary genes involved in the regulation of FSH and LH synthesis and found no significant changes. We found no changes in pituitary *Lhb* and *Fshb* mRNA levels suggesting that DEHP targets circulating FSH at the protein level. mRNA levels of

*Esr1*, *Esr2*, *Pgr* and *Gnrhr* were not affected, which indicates that the feedback machinery in the pituitary gland at the mRNA level was not targeted by *in utero* exposure to DEHP. We verified gene expression in the hypothalamus and similarly found that *Esr1, Esr2* and *Pgr* were unaffected. We also measured gene expression levels of *GnRH1*, *Kiss1* and *Kiss1r* and found no significant changes. Even though our findings show that gene expression in the pituitary and the hypothalamus was not affected, they do not rule out the possibility that DEHP might impact gonadotropin levels by influencing the amplitude or frequency of hypothalamic GnRH-I pulses to the pituitary.

In addition, ovarian hormonal feedback in the HPG axis directly affects mRNA levels in the pituitary and the hypothalamus; *Fshb* mRNA, for instance, is regulated by E2 and inhibin (152). The decrease in E2 at the proestrus stage together with unchanged gene expression in the pituitary indicates that the decrease in ovarian sex steroid was not substantial enough to cause the increase in circulating FSH. It is possible that DEHP's mode of action targets the secretion or half-life of FSH in lieu of its synthesis. GnRH receptor expression in the pituitary is also estrogen-sensitive and no change was seen in its gene expression. Furthermore, the findings of unaltered serum levels of inhibin B, inhibin A and activin A corroborate with the previous observations. Thus, in terms of the HPG axis, we hypothesized that DEHP alters levels of circulating FSH at the protein level rather than at the mRNA level of its feedback loop.

Furthermore, no significant change was seen in the number of large preovulatory follicles, or AMH serum levels. On the contrary, a significant decrease in *Amh* gene expression in the ovary starting at the 50 mg DEHP/kg/day dose was observed. Within the follicles, lower expression of *Amh* is associated with increased FSH sensitivity. In the clinic, circulating levels of AMH and FSH are often correlated to the magnitude of the ovarian oocyte reserve (153;154). In the case of

age-related ovarian failure, the expected phenotype would include elevated serum FSH and decreased serum AMH. Our data only depicts the former. The finding of normal circulating levels of AMH provides no evidence to suggest depletion of the ovarian reserve. The decrease in follicle development and the decrease in *Amh* expression suggest that the growing follicles are potentially more sensitive to FSH but are not responding correctly, even with exceedingly high gonadotropin levels and unaffected expression of the FSH receptor. We hypothesize that DEHP likely exerts a local effect on the ovaries and since the pituitary stores FSH prior to secretion, it is possible that it too is a target of DEHP.

We hypothesized that *in utero* exposure DEHP could affect reproduction in the female, however, overall pregnancy rate, litter size, pup weight and anogenital distance were not affected. This study was conducted at PND90, the optimal breeding age for rats. Although reproduction did not fail, the proposed events with regards to *Amh* expression and FSH levels depict a scenario in which the system is attempting to kick-start the recruitment of follicles. It is possible that flawed follicular development could potentially decrease the reproductive lifespan of the F1 female. A successive and long-term mating study would be required to attest to this. Our data show a reduction in first cycle pregnancy; less than 50% of the treatment groups got pregnant during the first estrous cycle with a male, compared to 75% of the control group. In addition, female behavior prior to ovulation has been well characterized and is thought to be dictated by P4 and E2 (155). As we have reported DEHP-induced alterations in E2 and P4, however unlikely, it is possible that DEHP may delay copulation by affecting the steroid-induced behavior in the female adult rat preceding ovulation. The effects of *in utero* exposure to DEHP on female behavior have yet to be studied.

It is important to note that the average human daily exposure to phthalates is estimated to be between 1.7 and 52.1 μg/kg/day (70;74;75). In our studies, we used DEHP doses that correspond to human equivalent doses. This is based on a formula that suggestively converts substance exposure across species by taking in to account many relevant features including body surface area and metabolism, which are thought to be more reliable conversion factors compared to body mass (76). Based on our calculations, human exposure corresponds to 0.18 to 62.5 mg DEHP/kg/day rat exposures. Our methods comprise DEHP doses ranging from 1 to 300 mg DEHP/kg/day, thus effects reported herein are within the window of environmentally relevant doses for humans.

In summary, our work proposes a mode of action in which *in utero* exposure to DEHP targets the ovary by obstructing follicular development. Figure 11 depicts a summary of all physiological outcomes reported. DEHP may also target the brain by affecting pituitary storage of FSH, although this has yet to be studied. Exposure to DEHP seems to perturb the steroid surges in the estrous cycle but is unlikely to target the HPG feedback mechanisms. Reproduction was not affected in the PND90 females, although the effect of DEHP on the reproductive lifespan in females has yet to be elucidated. Taken all together, our data supports the notion that *in utero* exposure to DEHP exerts a marginal effect on female reproduction in comparison to what has been previously studied in the male.

## Figures 4 to 11

**Figure 4. Reduction of E2 serum levels at proestrus in the adult following *in utero* exposure to DEHP**

Circulating E2 (E2) and progesterone (P4) (A) across the estrous cycle and averaged E2 (B) and P4 (C) levels from PND60 females staged accordingly at metestrus, diestrus, proestrus and estrus, exposed *in utero* to DEHP with concentrations ranging from 1-300 mg DEHP/kg/day. Results shown are means of PND60 *in utero*-treated females (n = 4). * Indicates a significant decrease in E2 by Bonferroni's post-test at the 300 mg DEHP/kg/day dose relative to the control.

**Figure 4. Reduction of E2 serum levels at proestrus in the adult following *in utero* exposure to DEHP**

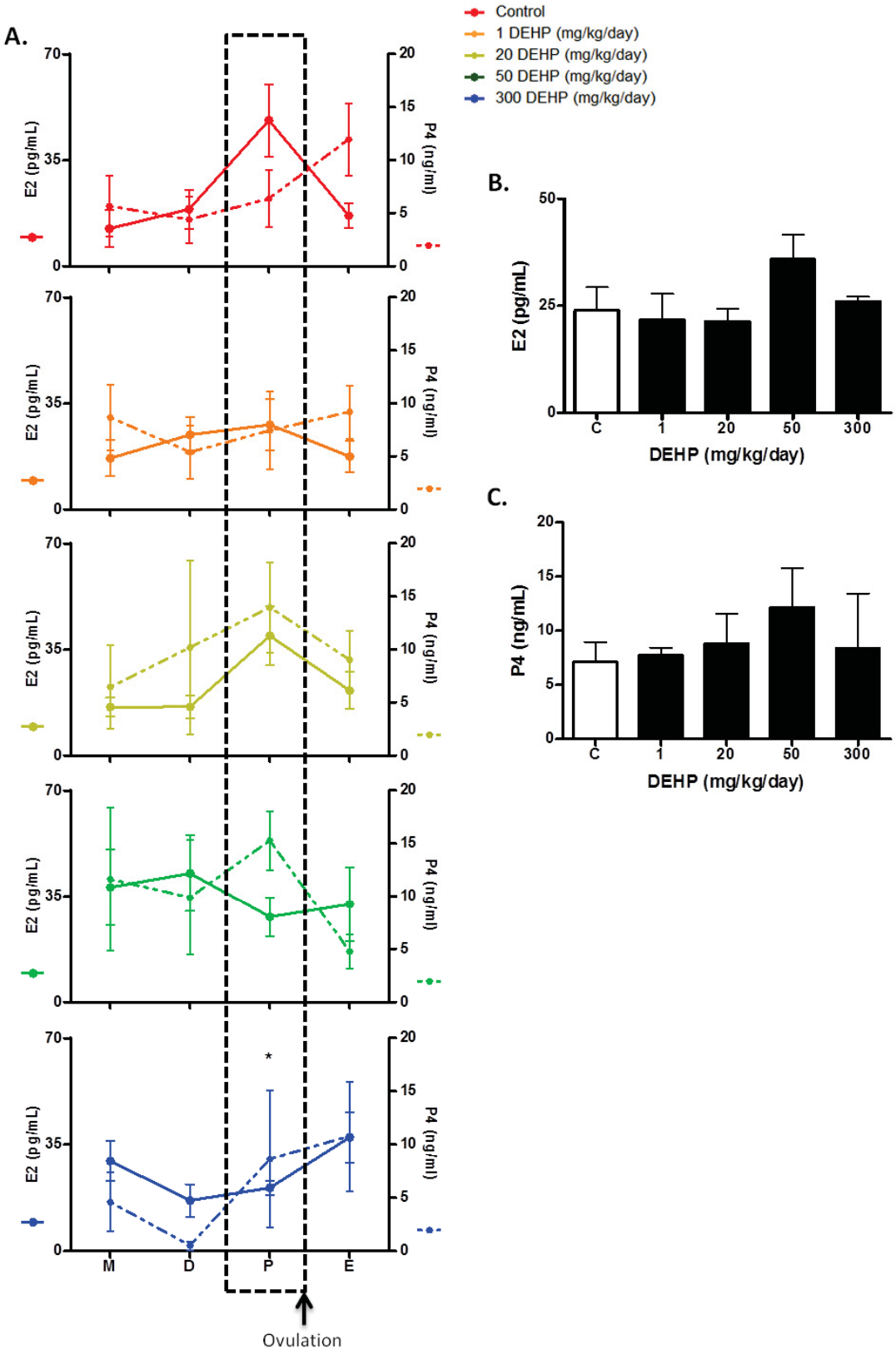


**Figure 5. Decrease in the enzyme required for androgen formation in adult ovaries following *in utero* exposure to DEHP**

Q-PCR gene expression analysis of enzymes involved in the steroidogenic pathway, *Cyp17a1, Cyp19a1* (A,B) in ovaries harvested from PND60 females staged at proestrus exposed *in utero* to DEHP with concentrations ranging from 1-300 mg DEHP/kg/day are depicted. Results shown are means of PND60 *in utero*-treated females (n = 4). *$P < 0.05$, indicates significant decrease relative to the control.

**Figure 5. Decrease in the enzyme required for androgen formation in adult ovaries following *in utero* exposure to DEHP**

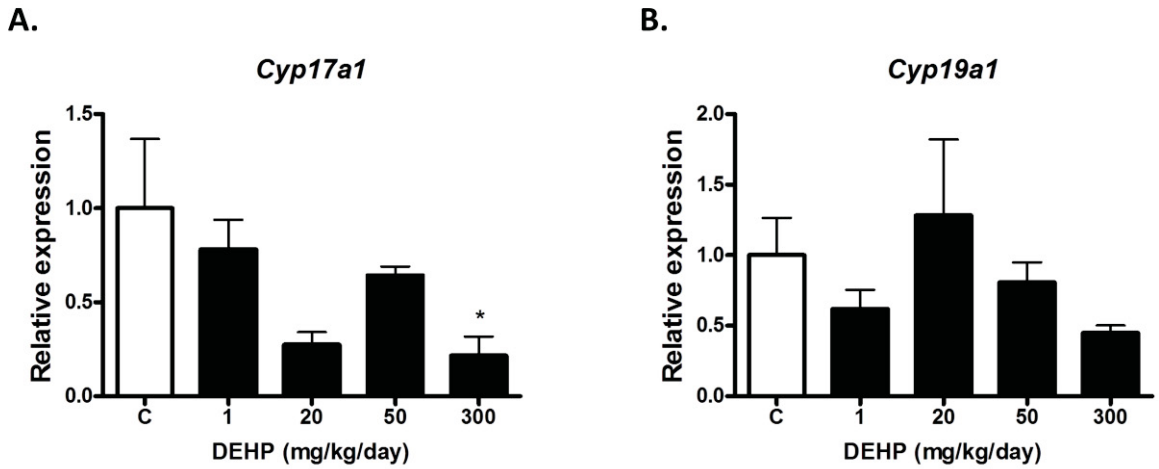
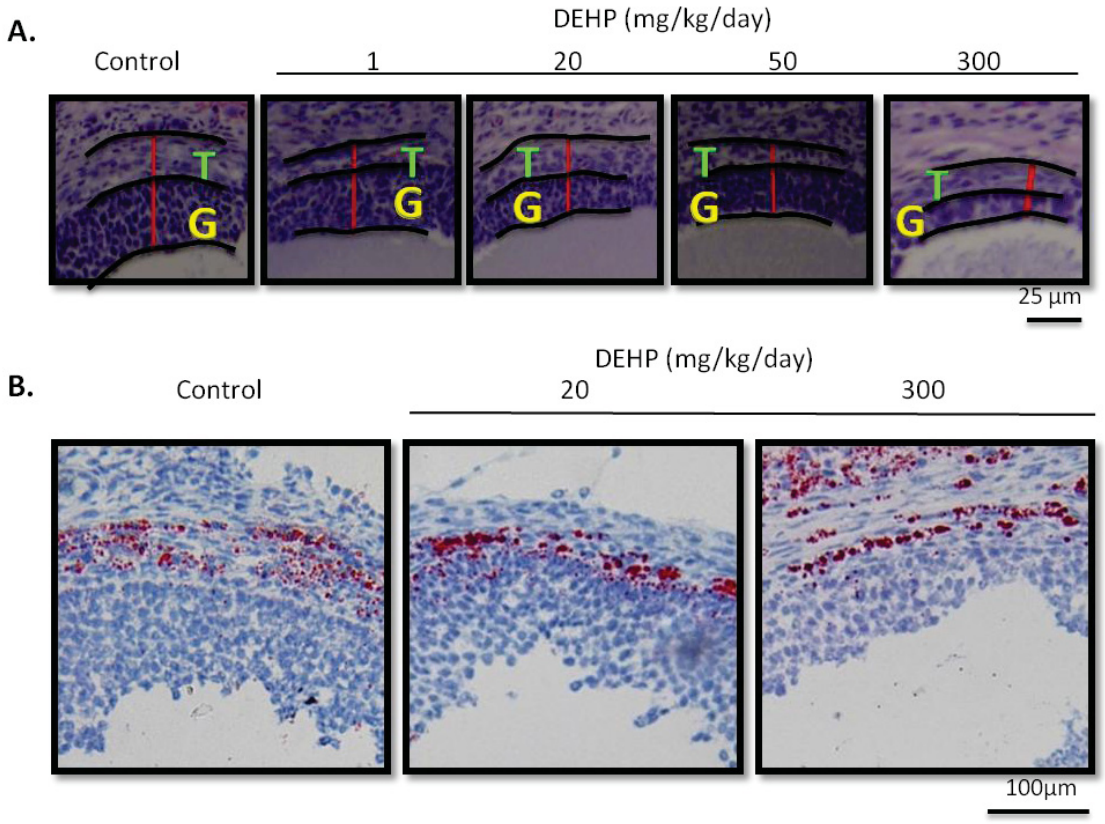
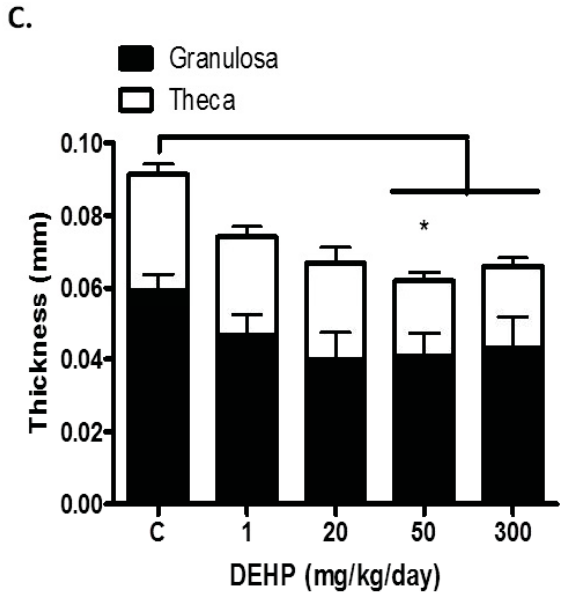


**Figure 6. Decrease in theca cell layer thickness in the ovarian follicles of DEHP *in utero* treated adults**

Ovaries obtained from PND60 females staged at proestrus at doses ranging from 1-300 mg DEHP/kg/day stained with hematoxylin and eosin (A), and Oil Red O (B). Theca and granulosa cell layers are identified with a T and G respectively. Granulosa cell layer thickness (black columns) and theca cell layer thickness (white column) (C) were measured three times per follicle, two follicles per section and two sections spaced 700 microns apart per ovary. * represents significant variations at the theca layer. Q-PCR of FSH and LH receptors (D, E). Results shown are means of PND60 *in utero*-treated females (n = 4-6). *$P < 0.05$.

**Figure 6. Decrease in theca cell layer thickness in the ovarian follicles of DEHP *in utero* treated adults**



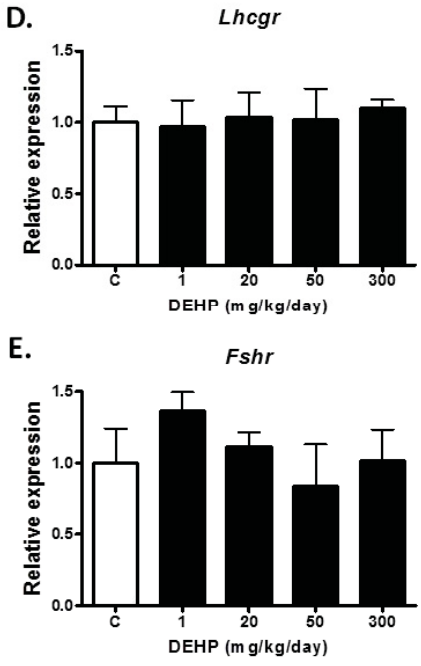



**Figure 7. Increase in FSH serum levels in the adult following *in utero* exposure to DEHP**

Circulating FSH (A) and LH (C) across estrous cycle and averaged FSH (B) and LH (D) levels from PND60 females staged accordingly at metestrus, diestrus, proestrus and estrus, exposed *in utero* to DEHP with concentrations ranging from 1-300 mg/kg/day. Serum levels of inhibin A, inhibin B and activin A staged at proestrus (E-G). Results shown are means of PND60 *in utero*-treated females (n = 4-6). □ # ● represent significance at 1, 50 and 300 mg DEHP/kg/day respectively, relative to the control. $*P < 0.05$, $***P < 0.001$.

**Figure 7. Increase in FSH serum levels in the adult following *in utero* exposure to DEHP**

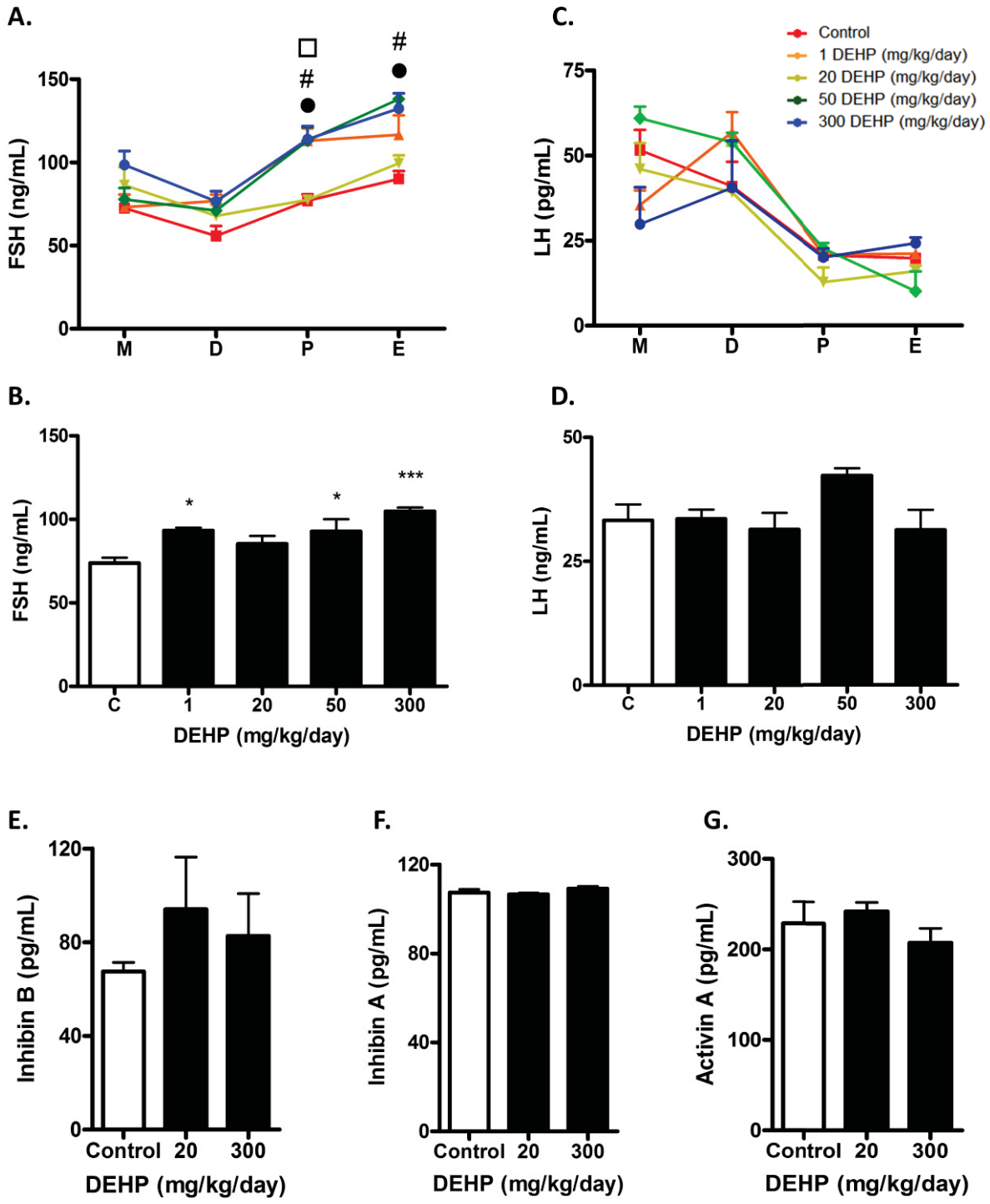


**Figure 8. Decrease in gene expression of *Amh***

Ovaries obtained from PND60 females staged at proestrus at doses ranging from 1-300 mg DEHP/kg/day and stained with hematoxylin and eosin. Mature follicles were counted at two sections spaced 700 microns apart (C). Q-PCR of anti-Müllerian-hormone (A) and serum levels (B). Results shown are means of PND60 *in utero*-treated females (n = 4-6). $*P < 0.05$.

**Figure 8. Decrease in gene expression of *Amh***

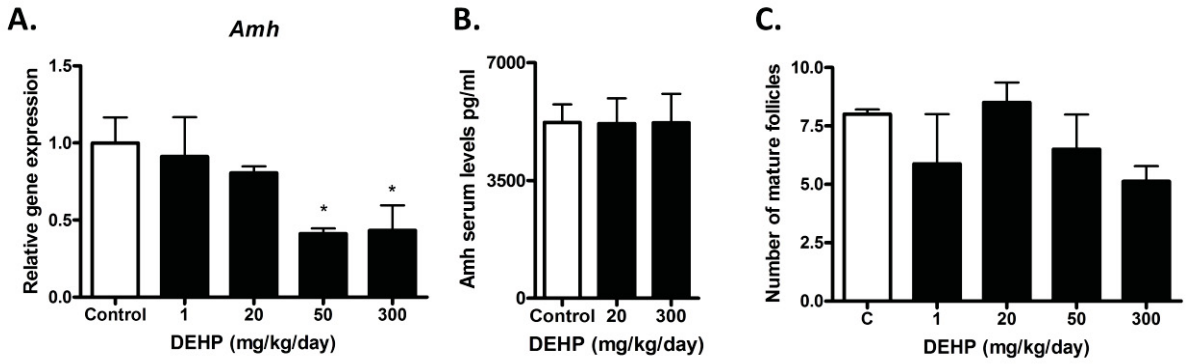


**Figure 9. Decrease in first cycle pregnancy**

Pregnancy rate of *in utero* treated PND90 females mated with control PND90 males. Fractions on the columns represent successful pregnancies over attempts per dose group. Overall pregnancy rate (A), first cycle pregnancy (C), litter size (D), pup weight (E), male and female litter percentage (F,G) and male and female anogential distances (H,I) are depicted. Results shown are means from the litters of PND90 *in utero*-treated female (n=6-8).

**Figure 9. Decrease in first cycle pregnancy**

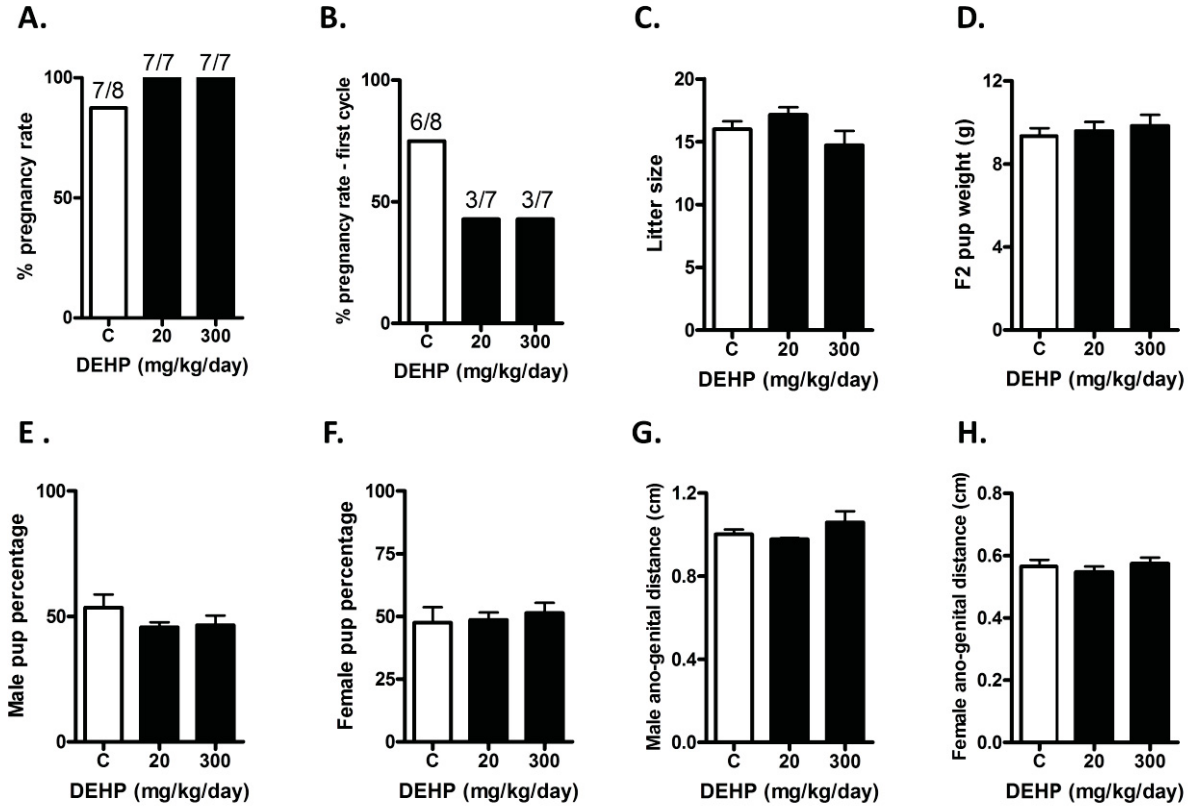


**Figure 10. Ovarian gene expression profile across treatment groups**

Microarray data from the ovaries of PND60 *in utero* treated females staged at proestrus. Venn diagram of the 20 (yellow) and 300 (purple) mg DEHP/kg/day treatment groups. Results shown are means of PND60 *in utero*-treated females (n = 3-4).

**Figure 10. Ovarian gene expression profile across treatment groups**

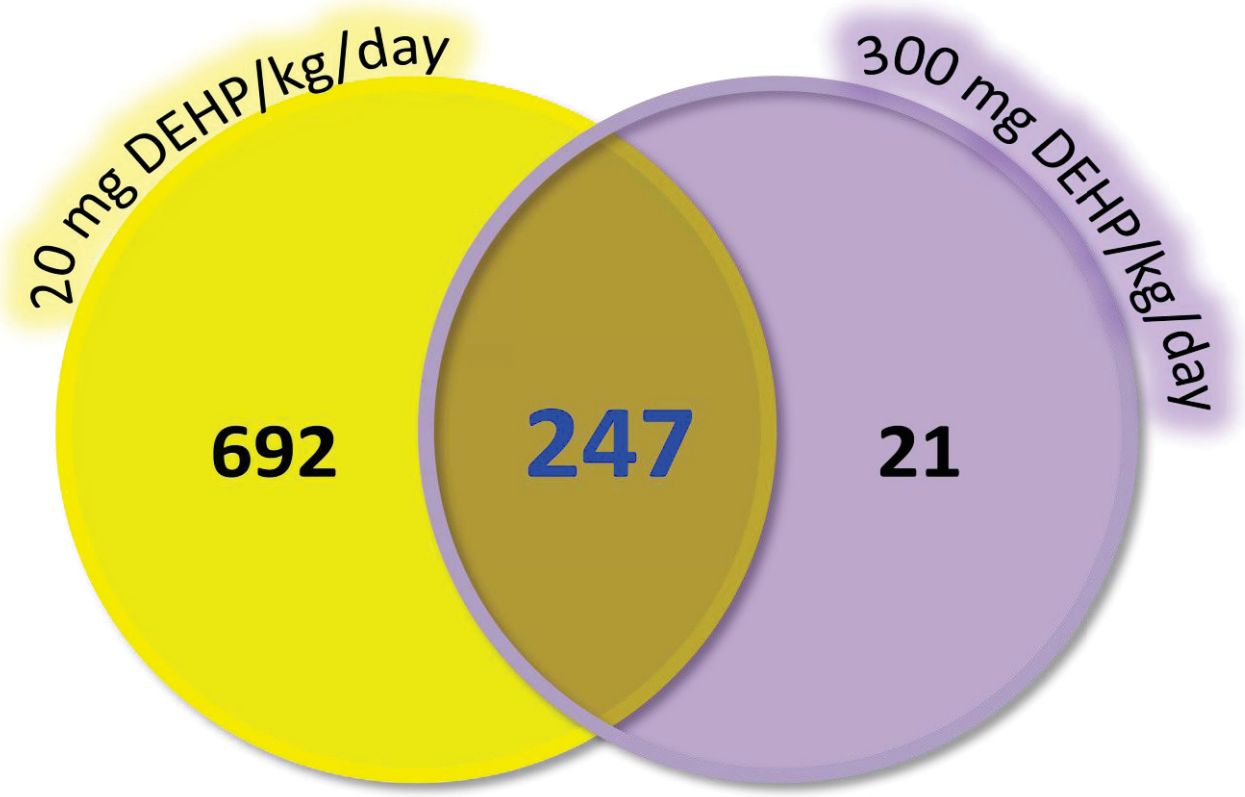
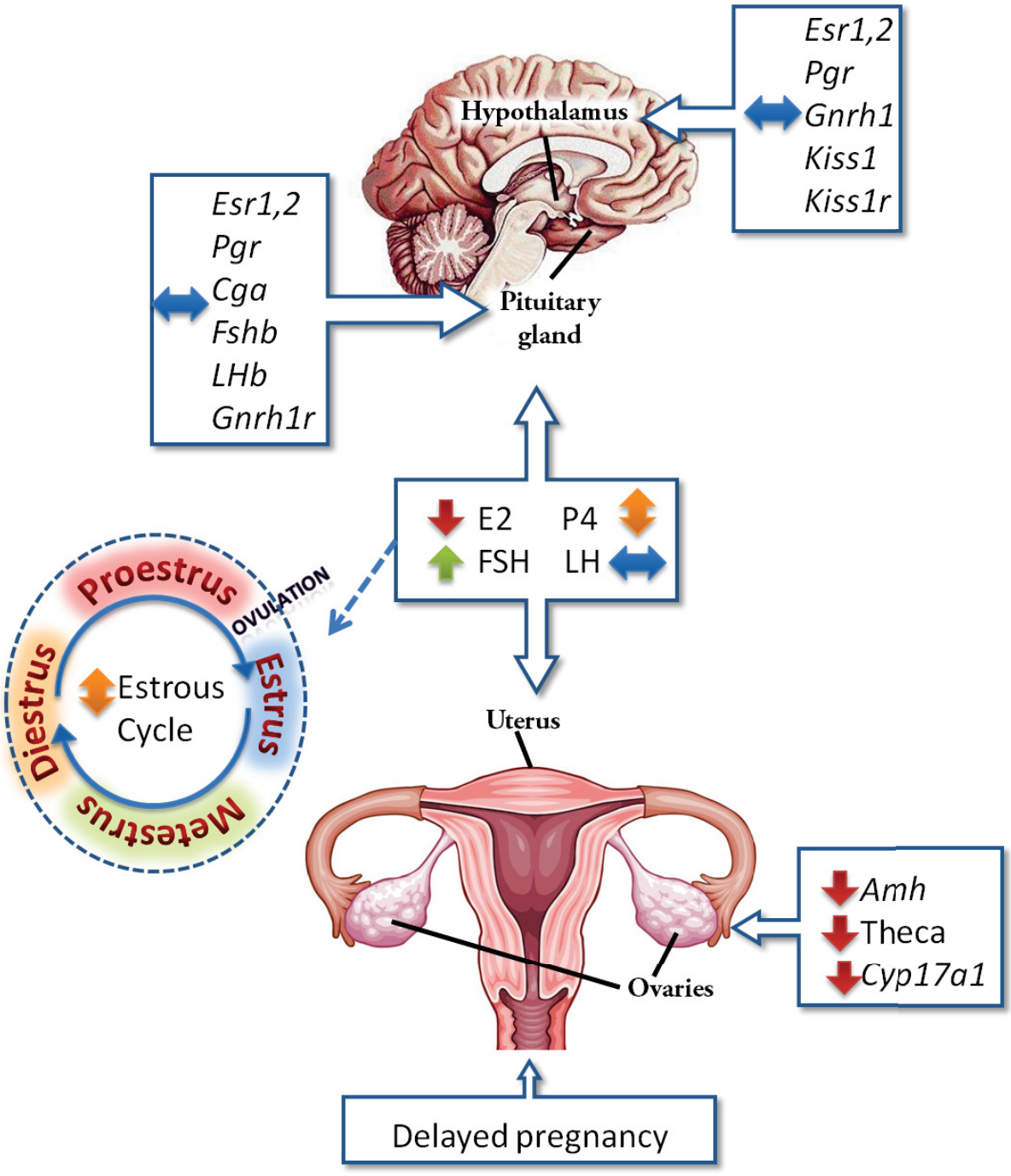


**Figure 11. Summary of the effects of *in utero* exposure to DEHP on the female adult**

Gene expression in the pituitary (*Esr1, Esr2, Pgr Cga, Fshb, Lhb, GnrhIr*) and hypothalamus (*Esr1, Esr2, Pgr, GnrhI, Kiss1, Kiss1r*) is unaffected. Serum levels of estradiol (E2) decrease, FSH serum levels increase, progesterone (P4) levels fluctuate throughout the cycle, and LH levels are unchanged. Estrous cycle periodicity is shifted. *Cyp17a1, Amh* expression and theca cell layer thickness are decreased in the ovary. *In utero* exposure to DEHP induces a delay in pregnancy.

**Figure 11. Summary of the effects of *in utero* exposure to DEHP on the female adult**



## Summary and conclusions

The effects of *in utero* exposure to DEHP on the female reproductive system have yet to be mechanistically elucidated. It is likely that gestational exposure to environmental chemicals such as DEHP results in long term health abnormalities in the offspring; this is the principle behind Barker's origin of disease hypothesis (98). Thus far, studies examining the reproductive outcome of developmental exposure to this phthalate in the female have indicated that the ovary is a probable target. Reports have shown changes in ovarian gene expression of critical enzymes in steroidogenesis and the HPG axis (119), variations in female sex steroid serum levels (123), increases in atretic follicle count and delayed onset of estrus (120;121).

The data presented herein provides evidence that *in utero* exposure to DEHP targets follicular development in the ovary. The ovarian sex steroid preovulatory surge was disrupted; there was an absence of the characteristic E2 peak at proestrus prior to ovulation. We counted the mature preovulatory follicles in the ovaries at proestrus and although there was no change in number, they exhibited a perturbation in development. We observed a decrease in the cell wall thickness of these large antral follicles with a significant decrease in theca layer thickness as well as a decrease in mRNA levels of *Cyp17a1*. Furthermore, we reported elevated levels of follicle stimulating hormone (FSH) at the proestrus and estrus stages. This increase suggests a requirement to kick-start follicular growth. Interestingly, gene expression of *Amh*, whose role is to suppress primordial follicle recruitment and to desensitize growing follicles to FSH, was found to be significantly decreased, while circulating levels were unchanged. As gene expression of the gonadotropin receptors was unaffected and the mature preovulatory follicle count did not vary significantly, the previous finding indicates an inability of the follicles to respond to FSH. The mating study we conducted with control males of known fertility resulted in no decrease in

overall pregnancy rate, however first cycle pregnancy was notably reduced in the treatment groups.

Although the increase in FSH seems not to be a direct result of a mechanistic problem in the pituitary, it is likely that DEHP directly targets multiple mechanisms in the ovary. The physiological profiles that the PND60 adult females exhibited after a brief window of exposure to DEHP from GD14 until birth demonstrate a disruption in ovarian follicular development and potential response to FSH.

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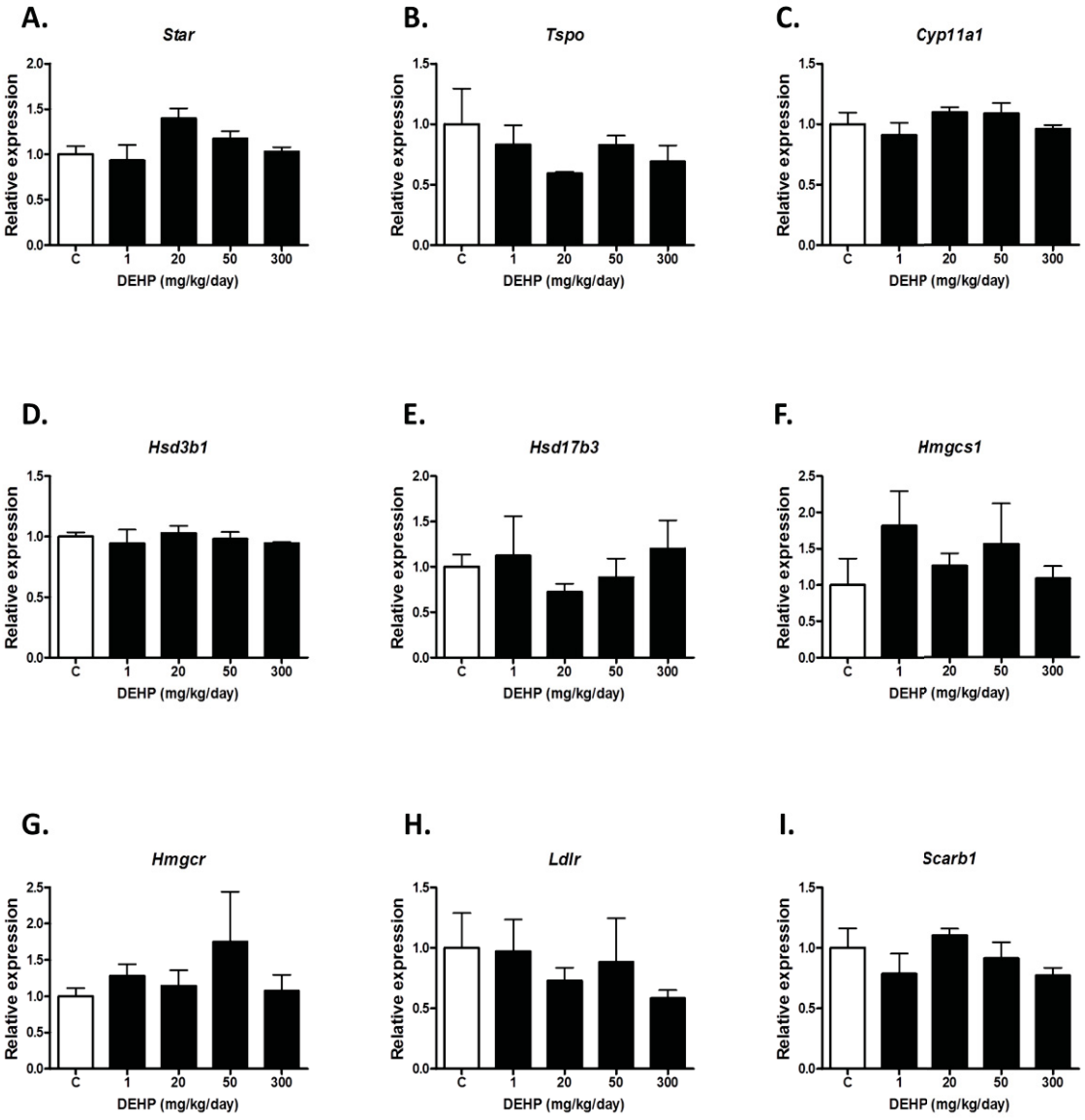
## Supplemental figures S1 and S2

**Supplemental figure S1. Gene expression profile in the ovaries staged at proestrus following *in utero* exposure to DEHP**

Q-PCR of genes involved in the steroidogenic pathway, *Star, Tspo, Cyp11a1, Hsd3b1, Hsd17b3* (A-G), cholesterol import, *Ldlr, Scarb1* (H,I) and de novo cholesterol biosynthesis, *Hmgcr, Hmgcs1* (J,K) from ovaries harvested from PND60 females staged at proestrus exposed *in utero* to DEHP with concentrations ranging from 1-300 mg DEHP/kg/day. Results shown are means of PND60 *in utero*-treated females (n = 4).

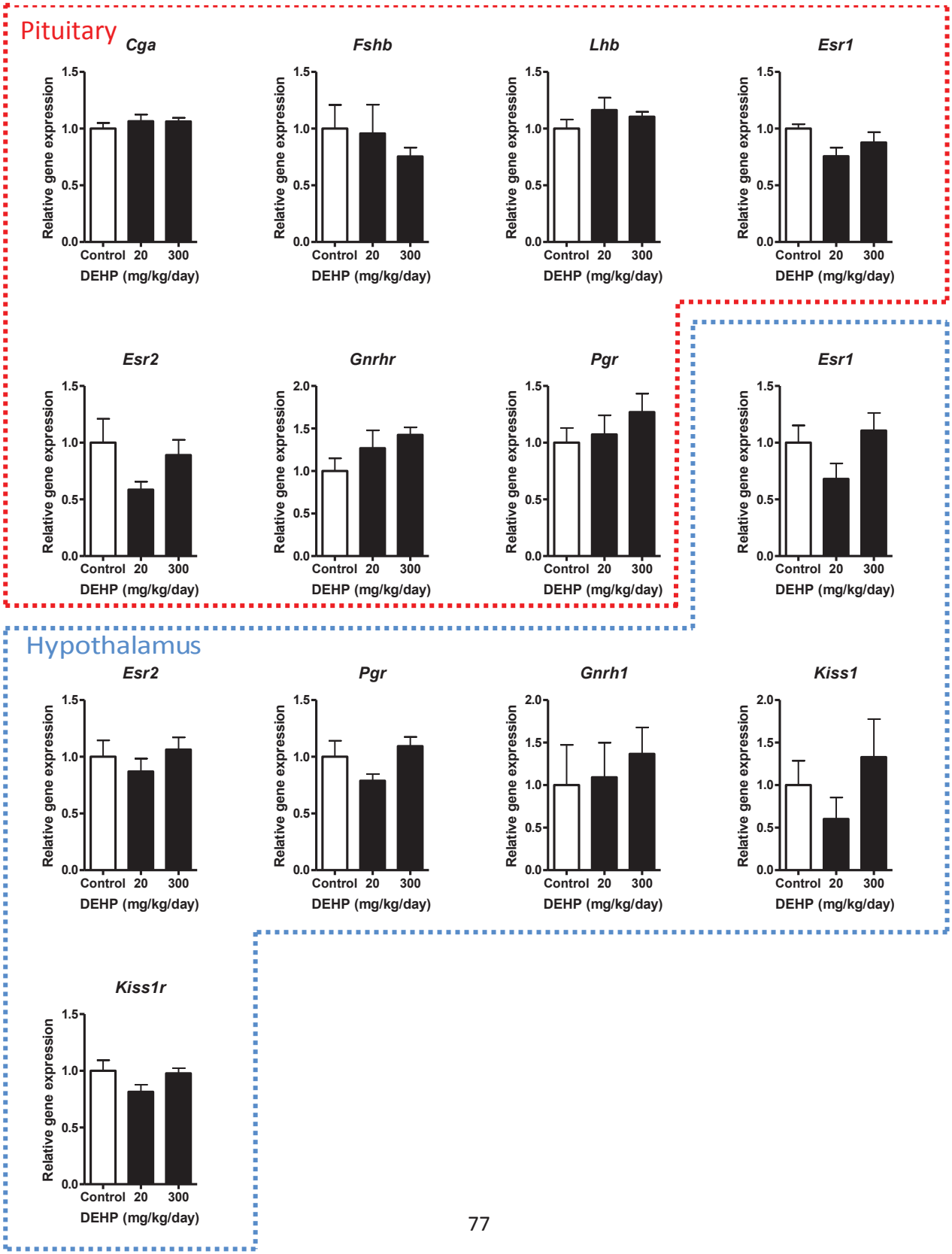
**Supplemental figure S1. Gene expression profile in the ovaries staged at proestrus following *in utero* exposure to DEHP**



**Supplemental figure S2. Q-PCR of genes involved in the gonadotropin secretion pathway in the pituitary and the hypothalamus of the adult rat following *in utero* exposure to DEHP**

Pituitary and hypothalamus were collected from PND60 *in utero* treated females from 20 and 300 mg DEHP/kg/day doses. Q-PCR of genes in the pituitary, *Cga, Fshb, Lhb, Esr1, Esr2, Gnrhr* and *Pgr* (A-G) and in the hypothalamus, *Esr1, Esr2, Gnrh1*, *Pgr, Kiss1* and *Kiss1r* (H-M). Results shown are means of PND60 *in utero*-treated females (n = 8).

**Supplemental figure S2. Q-PCR of genes involved in the gonadotropin secretion pathway in**



## Supplemental table T1

**Supplemental table T1. TacMan probes used for Q-PCR analysis.**

| Gene symbol | RefSeq | TacMan Reference |
|---|---|---|
| *Gapdh* | NM_017008.3 | 4352338E |
| *Star* | NM_031558.2 | Rn00580695_m1 |
| *Tspo* | NM_012515.2 | Rn00560892_m1 |
| *Cyp11a1* | NM_017286.2 | Rn00568733_m1 |
| *Hsd3b1* | NM_001007719.3 | Rn00820880_g1 |
| *Hsd17b3* | NM_054007.1 | Rn00588942_m1 |
| *Cyp17a1* | NM_012753.1 | Rn00562601_m1 |
| *Cyp19a1* | NM_017085.2 | Rn01422546_m1 |
| *Ldlr* | NM_175762.2 | Rn00598442_m1 |
| *Scarb1* | NM_031541.1 | Rn00580588_m1 |
| *Hmgcr* | NM_013134.2 | Rn00565598_m1 |
| *Hmgcs1* | NM_017268.1 | Rn00568579_m1 |
| *Fshr* | NM_199237.1 | Rn01648507_m1 |
| *Lhcgr* | NM_012978.1 | Rn00564309_m1 |

| | | |
|---|---|---|
| *Cga* | NM_053918.1 | Rn02532426_s1 |
| *Fshb* | NM_001007597.1 | Rn01484594_m1 |
| *Lhb* | NM_001033975.1 | Rn00563443_g1 |
| *Esr1* | NM_012689.1 | Rn00562166_m1 |
| *Esr2* | NM_012754.1 | Rn00562610_m1 |
| *Gnrhr* | NM_031038.3 | Rn00578981_m1 |
| *Pgr* | NM_022847.1 | Rn01448227_m1 |
| *Gnrh1* | NM_012767.2 | Rn00562754_m1 |
| *Kiss1* | NM_181692.1 | Rn00710914_m1 |
| *Kiss1r* | NM_023992.1 | Rn0056940_m1 |
| *Amh* | NM_012902.1 | Rn00563731_g1 |