MECHANISMS BY WHICH DI(2-ETHYLHEXYL) PHthalate AND MONO(2-ETHYLHEXYL) PHthalate DISRUPT OVARIAN FOLLICULOGENESIS AND STEROIDOGENESIS

BY

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DISSErTATION

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ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is a synthetic plasticizer used in the manufacturing of common polyvinyl chloride consumer, medical, and building products. Humans are exposed to DEHP on a daily basis because of its high production volumes, incorporation into widely used products, and its ability to leach out of the products. Daily exposure to DEHP results in measurable levels of the chemical and its metabolite mono(2-ethylhexyl) phthalate (MEHP) in human blood, urine, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluid samples. This is of public health concern because DEHP and MEHP are known endocrine disrupting chemicals and reproductive toxicants in the female.

The ovary is a primary regulator of the female reproductive and endocrine systems. Thus, proper regulation of ovarian function is required for reproductive health and steroid hormone controlled non-reproductive health. Two ovarian processes that are essential for normal ovarian function are folliculogenesis and steroidogenesis. Follicles are the functional units of the ovary containing the oocyte required for ovulation and fertilization and somatic cells responsible for synthesizing sex steroid hormones. Folliculogenesis is the process by which follicles undergo several irreversible developmental transitions starting from the most immature stage, the primordial follicle, to the most mature stage, the antral follicle. Steroidogenesis is the process by which somatic cells in the antral follicle enzymatically convert cholesterol to estradiol and other necessary precursor steroid hormones in a step-wise manner.

Improper regulation of ovarian folliculogenesis and steroidogenesis can lead to reproductive and non-reproductive complications. Specifically, aberrant regulation of folliculogenesis, indicated by an accelerated depletion or activation of primordial follicles, can lead to premature ovarian failure and infertility. This is because the primordial follicle reserve is set at birth and constitutes a female’s reproductive potential. Additionally, an increase in atresia, an apoptosis controlled process of follicular death, can also cause premature ovarian failure and infertility. Further, aberrant regulation of steroidogenesis, indicated by an inhibition of steroid hormone synthesis, can lead to infertility. This is because the hormones produced by the ovary act on the hypothalamus-pituitary-ovarian axis and the reproductive tract to regulate ovulation and the initiation of fertilization and pregnancy. Additionally, the hormones produced by the ovary act on non-reproductive tissues to regulate the cardiovascular, skeletal, and brain systems. Thus, defects in steroidogenesis are also associated with non-reproductive complications.
Very few studies have investigated the effects of environmentally relevant levels of DEHP and MEHP on ovarian folliculogenesis and steroidogenesis. Thus, the goal of my doctoral dissertation work was to investigate the ovotoxic effects of DEHP and MEHP on folliculogenesis and steroidogenesis and to elucidate the mechanisms by which these chemicals disrupt these essential processes. Specifically, I investigated whether and how DEHP and MEHP accelerate early folliculogenesis \textit{in vivo} and \textit{in vitro}. Further, I investigated how DEHP and MEHP disrupt the functionality of the antral follicle and inhibit estradiol production in adult antral follicles \textit{in vitro}.

I first tested the hypothesis that environmentally relevant levels of DEHP disrupt folliculogenesis \textit{in vivo} by over-activating the phosphatidylinositol 3-kinase (PI3K) signaling pathway leading to the acceleration of primordial follicle recruitment. Factors within the PI3K signaling pathway are regulators of primordial follicle survival, quiescence, and recruitment. Specifically, factors that inhibit PI3K signaling maintain primordial follicle quiescence, and factors that drive PI3K signaling promote primordial follicle recruitment. I found that DEHP exposure for 10 and 30 days accelerated primordial follicle recruitment, evident by a decrease in the percentage of primordial follicles and an increase in the percentage of primary follicles. Additionally, DEHP exposure for 10 and 30 days at doses that accelerate primordial follicle recruitment increased factors that drive PI3K signaling and decreased factors that inhibit PI3K signaling.

I next tested the hypothesis that DEHP directly alters regulators of the cell cycle, apoptosis, and the steroidogenic pathway in antral follicles \textit{in vitro} to disrupt antral follicle functionality. Antral follicles must grow, survive from atretic demise, and produce steroid hormones to function normally. I found that DEHP exposure initially increased the levels of cell cycle regulators in a compensatory manner; however, this compensation does not rescue the follicle from growth inhibition. Further, I found that DEHP exposure caused atresia by increasing the levels of pro-apoptotic factors. In addition to the effects on growth and atresia, DEHP exposure also inhibited steroidogenesis. Specifically, DEHP exposure decreased the levels of precursor sex steroid hormones, in the relative absence of changes in steroidogenic enzyme levels, at time-points prior to decreasing estradiol levels.

Further, I tested the hypothesis that MEHP directly mediates the DEHP-induced acceleration of primordial follicle recruitment \textit{in vitro}, and MEHP directly inhibits the synthesis of sex steroid hormones in antral follicles \textit{in vitro} via a different mechanism than DEHP. For the
folliculogenesis experiments, I found that MEHP exposure decreased the percentage of germ cells and increased the percentage of primary follicles by using a neonatal ovary culture system enriched in primordial and primary follicles. Similar to the mechanism in vivo, MEHP exposure also over-activated PI3K signaling by decreasing a PI3K inhibitor and increasing a PI3K activator. Meanwhile, DEHP exposure did not alter follicular dynamics or PI3K signaling factors. Thus, MEHP mediates the effects of DEHP on folliculogenesis in vivo. For the steroidogenesis experiments, I found that MEHP exposure inhibits estradiol production later than DEHP exposure. Specifically, MEHP exposure decreased the levels of steroidogenic enzymes, in the relative absence of prior changes in steroid hormone levels, at the same time-point in which estradiol levels are decreased. Thus, DEHP and MEHP differentially inhibit steroidogenesis in the antral follicle. Collectively, my doctoral dissertation data suggest that DEHP and MEHP disrupt ovarian folliculogenesis and steroidogenesis, which are essential processes for normal reproductive and non-reproductive health.
To my Mother, Father, Sisters, Grandparents, and Grace
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CHAPTER I

Overview

1.1 Overview
Di(2-ethylhexyl) phthalate (DEHP) is a synthetic plasticizer used in the manufacturing of common polyvinyl chloride consumer, medical, and building products [1, 2]. DEHP is produced in vast quantities because it is incorporated into a myriad of commonly used products, ranging from flooring, roofing, and carpeting to packaging for food and beverages and personal care products [2]. This is of concern because DEHP is non-covalently bound to the plastic and frequently leaches into the products that humans consume causing daily exposure via oral ingestion, inhalation, and dermal contact [2]. In fact, DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) are top contaminants present in human tissues including blood [3, 4], urine [1, 3-7], amniotic fluid [8-10], umbilical cord blood [11, 12], and breast milk [4].

From a public health standpoint, exposure to DEHP is concerning because it has been identified as a reproductive toxicant and endocrine disrupting chemical, often with toxicity mediated by its bioactive metabolite MEHP [1, 2]. Specifically, DEHP and MEHP have anti-androgenic effects in males indicated by tubular atrophy, testicular degeneration, and inhibition of testicular steroidogenesis [13, 14]. Further, DEHP and MEHP disrupt pregnancy outcomes indicated by an association with decreased rates of pregnancy and high rates of miscarriage in women [1] and reduced implantations, increased resorptions, and decreased fetal weights of offspring in laboratory animals [15, 16]. Interestingly, very few studies have investigated the effects of DEHP and MEHP on the ovary, even though the ovary is a regulator of these pregnancy outcomes, and MEHP is found in ovarian follicular fluid, indicating that these chemicals can reach the ovary [17].

The ovary is a heterogeneous organ primarily consisting of follicles, which are the functional ovarian units, at varying stages of development. Follicles contain the oocyte, which is the female germ cell, surrounded by granulosa and theca cells, which are somatic cells. The female is born with a finite number of primordial follicles, the most immature follicle type, which undergo several irreversible transitions to become primary follicles, preantral follicles, and then antral follicles, the most mature follicle type also containing a fluid filled antral space [18]. This
process of development is known as folliculogenesis and is an essential process for normal reproductive and non-reproductive health. Once matured, the antral follicles are the only follicles capable of ovulation and are the major producers of sex steroid hormones in the female. The production of steroid hormones, a process known as steroidogenesis, involves the enzymatic conversion of cholesterol to 17β-estradiol and other necessary sex steroid hormones by the theca and granulosa cells [19, 20]. Steroidogenesis is another essential process for normal ovarian function because the steroid hormones produced by the ovary are required for reproductive and non-reproductive health [21-38].

Previous studies indicate that the ovary may be a potential target of DEHP and MEHP toxicity. These studies suggested that DEHP at supraphysiological levels decreases serum estradiol levels, decreases aromatase levels, causes anovulation, and prolongs estrous cycles in vivo [39]. Further, studies using granulosa cell cultures suggested that MEHP decreases estradiol and aromatase levels [40-42]. However, the direct effects of these chemicals on the mature, antral follicle, and the mechanisms by which DEHP and MEHP inhibit estradiol levels are unknown. Further, aside from these few studies showing steroidogenic defects, the effects of DEHP and MEHP on ovarian folliculogenesis remain largely unknown. Thus, the overall goal of my doctoral dissertation work was to investigate the ovotoxic effects of DEHP and MEHP on folliculogenesis and steroidogenesis and to elucidate the mechanisms by which these chemicals disrupt these essential processes.

To achieve this goal, I specifically tested three main hypotheses: 1) Environmentally relevant levels of DEHP disrupt folliculogenesis in vivo by over-activating the phosphatidylinositol 3-kinase (PI3K) signaling pathway leading to the acceleration of primordial follicle recruitment, 2) DEHP directly alters regulators of the cell cycle, apoptosis, and the steroidogenic pathway in antral follicles in vitro to disrupt antral follicle functionality, and 3) MEHP directly mediates the DEHP-induced acceleration of primordial follicle recruitment in vitro, and MEHP directly inhibits the synthesis of sex steroid hormones in antral follicles in vitro via a different mechanism than DEHP. To test these hypotheses, I completed the following specific aims (Figure 1.1):
Specific Aim 1: Determine if environmentally relevant levels of DEHP over-activate PI3K signaling to accelerate primordial follicle recruitment in vivo.

To complete this aim, I orally dosed adult CD-1 mice with vehicle control (tocopherol-stripped corn oil) or DEHP (20 μg/kg/day, 200 μg/kg/day, 20 mg/kg/day, 200 mg/kg/day, and 750 mg/kg/day) daily for 10 and 30 days. Following dosing, I examined the effects of DEHP on follicular dynamics and the expression and protein levels of key PI3K signaling factors associated with folliculogenesis. The data indicate that DEHP exposure for 10 days decreased the percentage of primordial follicles and increased the percentage of primary follicles. Further, DEHP exposure for 30 days increased the percentage of primary follicles. In addition, DEHP exposure increased the ovarian mRNA levels of 3-phosphoinositide dependent protein kinase-1 (Pdpk1), mammalian target of rapamycin complex 1 (Mtorc1), and the ovarian protein levels of phosphorylated protein kinase B (pAKT), which are all factors that drive PI3K signaling and primordial follicle recruitment. Further, DEHP exposure decreased the ovarian mRNA and protein levels of phosphatase and tensin homolog (PTEN), which is a factor that inhibits PI3K signaling and maintains primordial follicle quiescence. Collectively, these data suggest that DEHP exposure accelerates primordial follicle recruitment potentially via over-activation of ovarian PI3K signaling in vivo. These data are presented in Chapter 3.

Specific Aim 2: Elucidate the mechanisms by which DEHP inhibits growth, induces atresia, and inhibits steroidogenesis is the antral follicle in vitro.

To complete this aim, I cultured antral follicles from adult CD-1 mice with vehicle control (dimethylsulfoxide, DMSO) or DEHP (1 μg/ml, 10 μg/ml, and 100 μg/ml) for 24-96 hr. Following each 24 hr time-point, I examined the effects of DEHP on the expression of cell cycle regulators, regulators of apoptosis, and steroidogenic enzymes. Further, I examined the effects of DEHP on the production of sex steroid hormones levels. The data indicate that DEHP inhibited antral follicle growth by altering the mRNA levels of cyclin D2 (Ccnd2), cyclin dependent kinase 4 (Cdk4), cyclin E1 (Ccne1), cyclin A2 (Ccna2), cyclin B1 (Ccnb1), and cyclin-dependent kinase inhibitor 1A (Cdkn1a) prior to growth inhibition. Further, DEHP induced antral follicle atresia by increasing the mRNA levels of pro-apoptotic factors prior to atresia, such as BCL2-associated agonist of cell death (Bad), BCL2-associated X protein (Bax), and BCL2-related ovarian killer protein (Bok). In addition, DEHP decreased estradiol production by
decreasing the levels of the precursor sex steroid hormones progesterone, androstenedione, and testosterone following 48 hr of exposure; as well as by decreasing the mRNA levels of the steroidogenic enzymes side-chain cleavage (Cyp11a1), 17α-hydroxylase-17,20-desmolase (Cyp17a1), 17β-hydroxysteroid dehydrogenase (Hsd17b1), and aromatase (Cyp19a1). These data are presented in Chapter 4.

Specific Aim 3: Compare and contrast the mechanisms by which DEHP and MEHP directly accelerate primordial follicle recruitment and inhibit steroidogenesis.

To complete this aim, for the folliculogenesis experiments, I cultured neonatal ovaries from CD-1 mice with vehicle control (DMSO), DEHP (0.2 μg/ml, 2 μg/ml, and 20 μg/ml), or MEHP (0.2 μg/ml, 2 μg/ml, and 20 μg/ml) from day four to day ten. Following culture, I examined the effects of DEHP and MEHP on follicular dynamics and PI3K signaling. To further complete this aim, for the steroidogenesis experiments, I cultured antral follicles from adult CD-1 mice with vehicle control (DMSO) or MEHP (0.1 μg/ml, 1 μg/ml, and 10 μg/ml) for 24-96 hr. Following each 24 hr time-point, I examined the effects of MEHP on the expression of steroidogenic enzymes and the production of sex steroid hormones. In the neonatal ovaries, MEHP, but not DEHP, decreased the percentage of germ cells and increased the percentage of primary follicles. In addition, MEHP decreased the levels of PTEN in the whole ovary, decreased the percentage of positively stained PTEN primordial follicle oocytes, and increased the percentage of positively stained pAKT primordial follicle oocytes. Meanwhile, DEHP treated ovaries had comparable levels of PTEN and pAKT to vehicle control treated ovaries. In the antral follicles, MEHP decreased the mRNA levels of 17α-hydroxylase-17,20-desmolase (Cyp17a1), 17β-hydroxysteroid dehydrogenase (Hsd17b1), and aromatase (Cyp19a1) following 96 hr, ultimately leading to a decrease in the levels of testosterone, estrone, and estradiol. These data are presented in Chapter 5.

In summary, Chapter 1 described the overview of my dissertation. Chapter 2 provides the background on phthalates, the ovary, ovarian toxicology, and the toxic effects of phthalates on ovarian function. Chapter 3 describes the work that tested the hypothesis that environmentally relevant levels of DEHP disrupt folliculogenesis in vivo by over-activating the phosphatidylinositol 3-kinase (PI3K) signaling pathway leading to the acceleration of primordial follicle recruitment. Chapter 4 describes the work that tested the hypothesis that DEHP directly
alters regulators of the cell cycle, apoptosis, and steroidogenic pathway in antral follicles in vitro to disrupt antral follicle functionality. Chapter 5 describes the work that tested the hypothesis that MEHP directly mediates the DEHP-induced acceleration of primordial follicle recruitment in vitro, and MEHP directly inhibits the synthesis of sex steroid hormones in antral follicles in vitro via a different mechanism than DEHP. Finally, Chapter 6 summarizes the findings of my dissertation work and outlines future research directions.
1.2 Figures and Legends

Figure 1.1 Model of DEHP- and MEHP-induced ovarian toxicity

The model depicts the proposed mechanisms by which DEHP and MEHP disrupt ovarian folliculogenesis and steroidogenesis. I propose that DEHP accelerates primordial follicle recruitment in vivo, by over-activating PI3K signaling (Specific Aim 1). In addition, I propose that DEHP inhibits growth, induces atresia, and inhibits steroidogenesis in antral follicles in vitro, by altering the levels of regulators of the cell cycle, apoptosis, and steroidogenesis (Specific Aim 2). Further, I propose that MEHP mediates the effects of DEHP on folliculogenesis, and MEHP inhibits steroidogenesis via a different mechanism than DEHP (Specific aim 3).
1.3 References


42. Lovekamp-Swan T, Jetten AM, Davis BJ. Dual activation of PPARalpha and PPARgamma by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells. Mol Cell Endocrinol 2003; 201:133-141.
CHAPTER II

The Effects of Phthalates on the Ovary

2.1 Abstract

Phthalates are commonly used as plasticizers in the manufacturing of flexible polyvinyl chloride products. Large production volumes of phthalates and their widespread use in common consumer, medical, building, and personal care products lead to ubiquitous human exposure via oral ingestion, inhalation, and dermal contact. Recently, several phthalates have been classified as reproductive toxicants and endocrine disrupting chemicals based on their ability to interfere with normal reproductive function and hormone signaling. Therefore, exposure to phthalates represents a public health concern. Currently, the effects of phthalates on male reproduction are better understood than the effects on female reproduction. This is of concern because women are often exposed to higher levels of phthalates than men through their extensive use of personal care and cosmetic products. In the female, a primary regulator of reproductive and endocrine function is the ovary. Specifically, the ovary is responsible for folliculogenesis, the proper maturation of gametes for fertilization, and steroidogenesis, and the synthesis of necessary sex steroid hormones. Any defect in the regulation of these processes can cause complications for reproductive and non-reproductive health. For instance, phthalate-induced defects in folliculogenesis and steroidogenesis can cause infertility, premature ovarian failure, and non-reproductive disorders. Presently, there is a paucity of knowledge on the effects of phthalates on normal ovarian function; however, recent work has established the ovary as a target of phthalate toxicity. This review summarizes what is currently known about the effects of phthalates on the ovary and the mechanisms by which phthalates exert ovarian toxicity, with a particular focus on the effects on folliculogenesis and steroidogenesis. Further, this review outlines future directions including the necessity of examining the effects of phthalates at doses that mimic human exposure.

2.2 Phthalates

Phthalates are ubiquitous environmental toxicants to which humans are exposed on a daily basis [1]. They are a group of synthetic chemicals composed of alkyl diesters of phthalic acid and are named based on their varying lengths of alkyl chains (Figure 2.1). Normally, phthalates in their pure form are colorless, odorless, oily liquids with high lipophilic properties and low solubility in water. Phthalates are predominantly used as plasticizers in polyvinyl chloride consumer, medical, and building products to impart flexibility, as matrices and solvents in personal care products, and as excipients in medications and dietary supplements. As plasticizers, phthalates are present in commonly used items such as flooring, roofing, carpeting, shower curtains, packaging equipment, food and beverage packaging, automotive parts, and even in children’s toys. Interestingly, di(2-ethylhexyl) phthalate (DEHP) is present in common medical devices such as tubing, blood and intravenous bags, dialysis equipment, and in the manufacturing of disposable and surgical gloves [2]. As matrices and solvents, phthalates are commonly found in consumer and cosmetic products ranging from hairsprays and perfumes to pesticides and wood finishes. Further, they are frequently used as adhesives, defoaming agents, and lubricants [3]. As excipients, some phthalates are incorporated in the enteric coating of oral medications and in dietary supplements ranging from certain fish oils to probiotics [4, 5]. Thus, there are multiple means of phthalate exposures due to their presence in a wide range of products used by humans on a daily basis.

Daily exposure to phthalates is also attributed to their widespread production. The global production and use of phthalates exceeds 18 billion pounds per year, in which the majority of phthalates are used in polyvinyl chloride products [6]. The most commonly used phthalate is DEHP, which belongs to a group of phthalates known as dioctyl phthalates. Domestic production of dioctyl phthalates exceeds 300 million pounds annually [7]. Dibutyl phthalate (DBP) and diethyl phthalate (DEP) are also produced in high volumes and are among the most commonly used phthalates in consumer products. Production and importation of DBP was estimated to be between 10 to 50 million pounds in the United States in 2006 [8]. Further, production of DEP reached 50 million pounds in the United States in 2005 [9].

Phthalates are non-covalently bound to plastics, meaning they frequently leach from these items into environmental sources such as in the atmosphere, soil and sediments, and natural water
bodies [10-12]. Phthalate contamination in the air can range from 1-50 ng/m³, but DEHP is often found at higher levels (up to 3640 ng/m³) [10]. Once in the air, phthalates typically bind to dust particles and are carried back to ground level [7]. Phthalates are also detectible in sediments (0.01-115 mg/kg), agricultural soil (0.02-264 mg/kg), and urban soil (0.01-30.1 mg/kg) [10]. Urban sewer wastewater is another source of phthalate accumulation in the environment. Median phthalate levels are 3.46 µg/l in industrial wastewater, 61.3 µg/l in residential wastewater, and 66.0 µg/l in man-made wastewater [10]. Further, phthalates are found in surface water, including freshwater, saltwater, and industrial water, at levels ranging from 0.29-1.24 µg/l [10, 13-15]. Based on their presence in natural water bodies, fish and other aquatic animals are also exposed to phthalates. Although few studies have measured the levels of phthalates in fish populations, levels of DEHP and DBP in freshwater fish are 1.8 µg/kg each [16]. Further, levels of phthalates in saltwater biota range from 0.0022-28.7 µg/g, and interestingly, phthalates do not appear to accumulate up trophic positions [17]. Although few studies have investigated the lifespan of phthalates in the environment, phthalates are considered fairly stable in the environment and can persist for quite a long time [7, 18-20]. Phthalates in the air and soil dissolve very slowly, whereas phthalates in surface water dissolve quicker in water with a half-life of 2-3 weeks [7, 18-20].

The widespread production of phthalates, their use in commonly used products, and their presence in the environment leads to daily human exposure via oral ingestion, inhalation, and dermal contact. The most common routes of exposure are via oral ingestion from food packaging and use of cosmetic products, but high levels of phthalates are also present in household dust [21, 22]. Based on large production volumes, widespread use, and environmental contamination, biomonitoring data suggest that 75-100% of the population is exposed to phthalates on a daily basis [23-25]. Thus, exposure to phthalates is ubiquitous in human populations.

Once consumed, phthalates are rapidly metabolized in the gut, liver, and blood by esterases and lipases. Initially, the phthalate diester is cleaved to its respective hydrolytic monoester where only one alkyl chain remains on the phthalic acid backbone, and interestingly, it is often the monoester metabolites that induce toxicity. Depending on the size of the remaining monoester metabolite, the alkyl chain can undergo further oxidative metabolism and ultimately glucuronidation depending on the species [21, 26]. These hydrolytic monoester and oxidative
monoester metabolites, in addition to the parent phthalates, are used as biomarkers to estimate daily human exposure levels [26]. Careful attention to the metabolite used for biomonitoring is essential for accurate estimations of daily exposure levels. For example, it is more accurate to measure oxidative monoester metabolites from high molecular weight phthalates, such as DEHP, than it is to measure the hydrolytic monoester metabolite [26]. In one study, the concentrations of the oxidative monoester metabolites of DEHP, mono(2-ethyl-5-oxohexyl) phthalate and mono(2-ethyl-5-hydroxyhexyl) phthalate, were found to be four-fold higher than the hydrolytic monoester metabolite, mono(2-ethylhexyl) phthalate (MEHP) [27]. Thus, some metabolites are more sensitive biomarkers than others.

As mentioned, the vast majority of the population is exposed to phthalates on a daily basis, but the level of exposure to each phthalate differs. It is estimated that the average total daily individual ambient exposure to DEHP ranges from 0.21-2.1 mg/day for the general population [28-32]. Thus, the estimated range of daily human exposure to DEHP is 3-30 µg/kg/day based on urinary metabolite concentrations; however, measurements of DEHP in household dust can reach up to 700 mg/kg, potentially increasing exposure levels in certain individuals [22, 29, 33]. Koch and Calafat compiled data from the United States and German populations where urinary metabolites were used to estimate daily exposure levels for other commonly used phthalates, such as DEP, butyl benzyl phthalate (BBP), DBP, and diisobutyl phthalate (DIBP). The estimated range of daily human exposure to DEP is 2.32-12 µg/kg/day, BBP is 0.26-0.88 µg/kg/day, DBP is 0.84-5.22 µg/kg/day, and DIBP is 0.12-1.4 µg/kg/day [26]. Based on these exposure levels, phthalates have been identified as top contaminants present in human tissues. As stated above, measureable levels of phthalates are found in human urine samples tested and in 95% of human blood samples tested [1, 22, 23, 25, 34]. In particular to reproduction and development, DEHP and its metabolites are present in 90-100% of amniotic fluid samples from second trimester fetuses, cord blood samples from newborns, breast milk from nursing mothers, and even in human ovarian follicular fluid, indicating their ability to reach the ovary [1, 23, 34, 35].

Interestingly, certain individuals are exposed to much higher levels of phthalates than the general population. Not surprisingly, the levels are much higher in humans occupationally exposed to phthalates. For example, it was estimated that in the 1980s, over 340,000 and 239,149 workers
were exposed to DEHP and DEP, respectively [36]. Further, the exposure level of DEHP to these workers was between 143-286 µg/kg/day [37].

The highest exposures to phthalates often result from medical therapies. Both DEP and DEHP are incorporated in medical equipment, and DEP and DBP can be found in the enteric coating of oral medications. Based on its use in medical equipment, levels of DEHP can reach 8.5 mg/kg/day following blood transfusions, 0.36 mg/kg/day following hemodialysis, and 14 mg/kg/day following extracorporeal membrane oxygenation procedures in neonates [38]. Additionally, infants in intensive neonatal care units had levels of DEHP metabolites that were 14 times higher than infants in a low-intensive unit [39]. Based on their use in oral medications, urinary levels of monoethyl phthalate (MEP), the monoester metabolite of DEP, and monobutyl phthalate (MBP), the monoester metabolite of DBP, in women of childbearing age were over 12 and 200 times higher, respectively, than in a reference population [40]. In another study, urinary measurements of MBP were 50 times higher in subjects that reported using oral medications containing DBP than in controls [5].

Important for the topic of this review, women have a phthalate exposure profile that is different than that in men. In fact, females at all ages have increased urinary phthalate metabolite levels when compared to men at that same age [23]. Compared to males, females have higher levels of MEP, MBP, monobenzyl phthalate (MBzP), and MEHP [23]. Interestingly, women of reproductive age have the highest exposure levels of MBP than any other age/sex group [41]. These findings are likely attributed to the widespread use of phthalates, in particular MBP, in common cosmetic and personal care products that females use on a daily basis, including perfume, lotion, nail polish, and hairspray.

Exposure to phthalates is a public health concern because several have been identified as reproductive and developmental toxicants and endocrine disrupting chemicals (EDCs). In females, chronic occupational exposure to high levels of phthalates has been associated with decreased rates of pregnancy and high rates of miscarriage [1, 42]. Further, high urinary phthalate levels are associated with pregnancy complications such as anemia, toxemia, and preeclampsia in women [43]. In laboratory animals, phthalates reduce implantations, increase resorptions, decrease fetal weights of offspring, and decrease incidence of pregnancy [44, 45]. The mechanisms by which phthalates disrupt these endocrine and reproductive events remain
unknown. Interestingly, the ovary is a critical regulator of these processes, and the effects of phthalates on ovarian function remain poorly understood. The next sections will provide background on the importance of normal ovarian function for reproductive and non-reproductive health and how EDCs, like phthalates, can disrupt ovarian function.
2.3 The Ovary

The ovary is the female gonad responsible for reproduction and is a primary component of the female endocrine system. This heterogeneous organ is comprised of a surface epithelium surrounding the ovary, an outer cortex region containing ovarian follicles, corpora lutea, and stroma, and an inner medulla region containing a vast network of blood vessels, lymphatic vessels, and nerves. The main functions of the ovary include maturation and ovulation of the female gamete (oocyte) for fertilization and secretion of sex steroid hormones necessary for reproductive and non-reproductive health.

Folliculogenesis

One of the primary functions of the ovary is the development and maturation of follicles to allow for ovulation of the oocyte for subsequent fertilization. The ovarian follicle is the functional unit of the ovary that consists of the oocyte surrounded by two somatic cell types termed the granulosa cells and the theca cells. Within the ovarian unit, follicles undergo several irreversible developmental transitions, and this process of follicular development is known as ovarian folliculogenesis (Figure 2.2).

In mammals, the female is born with a finite number of follicles; thus, the follicular reserve is set at birth and represents a female’s reproductive potential and reproductive lifespan [46]. These follicles are first formed during the later stages of fetal life in the human and during the early postnatal life in the rodent. The process of follicle formation is known as germ cell nest breakdown. During embryonic development, primordial germ cells, which will give rise to oocytes, migrate from the yolk sac to the genital ridge where the undifferentiated gonad resides [47]. These germ cells, now termed oogonia, massively proliferate via mitosis and develop in clusters or nests in which squamous pre-granulosa cells surround the oogonia [48]. Once established in the germ cell nests, mitosis of oogonia is ceased and meiosis begins. It is here that the oogonia become oocytes, and the oocytes progress through meiosis until they are arrested in the diplotene stage of meiotic prophase I [49].

Ovarian follicle assembly then occurs around the 6-9th month of gestation in the human and around post-natal day 3 in the rodent in which the most immature follicle type, the primordial follicle, is formed [50, 51]. For primordial follicle assembly to occur, the germ cell nests must
undergo programmed cell death of oocytes, primarily through regulation of the B-cell lymphoma/leukemia-2 (BCL-2) family members [52-56] and the actions of steroid hormone and intraovarian growth and transcription factors [57, 58]. The interaction of these molecular events leads to oocyte association with a single layer of flattened, squamous pre-granulosa cells, thus, the formation of the primordial follicle.

Once the primordial follicle population is established, the follicle is destined to three fates: to remain quiescent for varying lengths of time to constitute the ovarian reserve, to directly undergo atresia, which is follicular programmed cell death via apoptosis, or to activate into the growing population of follicles to become primary follicles, a process termed primordial follicle recruitment. Primordial follicle recruitment is a tightly regulated process controlled by multidirectional communication between the oocyte, granulosa cells, and surrounding somatic cells that will give rise to the theca cells. This process is gonadotropin-independent and relies on paracrine and autocrine regulation by multiple intrinsic ovarian growth factors that work through several different signaling pathways [46, 58-61]. Primordial follicle quiescence is maintained by factors that suppress follicle activation, whereas primordial follicle recruitment is initiated by factors that activate development. These stimulatory and inhibitory factors exist in a balance to maintain primordial follicle survival so that downregulation of inhibitory factors and/or overactivation of stimulatory factors favor an environment conducive for primordial follicle recruitment [62].

Once activated, primary follicles contain a larger oocyte that has initiated growth surrounded by a single layer of cuboidal granulosa cells. Primary follicles then develop into preantral follicles, also termed secondary and tertiary follicles, that contain the oocyte surrounded by at least two layers of cuboidal granulosa cells and two outer theca cell layers. Follicles at this stage, because of the presence of both granulosa and theca cells, are gonadotropin-responsive and begin synthesizing sex steroid hormones.

Preantral follicles then develop further into antral follicles, which are the most mature follicle type in the ovary. Antral follicles contain the oocyte surrounded by several layers of cuboidal granulosa cells with a fluid filled space, termed the antral space, and two outer theca cell layers.
Each fertile menstrual/estrous cycle requires the presence of a pre-existing antral follicle population that responds to cyclic gonadotropins, and this process is termed cyclic recruitment [60]. Therefore, folliculogenesis must remain dynamic to allow for the continual generation of antral follicles to undergo cyclic recruitment for potential ovulation. As antral follicles continue to mature, they produce estradiol and their receptivity to the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), increases. The increase in estradiol initiates the LH surge causing one or multiple follicles to ovulate depending on the species. Once the oocyte is released, the remaining granulosa and theca cells differentiate into large and small luteal cells respectively, and the remaining structure is termed the corpus luteum.

Not all follicles are destined to develop and ovulate, and in fact, approximately 99% of follicles undergo atresia. At birth, the human ovary contains approximately two million follicles, but by puberty, the number of follicles declines to roughly 400,000 due to atretic demise. Further, of the available follicles at puberty, only about 400 of them will ovulate throughout the reproductive lifespan, whereas the others undergo atresia [50]. Atresia is a coordinated process of follicle degeneration via hormonally controlled apoptosis [63]. Although atresia can occur at all stages of follicle development, early antral follicles are most susceptible to death in which apoptosis can occur in both somatic and germ cells. The regulation of follicular atresia involves a balance of pro- and anti-apoptotic factors. Specifically, gonadotropins, estrogens, insulin-like growth factor-I, and interleukin-1β are anti-apoptotic and help prevent follicles from undergoing atresia [64-70]. Conversely, tumor necrosis factor-α, Fas-Fas ligand, and androgens promote apoptosis and ultimately atresia [71-74]. The interplay of these pro- and anti-apoptotic factors primarily converge on the BCL-2 signaling pathway, with its own pro- and anti-apoptotic proteins, to regulate atresia [53-56].

**Ovarian steroidogenesis**

Another primary function of the ovary is to produce sex steroid hormones, a process termed ovarian steroidogenesis. Steroidogenesis is primarily conducted by the mature antral follicle and the corpus luteum following ovulation. The process of steroidogenesis involves the enzymatic conversion of cholesterol to 17β-estradiol and other necessary sex steroid hormones to regulate reproductive and non-reproductive health (Figure 2.3). Prior to the peri-ovulatory period, the antral follicle increases the synthesis of estradiol to promote the ovulatory surge of
LH. As ovulation approaches, the peri-ovulatory follicle increases production of progesterone to promote ovulation and formation of the corpus luteum. Once the follicle has transitioned to the highly vascularized corpus luteum, vast amounts of progesterone as well as estradiol are produced.

The steroid hormones produced by the ovary act on numerous target tissues associated with reproductive and non-reproductive function. For reproductive function, steroid hormones act on the ovary itself as well as the brain, pituitary, oviduct, uterus, cervix, vagina, and mammary gland. The actions of these steroid hormones include maintenance of the reproductive tract; establishment of a hormonal milieu for ovulation, fertilization, implantation, and pregnancy; and control of menstrual/estrous cyclicity by utilizing feedback loops in the brain and pituitary. These steroid hormones also act in non-reproductive tissues such as the brain, cardiovascular system, adipose tissue, skin, bone, and liver. Therefore, proper steroidogenesis is required for fertility as well as for maintenance of cardiovascular, brain, and skeletal health [75-91].

The generation of sex steroid hormones involves several enzymatic reactions in both the theca and granulosa cells. Specifically, cholesterol can either be transported into the theca cell cytoplasm via lipoprotein receptors or it can be synthesized de novo. Cholesterol is then internalized into the mitochondria via the steroidalogenic acute regulatory protein (STAR) [92-94]. Cholesterol is then converted to pregnenolone in the mitochondria via cytochrome P450 cholesterol side-chain cleavage (CYP11A1) [95, 96]. Pregnenolone then diffuses out of the mitochondria and is transported to the smooth endoplasmic reticulum where it is converted to progesterone or dehydroepiandrosterone (DHEA) via 3β-hydroxysteroid dehydrogenase (HSD3B) or 17α-hydrorxylase-17,20-desmolase (CYP17A1), respectively [97]. Progesterone and DHEA are then converted to the androgen androstenedione again via CYP17A1 or HSD3B, respectively [96]. Androstenedione can then be converted to either testosterone, another androgen, or estrone, a weak estrogen, via 17β-hydroxysteroid dehydrogenase (HSD17B) or aromatase (CYP19A1), respectively [96]. Testosterone and estrone are then converted to the most potent estrogen, estradiol, via CYP19A1 or HSD17B, respectively [96, 97]. Estradiol can be inactivated and metabolized in the ovary to 2-hydroxyestradiol via CYP1A1/2 and CYP3A4 or it can be broken down to 4-hydroxyestradiol via CYP1B1 [98, 99].
Interestingly, estradiol cannot be synthesized without the strict coordination of both theca cells and granulosa cells and the addition of pituitary derived FSH and LH. This is why ovarian steroidogenesis is known as the two-cell, two-gonadotropin theory [100, 101]. Theca cells in the early antral follicle only contain LH receptors, and upon receptor binding, LH stimulates the transcription of theca derived genes that encode the enzymes required for the conversion of cholesterol to the androgens [100, 101]. Once converted, androgens can diffuse from the theca cells through the basement membrane, which separates theca cells from granulosa cells, and into the granulosa cells. In contrast to theca cells, granulosa cells of the early antral follicle contain only FSH receptors, and in response to FSH binding, the transcription of granulosa derived genes that encode the enzymes necessary for the conversion of androgens to estrogens is stimulated [100, 101]. This distinct coordination is required because theca cells lack the CYP19A1 enzyme (which converts androgens to estrogens), and granulosa cells lack the CYP17A1 enzyme (which converts pregnenolone and progesterone to androgens). Luteal cells in the corpus luteum also utilize the two-cell approach to produce progesterone and estradiol [102, 103]. As is the case in the antral follicle, the small luteal cells that are derived from theca cells synthesize androgens from cholesterol, while the large luteal cells that are derived from granulosa cells convert androgens to estrogens.
2.4 Ovarian Toxicity of Endocrine Disrupting Chemicals

Because of its multifaceted roles, it is important to understand how ubiquitous EDCs, like phthalates, affect normal ovarian function, as defects in ovarian function have implications for health other than fertility. Normal ovarian function is essential for reproductive, cardiovascular, mood, brain, and skeletal health [75-91]. Due to their widespread production, extensive use, and ubiquitous presence in the environment, phthalates have the potential to target the ovary at all stages of development and in adulthood. These toxic effects can lead to premature ovarian failure, anovulation, infertility, and decreased steroidogenesis [104-107]. Thus, exposure to phthalates can disrupt normal ovarian function by several different mechanisms, leading to reproductive and non-reproductive abnormalities.

One way that EDCs can exert ovarian toxicity is through targeting follicles at different stages of folliculogenesis [104-107]. Specifically, chemicals can target the primordial, primary, preantral, or antral populations of follicles, or they can target corpora lutea. Once a particular population is targeted, the chemicals can induce atresia and deplete the follicles within that stage, they can arrest follicles within that stage, or they can promote accelerated development from that stage [104-107]. Each of these potential outcomes can have detrimental effects on fertility and/or non-reproductive health. Specifically, EDCs that deplete or accelerate the development of primordial follicles will cause permanent infertility caused by premature ovarian failure, or early onset of menopause [104-107]. This is because the primordial follicle pool is established at birth and is non-renewable [46]. Premature menopause is of concern because it is associated with increased risks of cardiovascular disease, osteoporosis, and premature death [75-78, 108-111]. EDCs can also target the later stages of folliculogenesis such as the antral follicle [104, 105]. Chemicals can cause atresia of antral follicles or inhibit the growth of antral follicles, leading to estrogen deficiency and anovulatory cycles and ultimately infertility [104, 105]. Similarly, EDCs that affect the process of luteinization, the developmental transition of a follicle to a corpus luteum, or the lifespan of the corpus luteum can affect progesterone production, implantation, and pregnancy, leading to infertility [104, 105].

Chemicals can also directly interfere with ovarian steroidogenesis and this can cause reproductive and non-reproductive complications. Steroidogenesis can be affected either by depletion of the antral follicles and/or corpora lutea, or it can be affected by disrupting the functionality of the steroidogenic units. Specifically, the loss of antral follicles or corpora lutea
from the ovary will result in a decrease in the available structures that are capable of producing steroids [104, 105]. Further, EDCs can disrupt the functionality of antral follicles by decreasing ovarian mRNA, protein, and/or activity of the enzymes responsible for generating estradiol and its precursor sex steroid hormones [104, 105]. Additionally, EDCs can increase mRNA, protein, and/or activity of the enzymes responsible for metabolizing estradiol; thus, rendering it inactive [104, 105]. The steroidogenic enzymes in the corpora lutea can also be affected in a similar manner, resulting in inadequate levels of necessary progesterone and estradiol to support a pregnancy [104, 105]. This disruption of hormone production can also alter normal menstrual/estrous cyclicity. A lack of ovarian derived steroid hormones will disrupt the hypothalamus-pituitary-ovarian axis, leading to infertile anovulatory or oligovulatory cycles by inhibiting the LH surge and/or altering FSH levels that are responsible for recruiting a cohort of antral follicles for ovulation [46, 101, 104, 105]. Defects in ovarian steroidogenesis are linked to infertility and an increased risk of heart disease, osteoporosis, mood disorders, and premature death [75-78, 108-111].

Removing or minimizing toxicant exposure may alleviate the ovotoxic effects, depending on the duration of exposure and population of follicles targeted by the chemical [106, 107]. For instance, if an EDC only targets the antral follicle causing ovarian toxicity, removal of the chemical has the potential to restore ovarian function [106, 107]. This is because the process of folliculogenesis from the primordial stage to the antral stage was unaffected. Once the EDC is removed, primordial follicles will develop to the antral stage as they had done previously, but now the detrimental effect will be alleviated [106, 107]. Reversal of toxic effects is nearly impossible when the primordial follicle pool is targeted for an extended period of time. Because the follicular reserve is nonrenewable, chronic exposure to an EDC that causes death of primordial follicles or accelerates primordial follicle recruitment will lead to permanent ovarian damage [106, 107]. Although removal of toxicant exposure may be beneficial in restoring ovarian function, exposure to many EDCs including phthalates cannot be completely removed. This adds to the public health concern of the use of phthalates, as even minimizing exposure can be a difficult task due to their ubiquitous use in common consumer products and presence in the environment.

Understanding the impact on ovarian function from exposure to phthalates is of great importance, particularly because the general population is constantly exposed to phthalates [23-
This importance is compounded in certain populations that are exposed to high levels of phthalates on a daily basis. These populations include patients undergoing medical care with phthalate containing medical devices and medications, women with careers in an industrialized environment, and women located near phthalate manufacturing and disposal sites [5, 36-41]. Often, women in today’s society postpone childbirth to prioritize career development during prime reproductive years. This leads to a longer period of exposure to phthalates, potentially leading to detrimental effects on fertility, especially when the female is aging. Because of the prevalent use and ubiquitous exposure to phthalates and the importance of the ovary for female reproductive and non-reproductive health, the goal of this review is to summarize what is currently known about the effects of phthalates on the ovary and the mechanisms by which phthalates exert ovarian toxicity, with a particular focus on the effects of phthalates on folliculogenesis and steroidogenesis.
2.5 Effects of Phthalates on Folliculogenesis

Effects of phthalates on oocyte development and primordial follicle assembly

Limited studies have investigated the effects of phthalates on folliculogenesis, but there is evidence suggesting that phthalates alter the formation and/or function of follicles at several stages of development. Specifically, phthalates have been shown to disrupt the earliest stages of folliculogenesis by altering ovarian and oocyte development. DEHP exposure in Japanese medaka during sexual development has been shown to inhibit oocyte development [112]. When given in an aqueous solution at 1-50 µg/l from hatching to 3 months of age, DEHP exposure decreased the percentage of completely matured oocytes in the ovaries, most likely via an anti-estrogenic mechanism of action [112]. MEHP exposure for 24 hrs at 250-500 µM has been shown to decrease murine fetal oocyte viability using an in vitro oocyte culture system [113]. This decrease in oocyte survival is attributed to an alteration in oocyte oxidative stress as the mRNA levels of mitochondrial respiratory chain protein (Nd1) were decreased and Cu-Zn superoxide dismutase (Sod1) were increased in the oocytes following MEHP exposure [113]. A decrease in Nd1 mRNA may lead to an increase in reactive oxygen species (ROS), which are toxic to the oocyte and are also associated with an increased risk of infertility [114]. The increase in the antioxidant Sod1 mRNA is most likely a compensatory response in detoxifying the increased ROS following MEHP exposure. DEHP exposure further affects oocyte development by causing heritable modifications in DNA methylation in mouse oocytes [115]. When given to pregnant mice during the length of gestation, DEHP exposure at 40 µg/kg/day reduced the methylation of CpG sites in the two critical imprinting genes, insulin-like growth factor 2 receptor (Igf2r) and paternally-expressed gene 3 (Peg3), in the primordial germ cells of the fetal ovary at gestational day 12.5 and the oocytes of the offspring by post-natal day 21 [115]. Interestingly, the decrease in oocyte DNA methylation of Igf2r and Peg3 is also evident in the oocytes of the F2 offspring, suggesting that the effects of DEHP on oocyte development are heritable [115]. Gestational exposure to a single intraperitoneal injection of DIBP resulted in architectural disarray of follicles in fetal rats [116]. Specifically, DIBP exposure at 0.375-1.25 ml/kg increased the numbers of degenerated oocytes and empty follicles without oocytes, and the blood vessels located in the stroma of the ovary appeared prominent and congested [116]. MEHP has also been shown to affect ovarian development in the human. Human ovaries from
gestational weeks 7 to 12 were cultured with MEHP at $10^{-4}$ M for 72 hrs and had dysregulated lipid/cholesterol synthesis as evidenced by an increase in the mRNA levels of liver X receptor alpha ($LXRa$) and sterol regulatory element-binding protein ($SREBP$) members [117]. Interestingly, oocyte numbers were not affected by MEHP treatment, but the same study suggests that phthalate toxicity to the developing human ovary may be mediated by nuclear receptor signaling [117]. It appears that phthalates disrupt early ovarian and oocyte development potentially leading to oocyte death and abnormal ovarian architecture [112, 113, 116]. The mechanisms by which phthalates alter the earliest stages of ovarian development appear to include an anti-estrogenic response [112], an increase in oxidative stress [113], and heritable modifications to the oocyte epigenome [115].

Phthalates have also been shown to affect germ cell nest breakdown and primordial follicle assembly. Newborn mouse ovaries cultured with DEHP for 72 hrs at 10-100 µM had an increase in oocytes contained in the germ cell nest, and there was a decrease in primordial follicle numbers [118]. Thus, germ cell nest breakdown and primordial follicle assembly were inhibited following DEHP exposure. Additionally, DEHP exposure increased apoptosis in the oocytes indicated by an increase in TUNEL positive oocytes and increased mRNA levels of pro-apoptotic BCL2-associated X protein ($Bax$) [118]. Further, DEHP decreased the mRNA levels of other factors associated with oocyte survival and primordial follicle formation, such as LIM homeobox 8 ($Lhx8$), factor in the germline alpha ($Figla$), spermatogenesis and oogenesis helix-loop-helix ($Sohlh2$), and newborn ovary homeobox ($Nobox$) [118]. Similar to previous reports, DEHP exposure affected oocyte DNA methylation by inhibiting the demethylation of CpG sites of $Lhx8$, a process required for early folliculogenesis [118]. These effects on primordial follicle formation can have lasting effects on folliculogenesis and fertility because primordial follicles serve as the female’s reproductive potential [46].

**Effects of phthalates on follicles across development**

Phthalates have also been shown to affect the rate in which primordial follicles are recruited to the growing population of follicles. In the adult mouse, oral exposure to DEHP for 10 and 30 days at 20 µg/kg/day-750 mg/kg/day accelerates primordial follicle recruitment, evident by a decrease in primordial follicles and an increase in primary follicles [119]. The mechanism by which DEHP accelerates primordial follicle recruitment is likely via overactivation of the
phosphatidylinositol 3-kinase (PI3K) signaling pathway, a pathway that regulates primordial follicle survival, quiescence, and recruitment. Specifically, DEHP exposure increased the ovarian mRNA levels of 3-phosphoinositide dependent protein kinase-1 (Pdpk1), mammalian target of rapamycin complex 1 (Mtorc1), which are factors that drive primordial follicle recruitment, and decreased the mRNA levels of phosphatase and tensin homolog (Pten) and tuberous sclerosis 1 (Tsc1), which are factors that maintain primordial follicle quiescence [119]. Additionally, DEHP exposure for 10 days increased phosphorylated protein kinase B (pAKT) protein in the whole ovary and in primordial and primary follicles, and decreased PTEN protein in the whole ovary, further suggesting that DEHP overactivates ovarian PI3K signaling to promote the acceleration of primordial follicle recruitment [119]. Similar effects on primordial follicle recruitment were observed following DEHP exposure during early postnatal life in mice. Following hypodermic injections during early postnatal life, DEHP at 20-40 µg/kg/day accelerated folliculogenesis by decreasing primordial follicles and increasing preantral and antral follicles when the ovaries were observed on post-natal day 15 and 21 [120]. Further, when the treated mice were allowed to breed, the F1 offspring had a similar decrease in primordial follicle numbers when the ovaries were observed in adulthood [120]. MEHP exposure in utero also accelerates folliculogenesis in mice. Oral exposure to MEHP via gavage from gestational days 17-19 at 100-1000 mg/kg/day resulted in an increase in preantral and antral follicles in the F1 generation [121]. These F1 females exposed to MEHP in utero also exhibited premature reproductive senescence by one month, likely attributed to the acceleration of folliculogenesis evident by the follicle count data [121]. Phthalates appear to accelerate primordial follicle recruitment by decreasing primordial follicle numbers and increasing the numbers of more mature follicle types, and this effect is consistent across timing and duration of exposure and the doses of phthalates used. Because the primordial follicle reserve is non-renewable, the above effects on primordial follicle recruitment can impact a female’s reproductive lifespan.

In addition to the effects of phthalates on immature follicle types, phthalates have also been shown to target and adversely affect more mature follicles. Exposure to DEHP alone and in combination with benzo[a]pyrene (B[a]P) via oral gavage decreased the population of primary and secondary follicles, potentially via induced follicular atresia in adult rats [122]. Specifically, DEHP alone (600 mg/kg/day) and in combination with B[a]P (10 mg/kg/day) induced granulosa cell apoptosis, resulting in an increase in the number of atretic follicles across developmental
stages [122]. Likewise, in utero and lactational exposure to DEHP from midgestation to weaning at 405 mg/kg/day increased the number of atretic preantral follicles in the rat offspring during adulthood [123]. A similar effect of increased atresia in growing follicles was seen in adult marine medaka. DEHP in an aqueous solution at 0.1-0.5 mg/l increased the numbers of atretic late-stage follicles resulting in reproductive dysfunction following exposure from hatching to adulthood [124]. A reduction in the growing population of follicles was also observed in DEHP exposed neonatal ovaries after transplantation into the kidney capsules of immunodeficient mice. Specifically, newborn mouse ovaries were cultured with DEHP for 72 hrs at 10-100 µM and were then transplanted into adult mice to observe if folliculogenesis was impaired. Contrary to control treated transplanted ovaries, DEHP treated transplanted ovaries had few, if any, growing follicles following 21 days post-transplantation [118]. Further, preantral follicles from rats cultured with MEHP in vitro for 10 days at 10-80 µg/ml had a lower survival rate and decreased rate of development to the antral stage [125]. Likewise, secondary follicles from rats cultured with MEHP at 100 µg/ml had suppression of follicular development accompanied by a decrease in follicular viability and an increase in granulosa cell apoptosis [126]. Prior to development to the antral follicle stage, phthalates appear to target primary and preantral to induce atresia at a wide range of doses. This effect on atresia is likely attributed to phthalate-induced apoptosis of granulosa cells [122, 126].

Phthalates also target mature antral follicles by adversely inhibiting their growth and maturation. Much of the work investigating the effects of phthalates on antral follicle growth utilizes the novel method of the whole antral follicle culture system [127, 128]. Using this method, DBP exposure for 168 hrs at 1000 µg/ml has been shown to inhibit antral follicle growth [129]. This inhibition of antral follicle growth is likely attributed to defects in the cell cycle, which is necessary for appropriate granulosa cell proliferation and follicle growth. Specifically, DBP exposure at 1-1000 µg/ml decreased mRNA levels of cyclin D2 (Ccnd2), cyclin E1 (Ccne1), cyclin A2 (Ccna2), and cyclin B1 (Ccnb1), and increased the mRNA levels of cyclin-dependent kinase inhibitor 1A (Cdkn1a) at a time-point prior to growth inhibition [129]. DBP treated follicles had greater numbers of cells in the G1 phase, fewer numbers of cells in the S phase, and a trend for fewer numbers of cells in the G2 phase, further indicating cell cycle arrest following 24 hrs of culture [129]. These defects in antral follicle growth potentially lead to the observed increase in atresia in DBP treated follicles [129]. Both DEHP at 1-100 µg/ml and MEHP at 0.1-
100 µg/ml also inhibit antral follicle growth in vitro. Specifically, both chemicals inhibit antral follicle growth following 72 hrs of culture, and this effect persists for the duration of the 96 hr culture [130-132]. Similar to DBP, DEHP at 100 µg/ml disrupts the cell cycle by decreasing the mRNA levels of Ccnd2 and cyclin-dependent kinase 4 (Cdk4), and MEHP at 10-100 µg/ml disrupts the cell cycle by decreasing the mRNA levels of Ccnd2, Ccne1 and Cdk4 [131, 132]. Further, MEHP exposure at 1-100 µg/ml increased the mRNA levels of pro-apoptotic Bax and apoptosis-inducing factor, mitochondrion-associated, 1 (Aifm1) and decreased the mRNA levels of anti-apoptotic Bcl2 and Bcl2-like 10 (Bcl2l10), leading to antral follicle atresia [131, 133]. Interestingly, DEHP and MEHP likely inhibit antral follicle growth and induce atresia via a mechanism involving oxidative stress. Specifically, DEHP at 10 µg/ml and MEHP at 1-100 µg/ml increased ROS levels in the treated follicles [130, 131]. This is accompanied by reduced expression and enzyme activity of SOD1 following DEHP exposure, and reduced expression and enzyme activities of SOD1 and glutathione peroxidase (GPX) following MEHP exposure [130, 131]. Supplementing the DEHP and MEHP treated follicles with estradiol (1-10 nM) or the antioxidant N-acetyl cysteine (NAC; 0.25-1 mM) only partially protects the follicles from phthalate-induced growth inhibition [130-132], but estradiol supplementation rescues the antral follicles from MEHP-induced atresia [133]. In additional studies, exposure to DEHP via oral gavage at 2 g/kg/day reduced preovulatory follicle size in rats, due to a reduced granulosa cell size and area [134]. Phthalates appear to directly target the antral follicle to inhibit growth via cell cycle inhibition [129, 131, 132], induction of atresia [129, 131, 133], and increased oxidative stress [130, 131]. Overall, the phthalate-induced inhibition of antral follicle growth can potentially impair ovulation and steroidogenesis [46, 101].

Effects of phthalates on ovulation and the corpus luteum

The process of oocyte maturation during the peri-ovulatory period is also affected by phthalate exposure. DEHP exposure in an aqueous solution at 0.02-40 µg/l in zebrafish inhibited oocyte germinal vesicle breakdown, which is a process required for the resumption of meiosis prior to ovulation [135]. This effect was accompanied by an increase in the levels of ovarian bone morphogenetic protein 15 (BMP15) and decreases in the levels of LH receptor (LHR) and membrane progesterone receptors (mPRs), which are factors that drive oocyte maturation [135]. Similar effects were seen using in vitro maturation assays with bovine oocytes. MEHP exposure
at 5-100 µM to denuded oocytes and cumulus-oocyte complexes for 22-24 hrs reduced the number of oocytes that resumed meiosis, indicated by an increase in the number of oocytes still in the germinal vesicle stage, and reduced the number of oocytes that progressed to metaphase II [136, 137]. Bovine oocytes exposed to MEHP at 50 µM during maturation also had a decrease in the mRNA levels of CCNA2, acid ceramidase 1 (ASAH1; an anti-apoptotic factor), and POU domain, class 5, transcription factor 1 (POU5F1; a factor responsible for pluripotency), which potentially led to the observed increased in apoptotic oocytes during culture [137]. These defects in oocyte maturation resulted in increased instances of poor quality early embryos [137]. Similar to the bovine model, DEHP exposure at 0.12-1200 µM inhibited oocyte maturation in the horse following an in vitro maturation assay [138]. Further, ROS levels and apoptosis were increased in the cumulus granulosa cells [138]. Additionally, BBP exposure at 100 µM to FSH-matured (10 ng/ml) porcine cumulus oocyte complexes inhibited cumulus cell expansion, a process required for normal ovulation, transport through the oviduct, and fertilization [139]. In the mouse, MEHP exposure at 200-400 µM in a maturation assay increased the number of oocytes in the germinal vesicle stage and decreased the number of oocytes that resumed meiosis in metaphase II [140]. A similar effect was seen with in utero and lactational exposure to DEHP in adult female offspring. Specifically, DEHP exposure through gestation and weaning at 0.05-5 mg/kg/day decreased the numbers of oocytes that reached meiosis II when the offspring were superovulated as adults [141]. Further, mouse oocytes that were matured in vitro had increased metaphase II spindle abnormalities following in vivo exposure to DEHP at 20-40 µg/kg/day, indicating that phthalates have the potential to alter post-meiotic resumption maturation processes [120]. Phthalates appear to inhibit germinal vesicle breakdown and resumption of meiosis in multiple different models, and these effects on oocyte maturation may be detrimental to ovulation and normal embryonic development.

Along with defects in oocyte maturation, phthalates disrupt the ovulatory process. Zebrafish exposed to DEHP in an aqueous solution at 0.02-40 µg/l had a significant reduction in ovulations likely attributed to a decrease in the mRNA levels of prostaglandin-endoperoxide synthase 2 (Ptgs2), which is an enzyme required for one of the final triggers of ovulation following the LH surge [135]. Further, the injection of DEHP inhibits ovulation in rats following equine chorionic gonadotropin (eCG)-induced ovulation (15-30 IU). Specifically, DEHP exposure at 500 mg/kg/day decreased the total number of rats that ovulated in response to eCG treatment, and
DEHP exposure reduced the total number of ovulated oocytes following eCG treatment [142]. Likewise, oral exposure to DEHP via gavage during metestrus at 2 g/kg/day delayed or suppressed ovulation by the first proestrus/estrus in rats [134]. In fact, 7 out of 10 rats did not ovulate by vaginal estrus in response to DEHP treatment [134]. These studies suggest that DEHP exposure is capable of inhibiting ovulation by decreasing the transcription of LH surge-response genes, even when the ovulatory process is chemically induced.

Phthalates have also been shown to disrupt the luteal transition and/or target corpora lutea. Perinatal exposure to diisononyl phthalate (DINP) at 20,000 ppm in the rat decreased the number of corpora lutea present in adulthood [143]. Adolescent rats exposed to DEHP via oral gavage for 28 days at 150-500 mg/kg/day also had a decrease in corpora lutea numbers [144]. A similar effect of decreased corpora lutea numbers is seen when DEHP alone (300-600 mg/kg/day) and in combination with B[a]P (10mg/kg/day) is administered to adult rats [122]. Because mechanistic studies were not conducted, it is unknown if the decreases in corpora lutea numbers are due to an inhibition of ovulation, an inhibition in the luteal transition, and/or a direct destruction of corpora lutea caused by phthalate exposure. However, previous studies have shown that phthalates inhibit ovulation [134, 135, 142].

A few studies suggest that phthalates may alter the functionality of corpora lutea. Marmosets exposed to DEHP via oral gavage from weaning to sexual maturity at 500-2500 mg/kg/day had abnormally large corpora lutea present in the ovary, which is a finding often seen in older female marmosets [145]. This increase in corpora lutea size likely causes the observed increase in ovarian weights following DEHP exposure [145]. Conversely, adult sheep treated with DEHP intramuscularly at 25-50 mg/kg/day had smaller corpora lutea and a decreased luteal phase of the estrous cycle [146]. These data suggest that phthalates have the potential to disrupt post-ovulatory ovarian processes, but further work must be done to elucidate the differences observed in the two studies.

Epidemiological links between phthalate exposure and alterations in folliculogenesis

Very few studies have investigated the link between phthalate exposure in humans and alterations in folliculogenesis. One study examined the association of phthalate exposure and prevalence of polycystic ovary syndrome (PCOS), which is a gynecological disorder often
associated with infertility and the presence of large, cystic follicles incapable of ovulating. Interestingly, lower urinary levels of MEHP, MEP, MBP and MBzP were associated with an increased likelihood of PCOS when compared to control patients and patients with higher levels of these phthalate metabolites [147]. Similarly, in the Western Australian Pregnancy Cohort Study, maternal serum levels of MEP and the sum of all phthalate metabolites were negatively associated with PCOS in the daughters [148]. However, this study did not investigate the prevalence of PCOS in the daughters whose mothers had low levels of phthalate metabolites. In the same study, maternal levels of MEP had a negative association with anti-Müllerian hormone (AMH), which is a hormone secreted by granulosa cells of maturing follicles to restrict primordial follicle activation [148]. Based on the paucity of available information, further epidemiology studies are warranted in investigating the effects of phthalate exposure on folliculogenesis in the human.

Ovarian folliculogenesis is an essential process for normal reproductive and non-reproductive health, and increasing evidence suggests that phthalates have the ability to adversely affect this process in numerous aspects. Specifically, phthalates have been shown to disrupt ovarian/oocyte development, accelerate primordial follicle recruitment, target growing follicles, inhibit growth of antral follicles, disrupt oocyte maturation and ovulation, and alter post-ovulatory processes (Figure 2.4). The mechanisms by which phthalates exert these toxic effects on folliculogenesis are unknown, but a few studies have begun to elucidate these mechanisms and have shown that phthalates can modulate genes associated with folliculogenesis (Table 2.1). It is clear that the majority of work investigating the effects of phthalates on folliculogenesis focus solely on DEHP and its metabolite MEHP. Future work should elucidate the mechanisms by which DEHP and MEHP disrupt folliculogenesis and should incorporate exposures to other commonly used phthalates. Further, the doses used in the reviewed animal studies rarely encompass the range of estimated human exposure. It would be advantageous to conduct experiments with levels of phthalates that fall within the range of human exposure, especially considering that phthalates exhibit non-monotonic dose responses [149, 150]. Additionally, experiments should be conducted to observe if these effects on folliculogenesis persist throughout the reproductive lifespan and if these effects directly cause infertility.
2.6 Effects of Phthalates on Steroidogenesis

*Effects of phthalate exposure on steroidogenesis in vivo*

Gestational exposure to phthalates has been shown to alter steroidogenesis in female offspring. Oral exposure to MEHP via gavage from gestational days 17-19 at 100-1000 mg/kg/day increased the levels of serum FSH and estradiol in female mouse offspring once they reached adulthood [121]. MEHP exposure in utero also decreased the mRNA levels of *Star* and *Cyp19a1* in the ovaries of the adult offspring [121]. Estrous cyclicity, a process controlled by ovarian derived hormones, was also altered in these offspring. MEHP exposed females exhibited a delay in the onset of cyclicity, and MEHP exposure increased the time spent in the estrus stage [121]. Oral exposure to DIBP via gavage from gestational days 7-21 at 600 mg/kg/day increased anogenital distance in female rat offspring, a steroid hormone regulated process, and increased the mRNA levels of *Cyp19a1* in the ovaries when the offspring were prepubertal [151]. A similar effect of increased anogenital distance was observed in female offspring following gestational BBP exposure via oral gavage to rats at 500 mg/kg/day [152]. Maternal exposure to DEHP in the diet at 0.05-5 mg/kg/day during the entire length of gestation through weaning decreased the mRNA levels of key steroidogenic enzymes and receptors in the ovaries of adult mouse offspring [141]. Specifically, ovaries from the adult offspring had decreased levels of *Cyp19a1*, *Cyp17a1*, progesterone receptor (*Pgr*), FSH receptor (*Fshr*), and LH receptor (*Lhr*) [141]. It is apparent that gestational exposure to phthalates results in defects in ovarian steroidogenesis across multiple developmental time-points by decreasing key steroidogenic enzyme levels.

Prepubertal exposure to phthalates has also been shown to disrupt ovarian steroidogenesis. DEHP exposure via inhalation from post-natal day 22-41 at 25 mg/m³ increased serum levels of cholesterol, LH, and estradiol in female rats following the duration of exposure [153]. When the exposure window was expanded from post-natal day 22-84, DEHP exposure increased the mRNA levels of ovarian *Cyp19a1*, advanced the age of vaginal opening and first estrous cycle, and increased the number of irregular estrous cycles [153]. The increase in *Cyp19a1* likely attributes to the increase in estradiol levels. Oral exposure to DEHP via gavage for 10 days at 500 mg/kg/day decreased the serum levels of progesterone and estradiol, and there was a trend of increased serum LH levels in prepubertal rats [154]. Further, granulosa cells from DEHP exposed prepubertal rats exhibited a decrease in *ex vivo* progesterone production even
following FSH and LH stimulation, which was likely attributed to a decrease in the required transport of endogenous cholesterol into the mitochondria to initiate steroidogenesis [154]. The discrepancy in the levels of steroid hormones following phthalate exposure is likely attributed to the route of exposure. When rats were exposed via inhalation, steroid hormone levels were increased [153]. On the contrary, when rats were exposed via oral ingestion, steroid hormone levels were decreased [154]. Further, the timing of exposure may explain why prepubertal-exposed animals had increased Cyp19a1 levels [153], but in utero-exposed animals had decreased Cyp19a1 levels [121, 141].

Additional in vivo studies indicate that phthalate exposure during adulthood targets the ovary and disrupts steroidogenesis. Exposure to DEHP via oral gavage for 8 days at 2 g/kg/day decreased serum estradiol levels in adult rats [134]. This suppression of estradiol led to secondary rises in FSH levels and was unable to induce the LH surge needed for ovulation [134]. Thus, DEHP exposure caused anovulation in the study [134]. Further, DEHP exposure prolonged the duration of the estrous cycle in the adult rats [134]. A similar study showed that DEHP exposure via oral gavage at 1000-3000 mg/kg/day also decreased serum estradiol levels in adult rats [155]. In addition, serum testosterone, progesterone, LH, and FSH were also decreased following DEHP exposure [155]. Similarly, DEHP exposure via oral gavage for 16 weeks at 500-2000 mg/kg/day prolonged the duration of estrous cycles, caused apoptosis and cell cycle arrest in granulosa cells, and decreased serum progesterone levels in adult mice [156]. A similar effect on estrous cyclicity was seen where oral exposure to DEHP for 10 and 30 days at 20 µg /kg/day-750 mg/kg/day increased the amount of time adult mice spent in the estrous stage [119]. Further, chronic DBP exposure from weaning, through puberty, mating, and gestation at 500-1000 mg/kg/day increased gestational ex vivo ovarian estradiol production and decreased gestational ex vivo ovarian progesterone production in adult rats [157]. Conversely, DEHP exposure via intramuscular injections at 25-50 mg/kg/day increased plasma concentrations of progesterone in the adult ewe [146]. DEHP exposure also decreased the duration of the ewe’s estrous cycle and increased the number of irregular estrous cycles [146]. The effects on progesterone production and estrous cyclicity are likely attributed to DEHP toxicity on the copora lutea [146]. Estradiol and progesterone mediated processes, such as uterine decidualization, are also affected by exposure to phthalates in the adult rat. BBP, DBP, and MBP exposure via gastric intubation at 750-1500 mg/kg/day suppressed uterine decidualization in the
adult rat, which is a required process for pregnancy and is controlled by ovarian derived steroid hormones [158-160]. Together, these studies provide evidence that phthalate exposure during adulthood alters ovarian steroidogenesis.

**Effects of phthalate exposure on steroidogenesis in vitro**

Several *in vitro* studies using multiple culture models confirm and expand upon the ability of phthalates to disrupt ovarian steroidogenesis. Importantly, some of these studies also provide essential insight into the mechanisms by which phthalates disrupt steroidogenesis. Isolated ovarian cell cultures have shown that phthalates directly target specific cell types in the ovary and disrupt steroidogenesis in animal models. Specifically, MEHP exposure for 48 hrs at 50-200 µM suppressed estradiol production in rat granulosa cells [161-163]. The decrease estradiol production from the granulosa cells was observed even with the supplementation of testosterone (a precursor for estradiol at 500 nM), FSH (an inducer and activator of aromatase for the conversion of testosterone to estradiol at 10 ng/ml), and 8-bromo cyclic adenosine monophosphate (a stable cAMP analogue, which is a secondary messenger for FSH signaling in granulosa cells at 1 mM). Thus, MEHP exposure disrupts estradiol production independent of FSH-cAMP signaling [161]. The mechanism by which MEHP suppresses estradiol in the culture system is via decreased mRNA levels, protein levels, and availability of aromatase [161, 162]. Further, it is likely that MEHP acts through peroxisome proliferator-activated receptors (PPARs) to decrease aromatase transcription [163]. PPARs are involved in granulosa cell differentiation, lipid metabolism, and even in the regulation of aromatase transcription and activity, and MEHP appears to activate PPARα and PPARγ in the granulosa cells to inhibit aromatase transcription [163]. Further, MEHP exposure for 24 hrs at 100 µM decreased progesterone production and FSH-induced cAMP accumulation in rat granulosa cells [164]. In contrast, MEHP exposure for 48 hrs at 100-250 µM in a different study increased basal steroidogenesis in rat granulosa cells, evident by increases in progesterone and protein levels of STAR [165]. Perhaps the discrepancy in the MEHP-induced defects in steroidogenesis can be attributed to the dosage and use of different rat strains and the different susceptibilities to phthalate toxicity across strains. MEHP exposure inhibited steroidogenesis in Fisher 344 rat granulosa cells [161-164], but it stimulated steroidogenesis in Sprague-Dawley rat granulosa cells [165]. This stimulation of steroidogenesis, evident by an increase in progesterone production, was also observed in KK-1 granulosa tumor
cells exposed to MEHP for 24 hrs at 25-100 µM [166]. Using another cellular model, DEHP exposure for 44 hrs at 1 µM increased the production of progesterone in FSH matured porcine cumulus oocyte complexes [139]. This effect can potentially disrupt the final maturation processes of the oocyte following ovulation. In isolated bovine granulosa cells and isolated luteal cells, DEHP and MEHP exposure for 72 hrs at 0.1-10 ng/ml increased the production of oxytocin [167]. Ovarian derived oxytocin plays a role in the regulation of the estrous cycle. These studies show that phthalates have a direct effect on disrupting steroidogenesis in specific ovarian cell types in multiple animal models. The mechanism by which phthalates inhibit steroidogenesis in granulosa cells appears to be via suppression of PPAR-mediated aromatase transcripion [161-163]. The mechanism by which phthalates stimulate steroidogenesis in granulosa cells appears to be via increased steroidogenic enzyme levels [165]. Future studies should aim to understand the differences in steroidogenesis in the different granulosa cell models.

Phthalates have also been shown to disrupt steroidogenesis in isolated human ovarian cell cultures. Similar to the previously mentioned study with rat granulosa cells [161-163], MEHP exposure at 0-500 µM/l decreased the production of estradiol in human granulosa-lutein cells isolated from women undergoing in vitro fertilization [168]. Likewise, this inhibition of estradiol production is independent of FSH-cAMP signaling; thus, it is attributed to a decrease in the mRNA levels and activity of aromatase [168]. In human luteal cells isolated from corpora lutea, DEHP, DBP, and BBP exposure for 24 hrs at 10^{-6}-10^{-9}M decreased basal and human chorionic gonadotropin-stimulated progesterone production [169]. In conjunction, DEHP, DBP, and BBP exposure decreased prostaglandin E2 (PGE2) secretion, and DEHP decreased prostaglandin F2α (PGF2α) secretion from the luteal cells [169]. Further, all three chemicals inhibited luteal cell release of vascular endothelial growth factor (VEGF) [169]. Prostaglandins and VEGF are regulators of corpora lutea survival. Specifically, PGE2 and VEGF are luteotrophic factors and PGF2α is a luteolytic factor. Another study has shown phthalate-induced defects in immortalized human granulosa cell lines. In detail, BBP exposure at 1 µM in HO23 cells increased the mRNA and protein levels of arylhydrocarbon receptor (AHR), arylhydrocarbon receptor nuclear translocator (ARNT), and cytochrome-P450 1B1 (CYP1B1), which are involved in estradiol metabolism, resulting in reduced cell viability and potential decreases in estradiol, though this was not directly tested [170]. Overall, phthalates appear to directly disrupt steroidogenesis by
decreasing steroid hormone and steroidogenic enzyme levels in human ovarian cells in a manner similar to animal studies \textit{in vitro}.

Expanding on the use of individual cell types, other culture systems utilizing the entire antral follicle and whole sections of ovaries have been used to investigate the effects of phthalates on steroidogenesis. This is important because steroidogenesis is a multi-cellular process involving both granulosa cells and theca cells. MEHP exposure for 48 hrs at 10-100 µg/ml increased the levels of progesterone and decreased the levels of androstenedione, testosterone, and estradiol in isolated rat secondary follicles [126]. Interestingly, even with the decreases in the three sex steroid hormones, the increase in progesterone promoted an increase in the combined level of all steroid hormones in response to MEHP exposure [126]. This suggests that MEHP potentially stimulates steroidogenesis in the secondary follicle, but it inhibits the conversion of progesterone to androstenedione [126]. Further studies using mouse preantral follicles show that MEHP exposure at 10-200 µM increased the levels of progesterone, testosterone, and estrone [127]. The discrepancies between testosterone production in these two studies can possibly be attributed to species differences and differences in culture methods. Using antral follicles, the most steroidogenically-active follicle type, isolated from mice, DEHP (10-100 µg/ml) and MEHP (1-100 µg/ml) exposure for 96 hrs decreased estradiol production via inhibition of \textit{Cyp19a1} transcription [132]. This effect on steroidogenesis coincides with the DEHP- and MEHP-induced inhibition of antral follicle growth, cell cycle arrest evident by alterations in \textit{Ccnd2}, \textit{Ccne1}, and \textit{Cdk4} mRNA levels, atresia evident by alterations in \textit{Bax}, \textit{Aifm1}, \textit{Bcl2}, and \textit{Bcl2l10} mRNA levels, and induction of oxidative stress evident by increases in ROS and altered SOD1 and GPX protein and activity [130-132]. However, it is unknown if the inhibition of steroidogenesis causes these other toxic events or is a secondary response to defects in cell cycle progression and/or oxidative stress. Interestingly, supplementing the media with estradiol (1-10 nM) and NAC (0.25-1 mM), an antioxidant, only partially protected the antral follicle from DEHP- and MEHP-induced growth inhibition and \textit{Cyp19a1} transcription [130-132], but estradiol completely rescued antral follicles from MEHP-induced atresia [133]. This likely suggests that the effects of MEHP on estradiol production precede and promote the incidence of atresia [133]. In another study, DBP exposure for 96 hrs at 1000 µg/ml decreased estradiol levels, and exposure for 168 hrs promoted atresia in cultured mouse antral follicles [129]. Similar to DEHP and MEHP, this effect on steroidogenesis coincided with DBP-induced (1-1000 µg/ml) inhibition of antral
follicle growth, cell cycle arrest evident by an increase in the number of follicular cells in the G\(_1\) stage and alterations in the mRNA levels of *Ccnd2*, *Ccne1*, *Ccna2*, *Ccnb1*, and *Cdkn1a*, and atresia evident by alterations in the mRNA levels of BH3 interacting-domain death agonist (*Bid*) and *Bcl2* [129]. These studies suggest that the entire follicle unit is a target for phthalate-induced disruption of steroidogenesis. Studies using secondary and preantral follicles have shown an increase in steroid hormone levels following phthalate exposure [126, 127]. Meanwhile, studies using more mature antral follicles have shown a decrease in steroid hormone levels following phthalate exposure, and the mechanisms by which phthalates inhibit steroidogenesis may involve an inhibition of antral follicle growth [129, 132], an induction of atresia [130-133], an increase in oxidative stress [130, 131], and decreases in steroidogenic enzyme levels [132].

Similar to the follicle culture, minced ovary cultures, containing all follicular cell types, have been used to investigate the effects of phthalates on steroidogenesis. DEHP exposure *in vivo* altered the steroidogenic profile of minced rat ovaries cultured for 1 hr depending on the stage of the estrous cycle. Specifically, DEHP exposure at 1500 mg/kg/day increased the minced ovary production of testosterone and estradiol when the rats were euthanized in diestrus [171, 172]. Conversely, when the rats were euthanized in estrus, cultured minced ovaries produced less estradiol [171, 172].

*Epidemiological links between phthalate exposure and alterations in steroidogenesis*

Exposure to phthalates has been shown to disrupt ovarian steroidogenesis and steroidogenic controlled processes. Though limited, there is epidemiologic evidence that phthalate exposure is associated with steroidogenic defects. Specifically in the Western Australian Pregnancy Cohort Study, serum from pregnant women during gestational week 18 was subjected to measurements of phthalate metabolites and hormones and found that several phthalate metabolites have a negative association with maternal sex hormone binding globulin, and MEP had a negative association with AMH in the adolescent daughter [148]. Further, the sum of DEHP metabolites was associated with a trend for an earlier age at menarche in the adolescent female offspring, which is a process heavily controlled by ovarian steroid production [148]. In another study, urinary levels of MEHP and the oxidative monoester metabolite mono(2-ethyl-5-hydroxyhexyl) phthalate in mothers were negatively associated with free testosterone levels and the free testosterone to estradiol levels ratio in the cord serum from female human
infants [173]. Similarly, the urinary levels of several phthalate metabolites were associated with decreased serum total testosterone levels in women aged 6-20 and 40-60 years from the National Health and Nutrition Examination Survey [174]. Further, urinary levels of DEHP metabolites and MBP were associated with decreased testosterone levels in pregnant women in the Study for Future Families [175]. Conversely, in utero exposure to MEP and MBzP was associated with increased testosterone levels in girls at ages 8-13 years from the Mexico City birth cohort [176]. Urinary levels of MEHP and MBzP in eight year old girls from Taiwan were also positively associated with increased serum progesterone levels, and urinary levels of MBzP and MBP were positively associated with increased serum FSH levels [177].

Proper regulation of ovarian steroidogenesis is vital for reproductive and non-reproductive health, and numerous studies indicate that phthalates have the ability to dysregulate steroidogenesis in multiple aspects (Figure 2.5). Specifically, phthalates have been shown to intervene in the production and secretion of multiple sex steroid hormones in both in vivo and in vitro systems to often lead to a decrease in estradiol levels. Further, phthalates have been shown to directly target several steroidogenic cell types in the ovary to elicit an adverse effect on steroid hormone production. These effects on steroidogenesis are likely attributed to alterations in the transcription of genes that synthesize and metabolize estradiol (Table 2.2). Importantly, the effects observed in animal studies moderately correlate to the effects observed in human ovarian cell types [161, 162, 164, 168, 169]. As is the case with studies investigating the effects of phthalates on folliculogenesis, future work investigating the effects of phthalates on steroidogenesis should incorporate exposures to other commonly used phthalates to expand upon what is known regarding DEHP and MEHP, which are the two most extensively studied phthalates. Further, the doses used in these studies should fall within the range of estimated human exposure. Most often, the doses used in the reviewed studies exceed human exposure levels. Because phthalates exhibit a non-monotonic dose response, the effects of phthalates on steroidogenesis at lower levels may be more toxic and/or have different mechanisms than at higher levels [149, 150]. Additionally, future work should elucidate the mechanisms by which phthalates disrupt steroidogenesis, and investigate whether the phthalate-induced disruption in steroidogenesis leads to infertility and non-reproductive complications.
2.7 Summary and Conclusions

Phthalates are a group of endocrine disrupting chemicals that target the ovary to adversely affect the two essential processes of folliculogenesis and steroidogenesis. This is concerning for public health because phthalates are used extensively in a wide variety of commonly used items, resulting in ubiquitous human exposure. Phthalates have been shown to alter ovarian and oocyte development, target specific follicle types, alter progression of follicular development, and disrupt the functionality of follicles and corpora lutea. Specifically, phthalates have been shown to inhibit germ cell nest breakdown and primordial follicle assembly [118], accelerate primordial follicle recruitment [119-121], inhibit antral follicle growth [129-132] and final oocyte maturation [120, 135-141] to potentially inhibit ovulation [134, 142], and induce atresia in follicles across several stages of development [122-124, 129-131, 133]. Further, increasing evidence suggests that phthalates disrupt the production, secretion, and action of several essential sex steroid hormones via altered mRNA, protein, and activity of multiple steroidogenic enzymes. These effects most commonly result in decreased estradiol levels [126, 129, 132, 134, 154, 155, 161-163, 168, 171, 172]; however, some studies suggest that phthalates stimulate steroidogenesis [121, 126, 139, 146, 165, 171, 172]. Regardless, these effects on folliculogenesis and steroidogenesis can have lasting effects on reproductive and non-reproductive health, as both of these processes are essential for fertility, maintenance of appropriately timed reproductive senescence, and the regulation of skeletal, cardiovascular, and brain health.

Further study is warranted in investigating the effects of phthalates on ovarian function. In particular, researchers should expand upon the dose ranges used in their studies to incorporate doses that mimic human exposure. The majority of the cited work focuses primarily on doses that exceed the range of estimated human exposure. Although these findings are important, observing the effects at levels that mimic human exposure would increase the translational nature of studies. Further, a unique characteristic of endocrine disrupting chemicals is that low doses often elicit different or more profound effects than high doses [149, 150]. To expand upon the use of doses that mimic human exposure, future studies should also consider realistic routes and lengths of exposure. Humans are predominantly exposed to phthalates via oral ingestion, not gavage, and are exposed throughout the duration of the day, not a single bolus. Thus, studies
should utilize oral dosing or exposure in the diet over multiple time-points in the day. Humans are also chronically exposed to phthalates from gestation through adulthood. Therefore, studies should investigate the effects of phthalate exposure, starting in utero and continuing in adulthood, on ovarian function across all stages of development. Additionally, humans are exposed to multiple phthalates and other environmental toxicants daily. The majority of the cited work understandably focuses on single phthalate exposures, but future studies should incorporate exposures to phthalate mixtures as well as a mixture of phthalates and other ubiquitous toxicants. The above suggestions would aid in translating the findings from animal studies to potential effects in humans. These future studies should also focus on the mechanisms of phthalate-induced ovotoxicity. Some studies suggest that phthalates exert toxicity via an estrogenic, anti-estrogenic, oxidative stress response, or PPAR activation depending on the model system, dose, and exposure window. However, further work must be done to elucidate the mechanisms by which phthalates disrupt folliculogenesis and steroidogenesis. This will aid in the treatment and/or prevention of phthalate-induced reproductive diseases.
2.8 Tables, Figures, and Legends

Figure 2.1 Chemical structures of common phthalates and their monoester metabolites that are mentioned in this introduction

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>Monoester metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di(2-ethylhexyl) phthalate (DEHP)</td>
<td>Mono(2-ethylhexyl) phthalate (MEHP)</td>
</tr>
<tr>
<td>Dibutyl phthalate (DBP)</td>
<td>Monobutyl phthalate (MBP)</td>
</tr>
<tr>
<td>Diethyl phthalate (DEP)</td>
<td>Monoethyl phthalate (MEP)</td>
</tr>
<tr>
<td>Butyl benzyl phthalate (BBP)</td>
<td>Monobenzyl phthalate (MBzP)</td>
</tr>
<tr>
<td>Diisobutyl phthalate (DIBP)</td>
<td>Monoisobutyl phthalate (MIBP)</td>
</tr>
<tr>
<td>Diisononyl phthalate (DINP)</td>
<td>Monoisononyl phthalate (MINP)</td>
</tr>
</tbody>
</table>

Chemical structures of parent phthalates are present on the left. Their respective monoester metabolites are present on the right.
The female is born with a finite number of primordial follicles that can mature through the primary, preantral, and antral stages of development. The follicle contains the gamete (oocyte) surrounded by granulosa cells (shown in red) and theca cells (shown in green), which are somatic cells. Following ovulation, the antral follicle differentiates into the corpus luteum, and the granulosa and theca cells become large and small luteal cells, respectively.
Steroidogenesis is primarily conducted by the mature antral follicle and the corpus luteum following ovulation. This process requires both the theca cells and granulosa cells, and involves the enzymatic conversion of cholesterol to 17β-estradiol and other necessary sex steroid hormones. The hormones produced by the ovary are listed in the white text boxes while the steroidogenic enzymes are listed in blue adjacent to the arrows between hormones.
Figure 2.4 Phthalates disrupt folliculogenesis

This figure is a summation of the major findings on the effects of phthalates on folliculogenesis. Text boxes above a particular follicle type outline the major effects of phthalates at that stage of development, while text boxes below transition arrows outline the major effects of phthalates on that developmental transition.
Table 2.1 Genes associated with folliculogenesis that are altered by phthalate exposure

<table>
<thead>
<tr>
<th>Phthalate (dose)</th>
<th>Model (duration of exposure)</th>
<th>Effect on gene (reference)</th>
<th>Gene name</th>
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<tr>
<td>DEHP (0.02-40 μg/l)</td>
<td>Adult zebrafish (21 days)</td>
<td>Decreased Ptx2 (155)</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
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<td>DEHP (100 μg/ml)</td>
<td>Mouse antral follicles (96 hrs)</td>
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<td>Cyclin D2</td>
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<td></td>
<td></td>
<td>Decreased Cdk4 (132)</td>
<td>Cyclin-dependent kinase 4</td>
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<td>Increased Bac (118)</td>
<td>BCL2-associated X protein</td>
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<td></td>
<td></td>
<td>Decreased Lhca9 (118)</td>
<td>LIM homeobox 8</td>
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<td></td>
<td></td>
<td>Decreased Figla (118)</td>
<td>Factor in the germline alpha</td>
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<td>Decreased Sox1/2 (118)</td>
<td>Spermatogenesis and oogenesis helix-loop-helix</td>
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<td>Decreased Nabo (118)</td>
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<td>DBP (1-1000 μg/ml)</td>
<td>Mouse antral follicles (24-108 hrs)</td>
<td>Decreased Crnad2 (129)</td>
<td>Cyclin D2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Cred2 (129)</td>
<td>Cyclin E1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Ckca (129)</td>
<td>Cyclin A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Ckcn1 (129)</td>
<td>Cyclin B1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased Cdkn1a (129)</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
</tr>
</tbody>
</table>

This table is a summation of the major effects of phthalates on genes involved with folliculogenesis.
Figure 2.5 Phthalates alter steroidogenesis

This figure is a summation of the major findings on the effects of phthalates on steroidogenesis. Black text boxes connected to hormones outline the major effects of phthalates on the levels of that hormone. Blue text boxes connected to steroidogenic enzymes outline the major effects of phthalates on the mRNA and/or protein levels of that enzyme.
Table 2.2 Genes associated with steroidogenesis that are altered by phthalate exposure

<table>
<thead>
<tr>
<th>Phthalate (dose)</th>
<th>Model (duration of exposure)</th>
<th>Effect on gene (reference)</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEHP (0.05-5 mg/kg/day)</td>
<td>Adult mouse, in utero (length of gestation-weaning)</td>
<td>Decreased Cyp19a1 (141)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Cyp17a1 (141)</td>
<td>Cytochrome P450 steroid 17-α-hydroxylase 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Pgr (141)</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Fshr (141)</td>
<td>FSH receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Lhr (141)</td>
<td>LH receptor</td>
</tr>
<tr>
<td>DEHP (100 µg/ml)</td>
<td>Mouse antral follicles (96 hrs)</td>
<td>Decreased Cyp19a1 (132)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td>DEHP (25 mg/m3)</td>
<td>Prepubertal rat (63 days)</td>
<td>Increased Cyp19a1 (153)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td>MEHP (10 µg/ml)</td>
<td>Mouse antral follicles (96 hrs)</td>
<td>Decreased Cyp19a1 (132)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td>MEHP (100-1000 mg/kg/day)</td>
<td>Adult mouse, in utero (gestational day 17-19)</td>
<td>Decreased Star (121)</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decreased Cyp19a1 (121)</td>
<td>Steroidogenic P450 aromatase</td>
</tr>
<tr>
<td>MEHP (50-200 µM)</td>
<td>Rat granulosa cells (48 hrs)</td>
<td>Decreased Cyp19a1 (161-163)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td>BBP (1 µM)</td>
<td>HO23 cells (24 hrs)</td>
<td>Increased AHR (170)</td>
<td>Arylhydrocarbon receptor</td>
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<tr>
<td></td>
<td></td>
<td>Increased ARNT (170)</td>
<td>Arylhydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased CYP1B1 (170)</td>
<td>Cytochrome-P450 1B1</td>
</tr>
<tr>
<td>DIBP (600 mg/kg/day)</td>
<td>Prepubertal rat, in utero (gestational day 7-21)</td>
<td>Increased Cyp19a1 (151)</td>
<td>Cytochrome P450 aromatase</td>
</tr>
</tbody>
</table>

This table is a summation of the major effects of phthalates on genes involved with steroidogenesis.
2.9 References

20. Registry USAfTSaD. Toxicological Profile for Di-nOctyl Phthalate (DNOP). In. Atlanta, GA; 1997.


121. Moyer B, Hixon ML. Reproductive effects in F1 adult females exposed in utero to moderate to high doses of mono-2-ethylhexylphthalate (MEHP). Reprod Toxicol 2012; 34:43-50.


123. Grande SW, Andrade AJ, Talsness CE, Grote K, Golombiewski A, Sterner-Kock A, Chahoud I. A dose-response study following in utero and lactational exposure to di-(2-
ethylhexyl) phthalate (DEHP): reproductive effects on adult female offspring rats. Toxicology 2007; 229:114-122.


164. Treinen KA, Dodson WC, Heindel JJ. Inhibition of FSH-stimulated cAMP accumulation and progesterone production by mono(2-ethylhexyl) phthalate in rat granulosa cell cultures. Toxicol Appl Pharmacol 1990; 106:334-340.


CHAPTER III

Daily Exposure to Di(2-ethylhexyl) Phthalate Alters Estrous Cyclicity and Accelerates Primordial Follicle Recruitment Potentially Via Dysregulation of the Phosphatidylinositol 3-Kinase Signaling Pathway in Adult Mice

3.1 Abstract

Humans are exposed daily to di(2-ethylhexyl) phthalate (DEHP), a plasticizer found in many consumer, medical, and building products containing polyvinyl chloride. Large doses of DEHP disrupt normal ovarian function; however, the effects of DEHP at environmentally relevant levels, the effects of DEHP on folliculogenesis, and the mechanisms by which DEHP disrupts ovarian function are unclear. The present study tested the hypothesis that relatively low levels of DEHP disrupt estrous cyclicity as well as accelerate primordial follicle recruitment by dysregulating phosphatidylinositol 3-kinase (PI3K) signaling. Adult CD-1 mice were orally dosed with DEHP (20 µg/kg/day-750 mg/kg/day) daily for 10 and 30 days. Following dosing, the effects on estrous cyclicity were examined and follicle numbers were histologically quantified. Further, the ovarian mRNA and protein levels of PI3K signaling factors that are associated with early folliculogenesis were quantified. The data indicate that 10 and 30 day exposure to DEHP prolonged the duration of estrus and accelerated primordial follicle recruitment. Specifically, DEHP exposure decreased the percentage of primordial follicles and increased the percentage of primary follicles counted following 10 day exposure and increased the percentage of primary follicles counted following 30 day exposure. DEHP exposure, at doses that accelerate folliculogenesis, increased the levels of 3-phosphoinositide dependent protein kinase-1, mammalian target of rapamycin complex 1, and protein kinase B and decreased the levels of phosphatase and tensin homolog, potentially driving PI3K signaling. Collectively, relatively low levels of DEHP disrupt estrous cyclicity and accelerate primordial follicle recruitment potentially via a mechanism involving dysregulation of PI3K signaling.

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3.2 Introduction

Phthalate esters are synthetic plasticizers added to plastics to impart flexibility. The most widely used phthalate ester is di(2-ethylhexyl) phthalate (DEHP), which is used in the manufacturing of common consumer, medical, and building products containing polyvinyl chloride. Products that contain DEHP include flooring, roofing, carpeting, food and beverage packaging, automobile upholstery, shower curtains, packaging equipment, medical bags and tubing, and personal care products. Although production volumes of DEHP alone are not quantified, DEHP belongs to a group of phthalates known as dioctyl phthalates. Domestic production of these phthalates reaches up to 300 million pounds per year [1]. DEHP is non-covalently bound to plastics, and therefore, leaching can occur after repeated use, heating, and/or cleaning of the products, allowing the toxicant to be ingested on a daily basis via oral ingestion, inhalation, and dermal contact [2]. In fact, the Agency for Toxic Substances and Disease Registry estimates that the range of daily human exposure to DEHP is 3-30 μg/kg/day, but measurements of DEHP in household dust can reach as high as 700 mg/kg [1, 3].

Exposure to DEHP represents a public health concern, partially because it has been identified as a top contaminant present in human tissues. DEHP and its metabolites are present in over 95% of human blood samples and nearly 100% of human urine samples tested [2-6]. In particular to reproduction, DEHP and its metabolites are present in 90-100% of amniotic fluid samples from second trimester fetuses, cord blood samples from newborns, breast milk from nursing mothers, and even in human ovarian follicular fluid, indicating its ability to reach the ovary [2, 4, 5, 7].

Continuous, daily exposure to DEHP, along with its prevalence in human tissues, is of major concern because DEHP has been designated as an endocrine disrupting chemical and reproductive toxicant for its ability to interfere with reproductive and hormone-regulated processes [8-10]. In males, DEHP exhibits anti-androgenic effects by inducing tubular atrophy and testicular degeneration and by inhibiting testicular steroidogenesis [11, 12]. To date, the majority of studies examining the effects of DEHP focus on male reproduction and development; however, in females, chronic occupational exposure to phthalates has been associated with decreased rates of pregnancy and high rates of miscarriage in women [2]. In laboratory animals, DEHP reduces implantations, increases resorptions, and decreases fetal weights of offspring [9,
Surprisingly, much less is known about the effects of DEHP on the adult ovary, but recent work suggests that the ovary may be a target of DEHP toxicity. Specifically, DEHP exposure has been shown to decrease serum and antral follicle-produced 17β-estradiol levels, decrease aromatase levels, cause anovulation, and disrupt estrous cyclicity [14-19]. Some of the doses of DEHP used in these previous studies, however, are not environmentally relevant; thus, the effects of DEHP at environmentally relevant levels on ovarian function remain unclear. Further, the mechanisms by which DEHP disrupts ovarian function remain unclear; however, DEHP is hypothesized to cause toxicity independent of steroid receptor signaling as both DEHP and its metabolite, mono(2-ethylhexyl) phthalate (MEHP), have weak to no binding to the androgen and estrogen receptor [20-25].

Understanding the effects of DEHP on ovarian function is vital because the ovary is an integral regulator of reproductive and non-reproductive health, and one of the most essential ovarian processes is folliculogenesis. The ovary is a heterogeneous endocrine organ containing ovarian follicles, which are the functional units of the ovary. In mammals, the female is born with a finite number of ovarian follicles; thus, the follicular reserve is set at birth and represents a female’s reproductive potential and reproductive lifespan [26]. Within the ovarian unit, follicles undergo several irreversible developmental transitions, starting from the primordial stage, in which the follicles are immature, dormant, and constitute the ovarian reserve, to the antral stage, in which the follicles are mature and capable of ovulation [26, 27]. This process of follicular development is known as ovarian folliculogenesis. Because the ovarian reserve is established at birth and folliculogenesis is an irreversible process, aberrant regulation of folliculogenesis can have adverse reproductive implications. In particular, the accelerated depletion of primordial follicles, either through apoptosis or irregular activation of development, can result in infertility and/or premature ovarian failure, or early onset of menopause. Although DEHP has been shown to disrupt normal ovarian function, its effects on folliculogenesis remain unclear.

Because ovarian folliculogenesis is essential for reproductive and non-reproductive health, and increasing evidence indicates that the ovary is a potential target of DEHP toxicity, the present study was designed to investigate the effects of in vivo DEHP exposure on normal ovarian function, as well as the role and mechanisms by which DEHP disrupts folliculogenesis. Specifically, the present study was designed to test the hypothesis that relatively low levels of
DEHP disrupt folliculogenesis by dysregulating the phosphatidylinositol 3-kinase (PI3K) signaling pathway, potentially leading to the acceleration of primordial follicle recruitment. We decided to investigate the effects of DEHP on the PI3K signaling pathway because PI3K signaling has recently been shown to be a critical regulator of early folliculogenesis, specifically in primordial follicle survival, quiescence, and recruitment [28-33]. In further detail, increased PI3K signaling is associated with the promotion of primordial follicle recruitment. To test our hypothesis, we orally dosed adult mice with DEHP daily for 10 and 30 days. Following dosing, we investigated reproductive endpoints and histologically examined the effect of DEHP on follicular dynamics. To provide insight into the mechanism of DEHP-induced defects in folliculogenesis, we measured the levels of key PI3K signaling factors that are associated with early folliculogenesis. Specifically, we examined the effects of DEHP on 3-phosphoinositide dependent protein kinase-1 (PDPK1), mammalian target of rapamycin complex 1 (mTORC1), mast/stem cell growth factor receptor (KIT), protein kinase B (AKT), and ribosomal protein s6 (rpS6), which are all factors that drive PI3K signaling. Additionally, we examined the effects of DEHP on forkhead box O3A (FOXO3A), phosphatase and tensin homolog (PTEN), and tuberous sclerosis 1 (TSC1), which negatively regulate PI3K signaling.
3.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Five stock solutions of DEHP were prepared using tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) as the vehicle. The doses used for these experiments were 20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day, and 750 mg/kg/day. To achieve these doses, the stock concentrations were 0.0125, 0.125, 12.5, 125, and 468.8 mg/ml for each respective dose.

The doses were chosen because they are much more environmentally relevant than doses used in previous DEHP ovotoxicity studies (2 g/kg/day) [15, 17, 34]. In particular, the Agency for Toxic Substances and Disease Registry estimates that the range of daily human exposure to DEHP is 3-30 µg/kg/day [1]. Additionally, they note that the no-observed-adverse-effect level (NOAEL) for DEHP is 5.8 mg/kg/day and the lowest-observed-adverse-effect level (LOAEL) is 140 mg/kg/day; however, potential reproductive effects of DEHP occur at lower levels ranging from 1-30 µg/kg/day [1, 2]. Further, the Environmental Protection Agency (EPA) reference dose for DEHP is 20 µg/kg/day, but it is possible that certain populations can be exposed to much higher levels than this EPA reference dose [3].

Animals

Cycling, adult CD-1 female mice at 39 days of age were obtained from Charles River Laboratories (Wilmington, MA) and were allowed to acclimate to the facility prior to dosing. The mice were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility. The mice were provided food and water *ad libitum*. Housing in a controlled animal room environment was established by the maintenance of temperature at 22 ± 1 °C and 12-hour light–dark cycles. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana-Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

In vivo dosing regimen

The mice were orally dosed with vehicle or DEHP daily for 10 and 30 days. For the 10 day dosing study, the mice received vehicle or DEHP at 20 µg/kg/day, 200 µg/kg/day, 20
mg/kg/day, 200 mg/kg/day, and 750 mg/kg/day (n=8/group). For the 30 day dosing study, the mice received vehicle or DEHP at 20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, and 200 mg/kg/day (n=8/group). Dosing volumes were determined daily by corresponding mouse weight, and the dose was given orally by inserting a pipet tip beyond the incisors towards the cheek pouch. Estrous cyclicity was monitored daily, and the mice were sacrificed in estrus following the 10 and 30 day dosing period.

**Body and organ weight analysis**

The mice were weighed daily to both calculate a dosing volume and monitor the effect of DEHP on body weight. Following 10 and 30 days of dosing, the mice were weighed and euthanized in estrus (n=8/group). Organs were aseptically removed, cleaned of interstitial tissue, and weighed. Percent body weight change was calculated by dividing the weight at the time of euthanasia by the weight at the start of dosing and multiplying that value by 100. Organ weights were recorded as whole organ weights or relative organ weights. Relative organ weights were calculated by dividing the weight of the organ by the body weight at the time of euthanasia and multiplying that value by 100.

**Estrous cyclicity analysis**

During dosing, the mice were subjected to daily vaginal smears to monitor estrous cyclicity (n=8/group). Stage of estrus was determined by vaginal cytology using a light microscope and was recorded based on previously defined and well-documented criteria [35-37]. Percentage of days in estrus was calculated by dividing the number of days in estrus by the number of days in the study and multiplying that value by 100. Percentage of days in metestrus/diestrus was calculated by dividing the number of days in either metestrus or diestrus by the number of days in the study and multiplying that value by 100.

**Histological evaluation of follicle numbers**

Following dosing, mice were euthanized in estrus and some ovaries were aseptically collected and fixed in Dietrich’s fixative for at least 24 hrs for histological evaluation of follicle numbers (n=3-8 ovaries/group). The ovaries were then transferred to 70% ethanol, embedded in paraffin wax, and serial sectioned (8 µM) using a microtome. The serial sections were mounted
on a glass slide and stained with hematoxylin and eosin. The numbers of oocyte containing follicles were counted in every tenth serial section using a light microscope, and the percentage of each follicle type was calculated by dividing the number of follicles of each specific type by the total number of follicles counted in every tenth serial section and multiplying that value by 100. Stage of follicular development was assessed using previously defined criteria [38, 39]. Briefly, primordial follicles contained the oocyte surrounded by a single layer of squamous granulosa cells, primary follicles contained the oocyte surrounded by a single layer of cuboidal granulosa cells, preantral follicles contained the oocyte surrounded by at least two layers of cuboidal granulosa cells and theca cell layers, and antral follicles contained the oocyte surrounded by multiple layers of cuboidal granulosa cells with a fluid filled antral space and theca cell layers. All primordial and primary follicles with oocytes, regardless of nuclear material in the oocytes, were counted; whereas, only preantral and antral follicles with visible nuclear material in the oocyte were counted to avoid the risk of double counting follicles large enough to span serial sections.

**Gene expression analysis**

Following dosing, mice were sacrificed in estrus and some ovaries were aseptically collected, snap frozen in liquid nitrogen, and stored at -80°C for quantitative real-time polymerase chain reaction (qPCR) analysis (n=3-5 ovaries/group). Total RNA was extracted from the ovaries using the RNaseasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol. Total RNA (1000 ng) was then reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer’s protocol. Each cDNA sample was diluted 1:4 with nuclease-free water prior to analysis. Analysis of qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and accompanying CFX Manager Software according to the manufacturer’s protocol. The machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. A standard curve was generated from six serial dilutions of three samples spanning different treatment groups to calculate the amplification efficiencies of each primer. Specific qPCR primers (Integrated DNA Technologies, Inc., Coralville, IA) for the genes of interest as well as the reference gene, beta-actin (Actb), which did
not differ across treatment groups, can be found in Table 3.1. All qPCR reactions were done in triplicate using 2 µl cDNA, forward and reverse primers (5 pmol) for Mtorc1, Pdpk1, Pten, Kit, Rps6, Tsc1, Foxl2, or Actb, in addition to a SsoFastEvaGreen Supermix qPCR kit for a final reaction volume of 10 µl. The qPCR program consisted of an enzyme activation step (95°C for 1 min), an amplification and quantification program (40 cycles of 95°C for 10 sec, 60°C for 10 sec, single fluorescence reading), a step of 72°C for 5 min, a melt curve (65°C –95°C heating 0.5°C per sec with continuous fluorescence readings), and a final step at 72°C for 5 min as per the manufacturer’s protocol. Expression data were generated using the mathematical model for relative quantification of real-time PCR data developed by Pfaffl [40]. The genes tested were chosen because they are associated with the regulation of early folliculogenesis, in particular primordial follicle recruitment [28, 29, 31, 41-43]. Additionally, each gene tested, except Foxl2, is involved in PI3K signaling [28-33, 44].

**Immunohistochemistry**

Following 10 days of dosing, mice were sacrificed in estrus and some ovaries were aseptically collected, fixed in 4% paraformaldehyde overnight, and transferred to 70% ethanol for immunohistochemical analysis (n=3/group). The ovaries were then embedded in paraffin wax, serial sectioned (5 µM), and five representative sections that span the entire length of the ovary were mounted on a glass slide. Following deparaffinization, the tissues were subjected to heat-induced antigen retrieval (10 mM sodium citrate buffer at pH 6.0) for 60 min followed by endogenous peroxide quenching in 3% hydrogen peroxide for 20 min. The tissues were then blocked in 5% goat serum and avidin for 30 min. In some experiments, the tissues were incubated with a rabbit anti-mouse phosphorylated-AKT (Ser473) (the active form of the protein) antibody (1:50) (Cell Signaling Technology, Inc., Boston, MA) with biotin for 60 min, while in other experiments, the tissues were incubated with a rabbit anti-mouse PTEN antibody (1:200) (Cell Signaling Technology, Inc., Boston, MA) with biotin for 60 min. The tissues were then incubated with a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA) for 20 min, followed by an incubation with an avidin biotin complex solution (Vector Laboratories, Inc., Burlingame, CA) for 20 min. ImmPACT NovaRED peroxidase substrate solution (Vector Laboratories, Inc., Burlingame, CA) was then applied until color optimally developed. Each sample was exposed to the chromogen for equal amounts of time. The
slides were then rinsed, counter-stained with a 1:10 dilution of hematoxylin, and coverslipped. A negative control was used in each experiment and was subjected to the same methods listed above, except instead of primary antibody, the negative control tissues were incubated with a negative control rabbit immunoglobulin fraction (Dako, Carpinteria, CA) at the same concentration of each primary antibody. Negative controls were confirmed to have no positive staining after exposure to the chromogen for equal amounts of time as the samples. Both pAKT and PTEN were chosen because they are integral regulators of the PI3K signaling pathway and have been associated with the regulation of primordial follicle recruitment [31, 43-46]. Specifically, pAKT drives PI3K signaling and ultimately promotes primordial follicle recruitment [31, 43-46]. Additionally, PTEN inhibits PI3K signaling and ultimately maintains primordial follicle quiescence [31, 43-46].

Analysis of the levels of protein staining

Following immunohistochemistry, the levels of pAKT and PTEN staining in the ovaries were quantified using published methods [47-49]. Briefly, images of whole ovaries and primordial and primary follicles were digitally captured using a Leica DFC 290 camera and analyzed using the ImageJ software (http://rsb.info.nih.gov/nih-image/). For whole ovarian analysis, 5 representative sections from each ovary were assessed from 3 separate animals per treatment group. For follicular analysis, 2-3 follicles of each type from the 5 representative ovarian sections were assessed from 3 separate animals per treatment group. Digital images were converted to 8-bit grayscale images and then converted to pseudocolored images. Colors were based on relative stain intensity, as defined digitally. Areas with no staining appeared black and blue, while areas with the most intense staining appeared deep yellow to orange. The pAKT and PTEN labeling index was defined as the percentage of positively stained area per whole ovary, primordial follicles, and primary follicles. The pixels of positively stained areas were divided by the total amount of pixels in the whole ovary, primordial follicles, and primary follicles, and that value was multiplied by 100.

Statistical analysis

All data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means ± standard error of the means (SEM). Multiple comparisons between
normally distributed experimental groups were made using one-way analysis of variance (ANOVA) followed by Tukey post-hoc comparison. Multiple comparisons between non-normally distributed experimental groups were made using Mann-Whitney and Kruskal Wallis tests when appropriate. Statistical significance was assigned at p ≤ 0.05.
3.4 Results

Effect of DEHP exposure on body and organ weights

To observe if DEHP is overtly toxic to the body and/or specific organs, we recorded body and organ weight changes in response to DEHP treatment. Importantly, DEHP exposure for 10 and 30 days had no effect on mortality of the mice and had no statistically significant effect on percent body weight change when compared to the vehicle control group (Table 3.2). However, DEHP exposure for 10 days significantly increased the whole and relative weight of the liver at the 750 mg/kg/day dose when compared to the vehicle control group (n=8/group, p ≤ 0.05). Additionally, DEHP exposure for 10 days significantly increased the whole and relative weight of the spleen at the 200 µg/kg/day and 20 mg/kg/day doses, respectively, when compared to the vehicle control group (n=8/group, p ≤ 0.05). In the 30 day dosing study, DEHP significantly increased the whole and relative weight of the uterus at the 20 µg/kg/day dose when compared to the vehicle control group (n=8/group, p ≤ 0.05). Again, these changes in organ weights had no effect on body weight change and mortality. Thus, the doses chosen for these studies were not overtly toxic to the body.

Effect of DEHP exposure on estrous cyclicity

DEHP exposure at high doses (2 g/kg/day) in adult rats previously has been shown to prolong estrous cycles, and DEHP treated antral follicles exhibit inhibition of estradiol production in vitro [15, 19]; thus, we decided to investigate if DEHP treatment altered estrous cyclicity in our dosing studies. In the 10 day dosing study, DEHP exposure significantly increased the percentage of days the mice were in estrus at the 20 µg/kg/day, and the 20, 200, and 750 mg/kg/day doses when compared to the vehicle control group (Fig. 3.1A, n=8/group, p ≤ 0.05). Similarly, DEHP exposure for 30 days significantly increased the percentage of days the mice were in estrus at the 200 mg/kg/day dose when compared to the vehicle control group (Fig. 3.1B, n=8/group, p ≤ 0.05). In both studies, DEHP exposure had no effect on the percentage of days the mice were in metestrus and diestrus when compared to the vehicle control group (Fig. 3.1A and 3.1B).
Effect of DEHP exposure on folliculogenesis

Because the ovary is a potential target of DEHP toxicity and ovarian folliculogenesis is essential for fertility and the maintenance of appropriately timed reproductive senescence, we histologically evaluated the effects of DEHP on follicular dynamics. In the 10 day dosing study, DEHP exposure significantly decreased the percentage of primordial follicles counted at the 20 and 200 mg/kg/day doses when compared to the vehicle control group (Fig. 3.2A, n=3-4/group, p ≤ 0.05). Likewise, DEHP exposure significantly increased the percentage of primary follicles counted at all selected doses of DEHP when compared to the vehicle control group (n=3-4/group, p ≤ 0.05). In accordance, DEHP exposure for 30 days significantly increased the percentage of primary follicles counted at the 200 µg/kg/day and 20 mg/kg/day doses when compared to the vehicle control group (Fig. 3.2B, n=8/group, p ≤ 0.05). These data suggest that DEHP exposure may affect early folliculogenesis, in that it may accelerate primordial follicle recruitment to the primary stage of development.

Effect of DEHP exposure on the mRNA levels of factors within the PI3K signaling pathway

Early folliculogenesis, specifically primordial follicle recruitment, relies on paracrine and autocrine regulation by intrinsic ovarian growth factors, and recent work has established the PI3K signaling pathway as an integral regulator of this process [26-33]. Thus, we measured the ovarian mRNA levels of several PI3K signaling factors that are associated with primordial follicle recruitment. In the 10 day dosing study, DEHP exposure significantly increased the mRNA levels of Mtorc1, a stimulator of the PI3K signaling pathway, at the 20 and 200 µg/kg/day doses, and at the 200 and 750 mg/kg/day doses when compared to the vehicle control group (Fig. 3.3A, n=4-5/group, p ≤ 0.05). Additionally, 10 day exposure to DEHP significantly increased the mRNA levels of Pdpk1, another stimulatory PI3K signaling factor, at the 200 and 750 mg/kg/day doses when compared to the vehicle control group (Fig. 3.3B, n=4-5/group, p ≤ 0.05). In the same study, DEHP exposure significantly decreased the mRNA levels of Pten, an inhibitory PI3K signaling factor, at the 200 µg/kg/day and 20 mg/kg/day doses when compared to the vehicle control group (Fig. 3.3C, n=4-5/group, p ≤ 0.05). However, 10 day exposure to DEHP had no effect on the mRNA levels of Kit, Rps6, Tsc1, or Foxl2 when compared to the vehicle control group.
In the 30 day dosing study, DEHP exposure significantly increased the mRNA levels of Kit, a stimulatory PI3K signaling factor, at the 20 µg/kg/day dose, but decreased its expression at the 200 mg/kg/day dose when compared to the vehicle control group (Fig. 3.4A, n=3-4/group, p ≤ 0.05). Similar to the 10 day dosing study, DEHP exposure for 30 days significantly decreased the mRNA levels of Pten at the 20 and 200 mg/kg/day doses when compared to the vehicle control group (Fig. 3.4B, n=3-4/group, p ≤ 0.05). DEHP exposure for 30 days also significantly decreased the mRNA levels of Rps6, a stimulatory PI3K signaling factor, at the 200 mg/kg/day dose when compared to the vehicle control group (Fig. 3.4C, n=3-4/group, p ≤ 0.05). Additionally, DEHP exposure significantly decreased the mRNA levels of Tsc1, another inhibitory PI3K signaling factor, at the 200 mg/kg/day dose when compared to the vehicle control group (Fig. 3.4D, n=3-4/group, p ≤ 0.05). Interestingly, DEHP exposure for 30 days altered factors outside of the PI3K signaling pathway that are also associated with primordial follicle recruitment. Specifically, DEHP significantly decreased the mRNA levels of Foxl2, an inhibitor of recruitment, at the 20 mg/kg/day dose when compared to the vehicle control group (vehicle: 1.1±0.3; 20 mg/kg/day: 0.5±0.1; n=4/group, p ≤ 0.05). However, DEHP exposure for 30 days had no effect on the mRNA levels of Pdpk1 and Mtorc1 when compared to the vehicle control group.

**Effect of DEHP exposure on protein levels of PI3K signaling factors**

DEHP exposure in the 10 and 30 day dosing studies selectively altered the mRNA levels of PI3K signaling factors that are associated with early folliculogenesis; however, some doses that exhibited phenotypic changes in folliculogenesis had unaltered mRNA expression. Additionally, certain factors within the PI3K signaling pathway are translationally and post-translationally regulated, thus the mRNA levels may not be indicative of the ovarian phenotypic changes observed in our dosing studies. Therefore, we measured protein levels of two key PI3K signaling factors that regulate primordial follicle recruitment in the 10 day dosing study. Representative images of pAKT, a stimulatory PI3K signaling factor involved in the promotion of primordial follicle recruitment, and PTEN, an inhibitory PI3K signaling factor involved in maintaining primordial follicle quiescence, staining in the ovary are found in Figure 3.5. Qualitatively, it appears that DEHP exposure increased the staining of pAKT (Fig. 3.5A-3.5D) and decreased the staining of PTEN (Fig. 3.5I-3.5L) in the whole ovary and in immature follicle
types (Fig. 3.5E-3.5H). When quantified, DEHP exposure significantly increased pAKT and significantly decreased PTEN staining in the whole ovary at all selected doses of DEHP when compared to the vehicle control group (Fig. 3.6A and 3.6B, n=3/group, p ≤ 0.05). To confirm that these effects were evident in the follicles of interest, we measured protein staining within the primordial and primary follicles themselves. DEHP exposure significantly increased pAKT staining in primordial follicles at all selected doses of DEHP and in primary follicles at the 20 and 200 µg/kg/day doses, and the 20 mg/kg/day dose when compared to the vehicle control group (Fig. 3.6C and 3.6D, n=3/group, p ≤ 0.05).
3.5 Discussion

We have shown that 10 and 30 day oral exposure to DEHP disrupts normal reproductive and ovarian function. Specifically, we provide evidence that relatively low levels of DEHP, including a dose within the estimated range of daily human exposure, alter estrous cyclicity and accelerate primordial follicle recruitment. Further, we are the first, to our knowledge, to provide mechanistic evidence by which adult exposure to DEHP accelerates early folliculogenesis. Our data show that DEHP exposure may interfere with normal PI3K signaling by altering the expression of key genes and/or the levels of key proteins.

An important note is that the selected doses of DEHP had no effect on mortality and very minor effects on organ weights. Thus, the adverse reproductive outcomes in response to DEHP exposure are most likely not due to overt toxicity. Additionally, the doses used in these studies are more environmentally relevant than doses used in several previous studies found within the literature [15, 17, 34]. Therefore, these findings provide some insight into the effects of DEHP on ovarian function at human exposure levels, though we acknowledge that investigating the effects at even lower levels would be beneficial.

Our data provide evidence that relatively low levels of DEHP disrupt estrous cyclicity, which may cause complications in reproductive function. DEHP exposure for 10 days increased the percentage of days that the mice were in estrus at the 20 µg/kg/day, and the 20, 200, and 750 mg/kg/day doses when compared to the vehicle control group. Likewise, but to a lesser degree, DEHP exposure for 30 days increased the percentage of days the mice were estrus at the 200 mg/kg/day dose when compared to the vehicle control group. These results correlate to previous reports suggesting that higher doses of DEHP (2 g/kg/day) disrupt estrous cyclicity, but also expand upon those findings, in that the lower levels of DEHP used in this study also exhibit effects on estrous cyclicity [15]. Interestingly, the effects of DEHP on estrous cyclicity exhibit a non-monotonic dose response and appear to be greater following 10 days of exposure than what is observed following 30 days of exposure. A non-monotonic dose response is typical among common endocrine disrupting chemicals, which suggests that different effects and mechanisms may exist at different doses. Further, we hypothesize that the ovary and/or the hypothalamus-pituitary-ovarian axis is able to compensate for the toxicity of DEHP between days 10 and 30. Additionally, it is possible that the biotransformation of DEHP is altered following a longer
period of dosing so that altered metabolism of the chemical helps protect the ovary from toxicity, but further work investigating this matter would need to be conducted to verify this statement.

Although DEHP has been shown to disrupt reproductive function in males and, to lesser extent, in females, the effect of DEHP on ovarian folliculogenesis, especially in the adult, is relatively unknown. For normal fertility and the appropriate timing of reproductive senescence, primordial follicles must undergo a strict and coordinated process known as primordial follicle recruitment. Under normal conditions, the primordial follicle is essentially destined for three fates: to survive in dormancy for varying lengths of time throughout reproductive life, to be recruited into the growing population of follicles, or to undergo death in the dormant state [50-52]. Our data show that 10 and 30 day exposure to DEHP accelerates this essential stage of early folliculogenesis. Specifically, 10 day exposure to DEHP decreased the percentage of primordial follicles counted at the 20 and 200 mg/kg/day doses and increased the percentage of primary follicles counted at all selected doses (20 µg/kg/day-750 mg/kg/day) when compared to the vehicle control group. A similar effect is observed following a longer period of dosing where 30 day exposure to DEHP increased the percentage of primary follicles counted at the 200 µg/kg/day and 20 mg/kg/day doses when compared to the vehicle control group. Thus, DEHP exposure at these two timepoints accelerates primordial follicle recruitment to the primary stage of development in the adult murine model. These results are consistent with those from other studies using different methods. Specifically, DEHP in vitro and in vivo alters early folliculogenesis and primordial follicle assembly in mice; however the exposure periods were limited to the neonate [53, 54]. Additionally, in utero exposure to high levels of MEHP, the metabolite of DEHP, accelerates early folliculogenesis in the F1 generation of mice [55]. Our data add to these existing studies by showing that adult exposure to relatively low levels of DEHP accelerates early folliculogenesis in the mouse.

Interestingly, in our study, the effects on early folliculogenesis appear to be more profound following 10 days of exposure than what is observed following 30 days of exposure. We attribute this observation to the ovary being able to compensate for the toxicity of DEHP between days 10 and 30 by potentially altering the biotransformation of DEHP. Although the defects in follicle numbers appear to be undergoing compensation, it is possible that the oocytes in follicles that undergo accelerated folliculogenesis may not be of fertilizable quality. In fact, previous work has determined that accelerated early folliculogenesis results in poor quality
oocytes and compromised fertility [56]. Further, it is possible that our follicle count data represent a single “snap-shot” following 10 and 30 days of DEHP exposure and that changes in follicle numbers may be observed at later time points. Additionally, it is possible that the excess primary follicles may undergo atresia which may be why we do not see changes in the later stages of folliculogenesis. In accordance with the estrous cyclicity results, we also observe a non-monotonic dose response for the effects of DEHP on early folliculogenesis. Future studies should examine the mechanisms underlying compensation and the non-monotonic dose response. Further, these studies should determine if accelerated primordial follicle recruitment has long term implications for fertility.

Primordial follicle recruitment is a gonadotropin-independent process that relies on paracrine and autocrine regulation by intrinsic ovarian growth factors [26, 27]. These factors either suppress activation and thus, maintain primordial follicle dormancy, or they activate primordial follicles and thus, promote recruitment. A balance exists between these inhibitory and stimulatory factors to maintain primordial follicle survival so that downregulation of inhibitory factors and/or overactivation of stimulatory factors favor an environment conducive for primordial follicle recruitment [43]. Although the exact mechanism by which follicles are recruited to the growing pool is unknown, recent work has established the existence of several key intrinsic ovarian factors that belong to multiple signaling pathways [41, 42, 50, 57]. Interestingly, several of these factors are involved in the PI3K signaling pathway, which indicates this pathway as a critical regulator of primordial follicle survival, quiescence, and recruitment to the primary stage of development [28-33].

The PI3K signaling pathway is involved in cell proliferation, survival, migration, metabolism, and most recently in early ovarian folliculogenesis [43, 45, 46]. Components of this signaling pathway that are also associated with primordial follicle recruitment and have been tested in these studies include PDPK1, mTORC1, KIT, pAKT, and rpS6, which are all factors that drive PI3K signaling. Additional components of this pathway that are associated with primordial follicle recruitment and have been tested in these studies include FOXO3A, PTEN, and TSC1, which negatively regulate PI3K signaling. Conditional deletion of Foxo3a, Pten, and Tsc1 from mouse oocytes results in global activation of all primordial follicles, leading to premature ovarian failure [31, 44, 58-61]. Thus, these inhibitors of PI3K signaling maintain primordial follicle dormancy. Acceleration of primordial follicle recruitment occurs because of
over-activation of the PI3K signaling pathway brought about by the unrestricted regulation of stimulatory PI3K factors, such as pAKT, mTORC1, and PDK1 [44, 59, 62, 63]. Therefore, proper regulation of PI3K signaling is needed for appropriate primordial follicle survival, quiescence, and recruitment; and dysregulation of this pathway may lead to the acceleration of primordial follicle recruitment and ultimately premature ovarian failure and infertility.

Our data provide evidence that 10 and 30 day DEHP exposure dysregulates the ovarian mRNA and protein levels of key PI3K signaling factors that are associated with primordial follicle recruitment. These findings are critical because PI3K signaling is an integral regulator of primordial follicle recruitment and because they provide mechanistic evidence by which DEHP disrupts folliculogenesis in an adult model. Specifically, in the 10 day dosing study, DEHP selectively increased the mRNA levels of the stimulatory PI3K signaling factors Mtorc1 and Pdk1 at doses that correspond to the acceleration of early folliculogenesis when compared to the vehicle control group. Additionally, DEHP exposure for 10 days selectively decreased the mRNA levels of the inhibitory PI3K signaling factor Pten at doses that correspond to the acceleration of early folliculogenesis when compared to the vehicle control group. Importantly, DEHP exposure for 30 days also decreased the mRNA levels of Pten at a dose that corresponds to the acceleration of early folliculogenesis when compared to the vehicle control group. Interestingly, DEHP exposure for 30 days also selectively altered the mRNA levels of stimulatory, Kit and Rps6, and inhibitory, Tsc1, PI3K signaling factors, but DEHP did so at doses in which early folliculogenesis was unaffected. Further, DEHP exposure altered the mRNA levels of factors outside the PI3K signaling pathway that also are involved in primordial follicle recruitment. In the 30 day dosing study, DEHP exposure decreased the mRNA levels of Foxl2 when compared to the vehicle control group. Thus, although we provide evidence that DEHP disrupts folliculogenesis by dysregulating the PI3K signaling pathway, the additive effects of other factors/signaling pathways in contribution to PI3K signaling cannot be discounted and should be examined in future studies.

Based on the selective ability of DEHP to aberrantly alter the mRNA levels of PI3K signaling factors that are associated with early folliculogenesis and that several PI3K signaling factors are translationally and post-translationally regulated, we decided to expand upon the gene expression findings by measuring protein levels of pAKT and PTEN. These proteins were chosen because they serve as integral regulators of PI3K signaling and are associated with
primordial follicle recruitment. Specifically, the phosphatase PTEN negatively regulates the PI3K-induced activation of the secondary messenger AKT; thus, PTEN lies upstream of AKT [45, 46]. Further, the loss of Pten in oocytes leads to global acceleration of all primordial follicles in the ovary via increased AKT-regulated PI3K signaling [44]. In the 10 day dosing study, DEHP exposure increased the staining of pAKT and decreased the staining of PTEN in the whole ovary at all selected doses of DEHP when compared to the vehicle control group. In confirmation that these effects were evident in the follicles of interest, DEHP exposure increased pAKT staining in primordial follicles at all selected doses of DEHP and in primary follicles at the 20 and 200 µg/kg/day doses, and the 20 mg/kg/day dose when compared to the vehicle control group. Thus, the mRNA and protein data provide evidence that DEHP may first downregulate PTEN leading to the overactivation of the PI3K/pAKT signaling cascade. Similar effects of DEHP-induced dysregulation of PI3K signaling have been noted in other tissues [64-66]. Our data expand upon these previous findings by showing DEHP-induced PI3K dysregulation in the ovary. Additionally, our data are consistent with our follicle count data in that increased pAKT and decreased PTEN would favor an environment conducive for primordial follicle recruitment. Furthermore, these data are fairly consistent with the mRNA data; however, not all of the gene expression changes correlate with the phenotypic changes in folliculogenesis. Additionally, the effects of DEHP exposure on gene expression exhibit a non-monotonic dose response. Thus, the protein data are more definitive than the mRNA data in that all doses that cause the acceleration of primordial follicle recruitment contain significant protein data.

Collectively, these data provide evidence that relatively low levels of DEHP alter estrous cyclicity and accelerate primordial follicle recruitment, which potentially can interfere with normal reproductive function and appropriate timing of reproductive senescence. Further, our data show that the DEHP-induced defects in early folliculogenesis may be mediated by dysregulation of the PI3K signaling pathway. This study is novel because we provide mechanistic evidence by which a ubiquitous endocrine disrupting chemical disrupts folliculogenesis, an essential ovarian process necessary for normal reproductive and non-reproductive health. However, further work is needed to completely elucidate the mechanism of DEHP-induced acceleration of primordial follicle recruitment. Additionally, although early folliculogenesis is aberrantly regulated in our dosing windows, it is of great interest to investigate if the primordial follicle pool is depleted at an accelerated rate in response to DEHP
exposure over an extended period of time or if DEHP exposure compromises oocyte quality in accelerated follicles, thus leading to the potential of premature ovarian failure and infertility.
### 3.6 Tables, Figures, and Legends

**Table 3.1 Sequences of primer sets used for gene expression analysis**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>5’-Forward-3’</th>
<th>5’-Reverse-3’</th>
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<tr>
<td>NM_011062</td>
<td>3-phosphoinositide dependent protein kinase-1</td>
<td>Pdpk1</td>
<td>AAAAGC AAGC CG9 TGGAAC</td>
<td>C TGG TGG GCAATTC CG CTTG</td>
</tr>
<tr>
<td>NM_007393</td>
<td>Beta-actin</td>
<td>Actb</td>
<td>GGCG ACAG TG TG GTG ACG</td>
<td>CTGG CACC ACAC TCC TAC</td>
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<tr>
<td>NM_012020</td>
<td>Forkhead box protein L2</td>
<td>Foxl2</td>
<td>GAAGG CCAGCTAC CCC GAGC</td>
<td>CGCC GGCTGG TGG TCC G</td>
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<tr>
<td>NM_920009</td>
<td>Mammalian target of rapamycin complex 1</td>
<td>Mtorl</td>
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<td>TCTTC AGTC CAC TG GCG AAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AACA ACA AAA AGA GC AAT CAG G CCC</td>
<td>TGA GACC ATC ACA AA TGAT C CC A T</td>
</tr>
<tr>
<td>NM_001122733</td>
<td>Fatty acid synthesis receptor</td>
<td>Fasn</td>
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<td></td>
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<tr>
<td>NM_008860</td>
<td>Phosphatase and tensin homolog</td>
<td>Pten</td>
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<td>AGGG GGC AAG GTAG GTAC G CAT</td>
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<tr>
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<td>TCC CCA ATATTC TAG CAG TCT</td>
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<tr>
<td>NM 001289575</td>
<td>Tubulin alpha 1</td>
<td>Tub1</td>
<td>ATCG TG CCT CG TGGG GGG TGA</td>
<td>AGGG TGAG AAC CATA ACC GCC A</td>
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</tbody>
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Specific primer sequences used for qPCR analysis of gene expression.
Table 3.2 Effect of DEHP exposure on body and organ weights

<table>
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<tr>
<th>Exposure Window</th>
<th>Treatment</th>
<th>Percent Body Weight Change (%)</th>
<th>Liver (g)</th>
<th>Relative Liver (%)</th>
<th>Uterus (g)</th>
<th>Relative Uterus (%)</th>
<th>Spleen (g)</th>
<th>Relative Spleen (%)</th>
<th>Adrenal Glands (g)</th>
<th>Relative Adrenal Glands (%)</th>
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<td><strong>10 days</strong></td>
<td>Vehicle</td>
<td>102.9</td>
<td>1.261</td>
<td>5.163</td>
<td>0.096</td>
<td>0.393</td>
<td>0.076</td>
<td>0.313</td>
<td>0.012</td>
<td>0.047</td>
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<td></td>
<td>20 µg/kg/day</td>
<td>106.0</td>
<td>1.244</td>
<td>5.111</td>
<td>0.120</td>
<td>0.495</td>
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<td>0.051</td>
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<tr>
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<td>200 µg/kg/day</td>
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<td>5.357</td>
<td>0.131</td>
<td>0.508</td>
<td><strong>0.100</strong></td>
<td>0.390</td>
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<tr>
<td></td>
<td>20 mg/kg/day</td>
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<td>1.293</td>
<td>5.347</td>
<td>0.119</td>
<td>0.485</td>
<td>0.095</td>
<td>0.393</td>
<td>0.009</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg/day</td>
<td>104.5</td>
<td>1.294</td>
<td>5.369</td>
<td>0.115</td>
<td>0.474</td>
<td>0.090</td>
<td>0.374</td>
<td>0.010</td>
<td>0.041</td>
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<tr>
<td></td>
<td>750 mg/kg/day</td>
<td>102.2</td>
<td><strong>1.461</strong></td>
<td><strong>5.972</strong></td>
<td><strong>0.094</strong></td>
<td>0.177</td>
<td>0.094</td>
<td>0.381</td>
<td>0.012</td>
<td>0.048</td>
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<tr>
<td><strong>30 days</strong></td>
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<td>1.561</td>
<td>5.269</td>
<td>0.175</td>
<td>0.601</td>
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<tr>
<td></td>
<td>20 µg/kg/day</td>
<td>123.2</td>
<td>1.470</td>
<td>5.123</td>
<td><strong>0.350</strong></td>
<td>1.235</td>
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</tr>
<tr>
<td></td>
<td>200 µg/kg/day</td>
<td>118.3</td>
<td>1.460</td>
<td>5.104</td>
<td>0.181</td>
<td>0.640</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>20 mg/kg/day</td>
<td>121.6</td>
<td>1.563</td>
<td>5.346</td>
<td>0.169</td>
<td>0.579</td>
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<tr>
<td></td>
<td>200 mg/kg/day</td>
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<td>1.604</td>
<td>5.178</td>
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</tbody>
</table>

Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day-750 mg/kg/day) daily for 10 and 30 days. Following dosing, percent body weight change, whole organ weights, and relative organ weights were calculated and compared in each treatment group. (n=8 mice/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 3.1 Effect of DEHP exposure on estrous cyclicity

Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day-750 mg/kg/day) daily for 10 (panel A) and 30 (panel B) days. Vaginal swabs were taken daily using phosphate buffered saline administered from a pipet tip. Stage of estrus was recorded based on previously defined criteria using a light microscope. Percentage of days in estrus and metestrus/diestrus were calculated and compared in each treatment group. Graph represents means ± SEM (n=8 mice/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 3.2 Effect of DEHP exposure on folliculogenesis

Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 μg/kg/day-750 mg/kg/day) daily for 10 (panel A) and 30 (panel B) days. Following dosing, ovaries were processed for histological evaluation of follicle counts. Percentages of each follicle type were calculated and compared in each treatment group. Graph represents means ± SEM (n=3-8 ovaries/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day-750 mg/kg/day) daily for 10 days. Following dosing, whole ovaries were subjected to qPCR for *Mtorc1* (panel A), *Pdk1* (panel B), and *Pten* (panel C). All values were normalized to *Actb*. Graph represents means ± SEM (n=4-5 ovaries/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 3.4 Effect of DEHP exposure for 30 days on the mRNA expression of key factors in the PI3K signaling pathway

Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day-200 mg/kg/day) daily for 30 days. Following dosing, whole ovaries were subjected to qPCR for *Kit* (panel A), *Pten* (panel B), *Rps6* (panel C), and *Tsc1* (panel D). All values were normalized to *Actb*. Graph represents means ± SEM (n=3-4 ovaries/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Panels A-H exhibit pAKT staining in the whole ovary (panels A-D) and in selected primordial and primary follicles (panels E-H). Panels I-L exhibit PTEN staining in the whole ovary. Panels A, B, E, F, I, and J were from vehicle control treated ovaries and follicles. Panels C, D, G, H, K, and L were from 20 µg/kg/day DEHP treated ovaries and follicles. Black arrow heads represent positive staining in the immunohistochemistry images, which translate to the yellow/orange pseudocolored pixels represented by red arrow heads. Scale bars are set as 500 µm for whole ovary images (panels A-D and I-L) and 100 µm for follicle images (panels E-H) and the same scale bars should be used across each row.
Figure 3.6 Effect of DEHP exposure on protein levels of PI3K signaling factors associated with early folliculogenesis

Adult CD-1 mice were orally dosed with vehicle (tocopherol-stripped corn oil) or DEHP (20 µg/kg/day-750 mg/kg/day) daily for 10 days. Following dosing, ovaries were subjected to immunohistochemistry for quantification of pAKT (panels A, C, and D) and PTEN (panel B) staining. Digital images were converted to 8-bit grayscale images and then converted to pseudocolored images. Colors were based on relative stain intensity. Percentage of positively stained pixels in the whole ovary (panels A and B) and in follicles (panels C and D) were calculated and compared in each treatment group. Graph represents means ± SEM (n=3/group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
3.7 References


25. Kruger T, Long M, Bonefeld-Jorgensen EC. Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology 2008; 246:112-123.


CHAPTER IV

Di(2-ethylhexyl) Phthalate Inhibits Antral Follicle Growth, Induces Atresia, and Inhibits Steroid Hormone Production in Cultured Mouse Antral Follicles

4.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental toxicant found in consumer products that causes ovarian toxicity. Antral follicles are the functional ovarian units and must undergo growth, survival from atresia, and proper regulation of steroidogenesis to ovulate and produce hormones. Previous studies have determined that DEHP inhibits antral follicle growth and decreases estradiol levels in vitro; however, the mechanism by which DEHP elicits these effects is unknown. The present study tested the hypothesis that DEHP directly alters regulators of the cell cycle, apoptosis, and steroidogenesis to inhibit antral follicle functionality. Antral follicles from adult CD-1 mice were cultured with vehicle control or DEHP (1-100μg/ml) for 24-96 hr to establish the temporal effects of DEHP on the follicle. Following 24-96 hr of culture, antral follicles were subjected to gene expression analysis, and media were subjected to measurements of hormone levels. DEHP increased the mRNA levels of cyclin D2, cyclin dependent kinase 4, cyclin E1, cyclin A2, and cyclin B1 and decreased the levels of cyclin-dependent kinase inhibitor 1A prior to growth inhibition. Additionally, DEHP increased the mRNA levels of BCL2-associated agonist of cell death, BCL2-associated X protein, BCL2-related ovarian killer protein, B-cell leukemia/lymphoma 2, and Bcl2-like 10, leading to an increase in atresia. Further, DEHP decreased the levels of progesterone, androstenedione, and testosterone prior to the decrease in estradiol levels, with decreased mRNA levels of side-chain cleavage, 17α-hydroxylase-17,20-desmolase, 17β-hydroxysteroid dehydrogenase, and aromatase. Collectively, DEHP directly alters antral follicle functionality by inhibiting growth, inducing atresia, and inhibiting steroidogenesis.

4.2 Introduction

Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used phthalate ester, and it is predominantly used in the manufacturing of a wide range of polyvinyl chloride consumer, medical, and building products to impart flexibility [1, 2]. Because of its incorporation in numerous commonly used consumer products, DEHP is produced in vast quantities. Domestic production of dioctyl phthalates, a subgroup of phthalate esters in which DEHP is classified, exceeds 300 million pounds annually [2]. Humans are exposed to DEHP on a daily basis via oral ingestion, inhalation, and dermal contact [1]. This is because DEHP is non-covalently bound to the plastic, allowing the chemical to frequently leach out into the environment and in the products that humans consume on a daily basis [1]. In fact, it is estimated that the range of daily human exposure to DEHP is between 3-30 μg/kg/day [3, 4]. Continuous, daily exposure to DEHP is a major concern because DEHP and its metabolite mono(2-ethylhexyl) phthalate (MEHP) have been identified in human blood samples [5, 6], urine samples [1, 5-9], amniotic fluid samples [10-12], cord blood samples from newborns [13, 14], breast milk samples [6], and in ovarian follicular fluid samples tested [15], indicating the ability of these chemicals to reach the ovary.

Important for public health, DEHP is a known endocrine disrupting chemical and reproductive toxicant [1, 2]. In women, chronic occupational exposure to phthalates is associated with an increased risk of miscarriage and a decreased rate of pregnancy [1]. In laboratory animals, DEHP causes pregnancy complications, including reduced implantations, increased resorptions, and decreased fetal weights of offspring [16, 17].

The mechanisms by which DEHP disrupts these endocrine and reproductive events remain unknown, but interestingly, antral follicles from the ovary are critical regulators of these processes. Follicles are the functional units of the ovary, and the antral follicle is the most mature follicle type. Antral follicles are the major sources of sex steroid hormone production in the female and are the only follicle type capable of ovulation [18]. Normal antral follicle function requires follicle growth, survival from atresia, and appropriate regulation of steroidogenesis [18].

Antral follicle growth is predominantly regulated by proliferation of granulosa cells and theca cells, which are somatic cells located in the follicle [18]. Proliferation of these cells, like
most mammalian cells, is regulated by cyclins, cyclin dependent kinases, and cyclin dependent kinase inhibitors [19-21]. Although some antral follicles are rescued from atresia and ovulate in a female’s reproductive lifespan, the vast majority of follicles (99%) are lost via atretic demise [18, 22]. The regulation of follicular atresia involves a balance of pro- and anti-apoptotic factors that signal through the B cell leukemia/lymphoma 2 (BCL2) signaling pathway promoting caspase-induced apoptosis [23-28]. Antral follicle production of sex steroid hormones, a process termed ovarian steroidogenesis, is also essential for reproductive and non-reproductive health [29-46]. Steroidogenesis is the enzymatic conversion of cholesterol to 17β-estradiol and other necessary sex steroid hormones.

Interestingly, recent work also suggests that DEHP targets the ovary and adversely affects antral follicle functionality. Specifically, in vivo studies using oral exposure to DEHP have shown that DEHP decreases serum estradiol levels, decreases aromatase levels, decreases antral follicle size, causes anovulation, and disrupts estrous cyclicity [47-51]. Further, studies using ovarian granulosa cell cultures and minced ovary cultures show that DEHP also decreases estradiol and aromatase levels [52, 53]. Our group has also reported that DEHP inhibits antral follicle growth and decreases estradiol and aromatase levels in a whole antral follicle culture system following 96 hrs of culture [54]. However, few studies have investigated the effects of DEHP on the precursor hormones and enzymes upstream of estradiol and aromatase. Further, the direct effect of DEHP on antral follicle atresia is unclear. Additionally, the mechanisms by which DEHP disrupts antral follicle growth, health, and steroidogenesis remain unknown.

The present study was designed to conduct a time-course study using an antral follicle culture system to investigate the initial and time specific DEHP-induced defects in antral follicle growth, atresia, and steroidogenesis upstream of the previously reported effects on estradiol. Specifically, we tested the hypothesis that DEHP directly alters regulators of the cell cycle, apoptosis, and steroidogenic pathway to inhibit antral follicle growth, induce atresia, and inhibit steroid production. To test this hypothesis, antral follicles were cultured with vehicle or DEHP for 24, 48, 72, and 96 hrs. Following culture, antral follicles were collected for histological evaluation of atresia and the measurements of mRNA levels of regulators of the cell cycle (Ccnb1, Ccna2, Ccne1, Ccnd2, Cdkn1a, and Cdk4), apoptosis (Bax, Bcl2, Bcl2l10, Casp3, and Casp8), and the enzymes responsible for generating estradiol (Star, Cyp11a1,
Hsd3b1, Cyp17a1, Hsd17b1, and Cyp19a1). Further, media were collected for the measurements of antral follicle produced progesterone, DHEA, androstenedione, testosterone, estrone, and estradiol.
4.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP were prepared using dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) as the vehicle in various concentrations (1.33, 13.3, and 133 mg/ml). This allowed for an equal volume of each stock to be added to the culture wells to control for vehicle concentration. Final concentrations of DEHP in culture were 1, 10, and 100 μg/ml, which is approximately equivalent to 2.77, 27.7, and 277 μM respectively.

The concentrations of DEHP were chosen based their ability to cause inhibition of antral follicle growth, decreased estradiol production from antral follicles, and decreased antral follicle Cyp19a1 expression [54, 55]. These concentrations of DEHP also have been determined to be clinically relevant in reproductive and non-reproductive cell and tissue cultures [56-58]. Additionally, the selected concentrations of DEHP are environmentally relevant. Plasma concentrations of DEHP in healthy women have been reported to be 0.18 μg/ml, and peritoneal fluid concentrations of DEHP in these women were reported to be 0.46 μg/ml, which are close to the lowest concentration in this study [59]. The no-observed-adverse-effect level (NOAEL) for DEHP is 5.8 mg/kg/day which equates to 14.3 μM [2]. In addition, patients undergoing consistent medical care have markedly higher levels of DEHP than healthy people due to the extensive use of DEHP in medical care products [60, 61]. In intensive neonatal care units, patients receiving blood transfusions have plasma levels of DEHP at 11.1 μg/ml [62]. Further, the lowest-observed-adverse-effect level (LOAEL) of DEHP is 140 mg/kg/day, which equates to 344.6 μM [2]. Each of the selected concentrations of DEHP falls below the LOAEL concentration.

Animals

Cycling, adult CD-1 female mice (34-37 days of age) were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in groups of 4 in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana-Champaign and were allowed to acclimate to the facility prior to experimentation. The mice were housed in a controlled animal room environment (temperature at 22 ± 1 °C and 12-hour light–dark cycles)
and were provided food and water *ad libitum*. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana-Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

**In vitro antral follicle culture**

Unprimed, female CD-1 mice were euthanized and their ovaries were aseptically removed for antral follicle isolation. Antral follicles were isolated from the ovary based on relative size (250-400 μm) and were cleaned of interstitial tissue using watchmaker’s forceps [63, 64]. At least 2-3 mice were used in each experiment, in which we could obtain approximately 20-30 antral follicles per mouse. Each treatment group contained 10-16 follicles. Isolated antral follicles were randomly and individually plated in wells of a 96-well culture plate containing unsupplemented α-minimal essential medium (α-MEM, Life Technologies, Grand Island, NY) prior to treatment.

Treatment groups included DMSO (vehicle control) and DEHP (1, 10, and 100 μg/ml) and were prepared in supplemented α-MEM. Supplemented α-MEM contained 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), 5 IU/ml human recombinant follicle-stimulating hormone (FSH; Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), and 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) as described previously [63, 64]. An equal volume of chemical (0.75 μl/ml of media) was added for each dose to control for the amount of vehicle in each preparation (final concentration of DMSO=0.075%). Each follicle was cultured in 150 μl of medium for 24–96 hr in an incubator at 37°C supplying 5% CO₂. Following each 24 hr culture window, media were collected for measurements of the levels of sex steroid hormones and follicles were collected, snap-frozen, and stored at −80°C for gene expression analysis. Further, follicles were collected following 96 hr of culture and processed for histological analysis of atresia. A time-course was conducted to observe when the effects of DEHP on antral follicle growth, atresia, and steroidogenesis begin and to determine which enzymes and/or hormones cause the inhibition of growth and reduction of estradiol production from antral follicles following 96 hr as reported previously [54].
Histological analysis of antral follicle atresia

Following 96 hr of culture, antral follicles were collected and processed for histological evaluation of atresia as described previously (n=10-16 follicles/3 separate experiments) [65, 66]. Briefly, each section of the follicle was observed for the presence of apoptotic bodies, which is a morphological sign of apoptosis. Based on the abundance of apoptotic bodies, the follicles were scored on a 1-4 scale. A rating of 1 indicated a healthy follicle without apoptotic bodies, a rating of 2 indicated the presence of apoptotic bodies encompassing 1-10% of the follicle section, a rating of 3 indicated the presence of apoptotic bodies encompassing 11-30% of the follicle section, and a rating of 4 indicated the presence of apoptotic bodies encompassing >30% of the follicle section. Atresia ratings were reported based on the average of all ratings throughout the follicle sections.

Analysis of gene expression

After 24, 48, 72, and 96 hr of culture, antral follicles were snap frozen in liquid nitrogen, and stored at -80°C for quantitative real-time polymerase chain reaction (qPCR) analysis (n=10-16 follicles/3-9 separate experiments). We elected to focus on mRNA gene expression analysis based on the limited amount of sample that can be retrieved from antral follicles. Total RNA (200 ng) was extracted from the follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol, and was then reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer’s protocol. Each cDNA sample was diluted 1:4 using nuclease-free water prior to qPCR analysis. Analysis of qPCR was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and accompanying CFX Manager Software according to the manufacturer’s protocol. All qPCR reactions were done in triplicate using 1 μl cDNA, forward and reverse primers (5 pmol) for Ccnd2, Cdk4, Ccne1, Ccna2, Ccnb1, Cdkn1a, Bad, Bax, Bak, Bcl2, Bcl2l10, Casp8, Casp3, Star, Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b1, Cyp19a1, Cyp1b1, or Actb, in addition to a SsoFastEvaGreen Supermix for a final reaction volume of 10 μl. Specific qPCR primers (Integrated DNA Technologies, Inc., Coralville, IA) for the genes of interest as well as the reference gene, beta-actin (Actb), which did not differ across treatment groups, can be found in Table 4.1. The genes tested were chosen because they are regulators of cell cycle (Ccnd2, Cdk4,
Ccne1, Ccna2, Ccnb1, and Cdkn1a) [19, 20, 67, 68], apoptosis (Bad, Bax, Bok, Bcl2, Bcl2l10, Casp8, and Casp3) [23-26, 69], and ovarian steroidogenesis (Star, Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b1, and Cyp19a1) [70-73].

The CFX96 machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. The qPCR program consisted of an enzyme activation step (95°C for 1 min), an amplification and quantification program (40 cycles of 95°C for 10 sec, 60°C for 10 sec, single fluorescence reading), a step of 72°C for 5 min, a melt curve (65°C−95°C heating 0.5°C per sec with continuous fluorescence readings), and a final step at 72°C for 5 min as per the manufacturer’s protocol. Expression data were generated using the mathematical standard comparative (ΔΔCt) method. The ΔCt was calculated by subtracting the Actb Ct value from the gene of interest Ct value. The ΔΔCt was calculated as the difference between the ΔCt between the treatment groups and the DMSO groups. The relative fold-change of expression was then equaled to $2^{(-ΔΔCt)}$ for each sample.

**Analysis of sex steroid hormone levels**

After 24, 48, 72, and 96 hr of culture, media were subjected to enzyme-linked immunosorbent assays (ELISAs) for the measurements of the levels of dehydroepiandrosterone (DHEA), progesterone, androstenedione, testosterone, estrone, and 17β-estradiol. ELISA kits were purchased from Diagnostics Research Group (DRG, Mountainside, NJ) (n=10-16 wells of pooled media containing follicles/3-9 separate experiments). The analytical sensitivity of each kit was 0.044 μg/ml for DHEA, 0.1 ng/mL for progesterone, 0.019 ng/mL for androstenedione, 0.083 ng/mL for testosterone, 6.3 pg/mL for estrone, and 9.71 pg/mL for estradiol. The assays were performed following the manufacturer’s protocol. All samples were run in duplicate and all intra- and inter-assay coefficients of variability were less than 10%. Some samples were diluted to match the dynamic range of each ELISA kit. Mean values for each sample were used in this analysis. These steroid hormones were chosen because of their necessity for normal female reproductive function and endocrinology. Further, these hormones serve as precursors for estradiol production, and estradiol production has been shown to be reduced in response to DEHP treatment [54].
Statistical analysis

All data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means ± standard error of the means (SEM), and at least three separate experiments were conducted for each treatment group prior to data analysis. Multiple comparisons between normally distributed experimental groups were made using one-way analysis of variance (ANOVA) followed by Tukey post-hoc comparison. Multiple comparisons between non-normally distributed experimental groups were made using the Kruskal Wallis test when appropriate. Statistical significance was assigned at p ≤ 0.05.
4.4 Results

Effect of DEHP on antral follicle growth and the mRNA levels of key regulators of the cell cycle

As previously reported, DEHP exposure for 72 and 96 hr inhibits antral follicle growth in vitro \([54, 55]\). To confirm this, antral follicles were cultured with vehicle control (DMSO) or DEHP (1-100µg/ml) for 96 hr, and follicle diameters were measured on a perpendicular axis every 24 hr. Similar to the previous reports, DEHP exposure for 72 hr inhibited antral follicle growth by roughly 10% at the 10 and 100µg/ml doses when compared to the vehicle control group (data not shown). Also comparable to the previous studies, DEHP exposure for 96 hr inhibited antral follicle growth by roughly 15% at all selected doses of DEHP when compared to the vehicle control group (data not shown).

Because DEHP has been shown to inhibit antral follicle growth in vitro beginning at 72 hr, we investigated the initial effects and potential mechanisms by which DEHP inhibits antral follicle growth. Specifically, we subjected antral follicles to qPCR following 24, 48, and 72 hr of culture to investigate the direct effects of DEHP on the mRNA levels of key cyclins, cyclin dependent kinases, and cyclin dependent kinase inhibitors, which are heavily involved in follicular cell proliferation and ultimately antral follicle growth \([18-20]\). Cyclins \(Ccnd2, Ccne1, Ccna2,\) and \(Ccnb1\) and cyclin dependent kinases \(Cdk4\) promote progression through the cell cycle, whereas cyclin dependent kinase inhibitors \(Cdkn1a\), also known as \(p21\) promote cell cycle arrest.

DEHP exposure for 24 hr significantly increased the mRNA levels of \(Ccnd2\) at the 1µg/ml dose when compared to the vehicle control group (Fig. 4.1A, \(n=3, p \leq 0.05\)). Similarly, DEHP exposure for 72 hr significantly increased the mRNA levels of \(Ccnd2\) at the 10µg/ml dose when compared to the vehicle control group (Fig. 4.1A, \(n=5-9, p \leq 0.05\)). DEHP exposure for 72 hr also increased the mRNA levels of \(Cdk4\) at the 10µg/ml dose when compared to the vehicle control group (Fig. 4.1B, \(n=5-9, p \leq 0.05\)). Further, DEHP exposure for 48 and 72 hr increased the mRNA levels of \(Ccne1\) at the 100µg/ml dose when compared to the vehicle control group (Fig. 4.1C, \(n=5-9, p \leq 0.05\)). The mRNA levels of \(Ccna2\) were significantly increased following 24 hr at the 1µg/ml dose and following 72 hr at the 10µg/ml dose when compared to the vehicle
control group (Fig. 4.1D, n=3-9, p≤0.05). Strikingly, DEHP exposure for 48 and 72 hr significantly increased the mRNA levels of Ccnb1 at all selected doses when compared to the vehicle control group (Fig. 4.1E, n=5-9, p≤0.05). The mRNA levels of the inhibitor Cdkn1a were significantly decreased by the 1 and 100µg/ml doses following 48 hr when compared to the vehicle control group (Fig. 4.1F, n=5-8, p≤0.05).

**Effect of DEHP on antral follicle atresia**

An important aspect of antral follicle health and functionality is survival from atresia, which is an apoptotic process. Although we have previously determined that DEHP exposure inhibits antral follicle growth and decreases antral follicle-produced estradiol levels [54, 55], the direct effects of DEHP on antral follicle atresia were unknown. Thus, we cultured antral follicles with vehicle control (DMSO) or DEHP (1-100µg/ml) for 96 hr and processed the follicles for histological evaluation of atresia. Atresia was rated by the presence of apoptotic bodies. A representative image of a DMSO treated follicle is displayed in Fig. 4.2A, whereas a representative image of a DEHP 100µg/ml treated follicle is exhibited in Fig. 4.2B. Qualitatively, it appears that DEHP treatment increased the presence and abundance of apoptotic bodies within the follicular unit when compared to the vehicle control treated follicles. When quantified, DEHP exposure for 96 hr significantly increased the number of apoptotic bodies, represented by a higher atresia rating, at the 10 and 100µg/ml doses when compared to the vehicle control group (Fig. 4.2C, n=3, p≤0.05).

**Effect of DEHP on the antral follicle mRNA levels of key regulators of apoptosis**

Although atresia is a natural occurrence in antral follicles, an increase in atresia can have negative impacts on ovarian and reproductive health [18, 22]. Because we observed an increase in antral follicle atresia following 96 hr of DEHP exposure, we investigated the initial effects and potential mechanisms by which DEHP induces atresia, or apoptosis, in the antral follicle. Specifically, antral follicles were cultured with vehicle control (DMSO) or DEHP (1-100µg/ml) for 24-72 hr. Following 24, 28, and 72 hr of culture, we subjected antral follicles to qPCR to investigate the direct effects of DEHP on the mRNA levels of factors that drive apoptosis to promote atresia (Bad, Bax, and Bok) and factors that inhibit apoptosis to promote follicle survival (Bcl2 and Bcl2l10). DEHP exposure significantly increased the mRNA levels of pro-apoptotic
Bad following 24 hr at the 1µg/ml dose and following 48 hr at the 100µg/ml dose when compared to the vehicle control group (Fig. 4.3A, n=3-8, p≤0.05). DEHP exposure also significantly increased the mRNA levels of pro-apoptotic Bax following 24 hr at all selected doses of DEHP and following 72 hr at the 1 and 10µg/ml doses when compared to the vehicle control group (Fig. 4.3B, n=3-9, p≤0.05). The mRNA levels of pro-apoptotic Bok were significantly increased following 24 hr at the 1µg/ml dose and following 72 hr at the 10µg/ml dose when compared to the vehicle control group (Fig. 4.3C, n=3-9, p≤0.05). Interestingly, DEHP exposure for 24, 48, and 72 hr significantly increased the mRNA levels of anti-apoptotic Bcl2 at the 1 and 10µg/ml doses when compared to the vehicle control group (Fig. 4.3D, n=3-9, p≤0.05). Similarly, the mRNA levels of anti-apoptotic Bcl2l10 were significantly increased following 48 hr at all selected doses of DEHP and following 72 hr at the 10 and 100µg/ml doses when compared to the vehicle control group (Fig. 4.3E, n=5-9, p≤0.05).

Apoptosis and cell survival rely on the balance of pro- and anti-apoptotic factors, and often the Bax/Bcl2 ratio is used to determine susceptibility to apoptosis [74, 75]. Following qPCR analysis, we calculated the Bax/Bcl2 ratio at 24, 48, and 72 hr of culture. Interestingly, DEHP exposure significantly decreased the Bax/Bcl2 ratio following 48 hr at the 1µg/ml dose when compared to the vehicle control group (Fig. 4.3F, n=5-8, p≤0.05).

**Effect of DEHP on the antral follicle mRNA levels of downstream apoptosis factors**

The present study indicates that DEHP exposure induced atresia in cultured mouse antral follicles; however, DEHP exposure also increased the mRNA levels of both pro- and anti-apoptotic BCL2 regulators of apoptosis. In addition, the Bax/Bcl2 ratio is relatively unchanged in response to DEHP exposure. In order to confirm the histological presence of atresia in the antral follicles, we measured the levels of key downstream facilitators of apoptosis. Specifically, we subjected antral follicles to qPCR following 24, 48, and 72 hr of culture to measure the mRNA levels of Casp8 and Casp3. These caspases are known executors of apoptosis that initiate the disassembly and degradation of the cell in response to pro-apoptotic BCL2 signaling [27, 28]. DEHP exposure significantly increased the mRNA levels of Casp8 following 72 hr at the 10 and 100µg/ml doses when compared to the vehicle control group (Fig. 4.4A, n=5-9, p≤0.05). Interestingly, DEHP exposure significantly increased the mRNA levels of Casp3 following 24 hr at the 1 and 100µg/ml doses when compared to the vehicle control group, but this effect was not
observed following 48 hr of exposure (Fig. 4.4B, n=3-9, \(p \leq 0.05\)). However, following 72 hr of exposure, DEHP significantly increased the mRNA levels of Casp3 at all selected doses of DEHP when compared to the vehicle control group (Fig. 4.4B, n=5-9, \(p \leq 0.05\)).

**Effect of DEHP on antral follicle-produced sex steroid hormone levels**

Previous studies have determined that DEHP exposure decreases estradiol levels both *in vivo* [47-50] and *in vitro* [52, 53]. Similar to previous studies, we have reported that DEHP exposure for 96 hr decreases estradiol levels in an *in vitro* antral follicle culture system [54]. However, the direct effects of DEHP at earlier time-points of exposure and on the estradiol precursor sex steroid hormones are relatively unknown. Thus, we conducted a time-course experiment to investigate the effects of DEHP upstream of estradiol production and to observe the initial effects of DEHP on antral follicle steroidogenesis. DEHP exposure significantly decreased the levels of progesterone following 48 and 72 hr at all selected doses of DEHP when compared to the vehicle control group (Fig. 4.5A, n=5-9, \(p \leq 0.05\)). DEHP exposure also significantly decreased the levels of DHEA following 72 hr at the 10\(\mu\)g/ml dose when compared to the vehicle control group (Fig. 4.5B, n=5-9, \(p \leq 0.05\)). Similarly, androstenedione levels were significantly decreased following 48 hr at all selected doses of DEHP, following 72 hr at the 10 and 100\(\mu\)g/ml doses, and following 96 hr at the 100\(\mu\)g/ml dose when compared to the vehicle control group (Fig. 4.5C, n=5-9, \(p \leq 0.05\)). Likewise, DEHP exposure significantly decreased the levels of testosterone following 48 hr at all selected doses of DEHP, following 72 hr at the 10 and 100\(\mu\)g/ml doses, and following 96 hr at all selected doses of DEHP when compared to the vehicle control group (Fig. 4.5D, n=5-9, \(p \leq 0.05\)). Similar to our previously published study [54], DEHP exposure significantly decreased estradiol levels following 96 hr at the 10 and 100\(\mu\)g/ml doses when compared to the vehicle control group; but interestingly and not previously reported, DEHP exposure for 72 hr also decreased estradiol levels at all selected doses of DEHP when compared to the vehicle control group (Fig. 4.5F, n=5-9, \(p \leq 0.05\)). In contrast, DEHP exposure had no effect on the levels of estrone at each time-point tested when compared to the vehicle control group (Fig. 4.5E, n=3-9).
Effect of DEHP on the antral follicle mRNA expression of key steroidogenic enzymes

Previous studies have determined that DEHP exposure decreases the ovarian mRNA levels of *Cyp19a1*, also known as aromatase, *in vivo* [47, 76] and in an *in vitro* antral follicle culture system following 96 hr [54]. Similar to the effects on the levels of steroid hormones, not much is known about the direct effects of DEHP on the steroidogenic enzymes upstream of *Cyp19a1*. Because we observed decreases in the levels of estradiol and its precursor sex steroid hormones across multiple time-points, we investigated the initial effects and potential mechanisms by which DEHP inhibits steroidogenesis. Specifically, antral follicles were cultured with vehicle control (DMSO) or DEHP (1-100µg/ml) for 24-96 hr. Following each 24 hr time-point, antral follicles were subjected to qPCR to investigate the direct effects of DEHP on the estradiol biosynthesis enzymes (*Star, Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b1*, and *Cyp19a1*), which convert cholesterol to estradiol in a step-wise manner. DEHP exposure significantly decreased the mRNA levels of *Cyp11a1* following 72 hr at the 10 and 100µg/ml doses when compared to the vehicle control group (Fig. 4.6B, n=5-9, p≤0.05). Beginning at 48 hr, DEHP exposure significantly increased the mRNA levels of *Hsd3b1* at the 100µg/ml dose when compared to the vehicle control group (Fig. 4.6C, n=5-8, p≤0.05). Further, DEHP exposure significantly decreased the mRNA levels of *Cyp17a1* following 96 hr at the 100µg/ml dose when compared to the vehicle control group (Fig. 4.6D, n=5-9, p≤0.05). Meanwhile, there was a trend for a significant decrease in the mRNA levels of *Cyp17a1* following 72 hr at the 100µg/ml dose and following 96 hr at the 1µg/ml dose when compared to the vehicle control group (Fig. 4.6D, n=5-9). Beginning at 48 hr, there was a trend for a significant increase in the mRNA levels of *Hsd17b1* at all selected doses tested, and this trend persists through 72 hr with statistical significance at the 10µg/ml dose (Fig. 4.6E, n=5-9, p≤0.05). However, by 96 hr, the mRNA levels of *Hsd17b1* were significantly decreased at the 100µg/ml dose when compared to the vehicle control group (Fig. 4.6E, n=5-9, p≤0.05). Similarly, there was a trend for a significant increase in the mRNA levels of *Cyp19a1* at all selected doses tested following 48 hr, with a statistical significance at the 1µg/ml dose when compared to the vehicle control group (Fig. 4.6F, n=5-8, p≤0.05). By 96 hr, there was a significant decrease in the mRNA levels of *Cyp19a1* at the 100µg/ml dose (Fig. 4.6F, n=5-9, p≤0.05). In contrast, DEHP exposure had no effect on the mRNA levels of *Star* at each time-point tested when compared to the vehicle control group (Fig. 4.6A, n=3-9).
4.5 Discussion

We utilized an \textit{in vitro} antral follicle culture system to investigate the effects of DEHP, a ubiquitous endocrine disrupting chemical, on antral follicle growth, atresia, and steroidogenesis. Our main findings suggest that DEHP exposure alters the follicular mRNA levels of cyclins, a cyclin dependent kinase, and a cyclin dependent kinase inhibitor to potentially impact the previously observed effect of DEHP-induced inhibition of antral follicle growth [54, 55]. Further, our work demonstrates that DEHP exposure directly induces antral follicle atresia as evident via histological evaluation and increased mRNA levels of caspases, and that alterations in the mRNA levels of BCL2 family members may be responsible for the DEHP-induced onset of atresia. Additionally, we have shown that DEHP directly impacts the production of sex steroid hormones upstream of estradiol and alters the mRNA levels of the estradiol biosynthesis enzymes upstream of \textit{Cyp19a1}. Importantly, we have established a time-course of the DEHP-induced defects on antral follicles, and we have determined the initial effects of DEHP on antral follicle growth, atresia, and steroidogenesis.

The use of this antral follicle culture system is vital in understanding the direct effects of DEHP on the most mature, and only, follicle type that is capable of ovulation and synthesis of steroid hormones. Previous work has investigated the effects of DEHP exposure on antral follicle growth and steroidogenesis \textit{in vivo} showing that DEHP decreased antral follicle diameter, and decreased the levels of serum estradiol produced by the antral follicles [47-50]. The limitation of these previous studies is that they did not determine whether the effects on antral follicle size and estradiol production were direct effects on the follicle or were indirect effects caused by toxicity in other organ systems, namely the hypothalamus-pituitary axis. To combat this limitation, previous studies have shown that DEHP and MEHP decreased estradiol production and \textit{Cyp19a1} levels in murine and human granulosa cell cultures \textit{in vitro} [53, 77-80]. However, the limitation of these studies is that they only utilized one somatic follicular cell type. Antral follicle growth, survival from atresia, and steroidogenesis rely on the multi-directional communication between the oocyte, granulosa cells, and theca cells [18, 22, 72, 73]. Thus, although previous studies have been instrumental in understanding the defects caused by DEHP, the \textit{in vitro} whole antral follicle culture system has proved to be a novel approach in understanding the direct effects of DEHP on antral follicle functionality [57, 81].
Our results indicate that DEHP directly inhibits antral follicle growth and dysregulates the expression of cell cycle regulators. We have previously reported that DEHP exposure decreased the antral follicle mRNA levels of Ccnd2 and Cdk4 following 96 hr [54]. Expanding upon these findings, we have established the time-course effects of DEHP on the antral follicle levels of several cyclins, Cdk4, and Cdkn1a. Contrary to the findings at 96 hr, DEHP exposure prior to 96 hr increased the mRNA levels of Ccnd2, Cdk4, Ccne1, Ccna2, and Ccnb1. Further, DEHP exposure decreased the mRNA levels of the inhibitor Cdkn1a. The earliest effect of DEHP on the regulators of the cell cycle was observed following 24 hr of exposure, where DEHP exposure increased the mRNA levels of Ccnd2 and Ccna2, but only at the lowest dose. The most striking finding is that DEHP exposure increased Ccnb1 following 48 and 72 hr at all selected doses. That, as well as the increases in the other cyclins and Cdk4, and decreases in Cdkn1a suggests that early exposure to DEHP may result in an initial push for cell cycle progression and proliferation to combat the toxicity of DEHP. This potentially is why we do not observe growth inhibition prior to 72 hr of DEHP exposure. This compensatory response, however, is not enough to prevent the DEHP-induced inhibition of growth following 72 hr, and this compensation is completely ablated by 96 hr of exposure because follicle growth is further inhibited and the mRNA levels of Ccnd2 and Cdk4 are decreased in response to DEHP treatment [54]. This compensation, nevertheless, may explain why we do not observe complete antral follicle growth inhibition, but rather the growth of the DEHP treated follicles is inhibited by roughly 15% of control treated follicles [55]. Interestingly, the 1µg/ml dose of DEHP is the only dose that does not inhibit antral follicle growth following 72 hr. This is likely because the 1µg/ml dose is the only dose to have compensatory increases in cyclins at the earliest time-point tested, while both the 10 and 100µg/ml doses show no signs of compensation at 24 hr of exposure.

For an antral follicle to function properly, it must remain viable by escaping atresia [18]. Atresia is a natural process by which follicles undergo demise via apoptosis [22]. Following 96 hr of treatment, DEHP exposure at the 10 and 100µg/ml doses directly increased the number of apoptotic bodies found in the antral follicles leading to an increase in the atresia rating. This increase in atresia dramatically affects the health of the antral follicle and possibly can have lasting impacts on reproductive and non-reproductive health. This expands upon previous studies, where exposure to DEHP and its metabolite MEHP induced apoptosis in ovarian cells in vivo [82, 83] and in vitro [84-86].
Follicular atresia is primarily regulated by the balance of pro-apoptotic factors (such as Bad, Bax, and Bok) and anti-apoptotic factors (such as Bcl2 and Bcl2l10) [23-26, 69]. Our findings that DEHP exposure may affect the expression of pro-apoptotic and anti-apoptotic factors expand upon previous studies that suggest DEHP causes oxidative stress in antral follicles following 24 hr of exposure [55]. Further, oxidative stress has been shown to be a regulator of atresia [87-92]. Therefore, it appears that DEHP exposure initially induces oxidative stress and increases pro-apoptotic factors or congruently induces oxidative stress and increases pro- and anti-apoptotic factors following 24 hr of exposure. Following 48 hr of exposure, the two anti-apoptotic factors tested are elevated to combat the initial increase in pro-apoptotic factors, and this occurs in the general absence of an increase in pro-apoptotic factors. However, pro-apoptotic factors again became elevated following 72 hr of exposure. It appears that the compensatory increases in Bcl2 and Bcl2l10 occur after the onset of oxidative stress and increases in pro-apoptotic factors, and this compensation is not enough to alleviate the atresia observed at the 10 and 100µg/ml doses following 96 hr of exposure. However, increases in BCL2 can potentially have pro-apoptotic implications. When BCL2 is cleaved by caspases, studies have reported that BCL2 may acquire pro-apoptotic properties [93]. In support of this hypothesis, both Casp8 and Casp3 are increased following DEHP treatment in the present study. Thus, even though DEHP increases anti-apoptotic factors, it is possible that these factors have pro-apoptotic activities, and DEHP increased downstream apoptotic caspases further suggesting an increase in apoptosis. Interestingly, atresia is not observed at the 1µg/ml dose, and this is likely attributed to the decrease in the Bax/Bcl2 ratio following 48 hr of exposure and the absence of DEHP-induced oxidative stress in the previously mentioned study at the 1µg/ml dose [55].

Proper regulation of antral follicle steroidogenesis is essential for reproductive and non-reproductive health because defects in hormone production, even upstream of estradiol, can lead to anovulation, infertility, premature ovarian failure, cardiovascular disease, osteoporosis, mood disorders, and even premature death [29-46]. We have previously reported that DEHP exposure directly decreased estradiol levels in the antral follicle culture system following 96 hr at the 10 and 100µg/ml doses, but few studies have investigated the effects of DEHP upstream of estradiol. [54]. Expanding upon previous findings and to understand the mechanism by which DEHP disrupts steroidogenesis, we established the time-course effects of DEHP on the levels of
antral follicle-produced sex steroid hormones. Prior to the observed decrease in estradiol levels, DEHP exposure decreased the levels of several precursor steroid hormones. The earliest time-point of the inhibition of steroidogenesis occurs following 48 hr of exposure, where DEHP decreased the levels of progesterone, androstenedione, and testosterone at all selected doses tested. This decrease persists following 72 hr, where DEHP exposure decreased the levels of progesterone, DHEA, androstenedione, testosterone, and even estradiol. Similarly, DEHP exposure following 96 hr decreased the levels androstenedione, testosterone, and estradiol. Estradiol production requires the presence of these upstream steroid hormones to be converted to downstream hormones and ultimately estradiol [18, 72, 73]. Thus, it is likely the lack of precursor hormones following 48 hr of exposure that leads to the decrease in estradiol levels following 72 hr and ultimately 96 hr of exposure.

Estradiol biosynthesis involves the strict coordination of the steroidogenic enzymes present in the theca and granulosa cells that convert the precursor sex steroid hormones to estradiol [72, 73]. We have previously reported that DEHP exposure directly decreased the antral follicle mRNA levels of Cyp19a1, which is the downstream enzyme that converts androgens to estrogens [54]. However, few studies have investigated the effects of DEHP upstream of Cyp19a1. Thus, we established the time-course effects of DEHP on the mRNA levels of each steroidogenic enzyme responsible for generating estradiol. The initial effects of DEHP exposure on the mRNA levels of the steroidogenesis occurred following 48 hr of exposure. DEHP exposure increased the mRNA levels of Hsd3b1 (which converts pregnenolone to progesterone and DHEA to androstenedione) at the 100µg/ml dose and Cyp19a1 at the 1µg/ml dose. Interestingly, DEHP increased the mRNA levels of Hsd17b1 (which converts androstenedione to testosterone and estrone to estradiol) following 72 hr of exposure at the 10µg/ml dose. Also following 72 hr of exposure, DEHP decreased the mRNA levels of Cyp11a1 (which converts cholesterol to pregnenolone). Following 96 hr of exposure, DEHP decreased the mRNA levels of Cyp17a1 (which converts pregnenolone to DHEA and progesterone to androstenedione), Hsd17b1, and Cyp19a1 at the 100µg/ml dose.

Initially, it appears that selected steroidogenic enzymes increase transcription to compensate for the decrease in steroid hormones following 48 hr of exposure. This compensation, however, is not adequate to restore the levels of androstenedione, testosterone,
and estradiol to control levels. Although there is not a difference in the levels of progesterone following 96 hr, it is not because the toxicity of DEHP on progesterone production is alleviated. In fact, progesterone levels remain constant throughout culture, and it is the vehicle control group that decreases production of progesterone from 72 to 96 hr of exposure. A similar phenomenon occurs with DHEA and androstenedione, where the levels of the hormones in the vehicle control group decrease from 72 to 96 hr of exposure. At this time-point in culture, it is likely that the antral follicles are shifting production of these precursor hormones to favor the production of the downstream hormones, testosterone, estrone, and estradiol. This effect was also seen in previous studies that investigated the time-course effects of bisphenol A on mouse antral follicles in vitro [94, 95]. Following 96 hr of exposure, the compensatory increase in the steroidogenic enzymes is lost at the 100µg/ml dose because of a decrease in the rate limiting enzymes, Cyp17a1, Hsd17b1, and Cyp19a1. However, this does not explain the decrease in estradiol levels at the 10µg/ml dose. It is most likely that the absence of precursor hormones leads to the decrease in estradiol following 96 hr of exposure at the 10µg/ml dose, whereas it is both the absence of precursor hormones and decrease in steroidogenic enzyme levels that lead to the decrease in estradiol levels following 96 hr of exposure at the 100µg/ml dose. Further, in the general absence of decreased steroidogenic enzyme expression, it is possible that ongoing apoptosis in the granulosa cells contributes to a loss of steroidogenically active cells, potentially leading to a decrease in precursor hormone levels. Conversely, even though the levels of precursor hormones are decreased at the 1µg/ml dose, the moderate increases in the mRNA levels of Hsd17b1 and Cyp19a1 following 48 and 72 hr of exposure at the 1µg/ml dose appear to be adequate in restoring estradiol levels to control levels following 96 hr of exposure.

In conclusion, we have utilized the novel antral follicle culture system to establish the direct and temporal effects of DEHP on antral follicle growth, atresia, and steroidogenesis. Collectively, our results indicate that DEHP inhibits follicle growth potentially via dysregulation of the cell cycle, induces atresia potentially via dysregulation of apoptosis, and inhibits steroidogenesis potentially via the lack of upstream sex steroid hormones and disruption of the steroidogenic enzymes. These effects of DEHP are of concern because they can negatively impact ovulation, initiation and maintenance of pregnancy, estrous cyclicity, maintenance of the reproductive tract, and non-reproductive health [29-46]. Although the processes of growth, atresia and steroidogenesis are linked in certain aspects, the mechanism of the temporal
relationship of the three aspects of follicular function has yet to be elucidated [18, 73]. Further, studies should be done to explore the reasons for the non-monotonic dose responses observed for some endpoints in response to DEHP.
4.6 Tables, Figures, and Legends

Table 4.1 Sequences of primer sets used for gene expression analysis

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<th>Accession No.</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
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<th>5’ Reverse-3’</th>
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<td>NM_009741</td>
<td>B cell leukemia/lymphoma 2</td>
<td>Blc2</td>
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<td>GGTATGCAACCAAGAGTGATC</td>
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<tr>
<td>NM_007522</td>
<td>BCL2-associated agonist of cell death</td>
<td>Bad</td>
<td>AAGTCCGATCCCGGAATCC</td>
<td>GCTCACTCGGCTCAAACTCT</td>
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<td>NM_007527</td>
<td>BCL2-associated X protein</td>
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<tr>
<td>NM_013479</td>
<td>Bcl-2-like 10</td>
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<td>GATCAAGTCCTGCCCCTG</td>
</tr>
<tr>
<td>NM_016778</td>
<td>BCL2-related ovarian killer protein</td>
<td>Bok</td>
<td>CTGCCCCTGGAGGAGCTTTG</td>
<td>CGGTCACCAACAGGCTGAGC</td>
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<tr>
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<td>Cytochrome P450 steroid 17α-hydroxylase</td>
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<td>NM_011489</td>
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<td>Star</td>
<td>CAAAGAGGGCCGCTGTAAGCA</td>
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Specific primer sequences used for qPCR analysis of gene expression.
Figure 4.1 Effect of DEHP on the antral follicle mRNA expression of key regulators of the cell cycle

Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100µg/ml) for 24-72 hr. Following each 24 hr time-point, antral follicles were pooled per treatment group and were subjected to qPCR for the measurements of Ccnd2 (panel A), Cdk4 (panel B), Ccne1 (panel C), Ccna2 (panel D), Ccnb1 (panel E), and Cdkn1a (panel F). All values were normalized to Actb. Graph represents means ± SEM from 3-9 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100 µg/ml) for 96 hr. Following 96 hr, antral follicles were processed for histological evaluation of atresia. A representative image of a DMSO treated follicle is found in panel A. A representative image of a DEHP 100 µg/ml treated follicle is found in panel B. Atresia ratings were assigned based on the presence of apoptotic bodies. A rating of 1 indicated a healthy follicle without apoptotic bodies, a rating of 2 indicated the presence of apoptotic bodies encompassing 1-10% of the follicle section, a rating of 3 indicated the presence of apoptotic bodies encompassing 11-30% of the follicle section, and a rating of 4 indicated the presence of apoptotic bodies encompassing >30% of the follicle section. Graph represents means ± SEM from 3 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100µg/ml) for 24-72 hr. Following each 24 hr time-point, antral follicles were pooled per treatment group and were subjected to qPCR for the measurements of Bad (panel A), Bax (panel B), Bok (panel C), Bcl2 (panel D), Bcl2l10 (panel E). All values were normalized to Actb. Further, ratios of Bax values/Bcl2 values were calculated (panel F). Graph represents means ± SEM from 3-9 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 4.4 Effect of DEHP on the antral follicle mRNA expression of downstream apoptosis factors

Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100µg/ml) for 24-72 hr. Following each 24 hr time-point, antral follicles were pooled per treatment group and were subjected to qPCR for the measurements of \textit{Casp8} (panel A) and \textit{Casp3} (panel B). All values were normalized to \textit{Actb}. Graph represents means ± SEM from 3-9 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100µg/ml) for 24-96 hr. Following each 24 hr time-point, media were pooled per treatment group and were subjected to ELISAs for the measurements of progesterone (panel A), dehydroepiandrosterone (panel B), androstenedione (panel C), testosterone (panel D), estrone (panel E), and estradiol (panel F). Graph represents means ± SEM from 3-9 separate experiments, with medium from 10-16 wells/treatment group in each experiment. Hormones with ND (not detectable) at certain time-points had levels of that hormone below threshold detection. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or DEHP (1-100 µg/ml) for 24-96 hr. Following each 24 hr time-point, antral follicles were pooled per treatment group and were subjected to qPCR for the measurements of *Star* (panel A), *Cyp11a1* (panel B), *Hsd3b1* (panel C), *Cyp17a1* (panel D), *Hsd17b1* (panel E), and *Cyp19a1* (panel F). All values were normalized to *Actb*. Graph represents means ± SEM from 3-9 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
4.7 References


57. Lenie S, Smitz J. Steroidogenesis-disrupting compounds can be effectively studied for major fertility-related endpoints using in vitro cultured mouse follicles. Toxicol Lett 2009; 185:143-152.


CHAPTER V

Mono(2-ethylhexyl) Phthalate Accelerates Early Folliculogenesis and Inhibits Steroidogenesis in Cultured Mouse Whole Ovaries and Antral Follicles

5.1 Abstract

Humans are ubiquitously exposed to di(2-ethylhexyl) phthalate (DEHP), which is an environmental toxicant present in common consumer products. DEHP potentially targets the ovary through its metabolite mono(2-ethylhexyl) phthalate (MEHP). However, the direct effects of MEHP on ovarian folliculogenesis and steroidogenesis, two processes essential for reproductive and non-reproductive health, are unknown. The present study tested the hypotheses that MEHP directly accelerates early folliculogenesis via over-activation of phosphatidylinositol 3-kinase (PI3K) signaling, a pathway that regulates primordial follicle quiescence and activation, and inhibits the synthesis of steroid hormones by decreasing steroidogenic enzyme levels. Neonatal ovaries from CD-1 mice were cultured for six days with vehicle control, DEHP, or MEHP (0.2-20μg/ml) to assess the direct effects on folliculogenesis and PI3K signaling. Further, antral follicles from adult CD-1 mice were cultured with vehicle control or MEHP (0.1-10μg/ml) for 24-96hr to establish the temporal effects of MEHP on steroid hormones and steroidogenic enzymes. In the neonatal ovaries, MEHP, but not DEHP, decreased phosphatase and tensin homolog levels and increased phosphorylated protein kinase B levels, leading to a decrease in the percentage of germ cells and an increase in the percentage of primary follicles. In the antral follicles, MEHP decreased the mRNA levels of 17α-hydroxylase-17,20-desmolase, 17β-hydroxysteroid dehydrogenase, and aromatase leading to a decrease in testosterone, estrone, and estradiol levels. Collectively, MEHP mediates the effect of DEHP on accelerated folliculogenesis via over-activating PI3K signaling and inhibits steroidogenesis by decreasing steroidogenic enzyme levels.
5.2 Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a widely used synthetic plasticizer incorporated in the manufacturing of common polyvinyl chloride consumer, medical, and building products in order to impart flexibility [1, 2]. DEHP is produced in vast quantities, up to 300 million pounds annually, to account for its high demand in products ranging from carpeting and roofing to food and beverage packaging [2]. This is of concern because DEHP is non-covalently bound to the plastic, meaning DEHP frequently leaches from the plastics into the environment and products that humans consume [1]. Based on its high production volumes, use in common items, and ability to leach out from the items, humans are exposed to DEHP on a daily basis via oral ingestion, inhalation, and dermal contact [1]. Specifically, the estimated range of daily human exposure to DEHP is 3-30 μg/kg/day [3, 4]. Exposure to DEHP represents a public health concern because DEHP and its metabolite, mono(2-ethylhexyl) phthalate (MEHP), have been identified as top contaminants in human tissues such as blood [5, 6], urine [1, 5-9], amniotic fluid [10-12], umbilical cord blood [13, 14], breast milk [6], and ovarian follicular fluid [15]. Important from a toxicological standpoint, it is hypothesized that the toxicity of DEHP is mediated by the bioactive metabolite MEHP in many organ systems, including the ovary [16-21].

Also important for public health, DEHP and MEHP are known endocrine disrupting chemicals and reproductive toxicants that potentially target the ovary [1, 2, 16-21]. Understanding the effects of these chemicals on normal ovarian function is crucial because the ovary is responsible for the maturation of follicles for ovulation, a process termed folliculogenesis, and for the synthesis of sex steroid hormones, a process termed steroidogenesis [22]. Defects in folliculogenesis and steroidogenesis, including accelerated depletion of follicles within the ovary and decreased estradiol production, can cause reproductive and non-reproductive complications, such as infertility, premature ovarian failure, cardiovascular disease, osteoporosis, mood disorders, and even premature death [23-34].

Interestingly, DEHP and MEHP have been shown to disrupt ovarian folliculogenesis. Specifically, we have previously reported that oral exposure to DEHP in adult mice accelerates primordial follicle recruitment following 10 and 30 days of dosing [35]. Primordial follicle recruitment is a gonadotropin-independent process involving strict regulation of intrinsic ovarian
growth factors [36-39]. Factors within the phosphatidylinositol 3-kinase (PI3K) signaling pathway have recently been implemented as regulators of primordial follicle survival, dormancy, and recruitment, where inhibition of PI3K signaling maintains primordial follicle dormancy and over-activation of signaling leads to primordial follicle recruitment [38, 40-45]. We have also previously reported that the DEHP-induced acceleration of primordial follicle recruitment is likely via over-activation of PI3K signaling, indicated by a decrease in ovarian phosphatase and tensin homolog (PTEN) levels, an inhibitor of PI3K signaling that maintains primordial follicle dormancy, and an increase in ovarian phosphorylated protein kinase B (pAKT) levels, a stimulator of PI3K signaling that promotes primordial follicle recruitment [35]. However, the limitation of that study is that we did not determine whether the effects on primordial follicle recruitment and PI3K signaling were direct effects on the ovary, or the effects were a secondary response caused by toxicity in other organ systems following exposure in vivo.

Additionally, DEHP and MEHP have been shown to disrupt ovarian steroidogenesis. Specifically, DEHP exposure has been shown to decrease estradiol levels and aromatase levels in vivo [46-48] and in vitro [16, 49, 50]. We have reported that the inhibition of steroidogenesis may be via a direct effect on antral follicle functionality, because DEHP exposure in cultured mouse antral follicles directly decreased the availability of precursor sex steroid hormone levels at time-points prior to decreasing estradiol levels [51]. This toxicity of DEHP may be mediated by MEHP, and MEHP exposure has also been shown to inhibit steroidogenesis in vitro [19-21, 52, 53]. Specifically, we have reported that MEHP exposure directly decreased the levels of estradiol and the mRNA levels of aromatase, Cyp19a1, in cultured mouse antral follicles following 96 hr of exposure [16]. Steroidogenesis is primarily conducted by the mature antral follicle and is a step-wise process involving the enzymatic conversion of cholesterol to estradiol and other necessary precursor steroid hormones that are essential for reproductive and non-reproductive health [54, 55]. However, few studies have investigated the effects of MEHP on the precursor steroid hormones and the steroidogenic enzymes upstream of estradiol and aromatase. Additionally, the temporal effects by which MEHP directly inhibits steroidogenesis in the antral follicle remain unknown.

The present study was designed to investigate the direct effects of DEHP and MEHP on early folliculogenesis and to compare the mechanisms by which these chemicals alter
folliculogenesis *in vivo* and *in vitro*. Further, the present study was designed to establish a time-course of the initial and time specific effects of MEHP on antral follicle steroidogenesis upstream of estradiol and aromatase and to compare the mechanisms by which MEHP and DEHP inhibit steroidogenesis in cultured antral follicles. Specifically, we tested the hypotheses that MEHP mediates the DEHP-induced acceleration of primordial follicle recruitment, and MEHP does so via a mechanism involving over-activation of PI3K signaling, which is the mechanism observed *in vivo*. Additionally, we hypothesized that MEHP directly inhibits the synthesis of sex steroid hormones to ultimately lead to a decrease in estradiol levels.
5.3 Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO), and MEHP (99% purity) was purchased from AccuStandard (New Haven, CT). Stock solutions of DEHP and MEHP were prepared using dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) as the vehicle in various concentrations (0.133, 0.267, 1.33, 2.67, 13.3, and 26.7 mg/ml). This allowed for an equal volume of each stock to be added to the culture wells to control for vehicle concentration. Final concentrations of DEHP in culture were 0.2, 2, and 20 μg/ml which is approximately equivalent to 0.54, 5.4, and 54 μM respectively. Final concentrations of MEHP in culture were 0.1, 0.2, 1, 2, 10, and 20 μg/ml, which is approximately equivalent to 0.34, 0.68, 3.4, 6.8, 34, and 68 μM respectively.

The concentrations of DEHP and MEHP were chosen based on their ability to disrupt ovarian steroidogenesis and folliculogenesis. Specifically, concentrations of these chemicals cause inhibition of antral follicle growth, decreased estradiol production from antral follicles, decreased antral follicle Cyp19a1 expression, and increased antral follicle atresia [16, 17, 56]. We have also previously determined that oral exposure to DEHP for 10 and 30 days accelerates primordial follicle recruitment at doses ranging from 200 μg/kg/day to 20 mg/kg/day [35]. These doses are approximately equivalent to 0.492-49.2 μM, which encompasses the doses ranging from 0.2-20 μg/ml. The concentrations of DEHP and MEHP selected in these experiments have also been determined to be clinically relevant [20, 52, 57-59]. Importantly, plasma concentrations of DEHP and MEHP in healthy women have been reported to be 0.18 μg/ml and 0.58 μg/ml respectively, and peritoneal fluid concentrations of DEHP and MEHP in these women were reported to be 0.46 μg/ml and 0.37 μg/ml respectively, which encompasses the lower selected concentrations [60]. In addition, the extensive use of DEHP in medical equipment leads to markedly higher levels of DEHP and MEHP in patients undergoing consistent medical care when compared to healthy people [61, 62]. Neonatal patients in intensive care units that receive blood transfusions have plasma levels of DEHP and MEHP at 11.1 μg/ml and 15.1 μg/ml respectively, which encompasses the higher selected concentrations [63]. Further, the lowest-observed-adverse-effect level (LOAEL) of DEHP is 140 mg/kg/day which is approximately
equivalent to 344.6 μM [2]. The selected concentrations of DEHP and MEHP in these experiments fall below the LOAEL concentration.

**Animals**

For the experiments investigating the direct effects of DEHP and MEHP on early folliculogenesis, cycling, adult CD-1 female mice (40-50 days of age) and mature, adult CD-1 male mice (40-50 days of age) were obtained from Charles River Laboratories (Wilmington, MA). Breeding groups (one male with two females) were housed in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana-Champaign to serve as a source for post-natal day (PND) 4 ovaries for culture. Once pregnant, the mice were individually placed in separate cages and allowed to give birth. On PND4, female pups were removed from the dam and used in the neonatal ovary cultures described below. For the experiments investigating the effects of MEHP on antral follicles, cycling, adult CD-1 female mice (35-39 days of age) were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in groups of four in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana-Champaign and were allowed to acclimate to the facility prior to experimentation. All animals in these experiments were housed in a controlled animal room environment (temperature at 22 ± 1 °C and 12-hour light–dark cycles) and were provided food and water ad libitum. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana-Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

**In vitro neonatal ovary cultures**

Whole ovaries were aseptically collected from CD-1 pups on PND4 and were used for ovarian culture as described previously [64, 65]. Briefly, each ovary was removed on PND4 and cleaned of excess fat, oviductal, and interstitial tissue under a light microscope. The ovaries were placed in media droplets on a piece of Millicell culture plate inserts (Millipore, Billercia, MA) that was floating on 500 μl of equilibrated (37°C) supplemented ovary culture media in a four-well culture plate. The treatment groups for the neonatal ovary culture experiments included vehicle control (DMSO), DEHP (0.2, 2, and 20 μg/ml), and MEHP (0.2, 2, and 20 μg/ml). The supplemented media contained DMEM/Ham F12 minus phenol red medium (Life Technologies, Grand Island, NY) with 1 mg/ml BSA (Sigma-Aldrich, St. Louis, MO), 1 mg/ml Albumax (Life
Technologies, Grand Island, NY), 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), 5 U/ml penicillin per 5 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 27.5 μg/ml transferrin (Sigma-Aldrich, St. Louis, MO) per well. Each ovary was cultured for 6 days until PND10 in an incubator at 37°C supplying 5% CO₂. The media were replaced daily with fresh, treated supplemented media, and one drop of medium was placed on the ovary to prevent the ovary from drying. Following culture, one ovary was collected for histological evaluation of follicle numbers, and the other ovary was subjected to immunohistochemistry as described below.

The neonatal ovary culture system and PND4-PND10 time-points were chosen because the neonatal ovary is enriched in primordial and primary follicles that are naturally undergoing the initial stages of folliculogenesis [66]. Since DEHP exposure (approximately equivalent to 0.492-49.2 μM or 0.2-20 μg/ml) for 10 and 30 days accelerates primordial follicle recruitment in vivo [35], this culture system provides a direct tool to assess the effects of DEHP and MEHP on early folliculogenesis. Specifically, At PND4, the neonatal ovary is completing germ cell nest breakdown; therefore, the ovary contains a population of follicles that are primarily in the primordial stage. At PND10, some primordial follicles naturally begin to undergo recruitment to the primary stage of development both in vivo and in vitro, and these follicles are structurally and functionally identical to those in the adult [22, 66].

**Histological evaluation of follicle numbers**

Following 6 days of culture, some PND10 neonatal ovaries were fixed in Dietrich’s fixative for at least 24 hrs for histological evaluation of follicle numbers (n=4-6 ovaries/group). The ovaries were then transferred to 70% ethanol, embedded in paraffin wax, and serial sectioned (5 μM) using a microtome. The serial sections were mounted on a glass slide and stained with hematoxylin and eosin. The numbers of oocyte containing follicles and germ cells were counted in every fifth serial section using a light microscope, and the percentage of each follicle type was calculated by dividing the number of follicles of each specific type by the total number of follicles counted and multiplying that value by 100. Stage of follicular development was assessed using previously defined criteria [67, 68]. Briefly, germ cells were identified by the presence of a single oocyte or group of oocytes without a defined granulosa cell layer encapsulating them, primordial follicles contained the oocyte surrounded by a single layer of squamous granulosa cells, and primary follicles contained the oocyte surrounded by a single
layer of cuboidal granulosa cells. Very few preantral and antral follicles are present at PND10; therefore, these follicle types were not included in the analysis. All germ cells, primordial and primary follicles with oocytes, regardless of nuclear material in the oocytes, were counted.

**Immunohistochemistry**

Following 6 days of culture, some PND10 neonatal ovaries were fixed in 4% paraformaldehyde overnight and transferred to 70% ethanol for immunohistochemical analysis for PTEN and pAKT (Ser473) (the active form of the protein) as described previously (n=3/group) [35]. Briefly, the ovaries were embedded in paraffin wax, serial sectioned (5 µM), and three representative sections that span the entire length of the ovary were mounted on a glass slide. Some tissue sections were subjected to staining for PTEN (1:200) (Cell Signaling Technology, Inc., Boston, MA), while other tissue sections were subjected to staining for pAKT (1:50) (Cell Signaling Technology, Inc., Boston, MA). The tissues were then incubated with a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA), followed by an incubation with an avidin biotin complex solution (Vector Laboratories, Inc., Burlingame, CA). ImmPACT NovaRED peroxidase substrate solution (Vector Laboratories, Inc., Burlingame, CA) was then applied until color optimally developed. Each sample was exposed to the chromogen for equal amounts of time. The slides were then rinsed, counterstained with a 1:10 dilution of hematoxylin, and coverslipped. A negative control was used in each experiment and was subjected to the same methods listed above, except instead of primary antibody, the negative control tissues were incubated with a negative control rabbit immunoglobulin fraction (Dako, Carpinteria, CA) at the same concentration of each primary antibody. Negative controls were confirmed to have no positive staining after exposure to the chromogen for equal amounts of time as the samples. Both PTEN and pAKT were chosen because they are integral regulators of PI3K signaling and have been associated with the regulation of primordial follicle recruitment [38, 43, 69-71]. Specifically, PTEN inhibits PI3K signaling and ultimately maintains primordial follicle dormancy [38, 43, 69-71]. Additionally, pAKT drives PI3K signaling and ultimately promotes primordial follicle recruitment [38, 43, 69-71]. Further, 10 day oral exposure to DEHP decreases ovarian PTEN and increases ovarian pAKT potentially driving the acceleration of primordial follicle recruitment that was observed in that study [35].
Analysis of the levels of protein staining

Following immunohistochemistry, the levels of PTEN and pAKT staining in the neonatal ovaries were quantified using published methods [35, 72-74]. Briefly, images of ovaries were digitally captured using a Leica DFC 290 camera and analyzed using the ImageJ software (http://rsb.info.nih.gov/nih-image/). For analysis, three representative sections from each ovary were assessed from three separate animals per treatment group. Digital images were converted to 8-bit grayscale images and then converted to pseudocolored images. Colors were based on relative stain intensity, as defined digitally. Areas with no staining appeared black and blue, while areas with the most intense staining appeared deep yellow to orange. Percentages of positively stained PTEN and pAKT pixels in the whole ovary and percentages of positively stained PTEN and pAKT primordial follicle oocytes were compared across treatment groups. For whole ovarian analysis, the pixels of positively stained PTEN and pAKT areas were divided by the total amount of pixels in the whole ovary, and that value was multiplied by 100. For primordial follicle oocyte analysis, positively stained PTEN and pAKT primordial follicle oocytes were quantified, divided by the total amount of primordial follicle oocytes, and that value was multiplied by 100.

In vitro antral follicle cultures

Unprimed, female CD-1 mice were euthanized and their ovaries were aseptically removed for antral follicle isolation. Antral follicles (250-400 μm) were isolated from the ovary and were cleaned of interstitial tissue using watchmaker’s forceps [75, 76]. At least 2-3 mice were used in each experiment, in which approximately 20-30 antral follicles per mouse were obtained. Each treatment group contained 8-16 follicles. Isolated antral follicles were randomly and individually plated in wells of a 96-well culture plate containing unsupplemented α-minimal essential medium (α-MEM, Life Technologies, Grand Island, NY) prior to treatment.

The treatment groups for the follicle culture experiments included vehicle control (DMSO) and MEHP (0.1, 1, and 10 μg/ml), and they were prepared in supplemented α-MEM. Supplemented α-MEM was prepared as described previously [75, 76]. Briefly, supplemented α-MEM contained 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), 5 IU/ml human recombinant follicle-stimulating
hormone (FSH; Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), and 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). An equal volume of chemical (0.75 µl/ml of media) was added for each dose to control that the amount of vehicle in each preparation was 0.075%. Each follicle was cultured in 150 µl of medium for 24–96 hr in an incubator at 37°C supplying 5% CO₂. Following each 24 hr culture window, media from cultured follicles were collected for measurements of the levels of sex steroid hormones, and cultured follicles were collected, snap-frozen, and stored at −80°C for gene expression analysis. A time-course was conducted to observe when the effects of MEHP on steroidogenesis begin, to determine which hormone/enzyme is causing the reduction of estradiol production from antral follicles following 96 hr as reported previously [16].

Analysis of sex steroid hormone levels

Following each 24 hr antral follicle culture window, culture media were subjected to enzyme-linked immunosorbent assays (ELISAs) for the measurements of the levels of sex steroid hormones. Levels of progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, estrone, and 17β-estradiol were measured following the manufacturer’s protocol using ELISA kits purchased from Diagnostics Research Group (DRG, Mountainside, NJ) (n=8-16 wells of pooled media containing follicles from 3-9 separate experiments). The analytical sensitivity of each kit was 0.044 µg/ml for DHEA, 0.1 ng/mL for progesterone, 0.019 ng/mL for androstenedione, 0.083 ng/mL for testosterone, 6.3 pg/mL for estrone, and 9.71 pg/mL for estradiol. All samples were run in duplicates and all intra- and inter-assay coefficients of variability were less than 10%. Some samples were diluted to match the dynamic range of each ELISA kit. Mean values for each sample were used in this analysis.

Analysis of antral follicle gene expression

Following each 24 hr antral follicle culture window, antral follicles were snap frozen in liquid nitrogen, and stored at −80°C for quantitative real-time polymerase chain reaction (qPCR) analysis (n=8-16 follicles/3-9 separate experiments). Total RNA (200 ng) was extracted from the follicles using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol, and was then reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the
manufacturer’s protocol. Prior to qPCR analysis, each cDNA sample was diluted 1:4 using nuclease-free water. The CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) and accompanying CFX Manager Software was used for analysis of qPCR according to the manufacturer’s protocol. All qPCR reactions were done in triplicate using 1 μl cDNA, forward and reverse primers (5 pmol), and a SsoFastEvaGreen Supermix for a final reaction volume of 10 μl. Specific qPCR primers (Integrated DNA Technologies, Inc., Coralville, IA) for the genes of interest as well as the reference gene, beta-actin (Actb), can be found in Table 5.1. Beta-actin was chosen as the reference gene because its expression did not differ across treatment groups. The genes tested were steroidogenic acute regulatory protein (Star), cytochrome P450 cholesterol side-chain cleavage (Cyp11a1), 3β-hydroxysteroid dehydrogenase (Hsd3b1), 17α-hydroxylase-17,20-desmolase (Cyp17a1), 17β-hydroxysteroid dehydrogenase (Hsd17b1), and aromatase (Cyp19a1). These genes were chosen because they synthesize estradiol and its precursor sex steroid hormones in the ovary.

The CFX96 machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. The qPCR program consisted of an enzyme activation step (95°C for 1 min), an amplification and quantification program (40 cycles of 95°C for 10 sec, 60°C for 10 sec, single fluorescence reading), a step of 72°C for 5 min, a melt curve (65°C–95°C heating 0.5°C per sec with continuous fluorescence readings), and a final step at 72°C for 5 min as per the manufacturer’s protocol. Expression data were generated using the mathematical standard comparative (ΔΔCt) method. The ΔCt was calculated by subtracting the Actb Ct value from the gene of interest Ct value. The ΔΔCt was calculated as the difference between the ΔCt between the treatment groups and the DMSO groups. The relative fold-change of expression was then equaled to $2^{-\Delta\Delta Ct}$ for each sample.

Statistical analysis

Analysis of data was conducted using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means and error bars represent the standard error of the means (SEM). Multiple comparisons between normally distributed experimental groups were made using one-way analysis of variance (ANOVA) followed by Tukey post-hoc comparison. Multiple
comparisons between non-normally distributed experimental groups were made using Kruskal Wallis tests when appropriate. Statistical significance was assigned at $p \leq 0.05$. 
5.4 Results

Effect of DEHP and MEHP on early folliculogenesis

Proper regulation of ovarian folliculogenesis is essential for fertility and the maintenance of appropriately timed reproductive senescence. Previously, we have shown that daily oral exposure to DEHP for 10 and 30 days disrupts folliculogenesis by accelerating primordial follicle recruitment to the primary stage of development [35]. However, that study utilized an in vivo dosing regimen and was unable to test if the effects on primordial follicle recruitment were due to direct ovarian toxicity caused by DEHP or the bioactive metabolite MEHP. Therefore, the direct effects of DEHP and MEHP on follicular dynamics were evaluated in this study. Following 6 days of culture, DEHP exposed ovaries had similar percentages of germ cells, primordial follicles, and primary follicles counted when compared to the vehicle control group (Fig. 5.1A, n=5-6/group). However, following 6 days of culture, MEHP exposure significantly decreased the percentage of germ cells counted at all selected doses when compared to the vehicle control group (Fig. 5.1B, n=4-6/group, p ≤ 0.05). MEHP exposure did not significantly alter the percentage of primordial follicles counted, but it significantly increased the percentage of primary follicles counted at all selected doses when compared to the vehicle control group (Fig. 5.1B, n=4-6/group, p ≤ 0.05).

Effect of DEHP and MEHP on protein levels of PI3K signaling factors

We have previously shown that the DEHP-induced acceleration of primordial follicle recruitment following 10 and 30 days of exposure may involve over-activation of the PI3K signaling pathway [35], but it was unknown if the DEHP-induced dysregulation of PI3K signaling was due to a direct ovotoxic effect of DEHP and/or MEHP. Thus, this study tested whether DEHP or MEHP directly alter the levels of PTEN and/or pAKT in the neonatal ovaries. Qualitatively, it appears that MEHP exposure decreased the staining of PTEN in the whole neonatal ovary (Fig. 5.2, A-D), decreased the percentage of PTEN positive oocytes (Fig. 5.2, E and F), and increased the percentage of pAKT positive oocytes (Fig. 5.2, G and H). When quantified, MEHP significantly decreased the levels of PTEN staining in the whole neonatal ovary at the 2 and 20 µg/ml doses when compared to the vehicle control group, while all selected doses of DEHP had comparable levels of PTEN staining to the vehicle control group (Fig. 5.3A,
n=3/group, p ≤ 0.05). Conversely, both MEHP and DEHP had comparable levels of pAKT staining in the whole ovary when compared to the vehicle control group (Fig. 5.3A, n=3/group). To ensure that the levels of the PI3K proteins were altered in the follicles of interest, the percentages of positively stained PTEN and pAKT oocytes in primordial follicles were quantified. MEHP significantly decreased the percentage of PTEN and significantly increased the percentage of pAKT positive oocytes in primordial follicles at all selected doses of MEHP when compared to the vehicle control group (Fig. 5.3B, n=3/group, p ≤ 0.05). Meanwhile, DEHP had comparable percentages of PTEN and pAKT positive oocytes to the vehicle control group (Fig. 5.3B, n=3/group).

**Effect of MEHP on sex steroid hormone production by antral follicles**

Previous studies have determined that DEHP exposure decreases estradiol levels in vivo [46-48] and in cultured ovarian cell types and follicles in vitro [16, 49-51]. Likewise, MEHP exposure has also been shown to decrease estradiol levels in cultured ovarian cell types and follicles in vitro [16, 19-21, 53]. However, the mechanism by which MEHP decreases estradiol levels is unknown. We have previously reported that MEHP exposure for 96 hr decreases estradiol levels produced by cultured mouse antral follicles [16]; but, the direct effects of MEHP on antral follicle steroidogenesis at earlier time-points of exposure and on the precursor steroid hormones upstream of estradiol are unknown. Thus, the temporal effects of MEHP on the levels of sex steroid hormones produced by the antral follicle were investigated by utilizing time-course culture experiments. Beginning with the most upstream precursor steroid hormones measured, MEHP exposure did not significantly alter the levels of progesterone or DHEA at any time-point tested when compared to the vehicle control group (Fig. 5.4, A and B, n=3-9/group). However, MEHP exposure significantly decreased the levels of androstenedione at the 10 µg/ml dose following 24 and 72 hr of exposure when compared to the vehicle control group (Fig. 5.4C, n=3-9/group, p ≤ 0.05). Further, MEHP exposure significantly decreased the levels of testosterone at the 10 µg/ml dose following 24 hr of exposure when compared to the vehicle control group (Fig. 5.4D, n=3/group, p ≤ 0.05). Likewise, MEHP exposure significantly decreased the levels of testosterone at all selected doses following 96 hr of exposure when compared to the vehicle control group (Fig. 5.4D, n=4-9/group, p ≤ 0.05). Further down the steroidogenesis pathway, MEHP exposure significantly decreased the levels of estrone at the 0.1 and 10 µg/ml doses.
following 96 hr of exposure when compared to the vehicle control group (Fig. 5.4E, n=4-9/group, p ≤ 0.05). Additionally, MEHP exposure significantly decreased the levels of estradiol at all selected doses following 96 hr of exposure when compared to the vehicle control group (Fig. 5.4F, n=4-9/group, p ≤ 0.05).

**Effect of MEHP on steroidogenic enzyme gene expression in antral follicles**

Previous studies have hypothesized that one potential mechanism by which DEHP and MEHP decrease estradiol levels is through a decrease in the ovarian mRNA levels of *Cyp19a1*, also known as aromatase, following exposure *in vivo* [46, 77] and *in vitro* [16, 19-21, 51]. However, not much is known about the direct effects of MEHP on the steroidogenic enzyme levels upstream of *Cyp19a1* in the antral follicle. Further, not much is known about the direct effects of MEHP on the enzyme levels prior to 96 hr of exposure. In addition, MEHP decreased the levels of sex steroid hormones upstream of estradiol across multiple time-points in the present study (Fig. 5.4). Thus, the temporal effects and potential mechanisms by which MEHP inhibits steroidogenesis were investigated. Following 48 hr of exposure, MEHP significantly decreased the mRNA levels of *Star* and *Cyp11a1* at the 1 and 10 µg/ml doses when compared to the vehicle control group (Fig. 5.5, A and B, n=3-5/group, p ≤ 0.05). Following 24 hr of exposure, MEHP significantly decreased the mRNA levels of *Hsd3b1* at the 10 µg/ml dose when compared to the vehicle control group (Fig. 5.5C, n=3/group, p ≤ 0.05). Following 96 hr of exposure, MEHP significantly decreased the mRNA levels of *Cyp17a1* at all selected doses when compared to the vehicle control group (Fig. 5.5D, n=4-9/group, p ≤ 0.05). Following 72 hr of exposure, MEHP significantly increased the mRNA levels of *Hsd17b1* at all selected doses when compared to the vehicle control group (Fig. 5.5E, n=4-9/group, p ≤ 0.05). However, following 96 hr of exposure, MEHP significantly decreased the mRNA levels of *Hsd17b1* at all selected doses when compared to the vehicle control group (Fig. 5.5E, n=4-9/group, p ≤ 0.05). MEHP exposure significantly increased the mRNA levels of *Cyp19a1* at the 0.1 and 1 µg/ml doses following 48 hr of exposure and at all selected doses following 72 hr of exposure when compared to the vehicle control group (Fig. 5.5F, n=3-9/group, p ≤ 0.05). However, following 96 hr of exposure, MEHP significantly decreased the mRNA levels of *Cyp19a1* at all selected doses when compared to the vehicle control group (Fig. 5.5F, n=4-9/group, p ≤ 0.05).
5.5 Discussion

We utilized a neonatal whole ovarian culture system and an antral follicle culture system to investigate the direct effects of DEHP and MEHP on early folliculogenesis and to investigate the direct and temporal effects of MEHP on steroidogenesis. Our main findings suggest that MEHP, but not DEHP, has a direct effect on over-activating PI3K signaling in the ovary to potentially lead to the acceleration of primordial follicle recruitment. Additionally, we have shown that MEHP directly inhibits the production of estradiol and upstream sex steroid hormones and alters the mRNA levels of Cyp19a1 and upstream estradiol biosynthesis enzymes. We have also established the initial and time specific effects of MEHP on antral follicle steroidogenesis. Importantly, we have determined that DEHP and MEHP have differential effects on the initial stages of folliculogenesis and on steroidogenesis.

The use of the whole neonatal ovarian culture system is essential for understanding the direct effects of chemicals on the earliest stages of folliculogenesis [64, 66]. Previous studies have shown that DEHP and MEHP accelerate primordial follicle recruitment in vivo [35, 78, 79]. The limitation of these studies is that they did not determine if these chemicals act directly on the ovary to elicit this response. A further study has shown that DEHP also affects early folliculogenesis in vitro, but that study only focused on germ cell nest breakdown and primordial follicle assembly [80]. Although these studies are influential in understanding the effects of DEHP and MEHP on early folliculogenesis, the effects of these chemicals on the activation of the dormant and already established primordial follicle pool remain unknown. Thus, we investigated the effects of DEHP and MEHP from PND4-PND10 on the neonatal ovary, a time-span when the follicular reserve is nearly established and primordial follicles naturally begin to undergo activation to the primary stage of development [22, 66]. Importantly, at PND10, the neonatal ovary contains a population of follicles that are primarily in the primordial and primary stages of development. Thus, we were able to primarily focus on the populations of follicles in which we observed DEHP-induced effects in vivo [35].

In the present study, MEHP, but not DEHP, directly decreased the percentage of germ cells counted and increased the percentage of primary follicles counted when compared to the vehicle control group. We have previously reported that oral exposure to DEHP for 10 and 30 days in adult mice decreased the percentage of primordial follicles counted and increased the
percentage of primary follicles counted at doses similar to the 0.492-49.2 μM range (0.2-20 μg/ml) used in this study [35]. Although we did not observe a change in primordial follicle numbers in the present study, we likely missed the time-point in which MEHP altered primordial follicle numbers. Our follicle count data represent a single “snap-shot” following 6 days of exposure; therefore, future studies should investigate the effects of MEHP on primordial follicle numbers prior to and after 6 days of exposure. However, our primary follicle count data correlate to those obtained in the in vivo studies [35]. Thus, these data suggest that MEHP has a direct effect on the ovary indicating that the observed effect in vivo is likely via a direct mechanism of action.

We have previously reported that the DEHP-induced acceleration of primordial follicle recruitment in vivo is likely via over-activation of the PI3K signaling pathway [35]. Primordial follicle recruitment is a tightly regulated process involving a balance between the factors that maintain primordial follicle quiescence and promote primordial follicle activation [36-39]. Interestingly, factors within the PI3K signaling pathway regulate primordial follicle survival, quiescence, and recruitment. Specifically, PTEN, an inhibitor of PI3K signaling, maintains primordial follicle quiescence, and pAKT, a stimulator of PI3K signaling, promotes development to the primary stage [38, 40-45]. Following 10 days of oral exposure to adult mice, DEHP at similar μM doses to those used in this study decreased the staining of PTEN in whole ovary and increased the staining of pAKT in the whole ovary and in the primordial and primary follicles [35]. Similarly, DEHP exposure decreased the mRNA levels of Pten in the whole adult ovary following 30 days of oral exposure [35]. In the present study, DEHP had no statistically significant effect on follicle numbers or PI3K signaling factors. However, MEHP exposure directly decreased PTEN staining in the whole ovary, decreased the percentage of PTEN positive primordial follicle oocytes, and increased the percentage of pAKT positive primordial follicle oocytes, again at doses that mimic those used in the in vivo studies. Thus, MEHP has a direct effect on over-activating ovarian PI3K signaling to promote an environment conducive for the acceleration of primordial follicle recruitment, and this mechanism is similar to the mechanism by which DEHP accelerates primordial follicle recruitment in vivo [35].

The antral follicle culture system used for the steroidogenesis experiments is an essential method to investigate the direct effects of MEHP on the antral follicle, which is the most mature
follicle type capable of ovulation and is the major producer of sex steroid hormones in the female. Previous studies have determined that MEHP exposure decreases estradiol levels \textit{in vitro}; however, these studies utilized secondary follicles or murine/human granulosa cells [19-21, 53]. Ovarian steroidogenesis is primarily conducted by the mature antral follicle, and it involves the strict coordination of theca and granulosa cells. Thus, the antral follicle culture system used in the present study expands upon the previously published data by using a more direct and sensitive approach [58, 81].

We have previously reported that MEHP exposure decreases estradiol levels and the mRNA levels of \textit{Cyp19a1} in cultured mouse antral follicles following 96 hr of exposure. However, the temporal effects of MEHP upstream of estradiol and CYP19A1 and the mechanism by which MEHP decreases estradiol levels were not known. Further, we have previously reported that DEHP exposure decreases estradiol levels following 72 and 96 hr of exposure in cultured mouse antral follicles via a mechanism involving depletion of available precursor steroid hormones beginning at 48 hr of exposure [51]. However, it was unknown if DEHP and MEHP exhibited similar mechanisms of steroidogenesis inhibition.

The initial effect of steroidogenesis was observed following 24 hr of exposure, where MEHP decreased the levels of androstenedione and testosterone at the 10 \textmu{g}/ml dose, and this is likely attributed to the decrease in the mRNA levels of \textit{Hsd3b1} at that same time-point and dose. Interestingly, these effects were ablated and no changes in the levels of steroid hormones were observed following 48 hr of exposure. However, MEHP decreased the mRNA levels of \textit{Star} and \textit{Cyp11a1} at that time-point, and a compensatory increase in the mRNA levels of \textit{Cyp19a1} was observed. The compensatory increase in \textit{Cyp19a1} levels persisted following 72 hr of exposure, and also at this time-point, a compensatory increase in the levels of \textit{Hsd17b1} was observed. Perhaps these compensatory increases in the levels of steroidogenic enzymes are present to combat the toxicity of MEHP; however, these increases do not lead to increases in steroid hormone levels. The increase in the levels of \textit{Hsd17b1} at the 72 hr time-point likely explains why we do not observe a statistically significant decrease in testosterone levels following 72 hr of exposure. Following 96 hr of exposure, the compensatory increases in the steroidogenic enzymes were ablated, and MEHP exposure decreased the mRNA levels of \textit{Cyp17a1}, \textit{Hsd17b1}, and \textit{Cyp19a1}. It is likely this decrease in steroidogenic enzyme levels led to the decrease in the
levels of testosterone, estrone, and estradiol following 96 hr of exposure. Thus, MEHP decreased the levels of immediate precursor hormones and estradiol via a mechanism involving depletion of the enzymes responsible for generating these hormones.

These findings on steroidogenesis also add to the existing literature that MEHP disrupts antral follicle functionality. Normal follicle function includes antral follicle growth, survival from apoptosis-induced atresia, and production of steroid hormones [22]. Previously, MEHP exposure for 72 and 96 hr has been shown to inhibit antral follicle growth in vitro, likely via disruption of cell cycle regulators [16, 17]. Additionally, MEHP exposure for 96 hr induced antral follicle atresia in vitro, likely via disruption of pro- and anti-apoptotic factors following 48 hr of exposure [56]. Further, MEHP exposure for 72 hr decreased the activity of glutathione peroxidase in antral follicles, which led to oxidative stress in the cultured follicles [17]. In the present study, it appears that that major defects in steroidogenesis begin following 96 hr of exposure. Thus, the defects in antral follicle growth, atresia, and oxidative stress prior to 96 hr of exposure may promote the compensatory increases in Hsd17b1 and Cyp19a1. Further, it appears that these defects in antral follicle functionality precede and may cause the decreases in Cyp17a1, Hsd17b1, and Cyp19a1, ultimately leading to the decreases in testosterone, estrone, and estradiol levels. Future studies should address how MEHP interferes with the cross-talk between antral follicle growth, atresia, and steroidogenesis.

Interestingly, DEHP and MEHP appear to exert toxicity differently depending on the system used. For the folliculogenesis studies, MEHP, but not DEHP, had a direct effect on accelerating primordial follicle recruitment via over-activation of the PI3K signaling pathway. For the steroidogenesis studies, both DEHP and MEHP have direct effects on the antral follicle. Specifically, we previously reported that DEHP exposure directly decreases precursor steroid hormones following 48 hr of exposure, and it is likely this decrease in the availability of precursor hormones that lead to the decrease in estradiol levels following 72 and 96 hr of exposure [51]. This is because the levels of the steroidogenic enzymes are relatively unchanged in response to 24-96 hr DEHP treatment [51]. However, in the present study, MEHP exposure decreased the levels of the steroidogenic enzymes following 96 hr of exposure, likely leading to the decrease in steroid hormone levels. We hypothesize that the differences between DEHP and
MEHP on folliculogenesis and steroidogenesis are attributed to ovarian metabolism of the chemicals.

The ovary contains enzymes, specifically aldehyde dehydrogenases, alcohol dehydrogenases, and lipoprotein lipases, responsible for converting DEHP to MEHP and MEHP to further metabolic derivatives [82-86]. Importantly, the levels and activity of these metabolic enzymes increase with age, meaning the metabolic capacity of the neonatal ovary is much lower than the adult [84-87]. Thus, the reason that we likely do not observe direct effects of DEHP on folliculogenesis in our neonatal ovary culture system is because the neonatal ovaries do not have the metabolic capacity or possess the enzymes needed to convert DEHP to the toxic metabolite MEHP. However, these metabolic enzymes are present in the antral follicles [84-87], and perhaps differential metabolism explains why we observe differences in the DEHP and MEHP steroidogenesis studies. Thus, the composition of the mixture between parent compound and metabolites is likely different between our DEHP and MEHP treated follicles, and it is possible that this composition dictates toxicity in the antral follicle. However, it is plausible that the parent compound and metabolite simply exert toxicity in different ways. Future studies should investigate the metabolic capacity of the antral follicle to shed insight into the differential mechanisms of DEHP- and MEHP-induced inhibition of steroidogenesis.

In conclusion, we have utilized the novel neonatal ovary and antral follicle culture systems to elucidate the mechanisms by which MEHP disrupts ovarian folliculogenesis and steroidogenesis. Collectively, our results indicate that MEHP has a direct effect on the neonatal ovary to accelerate primordial follicle recruitment, potentially via over-activation of ovarian PI3K signaling. Importantly, this mechanism in vitro is similar to the mechanism by which DEHP accelerates primordial follicle recruitment in vivo [35]. Additionally, our results indicate that MEHP has a direct effect on decreasing the levels of the steroidogenic enzymes responsible for generating estradiol and its immediate precursor steroid hormones. Interestingly, this mechanism is different than the mechanism by which DEHP inhibits steroidogenesis in vitro [51]. These effects of MEHP on folliculogenesis and steroidogenesis are of concern because ovarian controlled reproductive and non-reproductive processes have the potential to be compromised.
5.6 Tables, Figures, and Legends

Table 5.1 Sequences of primer sets used for gene expression analysis

<table>
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<tr>
<th>Accession No.</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
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<th>5'-Reverse 3'</th>
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<td>Beta-actin</td>
<td>Actb</td>
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Specific primer sequences used for qPCR analysis of gene expression.
Figure 5.1 Effect of DEHP and MEHP on early folliculogenesis

Ovaries from CD-1 mice were cultured from PND 4-PND 10 with vehicle (DMSO), DEHP (0.2-20 µg/ml) (panel A), or MEHP (0.2-20 µg/ml) (panel B). Following culture, ovaries were processed for histological evaluation of germ cell and follicle counts. Percentages of each stage of folliculogenesis were calculated and compared in each treatment group. Graph represents means ± SEM (n=4-6 ovaries/treatment group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 5.2 Representative images of immunohistochemistry and pseudocolored protein quantification

Panels A-F exhibit PTEN staining in the whole ovary (panels A-D) and at 20x magnification to observe staining in primordial follicles (panels E and F). Panels G and H exhibit pAKT staining at 20x magnification to observe staining in primordial follicles. Panels A, B, E, and G were from vehicle control treated neonatal ovaries, and panels C, D, F, and H were from MEHP (2 µg/ml) treated neonatal ovaries. In panels A-D, the black arrow heads represent positive staining in the immunohistochemistry images, which translate to the yellow/orange pseudocolored pixels represented by red arrow heads. In panels E-H, the black arrow heads represent positively stained oocytes in primordial follicles.
Figure 5.3 Effect of DEHP and MEHP on protein levels of PI3K signaling factors

Ovaries from CD-1 mice were cultured from PND4-PND10 with vehicle (DMSO), DEHP (0.2-2 µg/ml), or MEHP (0.2-20 µg/ml). Following culture, ovaries were subjected to immunohistochemistry for quantification of PTEN (the left side of panels A and B) and pAKT (the right side of panels A and B) staining. Digital images were converted to 8-bit grayscale images and then converted to pseudocolored images. Colors were based on relative stain intensity. Percentage of positively stained pixels in the whole ovary (panel A) and percentage of positively stained oocytes in primordial follicles (panel B) were calculated and compared in each treatment group. Graph represents means ± SEM (n=3/group). Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 5.4 Effect of MEHP on the antral follicle-produced sex steroid hormone levels

Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or MEHP (0.1-10 µg/ml) for 24-96 hr. Following each 24 hr time-point, media were pooled per treatment group and were subjected to ELISAs for the measurements of progesterone (panel A), dehydroepiandrosterone (panel B), androstenedione (panel C), testosterone (panel D), estrone (panel E), and estradiol (panel F). Graph represents means ± SEM from 3-9 separate experiments, with medium from 10-16 wells/treatment group in each experiment. ND indicates that hormone levels were not detectable because they were below the threshold level for detection. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
Figure 5.5 Effect of MEHP on the antral follicle mRNA levels of the steroidogenic enzymes

Antral follicles were isolated from adult CD-1 mice and were cultured with vehicle (DMSO) or MEHP (0.1-10 µg/ml) for 24-96 hr. Following each 24 hr time-point, antral follicles were pooled per treatment group and were subjected to qPCR for the measurements of the mRNA levels of Star (panel A), Cyp11a1 (panel B), Hsd3b1 (panel C), Cyp17a1 (panel D), Hsd17b1 (panel E), and Cyp19a1 (panel F). All values were normalized to Actb. Graph represents means ± SEM from 3-9 separate experiments, with 10-16 follicles/treatment group in each experiment. Asterisks (*) represent significant difference from vehicle control (p ≤ 0.05).
5.7 References


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58. Lenie S, Smitz J. Steroidogenesis-disrupting compounds can be effectively studied for major fertility-related endpoints using in vitro cultured mouse follicles. Toxicol Lett 2009; 185:143-152.


CHAPTER VI

Summary, Conclusions, and Future Directions

6.1 Summary, Conclusions, and Future Directions

The goal of my doctoral dissertation work was elucidate the mechanisms by which DEHP and MEHP disrupt ovarian folliculogenesis and steroidogenesis. DEHP is a highly produced plasticizer in widely used consumer, medical, and building products [1, 2]. DEHP frequently leaches out of the products that humans consume on a daily basis. Thus, DEHP and its metabolite MEHP are identified as top contaminants present in human tissues, including the ovary [1, 3-13]. This is a public health concern because DEHP and MEHP are known endocrine disrupting chemicals and reproductive toxicants [1, 2]. However, few studies have investigated the effects of DEHP and MEHP on ovarian folliculogenesis and steroidogenesis. Understanding the effects of these chemicals on these ovarian processes is crucial because proper regulation of folliculogenesis is needed for appropriate reproductive and non-reproductive health [14-31]. Collectively, my studies show that environmentally relevant levels of DEHP and MEHP accelerate primordial follicle recruitment, inhibit antral follicle growth, induce antral follicle atresia, and inhibit antral follicle steroidogenesis. A model of the effects on folliculogenesis (Figure 6.1) and steroidogenesis (Figure 6.2) can be found in Chapter 6 section 2.

In Chapter 3, I first hypothesized that environmentally relevant levels of DEHP disrupt folliculogenesis by accelerating primordial follicle recruitment in vivo. I found that DEHP exposure for 10 days decreased the percentage of primordial follicles at the 20 and 200 mg/kg/day doses and increased the percentage of primary follicles at all selected doses. Further, DEHP exposure for 30 days increased the percentage of primary follicles at the 200 μg/kg/day and 20 mg/kg/day doses with a trend for a decrease in the percentage of primordial follicles at those doses. The decrease in primordial follicles accompanied by an increase in primary follicles suggests that DEHP exposure accelerates primordial follicle recruitment in vivo. Because the female is born with a finite reserve of primordial follicles, acceleration of primordial follicle depletion can cause infertility and/or premature ovarian failure. Future studies should investigate if the effects of 10 and 30 day exposure to DEHP on primordial follicle recruitment are permanent. Further studies should investigate if chronic exposure to DEHP results in a similar
acceleration of primordial follicle recruitment. In addition, future studies should investigate if the effects on folliculogenesis cause infertility and/or premature ovarian failure.

In Chapter 3, I further hypothesized that DEHP exposure accelerates primordial follicle recruitment via over-activation of the PI3K signaling pathway. Factors within this signaling pathway are regulators of primordial follicle survival, quiescence, and recruitment [32-37]. Specifically, factors that inhibit PI3K signaling, such as PTEN, maintain primordial follicle quiescence, while factors that drive PI3K signaling, such as pAKT, promote primordial follicle recruitment [38]. I found that DEHP exposure at doses that accelerated primordial follicle recruitment altered the mRNA levels of PI3K signaling factors that are associated with folliculogenesis. Importantly, DEHP exposure at doses that accelerated primordial follicle recruitment decreased PTEN levels and increased pAKT levels in the whole ovary, primordial follicles, and primary follicles. Since the factors involved in PI3K signaling are heavily regulated translationally and post-translationally, the protein data expand upon the mRNA data. Further, I found that these signaling factors were altered in the primordial and primary follicle populations. Interestingly, increased levels of DEHP metabolites in serum and urine are associated with an earlier age of menopause in women [39]. Further, accelerated primordial follicle recruitment via over-activation of PI3K signaling is a mechanism also conserved in humans [33, 40]. Thus, the findings in these studies may be translational for women’s public health. Future studies should investigate the cross-talk between the PI3K signaling pathway and other signaling pathways associated with folliculogenesis to confirm if the effects on primordial follicle recruitment in the present study are direct effects on PI3K signaling.

In Chapter 4, I hypothesized that DEHP directly alters regulators of the cell cycle, apoptosis, and the steroidogenic pathway in antral follicles in vitro to inhibit growth, induce atresia, and inhibit steroidogenesis. The studies conducted in Chapter 4 provide the initial and time specific events by which DEHP induces toxicity in the antral follicle. I found that DEHP exposure initially increased the mRNA levels of cell cycle regulators perhaps in a compensatory manner to combat the toxicity of DEHP on the antral follicle. Specifically, DEHP increased the mRNA levels of Ccnd2, Cdk4, Ccne1, Ccna2, and Ccnb1 and decreased the levels of Cdkn1a prior to growth inhibition. However, following 72 hr of exposure, DEHP inhibited antral follicle growth, and by 96 hr of exposure, DEHP decreased the mRNA levels Ccnd2 and Cdk4 [41].
Although there is an initial compensatory alteration in these factors that control the cell cycle, the compensation is not adequate enough to rescue the growth inhibition. Perhaps, this is also why I did not observe complete inhibition of antral follicle growth, but rather, DEHP exposure inhibited antral follicle growth by 15% compared to controls.

Further, I found that DEHP exposure induces antral follicle atresia by increasing the levels of pro-apoptotic factors that regulate atresia. Specifically, DEHP increased the mRNA levels of Bad, Bax, and Bok prior to the observation of atresia at 96 hr of exposure. The increases in these pro-apoptotic factors were accompanied by increases in the mRNA levels of Bcl2 and Bcl2l10, which are anti-apoptotic factors. The increases in anti-apoptotic factors may be compensatory to combat DEHP toxicity; however, studies have shown that BCL2 can achieve pro-apoptotic properties in an apoptotic environment [42]. In addition, DEHP exposure increased the mRNA levels of Casp8 and Casp3, which are downstream regulators of apoptosis. In the ovary, 99% of all follicles undergo atresia [43, 44]. Thus, an increase in the incidence of antral follicle atresia can decrease sex steroid hormone levels and can cause infertility [45, 46]. The DEHP-induced increase in atresia can possibly have lasting effects of fertility and non-reproductive health. Future studies should investigate if DEHP exposure in vivo increases atresia, and these studies should investigate if the increase in atresia causes anovulation and defects in the brain, cardiovascular, and skeletal systems.

In addition to the defects in antral follicle growth and atresia, I found that DEHP exposure inhibits steroidogenesis by decreasing the levels of precursor sex steroid hormones available to be converted to estradiol. Specifically, DEHP exposure decreased the levels of progesterone, androstenedione, and testosterone following 48 hr of exposure, which is prior to the decrease in the levels of estradiol. These hormones serve as precursors to the generation of estradiol, but they are also required for reproductive and non-reproductive processes. The decrease in these hormones occurs in the general absence of a decrease in the levels of the steroidogenic enzyme, even though DEHP at 100 μg/ml decreased the mRNA levels of Cyp17a1, Hsd17b1, and Cy19a1. Thus, it is likely that the depletion of precursors at 48 hr of exposure led to the decrease in estradiol production following 96 hr of exposure. An inhibition of steroidogenesis can have lasting effects on reproductive and non-reproductive health [45, 46]. Specifically, decreasing these hormones can inhibit ovulation, disrupt the hypothalamus-
pituitary-ovarian axis, inhibit the maintenance of the reproductive tract, and cause mood disorders, osteoporosis, and cardiovascular disease [14-31]. Future studies should address if the inhibition of steroidogenesis is present following exposure in vivo, and if the DEHP-induced inhibition of steroidogenesis causes anovulation and non-reproductive disorders. Further, future studies should supplement progesterone in the antral follicle culture media, since progesterone is the most upstream hormone affected by DEHP exposure. These studies would provide insight into whether the effects of DEHP on steroidogenesis are treatable.

In Chapter 4, I provided evidence that DEHP directly alters antral follicle functionality. Antral follicles must undergo growth, survive atretic demise, and produce steroid hormones to remain viable for ovulation. DEHP exposure directly inhibits growth by altering cell cycle regulators, induces atresia by increasing pro-apoptotic factors, and inhibits steroidogenesis by depleting precursor steroid hormones. Interestingly, these effects occur rather congruently, so it is unknown which process is targeted first and whether the defects in one process promotes the defects in the others. Future studies should further investigate the time-specific effects of DEHP on growth, atresia, and steroidogenesis to determine which process is altered first. Additionally, future studies should investigate the cross-talk between antral follicle growth, atresia, and steroidogenesis in order to ascertain the interrelationship of these processes.

In Chapter 5, I first hypothesized that MEHP directly mediates the DEHP-induced acceleration of primordial follicle recruitment in vitro. I found that MEHP exposure at doses similar to those used in the in vivo experiments decreased the percentage of germ cell and increased the percentage of primary follicles in a whole neonatal ovary culture system. The neonatal ovary culture system was used because neonatal ovaries are enriched in primordial and primary follicles, which are the populations in which an effect is observed in vivo. Meanwhile, DEHP treated neonatal ovaries had comparable percentages of germ cells, primordial follicles, and primary follicles to control treated ovaries. These data suggest that MEHP directly accelerates primordial follicle recruitment, which is similar to the effect observed following DEHP treatment in vivo. I further hypothesized that MEHP directly accelerates primordial follicle recruitment via over-activation of the PI3K signaling pathway. I found that MEHP, but not DEHP, decreased the levels of PTEN in the whole ovary, decreased the percentage of
positively stained PTEN primordial follicle oocytes, and increased the percentage of positively stained pAKT primordial follicle oocytes.

These findings suggest that MEHP has a direct effect on over-activating ovarian PI3K signaling to potentially lead to the acceleration of primordial follicle recruitment. The mechanism by which MEHP accelerates primordial follicle recruitment is the same in which DEHP accelerates primordial follicle recruitment \textit{in vivo}. Thus, the effects on early folliculogenesis are direct on the ovary, and it is MEHP that mediates the effects of DEHP on accelerated primordial follicle recruitment. As previously mentioned, accelerated depletion of primordial follicles can cause infertility and premature ovarian failure. Further, increased levels of DEHP metabolites are associated with an earlier age of menopause, and the PI3K-regulated control of primordial follicle recruitment is conserved in women [33, 39, 40]. Future studies should incorporate the use of PI3K inhibitors in the culture system to investigate if MEHP is working exclusively through the PI3K signaling pathway. These studies would provide therapeutical insight by potentially rescuing the MEHP-induced acceleration of primordial follicle recruitment. Further, these findings would aid in the understanding of the mechanisms of primordial follicle activation, and provide novel techniques to manipulate the follicular reserve.

In Chapter 5, I further hypothesized that MEHP directly inhibits the synthesis of sex steroid hormones in antral follicles \textit{in vitro} via a different mechanism than DEHP. Similar to DEHP treated follicles, I found that MEHP exposure decreased the levels of testosterone and estradiol following 96 hr of exposure. In contrast to the DEHP treated follicles, I found that MEHP exposure also decreased the levels of estrone following 96 hr of exposure. Again, defects in antral follicle steroidogenesis can cause infertility and other non-reproductive disorders [14-31].

Interestingly, MEHP appears to inhibit steroidogenesis via a different mechanism than DEHP. Initially, MEHP exposure increased the mRNA levels of \textit{Hsd17b1} and \textit{Cyp19a1} following 48 and 72 hr of exposure. The increase in the levels of these steroidogenic enzymes is perhaps to compensate for the toxicity of MEHP; however, this compensatory increase did not lead to an increase in the levels of sex steroid hormones. Further, this compensatory increase is ablated following 96 hr of exposure where MEHP decreased the mRNA levels of \textit{Cyp17a1}, \textit{Hsd17b1}, and \textit{Cyp19a1}. Thus, it is likely this decrease in steroidogenic enzyme levels that led to
the decrease in testosterone, estrone, and estradiol following 96 hr of exposure. This mechanism is in contrast to the DEHP-induced inhibition of steroidogenesis. In Chapter 4, I concluded that DEHP decreased the levels of precursor steroid hormones beginning at 48 hr of exposure, in the general absence of a decrease in steroidogenic enzyme levels, which led to a decrease in estradiol levels. MEHP, meanwhile, relatively did not decrease the levels of precursor hormones prior to 96 hr of exposure. Thus, MEHP decreased the levels of the enzymes responsible for generating estradiol beginning at 96 hr of exposure.

Future studies should investigate the differential mechanisms by which DEHP and MEHP inhibit steroidogenesis. It is possible that the antral follicle differentially metabolizes DEHP to MEHP and MEHP to its further oxidative derivatives. The adult ovary and antral follicles contain the enzymes capable of metabolizing DEHP to MEHP [47-50]. Thus, the composition of the mixture between parent compound and metabolites is likely different between the DEHP and MEHP treated follicles, and it is possible that this composition dictates toxicity in the antral follicle. Future studies should investigate how the antral follicle metabolizes DEHP and MEHP. Additionally, these studies should use a composition of the DEHP metabolite mixture to investigate which metabolite and what levels of metabolites are responsible for defects in steroidogenesis. Further, the level and activity of these metabolic enzymes increase with age [47-50]. Thus, this may explain why DEHP did not affect early folliculogenesis in the neonatal ovary culture system. Additional studies should confirm that the metabolic capacity of the neonatal ovary is not adequate enough to metabolize DEHP to its toxic metabolites. Understanding ovarian metabolism is important from a physiological and therapeutical standpoint. Intervening in the ovarian metabolism of DEHP and MEHP may alleviate some of the toxic outcomes that were observed in my doctoral dissertation work.

Overall, my doctoral dissertation work indicates that environmentally relevant levels of DEHP and MEHP disrupt ovarian folliculogenesis and steroidogenesis. Specifically, DEHP, through its bioactive metabolite MEHP, accelerates primordial follicle recruitment via over-activation of PI3K signaling. Additionally, DEHP and MEHP differentially inhibit steroidogenesis in the antral follicle by decreasing the availability of precursor hormones and decreasing the levels of steroidogenic enzymes, respectively. These findings are important for
public health because humans are continuously exposed to these chemicals. Further, the ovary is an integral regulator of reproductive and non-reproductive health.
6.2 Figures and Legends

Figure 6.1 Mechanism by which DEHP and MEHP alter folliculogenesis

DEHP, through its bioactive metabolite MEHP, directly decreases PTEN and increases pAKT in the ovary to over-activate PI3K signaling. The over-activation of PI3K signaling leads to the acceleration of primordial follicle recruitment indicated by decreases in the percentages of germ cells and primordial follicles and increases in the percentage of primary follicles in vivo and in vitro.
DEHP and MEHP differentially inhibit the production of estradiol in cultured antral follicles. Specifically, DEHP decreases the precursors available to be converted to estradiol beginning at 48 hr of exposure, and MEHP decreases the levels of the steroidogenic enzymes responsible for generating estradiol beginning at 96 hr of exposure.
6.3 References


