THE IMMEDIATE AND LONG-TERM CONSEQUENCES OF TEMPORARY EXPOSURE DURING ADULTHOOD TO DI(2-ETHYLHEXYL) PHTHALATE AND DIISONONYL PHTHALATE ON FEMALE REPRODUCTION

BY

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DISSERTATION

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The ovary is a heterogenous structure that produces a variety of sex steroid hormones and participates in several feedback loops within the hypothalamic-pituitary-ovary axis. Further, it is the site of storage and maturation of the female gamete: the oocyte. Females are born with a finite number of oocytes in the form of the primordial follicle pool. Primordial follicles are composed of an oocyte surrounded by supporting somatic cells. Activated primordial follicles will grow through the primary, preantral, and antral follicle stages before ovulating. Proper maintenance of this follicular growth, termed folliculogenesis, is imperative for maintenance of proper fertility and overall somatic health. Female fertility relies on a steady stream of maturing follicles in addition to the proper maintenance of the cyclical rise and fall of sex steroid and peptide hormones. Further, sex steroid hormones play a role in maintenance of multiple aspects of somatic health, including the cardiovascular system, bone density, and neurological health. Thus, chemical exposures that target the ovary can lead to disruptions in reproductive and overall health.

The phthalate chemical family has several prominent members with endocrine disrupting capabilities. One of the most common phthalates is di(2-ethylhexyl) phthalate (DEHP), with up to 100 million pounds produced within the U.S. each year. DEHP is used as a plasticizer to impart strength and flexibility into products. DEHP can be found in products such as plastic food packaging, clothes, shower curtains, upholstery, and medical equipment. Because DEHP is noncovalently bound to products, it can leach out over time and be absorbed via inhalation, ingestion, and dermal contact. The estimated average daily intake of DEHP in humans is 3-30
µg/kg/day, and DEHP metabolites have been found in samples of human blood, urine, semen, amniotic fluid, cord blood, and ovarian follicular fluid. This is concerning because DEHP is a known reproductive toxicant and endocrine disrupting chemical. Although some studies have shown that DEHP targets the ovary, very few studies have investigated whether short-term exposure to DEHP during adulthood can have long-lasting impacts on the exposed individual.

Some manufacturers have elected to start replacing DEHP with other plasticizers. Unfortunately, these replacements are often understudied, and few studies have investigated the reproductive toxicity potential of these compounds. Diisononyl phthalate (DiNP) is one such understudied DEHP substitute. Few reproductive toxicity studies using DiNP have been conducted, and the majority focus on males. Further, studies that report data on the reproductive toxicity of DiNP in females tend to only include data on the effects of DiNP on the weights of reproductive organs. Although reproductive organ weights are an important and useful metric for assessing the reproductive toxicity for a particular chemical, these data do not provide a complete understanding of the potential reproductive toxicity of the chemical. Further, the present literature concerning the effects of DiNP on female reproduction often use doses well above the range of environmental relevance. It is imperative to investigate these chemicals at relevant doses because of the non-monotonic dose responses that some endocrine disrupting chemicals can elicit.

Together, information is lacking on the effects of DEHP and DiNP on female reproduction, especially as it concerns exposure during adulthood. Therefore, the purpose of my doctoral dissertation work was to investigate the effects of DEHP alongside a common DEHP
replacement chemical, DiNP, on the reproductive health over the lifetime of the exposed female mice at both high and environmentally relevant doses. Specifically, I investigated the effects of short-term exposure to DEHP and DiNP during adulthood on estrous cyclicity, fertility, sex steroid hormones, peptide hormones, and ovarian follicle populations at time points including immediately post-dosing and 3, 6, 9, 12, 15, and 18 months post-dosing.

First, I tested the hypothesis that short-term exposure (10 days) to DEHP and DiNP during adulthood in the mouse was sufficient to disrupt estrous cyclicity, fertility, and weights of reproductive organs at time points during the prime reproductive age of the mouse, including immediately post-dosing and 3 and 9 months post-dosing. Immediately post-dosing and 3 and 9 months post-dosing, fertility was assessed via breeding trials and weights of reproductive organs were measured. In addition, estrous cyclicity was monitored at 3 and 9 months post-dosing. Assessing estrous cyclicity disruptions and changes in weights of organs can give insight to possible avenues through which DEHP and DiNP may disrupt fertility. I found that 10 days of DEHP exposure was enough to significantly reduce the uterine weight immediately post-dosing. Additionally, at 3 months post-dosing, females treated with the lowest dose of DEHP and DiNP had significant difficulty achieving pregnancy from a mating, resulting in significantly fewer females producing offspring in these groups when compared to control. I also observed that several treatment groups had increased male to female ratios of pups at 9 months post-dosing. Further, 10 days of phthalate exposure was enough to significantly affect the estrous cyclicity at both 3 and 9 months post-dosing, indicating that short-term exposure can have long lasting impacts.
Next, I tested the hypothesis that short-term exposure (10 days) to DEHP and DiNP during adulthood in the mouse was sufficient to alter levels of sex steroid hormones, levels of the peptide hormones follicle-stimulating hormone (FSH) and inhibin B, and ovarian follicle populations at time points during the prime reproductive life of the mouse, including immediately post-dosing and 3, 6, and 9 months post-dosing. Proper maintenance of ovarian follicle maturation is necessary for proper fertility and maximization of the reproductive lifespan. Further, changes in circulating hormones can alter cyclicity and behavior and can directly impact fertility itself. I found that transient exposure to DEHP and DiNP during adulthood was sufficient to cause disruption of ovarian folliculogenesis by altering numbers of follicle types, percentages of follicle types, and percent of unhealthy follicles immediately post-dosing and up to 9 months post-dosing. Further, I found that DEHP and DiNP disrupted several hormones and that disruptions were present at all time points tested in this study.

Further, I tested the hypothesis that short-term exposure (10 days) to DEHP and DiNP during adulthood in the mouse was sufficient to disrupt fertility and estrous cyclicity and alter levels of sex steroid hormones, levels of the peptide hormones follicle-stimulating hormone (FSH) and inhibin B, and ovarian follicle populations at time points during late-life in the mouse, including 12, 15, and 18 months post-dosing. Maintenance of the finite pool of ovarian follicles is required for maximization of the reproductive lifespan in females. Dysregulation of the ovarian follicular pool or hormones could lead to abnormalities in reproductive behavior, fertility complications, and shortening of the reproductive lifespan. I found that DEHP and DiNP increased pregnancy loss, reduced fertility, and altered follicular populations and circulating hormone levels in late-life in female mice. Collectively, data from my dissertation research indicate that short-term
exposure to DEHP and DiNP during adulthood has consequences on a variety of aspects of female fertility at time points ranging from immediately post-dosing to 18 months following completion of the dosing period.
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To my family, my dogs, my friends, and my motorcycle (sorry, Mom)
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CHAPTER 1

Overview

1.1 Overview

The proper development of follicles within the ovary is critical for female fertility. Follicular development, often referred to as folliculogenesis, begins with maturation of a germ cell into a primordial follicle, then to a primary follicle, then a pre-antral, antral, and finally to a preovulatory follicle. Development through these stages and subsequent successful ovulation is necessary for proper female fertility [1, 2]. Thus, chemicals that cause dysregulation of folliculogenesis, or otherwise alter follicular numbers within the ovary, can alter the course of the reproductive life of the female in question. Endocrine disrupting chemicals (EDCs) are chemicals with the ability to alter aspects of the endocrine system within an organism [3]. Several EDCs have been found to have significant impacts on the ovary [4, 5]. Phthalates are a class of EDCs commonly used as plasticizers and stabilizers in several different kinds of consumer products, and they can be absorbed via inhalation, ingestion, and dermal contact [5-10]. Because of their widespread use, phthalate exposure is ubiquitous, as evidenced by the presence of phthalate metabolites in nearly all tested human urine samples [11]. Di(2-ethylhexyl) phthalate (DEHP), in particular, is a widely used phthalate with several million pounds produced annually in the U.S. alone [12]. Due to the mass production of DEHP, DEHP metabolites are found in the vast majority of urine samples taken from men, women, and children [13] and in a variety of bodily tissues [14]. This ubiquitous exposure to DEHP is of concern because DEHP, and its bioactive metabolite, mono(2-ethylhexyl) phthalate (MEHP), are known EDCs [4, 5, 14-16] and have been shown to accelerate folliculogenesis [16-18]. Further,
a previous study conducted in our laboratory reported that short-term DEHP exposure significantly reduced numbers of primordial follicles and total follicle numbers [17], but it is not known whether this decrease in ovarian reserve has any impact on female fertility and the reproductive lifespan.

Further, possibly due to the increased concern about the presence of DEHP in consumer products, there has been a rise in production and use of DEHP replacements such as diisononyl phthalate (DiNP) [19, 20]. Although DiNP has not been studied extensively, it has been shown to cause histopathological changes in the testes of perinatally exposed rats [21-23] as well as disruption of steroidogenesis in fetal rat testes in a similar manner as seen in DEHP exposure [23-25]. However, studies investigating the effects of DiNP on female reproduction are incredibly sparse, and studies that include data on reproductive toxicity in females consistently lack data on the effects of DiNP on reproductive parameters outside of effects on weights of the female reproductive organs. Although it is important to investigate the effects of DiNP on gross attributes of the reproductive organs, female fertility is a highly regulated and complex system with several interacting aspects. This results in an urgent need for more studies to investigate the effects of DiNP on female reproduction, especially in terms of consequences that occur after exposure during adulthood.

Thus, the goal of my doctoral dissertation work was to investigate the effects of short-term exposure to DEHP or DiNP during adulthood on female reproductive health at time points throughout the rest of the life of the mouse. Specifically, my doctoral dissertation work tested the hypothesis that 10 days of exposure to DEHP or DiNP during adulthood affects estrous
cyclicity, fertility, litter outcomes, ovarian hormones, peptide hormones, and ovarian follicular populations at time points throughout the life of the female CD-1 mouse. To test this hypothesis, I completed the following specific aims:

**Specific Aim 1: Determine if short-term exposure to DEHP and/or DiNP during adulthood alters estrous cyclicity, fertility, litter outcomes, and reproductive organ weights at time points during the prime reproductive life of the female CD-1 mouse.**

To complete this aim, I orally dosed adult (age 39-40 days) female CD-1 mice for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day). I investigated the effects of DEHP and DiNP on estrous cyclicity at 3 and 9 months post-dosing. Further, I performed breeding trials using untreated male CD-1 mice immediately post-dosing and 3 and 9 months post-dosing, wherein I assessed fertility of the dosed females and their litter outcomes. Lastly, separate groups of mice were dosed and cohorts were euthanized immediately post-dosing and 3 and 9 months post-dosing to examine changes in weight of reproductive organs. I found that multiple doses of DEHP decreased uterine weights and that DiNP disrupted estrous cyclicity at both tested time points, causing greater disruption than DEHP. Further, I found that DEHP and DiNP reduced overall fertility at 3 and 9 months post-dosing and that the lowest doses of DEHP and DiNP greatly reduced the ability of females to become pregnant at 3 months post-dosing. Treatment affected few litter outcomes, but multiple doses of DEHP and DiNP increased the male to female ratio of pups born at the 9 month post-dosing breeding trial. Collectively, these data show that short-term exposure to DEHP and DiNP during adulthood has
damaging effects on multiple aspects of female fertility throughout the prime reproductive life of the female CD-1 mouse. These data are presented in Chapter 3.

**Specific Aim 2: Determine if short-term exposure to DEHP and/or DiNP during adulthood alters ovarian follicle populations and circulating hormone levels at time points during the prime reproductive life of the female CD-1 mouse.**

To complete this aim, I orally dosed adult (age 39-40 days) female CD-1 mice for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day). Groups of mice were euthanized at different time points following completion of dosing, including immediately post-dosing and 3, 6, and 9 months post-dosing. Sera were collected and analyzed for levels of estradiol, progesterone, testosterone, FSH, and inhibin B. Ovaries were collected and histologically evaluated to assess numbers of different follicle types, percentages of different follicle types, percentages of unhealthy follicles, and the total number of follicles. I found that both DEHP and DiNP disrupted follicle numbers and percentages at multiple time points. Further, I found that DEHP and DiNP disrupted a variety of hormones at multiple time points and that the most severe effects were seen immediately post-dosing. Collectively, these data show that short-term exposure to DEHP and DiNP during adulthood has negative consequences on the ovary and levels of circulating hormones during the prime reproductive life of the female CD-1 mouse. These data are presented in Chapter 4.
Specific Aim 3: Determine if short-term exposure to DEHP and/or DiNP during adulthood alters estrous cyclicity, fertility, litter outcomes, ovarian follicle populations and circulating hormone levels at time points in the late-life of the female CD-1 mouse.

To complete this aim, I orally dosed adult (age 39-40 days) female CD-1 mice for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day). One group of mice was monitored for estrous cyclicity followed by a breeding trial at 12 and 15 months post-dosing to assess fertility and effects on litter outcomes. Further, additional groups of mice were euthanized at 12, 15, and 18 months post-dosing, and sera and ovaries were collected. Sera were analyzed for levels of estradiol, progesterone, testosterone, FSH, and inhibin B. Ovaries were histologically processed and evaluated to assess numbers of different follicle types, percentages of different follicle types, percentages of unhealthy follicles, and the total number of follicles. I found that DEHP disrupted estrous cyclicity at both time points tested. Further, I found that DEHP and DiNP increased pregnancy loss, decreased overall fertility, and decreased the percent of female pups born at 12 months post-dosing. Additionally, both DEHP and DiNP altered follicle populations and several hormones at many of the time points tested in this study. Collectively, these data show that short-term exposure to DEHP and DiNP during adulthood has long-term consequences that persist into late-life in the female CD-1 mouse. These data are presented in Chapter 5.

Taken together, my doctoral dissertation work shows that short-term insults such as exposure to DEHP or DiNP during adulthood has negative consequences on multiple aspects of female
reproduction. Further, my work shows that these effects can persist throughout the prime reproductive life of the female and that some effects remain after the transition into reproductive senescence and throughout late-life. Overall, my studies provide insight on the short-term and long-term consequences of transient exposure to DEHP and DiNP during adulthood on female reproduction.

In summary, Chapter 1 provides an overview of my doctoral dissertation work. Chapter 2 provides background information epidemiological evidence showing associations between adult exposure to a variety of environmental contaminants and reproductive outcomes in both men and women. Chapter 3 describes the work that tested the hypothesis that short-term exposure to DEHP and/or DiNP during adulthood alters estrous cyclicity, fertility, litter outcomes, and reproductive organ weights at time points during the prime reproductive life of the female CD-1 mouse. Chapter 4 describes the work that tested the hypothesis that short-term exposure to DEHP and/or DiNP during adulthood alters ovarian follicle populations and circulating hormone levels at time points during the prime reproductive life of the female CD-1 mouse. Chapter 5 describes the work that tested the hypothesis that short-term exposure to DEHP and/or DiNP during adulthood alters estrous cyclicity, fertility, litter outcomes, ovarian follicle populations and circulating hormone levels at time points in the late-life of the female CD-1 mouse. Lastly, Chapter 6 summarizes the findings of the work presented in this dissertation and suggests future studies and directions.
1.2 References


18. Hannon, P.R., J. Peretz, and J.A. Flaws, Daily exposure to Di(2-ethylhexyl) phthalate alters estrous cyclicity and accelerates primordial follicle recruitment potentially via


CHAPTER 2

Environmental Contaminants affecting Fertility and Somatic Health

2.1 Abstract

This review article summarizes the epidemiological findings published between 2011 and 2016 concerning bisphenol A (BPA), phthalates, dioxins, pesticides, air pollution, fracking chemicals, triclosan, and parabens and fertility parameters in men (i.e., semen volume, sperm concentration, sperm motility, sperm morphology) as well as fertility parameters in women (i.e., cyclicity, fertility treatment outcomes), pregnancy outcomes (i.e., preterm birth, miscarriage), and reproductive disorders (i.e., polycystic ovary syndrome, endometriosis, uterine fibroids). Overall, this review indicates that several environmental toxicants are significantly associated with reduced fertility parameters in men and women as well as several reproductive disorders in women. Although many studies report that the selected exposures are associated with adverse fertility outcomes, several studies report null associations. Thus, future studies are still needed to better elucidate the associations and potential mechanisms between these environmental chemicals and fertility outcomes in men and women.

2.2 Introduction

Humans are often exposed to a wide variety of chemicals in their everyday environments. This review focuses on epidemiological and clinical research conducted between 2011 and 2016 that investigates environmental chemicals and their associations with reproductive outcomes in both men and women (Figure 2.1 and 2.2). While the body of my doctoral dissertation work focuses on phthalate exposure in females and consequences of exposure during adulthood, humans are exposed to a wide variety of environmental chemical contaminants. Further, both males and females are affected by exposure to environmental contaminants. Thus, a variety of chemicals and chemical classes and their effects on both males and females are explored in this review. We chose to investigate bisphenol A (BPA), select phthalates, dioxins, pesticides, and air pollution because of the high rate of exposure of humans to these chemicals and their strong presence in the environment. We also chose to review fracking chemicals, triclosan, and parabens because of emerging literature and interest in the impact of these chemicals on reproductive outcomes. Several studies show contrasting results for the association between many environmental exposures and reproductive outcomes. Thus, this review summarizes positive, negative, and null associations.

2.3 BPA

BPA is a chemical used in a wide variety of products such as medical equipment, epoxy resins lining aluminum cans, polycarbonate plastics, and dental sealants [1-5]. Although BPA can be absorbed via inhalation, ingestion, and dermal contact, the most common route of exposure is via ingestion of food or beverages contaminated with BPA [1]. After exposure, BPA can be distributed throughout the body to various bodily fluids such as colostrum, ovarian follicular
fluid, breast milk, urine, semen, blood, and plasma [1, 5-7]. Due to BPA’s ability to interact with hormone receptors [2] and the extensive exposure of the general population to BPA, the associations between BPA exposure and reproductive outcomes have been widely investigated and the results are summarized below.

**Associations with Male Reproductive Outcomes**

BPA exposure has been associated with general male infertility [1, 6], but the results are equivocal [8-10] (Figure 2.1). High levels of BPA also have been associated with reduced sexual desire, increased erectile and ejaculatory difficulties, and increased severity of infertility [1, 6]. Further, seminal and urinary BPA levels have been negatively correlated with sperm density, total count, and concentration [1, 2, 6, 10, 11], motility [2, 6, 10, 12], and morphology [1, 6, 10, 11]. However, some studies reported no association between BPA exposure and these semen quality parameters [8, 10, 12]. In addition, although studies examining paternal exposure to BPA and assisted reproductive technologies (ART) are sparse, one study found that paternal urinary BPA levels were not associated with fertilization, embryo quality, implantation, or live birth [10]. Further, another study reported no association between paternal urinary BPA levels and couples fecundity [13].

**Associations with Female Reproductive Outcomes**

BPA exposure has been associated with fertility outcomes in women (Figure 2.2). Collectively, these studies have shown that elevated serum or urinary BPA levels were associated with anovulation [14], lower antral follicle counts [5, 15], and infertility [5]. Further, BPA exposure has been associated with miscarriage [16-18], shortened gestation [19], and preterm birth [2].
contrast, some studies report no association between urinary BPA levels and fecundity, time to pregnancy [5, 13], or spontaneous abortions [20].

As in males, BPA exposure has been associated with poor ART outcomes. Specifically, serum BPA levels were negatively associated number of oocytes retrieved, oocyte maturation, fertilization rates, as well as embryo quality in women undergoing in vitro fertilization (IVF) treatment [2, 5]. Additionally, urinary BPA levels were associated with a decrease in successful implantation rate in women undergoing IVF [2, 5]. However, some studies report null associations between urinary BPA levels and number of oocytes retrieved, embryo quality, and fertilization rates [5].

Several studies investigated BPA exposure and incidence of reproductive disorders such as polycystic ovarian syndrome (PCOS), endometriosis, and fibroids. The majority of these studies showed that women with PCOS had significantly higher levels of BPA in the blood [14, 21], serum [21, 22], and follicular fluid [22, 23] compared to women without PCOS. Similarly, women with endometriosis had higher levels of BPA than women without endometriosis [2, 24], and women with fibroids had higher levels of BPA than women without fibroids [25, 26]. However, some studies have found no associations between serum BPA concentrations and incidence of PCOS [27], endometriosis [28], or fibroids [29].

2.4 Phthalates

Phthalates are synthetic chemicals used in cosmetics, medications, plastic toys, and construction material. Phthalate exposure can occur via ingestion, inhalation, and dermal contact [3, 4, 30], and phthalate metabolites can be detected in over 99% of urine samples [30]. The phthalates
described here include di(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and benzylbutyl phthalate (BBP). DEHP is commonly found in materials such as surgical tubing and gloving as well as plastic food storage containers, roofing, and carpeting [31]. DEP and DBP are mostly found in personal care products such as lotions, nail polishes, and fragrances, whereas BBP is used in products such as vinyl flooring [32]. The associations between exposure to these phthalates and reproductive outcomes in men and women (Figure 2.1 and 2.2) are summarized below.

2.4.1 Di(2-ethylhexyl) Phthalate (DEHP)

Associations with Male Reproductive Outcomes

Several studies, but not all, have found that levels of DEHP or DEHP metabolites were associated with reduced fertility in men. Specifically, seminal DEHP levels and urinary levels of the DEHP metabolites mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), but not the DEHP metabolites mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), or mono-(2-carboxymethylhexyl) phthalate (MCMHP) have been associated with male infertility [13, 33, 34]. Further, serum concentrations of the sum of DEHP metabolites or the individual metabolites (MEOHP, MEHHP, and MECPP) have been significantly associated with decreased semen volume and sperm count [11, 35-38], sperm concentration [37, 39, 40], sperm motility [11, 36, 39-42], percent of morphologically normal sperm [36, 43], and acrosin activity [42]. In contrast, several studies report no associations between DEHP exposure and risk of subfertility [9], fecundity [13], sperm count, concentration, motility, or morphology [9, 35, 37-39, 43-45].
**Associations with Female Reproductive Outcomes**

Several studies have examined DEHP exposure and fertility in women. Overall, these studies have shown that urinary DEHP metabolites were significantly associated with decreased gestation length [19, 46] as well as increased gestational length [46], increased risk of preterm birth [30, 47, 48], and intra-uterine growth restriction [49]. Further, urinary DEHP metabolites were associated with reduced antral follicle counts, a potential indicator of subfertility [50]. However, some studies reported null associations when examining urinary levels of DEHP metabolites and fertility parameters such as time to pregnancy [51], infertility [34, 45], and fecundity [13, 34].

Similar to studies on BPA, DEHP exposure has been associated with ART outcomes. In women undergoing IVF, urinary levels of the sum of DEHP metabolites were significantly associated with reduced total oocyte yield and a reduced probability of achieving pregnancy and live birth [52]. In contrast, a Chinese study found no association between urinary MEHP levels and clinical pregnancy loss [53].

Additionally, some studies on DEHP have shown that urinary metabolites of DEHP were associated with reproductive disorders such as PCOS, endometriosis, and fibroids [2, 27, 46, 54]. In contrast, one study found that urinary levels of MEHP were inversely associated with endometriosis risk [55], and one study reported urinary levels of MEHP, MEHHP, or MEOHP were not associated with endometriosis [56]. Further, one study found that urinary levels of MEHP, MEOHP, MEHHP, and MECPP were not associated with uterine fibroids [25].
2.4.2 Diethyl Phthalate (DEP)

Associations with Male Reproductive Outcomes

A few studies have examined the associations between DEP exposure and fertility in men. Specifically, seminal levels of DEP and urinary levels of its metabolite monoethyl phthalate (MEP) have been significantly associated with male infertility [33, 45], reduced sperm count and concentration [37, 45], reduced sperm motility [37], and reduced numbers of morphologically normal sperm [36, 37, 45]. However, a few studies have found no associations between urinary levels of MEP and subfertility in men [9, 13], sperm count and concentration, sperm motility, sperm viability, and abnormal sperm morphology [9, 37, 43-45].

Associations with Female Reproductive Outcomes

Studies investigating DEP exposure and indicators of general fertility in women are scarce, but some studies have indicated that urinary MEP levels were significantly higher in females from infertile couples than females from fertile couples [45] and that urinary MEP levels were significantly associated with clinical pregnancy loss [53] and preterm delivery [57]. However, some studies have found no associations between urinary MEP concentrations and time to pregnancy [13, 51], clinical pregnancy loss [46], and preterm birth [46, 48]. Further, urinary levels of MEP were not associated with the probability of pregnancy or odds of live birth in women undergoing IVF or intra-cytoplasmic sperm injection (ICSI) [52].

Recent epidemiological studies investigating the associations between DEP exposure and reproductive disorders are limited in number, but one study reported that urinary concentrations of MEP were significantly associated with an increased incidence of PCOS [27]. Further, a few
studies report no associations between urinary MEP concentrations and endometriosis [46, 55] or uterine fibroids [25, 46].

2.4.3 Dibutyl Phthalate (DBP)

Associations with Male Reproductive Outcomes

A handful of studies have investigated the potential associations between DBP exposure and indicators of general fertility in men. These studies indicated that seminal levels of DBP [33] and urinary levels of one of its metabolites, mono-n-butyl phthalate (MnBP) [45], were significantly higher in infertile men when compared to fertile men. Further, urinary levels of MnBP, DBP, or another metabolite, monobutyl phthalate (MBP), were associated with lowered sperm concentration [37, 43-45, 58], reduced total sperm count [43], reduced sperm motility [37, 41, 44, 45, 58], reduced proportions of morphologically normal sperm [36, 42], and reduced acrosin activity [42]. Additionally, paternal urinary concentrations of MBP have been significantly associated with an increased time to pregnancy in couples [13]. Interestingly, some studies reported that urinary MnBP was significantly associated with increased semen volume and sperm concentration [45, 59]. In contrast, some studies show urinary MnBP was not associated with risk of subfertility [9] and that DBP exposure was not associated with sperm count, concentration, motility, or morphology [9, 37, 40-43, 45].

Associations with Female Reproductive Outcomes

Studies investigating the associations between DBP exposure and general indicators of female fertility are scarce. However, urinary MnBP levels were significantly higher in women from infertile couples than women from fertile couples [45], and urinary levels of MnBP from
pregnant mothers were significantly associated with clinical pregnancy loss [53]. Further, urinary MBP levels as well as cord blood concentrations of DBP were significantly associated with odds of preterm birth [30, 47, 48, 57]. Contrarily, some studies have found urinary MBP levels were not significantly associated with time to pregnancy [13, 51], gestational age [46], and pregnancy loss [46]. Similarly, one study reported no significant associations between urinary levels of MBP and probability of pregnancy or live birth in women undergoing IVF and live births resulting from ICSI [52].

Few studies have examined associations between exposure to DBP and PCOS, endometriosis, and fibroids. Interestingly, women with PCOS were more likely to have lower levels of urinary MBP than those without PCOS [27]. Further, urinary MBP levels were significantly associated with nearly a two-fold increase in odds of an endometriosis diagnosis [2], and urinary MnBP levels as well as plasma DBP levels were significantly associated endometriosis [46]. However, several studies report null associations between urinary MBP [46] and MnBP [46, 54, 55] and endometriosis. Similarly, studies consistently report no association between urinary MBP [46] or MnBP [25, 46] and uterine fibroids.

2.4.4 Benzylbutyl Phthalate (BBP)

Associations with Male Reproductive Outcomes

Few studies have examined the associations between exposure to BBP and fertility in men. These studies show that seminal levels of BBP were significantly higher in a group of infertile men when compared to a group of fertile men [33] and that paternal urinary levels of the metabolite monobenzyl phthalate (MBzP) were significantly associated with an increased time to
pregnancy [13], decreased sperm count and concentration [36, 42, 44], and reduced numbers of morphologically normal sperm [36, 42]. Interestingly, some studies reported that urinary MBzP levels were associated with higher semen volume [43, 59] as well as increased sperm concentration [45]. However, some studies reported no association between urinary MBzP and risk of subfertility [9, 45], semen volume, or sperm count, concentration, morphology, or motility [9, 37, 42-45].

Associations with Female Reproductive Outcomes

Very few recent studies have investigated the associations between BBP exposure and general indicators of female fertility. Although one study indicated that BBP exposure is associated with preterm birth [48, 57], some studies reported no association between urinary MBzP concentrations and risk of infertility [45], time to pregnancy [13, 51], and gestational age [46]. Further, urinary levels of MBzP were not associated with odds of pregnancy or live birth in women undergoing IVF or live births in women undergoing ICSI [52]. Finally, urinary MBzP levels were inversely associated with an increased odds of PCOS [27], but not with endometriosis [46, 54, 55] or uterine fibroids [25, 46].

2.5 Air Pollutants

Air pollution has significant harmful effects on environmental and human health worldwide. Inhalation exposure to air pollutants such as particulate matter (PM), ozone (O3), sulfur dioxide (SO2), nitrogen oxides (NOx), carbon monoxide (CO), polycyclic aromatic hydrocarbons (PAHs), and volatile organic compounds (VOCs) have been associated with various health problems, including reproductive disease [60-62].
Associations with Male Reproductive Outcomes

Several cross-sectional, longitudinal, and ecological studies have found that air pollution was significantly negatively associated with sperm quality, which may contribute to male-mediated infertility (Figure 2.1). For example, exposure to particulate matter 10 µM or less in size (PM10) was associated with increased sperm aneuploidy, DNA fragmentation, abnormal chromatin and sperm morphology, and decreased sperm motility [61]. Exposure to fine particulate matter less than 2.5 µM (PM2.5) was associated with sperm aneuploidy and decreased sperm motility, with a 2-3 month lag. Ambient O3 levels were significantly associated with low average sperm concentrations at all biologically relevant time points during spermatogenesis. Other gaseous pollutants such as SO2, NOx, and CO were associated with reduced sperm motility and kinetics and increased morphological abnormalities [61]. Further, traffic police and tollgate workers exposed to motor vehicle exhaust had an increased proportion of sperm with damaged chromatin and fragmented DNA compared to unexposed healthy men [62]. In contrast, one study found no associations between exposure to O3 or PM2.5 at levels below the current National Ambient Air Quality Standards and sperm quality parameters [61]. Similarly, although a few studies indicate that occupational exposure to the major components present in motor vehicle exhaust, including CO, NOx, O3, PM, VOCs and PAHs is associated with reduced sperm counts, total motility, progressive motility, and sperm kinetics, one study found that exposure to CO, NO2, and PM10 was not associated with sperm quality [62].

Associations with Female Reproductive Outcomes

Although limited information is available on air pollutants and female reproductive outcomes (Figure 2.2), one study indicated an association between residential proximity to major roads (as
an index of air pollution exposure) and preeclampsia, premature rupture of membrane, and preterm birth, but not gestational diabetes mellitus, placenta abruption, placenta previa, or preterm labor [63]. Similarly, exposure to ambient PM2.5 increased the risk of preterm birth, particularly if exposure occurred during early or mid-pregnancy [64]. Further, maternal exposure to emissions from municipal solid-waste incinerators throughout pregnancy was associated with preterm birth [65]. Ambient air pollution was also correlated spontaneous abortion [66] as well as decreased fecundability[67].

2.6 Dioxins

Dioxins and dioxin-like compounds (referred to from here on as dioxins) are a group of chemicals with similar structural and biological properties and include polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, and certain polychlorinated biphenyls. They are released into the environment as by-products of combustion (e.g., municipal and commercial waste incineration, burning of fuels, and forest fires), chemical manufacture (e.g., pesticide and herbicide production, and chlorine bleaching of paper and pulp), and metal industry operations. These compounds are highly stable, persistent pollutants in the environment that bioaccumulate in the food chain. Consumption of contaminated food and water is the major source of human exposure. Dioxins are a public health threat worldwide and have been associated with serious health issues, including reproductive problems [3, 68].

Associations with Male Reproductive Outcomes

Dioxin exposure has been associated with adverse male reproductive outcomes (Figure 2.1). Specifically, dioxin content in semen was associated with male infertility [69]. Further, 2,3,7,8-
tetrachlorodibenzo-p-dioxin (TCDD) content in semen was associated with abnormal spermograms (characterized by teratozoospermia, oligospermia, and asthenospermia)[69]. However, not all studies find associations between dioxin exposure and semen quality parameters or risk of subfertility in men [9].

**Associations with Female Reproductive Outcomes**

As in males, dioxin exposure in females has been associated with adverse reproductive outcomes (Figure 2.2). Maternal exposure to dioxins was associated with reduced fetal growth at intakes below the tolerable weekly intake of 14 picograms toxic equivalents/kilogram body weight/week [70]. However, the Seveso Women's Health Study, a retrospective cohort of TCDD exposure and reproductive health that assessed pregnancy outcomes over 30 years following a chemical explosion resulting in the highest known residential exposure to TCDD, found no significant associations between maternal exposure and spontaneous abortion, fetal growth, gestational length, or endometriosis [71]. Similarly, a Japanese study found no significant association between serum dioxin levels and endometriosis in infertile women diagnosed with the condition [72].

**2.7 Pesticides**

Pesticides, which include herbicides, insecticides, and fungicides play a major role in agriculture, horticulture, livestock production, and disease control. Their use has been associated with adverse effects on human and environmental health worldwide. Certain pesticides such as organochlorines are persistent pollutants in the environment and show bioaccumulation in the food web [73]. Exposure to pesticides and pesticide residues occurs through ingestion of
contaminated food, water, and soil, inhalation, as well as dermal and ocular contact. Pesticides are thought to act as endocrine disruptors and may be associated with reproductive problems [74].

**Associations with Male Reproductive Outcomes**

Collectively, studies show that various pesticide exposures have been associated with reduced sperm numbers, motility, or volume (Figure 2.1). Specifically, agricultural pesticide exposure was associated with a decline in semen quality, decreased sperm count, decreased sperm motility, and increased teratospermia [75]. Similarly, organophosphate pesticides were associated with reduced sperm motility, seminal volume, and increased sperm morphological abnormalities [75]. Further, occupational exposure to the herbicide 2,4-dichlorophenoxyacetic acid was associated with asthenospermia, necrospermia, and teratospermia [75]. Occupational exposure to the carbamate insecticide carbaryl was associated with low sperm counts and abnormal sperm morphology, and occupational exposure to dibromochloropropane was associated with male subfertility, oligospermia, and genetic alterations in sperm [74]. Exposure to organophosphate pesticides (dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate) was negatively associated with sperm concentration, total sperm count, and percentage of motile sperm [76]. Exposure to pyrethroid pesticides was positively associated with the percentage of medium sperm DNA fragmentation index and high sperm DNA fragmentation index [77] as well as abnormal sperm morphology and low sperm concentration [78]. Increased serum levels of oxychlordane (a metabolite of the organochlorine pesticide, chloradane) were associated with an increased risk of subfertility and decreased sperm concentration and motility [9]. Environmental exposures to dichlorodiphenyldichloroethylene
(DDE) and lindane were negatively associated with sperm motility [79], and DDE exposure was associated with increased sperm sex-chromosome disomy [80]. Finally, high plasma abamectin levels were associated with decreased sperm motility and sperm maturity [81].

**Associations with Female Reproductive Outcomes**

Similar to men, organochlorine pesticide exposures have been associated with adverse reproductive outcomes such as low fecundability, miscarriage, preeclampsia, intrauterine growth restriction, poor weight gain during fetal development, and preterm birth [73, 82](Figure 2.2). Further, organochlorine pesticide exposure has been associated with an increased risk of PCOS [22], endometriosis [83], and fibroids [84]. Interestingly, exposure to organochlorine pesticides was associated with shorter menstrual cycles, whereas exposure to non-organochlorine pesticides was associated with longer menstrual cycles or absence of cycles [14]. Similarly, exposure to the commonly used herbicide atrazine via drinking water was associated with menstrual cycle irregularity and longer follicular phases [85]. Finally, exposure to pyrethroids in women living in rural South Africa was negatively associated with plasma levels of anti-Müllerian hormone, a marker of ovarian reserve [86]. However, one study reported no association between glyphosate, another commonly used herbicide, and adverse reproductive effects at environmentally relevant concentrations [87].

### 2.8 Fracking Chemicals

Fracking chemicals are used and produced during the process of drilling and hydraulic fracturing for oil and gas manufacture. Fracking chemicals from oil and gas extraction, processing, transport, and disposal of wastewater contaminate the air, surface water, and ground water. Oral,
inhalation, and dermal exposure to these chemicals can occur through residential proximity or occupational exposure. Several fracking chemicals have been associated with endocrine disrupting activities and adverse reproductive outcomes [88, 89].

**Associations with Male Reproductive Outcomes**

A few studies reported that occupational exposure to fracking operations was associated with decreased sperm concentration and motility, and increased DNA damage [88](Figure 2.1). Further, exposures to fracking chemicals such as benzene, toluene, ethylbenzene, and xylene were associated with low sperm count, motility, and viability, abnormal sperm morphology, and abnormal semen viscosity [90].

**Associations with Female Reproductive Outcomes**

Several studies have reported a significant association between maternal exposure to fracking operations and preterm birth as well as miscarriage, but not the incidence of stillbirths [88](Figure 2.2). Further, the fracking chemical toluene was associated with reduced fecundity, difficulty to conceive and become pregnant, conception failure, miscarriage, and premature menopause [90].

**2.9 Triclosan**

Triclosan is a phenolic compound with antimicrobial activity that is commonly used in products like soaps, disinfectants, and toothpaste [8]. Exposure to triclosan occurs via oral, inhalation, and dermal routes [18]. Research on triclosan is sparse and epidemiological data regarding its association with infertility are lacking (Figure 2.1 and 2.2).
Associations with Male Reproductive Outcomes

A case control study which examined whether exposure to various endocrine disrupting chemicals was associated with subfertility in men found no associations between triclosan and semen quality parameters or risk of subfertility [9]. Another case control study which examined the relationship between exposure to phenols and idiopathic male infertility found that urinary triclosan concentrations were not significantly associated with idiopathic male infertility [8].

Associations with Female Reproductive Outcomes

Little information is available about the relationship between triclosan and female fertility. However, a retrospective time to pregnancy study in women recruited from the Maternal-Infant Research on Environmental Chemicals (MIREC) Study suggested that elevated urinary triclosan levels may be associated with decreased fecundity [51].

2.10 Parabens

Parabens are a group p-hydroxybenzoic acid esters that are commonly found in cosmetics as preservatives. They can also be found in foods where they are used as antimicrobials to help preserve foods and increase shelf life. Humans are most commonly exposed to parabens via dermal contact or ingestion [91]. Studies investigating the potential associations between parabens and fertility outcomes in men and women are sparse (Figure 2.1 and 2.2).

Associations with Male Reproductive Outcomes

Although limited information exists on parabens and male reproductive outcomes, one study reported that paternal urinary methylparaben concentrations were associated with a decrease in
live birth after intra-uterine insemination (IUI) [92]. In contrast, paternal urinary concentrations of methyl-, propyl- or butylparaben were not associated with fertilization rate, embryo quality, or odds of implantation in couples who underwent IVF [92]. Similarly urinary concentrations of methyl-, propyl-, and butylparaben were not associated with sperm concentration or motility [93].

**Associations with Female Reproductive Outcomes**

Few epidemiological studies have investigated parabens and female reproductive health. One such study found no significant associations between urinary concentrations of methyl-, propyl-, or butylparaben with total and mature oocyte yields, embryo quality, fertilization rates, implantation, clinical pregnancy, or live birth rates in women undergoing IVF [94].

### 2.11 Conclusions

The average person is exposed to environmental endocrine disruptors daily due to their ubiquitous presence in the general environment as well as their use in a wide variety of consumer products. Many of the studies published during the past five years demonstrate significant negative associations between exposure to the selected chemicals and reproductive outcomes in both men and women. In general, BPA exposure was associated with several negative reproductive factors in men and women with relative consistency. Similarly, dioxins and pesticides were significantly associated with reduced fertility parameters in men and women. However, not all studies reported significant associations between chemical exposures and reproductive outcomes in humans. These conflicting results could be due to the high variability of study population, sample size, methods of measuring exposure levels, as well as the
reproductive outcomes measured between studies. Thus, further studies are needed to clarify associations between exposures to these chemicals as well as further define the possible mechanisms through which these chemicals act.
A brief summation of epidemiological research investigating endocrine disruptors and their associations with aspects of infertility in men.
Figure 2.2 Associations with female reproductive outcomes

A brief summation of epidemiological research investigating endocrine disruptors and their associations with aspects of infertility and reproductive disease in women.
2.13 References


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CHAPTER 3

Subchronic Exposure to Di(2-ethylhexyl) Phthalate and Diisononyl Phthalate During Adulthood Has Immediate and Long-Term Reproductive Consequences in Female Mice

3.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in a wide variety of consumer products. This is concerning because DEHP is an endocrine disruptor and ovarian toxicant. Diisononyl phthalate (DiNP) is a DEHP replacement that is a rising human toxicant due to its increased use as a DEHP substitute. However, little is known about the effects of DEHP or DiNP exposure during adulthood on female reproduction. Thus, this study tested the hypothesis that DEHP or DiNP exposure during adulthood has long-term consequences for female reproduction in mice. Adult female CD-1 mice (39-40 days) were orally dosed with vehicle control (corn oil), DEHP (20 µg/kg/day - 200 mg/kg/day), or DiNP (20 µg/kg/day - 200 mg/kg/day) for 10 days. Females were paired with untreated male mice for breeding trials immediately post-dosing and again at three and nine months post-dosing. Immediately post-dosing, DEHP and DiNP did not affect fertility. At three months post-dosing, DiNP (20 and 100 µg/kg/day and 200 mg/kg/day) significantly disrupted estrous cyclicity, and DiNP and DEHP (20 µg/kg/day) significantly reduced the ability of females to get pregnant. At nine months post-dosing, DiNP significantly disrupted estrous cyclicity (100 µg/kg/day), reduced time to mating (100 µg/kg/day – 200 mg/kg/day), and borderline reduced percent of females who produced offspring (20 mg/kg/day). At nine months post-dosing, DEHP (200 µg/kg/day and 200 mg/kg/day) and DiNP (100

µg/kg/day and 20 and 200 mg/kg/day) increased numbers of male-biased litters. These data show that DEHP and DiNP exposure has long-term consequences for female reproduction, even long after cessation of exposure.

3.2 Introduction

Phthalates are chemicals commonly used as plasticizers and stabilizers in a wide variety of consumer products. One of the most common phthalates is di(2-ethylhexyl) phthalate (DEHP), with up to 100 million pounds produced within the U.S. each year [1, 2]. DEHP can be found in products such as surgical tubing, blood bags, building materials, clothes, and baby toys [1]. Because DEHP is noncovalently bound to the products it is used in, it can leach out over time and be absorbed via inhalation, ingestion, and dermal contact [1, 3, 4]. Due to the ubiquitous nature of DEHP environmental contamination, the estimated average daily intake of DEHP is 3-30 µg/kg/day [1].

Widespread and daily exposure of humans to DEHP is concerning because DEHP is a known reproductive toxicant and endocrine disrupting chemical [5-8]. DEHP exposure has been shown to reduce testosterone levels and decrease sperm quantity and quality in mice [9, 10] and it is associated with decreased sperm concentration and motility in humans [11, 12]. Further, DEHP exposure disrupts ovarian folliculogenesis and steroidogenesis in female mice [13, 14] and it is associated with preterm delivery in women and premature thelarche in girls [15, 16].

Possibly due to increased public awareness of the endocrine disrupting effects of DEHP, some manufacturers have elected to substitute DEHP with other plasticizers. Even with some
manufacturers replacing DEHP, the general population is still exposed to DEHP daily, and some populations are exposed to even higher levels due to occupation or medical treatment [17-20]. However, few studies have examined how DEHP affects female reproductive parameters overtime. Thus, it is critical to fill this gap in knowledge.

Although the movement to replace DEHP with a less toxic plasticizer is a step in the right direction, often these substitute chemicals have not been rigorously tested for toxicity, especially in regards to endocrine disruption. Diisononyl phthalate (DiNP) is one such understudied DEHP substitute. Few reproductive studies using DiNP have been conducted, but some studies have found that perinatal exposure to DiNP causes histopathological changes in the testes, and prenatal exposure to DiNP disrupts steroidogenesis in the testes in a similar manner to DEHP in rats [21-26]. In men, DiNP exposure has been associated with reduced testicular volume, semen volume, and testosterone levels [27-29].

Even fewer studies have been conducted on the effects of DiNP on female reproduction. One study found that DiNP reduced corpora lutea number and ovarian and uterine weight at puberty in rats [21]. Although these studies have shed some light on the effects of DiNP on reproduction, they often use doses well above the levels of human exposure. It is imperative to use environmentally relevant doses because effects may not be seen at these higher doses due to endocrine disrupting chemicals sometimes exhibiting non-monotonic dose response curves [30].

In this study, we aim to fill the gaps in knowledge concerning the short-term and long-term reproductive consequences of sub-chronic exposure to DEHP and DiNP in female mice. A
previous study in our laboratory has shown that 10 days of exposure to DEHP during adulthood is sufficient to alter folliculogenesis in female mice [31]. Thus, this study tested the hypothesis that 10 days of exposure to DEHP or DiNP during adulthood can significantly affect female fertility at time points immediately post-dosing, three months post-dosing, and nine months post-dosing.

3.3 Materials and Methods

Chemicals

DEHP and DiNP were purchased from Sigma-Aldrich (St. Louis, MO). Corn oil (vehicle control) was purchased from MP Biomedicals (Solon, OH). Dosing solutions were created by using the highest concentration dose of stock solution (200 mg/kg/day for both DEHP and DiNP) in serial dilutions to make lower dose stock solutions (20 µg/kg/day, 200 µg/kg/day, and 20 mg/kg/day DEHP and 20 µg/kg/day, 100 µg/kg/day, and 20 mg/kg/day DiNP). Stock solutions for DEHP doses were prepared at concentrations of 133.33, 13.33, 0.13, and 0.013 mg/mL from the highest to lowest dose. Stock solutions for DiNP doses were prepared at concentrations of 133.33, 13.33, 0.067, and 0.013 mg/mL from the highest to lowest dose.

These doses for DEHP were chosen because 20 µg/kg/day is the EPA reference dose and also falls within the range of estimated daily exposure to DEHP (3-30 µg/kg/day) [32, 33]. The 200 µg/kg/day DEHP dose falls within the estimated range for occupational exposure (143-286 µg/kg/day) [34]. The 20 and 200 mg/kg/day doses of DEHP were chosen because they were used in previous studies and provide a wide range to aid in observation of dose response effects [31, 35]. The doses for DiNP were chosen because 20 µg/kg/day falls within the range of
occupational exposure to DiNP (up to 26 µg/kg/day) [36]. The 100 µg/kg/day dose was chosen because it falls within the range of estimated daily exposure for infants chewing on plastic toys (up to 260 µg/kg/day) [37]. The 20 and 200 mg/kg/day doses were chosen because they allow for direct comparison of toxicity to DEHP throughout the study.

**Animals and Dosing**

Adult CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and were allowed to acclimate to the facility prior to the beginning of their dosing at age 39-40 days. Mice were given *ad libitum* access to reverse osmosis treated water and Teklad Rodent Diet (8604). Facilities were maintained at 21.1 ± 2.2˚C with humidity at 50 ± 20% and 12 hour light-dark cycles to provide a consistent and controlled housing environment. All animal handling and procedures used in this study were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (Protocol No.: 17079).

Mice were orally dosed with the vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, or 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 µg/kg/day, 20 mg/kg/day, or 200 mg/kg/day) daily for 10 consecutive days every morning (two hours following the start of the light cycle). Volume of dose was determined using a spreadsheet to find the corresponding volume given the weight of the mouse immediately before dosing each day. Mice were group housed three to a cage during the dosing period, with every mouse in a single cage receiving the same treatment. A single cohort of mice was designated for breeding trials and estrous cyclicity monitoring and this cohort was used continuously throughout the study at every time point. The sample size for this cohort was 24 mice in control and 12 mice for every dose of
DEHP and DiNP for every time point unless data were missing for any mouse/mice. For tissue collection, each time point used a separate cohort of mice. The sample size for these cohorts of mice designated for tissue collection was 12 mice in control and 6 mice for every dose of DEHP and DiNP for every time point unless data were missing for any mouse/mice.

_Estrous Cyclicity Monitoring_

Mice were group housed (three/cage) and vaginally lavaged with 1X phosphate-buffered saline daily (two hours following the start of the light cycle) for 14 days prior to each breeding trial occurring at three and nine months post-dosing (n = 12-24 mice/group per time point unless data were unavailable for any mouse/mice). The stage of the estrous cycle was determined by examining the lavage sample under a light microscope. The type, amount, and condition of the cells present was used to determine if the mouse was in estrus, metestrus, diestrus, or proestrus as previously defined [38]. Metestrus and diestrus were combined in the analyses because of their similarity in behavioral and hormonal profiles. Percent time spent in each stage of the cycle was determined by taking the number of days in each stage (proestrus, estrus, and metestrus/diestrus) divided by 14 and multiplied by 100.

_Breeding Trials and Fertility Indices_

Females were paired with an untreated CD-1 male mouse (age seven weeks) in a harem fashion, with two females plus a male in a new, clean cage. Females were checked in the morning and afternoon for the presence of copulatory plugs. If a copulatory plug was observed, the female was removed and placed in a new, clean cage. Females were group housed until 3-7 days before predicted birth. If no copulatory plug was observed, the female remained with the male until a
maximum of 14 days had passed. If a plug was found the morning immediately following the
day of male introduction, the time to mating was calculated as one day. From the time of pairing
until birth, females were weighed twice a week to monitor weight gain due to pregnancy. If a
weight gain of four grams was observed, the female was considered pregnant. Females were
checked morning and afternoon for birth. To calculate the length of gestation, the first day the
copulatory plug was observed was considered day 0.5 of the pregnancy. The fertility index was
calculated by dividing the number of pregnant females by the number of females who presented
with a copulatory plug and multiplying by 100. The gestational index was calculated by dividing
the number of females who gave birth to pups by the number of pregnant females and
multiplying by 100. The index for females who gave birth was calculated by dividing the
number of females who gave birth to pups by the number of total females in each treatment
group and multiplying by 100. If a female gave birth, the first day pups were observed was
considered to be postnatal day (PND) 0, and total pup number, number of females and males,
number of dead pups, and average weight of all live pups was recorded. Male to female ratio of
pups was calculated by dividing the number of male pups by the number of female pups. Pup
mortality was calculated by dividing the number of pups who died by the total number of pups in
the litter and multiplying by 100. Average live weight was measured by collectively weighing
all live pups on PND 0 and dividing the total weight by the number of live pups. If the number
of total pups or the number of males or females could not be determined due to cannibalism, the
data for these parameters were not used for that litter.
Tissue Collection

Mice were group housed (three/cage) until each cohort was collected immediately following dosing, three months following dosing, and nine months following dosing. Mice were euthanized via CO₂ asphyxiation in the diestrous stage of the estrous cycle. Ovaries were collected and the gonadal fat was trimmed away before weighing. The uteri were collected with the oviducts and the cervix attached and fat was trimmed away before weighing. The livers were collected and weighed for each animal.

Statistical Analysis

Data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). Data were presented as means ± standard error or as percentages. If data were normally distributed and met the assumption of homogeneity of variances, means were compared using a one-way analysis of variance (ANOVA) followed by Dunnett’s T test post hoc. If the data did not meet the statistical assumptions required for ANOVA, they were analyzed via Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U tests. Nominal data were analyzed using a 1-sided Fisher’s exact test. Statistical significance was assigned at p ≤ 0.05.

3.4 Results

Effects of DEHP and DiNP on body weight and organ weights

Immediately post-dosing, 200 µg/kg/day and 20 mg/kg/day DEHP significantly decreased uterine weight compared to control (Table 3.1, n = 6-12 mice/group, p ≤ 0.05). Additionally, 200 mg/kg/day DEHP borderline decreased uterine weight (Table 3.1, n = 6-12 mice/group, p = 0.08). No effects of phthalate treatment were seen for body weight, ovarian weight, or liver
weight at this time point. Additionally, no doses of DEHP or DiNP affected body weight, ovarian weight, uterine weight, or liver weight at three (Table 3.1, n = 5-12 mice/group) or nine months post-dosing (Table 3.1, n = 5-12 mice/group).

Effects of DEHP and DiNP on estrous cyclicity

At three months post-dosing, DEHP did not significantly affect the time spent in any stage of the estrous cycle. However, the 200 mg/kg/day dose of DEHP borderline decreased the time spent in proestrus compared to control (Fig. 3.1A, n = 12-22 mice/group, p = 0.09). In contrast, 20 µg/kg/day and 200 mg/kg/day DiNP significantly decreased the time spent in proestrus, and 100 µg/kg/day and 200 mg/kg/day DiNP significantly increased the time spent in metestrus and diestrus compared to control (Fig. 3.1A, n = 12-22 mice/group, p ≤ 0.05). At nine months post-dosing, DEHP did not significantly affect the time spent in any stage of the estrous cycle (Fig. 3.1B, n = 11-22 mice/group), but 100 µg/kg/day DiNP significantly increased the time spent in metestrus and diestrus compared to control (Fig. 3.1B, n = 11-22 mice/group, p ≤ 0.05).

Effects of DEHP and DiNP on time to mating

DEHP and DiNP did not significantly affect the time to mating following introduction of the male into the cage for breeding trials immediately post-dosing (Fig. 3.2A, n = 7-21 mice/group) or three months post-dosing compared to control (Fig. 3.2B, n = 7-18 mice/group). However, at nine months post-dosing, 200 mg/kg/day DiNP significantly decreased time to mating (p ≤ 0.05) and 100 µg/kg/day and 20 mg/kg/day DiNP borderline decreased time to mating compared to control (Fig 3.2C, n = 10-20 mice/group, 0.05 < p ≤ 0.10).
Effects of DEHP and DiNP on fertility index

DEHP and DiNP did not significantly affect the ability of females to become pregnant after a successful mating immediately post-dosing (Fig. 3.3A, n = 12-23 mice/group) or at nine months post-dosing compared to control (Fig. 3.3C, n = 10-22 mice/group). At three months post-dosing, however, the lowest doses of DEHP (20 µg/kg/day) and DiNP (20 µg/kg/day) significantly reduced the ability of females to become pregnant compared to control (Fig. 3.3B, n = 11-22 mice/group, p ≤ 0.05). Approximately half of successfully mated females treated with 20 µg/kg/day DEHP and one third of females treated with 20 µg/kg/day DiNP were unable to achieve pregnancy, whereas 95% of females achieved pregnancy in the control group (Fig. 3.3B, n = 11-22 mice/group).

Effects of DEHP and DiNP on gestational index

Treatment did not affect the gestational index. Specifically, DEHP and DiNP did not significantly affect the ability of females to carry their pregnancy to term immediately post-dosing (Fig. 3.4A, n = 11-23 mice/group), three months post-dosing (Fig. 3.4B, n = 6-21 mice/group), or nine months post-dosing compared to control (Fig. 3.4C, n = 6-18 mice/group).

Effects of DEHP and DiNP on gestation length

Immediately post-dosing, 200 mg/kg/day DiNP borderline increased length of gestation compared to control (Fig. 3.5A, n = 8-20 mice/group, p = 0.07). DEHP and DiNP did not significantly affect length of gestation at three months post-dosing (Fig. 3.5B, n = 3-17 mice/group) or at nine months post-dosing compared to control (Fig. 3.5C, n = 6-14 mice/group).
Effects on DEHP and DiNP on the ability of females to produce pups

Immediately post-dosing, DEHP and DiNP did not significantly affect the overall ability of females to produce offspring compared to control (Fig. 3.6A, n = 11-23 mice/group). Strikingly, after three months post-dosing, females in the lowest treatment groups (20 µg/kg/day) for DEHP and DiNP had a significantly impaired ability to give birth to pups compared to control (Fig. 3.6B, n = 11-22 mice/group, p ≤ 0.05). Additionally, at nine months post-dosing, females in the 20 mg/kg/day DiNP group had a borderline impaired ability to produce offspring compared to control (Fig. 3.6C, n = 11-22 mice/group, p = 0.07).

Effects of DEHP and DiNP on litter size and average live pup weight

DEHP and DiNP did not significantly affect litter size immediately post-dosing (Fig. 3.7A, n = 8-19 mice/group), three months post-dosing (Fig. 3.7B, n = 5-18 mice/group), or nine months post-dosing compared to control (Fig. 3.7C, n = 4-14 mice/group). Immediately post-dosing, the average live weight of pups on PND 0 was significantly increased by treatment with 20 mg/kg/day DEHP compared to control (Fig. 3.7D, n = 8-19 mice/group, p ≤ 0.05). At nine months post-dosing, treatment with 20 mg/kg/day DiNP borderline increased average live weight of pups on PND 0 compared to control (Fig. 3.7F, n = 4-15 mice/group, p = 0.06). DEHP and DiNP did not significantly affect the average live weight of pups on PND 0 at three months post-dosing compared to control (Fig. 3.7E, n = 5-16 mice/group).

Effects of DEHP and DiNP on pup mortality and sex ratio

DEHP and DiNP did not significantly affect pup mortality immediately post-dosing (Fig. 3.8A, n = 9-19 mice/group), three months post-dosing (Fig. 3.8B, n = 5-20 mice/group), or nine months
post-dosing compared to control (Fig. 3.8C, n = 3-13 mice/group). Additionally, DEHP and DiNP did not significantly affect the male to female ratio of pups immediately post-dosing (Fig. 8D, n = 9-18 mice/group) or at three months post-dosing compared to control (Fig. 3.8E, n = 4-14 mice/group). However, at nine months post-dosing, treatment with 20 and 200 mg/kg/day DEHP and 100 µg/kg/day DiNP significantly increased the male to female ratio of pups compared to control (Fig. 3.8F, n = 4-11 mice/group, p ≤ 0.05). Further, treatment with 20 and 200 mg/kg/day DiNP borderline significantly increased the ratio of male to female pups at nine months post-dosing compared to control (Fig. 3.8F, n = 4-11 mice/group, 0.05 < p ≤ 0.10).

3.5 Discussion

In this study, we investigated how sub-chronic exposure to DEHP or DiNP during adulthood affected female fertility. Previously, our research group showed that an acute exposure to DEHP (10 days) was sufficient to accelerate ovarian folliculogenesis immediately post-dosing and disrupt estrous cyclicity and steroidogenesis up to nine months post-dosing [31, 35]. The present study builds upon this knowledge by examining fertility at additional time points and testing the reproductive toxicity of a common DEHP replacement, DiNP. This study shows that exposure to DEHP and DiNP has negative impacts on female reproductive parameters several months after exposure has stopped. These negative impacts include changes in reproductive organ weights, disruption of estrous cyclicity, reduction in fertility, changes in mating behavior, and changes in litter outcomes.

Immediately post-dosing, 20 mg/kg/day DEHP significantly increased the average live weight of pups at PND 0 without concurrently decreasing litter size. DEHP has been shown to be
obesogenic [39]. Thus, it is possible that DEHP exposure increases adiposity and subsequently, birth weight of pups. Our finding contrasts with epidemiological data that find associations between phthalate exposure and low birth weight [40, 41]. The differences between the current study and epidemiological data may be due to different exposure times and species differences.

Immediately post-dosing, some doses of DEHP, but not DiNP, significantly decreased uterine weight. In a previous study in our laboratory, we did not observe any effects of DEHP exposure for 10 days on uterine weight [35]. This discrepancy may be due to animals being housed in groups for the current experiment and singly in the previous study. Group housing is known to suppress estrus in rodents, which may cause lower estradiol and subsequently, lower uterine weight [42, 43]. Additionally, DEHP has been shown to disrupt ovarian steroidogenesis in vivo and in vitro; thus, the observed decrease in uterine weight in this study may also be mediated by DEHP-induced decrease of estradiol [7]. Therefore, it is possible that the DEHP-induced reduction in uterine weight in this study was only statistically detectable once animals were group housed, causing them to be in a lower estrogen state than in the previous study.

Three months post-dosing, some doses of DiNP significantly disrupted estrous cyclicity compared to controls. At one dose, these effects persisted for as long as nine months following dosing. It is possible that DiNP may be affecting levels of sex steroid hormones which dictate the estrous cycle in the mouse [44]. DiNP has been shown to disrupt steroid hormone production in porcine granulosa cell cultures, indicating that DiNP maybe be targeting ovarian steroidogenesis and subsequently disrupting estrous cyclicity [45]. Other studies have found that perinatal exposure to DiNP does not affect estrous cyclicity during adulthood in rats [21, 46].
The difference in estrous cyclicity outcomes between these previous studies and the present study may be due to the use of different animal models as well as exposure windows. Previous studies assessed cyclicity at postnatal week (PNW) 8-11 or at PNW 8-9 and again at 19-20, whereas the mice in the present study were first monitored at approximately 19 weeks of age and again at approximately 44 weeks of age.

At three months post-dosing, we observed that treatment with 20 µg/kg/day of DEHP or DiNP, the lowest dose used in this study, significantly reduced the fertility index of females compared to control. A reduction in the fertility index indicates a reduction in the ability of females to become pregnant from a confirmed mating. Additionally, this significant reduction in the fertility index led to a significant reduction in the percent of females who produced pups. Because the affected females had complete infertility as opposed to reduced fertility (e.g. smaller litter sizes), it is possible that the underlying mechanism involves disruption of global uterine receptivity to implantation. Proper regulation of sex steroid hormones is necessary for the opening of the “implantation window,” the narrow time frame during which the uterus is receptive to embryo implantation [47]. In fact, DEHP has been shown to decrease implantation rates in mice by decreasing endometrial receptivity to the embryo [48]. However, one study found that rats exposed to DiNP via the diet prior to mating and during mating had no difficulty achieving pregnancy and producing pups [49]. The differences observed between this past study and the present study may be due to species differences as well as the use of different doses in these studies.
At nine months post-dosing, 200 mg/kg/day DiNP significantly reduced time to mating and 100 µg/kg/day and 20 mg/kg/day DiNP borderline reduced time to mating. The effects of DiNP on time to mating reported in the current study appear to be novel, as most studies investigate developmental exposure windows and do not measure time to mating as a fertility parameter. Sex steroid hormones are necessary for maintenance of proper reproductive behavior; thus, it is possible that DiNP disrupts circulating hormones, particularly estradiol and progesterone, in a manner that increases female sexual receptivity to males [50]. Although treatment did not affect the fertility index or gestational index of females at nine months post-dosing, 20 mg/kg/day DiNP borderline decreased the percent of females who gave birth. It is hard to specifically assign the cause of the DiNP-induced reduction in overall fertility to a reduction in ability to achieve pregnancy or ability to carry a pregnancy to term because neither the fertility index nor gestational index were statistically different from controls. It is likely that 20 mg/kg/day DiNP reduced both of these indices enough to cause a cumulative effect that can be seen in the reduction of percent of females who gave birth, but not enough to be statistically detectable for either of the individual fertility indices. It is possible that this overall reduction in fertility could be the beginning of the onset of reproductive senescence in these mice. Previous studies have shown that phthalate exposure has been associated with premature menopause in women as well as increased markers of reproductive aging in female mice [13, 31, 51].

At nine months post-dosing, 200 µg/kg/day and 200 mg/kg/day DEHP and 100 µg/kg/day DiNP significantly increased the male to female ratio of pups, and 20 and 200 mg/kg/day borderline increased the male to female ratio of pups. We find this result particularly interesting because it is unusual for preconception exposure to alter sex ratio, especially in females which are not the
heterogametic sex in mice. However, the present study is not the first to find that treatment of females can result in altered offspring sex ratio. Multiple animal-based studies have found that females fed diets supplemented with fats produce male-biased litters [52, 53]. One hypothesis that has existed for decades is that hormonal milieu affects offspring sex ratio in humans [54]. Supporting this hypothesis, one study found that women with fat deposition patterns indicative of higher testosterone were significantly more likely to produce male offspring than their counterparts with fat deposition patterns indicative of lower testosterone levels [55]. We speculate that the observed sex selection occurs at the level of the sperm and vaginal mucosa because of evidence that Y-bearing sperm are smaller and more motile and are subsequently able to navigate through cervical mucus faster than their X-bearing counterparts [56]. Because hormones are known to have a significant impact on mucus production and viscosity within the female reproductive tract, it is possible that DEHP and DiNP may be disrupting hormone levels, leading to a change in the mucosal environment of the female reproductive tract that selects favorably for Y-bearing sperm [57]. Further, we do not think the sex selection occurs at the level of the embryo because we did not observe any changes in litter size that would suggest altered resorption of embryos.

In conclusion, this study found that DEHP and DiNP disrupt several aspects of female fertility at time points up to nine months after cessation of exposure. Many of the results observed in this study may be due to disruption of the hormonal milieu due to treatment with DEHP or DiNP. Thus, future studies should investigate the effects of DEHP and DiNP on sex steroid hormones and gonadotropins. Additionally, future studies should investigate fertility at time points even further out from nine months post-dosing to follow females through their transition to
reproductive senescence and elucidate any effects of these phthalates on the reproductive lifespan. Collectively, these findings suggest that phthalates, which are ubiquitous environmental contaminants that humans are exposed to daily, may have long lasting consequences on the female reproductive system.
### 3.6 Table, Figures, and Legends

**Table 3.1 Effects of DEHP and DiNP on body weight and organ weights**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Treatment</th>
<th>Body Weight (g)</th>
<th>Ovary Weight (g)</th>
<th>Uterine Weight (g)</th>
<th>Liver Weight (g)</th>
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<tr>
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<td>Control</td>
<td>24.17 ± 0.55</td>
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<td>0.1050 ± 0.0065</td>
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<td>0.0767 ± 0.0036 *</td>
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<td>Immediately Post-Dosing</td>
<td>Control</td>
<td>30.95 ± 0.48</td>
<td>0.0212 ± 0.0002</td>
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<td>31.82 ± 1.76</td>
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<td>0.0210 ± 0.0017</td>
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<td>3 Months Post-Dosing</td>
<td>Control</td>
<td>48.78 ± 1.71</td>
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<td>2.0953 ± 0.0656</td>
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<td>200 µg/kg/day DEHP</td>
<td>44.50 ± 3.11</td>
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<td>20 mg/kg/day DEHP</td>
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<td>100 µg/kg/day DiNP</td>
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</tbody>
</table>

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were euthanized in diestrus either immediately post-dosing (n = 6-12 mice/group), 3 months post-dosing (n = 5-12 mice/group), or 9 months post-dosing (n = 4-12 mice/group) and body weight and organ weights were measured. Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 3.1 Effects of DEHP and DiNP on estrous cyclicity

A  Cyclicity at 3 Months Post-Dosing

B  Cyclicity at 9 Months Post-Dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were vaginally lavaged daily for two weeks to assess estrous cyclicity three months post-dosing (n = 12-22 mice/group) and again nine months post-dosing (n = 11-22 mice/group). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 3.2 Effects of DEHP and DiNP on time to mating

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing (Panel A, n = 7-21 mice/group), three months post-dosing (Panel B, n = 7-18 mice/group), and nine months post-dosing (Panel C, n = 10-20 mice/group) and checked every morning and afternoon until a copulatory plug was observed or until 14 days had elapsed. Time to mating was calculated as the days between male introduction and observation of a copulatory plug. Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing (Panel A, n = 12-23 mice/group), three months post-dosing (Panel B, n = 11-22 mice/group), and nine months post-dosing (Panel C, n = 10-22 mice/group). The fertility index was calculated by dividing the number of females who successfully achieved pregnancy by the number of females who presented with a copulatory plug and multiplying by 100. Data are represented as percentages. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing (Panel A, n = 11-23 mice/group), three months post-dosing (Panel B, n = 6-21 mice/group), and nine months post-dosing (Panel C, n = 6-18 mice/group). The gestational index was calculated by dividing the number of females who gave birth to pups by the number of females who successfully achieved pregnancy and multiplying by 100. Data are represented as percentages.
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing (Panel A, n = 8-20 mice/group), three months post-dosing (Panel B, n = 3-17 mice/group), and nine months post-dosing (Panel C, n = 6-14 mice/group). Gestation length was calculated by using the day a copulatory plug was observed as day 0.5 of pregnancy. Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing (Panel A, n = 11-23 mice/group), three months post-dosing (Panel B, n = 11-22 mice/group), and nine months post-dosing (Panel C, n = 11-22 mice/group). The percent of females who gave birth was calculated by dividing the number of females who produced pups by the total number of females in that treatment group and multiplying by 100. Data are represented as percentages. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing, three months post-dosing, and nine months post-dosing. Live and dead pups were counted to determine the litter size on PND 0 (Panels A, B, and C, n = 8-19 mice/group, n = 5-18 mice/group, and n = 4-14 mice/group, respectively). Live pups were weighed collectively and an average was taken on PND 0 to determine the average live weight (Panels D, E, and F, n = 8-19 mice/group, n = 5-16 mice/group, and n = 4-15 mice/group, respectively). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil), DEHP (20 µg/kg/day – 200 mg/kg/day), or DiNP (20 µg/kg/day – 200 mg/kg/day). Females were mated with untreated male mice for breeding trials immediately post-dosing, three months post-dosing, and nine months post-dosing. Pup mortality was calculated by dividing the number of dead pups by the total number of pups within the litter and multiplying by 100 (Panels A, B, and C, n = 9-19 mice/group, n = 5-20 mice/group, and n = 3-13 mice/group, respectively). The male to female ratio was calculated by dividing the number of male pups by the number of female pups for each litter and multiplying by 100 (Panels D, E, and F, n = 9-18 mice/group, n = 4-14 mice/group, and n = 4-11 mice/group, respectively). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
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expression levels in rat testes following in utero exposure to diethylhexyl phthalate,


CHAPTER 4

Exposure to Di(2-ethylhexyl) Phthalate and Diisononyl Phthalate During Adulthood Disrupts Hormones and Ovarian Folliculogenesis Throughout the Prime Reproductive Life of the Mouse

4.1 Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a phthalate commonly used for its plasticizing capabilities. Because of the wide production and use of DEHP, humans are exposed to DEHP on a daily basis. Diisononyl phthalate (DiNP) is often used as a DEHP replacement chemical, and because of the increased use of DiNP, humans are increasingly exposed to DiNP over time. Of concern is that DEHP and DiNP both exhibit endocrine disrupting capabilities, and little is known about how short-term exposure to either of these phthalates affects aspects of female reproduction. Thus, this study tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood has long-lasting consequences on ovarian follicles and hormones in female mice. Female CD-1 mice aged 39-40 days were orally dosed with either vehicle control (corn oil), DEHP (20 µg/kg/day–200 mg/kg/day), or DiNP (20 µg/kg/day–200 mg/kg/day) for 10 days. Ovarian follicle populations, estradiol, testosterone, progesterone, follicle stimulating hormone (FSH), and inhibin B were analyzed at time points immediately post-dosing and 3, 6, and 9 months post-dosing. The results indicate that 10 days of exposure to DEHP and DiNP changed the distribution of ovarian follicle populations, quality of follicles, and sex steroid hormones at multiple time points, including the last time point, 9 months post-dosing. Further, FSH was increased at multiple doses up to 6 months post-dosing. Inhibin B was not affected by treatment. These data show that short-term exposure to either DEHP or DiNP has long-term consequences that persist long after cessation of exposure.
4.2 Introduction

Maintenance of female fertility is a carefully controlled event with multiple inputs and feedback loops. Namely, female fertility is maintained by the cyclical rise and fall of a variety of hormones at key time points throughout the cycle. In females, the hypothalamus releases gonadotropin releasing hormone (GnRH), which travels to the pituitary via the hypophyseal portal system. Once GnRH arrives in the pituitary, it stimulates specialized cells called gonadotropes to release the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) [1]. FSH and LH are key components in proper development of the ovarian follicles, and LH initiates ovulation, which releases the mature oocyte for fertilization [2]. The follicle is also a major player in the maintenance of female fertility. The follicle is the primary site of production of sex steroid hormones, including estradiol, testosterone, and progesterone. These hormones also contribute to the cyclic nature of female fertility, and they can exert both positive and negative feedback at the level of the hypothalamus, pituitary, or even at the level of the ovary by acting on neighboring follicles [1, 3]. Additionally, they play roles in mating behavior as well as receptivity of the endometrial lining to an implanting embryo [4, 5]. Thus, disruption of the hormonal profiles may lead to disruption of follicular development and vice versa. In addition to disruption of fertility, altering levels of sex steroid hormones can elicit negative effects on somatic health. Disruption of the hormonal milieu in females has been linked to several negative health outcomes including cardiovascular disease, stroke, osteoporosis, and increased mortality [6-10], thus making reproductive health important for the overall health of the female [11].
Concerningly, a variety of environmental contaminants have been designated as endocrine disrupting chemicals that can impact the reproductive and overall health of exposed individuals. One such class of environmental endocrine disruptors is phthalates. Di(2-ethylhexyl) phthalate (DEHP) is a prominent member of the phthalate family and is commonly found in consumer products such as shower curtains, furniture and car upholstery, baby toys, medical tubing, and blood storage bags [12]. Because of the use of DEHP in a variety of consumer products, humans are ubiquitously exposed to DEHP on a daily basis at an estimated daily intake of 3-30 µg/kg/day for a 70kg adult [12]. This is concerning because DEHP is an endocrine disruptor. Multiple studies have found that DEHP is associated with reduced anogenital distance, a marker of fetal androgens, at birth in humans [13], and DEHP has been shown to transgenerationally disrupt female reproductive parameters in animal studies [14, 15]. Further, DEHP is associated with reproductive disorders in women [11, 16]. One study found that women diagnosed with endometriosis had higher serum levels of DEHP than women without endometriosis [17], and another study found that urinary DEHP metabolites were associated with increased uterine volume, which is used as a proxy for burden of uterine fibroids in women [18].

Diisononyl phthalate (DiNP) is another member of the phthalate family. Although a less prominent member of this family, DiNP production has been rising over the past several years [19], and subsequently, humans are being exposed to greater levels of DiNP as evidenced by rapidly increasing levels of DiNP metabolites in human urine samples [20]. Because DiNP is commonly used as a substitute for DEHP, it can be found in many of the same products [19, 21]. However, few studies have been conducted that explore the reproductive consequences of exposure to DiNP, especially in terms of exposure during adulthood, and the scarce literature that
is present indicates that DiNP has endocrine disrupting capabilities. For example, DiNP has been associated with reduced semen quality parameters and reduced testosterone in men in epidemiological studies [13]. Additionally, one animal study found that in utero exposure to DiNP reduces intratesticular testosterone in fetal rats [22], and previous studies have suggested the anti-androgenic effects of DiNP act through similar mechanisms of action as DEHP [23]. DiNP has also been shown to have negative effects in females. A study investigating reproductive outcomes in couples found that women with higher levels of serum DiNP had increased time to pregnancy [24]. Additionally, an animal study found that rats exposed through the maternal diet during gestation had reduced corpora lutea in the ovary at postnatal week 11 when compared to controls [25]. Although these aforementioned studies have investigated the effects of DiNP on both male and female reproduction, a significant lack of studies that utilize environmentally relevant doses exists, and few studies have investigated the effects of DiNP exposure on follicle populations within the ovary or hormone levels in exposed females.

This study was designed to fill the gap in knowledge concerning the effects of short-term exposure to DEHP and DiNP during adulthood on major reproductive hormones and follicle populations within the ovary in female mice. Previously, our laboratory has shown that short-term exposure to both DEHP and DiNP affects cyclicity and fertility for up to nine months following completion of dosing [26, 27]. However, it is still unknown if short-term exposure to DEHP or DiNP during adulthood affects follicle populations and hormones, including sex steroid hormones and peptide hormones. Thus, this study tested the hypothesis that short-term exposure to DEHP and DiNP during adulthood affects folliculogenesis and hormone levels in female mice.
4.3 Materials and Methods

Chemicals

DEHP and DiNP were purchased from Sigma-Aldrich (St. Louis, MO). Corn oil (vehicle control) was purchased from MP Biomedicals (Solon, OH). Dosing stock solutions were created by serially diluting from the highest dose down to the lowest dose. Batches of stock solutions were stored at room temperature away from light for up to a month.

Animals and Dosing

Female CD-1 mice were purchased from Charles River (Wilmington, MA) and housed in the College of Veterinary Medicine Animal Facility at the University of Illinois at Urbana-Champaign (Urbana, IL). Mice were allowed to acclimate to facilities for a minimum of 5 days before the dosing period started. Ambient temperature was maintained at 21.1 ± 2.2°C with humidity at 50 ± 20%. Mice were given ad libitum access to reverse osmosis-treated water and Teklad Rodent Diet (8604). Mice were kept on 12 hour light-dark cycles. All procedures involving animal handling were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (Protocol No.: 17079).

Mice were dosed orally via pipette at age 39-40 days with either vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day) for 10 consecutive days every morning at 2 hours following the start of the light cycle. Mice were weighed daily prior to dosing to determine the necessary dose volume each day. To minimize contamination between treatment groups, mice
were group housed 3 to a cage, and all 3 mice within a single cage were assigned to the same treatment group.

The doses above were chosen because the 20 µg/kg/day DEHP dose is within the estimated range for average daily exposure for a 70kg adult (3-30 µg/kg/day) [12]. The 200 µg/kg/day DEHP dose falls within the range for those that are occupationally exposed (143-286 µg/kg/day) [28]. The 20 and 200 mg/kg/day DEHP doses were chosen because they have been used in previous toxicological studies and also allow us to investigate a wide range of doses [15, 29, 30].

The doses for DiNP were chosen for similar reasons. The 20 µg/kg/day DiNP dose falls within the estimated range for occupational exposure (up to 26 µg/kg/day) [31]. The 100 µg/kg/day DiNP dose is within the estimated range of exposure for infants who are chewing on plastic toys (up to 260 µg/kg/day) [32]. Lastly, the 20 and 200 mg/kg/day DiNP doses were chosen because we wanted to directly compare the toxicity of DiNP to DEHP by using similar doses when possible.

**Experimental Design**

Groups of animals were euthanized via CO2 asphyxiation at different periods following completion of dosing; immediately post-dosing, 3 months post-dosing, 6 months post-dosing, and 9 months post-dosing. All animals were euthanized in the diestrous stage of the estrous cycle and tissues were collected immediately after euthanasia.
**Enzyme-Linked Immunosorbent Assays**

Blood was collected in a heparinized needle and allowed to clot at room temperature for a minimum of 15 minutes, followed by a minimum of 15 minutes in ice. Blood samples were then centrifuged at 4 °C at 14,000 RPM for 15 minutes. Sera that were separated out were collected and stored at -80 °C until use. Testosterone, progesterone, and estradiol ELISA kits were purchased from DRG®. Lypocheck® from Bio-Rad Laboratories was used as a control with known values for all DRG® ELISA kits. The limit of detection (LOD) for testosterone, progesterone, and estradiol was 0.083 ng/mL, 0.045 ng/mL, and 10.60 pg/mL, respectively. The inter- and intra-assay %CVs were ≤ 9.94 and ≤ 4.16, ≤ 9.96 and ≤ 6.99, and ≤ 14.91 and ≤ 9.23 for testosterone, progesterone, and estradiol, respectively. Remaining serum samples were aliquoted and sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of levels of FSH and inhibin B. The LODs for FSH and inhibin B were 1.6 ng/mL and 35 pg/mL, respectively. Intra- and inter-assay %CVs for FSH were 7.2 and 8.5, respectively, and intra- and inter-assay %CVs for inhibin B were 1.8 and 6.6, respectively (https://med.virginia.edu/research-in-reproduction/wp-content/uploads/sites/311/2019/04/2019-INTRA-INTER-ASSAY-CVs__030419.pdf). Samples below the LOD were analyzed using the LOD value specific to the assay divided by the square root of 2.

**Histological Evaluation of Ovarian Tissues**

Ovaries were dissected out of mice, cleaned of gonadal fat, and fixed in Dietrich’s solution. Tissues remained in the fixative overnight at minimum and then were transferred to 70% ethanol before being embedded into paraffin wax. Ovaries were serially sectioned at 8 µm width onto
microscope slides and stained with hematoxylin and eosin. Every 10th serial section was used to assess follicle populations using previously defined criteria [33, 34]. Primordial follicles were designated as oocytes with a single layer of squamous granulosa cells, primary follicles were designated as oocytes surrounded by a single layer of cuboidal granulosa cells, preantral follicles were designated as oocytes surrounded by more than one layer of cuboidal granulosa cells, and antral follicles were designated as oocytes surrounded by multiple layers of cuboidal granulosa cells with a distinct fluid filled antrum [15, 27]. Atretic follicles were follicles that had apoptotic bodies composing 10% or more of the area of the antral follicle. Only follicles with multinucleated oocytes, multiple oocytes, or fragmented oocytes were counted as abnormal. Nuclei must have been present in all abnormal follicle types and preantral and antral follicles to be counted to assure that one follicle was not counted twice across sections. Presence of the nucleus was not required for counting primordial or primary follicles because the follicles are too small to span multiple sections at this stage. Each ovary was assigned a unique ID with no information about treatment group to blind counters to treatment and prevent bias. Data were analyzed as raw counts, which give a perspective on total follicles in each category, and as percentages, which allows analysis of the rate of maturation of follicles. Percentages were achieved by taking the raw number of a particular follicle type, dividing by the total number of follicles and multiplying by 100. All follicle types, including unhealthy, were summed together to determine the total number of follicles. Ovarian follicle population data for the DEHP treatment groups in the immediately post-dosing time point are not included herein because a study conducted by our research group previously published data on the immediate consequences of 10 days of exposure to DEHP on folliculogenesis [29].
Statistical Evaluation

Outliers for normally distributed data were identified and removed from analysis via Grubb’s Test. If data met the assumptions of normality and homogeneity of variance, then a one-way analysis of variance (ANOVA) was used and followed by Dunnett T 2-sided tests. If data were non-parametric, not normally distributed, or lacked homogeneity of variance, then the Mann-Whitney U test was used to compare treatment to control. Significance was assigned at p ≤ 0.05.

4.4 Results and Discussion

This study tested the hypothesis that short-term exposure to DEHP and DiNP alters ovarian follicle populations and levels of circulating hormones. Previously, we have shown that short-term exposure to DEHP during adulthood disrupts estrous cyclicity up to 9 months following completion of dosing [27]. Additionally, a previous study from our research group showed that short-term exposure to DiNP can negatively impact female reproduction as long as 9 months following completion of dosing [26]. The current study builds upon this previous knowledge by assessing the effects of short-term exposure to DEHP and DiNP on ovarian follicle populations and hormone levels at time points where fertility has been disrupted in previous studies. Further, this study fills a gap in knowledge concerning the effects of DiNP on female reproduction.

4.4.1 Effects of DEHP and DiNP on ovarian follicle populations

Immediately Post-Dosing

A previous study in our lab found that 10 days of exposure to DEHP immediately disrupted folliculogenesis [29]. In the present study, we investigated the immediate effects of 10 days of exposure to DiNP. Immediately post dosing, 200 mg/kg/day of DiNP significantly decreased
raw numbers of primordial follicles (Fig. 4.1A, n = 5-11 mice/group, p ≤ 0.05) and borderline decreased the number of antral follicles when compared to control (Fig. 4.1A, n = 5-11 mice/group, p = 0.08). Analysis of follicles by percentages revealed that 20 µg/kg/day of DiNP borderline increased the percent of antral follicles when compared to control (Fig. 4.1B, n = 5-12 mice/group, p = 0.08). Additionally, 20 mg/kg/day of DiNP increased percent of abnormal follicle types when compared to control (Fig. 4.1C, n = 6-11 mice/group, p ≤ 0.05). DiNP did not affect total follicle numbers when compared to control (data not shown).

Female fertility is maintained by a steady stream of growing follicles, thus, disruption of folliculogenesis can alter female fertility. It is possible that DiNP is accelerating the rate at which preantral follicles are developing into antral follicles, leading to an abnormally high percent of antral follicles. Further, the decrease in primordial follicles caused by DiNP is concerning because females are born with a finite number of primordial follicles. Thus, targeting of the primordial follicle population can shorten the reproductive life of the affected individual. We find the differences between DiNP in the current study and DEHP in the previous study interesting [29]. The current findings suggest DEHP and DiNP target different follicles and/or different aspects of folliculogenesis, even when administered for the same time and at the same doses.

3 Months Post-Dosing

At 3 months post-dosing, DEHP and DiNP did not affect raw numbers of healthy follicle types compared to control (Fig. 4.2A, n = 4-11 mice/group). Interestingly, when follicle numbers were analyzed as percentages, 100 µg/kg/day of DiNP decreased the percent of primordial follicles
and increased the percent of primary follicles when compared to control (Fig. 4.2B, n = 4-11 mice/group, p \leq 0.05). Treatment with 200 µg/kg/day of DEHP decreased percentage of abnormal follicle types (Fig. 4.2C, n = 4-11 mice/group, p = 0.06), and 20 µg/kg/day of DEHP similarly decreased percent of atretic follicles when compared to control (Fig. 4.2C, n = 4-11 mice/group, p = 0.08). Treatment with DEHP and DiNP did not affect total follicle numbers when compared to control (data not shown).

The alteration of follicle population proportions induced by DiNP is characteristic of accelerated folliculogenesis, an event in which follicles mature at faster rates than is normal and may result in early entry into reproductive senescence. This is concerning because of the myriad of negative health that are outcomes associated with premature ovarian failure [6-9]. The reduction in abnormal and atretic follicles in some DEHP treatment groups contrasts with some literature that reported increased follicular atresia due to in vitro and lactational DEHP exposure [35, 36]. However, a previous study in our laboratory found that antral follicles treated with an environmentally relevant mixture of phthalates exhibited reduced atresia, possibly due to treatment-induced cell cycle arrest [37]. Thus, it is possible that DEHP may be acting on genes involved in cell cycle regulation and subsequently decreasing rates of atresia. It is also possible that DEHP may have upregulated metabolic enzymes and increased clearance of unhealthy and/or dying oocytes, thus giving the appearance of fewer unhealthy follicles, but in actuality only upregulating clearance. The discrepancies seen between the two chemicals further suggests that they may have different potencies or act through different mechanisms to alter folliculogenesis and follicular health. In a previous study, we found that treatment with 20 µg/kg/day of DEHP and DiNP disrupted the ability of females to achieve pregnancy at 3 months.
post-dosing. The lack of effects seen in those treatment groups on follicular populations and health at this time point suggests that the mechanism through which DEHP and DiNP disrupted female fertility in the previous study likely does not lie in alteration of follicle populations, follicular health, or folliculogenesis [26].

6 Months Post-Dosing

At 6 months post-dosing, 200 µg/kg/day DEHP and 100 µg/kg/day and 20 mg/kg/day DiNP increased numbers of primary follicles when compared to control (Fig. 4.3A, n = 5-11 mice/group, p ≤ 0.05). Effects were slightly different when follicles were analyzed as percentages. The 200 mg/kg/day dose of DiNP increased the percent of primary follicles (Fig. 4.3B, n = 4-11 mice/group, p ≤ 0.05) and borderline decreased the percent of preantral follicles when compared to control (Fig. 4.3B, n = 5-11 mice/group, p = 0.10). Further, 20 µg/kg/day DiNP (Fig. 4.3B, n = 5-11 mice/group, p ≤ 0.05) and 100 µg/kg/day DiNP (Fig. 4.3B, n = 5-11 mice/group, p ≤ 0.05) treatment groups had decreased percentages of antral follicles when compared to control. Additionally, 20 µg/kg/day DEHP (Fig. 4.3C, n = 5-11 mice/group, p = 0.08) and 200 mg/kg/day DiNP (Fig. 4.3C, n = 5-11 mice/group, p = 0.06) increased the percent of abnormal follicles in the ovary when compared to control. No effects were observed on total follicle numbers due to treatment (data not shown).

The observed increases in raw and percent of primary follicles suggest that some disruption of folliculogenesis still persists at 6 months post-dosing. It is possible that more primordial follicles are being recruited to grow into primary follicles and fewer primary follicles are being signaled to grow into preantral follicles, leading to a buildup of primary follicles. A delay in
advancement of follicles past the primary stage could also explain the decrease in the percent of preantral and antral follicles observed in some of the DiNP treatment groups.

The sudden reappearance of an increase in abnormal follicle types due to treatment was unexpected in light of the absence of effects on the percent of abnormal follicles at the 3 month post-dosing time point. It is possible that follicles that were exposed during the treatment period had continued to grow and began to display increasing rates of abnormalities as time elapsed, leading to an increase in abnormal follicles. Finding that DiNP elicited more effects at this time point than DEHP was surprising because DiNP has been purported to be less reproductively toxic when compared to DEHP [38]. Future studies should investigate potential alterations of expression of genes involved in folliculogenesis and maintenance of oocyte health to help elucidate the mechanism through which DEHP and DiNP are acting.

9 Months Post-Dosing

At 9 months post-dosing, 20 and 200 mg/kg/day of DEHP significantly increased the number of preantral follicles in treated mice when compared to control (Fig. 4.4A, n = 5-12 mice/group, p ≤ 0.05). No other effects due to treatment were seen on raw follicle numbers for other follicle types or total follicles. However, percent of follicles by type was affected by treatment at the 9 months post-dosing time point. Mice treated with 20 µg/kg/day of DiNP had significantly decreased percentages of primary follicles when compared to control (Fig. 4.4B, n = 5-12 mice/group, p ≤ 0.05). Further, 200 µg/kg/day (Fig. 4.4B, n = 5-12 mice/group, p = 0.10) and 20 and 200 mg/kg/day of DEHP (Fig. 4.4B, n = 5-12 mice/group, p ≤ 0.05) increased the percent of preantral follicles in treated mice when compared to control. Additionally, 20 µg/kg/day of
DEHP borderline decreased the percent of antral follicles when compared to control (Fig. 4.4B, n = 5-12 mice/group, p = 0.07). DEHP and DiNP did not affect percent of unhealthy follicles (Fig. 4.4C, n = 5-12 mice/group) total follicle numbers when compared to control (data not shown).

These results suggest that DEHP disrupts folliculogenesis and is altering the rate of advancement through the stages of follicular growth. Several genes are involved in the transition from the preantral to antral stage of the follicle, and during this transition, the follicles convert from FSH-independent growth to FSH-dependent growth. Thus, it is possible some genes involved in follicle development are affected by treatment in addition to potential disruption of the follicle’s ability to respond to FSH. Further studies should investigate the effects of DEHP and DiNP on the expression of genes involved in the transition of follicles through stages of growth.

4.4.2 Effects of DEHP and DiNP on hormone levels

Immediately Post-Dosing

Immediately following completion of dosing, DEHP and DiNP both altered the levels of several different hormones at multiple doses. Treatment with 20 and 100 µg/kg/day and 200 mg/kg/day of DiNP significantly reduced testosterone levels when compared to control (Fig. 4.5A, n = 6-12 mice/group, p ≤ 0.05). Treatment with 200 mg/kg/day of DEHP (Fig. 4.5B, n = 5-11 mice/group, p ≤ 0.05) and 20 mg/kg/day DiNP (Fig. 4.5B, n = 5-11 mice/group, p = 0.10) increased progesterone levels when compared to control. Additionally, DEHP (20 mg/kg/day) and DiNP (100 µg/kg/day and 200 mg/kg/day) significantly decreased levels of estradiol when compared to control (Fig. 4.5C, n = 5-12 mice/group, p ≤ 0.05), and 20 µg/kg/day of DiNP borderline decreased estradiol levels when compared to control (Fig. 4.5C, n = 5-12 mice/group,
Further, 20 µg/kg/day and 20 mg/kg/day of DEHP increased levels of FSH (Fig. 4.6A, n = 4-11 mice/group, p ≤ 0.05), and treatment with 100 µg/kg/day of DiNP borderline increased levels of FSH when compared to control (Fig. 4.6A, n = 4-11 mice/group, p = 0.09). No differences due to treatment were detected for inhibin B at this time point, although some treatment groups lacked adequate number of samples to statistically analyze due to insufficient volume of serum samples from some mice (Fig. 4.6B, n = 1 for 20 and 100 µg/kg/day DiNP treatment groups, all other groups n = 3-9 mice/group).

Although DEHP and DiNP tended to illicit similar effects, we found the differences in how these chemicals affected testosterone interesting. Previous studies conducted in our research group using in utero and in vitro exposure paradigms have shown that DEHP decreases levels of testosterone when compared to control [15, 36]. However, another study that orally dosed pregnant mice found an increase in maternal testosterone when compared to control [39]. Thus, the discrepancies observed between the present study and previous studies conducted in our laboratory are likely due to differences in timing of exposure. In addition, a study using a short-term exposure to DEHP during adulthood paradigm, but in dairy cattle, found that DEHP reduced levels of estradiol, further highlighting the different impacts exposure duration and timing can have on outcome [40]. In terms of DiNP, epidemiological literature agrees with the findings in this study that DiNP has anti-androgenic properties and is associated with lower testosterone in men [13]. A proposed mechanism of action of DEHP is that the primary bioactive metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), activates peroxisome proliferator-activated receptors (PPARs) α and γ, which both downregulate expression of aromatase, the enzyme found in the granulosa cell that is responsible for the conversion of
testosterone to estradiol [41]. Thus, it is possible that these phthalates may be acting through altering steroidogenic enzyme expression within the ovary. Interestingly, although we observed wide disruption of sex steroid hormones at this time point, in a previous study, we did not observe any effects on fertility endpoints in mice bred immediately post-dosing, suggesting that disrupting hormones alone is not sufficient to disrupt female fertility at this time point [26].

In terms of FSH, a previous study in our laboratory found that transgenerational exposure to DEHP affected FSH differently in different generations in mice that were one year of age [14]. Further, a study investigating the associations between urinary DEHP and DiNP metabolites and FSH in healthy Danish men found contrasting results and reported that both DEHP and DiNP metabolites were associated with reductions in FSH [42]. However, DEHP-induced increases in FSH were also observed in a study conducted in male mice orally dosed during adulthood, a paradigm that strongly resembles the one in the current study [43]. Although lack of serum volume prevented statistical analysis of inhibin B levels for some treatment groups, the lack of effects seen with inhibin B by treatment was not surprising due to the lack of dramatic effects seen on the follicle populations responsible for inhibin B production. Further, the previously mentioned study in Danish men also found no associations between urinary DEHP and DiNP metabolites and inhibin B [42]. Interestingly, DEHP and DiNP seem to follow similar trends in their effects, and they tend to have effects in similar directions when compared to control.

3 Months Post-Dosing

After 3 months following completion of dosing, there were fewer effects observed on the sex steroid hormones than at immediately post-dosing, but some effects persisted. The 100
µg/kg/day dose of DiNP significantly decreased levels of testosterone and progesterone when compared to control (Fig. 4.7A, n = 5-12 mice/group, p ≤ 0.05). Further, 20 µg/kg/day of DiNP borderline decreased levels of progesterone (Fig. 4.7B, n = 5-12 mice/group, p = 0.08), and 200 mg/kg/day of DiNP borderline increased levels of estradiol when compared to control (Fig. 4.7C, n = 5-12 mice/group, p = 0.08). Additionally, the 20 mg/kg/day dose of DiNP borderline increased levels of FSH when compared to control (Fig. 4.8A, n = 5-12 mice/group, p = 0.08), and no effects due to treatment were seen on levels of inhibin B (Fig. 4.8B, n = 4-11 mice/group).

Fewer overall effects on hormone levels were observed at 3 months post-dosing when compared to immediately post-dosing time point. However, the 100 µg/kg/day DiNP treatment group had persistently reduced testosterone, while other treatment groups had recovered. Interestingly, we also observed an inversion of the effects of treatment on progesterone and estradiol, and at this time point DiNP decreased levels of progesterone and increased levels of estradiol. A previous study using porcine granulosa cells in culture found that DEHP and DiNP did not affect basal levels of progesterone or estradiol, but did find that DiNP decreased FSH-induced estradiol when compared to control [44]. These results contrast with our study and highlight the different effects that can be elicited by different exposure paradigms and also suggest that DiNP may be affecting metabolism of these enzymes and not solely the ability of the granulosa cells to produce these hormones. Further, it is possible that species differences, along with differences in exposure paradigms, may attribute to the discrepancies observed between this study and the present study. The inversion of the effects of DiNP on estradiol (decreased immediately post-dosing and increased at 3 months post-dosing) is particularly perplexing. It is possible that estradiol
production was upregulated at the 3 month post-dosing time point to compensate for the reduction seen at the immediately post-dosing time point and that levels had yet to return to baseline.

At 3 months post-dosing, FSH was modestly increased in the 20 mg/kg/day DiNP treatment group, but otherwise was unaffected by treatment. We find the greater propensity of DiNP to increase FSH for a longer time than DEHP interesting because DiNP is thought to be less potent than DEHP [38]. In terms of inhibin B, results at 3 months post-dosing are similar to the immediately post-dosing time point, and inhibin B remained unaffected by treatment with either DEHP or DiNP.

6 Months Post-Dosing

Following 6 months after completion of dosing, 100 µg/kg/day of DiNP significantly reduced levels of testosterone in mice when compared to control, an effect seen at all previous time points (Fig. 4.9A, n = 6-12 mice/group, p ≤ 0.05). Additionally, 200 µg/kg/day of DEHP significantly increased circulating levels of estradiol when compared to control (Fig. 4.9C, n = 5-12 mice/group, p ≤ 0.05). However, no effects of DEHP and DiNP were seen on progesterone at any dose when compared to control (Fig. 4.9B, n = 6-12 mice/group). The 20 and 200 mg/kg/day doses of DEHP both significantly increased levels of FSH when compared to control (Fig. 4.10A, n = 4-12 mice/group, p ≤ 0.05). In contrast, inhibin B continued to be unaffected by treatment (Fig. 4.10B, n = 3-11 mice/group).
The DEHP-induced increase in estradiol contrasts with previous in vitro studies conducted by our research group [36, 45], but agrees with in vivo studies from our research group that have investigated the transgenerational effects of DEHP exposure [14, 15] as well as in vivo studies from other laboratories [46, 47]. In terms of DiNP, previous epidemiological studies have found that DiNP is associated with reduced testosterone [13], but we find this result particularly interesting because of its persistence and consistency in this treatment group.

We found the sudden and drastic reappearance of DEHP-induced increases in FSH surprising, especially in light of the lack of effects of DEHP on FSH observed at the previous time point. Because DEHP and DiNP are purported to act through similar mechanisms, the difference in effects between DEHP and DiNP was also surprising. This drastic increase in FSH was not accompanied by concurrent alterations of follicles nor sex steroid hormones, suggesting that the follicles may not be responding to FSH signals correctly. It is important for future studies to investigate the effects of phthalate exposure on expression of key female reproductive hormones and their receptors to better understand the mechanisms through which DEHP and DiNP may be acting.

9 Months Post-Dosing

At 9 months post-dosing, the only effect seen was a borderline increase in testosterone in the 200 µg/kg/day of DEHP treatment group when compared to control (Fig. 4.11A, n = 5-12 mice/group, p = 0.09). Progesterone, estradiol, FSH, and inhibin B were not affected by treatment at this time point (Figs. 4.11B, 4.11C, 4.12A, and 4.12B, n = 4-12 mice/group).
Interestingly, the observed effect deviates from all previous time points where, if an effect was observed, testosterone was decreased with treatment. Although a DEHP-induced increase in testosterone conflicts with previous transgenerational in vivo studies in our laboratory using a prenatal exposure window [14, 15], other studies have found that DEHP’s metabolite, MEHP, can increase levels of testosterone in culture [48, 49], further highlighting the different effects that can be elicited from exposure during different windows and through different routes. In terms of DiNP, we no longer observed the reduced testosterone levels in the 100 µg/kg/day DiNP group that had persisted until this time point, further suggesting that some effects can be recovered from given enough recovery time.

At 9 months post-dosing, neither FSH nor inhibin B were affected by treatment. This suggests recovery is possible given enough time passage from the exposure period. However, we were surprised inhibin B was not affected because we observed increases in preantral follicles, which are responsible for production of inhibin B. It is possible that the observed increase in preantral follicles in some treatment groups is not drastic enough to elicit detectable changes in inhibin B. Conversely, it is possible that treatment may be affecting the ability of these follicles to produce inhibin B. Future studies should investigate how DEHP may affect inhibin B production by small, growing follicles.

**4.4.3 Correlations between follicle numbers and hormones**

Interestingly, the hormone and follicle count data did not always correlate. This was surprising because antral follicles are the primary source of sex steroid hormones in females [3], and although estradiol and testosterone were reduced by treatment, the effects varied in whether it
decreased, increased, or did not affect antral follicle numbers. These data suggest that the mechanism through which DEHP and DiNP affect hormone levels is not solely through manipulation of antral follicle numbers, and that it may involve changing the expression or activity of enzymes responsible for the synthesis and/or breakdown of hormones. However, immediately post-dosing, the effects of DEHP and DiNP on sex steroid hormones and FSH are much more correlative. Treatment groups with reduced estradiol also tended to have increased levels of FSH, suggesting that the negative feedback on FSH production exerted by estradiol may be alleviated, resulting in increased FSH levels. Conversely, it is possible these phthalates may be interfering with the ability of FSH to stimulate production of estradiol. Previous studies have shown that MEHP in culture with rat granulosa cells decreased the levels of FSH-induced estradiol [41]. Thus, it is possible that the increased FSH observed in some treatment groups is compensatory and upregulated due to the lack of FSH-induced estradiol production by granulosa cells. Interestingly, the treatment groups that displayed increased progesterone were not the same ones that displayed an increase in FSH, indicating the treatment-induced levels of progesterone were likely not due to FSH-mediated mechanisms.

Similar to the immediately post-dosing time point, we again observed little correlation between our follicle population data and hormones at 3 months post-dosing. This further suggests that the mechanism through which these phthalates alter sex steroid hormones is not solely by altering the number of antral follicles in the ovary, and the answer likely lies in alteration of expression of key steroidogenic enzymes, metabolic enzymes, or the overall function of the steroidogenic cells. Further, neither the follicle population data nor the hormone data fully explain the decrease in fertility observed at 3 months post-dosing in a previous study conducted with an
identical dosing and exposure paradigm [26]. Thus, DEHP and DiNP must be affecting an aspect other than the follicles and hormones to reduce fertility. One study conducted in equine oocytes found that acute in vitro exposure to DEHP inhibits oocyte maturation [50], and another utilizing acute exposure during adulthood in dairy cattle reported that DEHP decreased developmental competence of oocytes [40]. Thus, it is possible that DEHP and DiNP may be affecting oocyte quality in a manner that is undetectable via histological evaluation.

At 6 months post-dosing, we observed increases in raw number and percent of primary follicles and decreases in percent of antral follicles. These changes in follicular populations did not always correlate with hormonal changes, except in the case of the 100 µg/kg/day DiNP treatment group that had both decreased antral follicles and decreased testosterone. In some DEHP groups, we observed an increase in FSH that did not coincide with a decrease in estradiol as was seen at the immediately post-dosing time point, suggesting that the elevation in FSH may be directly due to treatment as opposed to induced by lower levels of estradiol. However, increased FSH should instigate increased synthesis of both estradiol and progesterone, which was not observed in the treatment groups with elevated FSH. Subsequently, we suspect that treatment affected the ability of the follicles to respond to FSH, potentially through manipulation of expression of the receptor for FSH (FSHR). Although few studies have investigated this, some have found that DEHP decreases expression of FSHR [51, 52]. Further, one proposed mechanism of action of DEHP is that its metabolite, MEHP, may interfere with FSH binding to FSHR [41]. Thus, it is possible that the observed increases in FSH are compensatory and due to a lack of response of the follicle to normal levels of FSH. The lack of effects of treatment on inhibin B were not surprising
because treatment did not dramatically affect the follicles responsible for inhibin B production at this time point.

At 9 months post-dosing, the most striking effects were treatment-induced increases in preantral follicle populations. Interestingly, the follicle population effects were not reflected in sex steroid or peptide hormone levels, which was unexpected. This further suggests that the mechanisms through which DEHP and DiNP act to disrupt hormones and follicle populations are multifaceted and merit further investigation. Further, the observation of any effects at 9 months post-dosing, our latest time point, was surprising in itself. This is evidence that short term exposure during an adult exposure window has the ability to impart long-lasting effects on the exposed individual, and caution should be taken for exposure not only within developmental windows, but outside developmental windows as well.

4.5 Conclusion
Collectively, our data show that short-term exposure to both DEHP and DiNP have negative impacts on ovarian function and folliculogenesis throughout the reproductive life of the mouse. Interestingly, throughout our study we observed changes in and sometimes inversions of effects from timepoint to timepoint, indicating the mechanism or mechanisms through which these phthalates are acting are complex. Although the antral follicle is the primary source of sex steroid hormones in females, we did not always find that antral follicle changes correlated with hormone changes, suggesting that the mechanism of action does not solely involve changes to the antral follicle population. Some studies have found that phthalates can change steroidogenic enzyme expression within the ovary [45, 53, 54], and others have found that phthalates can
change how the ovary responds to other endocrine signals [41, 51, 52]. Further, DEHP has been shown to be capable of causing oxidative stress in the ovary and affecting oocyte development and competency [40, 50, 55]. Thus, there could be a combination of multiple pathways that may be contributing to the variety of effects seen in this study and the fertility effects seen in a previous study from our laboratory [26]. Future studies should investigate the mechanisms through which DEHP and DiNP act to disrupt these aspects of female reproduction.
4.6 Figures and Legends

Figure 4.1 Effects of DiNP on ovarian follicle populations immediately post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle immediately following dosing and the ovaries were collected and processed for histological evaluation of the number healthy follicle types (n = 5-11 mice/group) (A), percent of healthy follicle types (n = 5-12 mice/group) (B), and percent of unhealthy follicle types (n = 6-11 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.2 Effects of DEHP and DiNP on ovarian follicle populations at 3 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle 3 months post-dosing and the ovaries were collected and processed for histological evaluation of the number healthy follicle types (n = 4-11 mice/group) (A), percent of healthy follicle types (n = 4-11 mice/group) (B), and percent of unhealthy follicle types (n = 4-11 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.3 Effects of DEHP and DiNP on ovarian follicle populations at 6 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle 3 months post-dosing and the ovaries were collected and processed for histological evaluation of the number healthy follicle types (n = 5-11 mice/group) (A), percent of healthy follicle types (n = 4-11 mice/group) (B), and percent of unhealthy follicle types (n = 5-11 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.4 Effects of DEHP and DiNP on ovarian follicle populations at 9 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle 3 months post-dosing and the ovaries were collected and processed for histological evaluation of the number healthy follicle types (n = 5-12 mice/group) (A), percent of healthy follicle types (n = 5-12 mice/group) (B), and percent of unhealthy follicle types (n = 5-12 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.5 Effects of DEHP and DiNP on sex steroid hormones immediately post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of testosterone (n = 6-12 mice/group) (A), progesterone (n = 5-11 mice/group) (B), and estradiol (n = 5-12 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of FSH (n = 4-11 mice/group) (A) and inhibin B (n = 1-9 mice/group) (B). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.7 Effects of DEHP and DiNP on sex steroid hormones at 3 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of testosterone (n = 5-12 mice/group) (A), progesterone (n = 5-12 mice/group) (B), and estradiol (n = 5-12 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.8 Effects of DEHP and DiNP on FSH and inhibin B at 3 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of FSH (n = 5-12 mice/group) (A) and inhibin B (n = 4-11 mice/group) (B). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of testosterone (n = 6-12 mice/group) (A), progesterone (n = 6-12 mice/group) (B), and estradiol (n = 5-12 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.10 Effects of DEHP and DiNP on FSH and inhibin B at 6 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of FSH (n = 4-11 mice/group) (A) and inhibin B (n = 3-11 mice/group) (B). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 4.11 Effects of DEHP and DiNP on sex steroid hormones at 9 months post-dosing

Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day – 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of testosterone (n = 5-12 mice/group) (A), progesterone (n = 5-12 mice/group) (B), and estradiol (n = 5-12 mice/group) (C). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Adult female CD-1 mice were orally dosed for 10 days with either vehicle control (corn oil) or DiNP (20 µg/kg/day - 200 mg/kg/day). Mice were euthanized in the diestrous stage of the estrous cycle and sera were collected for analysis of FSH (n = 5-12 mice/group) (A) and inhibin B (n = 4-12 mice/group) (B). Data are represented as means ± standard error. Statistically significant difference when compared to control (p ≤ 0.05) is denoted with an asterisk (*). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
4.7 References


CHAPTER 5

Late-Life Consequences of Short-Term Exposure to Di(2-ethylhexyl) Phthalate and Diisononyl Phthalate During Adulthood in Female Mice

5.1 Abstract
Di(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental contaminant that has been shown to accelerate recruitment of primordial follicles from the resting follicle pool. In addition, diisononyl phthalate (DiNP) is a common DEHP replacement chemical. However, little is known about late-life consequences due to DEHP or DiNP exposure during adulthood. Thus, the current study tested the hypothesis that adult exposure to DEHP or DiNP negatively affects female reproductive parameters including cyclicity, ovarian follicle populations, fertility, and hormone concentrations during late-life in female mice. Female CD-1 mice aged 39-40 days were orally dosed with either vehicle control (corn oil), DEHP (20 µg/kg/day–200 mg/kg/day), or DiNP (20 µg/kg/day–200 mg/kg/day) for 10 days. Mice were aged with no additional phthalate exposure and cyclicity monitoring periods and breeding trials were conducted at 12 and 15 months post-dosing. Further, ovaries and sera were collected at 12, 15, and 18 months post-dosing for analysis of ovarian follicle populations and concentrations of estradiol, progesterone, testosterone, inhibin B, and follicle stimulating hormone. DEHP and DiNP disrupted estrous cyclicity, increased pregnancy loss, decreased overall fertility, and altered the sex ratio of pups. Further, DEHP and DiNP altered ovarian follicle populations and hormone levels at all time points. Taken together, these data show that short-term exposure to DEHP or DiNP during adulthood has long-term consequences in late-life, even after over a year of exposure cessation.
5.2 Introduction

Female mammals are born with a finite number of oocytes to use throughout their reproductive lives [1]. Once this reserve of oocytes has been depleted, the female enters reproductive senescence. During years of fertility, females undergo reproductive cycles in which species-specific numbers of oocytes are ovulated for fertilization in coordination with a consistent and predictable fluctuation in hormones. The primary hormones that take on a cyclic nature within females are the sex steroid hormones estradiol, progesterone, and testosterone as well as peptide hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), and inhibin A and B [2, 3]. Because the sex steroid hormones are primarily produced by structures only present in actively cycling females, namely the antral follicle and the corpus luteum (CL), high levels of sex steroid hormones are characteristic of fertile and actively cycling females. Additionally, some sex steroid hormones share antagonistic relationships with the peptide hormones [3]. Thus, as antral follicles, corpora lutea, and subsequently, sex steroid hormones decline with age, levels of some peptide hormones such as FSH increase as the negative feedback exerted by the sex steroid hormones is alleviated [4]. Additionally, inhibin B levels decline as the number of small growing follicles that are responsible for its production drop. Thus, the hormone profile of a normal, cycling female will have higher levels of sex steroid hormones and inhibin B and lower levels of FSH, and inverse characteristics will be present for acyclic females who have entered reproductive senescence. Taken together, data on levels of multiple hormones can be used as a metric for assessing female reproductive health and reproductive aging [4].
To prevent early entry into reproductive senescence, proper regulation of the activation and use of the ovarian reserve is imperative. Aberrant regulation can lead to early depletion of the ovarian reserve and, subsequently, early reproductive senescence [4]. In women, early menopause is associated with an increased risk of osteoporosis, stroke, cardiovascular disease, and increased mortality [5-9]. Concerningly, some studies have found that some environmental contaminants can lead to dysregulation of the ovarian reserve, and some studies have found markers of reproductive aging are altered by these chemicals as well [10-12]. One class of chemicals that has been shown to have endocrine disrupting capabilities is phthalates [13, 14].

Phthalates are a family of chemicals used widely for their plasticizing and stabilizing properties. Lower molecular weight phthalates can commonly be found in personal care products because they are used to maintain and stabilize fragrances and colors [15, 16]. Higher molecular weight phthalates are more often used for their plasticizing capabilities, resulting in their high use in plastic products such as polyvinyl chloride (PVC) goods and other consumer goods such as clothing and food packaging [16].

Di(2-ethylhexyl) phthalate (DEHP) is a high molecular weight phthalate and is often found in products such as plastic children’s toys, upholstery, construction materials such as floor tiles, and medical equipment such as blood bags and tubing [17]. Millions of pounds of DEHP are produced every year within the U.S., and this vast production and use has led to DEHP becoming a top environmental contaminant that humans are exposed to daily [17, 18]. The average person is estimated to be exposed to between 3-30 µg/kg/day [17], and some populations can experience even higher levels of exposure to DEHP due to occupation or medical exposure [19, 20]. Thus,
DEHP is a widespread contaminant that humans are exposed to on a daily basis, and some populations are vulnerable to even higher levels of exposure. This is a concern because DEHP has endocrine disrupting capabilities and has been associated with a variety of reproductive disorders in women in addition to affecting oocyte development, ovulation, and steroidogenesis [13, 14]. However, few studies have investigated how exposure to DEHP during adulthood affects reproductive aging in females.

Another phthalate of concern is diisononyl phthalate (DiNP), a phthalate often used as a DEHP replacement because it is thought to be less anti-androgenic than DEHP. Because of the increased use of DiNP as a DEHP replacement chemical, a steep increase in the levels of DiNP metabolites has been detected in human urine samples from the National Health and Nutrition Examination Survey (NHANES) [21]. This is concerning because of a paucity of information on the effects of DiNP on reproductive aging in females, especially in the context of exposure during adulthood. Further, studies from our own research group have found similar reproductive toxicity when comparing DEHP to DiNP [22]. Subsequently, this begs the question of whether DiNP is an adequate replacement for DEHP if the goal of substitution is decreased toxicity and increased safety for the consumer.

This study aims to fill the gap in knowledge concerning the effects of DEHP and DiNP exposure during adulthood on reproductive aging in the female mouse. Previously, our laboratory has shown that 10 days of exposure during adulthood to DEHP and DiNP is adequate to disrupt aspects of female fertility up to 9 months following completion of dosing [11, 22]. However, data on how DEHP and DiNP affect parameters of female fertility in late-life are still scarce.
Thus, this study tested the hypothesis that short-term exposure to DEHP and/or DiNP during adulthood alters markers of reproductive aging, including cyclicity, ovarian hormone populations, overall fertility, and circulating hormone levels.

5.3 Materials and Methods

Chemicals

DEHP and DiNP were purchased from Sigma-Aldrich (St. Louis, MO). Corn oil (vehicle control) was purchased from MP Biomedicals (Solon, OH). Dosing stock solutions were made via serial dilutions from the highest concentration dose. Stock solutions were stored at room temperature away from exposure to direct light for up to a month. If a month elapsed from the time the stock solutions were made, then a new batch of stock solutions was made.

Animals and Dosing

Female CD-1 mice were purchased from Charles River (Wilmington, MA) and housed in the animal facility at the College of Veterinary Medicine at the University of Illinois at Urbana-Champaign (Urbana, IL). Animals were allowed to acclimate to the facilities for a minimum of 5 days before the dosing period began. Facility temperature was maintained at 21.1 ± 2.2°C and humidity was maintained at 50 ± 20%. Mice were given ad libitum access to chow (Teklad Rodent Diet 8604) and reverse osmosis-treated water. Light-dark cycles were 12 hours light and 12 hours dark. All animal handling procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (Protocol No.: 17079).
Mice were dosed at age 39-40 days orally via insertion of a pipette tip into the mouth. Treatments consisted of vehicle control (corn oil), DEHP (20 µg/kg/day, 200 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day), or DiNP (20 µg/kg/day, 100 µg/kg/day, 20 mg/kg/day, 200 mg/kg/day) for 10 days. To determine dose volume, mice were weighed daily immediately before dosing. Mice were housed 3 to a cage with all mice within one cage receiving the same dose to minimize contamination.

*Experimental Design*

To determine how DEHP and DiNP affect fertility parameters, hormones, and ovarian follicle populations in late-life, groups of animals were dosed and data were taken at pre-determined time points. One group of mice was designated for cyclicity monitoring and breeding, and cyclicity was monitored at time points 12 and 15 months post-dosing. Breeding trials for this same group of mice commenced following completion of the cyclicity monitoring period. These mice were euthanized at 18 months post-dosing, and blood and ovaries were collected.

Additional groups of mice that were not used for cyclicity monitoring or breeding were dosed and euthanized in the same fashion at 12 months post-dosing and 15 months post-dosing, and blood and ovaries were collected. Collectively, this generated breeding and cyclicity data for 12 and 15 months post-dosing as well as hormone data and ovarian follicle population data for 12, 15, and 18 months post-dosing.
**Estrous Cyclicity Monitoring**

At time points 12 and 15 months post-dosing, mice were vaginally lavaged with 1X phosphate-buffered saline for 14 consecutive days, and the lavage samples were analyzed under a light microscope to assess stage of the estrous cycle using previously defined criteria [23]. Lavaging was conducted at 2 hours after the beginning of the light cycle. Lavage samples were analyzed no later than 3 days following collection, and samples were kept at 4 °C until analysis. Mice were housed in groups of 3 animals per cage. For statistical analysis, the metestrous and diestrous stages were combined into a single category because they are similar in mice [23]. Percent times spent in proestrus, estrus, and metestrus/diestrus were calculated by dividing the number of days spent in each stage of the cycle by 14 and multiplying by 100.

**Histological Evaluation of Ovarian Tissues**

Following euthanasia, ovaries were removed and fixed overnight in Dietrich’s solution. Following fixation, ovaries were transferred to 70% ethanol and then embedded in paraffin wax. Ovaries were sectioned serially on a microtome at 8 µm in width and mounted onto microscope slides. Slides were baked overnight at 50 °C and were stained the following day with hematoxylin and eosin. Every tenth section of the ovary was used to assess follicle populations as described previously [10]. In addition to normal follicle types, the percent of unhealthy follicle types, including abnormal and atretic follicles were assessed. Abnormal follicles were designated as follicles with either a fragmented oocyte, a multinucleated oocyte, or multiple oocytes. The nucleus must have been present in all follicle types to be counted to avoid double counting between sections, except for primordial and primary follicles which are too small to span more than 10 sections. To blind counters to treatment groups and avoid bias, ovaries were
given a unique histological ID with no relation to treatment group. Data that are reported as follicle numbers represent unaltered, raw number of follicles in each category. Data that are reported as percent of follicles were determined by dividing the raw number of follicles in each category by the total number of follicles within the ovary and multiplying by 100 to achieve a percentage. The total number of follicles was determined by summing all follicle types together, including unhealthy follicles.

*Breeding and Fertility Indices*

Following completion of the cyclicity monitoring periods, females were paired with an untreated CD-1 male mouse (age 7 weeks) in a new cage. Breeding pairs were kept in a harem fashion with 2 females and a single male. Following introduction of the male, females were checked every morning and afternoon for the presence of a copulatory plug, which indicated successful mating with the male. Females remained housed with the male until a copulatory plug was observed or if 14 days had passed. If a copulatory plug was noted or the female had been with the male for 14 days, the female was removed and single-housed in a new cage. To calculate time to mating, day one was considered the morning immediately after the introduction of the male. To calculate gestation length, the day the copulatory plug was noted was designated gestational day 0.5. Females were weighed twice a week starting from the introduction of the male until the litter was born. If a female gained 4 g or more in weight, she was considered pregnant. Around the time of litter bearing, females were checked in the morning and afternoon for presence of pups. Live pups on postnatal day (PND) 0 were weighed together and the weights were averaged, and live and deceased pups were counted and sexed, if possible. Percent of female pups was measured by dividing the number of female pups by the total number
of pups within the litter and multiplying by 100. If sex or number of pups could not be determined due to cannibalism by the dam, those data were not used in the statistical analysis.

Breeding trial indices were used to assess fertility success at multiple stages of the breeding trial. The fertility index measured the percent of females who successfully achieved pregnancy from a single, confirmed mating by dividing the number of pregnant females by the number of females with a copulatory plug and multiplying by 100. The gestational index measured the percent of females who carried their pregnancy to term and gave birth to pups by dividing the number of females who gave birth by the number of females that achieved pregnancy and multiplying by 100. The percent of females who gave birth was calculated by dividing the number of females who gave birth to pups by the total number of females within the treatment group and multiplying by 100.

**Steroid and Peptide Hormone Assays**

Groups of animals were euthanized via CO2 asphyxiation at different periods following completion of dosing: 12 months post-dosing, 15 months post-dosing, and 18 months post-dosing. All animals were euthanized in the diestrous stage of the estrous cycle. Immediately after euthanasia, blood was collected in a heparinized needle and allowed to clot in an Eppendorf® tube at room temperature for at least 15 minutes. Blood samples were then put on ice for at least 15 minutes. Following 15 minutes on ice, samples were centrifuged in a 4 °C room at 14,000 RPM for 15 minutes. Sera were collected and stored at -80 °C until use in enzyme-linked immunosorbent assays (ELISAs) or radioimmunoassays. ELISA kits for testosterone, progesterone, and estradiol were purchased from DRG®. The limits of detection
(LODs) for testosterone, progesterone, and estradiol were 0.083 ng/mL, 0.045 ng/mL, and 10.60 pg/mL, respectively. The inter- and intra-assay %CVs were ≤ 9.94 and ≤ 4.16, ≤ 9.96 and ≤ 6.99, and ≤ 14.91 and ≤ 9.23 for testosterone, progesterone, and estradiol, respectively. Lypocheck® from Bio-Rad Laboratories was used as a control with known values in all ELISAs in this study. Aliquots of sera were shipped to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. The facility used radioimmunoassays to measure levels of FSH and inhibin B. The LODs for FSH and inhibin B were 1.6 ng/mL and 35 pg/mL, respectively. Intra- and inter-assay %CVs for FSH were 7.2 and 8.5, respectively, and intra- and inter-assay %CVs for inhibin B were 1.8 and 6.6, respectively (https://med.virginia.edu/research-in-reproduction/wp-content/uploads/sites/311/2019/04/2019-INTRA-INTER-ASSAY-CVs__030419.pdf). Samples with values below the limit of detection (LOD) were statistically analyzed using the LOD value specific for each assay divided by the square root of 2.

Statistical Analysis

Data that were normally distributed were assessed for outliers using Grubb’s Test. If outliers were detected, they were not used in the following analysis. Data that were normally distributed and met the assumption of homogeneity of variance were analyzed via one-way analysis of variance (ANOVA). Dunnett’s 2-sided test was used post hoc. If data did not meet the assumption of normality and/or the assumption of homogeneity of variance or were non-parametric, the Mann-Whitney U test was used. Statistical significance was set at p ≤ 0.05.
5.4 Results

5.4.1 Effects of DEHP and DiNP on estrous cyclicity

12 months post-dosing

At 12 months post-dosing, 20 µg/kg/day DEHP-treated mice spent significantly more time in estrus (Fig. 1A, n = 9-22 mice/group, p ≤ 0.05) and borderline decreased time in metestrus/diestrus (Fig. 1A, n = 9-22 mice/group, p = 0.09) when compared to control. Females treated with 20 µg/kg/day of DiNP had borderline increased time spent in estrus when compared to control (Fig. 1A, n = 9-22 mice/group, p = 0.06).

15 months post-dosing

Following 15 months after dosing, females in the 20 µg/kg/day DEHP treatment group spent significantly more time in estrus (Fig. 1B, n = 8-20 mice/group, p ≤ 0.05) and borderline decreased time in metestrus/diestrus (Fig. 1B, n = 8-20 mice/group, p = 0.09) when compared to control. No other treatments affected estrous cyclicity when compared to control.

5.4.2 Effects of DEHP and DiNP on ovarian follicle populations

12 months post-dosing

At 12 months post-dosing, treatment with 20 and 200 mg/kg/day DiNP decreased numbers of primordial follicles when compared to control (Fig. 2A, n = 3-12 mice/group, p ≤ 0.05). Further, 20 mg/kg/day of DEHP significantly decreased numbers of primary follicles (Fig. 2A, n = 3-12 mice/group, p ≤ 0.05) and borderline decreased numbers of preantral follicles when compared to control (Fig. 2A, n = 3-12 mice/group, p = 0.10). Further, 20 mg/kg/day of DEHP borderline
decreased the total number of follicles within the ovary when compared to control (Fig. 2D, n = 3-12 mice/group, p = 0.06).

When follicle populations were analyzed as percentages, treatment with 20 and 200 mg/kg/day of DiNP significantly decreased the percent of primordial follicles when compared to control (Fig. 2B, n = 3-12 mice/group, p ≤ 0.05). Further, treatment with 200 mg/kg/day of DEHP (Fig. 2B, n = 3-12 mice/group, p = 0.07) and 20 µg/kg/day of DiNP (Fig. 2B, n = 3-12 mice/group, p ≤ 0.05) decreased the percent of primary follicles when compared to control. Additionally, 200 mg/kg/day of DiNP borderline increased the percent of preantral follicles when compared to control (Fig. 2B, n = 3-12 mice/group, p = 0.08), and 20 mg/kg/day of DiNP significantly increased the percent of antral follicles when compared to control (Fig. 2B, n = 3-12 mice/group, p ≤ 0.05).

Interestingly, we observed various effects on the percent of unhealthy follicles. Treatment with 200 mg/kg/day of DEHP (Fig. 2C, n = 3-12 mice/group, p ≤ 0.05), 100 µg/kg/day DiNP (Fig. 2C, n = 3-12 mice/group, p = 0.10), and 200 mg/kg/day DiNP (Fig. 2C, n = 3-12 mice/group, p ≤ 0.05) decreased percent of unhealthy follicles when compared to control. In contrast, 20 µg/kg/day of DiNP increased the percent of unhealthy follicles when compared to control (Fig. 2C, n = 3-12 mice/group, p = 0.10).

15 months post-dosing

At 15 months post-dosing, 200 µg/kg/day of DEHP and 100 µg/kg/day of DiNP both increased the number of preantral follicles when compared to control (Fig. 3A, n = 4-11 mice/group, p ≤
0.05). Additionally, 20 µg/kg/day and 200 mg/kg/day of DEHP decreased the number of antral follicles when compared to control (Fig. 3A, n = 4-11 mice/group, p ≤ 0.05). Conversely, no doses of DEHP or DiNP affected the total number of follicles when compared to control (Fig. 3D, n = 4-11 mice/group).

When follicles were analyzed as percentages, 200 µg/kg/day of DEHP borderline increased the percent of primary follicles when compared to control (Fig. 3B, n = 4-11 mice/group, p = 0.10) and borderline decreased the percent of antral follicles when compared to control (Fig. 3B, n = 4-11 mice/group, p = 0.10). Similarly, 20 mg/kg/day of DEHP and 100 µg/kg/day of DiNP significantly decreased the percent of antral follicles when compared to control (Fig. 3B, n = 4-11 mice/group, p ≤ 0.05). No doses of DEHP or DiNP affected the percent of unhealthy follicles when compared to control (Fig. 3C, n = 4-11 mice/group).

18 months post-dosing
At 18 months post-dosing, treatment with 100 µg/kg/day and 200mg/kg/day of DiNP significantly increased the number of primordial follicles when compared to control (Fig. 4A, n = 4-12 mice/group, p ≤ 0.05). Further, 20 µg/kg/day of DiNP significantly decreased the number of antral follicles (Fig. 4A, n = 4-12 mice/group, p ≤ 0.05), and in contrast, 100 µg/kg/day of DiNP borderline increased the number of antral follicles (Fig. 4A, n = 4-12 mice/group, p = 0.10). However, no doses of DEHP or DiNP affected total follicle numbers when compared to control at this time point (Fig. 4D, n = 4-12 mice/group).
When follicle populations were analyzed as percentages, 100 µg/kg/day of DiNP borderline increased the percent of primordial follicles when compared to control (Fig. 4B, n = 4-12 mice/group, p = 0.06). Further, both 20 µg/kg/day of DiNP (Fig. 4B, n = 4-12 mice/group, p = 0.08) and 200 mg/kg/day of DiNP (Fig. 4B, n = 4-12 mice/group, p = 0.10) decreased the percent of antral follicles when compared to control. In contrast, DEHP and DiNP did not affect the percent of unhealthy follicles when compared to control (Fig. 4C, n = 4-12 mice/group).

5.4.3 Effects of DEHP and DiNP on fertility

12 months post-dosing

Treatment with 20 µg/kg/day of DiNP resulted in borderline decreased time to mating when compared to control (Fig. 5A, n = 9-19 mice/group, p = 0.06). Treatment with 100 µg/kg/day of DiNP (Fig. 5B, n = 4-13 mice/group, p ≤ 0.05) and 200 mg/kg/day of DiNP (Fig. 5B, n = 4-13 mice/group, p = 0.06) increased the length of gestation when compared to control. DEHP and DiNP did not affect the mating index (Fig. 6A, n = 9-21 mice/group) or the fertility index (Fig. 6B, n = 8-20 mice/group) at 12 months post-dosing, indicating that treated females were able to mate and conceive pregnancies at rates comparable to control. However, treatment with 20 µg/kg/day (Fig. 6C, n = 6-16 mice/group, p = 0.07) and 200 mg/kg/day of DEHP (Fig. 6C, n = 6-16 mice/group, p = 0.10) borderline decreased the gestational index of females in these groups when compared to control. Additionally, females in the 20 mg/kg/day treatment group of DiNP had a significantly reduced gestational index when compared to control (Fig. 6C, n = 6-16 mice/group, p ≤ 0.05), indicating these females were less able to carry their pregnancy to term and give birth to pups when compared to control. Further, the overall ability of females to produce pups as measured by the percent of females in each group who gave birth was borderline
reduced in the 20 µg/kg/day treatment group for both DEHP (Fig. 6D, n = 8-21 mice/group, p = 0.06) and DiNP (Fig. 6D, n = 8-21 mice/group, p = 0.08) when compared to control.

15 months post-dosing

Treatment with DEHP and DiNP did not affect time to mating at 15 months post-dosing (Fig. 7A, n = 8-15 mice/group). At this time point, the natural and steep decline in fertility of CD-1 mice at this age made it difficult to collect data and perform statistical analysis for this breeding trial. However, the percent of females who produced pups was able to be statistically analyzed, and treatment with DEHP and DiNP did not affect the overall ability of females to produce pups when compared to control (Fig. 7B, n = 7-17 mice/group).

5.4.4 Effects of DEHP and DiNP on litter outcomes

12 months post-dosing

At 12 months post-dosing, 200 µg/kg/day of DEHP significantly decreased litter size in treated females when compared to control (Fig. 8A, n = 3-8 mice/group, p ≤ 0.05). Further, average live weight of pups on PND 0 was significantly increased by 200 µg/kg/day DEHP treatment (Fig. 8B, n = 3-9 mice/group, p ≤ 0.05) and borderline increased by 200 mg/kg/day of DEHP when compared to control (Fig. 8B, n = 3-9 mice/group, p = 0.07). However, too few litters were born to the 200 mg/kg/day DiNP treatment group that statistical analysis of average live weight of pups on PND 0 could not be performed (n = 2 mice). Interestingly, percent of female pups was borderline decreased in the 20 µg/kg/day group DEHP (Fig. 8C, n = 3-7 mice/group, p = 0.07) and significantly decreased in the 20 mg/kg/day DEHP and 20 µg/kg/day DiNP treatment groups when compared to control (Fig. 8C, n = 3-7 mice/group, p ≤ 0.05). Because of the reduced
number of litters born at this time point in addition to the inability to confidently sex pups due to cannibalization, sample size was too low for statistical analysis of the percent of female pups for the 200 mg/kg/day DEHP (n = 2 mice), 100 µg/kg/day DiNP (n = 2 mice), and 200 mg/kg/day DiNP groups (n = 1 mouse).

15 months post-dosing
Because the fertility of the CD-1 mouse naturally takes a sharp decline around this time in the life of the mouse, very few litters were born to both treatment and control. Thus, we were not able to assess whether treatment affected litter outcomes for the breeding trial conducted at this time point.

5.4.5 Effects of DEHP and DiNP on hormone levels

12 months post-dosing
Treatment with DEHP and DiNP did not affect testosterone and progesterone levels at 12 months post-dosing (Fig. 9A and 9B, n = 5-12 mice/group). However, 20 µg/kg/day of DEHP borderline decreased levels of estradiol when compared to control (Fig. 9C, n = 5-12 mice/group, p = 0.06), and 20 mg/kg/day of DiNP borderline increased levels of FSH when compared to control (Fig. 10A, n = 5-12 mice/group, p = 0.10). No doses of DEHP or DiNP affected levels of inhibin B when compared to control at this time point (Fig. 10B, n = 4-10 mice/group).

15 months post-dosing
Treatment with DEHP and DiNP did not affect levels of testosterone and estradiol at 15 months post-dosing when compared to control (Fig. 11A and 11C, n = 4-12 mice/group). In contrast,
200 µg/kg/day of DEHP borderline increased levels of progesterone in treated mice (Fig. 11B, n = 4-12 mice/group, p = 0.07). Further, 20 µg/kg/day of DEHP borderline increased levels of FSH when compared to control (Fig. 12A, n = 4-12, p = 0.09). No doses of DEHP or DiNP affected levels of inhibin B at 15 months post-dosing when compared to control (Fig. 12B, n = 4-10 mice/group).

18 months post-dosing

Treatment with DEHP and DiNP did not affect progesterone levels when compared to control at this time point (Fig. 13B, n = 7-16 mice/group). In contrast, treatment with 200 mg/kg/day of DEHP borderline increased levels of estradiol when compared to control (Fig. 13C, n = 7-16 mice/group, p = 0.07), and 100 µg/kg/day of DiNP significantly decreased levels of estradiol when compared to control (Fig. 13C, n = 7-16 mice/group, p ≤ 0.05). Further, 100 µg/kg/day of DiNP also decreased testosterone levels when compared to control (Fig. 13A, n = 7-16 mice/group, p ≤ 0.05). No effects of treatment were observed on levels of FSH when compared to control at this time point (Fig. 14A, n = 7-16 mice/group). In contrast, the 20 mg/kg/day DiNP treatment group displayed increased levels of inhibin B when compared to control (Fig. 14B, n = 7-16 mice/group, p ≤ 0.05).

5.5 Discussion

This study tested the hypothesis that short-term exposure to DEHP and DiNP during adulthood has long lasting consequences on fertility, estrous cyclicity, follicle populations, and circulating levels of hormones at time points just before, during, and after the transition into reproductive senescence in mice. Previously, we have shown that short-term exposure to DEHP and DiNP
can have negative consequences on fertility up to 9 months post-dosing [11, 22]. The current study builds upon existing knowledge by investigating the effects of short-term exposure to DEHP and DiNP during adulthood on time points that have not yet been studied, including those encompassing reproductive senescence in the mouse.

We observed that DEHP and DiNP disrupted estrous cyclicity at 12 and 15 months post-dosing wherein females spent more time in estrus and less time in metestrus/diestrus compared to control. Increased time in spent in estrus has been shown to occur in the aging rodent [24, 25]. Thus, this could be indicative of accelerated reproductive aging in the treatment groups displaying increased time spent in estrus. Previous studies in our laboratory have shown that both DEHP and DiNP have long-lasting effects on cyclicity up to 9 months post-dosing [11, 22]. In contrast, a study investigating exposure to DEHP during adulthood in mice reported that DEHP increased time spent outside of the estrous phase [26]. Further, a different study found that rats that were perinatally exposed to DiNP did not display disrupted estrous cycles [27]. However, the differences observed between these studies and the present study are likely due to differences in age at cyclicity monitoring and window of exposure.

At 12 months post-dosing, we found that DiNP decreased the number and the percent of primordial follicles and percent of primary follicles as well as increased percent of preantral and antral follicles. Further, DEHP decreased the number and percent of primary follicles and decreased the number of preantral follicles. Of note is the trend that both DEHP and DiNP reduced populations of more immature follicle types, such as primordial and primary follicles, and increased percent of more mature follicle types. Skewing the follicle populations from more
immature follicle types to more mature follicle types could be indicative of accelerated folliculogenesis, a phenomenon observed in a previous study conducted in our laboratory using the same dosing paradigm [10]. If not corrected, accelerated folliculogenesis can lead to early reproductive senescence.

We also observed reduced total follicle numbers in the mice treated with 20 mg/kg/day of DEHP in addition to reduced percent of unhealthy follicle types in both DEHP- and DiNP-treated groups. In contrast, 20 µg/kg/day of DiNP-treated mice displayed an increased percent of unhealthy follicle types. When comparing the total follicle numbers to the numbers of each follicle type, the reduction in total follicles observed in the 20 mg/kg/day DEHP treatment group can largely be attributed to the lower numbers of primordial and primary follicles when compared to control. Further, we found the reduction in unhealthy follicle types very interesting, and we suspect that treatment with DEHP and DiNP has upregulated clearance of unhealthy follicles, giving an appearance of fewer unhealthy follicles. This hypothesis could also explain the increase in unhealthy follicles observed in the lower treatment group of DiNP, which may not have been a high enough dose to induce clearance of unhealthy follicles.

At 15 months post-dosing, we primarily observed treatment-induced disruptions in preantral and antral numbers and percentages. This suggests that DEHP and DiNP altered the rate at which these follicles progress from the preantral to antral stage. The transition from the preantral to antral stage is regulated by a variety of hormones such as FSH and genes including Smad3 and Mad-related proteins (SMAD) family member 3 (Smad3) and members of the forkhead
transcription factors (Foxo) family [28]. Thus, it is possible that treatment with DEHP and DiNP caused aberrant regulation of the genes or receptors for hormones involved in this transition.

At 18 months post-dosing, only some doses of DiNP affected follicle numbers and percentages. We observed DiNP-induced increases in the number and percent of primordial follicles and disruption of antral follicle numbers and percentages. Interestingly, we observed contrasting effects between the 20 µg/kg/day and 100 µg/kg/day doses of DiNP on antral follicle numbers, with 20 µg/kg/day decreasing and 100 µg/kg/day increasing antral follicle numbers. This non-monotonic dose response is common with endocrine disrupting chemicals [29] and highlights the importance of the investigation of the mechanisms through which these chemicals act. The DiNP-induced increase in primordial follicles is particularly perplexing because previous in vitro and in vivo studies in our laboratory have shown that phthalates can accelerate primordial follicle recruitment [10, 30]. However, a previous study conducted in our laboratory has shown that exposure to a phthalate mixture can reduce atresia in antral follicles cultured in vitro [31]. Because females are born with a finite number of primordial follicles, an increase in primordial follicle numbers can only be explained by less atresia or less recruitment to the next stages of growth. The lack of increased numbers or percentages of primary follicles suggests that the increase observed in primordial follicle numbers is likely due to less atresia in the primordial follicle pool as opposed to increased primordial follicle recruitment. It is possible that DiNP is acting through a similar mechanism as seen in this previous study that observed reduced atresia. Thus, future studies should investigate the mechanisms through which DiNP may regulate follicular atresia at different stages of growth.
At 12 months post-dosing, we observed that DEHP and DiNP increased pregnancy loss and overall decreased the ability of treated females to produce offspring in multiple treatment groups when compared to control. The reductions in the fertility of the 20 µg/kg/day treatment groups for both DEHP and DiNP observed in the present study have been observed in a previous study in our laboratory that examined fertility at 3 months post-dosing [22], indicating these effects can arise at multiple time points throughout the life of the exposed individual. Further, these treatment groups both exhibited increased time spent in estrus is a marker of reproductive aging [25]. Thus, the combination of reduced fertility and increased time spent in estrus suggest the onset of reproductive senescence is occurring earlier in these treatment groups. Although few studies have investigated the late-life consequences of exposure to DEHP and DiNP during adulthood, previous studies have found that DEHP exposure during adulthood or through transgenerational exposure can impact markers of reproductive aging in mice [11, 12, 32]. Further, epidemiological evidence shows associations between phthalate exposure and early age at menopause [33]. Additionally, phthalate exposure has also been associated with increased pregnancy loss in women [34], and some animal studies have found that DEHP exposure disrupts placental growth and development, possibly providing insufficient support for the fetus [35, 36].

In terms of pup outcomes, we found that at 12 months post-dosing, DEHP and DiNP decreased the percent of female pups and that DEHP alone decreased litter size and increased average live weight of pups when compared to control. One study has shown that DEHP can interfere with receptivity of the uterine lining to implantation [37], which could explain the reduced litter size observed in the present study. The increase in average live weight at birth is a result we have
observed previously at earlier time points closer to the exposure window [22]. Although the decrease in litter size in the 200 µg/kg/day DEHP group may have led to decreased intrauterine growth restriction and subsequently, larger pups, this does not explain the increase in average live weight in the 200 mg/kg/day DEHP group. DEHP has been shown to have obesogenic properties, potentially through activation of peroxisome proliferator-activated receptor (PPAR) activation [38, 39]. Thus, it is possible DEHP increased the adiposity of the pups, leading to increased weight at birth. These results contrast with some epidemiological findings that phthalate exposure is associated with reduced weight at birth [40, 41], but some studies have found inverse results [42].

The alteration in percent of female pups born was surprising due to the pre-conception dosing paradigm of the study. However, sex ratio was altered in a previous study using the same dosing paradigm conducted in our laboratory at 9 months post-dosing [22]. In that previous study, we postulated that the difference in sex ratio may be due to treatment affecting hormones and, subsequently, sperm selection as the sperm traversed the female reproductive tract. However, we did not observe correlations between hormones and alterations in sex ratio in the current study, suggesting this is not the mechanism through which DEHP and DiNP alter sex ratio. Previous studies have shown that maternal age and condition can affect sex ratio of offspring [43-45]; thus, it is possible that treatment may lead to altered sex ratios by altering aspects not detectable through methods used in this study.

Overall, the effects of treatment on hormone levels were limited. However, alterations in hormones were present throughout the study through the last time point at 18 months post-
dosing. This indicates that hormones may be affected throughout the life of the exposed individual. Indeed, the most striking effects on hormones observed in this study were observed at 18 months post-dosing, wherein the 100 µg/kg/day DiNP treatment group exhibited reduced levels of both estradiol and testosterone. Interestingly, this same treatment group had increased number of antral follicles, indicating that reducing the antral follicle population is not necessary to reduce the levels of sex steroid hormones. It is likely that either the function of the antral follicles has been disrupted or that metabolism of these hormones is occurring at a faster rate than production when compared to control. The other effect seen in hormones at 18 months post-dosing was an increase in inhibin B levels in the 20 mg/kg/day DiNP treatment group when compared to control. We find this result surprising because inhibin B is a marker of ovarian reserve, and we observed no changes in any follicle types for this group at this time point. Thus, we suspect that this increase in inhibin B is not due to an increase in the ovarian reserve and that it may reflect disruptions in the metabolism or production of inhibin B itself.

We find the lack of correlation between the breeding outcomes and hormonal and follicle count data perplexing because a lack of fecundity can often be explained by disruptions to follicular populations or hormones. It is possible that the observed disruptions in fertility may be based in the uterus and not in the ovary or the production of ovarian hormones. Thus, future studies should investigate the effects of DEHP and DiNP on uterine function, especially as it concerns the support of pregnancy in light of the increased pregnancy loss observed in this study.

We also find the contrast between the hormone and follicle population data interesting. The lack of correlation between antral follicle numbers and changes in hormones at most time points
suggests that the answer may lie in alterations of metabolism or production of these hormones and not the absolute follicle numbers. Previous studies in our research group have shown that phthalates can alter expression of a variety of steroidogenic enzymes present within the ovary [31, 46]. Thus, it is possible that DEHP and DiNP cause altered expression or activity of the steroidogenic enzymes present in the ovary.

In conclusion, this study found that short-term exposure during adulthood has long-lasting consequences on estrous cyclicity, ovarian follicle populations, fertility, and hormones in the female mouse. The effects of DEHP and DiNP observed in this study occurred a year or longer after cessation of exposure, thus, future studies should investigate the mechanisms through which DEHP and DiNP may be imparting these long-lasting effects during such a short-term window of exposure.
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20μg/kg/day – 200 mg/kg/day), or DiNP (20μg/kg/day – 200 mg/kg/day). Females were vaginally lavaged daily for 14 days to assess cyclicity at 12 months post-dosing (n = 9-22 mice/group) and 15 months post-dosing (8-20 mice/group). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months following completion of the dosing period, females were euthanized and ovaries were collected and histologically evaluated for raw follicle numbers (n = 3-12 mice/group) (A), percent of follicles (n = 3-12 mice/group) (B), percent of unhealthy follicles (n = 3-12 mice/group) (C), and total number of follicles (n = 3-12 mice/group) (D). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.3 Effects of DEHP and DiNP on ovarian follicle populations at 15 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 15 months following completion of the dosing period, females were euthanized and ovaries were collected and histologically evaluated for raw follicle numbers (n = 4-11 mice/group) (A), percent of follicles (n = 4-11 mice/group) (B), percent of unhealthy follicles (n = 4-11 mice/group) (C), and total number of follicles (n = 4-11 mice/group) (D). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.4 Effects of DEHP and DiNP on ovarian follicle populations at 18 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 18 months following completion of the dosing period, females were euthanized and ovaries were collected and histologically evaluated for raw follicle numbers (n = 4-12 mice/group) (A), percent of follicles (n = 4-12 mice/group) (B), percent of unhealthy follicles (n = 4-12 mice/group) (C), and total number of follicles (n = 4-12 mice/group) (D). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months post-dosing, females were paired with an untreated male mouse directly after completion of the estrous cyclicity monitoring period. Females were checked every morning and afternoon for the presence of a copulatory plug. If a copulatory plug was observed or if 14 days had elapsed, the female was put into a new clean cage. If a female presented with a copulatory plug the day immediately after introduction of the male, the time to mating was considered 1 day (n = 9-19 mice/group) (A). The day the copulatory plug was observed was considered day 0.5 of gestation (n = 4-13 mice/group) (B). Females were checked morning and afternoon starting a week before the expected delivery date of the litter. Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months following the completion of the dosing period, breeding trials were conducted by pairing females with untreated male mice. The mating index (n = 9-21 mice/group) (A) is the number of females who presented with a copulatory plug divided by the total number of females in the group and multiplied by 100. The fertility index (n = 8-20 mice/group) (B) is the number of females who became pregnant divided by the number of females who presented with a copulatory plug and multiplied by 100. The gestational index (n = 6-16 mice/group) (C) is the number of females who gave birth divided by the number of females who became pregnant and multiplied by 100. Females who gave birth (n = 8-21 mice/group) (D) is the number of females who gave birth divided by the total number of females in the group and multiplied by 100. Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as percentages. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.7 Effects of DEHP and DiNP on time to mating and overall fertility at 15 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20μg/kg/day – 200 mg/kg/day), or DiNP (20μg/kg/day – 200 mg/kg/day). At 15 months post-dosing, females were paired with an untreated male mouse directly after completion of the estrous cyclicity monitoring period. Females were checked every morning and afternoon for the presence of a copulatory plug. If a copulatory plug was observed or if 14 days had elapsed, the female was put into a new clean cage. If a female presented with a copulatory plug the day immediately after introduction of the male, the time to mating was considered 1 day (n = 8-15 mice/group) (A). Females who gave birth (n = 7-17 mice/group) (B) was calculated as the number of females who gave birth divided by the total number of females in the group and multiplied by 100. Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error (A) and percentages (B). Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39–40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months following completion of dosing, females were paired with untreated male CD-1 mice for breeding trials. If females delivered pups, litter size on PND 0 was determined (n = 3–8 mice/group) (A), average weight of all live pups on PND 0 was measured (n = 3–9 mice/group) (B), and the percent of female pups was measured (n = 3–7 mice/group) (C). However, some groups lacked proper sample size to analyze outcomes, including average live weight for 200 mg/kg/day DiNP (n = 2 mice) and percent female pups for 200 mg/kg/day DEHP (n = 2 mice), 100 µg/kg/day DiNP (n = 2 mice), and 200 mg/kg/day DiNP (n=1). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected for analysis of testosterone (n = 5-12 mice/group) (A), progesterone (n = 5-12 mice/group) (B), and estradiol (n = 5-12 mice/group) (C) via enzyme-linked immunosorbent assay (ELISA). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 12 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected. Sera samples were shipped to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of follicle stimulating hormone (FSH) (n = 5-12 mice/group) (A) and inhibin B (n = 4-10 mice/group) (B) via radioimmunoassay (RIA). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.11 Effects of DEHP and DiNP on levels of sex steroid hormones at 15 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 15 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected for analysis of testosterone (n = 4-12 mice/group) (A), progesterone (n = 4-12 mice/group) (B), and estradiol (n = 4-12 mice/group) (C) via enzyme-linked immunosorbent assay (ELISA). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 15 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected. Sera samples were shipped to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of follicle stimulating hormone (FSH) (n = 4-12 mice/group) (A) and inhibin B (n = 4-10 mice/group) (B) via radioimmunoassay (RIA). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.13 Effects of DEHP and DiNP on levels of sex steroid hormones at 18 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 18 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected for analysis of testosterone (n = 7-16 mice/group) (A), progesterone (n = 7-16 mice/group) (B), and estradiol (n = 7-16 mice/group) (C) via enzyme-linked immunosorbent assay (ELISA). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
Figure 5.14 Effects of DEHP and DiNP on levels of follicle stimulating hormone and inhibin B at 18 months post-dosing

Female CD-1 mice were orally dosed at age 39-40 days for 10 days with either vehicle control (corn oil), DEHP (20µg/kg/day – 200 mg/kg/day), or DiNP (20µg/kg/day – 200 mg/kg/day). At 18 months following completion of dosing, mice were euthanized in the diestrous stage and sera were collected. Sera samples were shipped to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for analysis of follicle stimulating hormone (FSH) (n = 7-16 mice/group) (A) and inhibin B (n = 7-16 mice/group) (B) via radioimmunoassay (RIA). Statistical significance (p ≤ 0.05) is denoted with an asterisk (*). Data are represented as means ± standard error. Borderline statistical significance (0.05 < p ≤ 0.10) is denoted with a caret (^).
5.7 References


CHAPTER 6

Summary, Conclusions, and Future Directions

6.1 Summary, Conclusions, and Future Directions

The goal of my doctoral dissertation work was to investigate the short-term and long-term reproductive consequences of 10 days of exposure to DEHP or DiNP during adulthood in females. Phthalates are a class of chemicals commonly utilized as plasticizers and stabilizers in a wide variety of consumer goods [1, 2]. Due to this widespread use, humans are exposed to phthalates on a daily basis. DEHP is a particularly prominent member of the phthalate group, with several million pounds being produced within the U.S. every year [3]. Of concern is that DEHP has been shown to have endocrine disrupting capabilities in females [4-7]. Further, DEHP is commonly substituted with other chemicals which often lack adequate studies to determine their safety in terms of female reproductive toxicity. One common DEHP replacement is DiNP. Very few studies have investigated the reproductive toxicity of DiNP, but the few studies that have been conducted show that DiNP may be capable of endocrine disruption [8-10]. Thus, the safety of DiNP and, subsequently, its adequacy as a viable replacement for DEHP are equivocal. Compounding this concern is an overall lack of information on the effects of exposure to either DEHP or DiNP on female reproduction using an exposure window during adulthood. Very few studies have investigated the effects of exposure during this time frame and how the exposure may affect the exposed female throughout her life. To fill this gap in knowledge, my doctoral dissertation work investigated the effects of short-term exposure to DEHP and DiNP and the subsequent effects on female reproduction at various
timepoints following completion of dosing. The timepoints in my study ranged from immediately post-dosing to 18 months post-dosing. Collectively, my studies show that short-term exposure to DEHP and DiNP has long-term consequences on hormones, ovarian follicles, and various aspects of female fertility in mice.

In Chapter 3, I tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood affects estrous cyclicity and fertility during the prime reproductive life of the CD-1 mouse. Specifically, I investigated timepoints immediately post-dosing and 3 and 9 months post-dosing. I found that 20 µg/kg/day of DEHP or DiNP decreased the ability of females to become pregnant at 3 months post-dosing. Further, I found that multiple doses of DiNP disrupted estrous cyclicity at 3 months post-dosing and 100 µg/kg/day DiNP disrupted cyclicity at 9 months post-dosing. Results in Chapter 4 of my dissertation suggest that the reduction in fertility observed at the 3 months post-dosing breeding trial is not due to treatment-induced changes in follicle populations or circulating hormone levels. Because the decrease in fertility observed at 3 months post-dosing is largely attributable to the decrease in the ability of females to become pregnant, it is possible that DEHP and DiNP disrupted the implantation process or loss of pregnancy at an early enough stage that we were unable to determine if the female had become pregnant at all. Thus, future studies should investigate the effects of DEHP and DiNP on the uterus and implantation at 3 months post-dosing.

In Chapter 3, I also tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood affects litter outcomes. I observed that multiple doses of DEHP and DiNP caused male-biased litters at 9 months post-dosing. I was surprised to find that the preconception
exposure paradigm used in this study resulted in an alteration in the sex ratio of pups. Further, disruptions in sex ratios persisted as shown in Chapter 5. Results from Chapter 4 again suggest that this shift in sex ratio is not due to an alteration in hormone levels. Interestingly, studies have shown that maternal age and health can have influence on the sex of the offspring [11-13]. Thus, future studies should investigate the mechanism through which preconception exposure to DEHP and DiNP affects the sex ratio.

In Chapter 4, I tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood affect ovarian follicle populations and circulating hormone levels during the prime reproductive life of the CD-1 mouse. Specifically, I investigated timepoints immediately post-dosing and 3, 6, and 9 months post-dosing. I found that multiple doses of DEHP and DiNP caused significant disruption to hormones and follicle populations at all tested timepoints. Interestingly, I found a somewhat inverse relationship with phthalate-induced effects on follicle populations and hormones. Specifically, follicles were more disrupted at timepoints other than immediately post-dosing, but multiple doses of DEHP and DiNP disrupted almost every hormone assayed immediately post-dosing, with effects tapering off as timepoints progressed. Because the antral follicle is the primary source of sex steroid hormones in females, I expected follicle numbers, specifically those of antral follicles, to correlate with hormone levels, but this was not the case. Thus, it is likely that DEHP and DiNP acted through disruption of production or metabolism of sex steroid hormones. Therefore, future studies should investigate the function of the antral follicle by examining the expression levels of key sex steroid hormones within the ovary. Further, future studies should also investigate how DEHP and DiNP affect genes that
regulate folliculogenesis to shed light on the mechanisms through which DEHP and DiNP affect follicle numbers.

In Chapter 5, I tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood affects fertility and litter outcomes during the late-life of the CD-1 mouse. Specifically, I performed breeding trials at 12 and 15 months post-dosing. I found that DEHP and DiNP decreased percent of female pups born at 12 months post-dosing. The alteration in sex ratio of pups was surprising but not unprecedented, as this was a result that I observed in my research in Chapter 3, and again I found no correlation between sex steroid hormones and sex ratio. Some studies have suggested that maternal age and body condition can affect sex ratio of offspring [11-13]. Thus, future studies should investigate how DEHP and DiNP may affect the bodily health of the dam or how DEHP and DiNP may affect aging in general.

In Chapter 5, I also tested the hypothesis that short-term exposure to DEHP or DiNP during adulthood affects ovarian follicle populations and circulating hormone levels during the late-life of the CD-1 mouse. Specifically, I investigated timepoints 12, 15, and 18 months post-dosing. I found that both DEHP and DiNP disrupted follicle numbers and percentages and hormone levels at several timepoints. Interestingly, the most drastic changes in hormones occurred at 18 months post-dosing where I observed 100 µg/kg/day of DiNP decreased estradiol and testosterone compared to control. In contrast, multiple doses of DEHP and DiNP led to disruption of ovarian follicles at almost every timepoint. This coincides with results in Chapter 4 wherein disruptions in follicle numbers and percentages did not always correlate with hormone levels. Similarly to Chapter 4, this suggests that the changes in hormones are not due to treatment with DEHP or
DiNP directly affecting the absolute number of antral follicles. It is likely that DEHP and DiNP are dysregulating the production or metabolism of the sex steroid hormones. For example, it is possible DEHP and DiNP may be altering the expression or activity of the steroidogenic enzymes present in the antral follicle. Thus, future studies should investigate how DEHP and DiNP are affecting levels of circulating hormones, and further, future studies should investigate what possible mechanisms short-term exposures may act through to impart the observed long-lasting effects.

In Chapter 5, I also tested the hypothesis that short-term exposure to DEHP and DiNP during adulthood affects fertility at 12 and 15 months post-dosing. I observed that multiple doses of DEHP and DiNP increased pregnancy loss and that 20 µg/kg/day of DEHP and DiNP decreased overall fertility compared to control. I did not observe any correlation changes between treatment groups that experienced pregnancy loss and changes in progesterone, a hormone critical for maintenance of pregnancy. However, mice that contributed hormone data were non-pregnant and collected during the diestrous stage of the estrous cycle. Thus, it is possible that deficiencies in the production of progesterone may be to blame and such deficiencies may not be detectable in non-pregnant mice. Future studies should investigate the function of the corpus luteum during pregnancy at 12 months post-dosing to determine if DEHP and DiNP may affect hormone production and, subsequently, support of pregnancy.

In Chapter 5, I also tested the hypothesis that short-term exposure to DEHP and DiNP during adulthood accelerate reproductive aging. I observed that 20 µg/kg/day of DEHP increased the time females spent in the estrous stage of the cycle compared to control. The disruption in
estrous cyclicity and decrease in fertility described above are indicative of potential early reproductive aging, as females who enter reproductive senescence experience prolonged time in the estrous stage of the cycle in addition to becoming less fertile [14, 15]. In agreement, a previous study in our laboratory found that 10 days of exposure to DEHP during adulthood increased markers of reproductive aging [16]. With my studies and a past study showing increased markers of reproductive aging due to exposure to DEHP and DiNP, it is imperative that future studies investigate other markers of reproductive aging, such as increased inflammation and fibrosis to fully understand how DEHP and DiNP may be accelerating reproductive aging [17, 18].

Overall, my doctoral dissertation work shows that short-term exposure to DEHP and DiNP during adulthood disrupts a variety of aspects of female reproduction throughout the natural life of the exposed female. Specifically, 10 days of exposure to DEHP or DiNP decreased fertility at multiple timepoints, altered estrous cyclicity at all timepoints, altered sex ratio of pups born at multiple timepoints, disrupted ovarian folliculogenesis at all timepoints, and disrupted circulating hormones at all timepoints. My research provides important information on how short-term exposure during adulthood, a time frame often thought to not be critical or sensitive, affects fertility parameters of the exposed individual for the rest of their life. Further, my studies add crucial information to the scant body of literature concerning the reproductive toxicity of DiNP. Moreover, my research utilizes environmentally relevant doses for both DEHP and DiNP, heightening the relevance and importance of my findings. This work is important to public health because humans are exposed to phthalates on a daily basis, and proper reproductive function in females is critical for overall fertility as well as overall bodily health. Thus,
generating more knowledge on the deleterious effects of phthalate exposure is critical for a more fertile and healthy future human population.
6.2 References


Hormone Variability and Hot Flash Experience: Results from the Midlife Women’s Health Study²

A.1 Abstract

Objective: Hot flashes, common during midlife, are believed to be related to hormonal changes. However, hormonal fluctuations and the incidence of hot flashes has not been studied. The objective of this study is to determine hormone measurement summaries that best explain hot flash incidence in midlife women.

Methods: Data were from a cohort study of 798 midlife women over 1 to 7 years. Women were asked to provide 4 weekly blood samples annually and complete a survey detailing life history, ongoing behaviors, and menopausal symptoms. Estradiol, progesterone, and testosterone levels were measured from serum samples at each visit. Annual summary variables of each hormone consisted of median, mean, maximum, minimum, variance, and range. Association of these values with hot flash outcomes (incidence, severity, and frequency) was assessed using multivariable logistic regression and Bayesian Network analysis, controlling for smoking history and menopausal status.

Results: For most outcomes, the best-fit logistic regression model included progesterone variability; an increase in progesterone variance or range was correlated with a decrease in the

frequency and severity of hot flashes. In the Bayesian Network model, the maximum estradiol value was negatively correlated with many outcomes. The relationships between progesterone variability, maximum estradiol, maximum progesterone, and hot flashes outcomes indicate that the effects of progesterone variance on hot flashes outcomes are likely mediated through progesterone’s relationship with maximum estradiol.

Conclusions: These results indicate that variability of progesterone, as opposed to mean values, should also be used as an indicator for risk of hot flashes in midlife women.

A.2 Introduction
A future direction of my doctoral dissertation work is to investigate the associations between phthalates and hormones in midlife women. However, it is important to first characterize associations between hormone fluctuations and trends and quality of life outcomes. Investigating how hormones levels can affect quality of life will allow for analysis on how phthalates, through impacting hormone levels, can affect quality of life.

One significant factor in the quality of life of perimenopausal women is the experience of vasomotor symptoms (hot flashes) [1]. Up to 87% of perimenopausal women experience hot flashes, and these symptoms are associated with significant costs due to treatment (prescription, over-the-counter, alternative, and dietary), physician visits, laboratory testing, and loss of productivity; hormone therapy alone has been estimated to cost between $357 and $474 per patient per year [1, 2].
The reasons that perimenopausal women experience hot flashes (HF) are unclear; no single factor has been consistently identified as playing a major role [3]. Many studies have shown the relationship between HFs and the mean levels of hormones, particularly estrogen withdrawal [1]. However, it is generally believed that decreases in estrogen levels are the primary reason for HFs [4] as the menopausal transition is a time of fluctuations in hormones and estrogen supplementation has been the most effective treatment [5]. No previous studies have considered the effect of hormonal fluctuations on HFs.

The objective of this study is to determine the hormone measures that best describe the HF experience in a cohort study of midlife women. These include estradiol, progesterone, and testosterone, measured as the mean, median, maximum, minimum, and variance.

A.3 Materials and Methods

All participants gave written informed consent according to procedures approved by the University of Illinois and Johns Hopkins University Institutional Review Boards, which approved this research. The study design for the parent study is described in detail elsewhere [6]. Briefly, a cohort study of HFs among women 45-54 years of age was conducted starting in 2006 among residents of Baltimore and its surrounding counties. Women were recruited by mail and were included if they were in the target age range, had intact ovaries and uteri, and were pre- or perimenopausal. Exclusion criteria consisted of pregnancy, a history of cancer, exogenous female hormone or herbal/plant substance, and no menstrual periods within the past year. Participants made a baseline clinic visit, which included serum collection and completion of a detailed 26-page baseline survey. Participants then visited the clinic weekly for the next 3 weeks,
providing serum samples at each visit. After the baseline year, participants returned annually to complete a questionnaire, repeating all previous questions about hot flashes and smoking, and providing the 4 weekly serum samples as before. Blood samples were stored until measurement of hormone levels as described below.

The questionnaire asked if women had experienced HFs in the last year, in the last 30 days, and the severity and frequency of the majority of their HFs at the time of the visit. With regards to severity, descriptions were: mild (sensation of heat without sweating), moderate (sensation of heat with sweating), or severe (sensation of heat with sweating that disrupts usual activity). For this analysis, severity was also dichotomized into “moderate or severe” versus “none or mild”. With regards to frequency, descriptions were: every hour, every 2-5 hours, every 6-11 hours, every 12-23 hours, 1-2 days per week, 3-4 days per week, 5-6 days per week, 2-3 days per month, 1 day per month, less than 1 day per month, or never. For this analysis, frequency was categorized into “daily”, “weekly”, “monthly”, and “never” and dichotomized into “daily or weekly” versus “monthly or less”. Although self-report of HFs was not validated in this study against an objective measurement, self-report of HFs has been accepted as a valid measure by both the National Institute on Aging and the FDA [7, 8]. The questionnaire also asked women to report if they currently smoked, formerly smoked, or never smoked, as well as their age. Menopause status was defined as follows: premenopausal women were those who experienced their last menstrual period within the past 3 months and reported 11 or more periods within the past year; perimenopausal women were those who experienced 1) their last menstrual period within the past year, but not within the past 3 months, or 2) their last menstrual period within the
past 3 months and experienced 10 or fewer periods within the past year; postmenopausal women were those women who had not experienced a menstrual period within the past year.

**Hormone Variability**

Serum samples extracted from the collected blood samples were used to measure estradiol, testosterone, and progesterone levels in each sample using commercially available, previously validated enzyme-linked immunosorbent assay (ELISA) kits (DRG, Springfield, New Jersey, USA) [9-12]. The minimum detection limits and intra-assay coefficients of variation were as follows: estradiol 9.714 pg/ml; testosterone 0.083 ng/ml; and progesterone 0.045 ng/ml. The average inter-assay coefficient of variation for all assays was less than 5%. In the case of values lower than the detection limits for the assay, we used the limit of detection as the hormone value; of samples used for this analysis, 11/560 progesterone values were below the limit of detection, whereas no estradiol or testosterone levels were below the limit of detection. Each sample was measured in duplicate within the same assay. All estradiol values greater than 500 pg/ml (n=22), progesterone values greater than 40 ng/ml (n=1), and testosterone values greater than 10 ng/ml (n=7) were assumed to be laboratory error and removed from the data set.

Across each woman in each year, the following summary variables were calculated for each hormone: mean, median, maximum, minimum, range, and variance. All values were log10 transformed for analyses.
Univariable Analysis

The relationship between each summary value (mean, median, maximum, minimum, range, and variance) of each hormone (estradiol, progesterone, and testosterone) and each hot flash measurement was assessed using logistic regression (any in the last year, moderate/severe, weekly/daily, and in the last 30 days) or ordinal logistic regression (severity and frequency), with random effects to account for the year of the study and the individual.

Multivariable Analysis

The best fit multivariable model for each outcome was fit using receiver-operator characteristic (ROC)-based forward model selection, controlling for menopausal status and with and without the potential confounder of smoking history included in the model. Briefly, the model was fit with each of the potential variables added separately, and the area under the curve (AUC) of the ROC for each model was calculated. The model with the highest AUC was selected for that round, and the process was repeated additively until no additions produced a higher AUC.

Bayesian Network Analysis

Using data from the baseline visit, a Bayesian Network was fit for the variable nodes of hot flashes in the last year; hot flashes in the last 30 days; moderate/severe hot flashes; weekly/daily hot flashes; mean, maximum, minimum, and variance of all hormones; age; and smoking status. Smoking status was divided into two variables: ever vs. never smoked, and current smokers vs. not currently smoking. Menopausal status was divided into premenopausal vs. perimenopausal; there were no postmenopausal women at baseline. Hormone summary levels were not allowed to be parents for smoking status, and HF outcomes were not allowed to be parents for hormone
summary values. The number of parents for any variable was limited to 3. HFs in the last 30 days was forced to be a child to hot flashes in the last year. Best-fit models were plotted to include all parental nodes to the outcomes. Vectors were plotted with the coefficient of the statistical relationship between the parent and child node. For categorical variables, these relationships are based on a logistic regression model. For continuous variables, these relationships are based on a linear regression model.

**Model Fitting**

Logistic regression models were fit using the lme4 package\(^1\) in R 3.4.2 \(^2\). Ordinal logistic regression models were fit using the multgee package\(^3\) in R 3.4.2 \(^2\). The AUC of the ROC curve for the model predictions was calculated using the pROC package\(^4\) for logistic regression models and the HandTill2001 package\(^5\) for ordinal logistic regression models. Bayesian Network models were fit using the abn package\(^6\) in R version 3.0.3 \(^2\).

**A.4 Results**

**Hormone Variability**

The number of samples available to analyze for each of the hormones measured (estradiol, progesterone, and testosterone) are shown in Table 1. The distribution of the summary variables relative to HF experience is shown in Figure 1. Individuals providing only one sample in a year were not included in the variance and range variables.
Univariable Analysis

All summary variables for estrogen and progesterone were significantly associated with all outcomes (p<0.001 for all comparisons), with increases in any summary variable associated with decreases in the probability of any output (data not shown). The range of testosterone values was only significantly associated with the two outcomes measuring severity (p=0.029 for the ordinal outcome and p=0.005 for the dichotomous outcome, data not shown). All other summary variables for testosterone were significantly associated with all outcomes (p<0.05), with increases in any summary variable associated with decreases in the probability of any output (data not shown).

Multivariable Analysis

The results of the multivariable logistic analysis are shown in Tables 2-5. Smoking status was correlated with several HF outcomes. Specifically, never smoking and former smoking was correlated with a decrease in HF frequency, HF severity, experiencing HFs in the last year, and experiencing HFs in the last 30 days when compared to current smokers (Tables 2-5). Menopausal status was correlated with all measured HF outcomes. Pre-menopausal status was negatively correlated with all HF outcomes, whereas post-menopausal status was positively correlated with all HF outcomes when compared to peri-menopausal status (Tables 2-5). Progesterone variance was correlated with decreased frequency and severity of HF (Tables 4 and 5).
Bayesian Network Analysis

The result of the Bayesian Network analysis are shown in Figure 2. Variables that were not correlated with HF outcomes are not shown. Smoking was associated with probability of any HFs. Higher probability of any HFs was associated with lower maximum estradiol as well as menopausal status. The relationship between maximum progesterone and frequency of HFs was mediated through maximum estradiol levels, which were negatively associated with experiencing any HFs. Menopausal status was associated with hormonal outcomes as well as HF outcomes. The relationship menopausal status shares with HF outcomes was partially mediated by the negative association between maximum estradiol and probability of any HFs, while the relationship between menopausal status and the frequency of hot flashes was partially mediated by the negative association between maximum progesterone and the frequency of HFs.

A.5 Discussion

Our results found that progesterone variance was the most important hormonal measurement in terms of HF experience. Specifically, when controlling for menopausal status and smoking history, an increase in progesterone variance was associated with a decrease in frequency and severity of HFs. Using a multivariate machine learning approach, most HF outcomes were ultimately explained by the variance of progesterone, history of smoking, and menopausal status.

Previous analyses of these data [19-22] and similar studies [3, 9] have found associations between mean hormone levels and HF experience, but this study shows that the predictive ability of the models was maximized by using summary values more related to variability. It is known that estradiol variability is associated with depressive symptoms during the menopausal
transition [23], but to our knowledge, this is the first analysis to show that the variability of progesterone is highly associated with HF symptoms. Progesterone has long been suspected to play a role in vasomotor symptoms, as progesterone receptors have been found in the hypothalamus and progesterone can alter gene expression within serotonin neurons [24]. Progesterone therapy is known to reduce vasomotor symptoms, and may improve the efficacy of low-dose estrogen therapy [5]. It is possible that the associations we found between progesterone variability and decreased HF symptoms may be explained by the variance of progesterone throughout the menstrual cycle, indicating that these women with increased progesterone variability and, subsequently, decreased HF symptoms, may still be experiencing full menstrual cycles. The menstrual cycle is commonly divided into the follicular phase and the luteal phase [25]. In the follicular phase, follicles within the ovary begin to mature and a dominant follicle arises and stimulates the preovulatory luteinizing hormone (LH) surge, leading to ovulation and release of the oocyte from the follicle. Following ovulation, the corpus luteum (CL) is formed and the luteal phase begins. During this time, the CL produces large of amounts of progesterone as well as estradiol. Progesterone and estradiol production both decline with the degeneration of the CL and the beginning of the new follicular phase, whereas estradiol will slowly begin to rise again with the growth of the dominant follicle. Because estradiol is present in both the follicular and the luteal phase in addition to the preovulatory estradiol spike being relatively brief, it is perhaps not surprising that maximum estradiol, as opposed to variance of estradiol, was associated with reduced HF symptoms in women who donated samples once a week for four weeks. In contrast, progesterone is produced in very low quantities in the follicular phase and very high quantities in the luteal phase, possibly leading to samples over the course of four weeks from an actively cycling woman (i.e., a woman not likely to be
experiencing more severe HF symptoms) having more variable readings of progesterone. Thus, it is possible that variance of progesterone in addition to measurements of maximum estradiol and maximum progesterone can be used as an indicator of cycling status in midlife women and, therefore, a predictor of their risk of experiencing HFs.

Many possible summary variables were associated with many possible HF-related outcomes in this study, which raised a potential for false association due to multiple testing. However, some models found an association with the variance of progesterone, which indicates that this measure is associated with the experience of HFs. In addition, the Bayesian Network approach, which is designed to allow the discovery of complex relationships with many factors, found that the connection between hormone values and HF experience was limited to a few summary variables: progesterone variance, maximum progesterone, and maximum estradiol.

Many of the hormone variables were highly correlated, which could lead to multicollinearity in model fitting. However, the forward-stepping model selection procedure allowed for addition of the variables most improving the predictive ability of the model; highly correlated variables would not be likely to improve the model prediction.

The findings of this study indicate that one hormone measurement, or summarizing multiple hormone measurements by averaging them, is not the best way to predict HF experience. Given that there is evidence that hormone fluctuations are also important predictors of depressive symptoms during menopause [26], measurement of hormones in studies of perimenopausal women should be longitudinal in nature.
### Table A.1a Number of woman-year combinations providing multiple samples analyzed for hormone levels

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<td>Level</td>
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<tr>
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<tr>
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<td>max</td>
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### Table A.2 Multivariable results for hot flashes in the last year. Area under the ROC curve = 0.802

|                      | OR         | Estimate | Std. Error | z value | Pr(>|z|) |
|----------------------|------------|----------|------------|---------|----------|
| Former smoker        | 0.68 (0.47, 0.98) | -0.39    | 0.19       | -2.09   | 3.66E-02 |
| Never smoker         | 0.44 (0.31, 0.62)  | -0.83    | 0.18       | -4.58   | 4.76E-06 |
| Post-menopause       | 2.12 (1.49, 3.03)  | 0.75     | 0.18       | 4.15    | 3.29E-05 |
| Pre-menopause        | 0.18 (0.14, 0.22)  | -1.71    | 0.11       | -15.30  | 7.09E-53 |

### Table A.3 Multivariable results for hot flashes in the last 30 days. Area under the ROC curve = 0.797

|                      | OR         | Estimate | Std. Error | z value | Pr(>|z|) |
|----------------------|------------|----------|------------|---------|----------|
| Former smoker        | 0.57 (0.4, 0.82)   | -0.56    | 0.18       | -3.06   | 0.0022   |
| Never smoker         | 0.39 (0.27, 0.55)  | -0.94    | 0.18       | -5.29   | 1.25E-07 |
| Post-menopause       | 2.61 (1.86, 3.66)  | 0.96     | 0.17       | 5.58    | 2.42E-08 |
| Pre-menopause        | 0.21 (0.16, 0.26)  | -1.58    | 0.11       | -14.14  | 2.21E-45 |

### Table A.4 Multivariable results for hot flash severity. Area under the ROC curve = 0.505

|                      | OR         | Estimate | Std. Error | z value | Pr(>|z|) |
|----------------------|------------|----------|------------|---------|----------|
| Former smoker        | 0.6 (0.448 - 0.814) | 0.504    | 0.152      | 3.316   | 0.001    |
| Never smoker         | 0.37 (0.275 - 0.489) | 1.002    | 0.146      | 6.85    | <0.001   |
| Post-menopause       | 1.28 (1.001 - 1.645) | -0.249   | 0.127      | -1.965  | 0.049    |
| Pre-menopause        | 0.31 (0.244 - 0.386) | 1.18     | 0.116      | 10.132  | <0.001   |
| Variance of Progesterone | 0.82 (0.772 - 0.877) | 0.195    | 0.032      | 6.005   | <0.001   |

### Table A.5 Multivariable results for hot flash frequency. Area under the ROC curve = 0.508

|                      | OR         | Estimate | Std. Error | z value | Pr(>|z|) |
|----------------------|------------|----------|------------|---------|----------|
| Former smoker        | 0.57 (0.42, 0.77)    | 0.57     | 0.16       | 3.58    | <0.001   |
| Never smoker         | 0.44 (0.32, 0.59)    | 0.83     | 0.16       | 5.27    | <0.001   |
| Post-menopause       | 1.48 (1.13, 1.95)    | -0.39    | 0.14       | -2.8    | 0.01     |
| Pre-menopause        | 0.26 (0.21, 0.33)    | 1.35     | 0.12       | 11.7    | <0.001   |
| Variance of Progesterone | 0.82 (0.74, 0.91)    | 0.2      | 0.05       | 3.89    | <0.001   |
| Minimum Progesterone | 0.81 (0.58, 1.12)    | 0.21     | 0.17       | 1.29    | 0.2      |
| Median Progesterone  | 0.9 (0.65, 1.26)     | 0.1      | 0.17       | 0.6     | 0.55     |
Distribution of parameter values among women experiencing (blue, right) or not (purple, left) hot flashes in the last year. Values are shown as log-transformed. Bars indicate the median value of each distribution.
Figure A.2 Bayesian Network for hot flash experience.

Black/solid lines are outcomes, red/dotted lines are progesterone, blue/dashed lines are estradiol, and purple/dash-dot lines are the potential confounders smoking history and menopausal status. Lines leading into progesterone and estradiol boxes are coefficients from linear regression models. Lines leading into outcome boxes are coefficients from logistic regression models.
A.7 References


18. Lewis, F.I., abn: Data Modelling with Additive Bayesian Networks. 2014.


