Phthalates and Male Reproductive Health: Estimation of Daily Intake Doses in Pregnant Women from an Epidemiologic Study

by

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Chapter 1: Introduction and Background

The contribution of environment to human health has received increasing attention in recent years. There are various reasons for this increased focus on environmental health, including mounting evidence that environmental factors contribute to acute and chronic diseases, rising incidence of a wide variety of diseases that may be associated with environmental factors, and the evolution of technologies that allow investigators to more accurately characterize individuals’ exposures to specific environmental toxins.

Evidence linking environmental factors to health outcomes is continuously mounting. Additionally, evidence suggests that early life stages are critical periods of development (Makri et al. 2004; Selevan et al. 2000). Investigators deem the critical or most susceptible time period for many environmental exposures to be in utero to two years of age (Needham et al. 2005). A burgeoning scientific field, labeled the “developmental basis of health and disease”, suggests that susceptibility to many diseases that may arise later in life is set in utero or neonatally as a result of nutrition and exposures to environmental stressors and toxicants (Heindel 2006). Prenatal exposure to a wide variety of environmental contaminants has been linked to various adverse effects in humans. Prenatal exposures to even low levels of lead and methyl mercury (Lanphear et al. 2005; NRC 2000), PCBs (Jacobson and Jacobson 1996; Stewart et al. 2000; Stewart et al. 2003), and pesticides (Young et al. 2005) have been associated with neurobehavioral impairment in humans. For example, in one recent study, prenatal, but not postnatal, PCB levels were associated with impaired intellectual function during infancy, preschool, and at 11 years of age in a group of children born to mothers who
frequently ate PCB-contaminated fish (Jacobson and Jacobson 2002). Investigators have calculated that the neurodevelopmental impacts of methyl mercury – specifically loss of intelligence – cost Americans $8.7 billion annually in terms of lost productivity (Trasande et al. 2005), and a recent National Academy of Sciences study (National Academy of Sciences 2000) suggests that at least 28% of developmental disabilities in children may be due to environmental exposures acting alone or in concert with genetic susceptibility. Emerging evidence suggests that certain types of childhood leukemia have a prenatal origin (McHale et al. 2003; McHale et al. 2003; Smith et al. 2005) and that exposure to household insecticides during the prenatal period and first year of life, but not later, are associated with leukemia risk (Ma et al. 2002).

Fueling this growing body of research are emerging technologies, such as biomonitoring, which allow investigators to more accurately examine the links between environmental exposures and health. Biologic monitoring, or biomonitoring, can be defined as the measurement of environmental chemicals or their metabolites in human samples such as urine, blood, breast milk, or fatty tissue, with the purpose of acquiring information regarding environmental exposures (National Center for Environmental Health 2005). A more expansive definition of biomonitoring may include additional biomarkers, such as genomic, proteomic, or other biomarkers associated with the emerging “omics” technologies. The Centers for Disease Control and Prevention (CDC) conducts the most extensive biomonitoring program in the country. They began systematically sampling nationally representative populations in 1999 for a variety of environmental chemicals, and their latest report, released in 2005, presents data on over 200 different chemicals in over 5000 participants (National Center for Environmental
Health 2005). Scientists have used biomonitoring techniques on a smaller, more focused, scale to characterize exposures in research studies, while several non-governmental and media organizations have undertaken small pilot biomonitoring studies. The National Academy of Sciences recently released a report that reviews current practices and discusses future directions of biomonitoring (National Academy of Sciences 2006).

Further contributing to an increased focus on environmental health is the knowledge that many diseases have risen dramatically in incidence in recent decades without any clear explanation for the changes. While there is a lack of epidemiologic evidence linking most of the conditions to any specific causes of the increasing rates, exposure to environmental chemicals is a leading candidate, often supported by evidence from animal toxicology studies and other venues. A number of childhood disorders have increased in recent decades, including neurobehavioral disorders such as autism, attention-deficit and hyperactivity disorder (ADHD), and mental retardation; childhood cancers such as brain cancers and acute lymphocytic leukemia; birth defects; and asthma (Woodruff et al. 2004).

Introduction to Chapters 2 and 3

One realm of human health that has received particular attention in relation to environmental factors is that of reproductive health, especially male reproductive health. Evidence suggests that incidence of male congenital malformations and testicular cancer have increased dramatically in recent decades, while sperm concentrations and quality have decreased over the same period. A wealth of toxicology evidence has implicated a variety of pesticides and industrial chemicals as causes of male reproductive
malformations in animals, while evidence linking these chemicals to human effects remains elusive and contradictory. Meanwhile, biomonitoring techniques and increasing awareness among researchers are fueling further inquiries into the links among environmental exposures and male reproductive health.

In the late 1990s and after the year 2000, a particular group of chemicals, the phthalates, has been increasingly associated with male developmental reproductive disorders in animals. Prenatal exposures to certain phthalate chemicals cause testicular and other reproductive malformations in male offspring. Meanwhile, investigators have compiled epidemiologic and biological evidence that suggests that there is a human “testicular dysgenesis syndrome,” comprised of four distinct but possibly overlapping reproductive traits. Studies have indicated that the effects of phthalates in animals resemble the traits that comprise the human testicular dysgenesis syndrome.

In 2005 a published study suggested that developmental effects caused by phthalates in animals may also be present in boys whose mothers are exposed to phthalates during pregnancy. This new research indicated that the U.S. Environmental Protection Agency’s phthalate reference doses, which were finalized in the early 1990s, may have been outdated estimates of chemical toxicity levels in humans. I adapted mathematical models to estimate daily exposure doses of phthalate chemicals based on the urinary metabolite concentrations, in order to inform future reference doses of phthalate toxicity based on recent human epidemiologic data. This work has been published (Marsee et al. 2006).

In this thesis I address the question, “Do the U.S. Environmental Protection Agency reference doses for the specific phthalate chemicals adequately protect the public
from the risk of altered male reproductive development (specifically reduced anogenital distance) from prenatal phthalate exposures?"

In Chapter 2 of the thesis, I will outline the relationship between the phthalate chemicals and male reproductive health. I will begin with a discussion of the normal physiology of male sexual development and differentiation, and highlight its vulnerability to endocrine disruption. I will continue with a discussion of the evidence for the testicular dysgenesis syndrome, a collection of congenital male reproductive conditions that have been increasing in incidence for decades, and for which there is some evidence of environmental causes. Finally, I will discuss the various phthalate chemicals in depth, and will summarize animal toxicology and human epidemiologic evidence for linkages between phthalate exposures and male reproductive health effects.

In Chapter 3, I will highlight the work that I performed using a data set collected by epidemiologists who have published a study indicating that prenatal phthalate exposures may be associated with adverse reproductive effects in human newborn boys. I will begin the chapter with a discussion of the how the U.S. Environmental Protection Agency (U.S. EPA) performs a standard risk assessment for non-carcinogenic effects of chemicals, and will then discuss the U.S. EPA’s current reference doses for the phthalate chemicals. I will then focus on a model that I developed to enable utilization of the data available from the epidemiologic study in a format that can inform a risk assessment analysis for important phthalate chemicals. I will conclude with a discussion of limitations, conclusions, and suggested next steps.
Chapter 2: Phthalates and Male Reproductive Development

I. ENDOCRINE SYSTEM AND MALE REPRODUCTIVE DEVELOPMENT

Since aviation crop-dusters who handled DDT were found to have reduced sperm counts in the mid-20th century, investigators have learned more and more about chemicals that can disrupt the normal function of hormones in humans and wildlife (Amaral Mendes 2002). These chemicals, labeled endocrine disruptors, act by a variety of mechanisms. They may: (a) mimic the effect of endogenous hormones; (b) antagonize the effect of endogenous hormones; (c) disrupt the synthesis or metabolism of endogenous hormones; or (d) disrupt the synthesis of hormone receptors (Amaral Mendes 2002). Endocrine disruptors can affect various hormone systems in the body, including thyroid, parathyroid, CNS and neuroendocrine, adrenalal, pancreatic, pineal, and gonadal. Researchers have focused on the interference of male sex differentiation by endocrine disruptors for various reasons.

In contrast to female sex differentiation, which is largely hormone-independent, male sex differentiation, “masculinization”, is hormone-dependent, and thus especially vulnerable to endocrine disruption (Sharpe 2006). Additionally, evidence that a variety of pesticides and industrial pollutants affect male reproductive development in animals (Gray et al. 2006), coupled with rising human trends of some male reproductive disorders of unknown cause (Bay et al. 2006; Fisher 2004), have further directed attention to male reproductive health and endocrine disruptors. In this section I will characterize the vulnerability of the developing male reproductive organs to endocrine disruption. I will begin by reviewing the normal development of the male reproductive organs, will
continue with a brief discussion of the pesticides and pollutants for which there is evidence of adverse effects on male reproductive development, and will conclude with a discussion of the proposed human “testicular dysgenesis syndrome.”

**Development of the male reproductive tract**

The development of the male reproductive organs, which actually occurs as a continuum of processes, is traditionally separated into two distinct events. The first, labeled sex “determination”, involves the formation of the testis from the genital ridge, and is considered to be more or less hormone-independent (Brennan and Capel 2004; Sharpe 2006). The second event, sex differentiation, comprises the further development of the testes and the “masculinization” of the rest of the reproductive organs, a process which is almost entirely hormone-dependent (Sharpe 2006).

**Sex determination**

In mammals the preset program is for a fetus to develop into a female. Thus, without specific genetic intervention, development will proceed upon the pathway in which the Mullerian ducts persist internally and develop into the fallopian tubes, uterus, and upper part of the vagina, while the Wolffian ducts, which would otherwise develop into the epididymis, seminal vesicles, and vas deferens, will spontaneously degenerate (Sharpe 2006).

The intervention required to set the fetus on the pathway to becoming a male lies in the activation of the Sry gene on the Y chromosome (Brennan and Capel 2004). Activation of this gene results in differentiation of the Sertoli cells, followed by a series of events that result in formation of seminiferous cords, Leydig cells, and ultimately, the testes (Sharpe 2006). Once the testes form, a complicated interplay of reproductive
hormones drives the continued differentiation along the male pathway of development. I describe this hormone-dependent process in the following section.

Hormone-dependent male differentiation

The first hormone to be secreted following testis development is Anti-Mullerian hormone (AMH), also known as Mullerian-inhibiting substance. The AMH, secreted from the Sertoli cells into the bloodstream, reaches the type II AMH receptors in the epithelium surrounding the Mullerian ducts, and induces the regression of the ducts via apoptosis of the epithelial cells (Xavier and Allard 2003). While this is the main function of AMH, investigators have demonstrated that AMH contributes to development of adult Leydig cell populations postnatally in rats (Salva et al. 2004; Wu et al. 2005), and it is likely that there are other currently uncharacterized functions of this hormone (Sharpe 2006). Failure of the Mullerian ducts to regress is a relatively rare event, and no chemical that disrupts AMH has, as of yet, been identified. Therefore, this hormone has not received much attention as a potential target of endocrine disruptors (Sharpe 2006).

Secretion of testosterone by the fetal Leydig cells is the most important process of masculinization. Testosterone delivered locally from the testes prevents the programmed degeneration of the Wolffian ducts and allows them to further develop into the epididymides, vas deferens, and seminal vesicles. In contrast to this local hormonal circuit, masculinization of other parts of the body results from testosterone that enters the bloodstream. While local testosterone itself prevents Wolffian degeneration, testosterone in the bloodstream generally acts through intermediary species that can amplify the relatively low blood concentrations of testosterone. For example, the enzyme 5α-reductase converts testosterone into 5α-dihydrotestosterone (DHT), which has a 10-fold
higher potency than testosterone for activating the androgen receptor (AR). Tissues that depend on masculinization typically have relatively high concentrations of 5α-reductase, and are thus able to convert testosterone to its more potent analog, DHT. Acting through this mechanism, DHT drives the formation of the external genitalia, including the penis and scrotum, and the ventral prostate (Gray et al. 2000; Sharpe 2006). The androgens, testosterone and DHT, also act in the masculinization of more distant parts of the body, including the brain and skin.

Testosterone can alternatively be converted into estradiol by the enzyme aromatase. Estradiol created in target tissues may interact with estrogen receptors, and it is likely that this hormone contributes to masculinization in some areas of the body. It is, however, unclear to what degree locally produced estradiol contributes to masculinization relative to the androgens (Sharpe 2006).

Sex determination, which involves the formation of the testes, is considered a largely hormone-independent event. It is important, however, to mention that recent evidence suggests that this may be an oversimplification, and that adequate androgen activity is essential for normal development of the testes. Humans with complete androgen insensitivity syndrome, due to an inactivation mutation of the AR, may have some degree of testicular dysgenesis (Rutgers and Scully 1991). Furthermore, rats with ablated AR or treated with the AR antagonist flutamide have 30-50% reductions in Sertoli cell numbers at birth, and more severe reductions (60-75%) in adulthood (Atanassova et al. 2005; Tan et al. 2005). This reduction in Sertoli cells in turn causes a reduction in adult Leydig cell numbers by as much as 80% (De Gendt et al. 2005), and, importantly, Sertoli cell number per testis determines how many germ cells may be
supported into development as spermatozoa, suggesting that any mechanism that causes a reduction in Sertoli cells would have important effects on future sperm quality and fertility (Sharpe 2006).

The third hormone important in masculinization is insulin-like 3 hormone (insl3), which is produced by fetal Leydig cells to stimulate the growth of the gubernacular ligaments in order to guide testicular descent (Sharpe 2006). In addition to the differentiation of the male sex organs, the placement of the testes in the scrotum, rather than the abdominal cavity, is an important step for male development and fertility. This sexually dimorphic placement of the gonads relies on differential development of two different ligaments. In females the ovaries are supported in their position lateral to the kidneys by the cranial suspensory ligament (CSL), whereas in males the testes are more strongly attached caudally to the inguinal region by the gubernacular ligaments (Adham and Agoulnik 2004). Investigators have recently discovered that the factor that promotes gubernacular development in males is the hormone insl3. Mice with knockout insl3 genes have bilateral cryptorchidism (undescended testes), with testes that are freely mobile (unattached to a gubernaculum) within the abdominal cavity (Nef and Parada 1999; Zimmermann et al. 1999). Furthermore, overexpression of insl3 in females results in descent of the ovaries and inguinal hernias (Adham et al. 2002). Further evidence has revealed that, while insl3 is the principal factor that guides the differentiation of the gubernaculum, androgens play a complementary role in the process as well. Androgens cause the degeneration of the CSL, as well as support the growth of the gubernaculum (Adham and Agoulnik 2004; Emmen et al. 2000).
To summarize this section, three main hormones are important in the masculinizing differentiation of the reproductive tract. Anti-Mullerian hormone, produced by Sertoli cells, induces the regression of the Mullerian ducts, the precursor to the female reproductive tract. The fetal Leydig cells produce two important hormones, testosterone and insulin-like factor 3 (insl3). Testosterone, the most important of the reproductive hormones, stimulates development of the Wolffian ducts directly, and masculinizes the rest of the reproductive tract and body, mainly through peripheral conversion to DHT or estradiol. Testosterone also contributes to the development of the mature testis, and inhibition of its function results in reduced Sertoli cell and Leydig cell numbers. The hormone insl3 promotes gubernacular development, which is essential for the descent of the testes into the scrotum. Because of the significance of the hormonal events that contribute to male sex differentiation, the vulnerability of this process to endocrine disruption is clear.

Any process that interferes with the three hormones, or with their receptors, could have profound effects on the masculinization of the reproductive tract. The enzymes that convert testosterone to DHT and estradiol, 5α-reductase and aromatase, respectively, may also act as targets for endocrine disruptors. Finally, any insult that interferes with the complicated process of testis and seminiferous cord differentiation, considered to be mainly hormone-independent, will result in some degree of testicular dysgenesis that will lead to abnormal hormone production and an increased incidence in downstream disorders of hormone-dependent processes. Thus, a chemical that has no intrinsic anti-hormone function may result in lowered hormone production through its toxic effects to the fetal testis. Sharpe (2006) hypothesizes that this is the mechanism by which the
phthalate esters cause aberrations of hormone-dependent processes, such as masculinization, testicular descent, and Sertoli cell proliferation, in rats. The following diagram summarizes the complex events of male sex differentiation.
Figure 1: Steps of masculinization; adapted from (Sharpe 2006)

- Sertoli Cells in seminiferous cord
  - Anti-Mullerian Hormone (AMH)
  - Testosterone (T) insl3
  - Blood vessels
    - 5α-reductase
    - Estradiol
    - insl3 T DHT
      - Regression of Mullerian Ducts
      - Aromatase
      - Masculinization of body tissues and systems
      - Masculinization of external genitalia and prostate; Masculinization of the brain
      - Masculinization of Wolffian duct
      - DHT
      - Gubernacular development and testis descent into scrotum
Environmental chemicals that are antiandrogens

In the previous section I briefly discussed the processes of male sex determination and differentiation, in part to emphasize the importance of hormones in male sex differentiation and thus the vulnerability of this process to endocrine disruption. In this section I will briefly summarize the experimental evidence indicating that some pesticides and industrial chemicals interfere with normal development and differentiation of the male reproductive organs.

In the late 1970s researchers introduced the "estrogen hypothesis", based on evidence that in utero exposure to synthetic estrogens [specifically, diethylstilbestrol (DES)] increases the risk for congenital reproductive abnormalities in male offspring (Coscrove et al. 1977; Henderson et al. 1976). Since then, evidence that in utero exposures to estrogens have a weaker effect on male reproductive development than initially hypothesized, combined with the discovery of other mechanisms by which reproductive development may be altered, have shifted the focus from estrogens toward chemicals that cause a distortion of the normal androgen actions (Bay et al. 2006). Specifically, investigators have demonstrated that various chemicals, termed "antiandrogens", may impair the actions of androgens (testosterone and DHT) in experimental animals, and that other chemicals may mimic the effects of androgens during development (Gray et al. 2006).

Gray et al. (2006) review the pesticides and industrial chemicals for which experimental evidence demonstrates impairment of androgen function and male reproductive development with in utero exposures in animal models. Chemicals from a variety of chemical classes appear to exert their antiandrogenic effects through two main
mechanisms: antagonism of the androgen receptor (AR) or inhibition of fetal Leydig cell testosterone production. Additionally, at least one chemical increases male fetal progesterone production, while another chemical, used as a beef cattle growth stimulant, acts as an AR agonist. I will briefly discuss these chemicals and their effects on male reproductive development below.

Investigators have demonstrated that in utero exposure to a number of chemicals from various chemical classes may adversely affect male reproductive development by acting as antagonists of the androgen receptor (AR). I described in the previous section that the androgens, DHT and testosterone, interact with the AR to drive the maintenance and development of the Wolffian ducts, the development of the external genitalia, and the masculinization of the reproductive organs and other body parts, and are essential for complete development of healthy testes. Two dicarboximide fungicides, vinclozolin and procymidone, are examples of chemicals that competitively inhibit binding of androgens to the AR, resulting in an inhibition of androgen-dependent gene expression in animals (Earl Gray et al. 2006). Peripubertal administration of these chemicals can delay pubertal maturation (Monosson et al. 1999), and administration during sexual differentiation demasculinizes male rat offspring such that treated males display shortened (female-like) anogenital distance at birth, retained nipples, hypospadias, and small to absent sex accessory glands (Gray et al. 1999; Gray et al. 1999; Ostby et al. 1999). Another pesticide that has similar effects to those described above is p,p’DDE, which is a metabolite of the pesticide DDT. It also acts by antagonizing the AR, and can cause a delay in puberty and developmental aberrations similar to those described for vinclozolin and procymidone (Gray et al. 2006; Gray et al. 1999). Additionally, while they haven’t
yet been extensively studied, evidence suggests that some polybrominated diphenyl ether (PBDE) flame retardants may also act as AR antagonists (Stoker et al. 2005).

Animal evidence suggests that some chemicals may inhibit male reproductive development by multiple mechanisms. Linuron, for example, is an herbicide that acts both as an AR antagonist and by reducing fetal testosterone production (Gray et al. 2006; Wilson et al. 2004). While malformed external genitalia and undescended testes were rare with in utero exposure, epididymal and testicular abnormalities were very common in treated males (Gray et al. 1999). Another fungicide, prochloraz, disrupts reproductive development by many different mechanisms. It is an AR antagonist and inhibits steroidogenic enzymes and aromatase. In utero exposure reduces fetal testosterone production and increases progesterone production 10-fold, and results in reduced anogenital distance, female-like areolas, and hypospadias (Gray et al. 2006; Wilson et al. 2004).

Finally, phthalates are the only chemicals known to disrupt reproductive development by inhibiting both testosterone and insl3 production, resulting in a variety of malformations that will be discussed in a later section. The preceding brief discussion should provide an idea of the potential for chemicals to interfere with the highly hormone-dependent process of male sex differentiation. In the next section, I will conclude the discussion of the vulnerability of the developing male reproductive tract by discussing the hypothesis that male human health may be vulnerable to a rising incidence of developmental abnormalities that constitute a single syndrome, labeled “testicular dysgenesis syndrome”.

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The Human Testicular Dysgenesis Syndrome

In 2001 Skakkebaek and others (Skakkebaek et al. 2001) postulated that four male reproductive abnormalities, cryptorchidism, hypospadias, poor semen quality, and testicular cancer, may actually comprise a single underlying entity, the "testicular dysgenesis syndrome." The investigators provided epidemiological and biological evidence in support of their theory, noting that the four conditions are common and are likely increasing in incidence, the conditions are risk factors for each other and often occur together in individual patients, and the conditions all appear to have an origin in fetal development. Additionally, the authors hypothesized that the treatment of these individual disorders by different medical specialists had contributed to the lack of the identification of a single syndrome, and that the rapid increase in the conditions' incidences implicated lifestyle and environmental conditions as the most likely causes of the disease (Skakkebaek et al. 2001). In this section I will discuss the epidemiologic and biologic evidence for the existence of the testicular dysgenesis syndrome (TDS), and will discuss the limited evidence that endocrine-disrupting chemicals (EDCs) may play a role in its increasing incidence.

Trends in cryptorchidism and hypospadias

As early as the mid-1980s researchers began to provide evidence that congenital malformations of the male reproductive organs, including cryptorchidism (undescended testes) and hypospadias (presence of the urethral meatus on the ventral aspect of the penis), were increasing in incidence (Chilvers et al. 1984; Matlai and Beral 1985). More recent registry studies, such as those that used data from the International Clearinghouse
for Birth Defects Monitoring Systems (ICBDMS), suggest that hypospadias increased in multiple countries over different time periods (Paulozzi 1999; Toppari et al. 2001). For example, hypospadias incidence increased in the U.S. from the 1970s to the 1990s, while it increased in the Scandinavian countries during the 1960s and into the 1970s and 1980s for some individual countries (Toppari et al. 2001). The studies also reported increasing trends of cryptorchidism, and reported significant geographical differences among the various malformations. Unfortunately, while cryptorchidism and hypospadias are the most common congenital malformations of the male urogenital organs, they have often been considered relatively minor malformations and are often not identified by malformation registries (Toppari et al. 2001). While the hypospadias data may be more reliable than those for cryptorchidism, it is still difficult to compare incidence of the malformations among countries that have different reporting requirements.

Cohort studies with preset reporting and diagnostic criteria have recently been undertaken in order to more adequately characterize the trends in hypospadias and cryptorchidism. Recent cohort studies demonstrated that the birth prevalence of hypospadias was 1.03% in Denmark from 1997 to 2004, and was significantly higher than in Finland (0.27%), which has been reported to have fewer cases of testicular cancer and other reproductive malformations when compared to Denmark (Boisen et al. 2005; Boisen et al. 2004; Skakkebaek et al. 2001). Additionally, the birth prevalence of cryptorchidism was significantly higher in the Danish than the Finnish cohort, and the authors postulated that the geographic differences could more likely be explained by lifestyle and environmental than genetic factors (Boisen et al. 2005; Boisen et al. 2004). More prospective studies are currently underway in an effort to further characterize
geographic and temporal variations in incidence of malformations, and to characterize associations with lifestyle and environmental factors (Fisher 2004). Good retrospective surveillance of data with adequate reporting requirements would also contribute to the debate over the rising incidence of cryptorchidism and hypospadias.

**Trends in semen quality**

The third biologic component of the testicular dysgenesis syndrome is impairment of semen quality. In the early 1990s Carlsen and others reported that human semen quality had declined by about 50% from 1930 to 1991 (Carlsen et al. 1992). This study raised significant controversy, and was frequently criticized and reanalyzed. Its findings were supported by some studies but not by others (Fisher 2004). The most recent reanalysis, however, confirmed the findings of the Carlsen study, and even suggested that some of the declines in sperm concentration may have been underestimated, after controlling for additional factors (Swan et al. 2000). Nevertheless, while these meta-analyses strongly suggest that sperm density and semen quality are declining at alarming rates, they are constrained by the retrospective nature of the studies and are subject to many of the faults discussed above in regard to registry studies involving cryptorchidism and hypospadias, since protocol for collecting and analyzing sperm have not been consistent across time and geography.

Investigators have initiated numerous prospective cohort studies since the publication of the studies discussed above. While the cohort studies have not been of long enough duration to elucidate long-term trends in semen quality, they have uncovered a significant degree of geographic variability in semen quality. In the Nordic-
Baltic region of Europe there is an east-west gradient in semen quality, with men in Norway and Denmark experiencing significantly lower sperm counts than those in Finland and Estonia (Jorgensen et al. 2002). This gradient mirrors a similar gradient in incidence of testicular cancer in these countries (Jorgensen et al. 2002). Danish men, especially, appear to be at risk of sub-optimal semen quality, as a recent study of young Danish men attending medical examinations for compulsory medical service between 1996 and 1998 demonstrated that 48% had sperm concentrations below those associated with decreased fecundity, while 25% had counts below those deemed abnormal by the World Health Organization (Andersen et al. 2000; Bonde et al. 1998; Fisher 2004). Some limited data suggest that geographic variation in semen quality may occur in the U.S. The sperm counts in an agricultural town in Missouri were significantly lower than in New York, Los Angeles, and Minneapolis (Swan et al. 2003). The authors demonstrated that increased exposure to pesticides, as measured by sampling metabolites in biologic samples, was associated with lower sperm counts in a subset of the participants (Swan 2006).

While a number of researchers have hypothesized that endocrine-disrupting chemicals may be responsible for the declines in semen quality, these associations have not been effectively demonstrated in epidemiologic or experimental studies. Furthermore, a variety of lifestyle characteristics, including maternal diet and smoking habits, sedentary lifestyle, season, and frequency of ejaculation, can have important effects on sperm concentrations (Sharpe and Franks 2002), further obscuring both the characterization of and the potential causes of declines in semen quality.
Trends in testicular cancer

While the hypotheses that semen quality is declining and the incidences of cryptorchidism and hypospadias are increasing remain relatively contentious, increases in testicular cancer are well established (Bay et al. 2006). Testicular cancer is the most common cancer of 20-34 year old males, and the lifetime risk is 0.5-1% (Hoei-Hansen et al. 2005). Investigators have characterized the geographic and temporal trends in industrial European nations particularly well. There is marked variation in testicular cancer rates among various countries, with a five-fold variation in incidence among 12 European countries (Bray et al. 2006). Testicular cancer incidence has been increasing since 1945 in at least six European countries (Adami et al. 1994; Bergstrom et al. 1996), with an annual increase in incidence ranging from 1-6% (Bray et al. 2006). Testicular cancer incidence is still increasing in Europe, although it appears that in Denmark, which has the highest documented rates of testicular cancer in Europe, the increase may be leveling off or even decreasing (Bray et al. 2006; Jacobsen et al. 2006; Richiardi et al. 2004).

The upward trends are similar in the United States. Data from a National Cancer Institute database indicate that testicular cancer incidence increased 51% between 1973 and 1995, and that the peak age at diagnosis decreased during this time period (McKiernan et al. 1999). Interestingly, there is a strong association in both the U.S. and European data between birth cohort and the increase in testicular cancer. Birth cohort is a stronger predictor of testicular cancer risk than is calendar time alone (Bergstrom et al. 1996; Bray et al. 2006; Jacobsen et al. 2006; McKiernan et al. 1999), which supports the
hypothesis that the etiology of testicular cancer is associated with some insult that affects fetal development.
Evidence that the four factors comprise a single testicular dysgenesis syndrome

Cryptorchidism, hypospadias, impaired sperm quality, and testicular cancer may all originate during fetal life, which may support the view that these four conditions
comprise a single underlying entity (Bay et al. 2006; Skakkebaek et al. 2001). As described in a previous section, proper descent of the testes is mediated by the Leydig cell hormones, insl3 and the androgens, during the second and third trimesters of gestation (Toppari and Kaleva 1999). The etiology of most cases of hypospadias are unknown. Nevertheless, the deformity is present at birth, and therefore must have a prenatal cause, possibly associated with an embryonic hormonal disturbance (Baskin 2000).

Although the fetal origins of hypospadias and cryptorchidism are clear, it may be conceptually more difficult to understand how testicular cancer and poor semen quality, both of which manifest after puberty, may have fetal origins. However, testicular cancer almost certainly arises from a malignant transformation that takes place in utero. Over 95% of testicular cancers arise from germ cells, and are known as testicular germ cell tumors (Hoei-Hansen et al. 2005). These cancers arise from a cell known as the carcinoma in situ (CIS) cell, which is generally believed to arise from gonocytes during fetal development. The true prevalence of CIS is unknown, since testicular biopsy is the only current method of diagnosis, but investigators believe that almost all cases of CIS develop into testicular cancer following puberty (Hoei-Hansen et al. 2005).

There is also evidence that some cases of sub-optimal semen quality may be attributable to the embryonic environment as well (Bay et al. 2006). This is could be due to impaired germ cell development, decreased testosterone secretion, and genital malformation. Additional evidence that the four conditions have a fetal origin is that they all share a number of risk factors that are tied to embryonic development, such as low
birth weight, maternal age, premature birth, and low parity (Bay et al. 2006; Bray et al. 2006).

In addition to sharing a fetal origin, the other major factor that the original authors postulated in support of the singular TDS is that the four conditions are epidemiologic risk factors for each other and occur concurrently in many individuals (Bay et al. 2006; Skakkebaek et al. 2001). Patients often present with more than one trait of TDS (Bay et al. 2006). For example, individuals diagnosed with testicular cancer often have reduced semen quality and decreased fertility that precedes the cancer (Jacobsen et al. 2000; Moller and Skakkebaek 1999). Furthermore, men with unilateral testicular cancer frequently have histologic abnormalities of the contralateral testis suggestive of testicular dysgenesis, along with decreased spermatogenesis (Hoel-Hansen et al. 2003).

Additionally, cryptorchidism is a risk factor for hypospadias, testis cancer and poor semen quality, while hypospadias is in turn associated with an increased risk for cryptorchidism (Bay et al. 2006).

In summary, investigators have postulated the existence of a singular disorder, testicular dysgenesis syndrome (TDS), that comprises four different male reproductive conditions: cryptorchidism, hypospadias, poor semen quality, and testicular cancer. Various types of evidence support a linkage among these four conditions, including that they have all been increasing in incidence for decades, they share significant geographic variation, they all have an origin based in fetal development, and they are all risk factors for each other. According to the investigators that initially postulated the existence of TDS, the disorder exists as a continuum with wide variation in clinical expression. The mildest and most common form consists mainly of reduced sperm concentration, while
the more severe cases consist of hypospadias, cryptorchidism, and an increased risk of testicular cancer (Bay et al. 2006).

Possible role of endocrine disruptors in TDS

The original TDS investigators (Bay et al. 2006; Skakkebaek et al. 2001) and others (Fisher 2004) have hypothesized that TDS may be caused by exposure to endocrine disrupting chemicals (EDCs). The investigators, however, only provide circumstantial evidence for an association between EDC exposure and development of TDS, while pointing out that it can be difficult to obtain data to support causal relationships in humans for obvious ethical reasons (Bay et al. 2006).

A key piece of evidence cited by investigators is the existence of an animal model that mirrors the situation seen in human TDS. Animals exposed in utero to di-n-butyl phthalate, a ubiquitous industrial chemical, exhibit anatomical and histological malformations similar to those seen in the human TDS (Fisher et al. 2003; Mahood et al. 2005). The details of this relationship will be discussed in depth in a later section.

A number of epidemiological studies have exhibited correlations between risk of maternal or paternal exposure to EDCs and incidence of male reproductive malformations. Maternal and paternal pesticide exposure, estimated using occupational and geographic data, is associated with increased incidence of cryptorchidism in sons (Bay et al. 2006).

Hardell et al (Hardell et al. 2006) recently demonstrated that the presence of some persistent organic pollutants in mothers were associated with an increased risk of testicular cancer in sons. However, the biologic measurements of the chemicals were
obtained from the mothers many years after the potential *in utero* exposures would have taken place, obscuring the connection.

A recent cohort study compared male newborns in Finland, which has among the lowest reported rates of male reproductive disorders, and in Denmark, which has the highest reported incidence of testicular cancer and reproductive malformations in Europe. Finnish newborns had significantly larger testis volume at birth and larger testis growth from birth to three months when compared to Danish newborns (Main et al. 2006). Finnish newborns also had significantly higher levels of certain reproductive hormones, including inhibin B, which was significantly positively correlated to testis volume. The investigators concluded that, while the differences may be explained by genetic differences between the two countries, environmental factors affecting testicular development may also explain the differences (Main et al. 2006).

To summarize, epidemiologic studies currently demonstrate only relatively weak correlations among EDC exposure and male reproductive health outcomes. More prospective studies that utilize biomonitoring data to demonstrate chemical exposures are needed to better assess chemical exposures and male reproductive health. In the next section I will discuss the class of chemicals most closely linked to male reproductive malformations in animals and most frequently postulated as a potential contributor to human TDS: the phthalates.

**II. INTRODUCTION TO THE PHTHALATE CHEMICALS**

Phthalates are industrial chemicals frequently used as plasticizers. People are regularly exposed to phthalates through a variety of pathways, and some of the phthalate
chemicals have been shown to have important health effects in animals, although effects in humans have not been well characterized. The following section will discuss various traits of the phthalates in the context of environmental health. The section will focus on individual phthalates of interest, beginning with a relatively in-depth discussion of di-n-butyl phthalate, followed by more abbreviated discussions of additional phthalates. I will discuss evidence for phthalate effects on human health in a later section. The following table details the various phthalate metabolites, along with their parent chemicals, that were analyzed in the 2001-2002 NHANES sample.
<table>
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<th>Abbreviations</th>
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<th>Abbreviations</th>
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<td>DMP</td>
<td>Mono-methyl phthalate</td>
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<tr>
<td>Diethyl phthalate</td>
<td>DEP</td>
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</tr>
<tr>
<td>Di-n-butyl phthalate</td>
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<td>Di-isobutyl phthalates</td>
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</tr>
<tr>
<td>Di-2-ethylhexyl phthalate</td>
<td>DEHP</td>
<td>Mono-2-ethylhexyl phthalate</td>
<td>MEHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mono-(2-ethyl-5-oxohexyl) phthalate</td>
<td>MEOHP</td>
</tr>
<tr>
<td>Substance</td>
<td>DOP</td>
<td>Other Substance</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------------------</td>
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<td>----------------------------------------</td>
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</tr>
<tr>
<td>Di-n-octyl phthalate</td>
<td></td>
<td>Mono-(2-ethyl-5-hydroxyhexyl) phthalate</td>
<td>MEHHP</td>
</tr>
<tr>
<td></td>
<td>DOP</td>
<td>Mono-n-octyl phthalate</td>
<td>MOP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mono-(3-carboxypropyl) phthalate</td>
<td>MCPP</td>
</tr>
<tr>
<td>Di-isononyl phthalate</td>
<td></td>
<td>Mono-isononyl phthalate</td>
<td>MiNP</td>
</tr>
</tbody>
</table>
Di-n-butyl phthalate

Di-n-butyl phthalate (DBP) is a colorless to faint yellow oily liquid that is primarily used as a "plasticizer", a compound used to make plastic products more flexible (ATSDR 2001). It is present in a number of consumer products, including home furnishings, paints, clothing, and cosmetic products. It was formerly used as a plasticizer in polyvinyl chloride (PVC), but is no longer used for that purpose. In 1994, U.S. manufacturers produced over 17 million pounds (7.7 million kilograms) of DBP, and the chemical has been identified in at least 471 of the 1585 hazardous waste sites that have been proposed for inclusion on the EPA National Priorities List (ATSDR 2001). It is still produced in large quantities.

DBP is widespread in the environment and has been identified at low levels in all types of environmental media (ATSDR 2001). Humans may be exposed to DBP through food or water, through inhaled air or attached to dust particles, or through dermal contact with products containing DBP. Consumption of contaminated food, including butter, fish, cereal products, and produce, is likely to be the most significant source of exposure to DBP (Kavlock et al. 2002). DBP can enter food either by environmental contamination during crop cultivation, or by migration from packaging or processing materials, which is possible because phthalates are not irreversibly bound to the products that contain them (ATSDR 2001; Kavlock et al. 2002). Additionally, concentrations of DBP and other phthalates in personal air samples are significantly correlated with urinary concentrations of the corresponding phthalate monoesters (Adibi et al. 2003). Once a person is exposed to DBP, the chemical is rapidly absorbed and widely bioavailable (ATSDR 2001). It is rapidly metabolized to its monoester (mono-n-butyl phthalate,
MBP) in the gut and rapidly excreted in urine as MBP and the glucuronide conjugate of MBP. The bulk of DBP metabolites are excreted in the first 24 hours following exposure (Anderson et al. 2001). While dermal exposure to DBP has not been well studied, it is likely that the kinetic profile differs from oral or inhalation exposure. The rate of dermal absorption of various phthalate diesters is slower in humans than in rats (Scott et al. 1987), but one limited study indicates that metabolism of phthalate diesters following absorption through the skin in rats takes place slowly over the course of seven days, which contrasts with the rapid excretion following oral ingestion (Elsisi et al. 1989). It is unclear how the slower dermal absorption might influence the potential health effects of exposure.

In spite of the fact that phthalates are rapidly metabolized and excreted from the body, recent biomonitoring studies indicate that most, if not all, people have detectable levels of phthalate metabolites in their body. Biomonitoring for phthalates has focused on measuring monoester metabolites in urine for various reasons. The high concentrations of metabolites in urine relative to serum contributes to the ease of sampling. Perhaps more importantly, sampling of metabolites rather than parent phthalates allows investigators to avoid sample contamination by using blood extraction equipment that may contain diester phthalates that could contaminate samples (Blount et al. 2000; National Center for Environmental Health 2005). In the 1999-2000 sample population of the CDC’s National Health and Nutrition Examination Survey (NHANES), 99% of human urine samples had detectable levels of MBP (Silva et al. 2004). Samples from 2001-2002 had similar levels (National Center for Environmental Health 2005), indicating that almost all Americans experience detectable, and probably relatively continuous, exposures to DBP.
There is an extensive literature of animal studies documenting effects of DBP. While liver, hematologic, and renal effects, as well as changes in body weight, have been observed in exposed rats, the primary health effects of DBP exposure are developmental and reproductive alterations (ATSDR 2001). Developmental effects include increases in postimplantation losses, decreased male and female fetal/pup weight, and increases in incidences of external, skeletal, and internal malformations (Ema et al. 1993; Ema et al. 1994; Ema et al. 1997; Ema et al. 1995; Ema et al. 1996; Ema and Miyawaki 2001; Ema et al. 1998; Ema et al. 2000; Gray et al. 1999; Mylchreest et al. 1998).

At levels of exposure below those which cause the developmental abnormalities described above, in utero exposures to DBP cause abnormalities in the development of the male reproductive tract. These abnormalities include testicular atrophy, reduced sperm production, absent or undeveloped epididymis, hypospadias, cryptochoirdism (undescended testes), absence of prostate gland and seminal vesicles, reduced anogenital distance (AGD), and retention of thoracic nipples (Ema and Miyawaki 2001; Mylchreest et al. 1998; Mylchreest et al. 2000). It is likely that monobutyl phthalate (MBP), the monoester metabolite of DBP, causes the toxic effects of DBP (ATSDR 2001; Ema et al. 1995; Ema et al. 1996; Ema and Miyawaki 2001). Ema et al. (Ema et al. 2000) determined that days 15-17 of rat gestation were the most critical time period for DBP exposure to result in testicular toxicity of the male offspring.

DBP produces its testicular effects by interfering with reproductive hormones and Leydig and Sertoli cell function. Mylchreest et al. (Mylchreest et al. 2002) demonstrated that in utero DBP exposure reduces fetal testosterone production on gestational days 18-21, resulting in reproductive tract malformations and a compensatory increase in Leydig
Cell concentrations. Exposure levels lower than those that result in reduced testosterone levels cause a reduction in the expression of key genes and proteins involved in cholesterol transport and steroidogenesis (Lehmann et al. 2004). In addition to reducing testosterone levels, DBP reduces the gene expression of insulin-like hormone 3 (insl3) (Wilson et al. 2004). Exposed rats had gubernacular lesions and higher incidences of undescended testes (cryptorchidism).

While the animal studies conclusively demonstrate the reproductive and developmental toxicity of DBP, the data on the effects of DBP in humans are very limited. The lack of human data contributed to the belief that, in spite of the demonstrated toxicity of DBP and other phthalates, humans posed no significant risk from phthalate exposure, mainly because they experienced much lower exposures than those that caused adverse effects in animal studies. A National Toxicology Program panel had “negligible concern for adult reproductive toxicity” and “minimal concern about effects to human development and development of the reproductive system from current estimated exposure to DBP” (Kavlock et al. 2002). Recent epidemiologic evidence, to be discussed in a later section, has contributed to increasing doubt that there is no reason to worry about potential effects from human exposures to DBP.

**Butyl benzyl phthalate**

Butyl benzyl phthalate (BBzP) is a plasticizer used in PVC, and is included in vinyl tiles, construction materials, automotive materials, and food conveyor belts (Kavlock et al. 2002). Consumption of contaminated food is believed to be the most significant source of human exposure, although dermal and inhalation routes are not well
studied and may also be important. While BBzP is not easily volatilized (Kavlock et al. 2002), concentrations of BBzP in personal air samples are significantly correlated with urinary metabolite concentrations (Adibi et al. 2003). With oral ingestion, BBzP is rapidly metabolized to its monoester metabolites, monobenzyl phthalate (MBzP) and mono-n-butyl phthalate (MBP), in the gut, and excreted in the urine mainly as the monoester metabolites and their glucuronide conjugates (Kavlock et al. 2002). While MBP, the main metabolite of DBP, is one of the products of B3zP metabolism, excretion of MBP represents only 6% of the initial BBzP exposure, and MBzP is considered the main metabolite (Anderson et al. 2001; National Center for Environmental Health 2005).

Subchronic and chronic dietary studies demonstrated adverse effects on body weight, and in the liver, kidney, and testes (Kavlock et al. 2002). The developmental and reproductive effects of BBzP are similar to those observed with DBP exposure. The developmental effects include increased embryo death (including preimplantation and postimplantation loss) and decreased fetal/pup weight, while in utero exposure is also toxic to the male reproductive tract, resulting in increased incidence of cryptorchidism and decreased anogenital distance (Ema et al. 1992; Ema et al. 1992; Ema et al. 1993; Ema et al. 1994; Ema and Miyawaki 2002; Ema et al. 1998; Ema et al. 1999). The same developmental and reproductive effects caused by BBzP are also induced by exposure to MBzP (Ema et al. 1996; Ema et al. 1996; Ema et al. 2003), indicating that this metabolite may be the mediator of BBzP's adverse effects.

The mechanism of action for BBzP is not as well characterized as that for DBP, but there is evidence that BBzP exposure causes a reduction in testosterone and insulin production, acting via an antiandrogenic mechanism similar to DBP (Wilson et al. 2004).
Additionally, humans are exposed to BBzP at high levels; 97% of participants in the 1999-2000 NHANES study had detectable levels of MBzP in their urine, and levels were roughly similar in the 2001-2002 samples (National Center for Environmental Health 2005; Silva et al. 2004).

**Di-2-ethylhexyl Phthalate**

Di-2-ethylhexyl phthalate (DEHP) is a plasticizer commonly used in the manufacture of flexible polyvinyl chloride (PVC) products, such as floor tiles, shower curtains, raingear, clothes, toys, medical equipment, food packaging, automobile parts, and clothing (ATSDR 2002). Due to health concerns, DEHP has been removed or replaced from most baby toys intended for mouthing and from food packaging used in the U.S. (ATSDR 2002; Kavlock et al. 2002; National Center for Environmental Health 2005). As of 2002, annual production of DEHP was nearing 260 million pounds (Kavlock et al. 2002), and it is widely dispersed in the environment. Individuals are exposed to DEHP during manufacture of products, migration from products during use, and from disposal in landfills and incineration. Oral ingestion is probably the most important source of exposure (ATSDR 2002; Kavlock et al. 2002), although volatization and inhalation may be an important route of exposure as well. DEHP is the primary plasticizer in medical equipment, such as blood storage bags and infusion devices, hemodialysis equipment, and surgical products (ATSDR 2002; Kavlock et al. 2002). Investigators have demonstrated that patients with extensive exposure to PVC-containing medical devices, including hemodialysis patients and infants in the NICU, experience
elevated exposures to DEHP, and may have levels several-fold higher than the general population (Calafat et al. 2004; Faouzi et al. 1999; Green et al. 2005; Pollack et al. 1985).

The metabolism of DEHP, a relatively high-weight phthalate, is much more complicated than that of the lower-weight compounds, such as DBP or BBzP. While the lower-weight compounds are metabolized mainly to their corresponding monoester and its glucuronide conjugate, DEHP metabolism consists of several additional steps. Following metabolism to its monoester, mono-2-ethylhexyl phthalate (MEHP), it is further oxidized to a number of other compounds, as shown in Figure 3 (Koch et al. 2005). Since some of these oxidative metabolites are present in the urine at concentrations an order of magnitude higher than MEHP, investigators originally suggested that two additional metabolites, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), would be better biomarkers for exposure than MEHP (Barr et al. 2003; Kato et al. 2004). Recent studies have raised the possibility that other metabolites may be effective biomarkers as well (Koch et al. 2005; Silva et al. 2006; Silva et al. 2006).
Health effects of DEHP have been extensively documented in animals. Chronic dosing studies cause testicular and liver toxicity in adult animals, but the mechanism of action for liver damage in rats is thought to be irrelevant in humans (ATSDR 2002; Kavlock et al. 2002), although this may be controversial. As with other phthalates, the most important health effects are its developmental and reproductive effects. Developmental effects include embryo death, reduced fetal/pup weight, developmental delay, and malformations of the skeleton, cardiovascular system, eye, and neural tube (ATSDR 2002; Kavlock et al. 2002). Perinatal, especially in utero, exposures to DEHP cause testicular atrophy, decreased AGD, retention of thoracic nipples, Sertoli and Leydig cell lesions, decreased spermatogenesis, and decreased prostate and seminal vesicle weight (Gray et al. 2000; Gray et al. 1999), and it is likely that the monoester metabolite, MEHP, and possibly other metabolites, are the mediators of the adverse effects (Gray and Beamand 1984; Li et al. 1998).
The mechanism of action for DEHP and its metabolites appears to be similar to that for DBP. Exposure to DEHP reduces fetal testosterone synthesis (Parks et al. 2000), and investigators (Borch et al. 2004; Borch et al. 2006; Wilson et al. 2004) have demonstrated that the anti-androgen effects of DEHP may be caused by a reduction in the expression of genes and proteins involved in steroidogenesis, a reduction in the expression of the hormone insl3.

While the animal data for the reproductive effects of DEHP exposure paint a conclusive picture, there are few data on effects of DEHP in humans. Nevertheless, the U.S. population is exposed to significant levels of DEHP. In the 1999-2000 NHANES population, 78% had detectable levels of MEHP in their urine (Silva et al. 2004). However, it is likely that other metabolites are better biomarkers for exposure to DEHP (Barr et al. 2003; Silva et al. 2006), and the levels of MEHHP and MEOHP in the 2001-2002 NHANES population (the first period in which they were tested in NHANES), are an order of magnitude higher than the levels of MEHP (National Center for Environmental Health 2005). Given the high concentrations of these additional metabolites, it is likely that more than 78% of the population experiences regular exposures to DEHP.

**Additional Phthalates of Interest**

Diethyl phthalate (DEP) is used as a plasticizer, and is especially present in a variety of cosmetic products, including nail polish, bath preparations, perfumes and fragrances, nail extenders, and detergents. The concentration of DEP in some of these preparations can be as high as 25-50% (ATSDR 1995; National Center for
Environmental Health 2005). It is also present in aspirin coating, insecticides, mosquito repellants, and plastic packaging. Contrary to some other phthalates, DEP does not appear to affect male reproductive development when the fetus is exposed (ATSDR 1995; Lamb et al. 1987). Gray et al. (Gray et al. 2000) demonstrated that while BBzP, DEHP, and DiNP caused a reduction in AGD and testicular weight, and caused an increased retention of thoracic nipples, DEP did not affect these or other reproductive measures. The investigators postulated that the antiandrogen effects of the phthalate esters are related to their chemical structures; phthalates with long ester side chains (between four and six carbons) in the ortho position, such as DEHP, BBzP, DBP, and some DiNP, are antiandrogenic, while phthalates with short ester side chains, such as DEP (C2, ortho position), or ester side chains not in the ortho position, would likely not be antiandrogenic. In spite of the lack of convincing data regarding the health effects of DEP, humans are exposed to DEP in relatively large quantities. The urinary concentrations of mono-ethyl phthalate (MEP) in the U.S. population are one to two orders of magnitude higher than the levels of other phthalate metabolites (National Center for Environmental Health 2005), and 100% of those tested in the 1999-2000 NHANES study had detectable levels of MEP (Silva et al. 2004).

Di-isononyl phthalate (DiNP) is a complex mixture of as many as 100 isomers of mainly nine-carbon branched chain dialkyl phthalates (National Center for Environmental Health 2005; Silva et al. 2006). It is widely used in many plastic products, and has been used to replace DEHP in many cases (National Center for Environmental Health 2005). Animal studies demonstrate some renal and hepatic toxicity due to high-dose DiNP exposure (Kavlock et al. 2002), but the relevance to humans is unclear. DiNP
does have some reproductive toxicity when animals are exposed in utero, but DINP was an order of magnitude less active as an antiandrogen than BBzP or DBP (Gray et al. 2000). Human exposure to DiNP has been difficult to characterize, mainly due to the use of its monoester metabolite, mono-isonyonyl phthalate (MiNP), as the principal biomarker. MiNP is weakly water-soluble, and has been detected only at the 95th percentile in the NHANES study (National Center for Environmental Health 2005).

Similar to DEHP, it is likely that other oxidized metabolites of DiNP would make better biomarkers of exposure, although they have not been evaluated yet in NHANES (Silva et al. 2006). Nevertheless, it is likely that the use of additional biomarkers will indicate that humans are more widely exposed to DiNP than is currently thought. It is also possible that DiNP plays an important role in human reproductive effects, especially when present with other phthalates.

Di-isobutyl phthalate (DiBP) is a type of dibutyl phthalate that is very similar to di-n-butyl phthalate (DBP). In fact, prior to the 2001-2002 NHANES sampling period, the monoester metabolites of the two dibutyl phthalates, mono-isobutyl phthalate (MiBP) and MBP, were not analytically distinguished from each other (National Center for Environmental Health 2005). DiBP is used in similar products to those of DBP, and is increasingly being used as a substitute for DBP in a variety of consumer products (Borch et al. 2006). Toxic effects of DiBP are not well characterized in animals or humans, but a recent study by Borch and others (2006) demonstrated that in utero exposures to DiBP cause adverse effects on the reproductive tract similar to those seen with DBP and DEHP, such as reduced AGD, reduced testicular testosterone production, and testicular and other reproductive lesions. The urinary concentrations of MiBP in the U.S.
population were an order of magnitude lower than those for MBP (National Center for Environmental Health 2005), but the increased use of DiBP in place of DBP, combined with recent toxicological evidence, raises worries regarding potential adverse effects in humans.

**Summary of phthalate characteristics, and mechanisms for testicular dysgenesis**

In this section I summarize the phthalate information described for individual phthalates above, and further characterize the mechanisms by which the phthalates may lead to testicular dysgenesis. To summarize, phthalates are present in all environmental media, and humans are exposed to regular doses of various phthalate diesters. Consumption of phthalates in food is probably the most important pathway of exposure, but dermal absorption and inhalation exposures have been poorly studied in people and animals. The following table summarizes the parent phthalates described above, along with their main metabolites and a note describing their linkage to developmental reproductive health effects in male animals.
<table>
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<tr>
<td>Di-2-ethylhexyl (DEHP)</td>
<td>Mono-2-ethylhexyl (MEHP), MEHHP, MEOHP, others</td>
<td>Diester linked to male reproductive malformations <em>in utero</em></td>
</tr>
<tr>
<td>Butyl benzyl (BBzP)</td>
<td>Monobenzyl (MBzP), mono-n-butyl</td>
<td>Metabolite and diester linked to male reproductive malformations <em>in utero</em></td>
</tr>
<tr>
<td>Di-isobutyl (DiBP)</td>
<td>Mono-isobutyl (MiBP)</td>
<td>Diester linked to male reproductive malformations <em>in utero</em></td>
</tr>
<tr>
<td>Di-isononyl (DiNP)</td>
<td>Mono-isononyl (MiNP), other oxidative metabolites</td>
<td>Diester more weakly linked to male reproductive malformations <em>in utero</em></td>
</tr>
<tr>
<td>Di-ethyl (DEP)</td>
<td>Monoethyl (MEP)</td>
<td>No links to male reproductive malformations</td>
</tr>
</tbody>
</table>

Scientists postulated that phthalates might be environmental and human contaminants as early as the 1970s (Mayer et al. 1972), and soon thereafter began examining their health effects in rat experiments (Nikonorow et al. 1973). These studies
indicated that phthalate esters were associated with a number of reproductive effects, including increased fetal resorptions, decreased fetal weight, and decreased numbers of weaned pups (Nikonorow et al. 1973; Peters and Cook 1973). In the early 1980s researchers linked phthalate exposures to testicular atrophy and Sertoli cell abnormalities by dosing adult rats and cell cultures with phthalate esters (Foster et al. 1981; Foster et al. 1981; Gray and Beamand 1984; Gray and Butterworth 1980; Heindel et al. 1989).

Beginning with numerous studies in the latter half of the 1990s and early 21st century, scientists have further elucidated the cellular mechanisms by which phthalates are toxic to the developing male reproductive tract. It is now clear that some phthalate diesters cause a number of abnormalities in the developing male reproductive tract when rats are exposed during lactation, and especially, in utero. These abnormalities, as reviewed by Hauser et al. (Hauser and Calafat 2005), include epididymal malformations or absence of the epididymis, increased incidence of hypospadias, cryptorchidism, decreased AGD, delayed preputial separation (which is a pubertal milestone), retention of thoracic nipples, reduced prostate and seminal vesicle volume, and testicular lesions in male offspring of female rats exposed to phthalates. Some, but not all, phthalates have been associated with these defects. Investigators have conclusively documented that at least three phthalates (DEHP, DBP, and BBzP) are relatively potent antiandrogens, while DINP is an order of magnitude less potent as an antiandrogen. An additional phthalate, DiBP, likely has antiandrogenic effects similar to other phthalates, but it has not been as well-studied as the other chemicals. It is likely that the monoester, and perhaps additional, metabolites of the phthalate diesters are the mediators of the toxic effects, and numerous researchers have demonstrated that a critical window during late gestation is
the most sensitive time period for disruption of reproductive development by phthalate exposure.

While researchers originally postulated that phthalates might be “estrogenic”, or alternatively might bind competitively to androgen receptors, thus causing their “feminizing” effects, investigators discovered in the late 1990s and in 2000 that exposure to phthalates causes a reduction in the fetal production of testosterone and other reproductive hormones. Dosage with DEHP reduces testosterone production in fetal and postnatal male rats to female levels, and causes a corresponding 36% reduction in AGD, while neither DEHP nor its metabolite, MEHP, exhibits significant affinity for androgen receptors (Parks et al. 2000). Mylchreest et al. (Mylchreest et al. 2002) demonstrated that in utero DBP exposure causes “feminizing” changes, such as testis atrophy; markedly reduced male fetal testosterone levels; and compensatory Leydig cell hyperplasia without significantly interacting with androgen receptors.

In the past couple of years, investigators have attempted to determine the mechanism of action by which the phthalate esters modulate fetal reproductive hormone production. Lehmann et al. (Lehmann et al. 2004) first presented data demonstrating that in utero exposure to DBP at levels lower than those associated with observable physiological defects reduces the expression of genes and proteins involved in cholesterol transport and steroidogenesis, in turn reducing fetal testosterone production. Other phthalates, in addition to DBP, appear to affect expression of genes and proteins involved in cholesterol transport and steroidogenesis as well. Exposure to DEHP is associated with reductions in many of the same genes and proteins that are affected by DBP (Borch et al. 2006).
It is still unclear, however, whether the phthalates are directly downregulating these important genes and proteins, or whether the phthalates are in some manner toxic to Leydig cells, thus resulting in lowered hormone production by these cells. Wilson et al. (Wilson et al. 2004) demonstrated that in utero exposure to DEHP, BzBP, and DBP, in addition to being associated with reduced testosterone production, also causes a reduction in the production of fetal insl3. Loss of insl3, and in turn the gubernacula, is associated with cryptorchidism in animals. Wilson et al. (Wilson et al. 2004) hypothesized that phthalates, by an undetermined mechanism, disrupt the autocrine and paracrine environment of the fetal testes to cause a delay in the maturation of Leydig cells, which causes them to proliferate without differentiating, resulting in reduced production of testosterone and insl3. The hormone reductions are responsible for the physiological effects seen in the animal experiments.

While investigators hypothesized that the testosterone reductions associated with phthalate exposures resulted in Leydig cell hyperplasia (Mylchreest et al. 2000; Wilson et al. 2004), recent work by other researchers indicates that the histologic scenario is a little more complicated. The changes seen on a cellular level with prenatal exposure to DBP are similar to those seen with the hypothesized human testicular dysgenesis syndrome (TDS). These physiologic changes of DBP exposure include post-natal development of focal areas of dysgenesis, comprising malformed seminiferous cords/tubules with intracordal/intratubal leydig cells (ITLCs) and immature Sertoli cells, surrounded by otherwise normal tubules exhibiting complete spermatogenesis (Fisher et al. 2003; Mahood et al. 2005). Mahood et al. (Mahood et al. 2005) present evidence that phthalate exposure is not associated with Leydig cell hyperplasia, but rather causes
Leydig cells to abnormally aggregate in focal areas centrally in the fetal testis. It is likely that large clusters of Leydig cells “trap” Sertoli and other cells during development, and that the “dysgenetic areas” form when these groups of intermingled cells attempt to form seminiferous tubules (Mahood et al. 2006). Mahood et al. (Mahood et al. 2005) further hypothesize that the presence of ITLCs within the seminiferous tubules interferes with Sertoli cell development and cell–cell interactions, such that the long-term survival of germ cells and the progression of normal spermatogenesis are not possible, leading to the development of Sertoli-cell-only (SCO) tubules in adulthood. Indeed, Sharpe et al. (Sharpe 2006) hypothesize that the testicular toxicity of the phthalate diesters, which results in aberrant testicular development and testicular dysgenesis, results in a downstream deficit in hormone production by the fetal testis and the associated pathologies of hormone-dependent processes, such as cryptorchidism and malformations of the external genitalia and internal reproductive organs.

In summary, extensive animal literature demonstrates that phthalate diesters with long-chain esters in the ortho position suppress male fetal testosterone secretion and insulin-like hormone gene expression, resulting in a variety of “feminizing” pathological alterations, such as cryptorchidism, hypospadias, atrophied testes, retained thoracic nipples, epididymal malformations, testicular lesions, and reduced anogenital distance. The cellular changes seen with DBP exposure mirror those observed in testicular dysgenesis syndrome, and likely result in impaired long-term survival of germ cells and progression of spermatogenesis. The concept map in Figure 4 [adapted from (Borch et al. 2006; Mahood et al. 2005; Mahood et al. 2006; Wilson et al. 2004)] further illustrates this hypothesized process.
Figure 4: Proposed mechanism of phthalate action on reproductive organs; adapted from (Borch et al. 2006; Mahood et al. 2005; Mahood et al. 2006; Wilson et al. 2004)

- Phthalate ingestion by animal
  - Absorption of monoester, and other, metabolites
  - Transplacental exposure of fetus
  - Altered autocrine and paracrine environment in fetal testes

- Delay in Leydig maturation; focal aggregations of immature Leydig cells
- Decreased gene/protein expression of steroidogenic factors
- Misshapen tubules with trapped Sertoli cells, resulting in postnatal decrease in spermatogenesis

- Decreased fetal testosterone
- Malformations of androgen-dependent tissues
- Reduced ins13 production
- Gubernacular agenesis and undescended testes
III. HUMAN EVIDENCE FOR REPRODUCTIVE EFFECTS OF PHTHALATES

Although the animal experiments paint a convincing and detailed picture of the effects of prenatal phthalate exposures to male reproductive health, there is a relative dearth of evidence linking phthalate exposures to reproductive effects in humans. This appears to be changing, however, as more and more researchers, perhaps encouraged by animal toxicological results and the evidence of ubiquitous human exposures to phthalates, have begun to investigate the linkages among phthalates and human health. This section concludes the second chapter with a discussion of the handful of epidemiologic studies published within the last few years that address the effects of phthalate exposures on human male reproductive health.

Phthalates and sperm concentration and quality

Some of the first epidemiologic studies investigated the links between phthalate exposures and semen quality in adult males. While a few studies have examined these connections, the results are inconclusive and contradictory.

Duty et al. (Duty et al. 2004; Duty et al. 2003) examined associations among urinary phthalate metabolite concentrations and human semen parameters in males that presented to an infertility clinic in Massachusetts. Among 168 men, there were dose-response relationships between MBP and sperm motility and sperm concentration, and between MBzP and sperm concentration (Duty et al. 2003). A more recent study by the same authors confirmed dose-response relationships between DBP and decreased sperm concentration and motility in 463 men (Hauser et al. 2006). Metabolite levels of six additional phthalates, including MEP and MEHP, were not significantly associated with
semen parameters. Another study by the same authors demonstrated a small but significant association between MEP levels and sperm DNA damage, but no associations with other phthalates (Duty et al. 2003).

Another recent study performed in Sweden failed to find associations between phthalate levels and semen parameters (Jonsson et al. 2005). The researchers measured urinary metabolite levels of MEHP, MEP, MBP, and MBzP from 234 young men presenting for a mandatory medical conscript examination. Unlike the Duty et al. studies, there were no relationships between MBP or MBzP levels and any of the semen parameters. Individuals in the highest quartile of MEP levels had fewer motile sperm, but there were no other significant associations. While these results contradict those observed in the Duty et al. studies, it is important to note that the study designs differed in substantial ways. Perhaps most importantly, the Jonsson et al. study examined young men (18 – 21 years of age) from the general population while the Duty et al. studies examined older men (mean age of 35 years) who had presented to an infertility clinic. It is also likely that a sample size of 234 men is inadequate to accurately assess variations in semen quality and phthalate exposures. Regardless of the etiology, the conflicting results highlight the inability to conclusively connect phthalate exposures to semen quality in adult men.

**Phthalates in breast milk and reproductive hormone levels**

Phthalate concentrations have also been measured, along with reproductive hormone levels, in breast milk and young boys. A recent publication used biologic samples from a Danish-Finnish prospective cohort study on cryptorchidism to examine
associations among breast milk concentrations of phthalate metabolites and reproductive hormone levels of newborn boys at three months of age (Main et al. 2006). The investigators analyzed individual breast milk samples collected from 1-3 months postnatally for MEP, MMP, MBP, MEHP, MBzP, and MiNP, and analyzed boys’ serum samples for gonadotropins, sex-hormone binding globulin (SHBG), testosterone, and inhibin B.

Various associations among phthalate monoester concentrations and hormone levels were found. MBP was negatively correlated with free testosterone, while MMP, MEP, and MBP were positively correlated with LH: free testosterone ratio. MEP and MBP were positively correlated with SHBG, while MiNP was positively correlated with LH.

Importantly, these results indicate that phthalates at environmental concentrations may have antiandrogenic activity in human boys, similar to that observed in animal studies. Of note, increased SHBG is an indirect sign of reduced androgenic activity, while elevated LH levels, together with reduced testosterone and increased LH: testosterone ratio, are consistent with a reduced biologic androgenic effect (Main et al. 2006). There is negative feedback between serum testosterone levels and pituitary secretion of LH. While additional studies are needed to confirm these results, this study supports the prospect that environmental phthalate exposures may be significant enough to affect male reproductive development.

**Prenatal phthalate exposures and anogenital distance**
The study that has probably garnered the most attention regarding the human health effects of phthalate exposures was published in 2005 and linked elevated prenatal levels of four phthalate metabolites to reduced AGD in newborn boys (Swan et al. 2005). Investigators measured AGD and anogenital index (AGI = AGD/weight), measurements that correlate with prenatal androgen activity, in boys 2-36 months of age. They measured nine phthalate metabolites in prenatal maternal urine samples as predictors of age-adjusted anogenital index (AGI) in 85 mother-infant pairs.

Concentrations of four of the metabolites, MEP, MBP, MBzP, and MiBP, were inversely correlated to AGD and AGI in infant boys. The oxidative metabolites of DEHP (MEOHP and MEHHP) exhibited a similar relationship but were not statistically significant. AGD was significantly correlated with penile volume and proportion of boys with incomplete testicular descent. AGD was one of the most sensitive endpoints in the animal toxicology studies. Prenatal exposure to many of the phthalates, including DBP, DEHP, and BBzP, caused a reduction in AGD along with additional reproductive lesions.

The discussion of the AGD study serves as an appropriate launching pad for the transition to chapter 3 of this thesis. Given a few important characteristics of the study, some colleagues in the Office of Policy, Economics and Innovation at the U.S. Environmental Protection Agency (EPA) and I, determined that this study should be considered in any government risk analysis for the important phthalate chemicals. First, the study provided physiological evidence that environmental levels of phthalates may effect reproductive development in humans. Second, it provided the first examination of the effects of phthalate exposures during the prenatal period in humans, the period of development that is most sensitive to disruption by phthalates in animal studies. Given
this important information, along with the knowledge that the U.S. EPA was working on at least one draft risk assessment for a phthalate chemical at the time of publication of the Swan study, we decided that it was important to work with the data from the AGD study so that it could be presented in a format that would allow it to be utilized in the customary form of a government risk assessment.

Chapter 3 of this thesis discusses work that I did, in collaboration with the authors of the original AGD study, to convert the AGD data so that it could inform a risk assessment analysis. Risk assessment often uses toxicological studies, with specific daily dosing regimens, to inform regulators of the chemical doses that could lead to abnormal physiological effects in humans. This provides a guideline for acceptable limits of daily exposure to the chemicals of interest. These risk assessments are used to help set policy limits for acceptable levels of chemicals released in environmental media and, in turn, levels that come into contact with public citizens. Since the AGD study had exposure data only in terms of urinary phthalate metabolite data, it could not be used directly to inform a risk assessment analysis with daily dosing data. Chapter 3 begins with a more detailed discussion of the EPA risk assessment process, followed by an in-depth discussion of the model that I adapted to estimate daily doses of individual phthalate chemicals based on urinary metabolite concentrations. Chapter 3 concludes with a discussion of conclusions and limitations of the study, as well as suggested next steps for continuing evaluation of phthalate effects in people.
CHAPTER 3: Estimated Daily Phthalate Exposures in a Population of Mothers of Male Infants Exhibiting Reduced Anogenital Distance

I. BACKGROUND ON THE U.S. EPA RISK ASSESSMENT PROCESS

Understanding the process by which the U.S. EPA performs risk assessments for chemicals with non-carcinogenic effects will help clarify why it was useful to convert the epidemiologic data from Swan et al. (Swan et al. 2005) into daily dose estimates. This section presents background on how a U.S. EPA risk assessment is performed, and will then discuss the current “reference doses” for the phthalate chemicals.

When undertaking a risk assessment, the U.S. EPA is attempting to “assess the human health risk from chemical exposures” (Harvey et al. 1995). In other words, a risk assessment permits an “estimate to be made of the present or potential health risk” to a certain group of people (U.S. EPA). While risk assessment can be thought of as information on a health risk, risk management encompasses the action that is taken in order to protect the public health. Risk assessment information is used in the risk management process in deciding how to protect public health (U.S. EPA).

The process of a risk assessment for a given chemical or mixture of chemicals can be divided into four main parts: 1) Hazard identification, 2) Dose-response assessment, 3) Exposure assessment, and 4) Risk characterization. These four processes will be described below.

“Hazard identification is the identification of chemical and biological contaminants that are suspected to pose hazards and a determination of levels at which they are found in the environment. It includes a description of specific
forms of toxicity evoked by these chemicals or mixtures and a *qualitative*
assessment of whether these contaminants are causally associated with adverse
health effects in exposed humans.

*Dose-response assessment* addresses the question of how much biological
exposure to a contaminant is necessary for adverse health effects to result in
humans and/or how humans may be expected to respond to different doses or
concentrations of contaminants. The dose-response component often involves
analyses of sensitive human subpopulations, interspecies comparisons of toxicity
(using, for example, toxicokinetics) and extrapolation of dose or concentration,
the time of onset and progress of the adverse effects, and the overall toxicity
database to determine data gaps.

*Exposure assessment* includes identification of the individuals and
population(s) exposed, the routes of actual and potential exposures, and the
estimation of duration and concentration of such exposures. Considerations such
as stage of development during exposure (e.g. fetal, neonatal, adult) and
bioavailability that may influence a toxicological response are also studied.

*Risk characterization* involves the integration of each of the human health
components described above, with the goal of determining whether specific
exposures to an individual or population are likely to result in adverse health
effects. Risk characterization includes a full discussion of the uncertainties and
alternative choices associated with the overall risk assessment” (Harvey et al.
1995).
If a decision is made to institute any regulation, risk characterization can be the transitional step to risk management (U.S. EPA).

Table 3: Summary of the stages of a U.S. EPA risk assessment, adapted from (Harvey et al. 1995)

<table>
<thead>
<tr>
<th>Stage of Risk</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard</td>
<td>Qualitative assessment of which contaminants may pose a hazard, and determination of environmental levels.</td>
</tr>
<tr>
<td>Identification</td>
<td>Quantitative determination of the effects of varying doses of a contaminant on human physiology. This may include sensitive subgroups.</td>
</tr>
<tr>
<td>Exposures</td>
<td>Identification of populations exposed, and determination of methods of exposure.</td>
</tr>
<tr>
<td>Assessment</td>
<td>Determination whether specific exposures are likely to result in adverse effects.</td>
</tr>
</tbody>
</table>

One approach to risk assessment is to determine a reference dose (RfD) for the chemical of interest. The RfD, as defined by U.S. EPA, is intended to be a dose for which daily exposure to the human population (including sensitive subgroups) is likely to be without an appreciable risk of deleterious effects during a lifetime (Barnes and Dourson 1988). The RfD is determined during the dose-response portion of the risk assessment. In formulating the RfD, the risk assessor must choose the critical study and the critical effect that will be used. Data from experimental studies of animals are often used, since available human data are usually insufficient (U.S. EPA 1993). The selection of the
critical study and effect can be idiosyncratic. The risk assessor often chooses the study that has the most sensitive response or is believed to most closely mirror a human model (U.S. EPA 1993). The critical study will then be used to determine the dose at which there are no observed adverse effects – the NOAEL, or "no observed adverse effects level." The RfD will be formulated based on the NOAEL divided by a set of uncertainty factors that are meant to account for uncertainty in interspecies, within-species, and other types of variability. For example, an NOAEL from a valid animal study will typically be divided by a factor of 100 to determine the RfD for humans (U.S. EPA 1993). In the next section, I will discuss how this process was performed for a few of the phthalate chemicals.

Risk assessors do not always rely on experimental animal studies when determining an RfD for a specific chemical. In fact, the U.S. EPA has stated that human data, when available, should be given priority over animal data, because then there is no need to extrapolate from animals to humans (Barnes and Dourson 1988). A recent comparison of risk assessments using human and animal data demonstrated that reference doses that used animal data with the associated uncertainty factors were often higher than associated reference doses that used human data, which raised the question of whether these animal-based RfDs were sufficient to protect human health (Dourson et al. 2001).

Current Reference Doses for Phthalate Chemicals
This section will discuss the current status of the U.S. EPA RfDs for the phthalates, and examine whether they adequately take into account relatively recent research that has demonstrated that the phthalates are reproductive toxicants.

The U.S. EPA lists the chemicals for which it has adapted a toxicity value, such as a reference dose or a cancer potency estimate, in its Integrated Risk Information System (IRIS) database, which is publicly available online (http://www.epa.gov/iris/index.html). Full assessments of non-cancerous effects have been performed for four of the phthalate chemicals: dibutyl phthalate (DBP), benzyl butyl phthalate (BzBP), diethyl phthalate (DEP), and di(2-ethylhexyl) phthalate (DEHP). The IRIS entry for each of these substances describes the individual risk assessment process, including a discussion of the key study and data that were chosen, the determination of the NOAEL or “lowest observed adverse effects level (LOAEL)”, and the application of uncertainty factors to determine a final RfD (U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005). This information is summarized in Table 4.

A few themes emerge upon analysis of the phthalate RfD information. First, all four of the risk assessments were finalized in the early 1990s, ranging from 1990 for DBP to 1993 for DEP and BzBP. Since much of the research on developmental reproductive effects in both animals and humans was performed in the late 1990s and after 2000, it was not included in the formulation of the current RfDs. In fact, the key studies used to inform the RfDs for DBP and DEHP were published in 1953 (Carpenter et al. 1953; Smith 1953), while the key studies for DEP and BzBP were published or released in 1978 and 1985, respectively (Brown et al. 1978; National Toxicology Program 1985).
Second, the current body of research (as discussed in Chapter 2 of this thesis) has demonstrated that the prenatal period is the most sensitive period for phthalate exposure and that male reproductive malformations the most sensitive endpoint. None of the key studies used to set the RfDs for the four phthalate chemicals examined prenatal exposures or male reproductive malformations as endpoints. Instead, the key studies utilized much higher daily dosing regimens than those used in several more recent studies (Lehmann et al. 2004; Mylchreest et al. 1998; Mylchreest et al. 2002; Mylchreest et al. 2000), and focused on gross abnormalities, such as increased liver weight, overall mortality, and decreased growth rates. While these findings are certainly important, lack of information on the most sensitive endpoints and sampling time periods make it unlikely that the current RfDs adequately consider effects of low-level prenatal exposures.
Table 4: Current reference doses (RfDs) for the phthalates

<table>
<thead>
<tr>
<th>Chemical</th>
<th>RfD</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutyl phthalate (DBP)</td>
<td>0.10 mg/kg/day</td>
<td>Key study (Smith 1953) demonstrated increased mortality in male rats with an NOAEL of 125 mg/kg/day. An uncertainty factor (UF) of 1000 was applied for deficiencies in the study (10-fold), interspecies variation (10-fold), and protection of sensitive human subpopulations (10-fold).</td>
</tr>
<tr>
<td>Butyl Benzyl Phthalate (BBzP)</td>
<td>0.20 mg/kg/day</td>
<td>Key study (National Toxicology Program 1985) demonstrated liver enlargement in rats with an NOAEL of 159 mg/kg/day. A UF of 1000 was applied.</td>
</tr>
<tr>
<td>Diethyl phthalate (DEP)</td>
<td>0.80 mg/kg/day</td>
<td>Key study (Brown et al. 1978) demonstrated decreased growth rates and food consumption and altered organ weights in rats with an NOAEL of 750 mg/kg/day. A UF of 1000 was applied for interspecies variation, protection of sensitive human subpopulations, and extrapolation for subchronic to chronic doses.</td>
</tr>
<tr>
<td>Di(2-ethylhexyl) phthalate (DEHP)</td>
<td>0.02 mg/kg/day</td>
<td>Key study (Carpenter et al. 1953) demonstrated increased liver weights in guinea pigs with a lowest observed adverse effects level (LOAEL) of 19 mg/kg/day. A UF of 1000 was applied.</td>
</tr>
</tbody>
</table>
This section demonstrates that the phthalate risk assessments did not consider prenatal exposures or male developmental reproductive effects in formulating the current phthalate RfDs. Further updates of the phthalate RfDs are warranted to account for the important body of research on prenatal exposures and male reproductive effects. Since the Swan et al. study demonstrated the association of subtle developmental effects with phthalate metabolite concentrations in human newborn boys, it could inform risk assessments for the phthalate chemicals of interest in the study. As discussed in the preceding section, dose-response assessment and the formulation of an RfD are critical steps in a chemical risk assessment. Studies used in such assessment customarily provide information on the daily dose to which animals or people were exposed. To make this study accessible for U.S. EPA risk assessments, I adapted models to estimate daily doses of phthalates based on human urinary metabolite concentrations.

II. EPIDEMIOLOGIC DATA AND PHTHALATE REFERENCE DOSES

In summary of information presented in Chapter 1 of this thesis, phthalates are used in a variety of industries and are present in many consumer products, such as soaps, perfumes, cosmetics, shampoos, building products, shower curtains, aerosols, plastic toys, and in plastic packaging (ATSDR 1995; ATSDR 2001; ATSDR 2003). Di(2-ethylhexyl) phthalate (DEHP) is the primary plasticizer in polyvinyl chloride, while diethyl phthalate (DEP) and dibutyl phthalates (DBP) are commonly used in consumer and personal care products such as lotions, fragrances, cosmetics, deodorants, and pharmaceutical coatings (ATSDR 1995; ATSDR 2001; ATSDR 2003). The reproductive and developmental toxicities of some phthalates have been demonstrated extensively in
animal studies. Prenatal exposures to DEHP, DBP, butyl benzyl phthalate (BBzP), and, more weakly, di-isononyl phthalate (DiNP) cause a reduction in testosterone production in fetal testes (Lehmann et al. 2004; Mylchreest and Foster 2000; Mylchreest et al. 2002; Parks et al. 2000), which can result in incomplete development of the male reproductive tract and malformations of the external genitalia (Ema and Miyawaki 2001; Ema et al. 2003; Foster et al. 2000; Gray et al. 2000; Mylchreest et al. 1998).

In a recent study, Swan et al. (Swan et al. 2005) provided the first demonstration of subtle developmental effects, similar to those seen in animal studies, in human male infants exposed prenatally to phthalates. The study population for Swan et al. included 134 women and their male infants. For 85 of the women, a urine sample during pregnancy was available. These prenatal maternal urine samples were analyzed for nine phthalate metabolites commonly used as biomarkers of exposure to phthalates, using an analytical method described before (Silva et al. 2004). The male infants (n = 134), including 49 for whom no maternal prenatal urine sample had been collected, were physically examined to determine AGD – a marker for prenatal anti-androgen exposure – and other reproductive organ measurements. Of nine urinary phthalate metabolites, Swan et al. (2005) found that prenatal maternal urinary levels of monoethyl phthalate (MEP; a metabolite of DEP), monobenzyl phthalate (MBzP; a metabolite of BBzP), mono-n-butyl phthalate (MBP; a metabolite of di-n-butyl phthalate, DnBP) and mono-isobutyl phthalate (MiBP; a metabolite of di-isobutyl phthalate) were significantly associated with reduced AGD and AGI in male infants.

While none of the 134 boys examined showed frank malformations or disease, and 86.6% of these boys had both testicles classified as normal, AGI was significantly
correlated with degree of testicular descent, as well as penile volume and scrotal size (Swan et al. 2005). The median concentrations of phthalate metabolites (Table 5) in the Swan study associated with short AGI and incomplete testicular descent were similar to the median concentrations found in the female population of the United States, based on the 2001-2002 National Health and Nutrition Examination Survey (NHANES) (National Center for Environmental Health 2005).

The current U.S. Environmental Protection Agency (U.S. EPA) RfDs for DEP, DBP, and BBzP were formulated in the early 1990s using older animal studies (DBP was completed in 1990, DEP and BBzP in 1993) (U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005). The RfD, as defined by U.S. EPA, is intended to be a dose for which daily oral exposure to the human population (including sensitive subgroups) is likely to be without an appreciable risk of deleterious effects during a lifetime. Because the data presented by Swan et al. suggested subtle human developmental effects at levels of exposure similar to those observed in the general population, the Swan et al. study provides important information when considering any future updates to RfDs for phthalates. In order for this study to be useful for this purpose, it is necessary to estimate the average daily exposures of phthalates for the study individuals.

I applied a simple pharmacokinetic model, initially proposed by Kohn et al. (Kohn et al. 2000) and later used by Koo et al. (Koo et al. 2002) to estimate the individual daily exposure of phthalate diesters in the pregnant women in the Swan et al. study population. I also used a second model, initially proposed by David (David 2000), to provide comparisons for the exposure estimates generated by the first model.

**Study Methods**
Study Population

Women included in this study were originally recruited into the Study for Future Families (SFFI), a multicenter pregnancy cohort study, at prenatal clinics in Los Angeles, California (Harbor-UCLA and Cedars-Sinai), Minneapolis, Minnesota (University of Minnesota Health Center), and Columbia, Missouri (University Physicians), from September 1999 through August 2002. Details of study participation are given in Swan et al. (Swan et al. 2005). I had no involvement in the recruitment or data collection in this cohort. All participants completed a questionnaire, and after urine collection was added midway through the study, most gave a urine sample. Eighty-five percent of SFFI participants agreed to be recontacted, and these mothers were invited to take part in the follow-up study (SFFII) (Swan et al. 2005). Institutional review boards at all participating institutions approved the SFFI and SFFII, and all participants signed an informed consent for each study.

In the original study (Swan et al. 2005) the authors reported on results in boys for whom a first prenatal visit had been completed by Dec 17, 2004. These included 172 boys, 134 of whom had complete data for AGD, age and weight. Urinary phthalate metabolite concentrations in 214 mother-infant pairs were also obtained (girls and boys), of whom 85 were boys with measurements of AGD and complete data on age and weight, and whose mother had a prenatal urine sample. I used the urinary phthalate monoester concentrations from the study population of 214 mother-infant pairs in order to calculate daily exposure estimates. The monoester concentrations for the complete study population (n = 214) are shown in Table 5. This study population has urinary monoester concentrations very similar to those found in the subset of this population (n =
85) used by Swan et al. (Swan et al. 2005) (see note to Table 1). I evaluated the larger sample as it provides more information on the distribution of phthalate exposures. I calculated daily exposure for the phthalate metabolites that were statistically significantly associated with reduced AGI in the Swan et al. study, and the metabolites of DEHP. Although metabolites of DEHP were not significantly associated with AGI in the Swan et al. study, the associations for two oxidative metabolites of DEHP ((mono(2-ethyl-5-oxohexyl) phthalate, MEOHP and mono(2-ethyl-5-hydroxyhexyl) phthalate, MEHHP)) were of comparable magnitude with those for metabolites of DBP and BBzP. Moreover, there is an extensive animal literature showing DEHP-mediated androgen-related effects.

**Daily Exposure Estimates**

Kohn et al. calculated the daily exposure for each individual in the population by using a linear two-compartment model. The normalized integrated rate equations for fractional excretion are:

\[ FE = 1 - \exp(-k_{total}t) \]  

[1]

\[ FU = \frac{k_u}{k_{total}}[1 - \exp(-k_{total}t)] \]  

[2]

where FE is the total fraction and FU is the urinary fraction of the dose eliminated in time t, and \( k_{total} \) and \( k_u \) are the apparent first-order rate constants for total elimination and urinary elimination of monoester, respectively. I calculated the two rate constants, \( k_{total} \) and \( k_u \), by using previously published values for the excreted fractions of each parent diester (Kohn et al. 2000; Koo et al. 2002). Values of FE and FU from Kohn et al.,
originally calculated from animal and human studies, were used for all metabolites reported by Swan et al., except for MiBP, which was not considered by Kohn et al. I assumed that the FE and FU for MiBP and its parent diester were equal to those calculated for MBP and DnBP. The excretion rate equations are used to estimate $k_{\text{total}}$ and $k_u$ for input into the equation from Kohn et al. that estimates phthalate exposure (Kohn et al. 2000).

Kohn et al. provide the following equation for the exposure rate for an individual, assuming steady-state exposure and metabolic clearance of the diester:

$$\text{Daily intake (ug/kg/day)} = \frac{\text{ME (ug/g)} \times \text{CE (mg/kg/day)}}{f \times 1000 \text{(mg/g)}} \times \frac{\text{MW}_d}{\text{MW}_m} \quad [3]$$

where ME is the urinary concentration of monoester per g creatinine, CE is the creatinine excretion rate normalized by body weight, $f$ is the ratio of urinary excretion to total elimination ($k_u/k_{\text{total}}$), and $\text{MW}_d$ and $\text{MW}_m$ are the molecular weights of the diesters and monoesters, respectively. I used a value of 18 mg/kg/day for CE (Kohn et al. 2000), and creatinine-adjusted concentrations (ME) for each subject in the study. The unadjusted and creatinine adjusted phthalate urinary concentrations from the 214 samples from the Swan et al. study are shown in Table 1.

For comparison, I also estimated the daily exposure using a second formula published by David (David 2000), and later used by Koch et al. (Koch et al. 2003):

$$\text{Daily intake (ug/kg/day)} = \frac{\text{ME (ug/g)} \times \text{CE (mg/kg/day)}}{\text{F}_{\text{UE}} \times 1000 \text{(mg/g)}} \times \frac{\text{MW}_d}{\text{MW}_m} \quad [4]$$
This formula is an alternate version of the Kohn method, and results in similar exposure values (Koch et al. 2003). The variables used are the same as those used in the Kohn formula, except $F_{UE}$ is the molar fraction of the urinary excreted monoester related to the parent diester. This is used in place of $f$ in the Kohn formula. The fractional urinary excretion values for DBP (0.69) and BBzP (0.73) were taken from published human data (Anderson et al. 2001). For DEP, I presumed that the excretion factor is the same as that for DBP, as done by Koch et al. (Koch et al. 2003) and Kohn et al. (Kohn et al. 2000). The fractional excretion data for the three DEHP metabolites measured in the Swan et al. study were taken from recently published human data (Koch et al. 2005). The values for mono(2-ethylhexyl) phthalate (MEHP), MEHP, MEHHP, and MEOHP are 0.059, 0.233, and 0.150, respectively.

I calculated DEHP exposures based on each of the three metabolites independently, and also based on the averages of the exposures calculated using the secondary metabolites (MEHHP and MEOHP). DEHP is initially metabolized to MEHP, which is then further metabolized to various other products, including MEHHP and MEOHP. All three metabolites are thought to be toxic (Koch et al. 2005), and estimating DEHP exposure based on the three different metabolites allows for comparison of the various estimates. I treated the concentrations of MBP and MiBP as one combined measure of exposure to DBP. This makes my estimates consistent with previous literature on DBP, which did not distinguish between iso and n-butyl isomers. I considered MBzP to be the main metabolite of BBzP. MBP is a minor metabolite of BBzP, but only 6% of the ingested BBzP diester is excreted as MBP (Anderson et al. 2001).
Table 5: Urinary phthalate monoester concentrations (ng/mL urine, µg/g creatinine) from a study population of 214 pregnant women from Swan et al. (Swan et al. 2005).

<table>
<thead>
<tr>
<th>Phthalate</th>
<th>25&lt;sup&gt;th&lt;/sup&gt; perc.</th>
<th>Median</th>
<th>75&lt;sup&gt;th&lt;/sup&gt; perc.</th>
<th>95&lt;sup&gt;th&lt;/sup&gt; perc.</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MEP) ng/mL</td>
<td>50</td>
<td>117</td>
<td>466</td>
<td>3199</td>
<td>30528</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>71.1</td>
<td>108</td>
<td>506</td>
<td>3015</td>
<td>33932</td>
</tr>
<tr>
<td>Benzyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MBzP) ng/mL</td>
<td>3.6</td>
<td>9.3</td>
<td>20.9</td>
<td>57.8</td>
<td>436</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>6.5</td>
<td>11.7</td>
<td>21.6</td>
<td>58</td>
<td>364</td>
</tr>
<tr>
<td>n-butyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MBP) ng/ml</td>
<td>7.4</td>
<td>16.2</td>
<td>29.6</td>
<td>64.5</td>
<td>337</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>13.8</td>
<td>20.6</td>
<td>32.2</td>
<td>57.3</td>
<td>144</td>
</tr>
<tr>
<td>Isobutyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Unit/Value 1</td>
<td>Unit/Value 2</td>
<td>Unit/Value 3</td>
<td>Unit/Value 4</td>
<td>Unit/Value 5</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>MiBP ng/mL</td>
<td>&lt;LOD</td>
<td>2.5</td>
<td>4.7</td>
<td>13.1</td>
<td>39.8</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>&lt;LOD</td>
<td>2.9</td>
<td>5.1</td>
<td>10.0</td>
<td>71.1</td>
</tr>
<tr>
<td>2-ethylhexyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEHP ng/mL</td>
<td>1.5</td>
<td>4.25</td>
<td>11.0</td>
<td>38.6</td>
<td>206.8</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>2.15</td>
<td>5.53</td>
<td>14.0</td>
<td>39.2</td>
<td>172.8</td>
</tr>
<tr>
<td>2-ethyl-5-hydroxyhexyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEHHP ng/mL</td>
<td>5.6</td>
<td>10.8</td>
<td>21.7</td>
<td>76.4</td>
<td>2108</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>8.4</td>
<td>13.0</td>
<td>26.9</td>
<td>88.9</td>
<td>1254</td>
</tr>
<tr>
<td>2-ethyl-5-oxohexyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEOHP ng/mL</td>
<td>5.1</td>
<td>9.75</td>
<td>21.0</td>
<td>65.0</td>
<td>1677</td>
</tr>
<tr>
<td>µg/g creatinine</td>
<td>7.7</td>
<td>12.6</td>
<td>23.1</td>
<td>80.5</td>
<td>998</td>
</tr>
</tbody>
</table>
Results

The results for the exposures of DEP, DBP, and BBzP of the women in the Swan et al. study, as calculated using the Kohn et al. method, are presented in Table 6. The relevant monoesters are presented with their parent diesters. Using the Kohn formula, I estimated the median and 95th percentile of daily exposures for: DBP of 0.99 µg/kg/day and 2.68 µg/kg/day respectively; for DEP of 6.64 µg/kg/day and 112.3 µg/kg/day, respectively; for BBzP of 0.50 µg/kg/day and 2.47 µg/kg/day, respectively; and for DEHP of 1.32 µg/kg/day and 9.32 µg/kg/day, respectively.

The estimated exposures calculated using the David formula are compared to the Kohn method estimates in Table 7. The David method produces exposure estimates that are typically about 20% lower than the estimates using the Kohn formulas. The exception is DEHP exposure estimates, which are about 30-80% higher based on the David method, depending on which metabolites are used for the calculation.
Table 6: Estimated phthalate exposure (µg/kg/day), calculated using the Kohn et al. method, for 214 pregnant women from Swan et al. (Swan et al. 2005). The phthalates shown are those that were significantly associated with reduced AGD and AGI (Swan et al. 2005), along with MEHP\(^a\).

<table>
<thead>
<tr>
<th>Monoester</th>
<th>Diester</th>
<th>25(^{th}) perc.</th>
<th>Median</th>
<th>75(^{th}) perc.</th>
<th>95(^{th}) perc.</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>DEP</td>
<td>2.65</td>
<td>6.64</td>
<td>18.82</td>
<td>112.3</td>
<td>1263</td>
</tr>
<tr>
<td>MBzP</td>
<td>BBzP</td>
<td>0.28</td>
<td>0.50</td>
<td>0.92</td>
<td>2.47</td>
<td>15.53</td>
</tr>
<tr>
<td>MBP</td>
<td>DnBP</td>
<td>0.56</td>
<td>0.84</td>
<td>1.31</td>
<td>2.33</td>
<td>5.86</td>
</tr>
<tr>
<td>MiBP</td>
<td>DiBP</td>
<td>NA(^b)</td>
<td>0.12</td>
<td>0.21</td>
<td>0.41</td>
<td>2.90</td>
</tr>
<tr>
<td>MiBP+MBP</td>
<td>DnBP+DiBP</td>
<td>0.63</td>
<td>0.99</td>
<td>1.53</td>
<td>2.68</td>
<td>5.98</td>
</tr>
<tr>
<td>MEHP</td>
<td>DEHP</td>
<td>0.51</td>
<td>1.32</td>
<td>3.32</td>
<td>9.32</td>
<td>41.10</td>
</tr>
</tbody>
</table>

\(^a\) Current USEPA Reference Doses are 100 µg/kg/day (dibutyl phthalate, DBP), 200 µg/kg/day (butylbenzyl phthalate, BBzP), 800 µg/kg/day (diethyl phthalate, DEP), and 20 µg/kg/day (di-2-ethylhexyl phthalate, DEHP) (U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005).

\(^b\) NA: The daily exposure was not estimated when the urinary concentration of the phthalate metabolite was <LOD.
Table 7: Estimated daily exposure values of phthalates to the pregnant women of Swan et al. study population based on the Kohn et al. (Kohn et al. 2000) and the David\textsuperscript{a} (David 2000) methods\textsuperscript{b}.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Diester</th>
<th>Kohn Method</th>
<th>David Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>95\textsuperscript{th} perc.</td>
<td>Range</td>
</tr>
<tr>
<td>Ethyl</td>
<td>DEP</td>
<td>6.64</td>
<td>112.3</td>
</tr>
<tr>
<td>(MEP)\textsuperscript{c}</td>
<td>BBzP</td>
<td>0.50</td>
<td>2.47</td>
</tr>
<tr>
<td>Benzyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MBzP)\textsuperscript{c}</td>
<td>DnBP</td>
<td>0.84</td>
<td>2.34</td>
</tr>
<tr>
<td>n-butyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MBP)\textsuperscript{c}</td>
<td>DiBP</td>
<td>0.12</td>
<td>0.41</td>
</tr>
<tr>
<td>Compound Description</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>n-butyl + isobutyl</td>
<td>0.99</td>
<td>2.68</td>
<td>0 - 5.98</td>
</tr>
<tr>
<td>(MBP+MiBP) DBP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ethyl-5-hydroxyhexyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MEHHP) DEHP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ethyl-5-oxohexyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MEOHP) DEHP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. (^d) DEHP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound Description</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
<th>Value 4</th>
<th>Value 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-ethylhexyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MEHP) DEHP</td>
<td>1.32</td>
<td>9.32</td>
<td>0 - 41.1</td>
<td>2.37</td>
<td>16.8</td>
</tr>
</tbody>
</table>

\(^a\) F<sub>ue</sub> for MEHP, MEHHP, and MEOHP are 0.059, 0.233, and 0.150 respectively based on human data from Koch et al. (Koch et al. 2005).\(^b\) Current USEPA Reference Doses are 20 μg/kg/day (di(2-ethylhexyl) phthalate, DEHP), 100 μg/kg/day (dibutyl phthalate, DBP), 200 μg/kg/day (butylbenzyl phthalate, BBzP), and 800 μg/kg/day (diethyl phthalate, DEP) (U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005; U.S. EPA 2005).

\(^c\) Statistically significantly associated with reduced anogenital index in the Swan et al. study (Swan et al. 2005).

\(^d\) Avg: the average of the exposure estimates using MEHHP and MEOHP
Discussion

I estimated exposures to a variety of phthalate diesters using data from a population of mother-infant pairs in which subtle developmental effects were associated with prenatal urinary phthalate metabolite concentrations. The models I used to estimate exposures make no assumptions regarding the route of exposure. There are multiple possible routes of exposure to phthalates, including dermal (Duty et al. 2005), ingestion (Clark et al. 2003), and inhalation (Adibi et al. 2003). Furthermore, phthalate diesters and their metabolites are cleared from the body within a few days, with the bulk of the dose cleared within the first 24 hours (Anderson et al. 2001; Koch et al. 2005). There were relatively few urine samples with non-detectable levels of phthalate metabolites from this population, indicating that exposures of the levels observed in this study reflect relatively continuous daily exposures.

The median estimated exposures for DBP and BBzP in the Swan et al. (Swan et al. 2005) study population (n = 214) are on the order of 1 μg/kg/day, and for DEP are on the order of 6 μg/kg/day. Current U.S. EPA RfDs are 100 μg/kg/day (DBP), 200 μg/kg/day (BBzP), and 800 μg/kg/day (DEP), which are all more than 100 times greater than the median exposures in the Swan et al. population.

The exposures within this study population of pregnant women are similar or somewhat lower than those documented in other populations of women of reproductive age. Median fcmalc (all ages above 6 years) MBP concentrations in the NHANES 2001-2002 (21.5 μg/g creatinine) compare closely to the MBP concentrations in the Swan et al. study population (20.6 μg/g creatinine) and median 2001-2002 NHANES MBzP concentrations (15.1 μg/g creatinine) were similar to the Swan et al. study population...
(11.7 μg/g creatinine) (National Center for Environmental Health 2005). In the population of 97 women aged 20-40 years that was evaluated by Kohn et al. (Kohn et al. 2000), the median concentration of MBP, MBzP, MEP and MEHP were greater than the respective median concentrations in the Swan et al. study population. A population of 25 pregnant women in New York City exhibited median MBP, MEP and MBzP urinary concentrations within the same order of magnitude, but higher than those observed in the Swan et al. study population (Adibi et al. 2003). With the exception of the Adibi study (Adibi et al. 2003), the studies mentioned above deal primarily with women who are not pregnant, while the women in the Swan et al. (Swan et al. 2005) study population were pregnant. Differences in fluid level and metabolism between pregnant and non-pregnant state may account for some of these differences. Alternatively, the differences among the study populations may represent temporal differences in exposures to phthalate-containing materials. The Kohn study (Kohn et al. 2000) evaluated samples collected from 1988-1994 (NHANES III), while the Adibi study (Adibi et al. 2003) evaluated samples collected in 2000. Samples from the Swan et al (Swan et al. 2005) study population were collected from 2000-2003.

As discussed in the Swan et al. study, the observed relationship between prenatal exposure to phthalates and AGD in male infants is similar to what has been observed in animal studies, except that antiandrogenic effects have only been seen at higher doses in animals (Swan et al. 2005). For DBP, a study by Mylchreest et al. (Mylchreest et al. 2000) finds androgen-dependent effects from exposure in rats, such as decreased AGD, retained areolas or nipples and reproductive tract malformations. The most sensitive endpoint observed was a dose-dependent increase in the incidence of thoracic areola and
nipple development. When compared to the control animals, the lowest statistically significant dose group was at 100 mg/kg/day (100,000 µg/kg/day). This is well above the values obtained from the Swan study. Some of the difference could be attributed to the difference in study design, in which Mylchreest only compared each dose group to the controls and did not present an overall test for trend among the doses, in contrast to Swan et al., which looked at a continuous dose-response function. It also suggests that humans could be more sensitive than animals to exposures to phthalates. A separate study (Lehmann et al. 2004) demonstrated statistically significantly reduced fetal testicular testosterone production with daily DnBP administration as low as 50 mg/kg/day in experimental rats. Alterations to the activity of enzymes involved in the production of testosterone were observed at DnBP levels as low as 0.1 mg/kg/day. Given the small sample size of the study (four to five fetuses per treatment group), it is possible that effects at DnBP doses lower than 50 mg/kg/day might significantly reduce fetal testosterone production in animal models.

In addition, the observed associations in the Swan et al. study at the lower concentrations could reflect the “real-world” scenario that occurs in the human population, where exposure to any individual chemical of interest occurs simultaneously with exposures to other environmental factors which could affect the dose at which effects are seen. In the Swan et al. study, multiple phthalates were detected in the women, many of which have androgen-related effects (Gray et al. 2000; Lehmann et al. 2004; Mylchreest et al. 2000; Parks et al. 2000). In the animal studies, only one chemical is assessed at a time, and cannot account for the effect of multiple exposures that occur in the human population.
Indeed, recent evidence supports the idea that mixtures of low doses of chemicals may have more physiologic activity than higher doses of single chemicals. Hayes et al. (Hayes et al. 2006) demonstrate that mixtures of low ecologically-relevant concentrations of common pesticides had much greater effects than individual pesticides in limiting larval growth and metamorphosis and in inhibiting immune function in frog species. While it was unclear what contribution each of the nine pesticides made to the physiological effects, the adverse effects may have been mediated by increased plasma levels of the stress hormone corticosterone. The authors concluded that “using studies that examine only single pesticides at high concentrations may lead to gross underestimations of the role of pesticides in amphibian declines” (Hayes et al. 2006).

In another study of chemical mixtures, Rajapakse et al. (Rajapakse et al. 2002) demonstrate that mixtures of synthetic estrogenic chemicals at levels much lower than their individual no-observed-effect concentrations significantly enhance the physiologic effects of the potent steroidal estrogen, 17β-estradiol. A mixture of 11 xenoestrogens interacted in an additive fashion with a single human estrogen receptor in a yeast reporter gene assay, indicating that, while the contribution of individual xenoestrogens in environmental concentrations is small, the potentially large amounts of xenoestrogens in humans and wildlife may have physiologic effects. The authors concluded that ignoring combination effects likely underestimates the effects of exposure to xenoestrogens (Rajapakse et al. 2002). Additionally, recent research indicates that effects of exposure to phthalate chemical mixtures may be larger than with exposures to single phthalates. Rats dosed with mixtures of chemical “antiandrogens”, including DBP, DEHP, BBzP, and four different herbicides, indicates that all tested mixtures of these
chemicals acted to produce cumulative, apparently dose-additive effects on androgen-dependent tissues (Gray et al. 2006). Mixtures with the phthalate chemicals induced dose-additive reductions in anogenital distance, and also induced agenesis of the insl3-dependent gubernacular ligaments.

Furthermore, there is evidence that, even individually, certain endocrine-disrupting chemicals may have more pronounced physiological effects at low doses than at higher doses. Welshons et al. (Welshons et al. 2003) discuss the mechanisms by which endocrine-disrupting chemicals with estrogenic activity (EEDCs) could have large physiologic effects from small exposures. They argue that a relatively detailed understanding of estrogen mechanisms of action allow for prediction of the low-dose effects of EEDCs. The investigators demonstrate that a type of estrogen (E2) acts by a non-monotonic (i.e., "inverted U") dose response relationship in which low doses have larger physiological effects than substantially larger doses. This effect is due, in large part, to the fact that receptor occupancy and responses to hormones saturate as hormone doses increase (Welshons et al. 2003). The same investigators demonstrate that a particular estrogenic chemical, bisphenol A, stimulates human and rodent tissues at concentrations below those detected in human blood (Welshons et al. 2006). The authors predict that current risk assessment assumptions "can lead to a dramatic underestimation of responses (and thus risk) associated with exposures to low doses of EEDCs, particularly during development when the effects of very small changes in hormonal activity are permanent" (Welshons et al. 2003). In particular, "the practice of examining only a few very high doses and then extrapolating to predict effects of doses thousands or millions of times below those being studied is especially problematic for endocrine
disruptors” (Welshons et al. 2003). While the mechanisms of action for the phthalate chemicals are not known with certainty, it is possible that they act by non-monotonic relationships similar to those observed with estrogens. If this is the case, it could partially explain increased physiologic activity of phthalates at lower human levels compared to the higher doses seen in animal studies.

Study Limitations, Conclusions, and Proposed Next Steps

There are potential sources of uncertainty in the Kohn et al. and David formulas. Creatinine excretion rates are known with 10% accuracy (Kohn et al. 2000). Furthermore, Kohn et al. discuss the potential uncertainty within the total and urinary excretion values (FE and FU). These values may be more uncertain in this population of pregnant women, who have different fluid levels and metabolism than the general population. Because Kohn et al. used animal excretion data for some of the metabolites, they estimated that their FE values were accurate to approximately 50%, while the FU values could vary by 15-fold among species with humans in the middle. However, I used fractional urinary excretion values obtained from human studies in the calculations using the David formula. There has been much scientific debate regarding the appropriate use of $F_{UE}$ values when using the David formula to calculate DEHP exposure values. David (David 2004) has argued in favor of using an $F_{UE}$ for MEHP of 13%, calculated from human excretion data provided by Anderson et al. (Anderson et al. 2001). In a reply to David (David 2004), Koch et al. (Koch et al. 2004) support their use of an $F_{UE}$ for MEHP of 2.4%, and also provide a mathematical argument against the feasibility of 13% as the $F_{UE}$ for MEHP. The choice of $F_{UE}$ values is important because it affects the results of the
exposure calculations. I used $F_{UE}$ values from the most recent human excretion data on DEHP (Koch et al. 2005). My MEHP $F_{UE}$ of 5.9% falls in between the values previously proposed by David and Koch et al. The exposure calculations using this value are in close agreement with the calculations using the oxidative metabolites of DEHP (MEHHP and MEOHP), and with the calculations using the Kohn method, which does not use $F_{UE}$ values. The exposure estimates from the Kohn et al. and David formulas are similar, suggesting reasonable agreement between the models and parameters used.

Another source of uncertainty is the use of “spot” urine samples, which are taken at different times of the day in different individuals. It is generally accepted that collecting the “first morning void” of urine results in more consistent measurement of relatively short-lived contaminants in individuals than when using spot urine samples (Kissel et al. 2005). Additionally, since urine samples were taken once from each woman during pregnancy, it is unclear if the concentrations of these single urine samples are in fact representative of phthalate exposures throughout the entire pregnancy. Since phthalates are metabolized and excreted within 24 hours, metabolite concentrations in a single urine sample represent a small window of time. Evidence suggests that phthalate metabolite concentrations may be somewhat consistent within individuals over time, but the degree of consistency varies for the different metabolites and is not well established (Hauser et al. 2004).

It is currently unclear what conclusions can be drawn from the epidemiologic data of the Swan study and my subsequent estimates of the phthalate daily doses. Given the relatively small sample size of mothers and infants (less than 200), the results linking phthalate exposures to reduced AGD should be replicated in additional larger studies.
Another important question is whether the changes in AGD observed in the Swan study represent an actual "adverse" effect. It is possible, for example, that the decreases in AGD fall within the normal human range, and therefore should not be considered adverse effects. On the other hand, investigators have suggested that "statistically significant changes in specific end points that have been considered indicators of perturbed androgen status may be permanent and thus in their own right be considered adverse effects" (Foster and McIntyre 2002). Various animal studies have demonstrated that antiandrogen-induced reductions in AGD are often permanent, and are predictive of other serious reproductive tract malformations, such as epididymal malformations and testicular atrophy (Barlow et al. 2004; Hotchkiss et al. 2004; McIntyre et al. 2002).

Anogenital distance has been studied much less frequently in humans as in animals. It is unclear if AGD reductions in humans, such as those seen in the Swan study (Swan et al. 2005), will be permanent and/or associated with other more serious reproductive tract malformations. Nevertheless, the permanence of AGD reductions and the correlations with more serious malformations in animals should elevate concern that AGD reductions are adverse events in their own right in humans.

Our research indicates that a variety of additional steps are warranted in the analysis of human effects of phthalates. The associations between phthalate exposures and reduced AGD in boys should be replicated by larger studies, and the possible permanence of reduced AGD and its association with reproductive impairments should be characterized. Other environmental and lifestyle factors, such as parental smoking and alcohol intake, older paternal and maternal age of conception, and environmental toxins
in addition to the phthalates, may also be associated with reduced AGD in boys. Associations of these factors to AGD status should be further characterized in people.

It is possible that prenatal phthalate exposures may have biological effects in addition to those observed with reproductive development. In addition to masculinizing the reproductive organs, testosterone is responsible for modulating development of other parts of the body, including the brain. Further studies are necessary to determine if phthalates reduce human testosterone production in utero, a relationship which occurs in animals. If testosterone secretion is indeed reduced, developmental processes in addition to those involving the reproductive organs could be affected. Along these lines, a recent study indicated that rat brain aromatase levels (the hormone that converts testosterone to estradiol) were altered with prenatal exposure to both low and high doses of DEHP (Andrade et al. 2006).

The need for additional inquiry extends beyond science and academia to the regulatory and policy world. More extensive surveillance of environmental exposures in the nation’s communities is warranted. Given evidence of the importance of prenatal and early-life exposures, more extensive monitoring of these life periods is particularly necessary. The nation’s largest biomonitoring program, run by the CDC’s National Health and Nutrition Examination Survey, does not analyze blood or urine samples from children younger than six years of age (National Center for Environmental Health 2005). Furthermore, our phthalate research suggests that the customary way of assessing a chemical’s risk by formulating a reference dose, below which there is “no risk” to people, may be outdated. Reference doses, as they are customarily calculated, do not adequately address a chemical’s toxicity in the real-world milieu of contaminant
mixtures that may have synergistic effects. Given the emerging evidence of biological
effects at low doses of chemical exposures, and the difficult nature of acquiring data that
demonstrate causal relationships in humans, it may be appropriate to adopt a more
precautionary approach that seeks to minimize chemical exposures without waiting for
demonstrations of health effects.

In summary, Swan and others (Swan et al. 2005) found subtle developmental
effects associated with phthalate exposures in a human population. It is uncertain if
reduced anogenital distance will be permanent, or if this reduction is associated with
other developmental effects in human males. However, in animals exposed to phthalates,
AGD reductions are permanent and associated with other reproductive and
developmental effects. These animal data indicate that reduced AGD itself should be
considered an adverse effect. The maternal phthalate metabolite levels that are associated
with developmental reproductive effects in male infants are comparable to exposures
observed in other female populations in the United States (Adibi et al. 2003; Blount et al.
2000; National Center for Environmental Health 2005; Silva et al. 2004). Dose estimates
for the phthalates associated with health effects in this study population are two orders of
magnitude lower than the doses deemed protective by the U.S. EPA. The values of these
dose estimates are in close agreement when calculated using two different models and
different excretion factors. This information demonstrates that the current phthalate
reference doses are out of date. This work will be an asset to any future assessments of
the toxicity of the phthalate chemicals.
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