A comparative assessment of phthalate effects on gonadal steroidogenesis in male rats.

by

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Abstract

Although it is a known endocrine disrupting chemical (EDC) of the male reproductive tract that is ubiquitous in the environment, di (2-ethylhexyl) phthalate (DEHP) has remained the most utilized industrial plasticizer on Earth. Government regulations that are based on public health concerns, however, have promoted the search for an alternative to DEHP in consumer products. In the present study, we evaluated diisononyl phthalate (DINP), a structural analog of DEHP, as a potential replacement chemical for DEHP. To accomplish this goal, we compared the individual (0, 5, 10, or 15 µg/L) and combined (5 µg/L DINP + 5 µg/L DEHP; DINP+DEHP) effects of these chemicals on the serum concentrations and testicular production of testosterone (T) and estradiol (E2) in weanling male Long-Evans rats. We performed two sets of 14- and 28-day experiments, i.e., 14-d_A and 28-d_A and 14-d_B and 28-d_B, during which rats were exposed to DINP, DEHP, or DINP+DEHP in drinking water (Table 1). The results of experiments 14-d_A and 28-d_A demonstrated that exposure to DEHP, but not to DINP or DINP+DEHP, affected testicular T and E2 production in male rats. Results from the second set of experiments showed that DINP and DEHP individually affected serum E2 concentration and testicular E2 production, but only DEHP and DINP+DEHP exposures affected testicular T production in male rats. Furthermore, the data showed that DINP exerted an additive effect on the cellular effects of DEHP. Collectively, the results of our study demonstrated that DINP caused a lesser testicular toxicity in prepubertal male rats than DEHP, but the results also showed that the prepubertal rat testis is targeted by DINP, which affected serum T and E2 secretion after 14- and 28 day-exposures. Future studies will identify the molecular mechanisms associated with the estrogenic and antiandrogenic effects of DINP and/or DEHP in prepubertal male rats.

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This Master's thesis is dedicated to my late grandparents, John F. Fischer, Sr., and Donna L.R. Fischer. I could not have accomplished my goals without their undying love, guidance, and support. May they eternally rest in peace.

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List of Abbreviations

ABG	Androgen-binding protein
AGD	Anogenital distance
APG	Anterior pituitary gland
AR	Androgen receptor
BBP	Benzyl butyl phthalate
B-PAE	Branched-chain phthalate alkyl ester
bw	Body weight
°C	Degrees Celsius
d	Day
DBP	Dibutyl phthalate
DEHP	Di(2-ethylhexyl)phthalate; bis(2-ethylhexyl) phthalate
DEHP-5	5.00 µg/L DEHP
DEHP-10	10.00 µg/L DEHP
DEHP-15	15.00 µg/L DEHP
DEP	Diethyl phthalate
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate
DINP	Diisononyl phthalate; diisononylphthalate; bis(7-methyloctyl) phthalate
DINP-5	5.00 µg/L DINP
DINP-10	10.00 µg/L DINP
DINP-15	15.00 µg/L DINP
DINP/DEHP	DINP + DEHP

DMP	Dimethyl phthalate
DMSO	Dimethyl sulfoxide
DNHP	Di-n-hexyl phthalate
DnOP	Di-n-octyl phthalate
ED(s)	Endocrine disruptor(s)
EDC(s)	Endocrine disrupting chemical(s)
E2	Estradiol
ER	Estradiol receptor
g	Gram
HMW	High molecular weight
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
HYPO	Hypothalamus
Ih-B	Inhibin B
kg	Kilogram
KO	Knockout
LC	Leydig cell
LH	Luteinizing hormone
LMW	Low molecular weight
µg/L	Micrograms per liter
µL	Microliter
mg/kg testis	Milligrams per kilogram testis
MEHP	Mono(2-ethylhexyl)phthalate

MEI	Monoester intermediate
MINP	Monoisononyl phthalate
MIS	Mullerian Inhibiting Substance; anti-Mullerian hormone
MW	Molecular weight
ng/mg	Nanograms per milligram
ng/ml	Nanograms per milliliter
PA	Phthalic Anhydride
PAE(s)	Phthalate alkyl ester(s); phthalate diester(s); phthalate(s)
PAH	Phthalic Acid
PND	Post-natal day
RIA	Radioimmunoassay
R-OH	Alcohol; monohydric alcohol; alkanol
SC	Sertoli cell
S-PAE	Straight-chain phthalate alkyl ester
T	Testosterone

Chapter 1: Literature Review

Introduction

An endocrine disrupting chemical (EDC), or an endocrine disruptor (ED), is any exogenous chemical that interferes with the ability of an endogenous hormone to perform its physiological and/or developmental functions (1). Most EDCs, such as phthalates (phthalic acid esters, phthalate esters, or phthalate diesters; PAEs), are synthetic industrial chemicals that are not only present among the materials utilized to manufacture the vessels in which foods and beverages are stored, but they are also present within a wide range of manufactured goods and all environmental mediums (2). Consequently, humans and animals are inevitably exposed to EDCs.

In general, PAEs are a class of synthetic chemicals that are utilized as industrial plasticizers, or in solvents, to manufacture a wide variety of products (2). These chemicals are internationally mass-produced with the purpose of subsequently being utilized to manufacture a multitude of non-PVC- and PVC-based commercial goods. Consequently, the physiological and developmental effects of the most commonly utilized PAEs, such as di(2-ethylhexyl phthalate) (DEHP) and dibutyl phthalate (DBP), were extensively investigated to determine whether these industrial chemicals are toxic to humans and animals (2). The primary goals of these PAE exposure studies have been to (a) identify any pre- and/or postnatal effects due to PAE exposures and (b) determine the mechanism(s) associated with specific reproductive effects.

Moreover, most PAE exposure studies have primarily focused on investigating the effects of DEHP exposures because it is the most utilized industrial plasticizer in the world and it is an environmentally ubiquitous pollutant. DEHP has been estimated to have a no-observed-adverse-effect level (NOAEL) of 4.8 mg/kg bodyweight/day and a tolerable daily intake (TDI) of 48 mg/kg bodyweight/day in humans (3,4). Previous studies collectively demonstrated that the

exposure to DEHP negatively affects the hormone-regulated processes of testicular steroidogenesis and spermatogenesis in male rat testes by disrupting luteinizing hormone (LH)-LH receptor (LHR) signaling in Leydig cells (LC), follicle-stimulating hormone (FSH)-FSH receptor (FSHR) signaling in Sertoli cells (SC), inhibin B (Ih-B) production and secretion, and the expression of multiple steroidogenic enzymes (5). Consequently, these studies have identified DEHP as an EDC of the male rat reproductive tract.

Due to the large number of biomedical studies that have collectively demonstrated that exposures to DEHP and other PAEs disrupted development and physiological functions of the male reproductive tract in neonatal rats, the European Union (EU) and the United States (US) House of Congress (US HoC) passed separate laws that have begun to regulate the use of DEHP in industrial manufacturing. On August 14, 2008, the US HoC passed the *Consumer Product Safety Improvement Act of 2008* (*CPSIA*), which was almost identical to the regulations that the EU had already voted into law on December 18, 2006 (*Registration, Evaluation, Authorization, and Restriction of Chemicals Regulation (REACH) No 1907/2006* (REACH (No 1907/2006))) (6,7). Both laws indefinitely restricted DEHP, DBP, and benzyl butyl phthalate (BBP) from exceeding a concentration of 0.10% by composition in any commercial product that is specifically manufactured for children (e.g., toys, clothing, diapers, pacifiers, baby formula bottles, etc.), who, according to both laws, are defined as being three years of age and younger (*CPSIA of 2008* and REACH (No 1907/2006)) (6,7). Furthermore, both laws also temporarily prohibited (i.e., placed an interim prohibition on) diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), and di-n-octyl-phthalate (DnOP) from exceeding a concentration of 0.10% by composition in all childcare items, and in any toys that a child could fit into their mouth (6,7). In 2017, however, US HoC added an amendment to the *CPSIA of 2008*, which caused US's interim

prohibition of DINP, DIDP, and DnOP in childcare products to become an indefinite prohibition of these chemicals (8). Unsurprisingly, in December 2018, the EU also amended *REACH (No 1907/2006)* in the same manner (9).

Furthermore, the laws prohibiting the use of DINP to manufacture childcare products and toys are perplexing because DINP has not been classified as a reproductive toxicant (10). Regardless of these legal restrictions, however, most of the commercial goods that are sold within the US and EU are still lawfully permitted to contain DEHP and other known EDCs. As a result, although the US government and EU have begun to restrict their use in consumer items, a plethora of products still contain phthalates at concentrations that are much higher than 0.10% by composition.

Recently, however, a growing interest to identify a less reproductively toxic alternative to DEHP has resulted in an increase in the number of studies that have investigated the reproductive effects of DINP in male rats. These studies were based on the hypothesis that DINP is less toxic than DEHP (11). Consequently, after a general overview on the hormonal regulation of the male hypothalamic-pituitary-gonadal (HPG) axis, this review will (a) discuss the literature that describes the synthesis and physical-chemistry of phthalates, (b) demonstrate that DEHP is an EDC of the male reproductive tract, and (c) indicate that DINP is a potential alternative to DEHP in the manufacturing industry.

Chapter 1.1: Hormonal Regulation of the Male Hypothalamic-Pituitary-Gonadal (HPG) Axis

Results of many studies that investigated the effects of DEHP exposure on the reproductive tract in male rats showed that DEHP and its bioactive metabolites disrupted the processes of steroidogenesis and spermatogenesis by targeting the testes, which is one of the three organs that form the male HPG axis. In addition to the testes, this endocrine axis also includes the hypothalamus (HYPO) and anterior pituitary gland (APG). Collectively, the three organs of the HPG axis produce and secrete the hormones that serve to regulate the axis through a negative feedback loop (12). To fully understand the discussion on how DEHP exposures affect the principal functions of male rat testes, this section provides an overview of the hormonal regulation of the HPG axis under physiological conditions in male rats.

The HYPO is a neuroendocrine gland that rests within the sella turcica of the skull's sphenoid bone and is located between the thalamus and pituitary in the limbic system of the brain (12-16). This gland contains gonadotropin-secreting hormone (GnRH) neurons, which secrete GnRH in a pulsatile manner and are gradually inhibited as peripheral gonadal hormone concentrations increase (12-14,16). Upon detecting decreases in the direct stimulation of testosterone (T) and estradiol (E2), and/or decreases in their indirect stimulation by T-activated kisspeptin/neurokinin B/dynorphin (KNDy) neurons within the arcuate nucleus of the brain, GnRH neurons upregulate GnRH secretion in the HYPO (12,13,17-21). This neuroendocrine hormone is secreted into the hypophyseal portal vasculature of the median eminence (ME) and is then transported in the circulating blood from the HYPO to the APG (12-14,16,22). When GnRH stimulates the gonadotrophs of the APG, these cells secrete both FSH and LH into systemic circulation, which then transports these gonadotropins to the testes (12-14,22,23).

Upon being transported to the testes, LH induces steroidogenesis by binding to and activating LHRs, which are G protein-coupled receptors (GPCRs) located on LC surfaces (12,13,22,24-26). LH-activated LHRs (+LHRs) induce an intracellular cascade by activating the $G\alpha_s$ subunit of its G protein, which then activates adenylyl cyclase (AC) to elicit increases of cyclic adenosine monophosphate (cAMP) (12,13,22,24-26). Subsequently, intracellular cAMP activates protein kinase A (PKA), which drives the steroidal acute regulatory protein (STAR)- and the translocator protein (TPSO)-mediated translocation of cholesterol into the mitochondrial inner membrane of LCs (12,13,22,24-27). Within the mitochondrial inner membrane of LCs, cholesterol is first converted into pregnenolone by cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), which is then utilized by the mitochondrion to produce progesterone via the enzymatic action of 3β-hydroxysteroid dehydrogenase (3β-HSD) (12,13,22,24-27). Progesterone is then transported to the smooth endoplasmic reticulum (SER) of the LCs where the conversion of (a) progesterone into androstenedione and (b) androstenedione into T occurs by the action of 17α-hydroxylase 17,20-lyase (CYP17A1) and 17β-HSD (17β-HSD), respectively (12,13,22,23,25-30). Collectively, the signal-transduction pathway of testicular steroidogenesis demonstrates that LH is the primary driver of testicular steroidogenesis because it activates LHRs on LC surfaces to induce the downstream cascade of enzymatic reactions that ultimately result in T production in the testes.

Evidence that LH is the primary regulator of LC steroidogenesis is provided by the finding that the absence of LHRs on LC surfaces caused abnormalities of the male reproductive tract. By knocking out LHRs (LHRKO) on LCs, these studies demonstrated that the absence of +LHRs simultaneously decreased the total number of testicular LCs and suppressed testicular steroidogenesis (31,32). Furthermore, the absence of +LHRs in LCs suppressed the development

of pre- and post-natal testes in male rats and decreased their testes weights and anogenital distances (AGD) (31,32). Altogether, the evidence provided indicates that LH is required for normal development of the testes and the HPG axis (31,32).

In general, T is the primary sex steroid hormone in males, and it is predominantly produced by steroidogenesis within testicular LCs. After this hormone is produced, it remains within the testes (intratesticular T) where it is utilized during various processes, converted into E2 and/or dihydrotestosterone (DHT), and/or secreted into systemic circulation. Within the testes, T is required for the proper maturation and maintenance of LCs, SCs, and the epididymis; testicular production of E2; induction of spermatogenesis; and the maturation of spermatozoa (12,13,22,25,28). Serum T, or the T that is released into systemic circulation from the testes, however, is essential for (i) the development and maintenance of secondary sex characteristics (e.g., body and facial hair) and accessory sex organs (e.g., the prostate); (ii) the prepubertal priming of the liver and brain; (iii) and promoting muscle and bone growth (12,13,22,24,25,30,33,34). Serum T is also required to properly regulate the HPG axis because it directly controls hypothalamic GnRH secretion and adenohypophyseal LH/FSH secretion, implying that serum T indirectly regulates its own production within the male gonads. As serum T increases to levels that define the maximum concentration required for physiological function, it then negatively feeds back on the HYPO and APG to inhibit the release of GnRH, LH, and FSH, which ultimately inhibits testicular steroidogenesis and T production (12,13,22,25,26,28).

Pituitary FSH is a gonadotropic hormone that is required for maintaining normal testis function and development. FSH binds to the FSHRs present in SCs and subsequently activates a $G\alpha_s$ signal transduction pathway that ultimately promotes germ cell development and stimulates the production and release of androgen binding protein (ABP), both of which are functions that

are required for spermatogenesis (13,22,26,33,35). Spermatogenesis, however, is not exclusively stimulated by FSH and ABPs. The T produced by LCs and FSH work in concert to stimulate the production of ABPs by binding to androgen receptors (ARs) in the nuclei of SCs (13,22,33,35,36). Once the ABPs are released from SCs, they bind to T, which elevates seminiferous tubule T to levels at which spermatogenesis can occur (22,26,33,35). This paracrine relationship between LCs and SCs is required to produce the ABPs needed to initiate spermatogenesis. Many studies have shown that a decrease in FSH and/or T stimulation downregulated spermatogenesis and decreased the number of SCs and germ cells (22,26,33,35-37). Additionally, inhibition of T action prevented the maturation of the testes and decreased serum FSH-inhibited LC T secretion (22,26,33,35-37). Altogether, LH-stimulated T secretion from LCs, FSH- and T-induced secretion of ABPs from SCs, and the ABP-induced increase in seminiferous tubular T are all required to initiate the process of spermatogenesis.

In addition to stimulating the release of ABPs from SCs, FSH also stimulates the secretion of inhibin (Ih) from SCs. Just as with T, Ih (called Ih-B in rats), is a gonadal peptide that acts to suppress GnRH secretion from the HYPO (38). In contrast to T, however, Ih also specifically inhibits FSH release from the APG (13,33). Consequently, increased FSH secretion results in a subsequent increase in Ih secretion, which then augments the negative feedback signal on the HYPO and APG to decrease GnRH and FSH secretion, respectively (13,33,38). Due to its actions, Ih is thus defined as an essential regulatory hormone of the HPG axis.

Furthermore, contrary to traditional social beliefs, the presence of estrogen (E2), which is known as estradiol in rats, is also essential to the physiological functions of the male mammalian reproductive tract (20,39). In males, the reproductive significance of this hormone is attributed to how E2 receptors (ERs) are present in many testicular cell types, which is supported by how

studies have exhibited that reproductive abnormalities are caused by their ablation (39-41). For example, one study demonstrated that E2 regulated the amount of T produced by LH/LHR-stimulated LCs because, when ERα was knocked out, testicular T secretion was greater compared to the control (40). This increase in T secretion negatively affected this hormone's ability to accomplish its peripheral functions and adversely affected spermatogenesis (40). These effects occurred because the increase in T over a shorter time period produced a much stronger signal that negatively fed back on the APG and HYPO (39-41). Therefore, it appears that E2 at low concentrations is required for the maintenance of reproductive homeostasis and testicular T production.

Collectively, the physiological roles of the endocrine organs and hormones of the male HPG axis demonstrated that it is a highly specialized endocrine axis in which GnRH is released by the HYPO in a pulsatile manner to stimulate the release of LH and FSH from the gonadotrophs of the APG, which promotes the testicular production and secretion of T and E2. Both FSH and intratesticular T then induce spermatogenesis and FSH alone stimulates SC-mediated Ih production and secretion. Subsequently, the increase in serum T and Ih inhibit the APG from producing LH and FSH, respectively. Indeed, all three gonadal hormones (T, E2, and Ih) attenuate the release of GnRH from the HYPO. Additionally, the separate processes that are responsible for producing T and ABPs are what indirectly links the functions of LH and FSH, which are also regulated by a low intratesticular E2 concentration. Consequently, the HPG axis is a highly modified endocrine axis that regulates the development and functions of the male reproductive tract.

Chapter 1.2: Phthalates

PAEs are defined as a class of synthetic chemicals that are industrially utilized as plasticizers or in solvents. Excluding BBP, which possesses two ester substituents with structurally different alkyl side-chains (ASCs; R and R'), the basic chemical structure of all PAEs consists of two structurally identical ester substituents (2R) that are individually and covalently bound to adjacent carbons on a benzene ring (Figure R1) (42-48). Furthermore, all PAEs are subclassified by molecular weight (MW); as being either high molecular weight PAEs (HMW PAEs) or low molecular weight PAEs (LMW PAEs) (44,46,47,49-51). The chemical structures, MWs, and physicochemical properties of PAEs are all derived from the process by which they are synthesized.

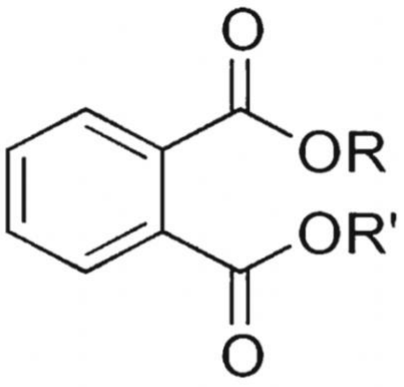

Figure R1 The structural skeleton of PAEs (42). In respect to the chemical structures of almost all PAEs, R = R' (2R). A notable exception to this rule, however, is benzyl butyl phthalate (R ≠ R'). Additionally, in respect to the chemical structures of PAE monoesters, –OR' = –OH.

In general, all PAEs are synthesized by a two-step reaction mechanism that occurs between 1 mole (mol) of phthalic anhydride (PA; $C_8H_4O_3$ or $C_6H_4(CO)_2O$) and 2 mols of one of the many different monohydric alcohols (R-OH) that are responsible for individually adding a branched or straight alkyl chain to what will then become the ester (or non-carbonyl) oxygen of each PAE substituent (Figure R1 and Figure R2a) (45,47,52-56). During the first reaction step of

PAE synthesis, which is rate-limiting and the only reaction step that requires an acid catalyst, 1 mol of PA and 1 mol of R-OH undergo a ring-opening esterification reaction, which produces a mono-ester intermediate (MEI) product (i.e., PA + R-OH $\xrightarrow[\text{acid (catalyst)}]{}$ MEI) (53). The second step of PAE synthesis is also an esterification reaction; however, this reaction, which yields the final PAE product and the H_2O byproduct, occurs between the MEI product of the first reaction step and the second mol quantity of the ROH reactant (i.e., MEI + R-OH → PAE + H_2O) (53). Consequently, the specific PAE produced during PAE synthesis is dependent on the R-group of the R-OH species that reacts with PA and the MEI.

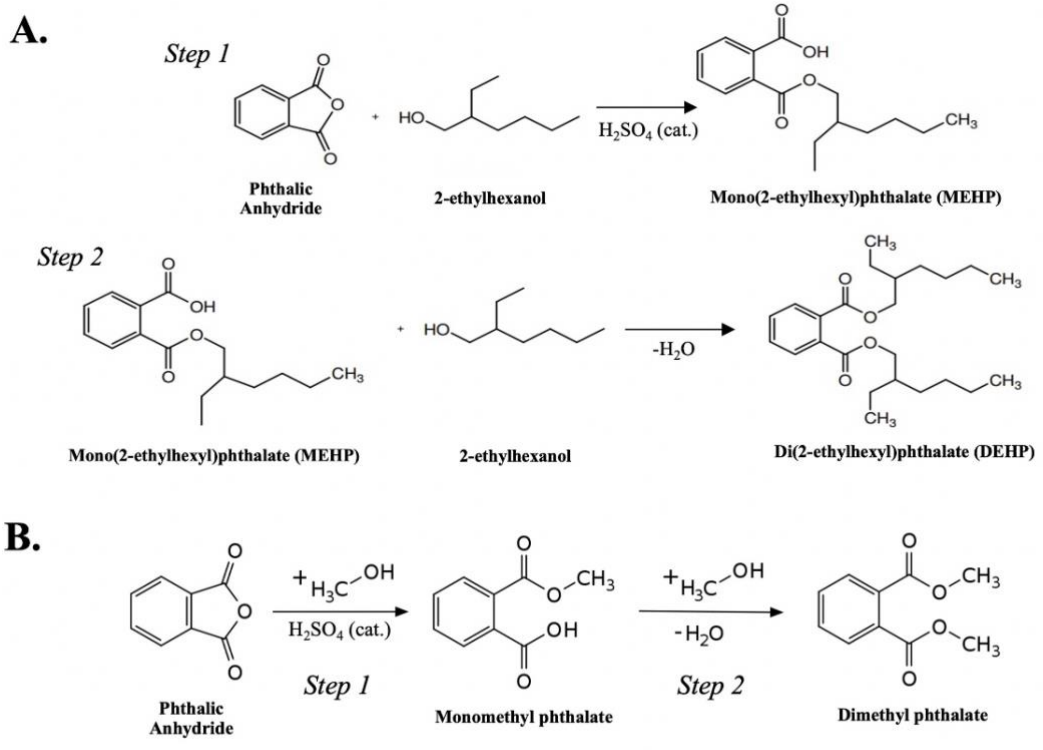

Figure R2 PAE Synthesis: the reaction mechanisms of **(a)** DEHP and **(b)** DMP Synthesis (54-56).

Furthermore, PAEs are non-polar, H_2O insoluble, lipophilic industrial chemicals that have very low melting points (MPs; all PAE MPs ≤ 0ºC at 760 mmHg; *NOAA CAMEO*

Chemicals) and relatively high boiling points (BPs; all PAE BPs ≥ 286.2ºC at 760 mmHg; *NOAA CAMEO Chemicals*) (43,44,48). When analyzed in increasing order of MW, it is clear that these physicochemical properties of PAEs are either directly or indirectly proportional to alkyl side-chain MW (ASC-MW), which is what structurally and, thus, physicochemically dictates whether a PAE is subclassified as a LMW PAE or HMW PAE (47). LMW PAEs, such as DMP and diethyl phthalate (DEP), contain ester substituents with ASCs that range from 1-C to 4-C in length (DMP to DIBP) (45-47). The compounds belonging to this subclass of PAEs are primarily used to manufacture non-PVC products, such as resins, paints, toiletries, perfumes, cosmetics, and even the outer coating of orally administered capsules that contain a pharmaceutical drug (45,46,58-63). Conversely, HMW PAEs, such as DEHP and DINP, contain esters with ASCs that are 6-C to 13-C in length (diisohexyl phthalate (DIHP) to diisotridecyl phthalate (DITP)) (45-47). Compared to LMW PAEs, HMW PAEs have lower volatilities and water solubilities, higher hydrophobicities (lipophilicities), and stronger resistances to migration from polyvinyl chloride (PVC) and other host polymers (44,45,48,58,59). These physicochemical properties are what characterize HMW PAEs as being efficient industrial plasticizers; and, therefore, the more efficient HMW PAE plasticizers, such as DEHP, are mass-produced to soften, and increase the durability and elasticity of, PVC products and many non-PVC plastic products (45,46,48,64). These products collectively include, but are not limited to, medical tubing, medical-grade plastic masks, plastic syringe plungers, prescription bottles, and dental sealant; jarred, canned, and bottled non-perishable food items with a high fat content; plastic wrap used to pack meat and cover/air-lock microwavable meals; plastic bags used to air-lock cereals, snack foods, rice, and other non-perishable food items; household cleaners, detergents, and dryer sheets; floor tiles; footwear; eyeglass and sunglass frames; waterproof and

water-resistant products; swimming pool liners; and, most infamously, disposable plastic bottles and many re-usable plastic water bottles (45,46,48,49,59,62,63,65-70).

Altogether, PAEs are utilized to produce a wide variety of commercial products that are intended for daily use, which also supports the claim that most humans and animals around the world are exposed to both LMW and HMW PAEs every day. Additionally, their synthesis protocol predicts the classification, physicochemical properties, and plasticizing efficacy; and, therefore, are responsible for how: (i) LMW phthalates are generally utilized in solvents to produce cosmetics, perfumes, pesticides, paints, and many other common household products; and (ii) HMW phthalates are primarily used as plasticizers in many PVC and non-PVC products.

Chapter 1.3: The pharmacokinetics of orally administered DEHP and DINP in rats.

Although DEHP or DINP exposure in mammals has been demonstrated to occur through dermal absorption and inhalation, the primary exposure route, and, thus, the predominant pharmacokinetic route, of DEHP or DINP exposure is through oral ingestion (51,71-74). The pharmacokinetics of DEHP have been extensively investigated, and the metabolism of HMW PAEs are thought to be influenced by the hydrophobicity and length of a PAE's ASCs (72). Since PAE hydrophobicity increases with increasing ASC length (and decreasing solubility), both the similarly high hydrophobicities and long ASCs of DEHP and DINP have led the scientific community to hypothesize that their general pharmacokinetic pathways are similar (51,72). Consequently, this section will focus on the pharmacokinetics of orally administered DEHP in male rats.

Upon ingestion, DEHP is transported down the gastrointestinal tract (51,75,76) and may remain within the lumen of the gastrointestinal tract until it is later excreted in feces and/or is absorbed by enterocytes that line the small intestine (51,72,76). Within the enterocytes, esterases rapidly hydrolyze DEHP into its less hydrophobic monoester metabolite, MEHP, creating a mixture of intact and partially metabolized DEHP (DEHP/MEHP) (51,72,76). Esterases are also present in blood and liver tissues; and, therefore, the hydrolysis of intact DEHP is not only continued within the liver, but also occurs during transportation of DEHP/MEHP in blood from the enterocytes to the liver through the hepatic portal system (51,75,76).

Furthermore, the hepatic metabolism of MEHP includes oxidation to form secondary metabolites of DEHP and is, to a lesser extent, hydrolyzed into PA (76,77). Most of the MEHP that enters the liver, however, is oxidized by ω- or (ω-1)-oxidation before undergoing β-oxidation because the process by which the secondary metabolites of DEHP are prepared for

renal excretion is more energetically favorable than that of its more hydrophobic/less soluble primary metabolites (77). Although DEHP has many secondary metabolites, the studies that have investigated the hepatic metabolism of DEHP and MEHP in rats have concluded that the liver predominantly metabolizes MEHP into 2-ethyl-5-hydroxy-hexylphthalate (5-OH MEHP), 2-ethyl-5-oxy-hexylphthalate (5-oxo MEHP), 2-ethyl-5-carboxy-pentylphthalate (5-carboxy MEPP), and 2-carboxymethy-hexylphthalate (2-carboxy MMHP) (76). Contrary to how MEHP was once thought to be the most reproductively toxic DEHP metabolite, these secondary bioactive metabolites are believed to have a higher toxicity during prenatal development (78).

The mixture of intact DEHP and its various metabolites are then transported from the liver and circulated through the heart and lungs before being further metabolized in other tissues, such as the testes, brain, kidneys, adrenals, muscle, adipose and thyroid (72,75,76,78,79). The mechanisms by which PAE partitioning occurs, however, is still unknown. Prior to excretion in urine, DEHP metabolites undergo phase II conjugation by glucuronidation (51,75,80). This process is most critical to the renal clearance of DEHP metabolites because, in contrast to intact DEHP, MEHP, and their secondary metabolites, the conjugated metabolites are soluble in water; and, therefore, are readily excreted in urine (51,75,80).

Chapter 1.4: DEHP exposure affects steroidogenesis in male rats.

The reproductive effects of DEHP exposure on the reproductive tract in male rats is a research topic that has been extensively investigated for over 50 years. Several studies have investigated the specific mechanisms by which DEHP disrupts testicular steroidogenesis (81-91). The results reported by *Akingbemi et al. 2001* demonstrated that the reproductive effects due to 14-d and 28-d DEHP exposures in prepubertal male rats provided evidence of a paradoxical paradigm, which was related to the concentration and duration of DEHP exposure and the life-stage during which initial DEHP exposure occurred (84). For example, prepubertal male rats were gavaged with DEHP (0, 1, 10, 100, or 200 mg/kg/day) from postnatal day (PND) 21 to 34 (14 days; samples acquired on PND 35), 35 to 48 (14 days; samples acquired on PND 49), and 21 to 48 (28 days; samples acquired on day 49) (84). At the end of each exposure period, serum samples and testicular explants were collected from all animals to assess serum LH and T concentrations, and basal and LH-stimulated testicular T production were respectively assessed in all exposure groups (84).

After both 14-day (14-d) experiments (PND 21-34 and PND 35-48), serum T and LH were unaffected by DEHP at all exposure concentrations in prepubertal male rats (84). DEHP exposure (PND 21-34: 100 and 200 mg/kg/day DEHP; and PND 35-48: 10, 100, and 200 mg/kg/day DEHP), however, decreased both basal and LH-stimulated testicular T production (84). These results demonstrated that DEHP directly targeted the testes to inhibit LC-mediated T production in prepubertal male rats after 14-d DEHP exposures.

The effects of DEHP exposure from PND 35-48 on the activity of the following four steroidogenic enzymes in LCs were also investigated: cytochrome P450 cholesterol side-chain cleavage enzyme ($P450_{SCC}$); cytochrome P450 17α-hydroxylase/17,20 lyase ($P450_{17\alpha}$); 3ß-HSD;

and 17ß-HSD (84). These enzymes play critical roles in LC-mediated testicular T production. Therefore, any observed DEHP-induced effect(s) on the transcription and translation of these enzymes were critical in providing insight into elucidating the specific molecular mechanisms by which DEHP disrupts LC-mediated T production. The results indicated that 14-d DEHP exposures during this time period effectively reduced the enzymatic activity of $P450_{SCC}$ (100 and 200 mg/kg/day DEHP; $P < 0.05$), 3ß-HSD (100 and 200 mg/kg/day DEHP; $P < 0.05$), $P450_{17\alpha}$ (200 mg/kg/day DEHP; $P < 0.05$), and 17ß-HSD (10, 100, and 200 mg/kg/day DEHP; $P < 0.05$) (84). Consequently, DEHP exposures decreased the steroidogenic capacity of LCs by inhibiting steroidogenic enzyme protein expression and LC T production in a dose-dependent manner.

Results from the 28-day DEHP exposures indicated that DEHP had androgenic and/or antiandrogenic effects in prepubertal rat testes because it increased basal and LH-stimulated testicular T production in association with elevated serum LH and T levels (84). Although the data collected during the 14-d experiment initially appeared to contradict results from the 28-d experiments, these findings were presumably related to exposure concentration, duration of DEHP exposure, and life stage at exposure (84). The authors suggested that, since serum LH and T are inversely related under physiological conditions, the observed increases of both steroid hormones in serum, as well as the increases in testicular T production, were likely caused by DEHP-induced increases in LC number (testicular hyperplasia), which the authors later confirmed in a separate study (84,92).

Another study conducted by *Akingbemi et al. 2001* investigated the effects of 28-d DEHP exposures (1, 10, 100, or 200 mg/kg/day DEHP) in adult male rats (PND 62-89) (84). In contrast to the results of developmental exposure paradigms, serum LH and T levels, and testicular T production, were unaffected by all DEHP exposure compared to the control (0 mg/kg/day

DEHP) (84). Consequently, in addition to exposure concentration and duration, the results of this experiment supported the conclusion that the effects of DEHP in male rats were also dependent on the specific life stage during which DEHP exposure occurred.

Additionally, in agreement with the results presented by *Akingbemi et al.* (*2001 and 2004*), and after Long-Evans rats were exposed to DEHP (0, 10, 500, or 750 mg/kg bw DEHP) for 28-d (PND 21-49), the data presented in *Ge et al. (2007)* exhibited that exposure to 10 mg/kg DEHP prematurely induced puberty (39.7 ± 0.1 days) and increased serum T (3.13 ± 0.37 ng/mL) compared to the control (0 mg/kg DEHP; 41.5 ± 0.1 days, 1.98 ± 0.20 ng/mL). Exposure to 750 mg/kg DEHP, however, decreased serum T production (1.18 ± 0.18 ng/mL) and delayed the onset of puberty (46.3 ± 0.1 days) (87). Altogether, 28-d exposure paradigms collectively demonstrated that low-dose DEHP exposures had androgenic and/or antiandrogenic effects in prepubertal male rats, which were associated with DEHP-induced LC hyperplasia, increased T secretion, and non-responsiveness of the hypothalamus-pituitary axis to circulating androgen levels.

Similar to the studies by *Akingbemi et al. 2001*, other studies have concluded that DEHP strictly had antiandrogenic effects on male rats at all developmental life stages. These reports suggest that DEHP targeted the APG and testes to inhibit both serum T and testicular T production in pubertal male rats (86,88). For example, after rats (PND 16) were exposed to DEHP for 30 days, the authors of one study observed that DEHP targeted the APG to decrease serum LH and the testes to downregulate the gene expression of steroidogenic enzymes, induce oxidative stress, activate the ERK-pathway, and upregulate the expression of 5α-reductase 2 in Sprague-Dawley rats (88). Furthermore, a more recent study investigated the reproductive effects of 28-d DEHP exposures (0, 250, 500, 1000 mg/kg/d) on the STAT/p53-mediated mitochondrial

apoptosis pathway and autophagy in prepubertal Sprague Dawley rat testes (86). The results showed that exposure of male rats to 500 and 1000 mg/kg/d DEHP disrupted the STAT/p53-mediated mitochondrial caused testis injury and decreased serum T (86). Additionally, DEHP increased pro-autophagy proteins (phosphorylated ULK1, Beclin-1, Atg7, and LC3-II) in association with inhibition of the P13K-Akt-mTOR pathway (84,86). Although *Akingbemi et al. 2001* reported that 28-d DEHP exposures had androgenic effects on prepubertal male rats, other studies have reported that it had antiandrogenic effects on prepubertal male rats. Altogether, it appears that any conflicting results of reproductive effects due to DEHP exposures in male rats were related to variations in use of exposure paradigms (71,84,87,89,91). Nevertheless, these observations collectively provide insight into our understanding of the basis for the cellular effects of DEHP and the associated molecular mechanisms in the male gonad. This thesis extends the investigation of the cellular and molecular mechanisms by which DEHP exposure affects the testes in male rats.

Chapter 1.5: DINP exerts lesser reproductive toxicity than DEHP.

Since DEHP has been empirically demonstrated to be an EDC of the male reproductive tract, it has become evident that there is a critical need to identify a non-toxic, or, at least, a much less toxic, chemical to replace it in industry. DINP has been hypothesized as being a less reproductively toxic analog of DEHP, and, therefore, a suitable industrial replacement for DEHP (93). Consequently, some companies have already started to use DINP instead of DEHP to manufacture PVC and non-PVC products. Conversely, there are also studies in which the authors have hypothesized that the reproductive effects of DEHP and DINP exposures in male rats are similar, which is based on how their MWs, chemical structures, physicochemical properties, and pharmacokinetics are also similar (52,93-97); but, overall, the reproductive effects due to DINP in male rats are still unclear. Furthermore, if DEHP is eventually replaced by DINP altogether, it will be expected that DINP has little or no effect on product quality and, thus, that both DINP and DEHP have similar plasticizing properties.

Chapter 1.5a: DINP exposure and the reproductive tract in male rats.

Although some studies, such as *Waterman et al. (2000)* observed no reproductive effects in male rats that were exposed to DINP, its reproductive toxicity has remained ambiguous (52,93-97). The similarity between DEHP and DINP in physicochemical properties and pharmacokinetics has led many studies to suggest that exposures to DINP and DEHP would also have similar reproductive toxicity in male rats (93). Therefore, very few studies have investigated the effects of DINP exposure in male rats. Of the small number of DINP exposure studies that have been conducted, even fewer studies conducted hormone assays to detect any DINP-related impacts on testicular steroidogenesis in male rats (93). (97). Consequently, additional DINP exposure studies are warranted to increase the overall confidence in the current

literature.

Interestingly, the studies by *Borsch et al. (2004)* and *Gray et al. (2000)* concluded that DINP elicited weakly antiandrogenic effects in male rats. Additionally, *Borsch et al. (2004)* also investigated the reproductive toxicity of exposures to single and combined phthalates, which included exposures to DEHP (300 mg/kg bodyweight (bw) per day (d)), DINP (750 mg/kg bw/d), and a combination of DEHP (300 mg/kg bw/d) and DINP (750 mg/kg bw/d; DINP/DEHP) from gestational day (GD) 7 to 21 in male Wistar rats (93). Compared to the control (vehicle; peanut oil), all three chemical exposures decreased testicular T production (ng/testis/3h; $P_{DEHP} < 0.01$; $P_{DINP} < 0.05$; $P_{DINP/DEHP} < 0.001$) and testicular T content (ng/testis; $P_{DEHP} < 0.01$; $P_{DINP} < 0.01$; $P_{DINP/DEHP} < 0.001$) (93). Among these chemical exposures, however, their antiandrogenic effects were in the following decreasing order of strength: DINP/DEHP > DEHP > DINP (93). Furthermore, exposure to DINP/DEHP, but not to DINP or DEHP, decreased plasma T (pg/mL; $P < 0.05$) and increased plasma LH (ng/mL) (93). The effects of the DINP/DEHP exposure, however, were not identified as being evidence of any DINP-induced modulation of DEHP exposure effects, but they did suggest that the exposure to this PAE combination had accumulating effects on testicular steroidogenesis and serum LH (93). Collectively, the results showed that, compared to DEHP and DINP/DEHP exposures, the weakest antiandrogenic effects were elicited by DINP exposure; and, compared to the DINP and DEHP mono-chemical exposures, the effects of DINP/DEHP on testicular steroidogenesis in male rats were additive.

Additionally, *Gray et al. (2000)* investigated the reproductive effects of DINP, DEHP, BBP, DEP, DMP, and dioctyl terephthalate (DOTP) exposures in perinatal Sprague-Dawley rats. Pregnant dams were divided into six groups, and each group was orally-administered a daily

dose (0.75 g/kg) of one of the six chemicals of interest from GD 14 to PND 3 (97). In contrast to *Borsch et al. (2004)*, however, *Gray et al. (2000)* did not conduct hormone assays, but rather measured AGD, testis weight, areola presentation, testis malformations, and malformations of androgen-dependent tissues in order to investigate the effects of DINP exposure in reproductive tissues within male rats (93,97).

Although only DEHP and BBP exposures reduced AGDs and testis weights, *Gray et al. (2000)* concluded that all three chemicals separately had antiandrogenic effects in male pups (97). The authors of this study reported that 87% ($p < 0.01$) and 70% ($p < 0.01$) of male pups that were exposed DEHP or BBP, respectively, presented with feminized areolas, but this anatomical effect was only observed in 22% ($p < 0.01$) of male pups that were exposed to DINP (97). Similarly, malformations of androgen-dependent tissues were identified in 82%, 84%, and 7.7% of male rat pups exposed to DEHP ($p < 0.0001$), BBP ($p < 0.0001$), or DINP ($p < 0.04$), respectively (97). Following exposure to DINP from GD 14 to PND 3, histopathological analysis of testis tissue showed decreased T production (and/or increased testicular E2 production) associated with testicular and epididymal atrophy, atrophic tubules lacking sperm, and a low epididymal sperm count (97). Although no hormone assays were conducted, the data presented by *Gray et al. (2000)* indicated that perinatal exposure to DINP elicited antiandrogenic effects in male rat pups and that those effects were weaker than the effects induced by DEHP and BBP (97), thereby reinforcing the view that DINP may be a potential industrial replacement for DEHP.

1.5b: The relative reproductive toxicities of various PAEs

Some reports have suggested that there is a structure-activity relationship (SAR) for phthalate-mediated disruption of testicular T production in male rats (98). In this regard, *Li et al.*

(2019) reviewed the results of more than 75 PAE exposure studies which focused on various factors that contribute to the presentation of testis dysgenesis in fetal male rats (46,99,100). *Li et al. (2019)* concluded that the potency of PAE-inhibited T production in fetal rat testes was dependent on the combination between the ASC lengths of PAEs and whether their ASCs are straight (S-) or branched-chained (B-) (46). The data indicated that LMW PAEs with S-ASCs (DMP, DEP, DPrP, DBP, DPP, DNHP, DHP, DOP, DUP, and DTDP) were less toxic to male rat testes than their B-PAE analogs (DIBP, DIHP, DEHP, and DINP) (46). Furthermore, exclusively among the HMW B-PAEs, an increase in testicular toxicity was directly correlated to an increase in the extent of branching, i.e., the testicular toxicity of the more extensively branched PAEs (e.g., DEHP and DIBP) was greater than the PAEs containing ASCs that are not as extensively branched (e.g., DINP and DIHP) (46). Using these parameters, DINP was found to be the least toxic, which further supports the view that it may be a potential alternative to DEHP in industry.

Chapter 1.5c: Industrially Replacing DINP with DEHP

The plasticizing performance of a PAE is evaluated by the following three parameters: (i) its compatibility with a host polymer, (ii) migration resistance, and (iii) plasticizing efficacy (44,64). The compatibility of a PAE with a host polymer, such as PVC, is determined by both their structural (or molecular) and thermodynamic compatibilities, and the structural compatibility of a PAE with PVC decreases as ASC length of the PAE increases (64,101). In a study that evaluated all three of the plasticizing performance parameters of DEHP and the five PAEs that most closely resemble the structure and physicochemical properties of DEHP, their molecular compatibilities with PVC were reported as being in the following descending order: DIBP (4-C ASCs) > DEHP (8-C ASCs) > DIOP (8-C ASCs) > DINP (9-C ASCs) > DIDP (10-C ASCs) > DITP (13-C ASCs) (64). Interestingly, although DEHP and DIOP both contain a pair of

8-C B-ASCs, the molecular compatibility of DEHP with PVC was greater than the molecular compatibility of DIOP with PVC (64). The authors attributed this difference in molecular compatibility to DEHP as a HMW PAE with B-ASCs that each contain an ethyl group structurally close to the carboxyl ester of their respective B-ASC (Figure 2a) (64). Essentially, in contrast to the structures of DIOP B-ASCs, the structural locations of the ethyl groups on DEHP B-ASCs allow this plasticizer to have stronger and closer interactions with the CH(Cl) groups that are located along the PVC backbone, which grants DEHP the ability to increase the flexibility of the PVC backbone by blocking the intermolecular forces that promote its rigidity (64,101). Consequently, the molecular compatibility of DEHP with PVC was greater than the molecular compatibility of DIOP with PVC. Moreover, the authors of this study then evaluated the thermodynamic compatibilities of each of the six PAEs with PVC, which increases as the heat of mixing (ΔH, J/g) of a PAE with PVC decreases (64,101-103). According to the data presented by this study, DIBP had the lowest ΔH, followed by DEHP, DIOP, DINP, DIDP, and then DITP (64). Also, the thermodynamic compatibility of a PAE with PVC is also indicative of the migration resistance of a PAE from PVC because PAE migration decreases as the thermodynamic affinity of a PAE with PVC increases (64,101). Consequently, the overall compatibilities of these six PAEs with PVC, as well as their migration resistances, were in the following descending order: DIBP > DEHP > DIOP > DINP > DIDP > DITP (64). To determine the plasticization efficacies of the six PAEs, the glass transition temperatures (T_g, K) of pure PVC and six PAE/PVC mixtures were experimentally acquired because plasticization efficiency decreases as the value of T_g increases (64,101). The results showed that T_g increased with ASC length (64). Consequently, according to the data reported in this study, DIBP had the highest plasticization efficacy, followed by DEHP, DIOP, DINP, DIDP, and then DITP (64). The

plasticizer performance [i.e., the combination among the (molecular and thermodynamic) compatibility of a PAE with PVC, a PAE's resistance to migration from PVC, and plasticization efficacy] of DEHP and the five PAEs that most closely resemble the structure and physicochemical properties of DEHP, are in following descending order: DIBP > DEHP > DIOP > DINP > DIDP > DITP (64). Although these results indicated that DIBP and DIOP are more suitable candidates for industrially replacing DEHP, both PAEs are potent EDCs of the male reproductive tract. As a result, in terms of plasticizer performance and potentially decreased toxicity, DINP is a suitable candidate for industrially replacing DEHP.

Conclusion

In summary, the bulk of the literature collectively indicated that both DINP and DEHP are EDCs of the male reproductive tract that interfere with LC-mediated steroidogenesis in rats following prenatal and postnatal exposures. Interestingly, the testicular effects of DEHP and DINP were due to antiandrogenic activity in testes of male rats, but the mechanisms and pharmacokinetics related to these effects have remained unclear. The studies reported herein focus on the effects of DEHP and DINP and their combinations on serum LH and E2 and testicular T and E2 production.

Chapter 2: Comparative assessment of phthalate effects on steroidogenic capacity in the male rat gonad

Chapter 2.1: Introduction and Objectives

Di (2-ethylhexyl) phthalate (DEHP) is an industrial plasticizer that was originally synthesized and utilized in Japan before it became the most utilized industrial plasticizer within the US during the early 1950s. Since entering US commerce, DEHP quickly became, and has since remained, the most utilized industrial plasticizer on Earth. Subsequently, however, this high molecular weight phthalate (HMW PAE) has also been identified as an endocrine disrupting chemical (EDC) of the male reproductive tract and an environmentally ubiquitous pollutant (47,104,105). Just as other HMW PAEs, the primary industrial use of DEHP is to soften, and increase the flexibility and durability of, polyvinyl chloride(PVC)-based and non-PVC products, which encompasses an extraordinarily wide range of commercial goods that are regularly used by the majority of the world's population (e.g. plastic bottles and containers, rainwear, eyeglass frames, shampoos, household cleaners, swimming pool liners, inner lining of automobiles, plastic wrap that is used to package meat, and vinyl flooring) (47,86,104,105). The primary concern attributed to the extensive use of DEHP, however, is that it is a known EDC that is able to chemically migrate or leach into consumer products (47,105). For example, if a plastic bottle containing a beverage was manufactured with DEHP, this known EDC of the male reproductive tract would not only gradually leach into the surrounding environment, but also into the beverage that is contained within it – especially at higher temperatures and/or in warmer climates. The primary route of DEHP exposure in humans and animals is through oral ingestion and, to a lesser extent, inhalation (e.g., dust, contaminated air, and aerosolized products that contain DEHP) and dermal absorption (47,104,105).

As an EDC, DEHP is an exogenous chemical with the capacity to disrupt the function of multiple hormones (1-5, 10-11,24-25). Importantly, DEHP is an EDC of the male reproductive tract that is known to interfere with the functions of reproductive hormones (e.g., luteinizing hormone (LH), testosterone (T), and estradiol (E2)) that are required for the development and maintenance of the reproductive tract in male rats (82,84,85,87,105-110). For example, when orally administered in low doses over a short period of time, DEHP decreased serum LH and T in prepubertal and peripubertal male rats (84,109). Conversely, when administered at greater doses and over longer periods of time, DEHP increased serum LH and T in prepubertal and peripubertal male rats (84,87).

Although some studies have concluded that DEHP exposures had no effect on the adult male reproductive tract, the results of other DEHP exposure studies have indicated that this EDC impairs the reproductive tract in fertile adult male rats, but its reproductive toxicity in adult male rats was less severe (47,90,104). Consequently, although DEHP is a known EDC of the male reproductive tract during pre- and post-natal reproductive development, its effects on the adult male reproductive tract have remained controversial.

Due to its wide-spread industrial use, environmental ubiquity, and high reproductive toxicity in males, identifying a less toxic replacement chemical for DEHP is critical. One potential replacement chemical for DEHP is diisononyl phthalate (DINP), which is a structural analog of DEHP (47,93,111). According to US federal law, DEHP has been prohibited from being used in commercial products that are specifically manufactured for children (3 years old and younger), and that these specific products must not contain DINP at a quantity that exceeds a concentration of 0.10% by composition (47). Thus, the US government still permits the industrial utilization of DEHP during the production of all other relevant commercial products, which,

according to the law itself, is due to the lack of empirical evidence to support a ban on the industrial use of DEHP altogether.

Both DEHP and DINP are HMW PAEs that are used as plasticizers; and some reports have shown that, compared to DEHP, DINP had antiandrogenic effects on the male reproductive tract that were less severe, but the reproductive toxicity of DINP in the male reproductive tract has received very little attention (47,93,111). The present study was designed to compare the effects of DEHP, DINP, and a phthalate mixture (DINP/DEHP) on the steroidogenic capacity of the testes in weanling male Long-Evans rats. At the conclusion of all exposure periods, animals were sacrificed, and blood and testis tissue samples were collected to measure the serum concentrations and testicular production of T and E2 by radioimmunoassay (RIA).

Chapter 2.2: Materials and Methods

Experimental Design: 14-d and 28-d Single Phthalate Exposures

To investigate the effects of 14- and 28-d exposures to DINP and DEHP, we chose weanling male Long-Evans (LE) rats (Charles River, Wilmington, MA) as our animal model, which is commonly used in studies that investigate the effects of EDCs *ex vivo* and/or *in vivo*. Members of the husbandry staff at Auburn University College of Veterinary Medicine then randomly grouped these rats into chemical-free cages (see Table 1 for *n* per experiment), which were equipped with an anti-drip glass water bottle, rodent bedding, and a dry chow dispenser. After an initial 48-hour acclimation period, each group was assigned to one of six treatment (i.e., exposure) groups (Table 1) showing, (i) the duration of exposure, (ii) the specific phthalate(s) to which they were exposed, and (iii) the concentration(s) at which they would be exposed to test chemical(s).

All rats were orally administered their assigned chemical treatment in drinking water, which was replenished at 2-d intervals throughout the exposure period. All exposure concentrations were chosen according to the lowest observed effect level (LOEL) of DEHP in male LE rats from PND 21-35 and PND 21-49, which were previously determined (84,87). In respect to its concentration in the drinking water administered to the control groups, a proportional concentration of dimethyl sulfoxide (DMSO) was also added to the drinking water administered to the experimental groups.

Additionally, at the same rate that their water was replaced, all rats were weighed (data not shown), and their food dispensers were refilled with 100 grams (g) of normal dry chow. Upon the conclusions of the 14-d and 28-d periods of chemical exposures, all cohorts were sacrificed to collect serum and testis samples.

Measurement of sex steroid hormones by Radioimmunoassay (RIA)

Blood samples were stored overnight (4°C) before being separated by centrifugation (3000rpm for 15min at 4°C). Serum supernatants were then isolated with transfer pipettes before samples were analyzed for serum T and E2 by radioimmunoassay (RIA). Testis explants were placed in media, containing either 48.75-mL Leydig Cell Media (LCM) and 1.25 mL Lipogro® (basal), or 48.75-mL LCM, 1.25 mL Lipogro® and 100 ng/mL ovine LH (LH-stimulated) for 3h at 34ºC. Steroid hormones (T, E2) were separately measured in serum samples and spent media of testicular explants, respectively, by a tritium-based RIA. This assay was validated with rat antiserum and conducted just as described in studies that we have previously conducted (84,87,92,112).

Statistical Analysis

Prior to analyzing any given data set for significance via ordinary one-way ANOVA, the data acquired via RIA were interpolated via GraphPad Prism v.8. Interpolated data points (derived from serum samples or testicular explants) were adjusted for aliquot size (Microsoft Excel; data not shown), the RIA data derived from testicular explants were adjusted for the tissue weights of the testicular explants, and then each data set was individually normalized via GraphPad Prism v.8 (data not shown). Furthermore, all data sets were then individually analyzed by the ROUT method, which is a statistical test that identifies any outlying data points within a data set. The ROUT method (GraphPad Prism v.8) was conducted at a false discovery rate, Q, equal to 1.00%; and, therefore, the probability that a data point was falsely identified as an outlier is less than 1.00% (GraphPad Prism v.8). In any data set that included a statistically significant effect caused by DINP/DEHP exposure, paired t tests were conducted to separately compare the DINP/DEHP exposure group to the low-dose DINP and DEHP exposure groups in the same data set. Lastly, data were analyzed by ordinary one-way ANOVA and are presented as the mean ± SEM of each exposure group (GraphPad Prism v.8).

Table 1: Experimental Approach for 14-d and 28-d Phthalate Exposures in Prepubertal Male Rats

Experiment	*N*	n_{DINP}, n_{DEHP}, $n_{DINP/DEHP}$	Age on First-Last Days of PAE Exposure Period (PND)	# of Control Groups, # of Experimental Groups	DINP Exposures (µg/L)	DEHP Exposures (µg/L)	Parameters
14-d_A	30	6, 4, 0	21-35	2, 4	0	0	Serum [T]
					5	5	
					15	15	Serum [E2]
28-d_A	30	4, 4, 5	21-49	1, 5	0		Basal Testicular T Production
					5	5	
					15	15	
					5 DINP + 5 DEHP		LH-Stimulated Testicular T Production
14-d_B	30	5, 5, 5	21-35	1, 5	0		
					5	5	Basal Testicular E2 Production
					10	10	
					5 DINP + 5 DEHP		
28-d_B	30	5, 5, 5	21-49	1, 5	0		LH-Stimulated Testicular E2 Production
					5	5	
					10	10	
					5 DINP + 5 DEHP		

Chapter 2.3: Results

14-d Experiment A ($14d_A$): Exposure to DINP or DEHP in drinking water.

First, we conducted a 14-day [14-d] experiment to investigate the effects of DINP and DEHP (0-, 5.0-, or 15.0µg/L in drinking water) on serum testosterone (T) concentration (serum [T]) and testicular T production in male weanling Long-Evans rats (PND 21-35; $N = 30$; $n_{DINP} = 6$; $n_{DEHP} = 4$). After the 14-d period of chemical exposures, serum T remained unaffected in all exposure groups (Figure 1A; $P > 0.05$). By contrast, the data presented in Figure 1B demonstrates that 14-d exposures to 5.0µg/L DEHP (DEHP-5) and 15.0µg/L DEHP (DEHP-15) decreased basal testicular T production (DEHP-5: $\bar{x} = 97.967$ ng/mg testis; DEHP-15: $\bar{x} = 73.178$ ng/mg testis) in male rat testes ($P_{DEHP\text{-}5} = 0.0495$; $P_{DEHP\text{-}15} = 0.0010$). Similarly, exposure to DEHP-15 also decreased LH-stimulated testicular T production (Figure 1C; DEHP-15: $\bar{x} = 158.486$ ng/mg testis, $P = 0.0074$) in male rat testes. Testicular T production in rats exposed to 5.00-µg/L DINP (DINP-5) and 15.00-µg/L DINP (DINP-15), however, remained unaffected altogether (Figure 1C-D, $P > 0.05$). Consequently, after 14 days, serum [T] remained unaffected in all groups; and DEHP, but not DINP, affected testicular T production in male rat testes compared to the control ($\bar{x}_{Basal} = 128.715$ ng/mg testis; $\bar{x}_{LH} = 158.486$ ng/mg testis).

We then investigated the effects of 14-d DEHP and DINP exposures on the serum concentration and testicular production of E2 in male rats from PND 21 to 35 days of age. Just as serum [T], serum [E2] was unaffected in male rats after all 14-d_A chemical exposures (Figure 2A; $P > 0.05$). Exposures to DINP-5 and DINP-15 also did not affect testicular E2 production in male rat testes (Figure 2B-C; $P > 0.05$). DEHP-5 and DEHP-15, however, decreased basal testicular E2 production (DEHP-5: $\bar{x}_{Basal} = 52.0591$ ng/mg testis, $P = 0.0485$; DEHP-15: $\bar{x}_{Basal} = 42.184$ ng/mg testis, $P = 0.0012$) and LH-stimulated testicular E2 production (DEHP-

5: $\bar{x}_{LH} = 82.3431$ ng/mg testis; DEHP-15: $\bar{x}_{LH} = 73.5867$ ng/mg testis) in male rats compared to the control ($\bar{x}_{Basal} = 65.7193$ ng/mg testis; $\bar{x}_{LH} = 158.145$ ng/mg testis) (Figure 2B-C). Collectively, the results of experiment $14d_A$ indicated that both serum T and E2 were unaffected in male rats after all $14d_A$ phthalate exposures; and exposure to DEHP, but not to DINP, decreased testicular T and E2 production in male rats ($P<0.05$).

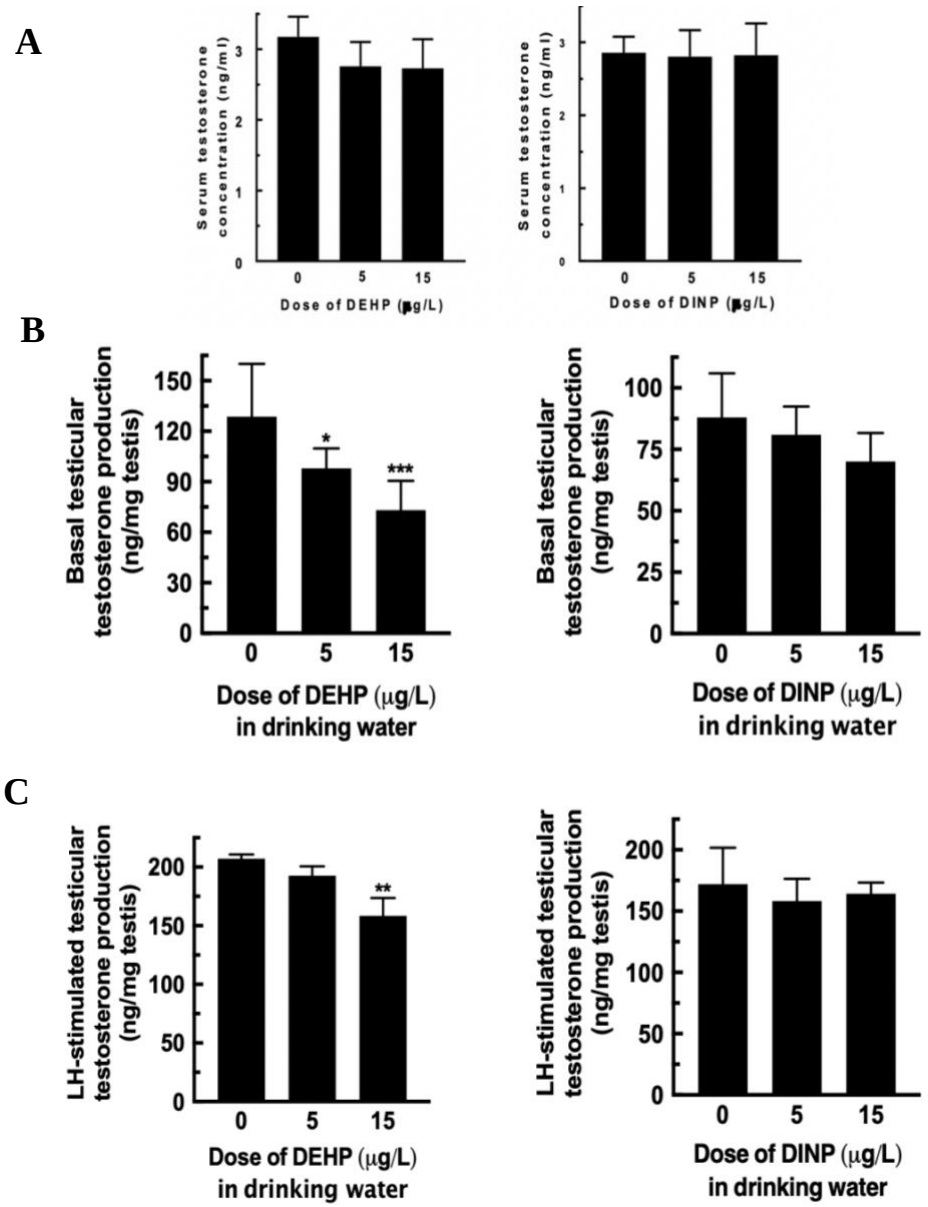

Figure 1. Individual effects of 14-d_A DEHP and DINP exposures (0 ug/L + DMSO, 5.00 µg/L, or 15.00 µg/L) on the serum concentration and testicular production of T in male rats (n_{DINP} = 6 and n_{DEHP} = 4). After the 14-day period of chemical exposure (PND 21-35), **(A)** serum [T] and **(B-C)** testicular T production in male rats were assessed by radioimmunoassay (RIA). The data were analyzed by ordinary one-way ANOVA and are presented as the mean ± SEM of each exposure group. *P < 0.05 vs. control, **P < 0.01 vs. control, and ***P < 0.001 vs. control.

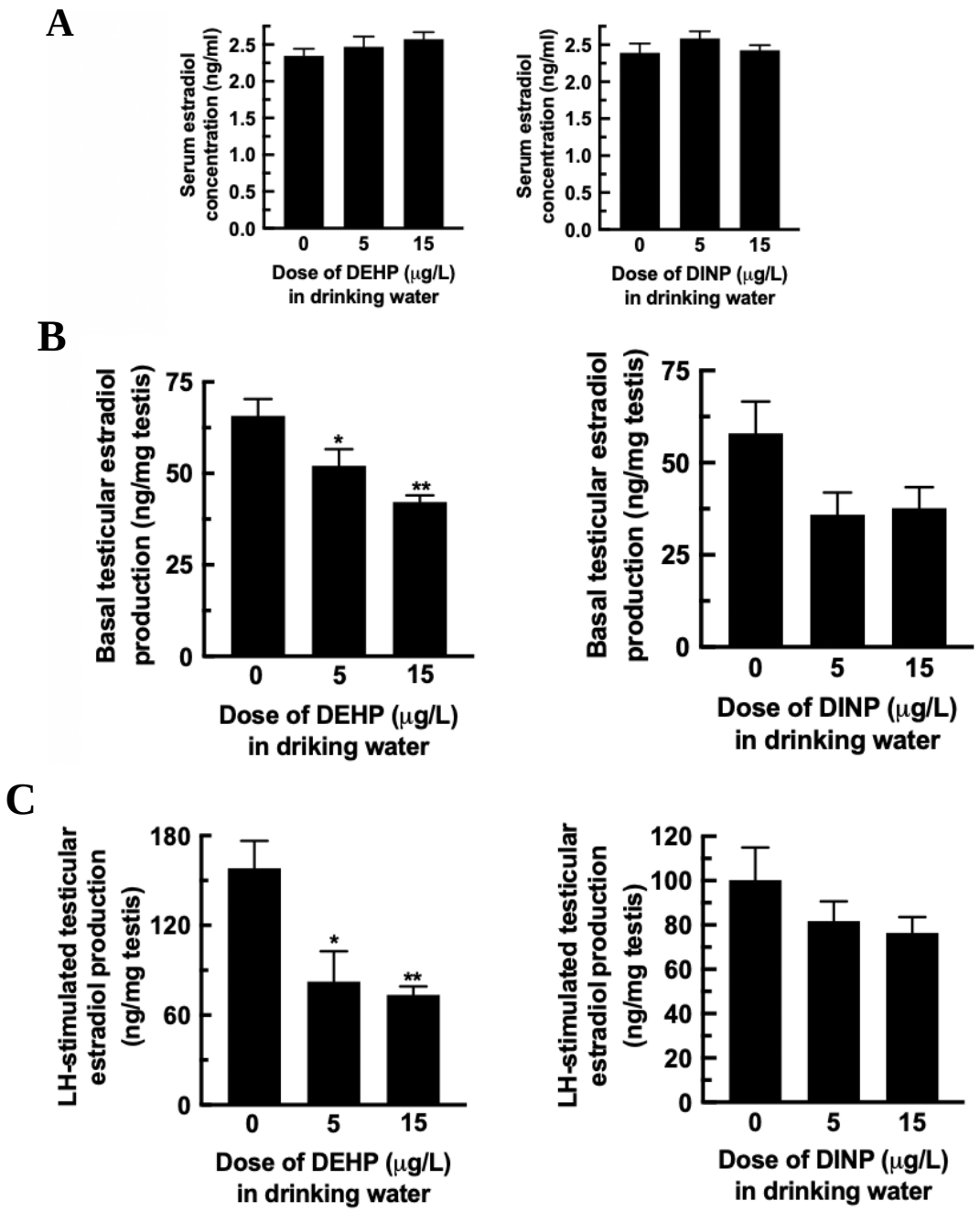

Figure 2. Individual effects of 14-d$_A$ DEHP and DINP exposures (0 ug/L + DMSO, 5.00 µg/L, or 15.00 µg/L) on the serum concentration and testicular production of E2 in male rats (n_{DINP} = 6 and n_{DEHP} = 4). After the 14-day period of chemical exposure (PND 21-35), **(A)** serum [E2] and **(B-C)** testicular E2 production in male rats were assessed by radioimmunoassay (RIA). *P < 0.05 vs. control and **P < 0.01 vs. control.

28-day Experiment A (28-d_A): Exposure to DINP, DEHP, or DEHP/DINP in drinking water.

Upon the conclusion of experiment 14-d_A, we then conducted another dose-finding experiment during which cohorts of weanling male Long-Evans rats (N = 30, n = 4) were exposed to DINP (DINP-5 or DINP-15) or DEHP (DEHP-5 or DEHP-15). In contrast to the 14d_A experiment, however, this second experiment was conducted over 28 days (28d_A), which included only one collective control group (0-µg/L PAE; deionized H_2O) and an experimental group that was exposed to a PAE mixture that consisted of 5-µg/L DINP + 5-µg/L DEHP (DINP-5/DEHP-5; DINP/DEHP) in drinking water. Consequently, experiment 28d_A was conducted to evaluate the effects of exposure to DEHP, DINP, or DINP/DEHP on the serum concentration and testicular production of T and E2 in weanling male Long-Evans rats after 28 days (PND 21 to PND 49).

Surprisingly, following the 28-d_A exposure period (PND 49), serum T and E2, basal testicular E2 production, and LH-stimulated T and E2 production in male rats remained unaffected by all phthalate exposures (Figure 3 and Figure 4; $P > 0.05$). Exposure to DEHP-15, however, tripled basal testicular T production ($\bar{x}_{control} = 79.1757$ ng/mg testis; $\bar{x}_{DEHP-15} = 237.96$ ng/mg testis) compared to control (Figure 3B; $P = 0.0209$). Collectively, these results (Figures 3 and Figure 4) demonstrated that the only parameter affected during experiment 28d_A was basal testicular T production, which was increased in the testes of rats that were exposed to DEHP-15 in drinking water for 28 days.

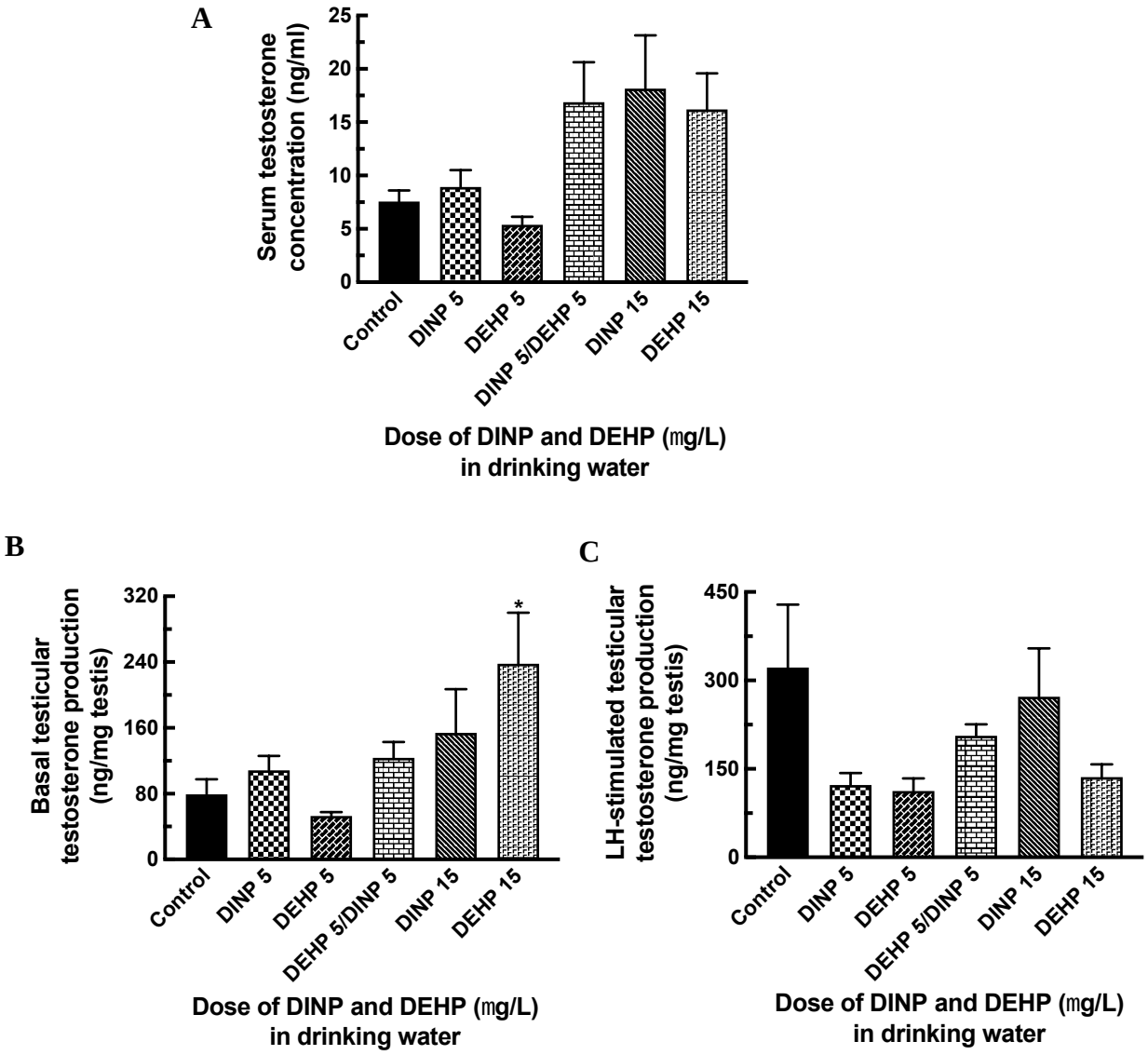

Figure 3. Individual and combined effects of 28-d$_A$ DINP (5.00 µg/L or 15.00 µg/L), DEHP (5.00 µg/L or 15.00 µg/L), or DINP 5/DEHP 5 (5.00 µg/L DINP + 5.00 µg/L DEHP) exposure on the serum concentration and testicular production of T in male rats (n_{DINP} = 4, n_{DEHP} = 4, and $n_{DINP/DEHP}$ = 5). After the 28-day period of chemical exposure, **(A)** serum [T] and **(B-C)** testicular T production in male rats were assessed by radioimmunoassay (RIA). *P < 0.05 vs. control.

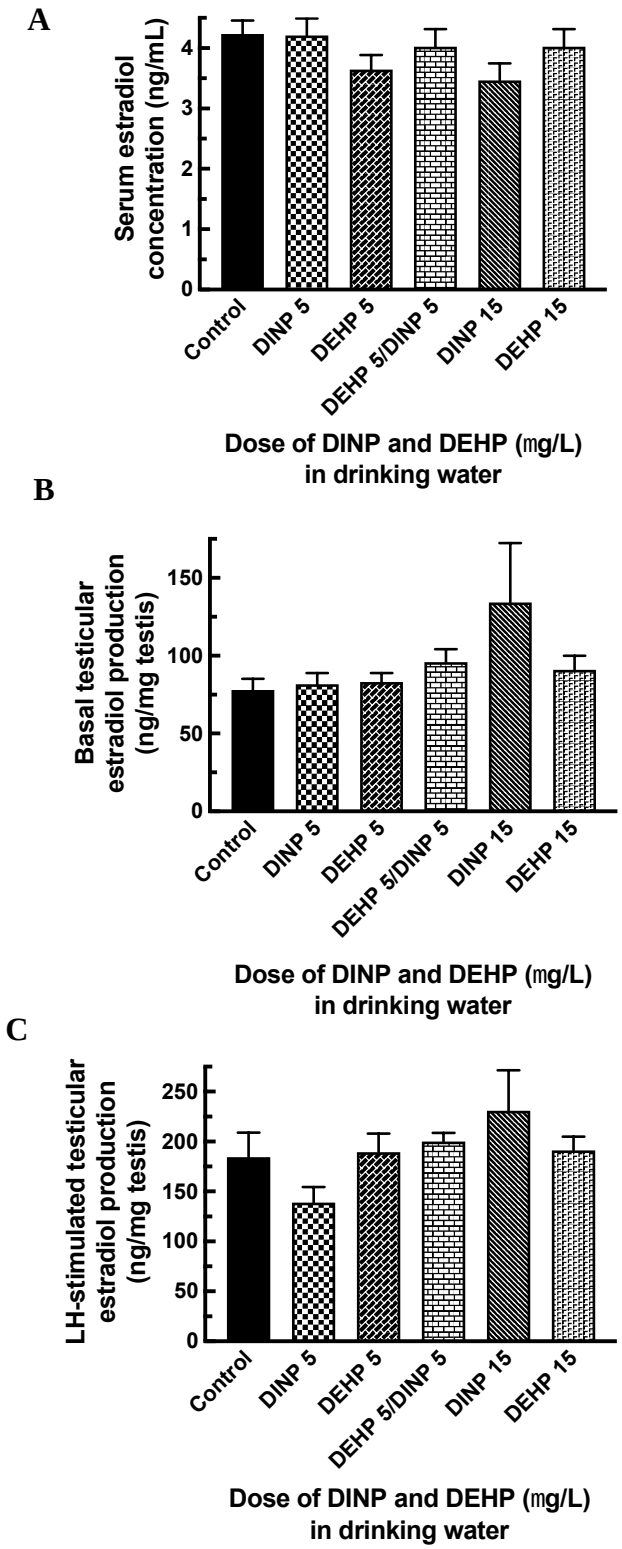

Figure 4. Individual and combined effects of 28-d$_A$ DINP (5.00 µg/L or 15.00 µg/L), DEHP (5.00 µg/L or 15.00 µg/L), or DINP 5/DEHP 5 (5.00 µg/L DINP + 5.00 µg/L DEHP) exposures on the serum concentration and testicular production of E2 in male rats (n_{DINP} = 4, n_{DEHP} = 4, and $n_{DINP/DEHP}$ = 5). After the 28-day period of chemical exposure, **(A)** serum [E2] and **(B-C)** testicular E2 production in male rats were assessed by radioimmunoassay (RIA). *P < 0.05 vs. control.

14-d Experiment B ($14d_B$): Exposure to DINP, DEHP, or DINP/DEHP in drinking water.

Since only one PAE-exposure-effect was detected in male rats after 28 days during experiment $28d_A$, we then conducted another set of 14-d and 28-d exposure experiments and subsequently we assessed the effect of phthalates on serum concentration and testicular production of T and E2 in male rats after all cohorts underwent one of the following $14d_B$ exposures: 0-µg/L PAE + DMSO (control), DINP-5, DEHP-5, 10-µg/L DINP (DINP-10), DINP-5/DEHP-5 (DINP/DEHP), and 10-µg/L DEHP (DEHP-10) (n = 5). At the end of treatment, both DEHP-5 and DINP-10 decreased serum T production, but DINP-5, DINP/DEHP, and DEHP-10 had no effects (Figure 5). Additionally, only DEHP-5 and DINP/DEHP, inhibited basal testicular T production while LH-stimulated testicular T production was unaffected.

Similarly, serum E2 was upregulated in male rats by DINP-10 (P = 0.0005; $\bar{x}_{\mathrm{DINP-10}}$ = 58.1947 ng/mL) and DEHP-10 (P = 0.0156; $\bar{x}_{\mathrm{DEHP-10}} = 50.7335\ \mathrm{ng/mL}$) compared to the control ($\bar{x}_{\mathrm{control}} = 30.7265\ \mathrm{ng/mL}$), but no effects were observed in rats exposed to DINP-5, DEHP-5, DINP/DEHP (Figure 6A; $P > 0.05$). Exposure to DEHP-10, but not to the other chemical doses, augmented basal testicular E2 production (Figure 6B). Conversely, however, both DINP-10 and DEHP-10 upregulated LH-stimulated T production in male rat testes compared to control (Figure 6C).

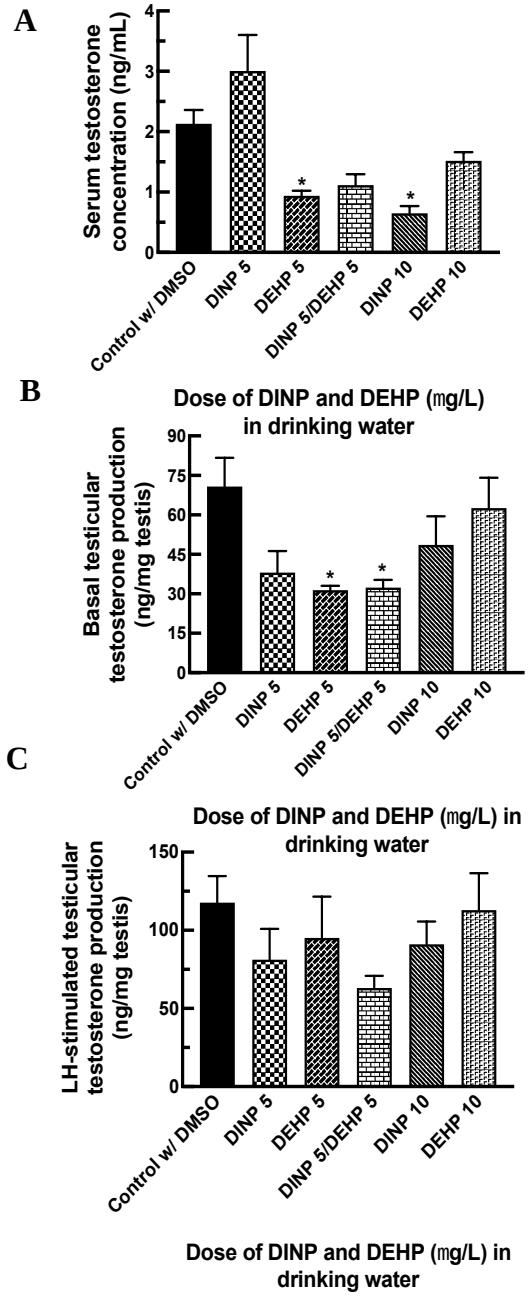

Figure 5. Individual and combined effects of 14-d_B DINP (5.00 µg/L or 10.00 µg/L), DEHP (5.00 µg/L or 10.00 µg/L), or DINP/DEHP exposures (5.00 µg/L DINP + 5.00 µg/L DEHP) on the serum concentration and testicular production of T in male rats (n = 5). After the 28-day exposure period, **(A)** serum [T] and **(B-C)** testicular T production in male rats were assessed by radioimmunoassay (RIA). A mixture of drinking water and DMSO was administered to the control group. Experimental groups received drinking water that contained their respective PAE treatments and DMSO. *P < 0.05 vs. control.

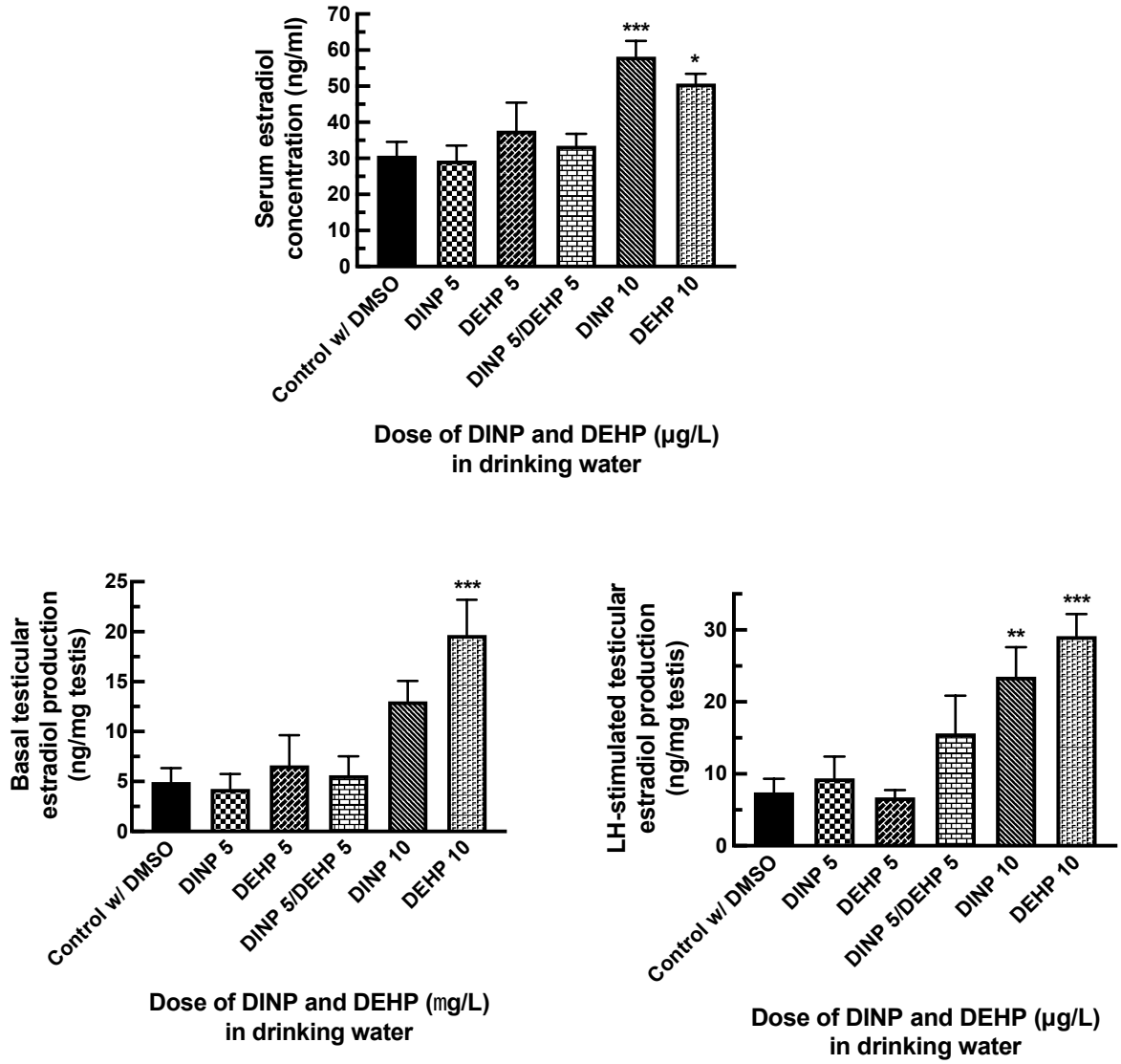

Figure 6 Individual and combined effects of 14-d_B DINP (5.00 µg/L or 10.00 µg/L), DEHP (5.00 µg/L or 10.00 µg/L), or DINP/DEHP exposures (5.00 µg/L DINP + 5.00 µg/L DEHP) on the serum concentration and testicular production of E2 in male rats (n = 5). After the 28-day exposure period, **(A)** serum [E2] and **(B-C)** testicular E2 production in male rats were assessed by radioimmunoassay (RIA). DMSO was added to the drinking water administered to the control group and to the PAE-treated drinking water administered to the experimental groups. *P < 0.05 vs. control, **P < 0.01 vs. control, ***P < 0.001 vs. control.

28-d Experiment B ($28d_B$): Exposure to DINP, DEHP, or DINP/DEHP in drinking water.

A final set of experiments were conducted utilizing 28-d chemical exposing ($28d_B$). Male weanling Long-Evans rats were exposed to DINP (DINP-5 or DINP-10), DEHP (DEHP-5 or DEHP-10), or to a mixture of both PAEs (DINP/DEHP) in drinking water (n = 5). After chemical exposures were terminated, serum concentrations and testicular production of T and E2 from all 28-d_B exposure groups were analyzed by RIA. Compared to the control group (water + DMSO), serum T and LH-stimulated testicular T production remained unaffected in male rats after all 28-d_B chemical exposures (Figure 7A and Figure 7C; $P > 0.05$). The DINP/DEHP exposure paradigm, however, increased basal testicular T production in male rats (Figure 7B; $P = 0.0019$).

The effects of DEHP, DINP, or DINP/DEHP on serum E2 and testicular E2 production were also analyzed by RIA. The data presented in figure 8A shows that serum E2 remained unaffected after all 28-d chemical exposures. DINP-10 and DEHP-10, however, increased basal testicular E2 production and decreased LH-stimulated testicular E2 production (Figure 8B-C)($P > 0.05$).

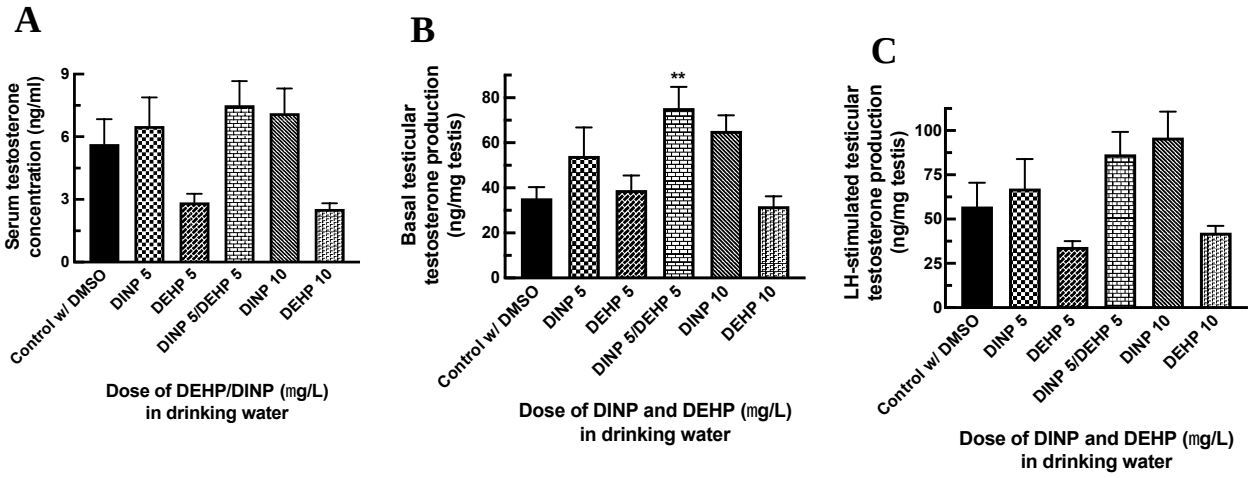

Figure 7 Individual and combined effects of 28-d_B DEHP and DINP exposures (0 µg/L + DMSO, 5.00 µg/L, or 10.00 µg/L) on the serum concentration and testicular production of T in male rats (n = 5). After the 28-day period of chemical exposure, **(A)** serum [T] and **(B-C)** testicular T production in male rats were assessed by radioimmunoassay (RIA). A mixture of drinking water and DMSO was administered to the control group. Experimental groups received drinking water that contained their respective PAE treatments and DMSO. *P < 0.05 vs. control and **P < 0.01 vs. control.

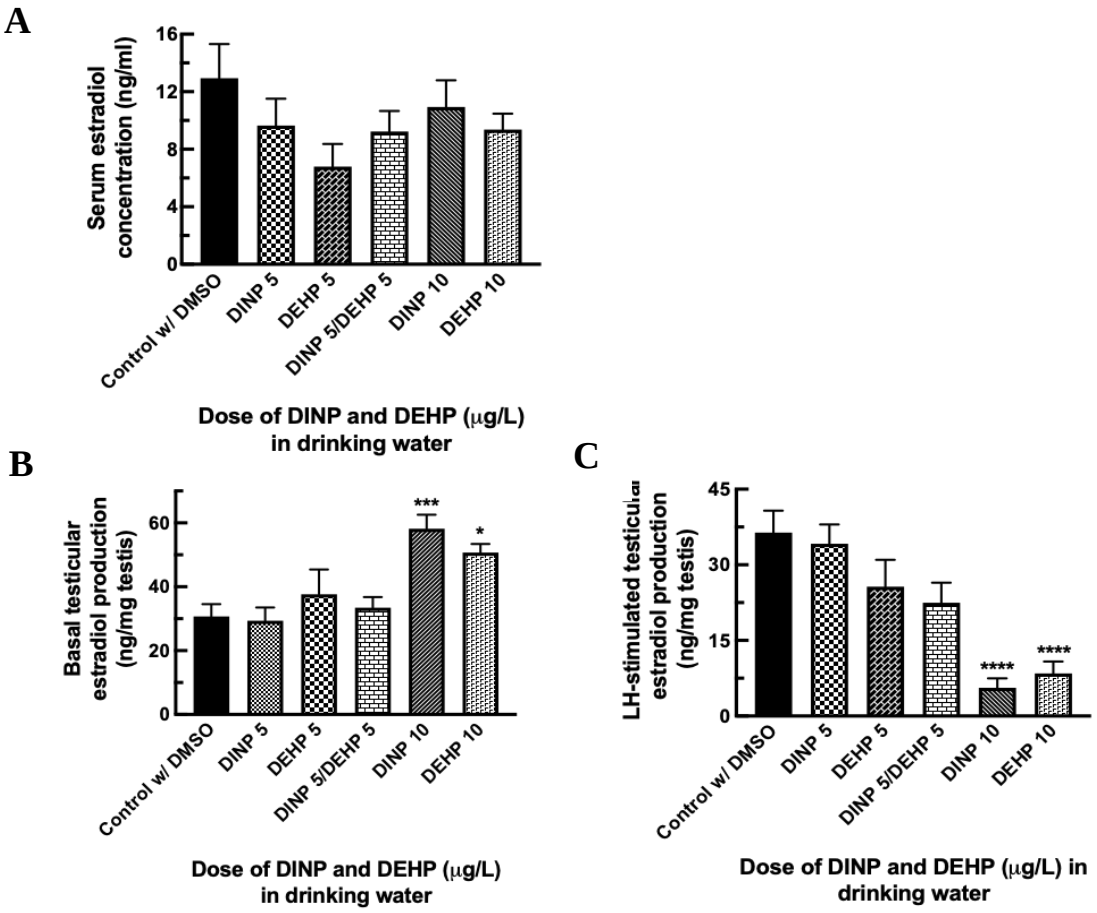

Figure 8 Individual and combined effects of 28-d_B DINP (0 + DMSO, 5.00 µg/L, or 10.00 µg/L), DEHP (0 + DMSO, 5.00 µg/L, or 10.00 µg/L), or DINP/DEHP exposures (5.00 µg/L DEHP + 5.00 µg/L DINP) on the serum concentration and testicular production of E2 in male rats (n = 5). After the 28-day period of chemical exposure, **(A)** serum [E2] and **(B-C)** testicular E2 production in male rats were assessed by radioimmunoassay (RIA). A mixture of drinking water and DMSO was administered to the control group. Experimental groups received drinking water that contained their respective PAE treatments and DMSO. *P < 0.05 vs. control, **P < 0.01 vs. control, ***P < 0.001 vs. control, and ****P < 0.0001 vs. control.

Chapter 3: Discussion of Results

Experiments 14-d_A and 28-d_A: Exposure to DEHP, but not to DINP, regulated steroidogenic capacity in prepubertal male rats.

Results of our initial studies indicated that testicular steroidogenesis was unaffected in prepubertal male LE rats that were exposed to DINP in drinking water. Also, previous DINP exposure studies have suggested that the reproductive effects caused by DEHP or DINP exposure only differed in amplitude (DINP < DEHP). The present results, therefore, compared favorably to data from past studies showing that exposure to DINP caused less toxicity in the developing testis when compared to DEHP (52,93,97,113). For example, *Driesche et al. (2020)* compared the effects of prenatal exposures to DBP or DINP (125 mg/kg bw/day or 750 mg/kg bw/day) in fetal (e17.5 or e21.5) and adult (PND 90) male Wistar rats. In agreement with the results of experiments 14-d_A and 28-d_A, *Driesche et al. (2020)* showed that serum T and testicular T production in both fetal (e17.5 or 21.5) and adult (PND 90) male Wistar rats were unaffected following prenatal DINP exposures. To our knowledge, however, the present study is the first to investigate the effects of postnatal DINP exposures on serum E2 and testicular E2 production in prepubertal male rats.

Furthermore, although serum T and E2 remained unaffected after all 14-d_A chemical exposures, dose-dependent decreases in testicular T and E2 production were detected in prepubertal LE rats exposed to 14-d_A DEHP. This finding is consistent with the data reported by previous 14-d DEHP exposure studies and have been attributed to the antiandrogenic effects of DEHP and/or downregulation of steroidogenic enzyme activity (84,114). DEHP has been shown to suppress the production of T and E2 in LCs by downregulating 17β-hydroxysteroid dehydrogenase (17β-HSD) activity (114). Alternatively, DEHP may stimulate the upregulation

of T 5α-reductase (T5α-R) expression and then downregulate aromatase expression to suppress the production of T and E2 in LCs (114).

Interestingly, the DEHP-15 dose increased basal testicular T production (>300% versus control) in prepubertal male LE rats after a 28-d_A (PND 49) exposure. Although this DEHP-induced effect was consistent with data from past 28-d exposure studies, LH-stimulated testicular T production was unaffected (84,87,89,91,92). According to the study by *Gunnarsson et al. (2008)*, however, the increase in basal testicular T production is probably caused by the primary metabolite of DEHP, i.e., MEHP, which activated the LH-LHR-$G\alpha_s$-cyclic adenosine monophosphate (cAMP)-StAR (LH-LHR-$G\alpha_s$-cAMP-StAR) signal transduction pathway (115). The authors also reported that, prior to the synthesis of pregnenolone in the mitochondria of LCs, MEHP exposure induced an upregulation of hormone-sensitive lipase (HSL) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (115). In the context of the present study, the aforementioned results are relevant because HSL mobilizes stored fats in LCs, and HMG-CoA controls the rate-limiting step of LC-mediated cholesterol biosynthesis, which collectively promote the induction of cAMP- and StAR-independent testicular steroidogenesis (115).

Moreover, very few studies have investigated the effects of oral PAE exposures on serum E2 and testicular E2 production in prepubertal rats. To our knowledge, this is the first study to investigate the effects of 28-d DINP exposures on serum E2 and testicular E2 production in prepubertal rats exposed to DEHP and/or DINP. The data demonstrated that serum E2 and testicular E2 production were unaffected after a 28-d exposure to DEHP and DINP acting alone. Interestingly, these results contradicted several previous reports, which showed that serum E2 levels and testicular E2 production were increased in male rats that were exposed to DEHP for 28 days (82,84,92,116). The estrogenic effects of DEHP were thought to result from DEHP-

induced upregulation of aromatase expression in LCs (82,84,92,116). In contrast, testicular steroidogenesis was unaffected in prepubertal male rats subjected to 14-d_A or 28-d_A DINP exposures (Figures 1-4). Thus, the data seem to support the view that DINP exerts lesser toxicity in the testis compared to DEHP.

Experiments 14-d_B and 28-d_B: Exposure to DEHP or DINP disrupted steroidogenic capacity in prepubertal male rats.

Data from the second set of experiments (14-d_A and 28-d_A) showed that DINP and DEHP acting alone disrupted testicular steroidogenesis in prepubertal LE rats. This conclusion is supported by the observation that 14 day-exposures to DINP -10 and DEHP -5 decreased serum T while the DINP (5 µg/L) and DEHP (5 µg/L) mixture increased both serum E2 and testicular E2 production in prepubertal LE rats (Figures 5 and 6). Also, the DEHP-5 and the DEHP/DINP group showed decreased basal testicular T production (Figure 5). Together, these results indicated that the testicular toxicity of the DINP and DEHP mixture was estrogenic as related to increased E2 production.

In agreement with previous DEHP exposure studies the estrogenic effects of 14-d_B DINP-10 were indicative of a DINP-induced increase in the expression of the *CYP19A1* gene which regulates LC expression of aromatase and conversion of T into E2 (20,21). This aromatase-catalyzed conversion of T into E2 is important because E2 produced by LCs is required for the maintenance and regulation of testicular steroidogenesis and spermatogenesis (20,21,41,92). It is likely that upregulation of aromatase expression increased testicular E2 production and serum E2 became elevated followed by a decrease in serum T levels (20,21,39,41,116). This type of imbalance in the T/E2 ratio has been shown to impact the process of spermatogenesis and cause infertility or premature reproductive senescence (41). Moreover, the disrupted T/E2 ratios interfere with the negative feedback loop of the HPG axis along with the compensatory mechanisms that maintain sex steroid hemostasis (21,39,41,116).

The results of 28-d_B exposures (Figure 7 and 8) showed that both serum T and E2 remained unchanged. However, DEHP-10 and DINP-10 each acting alone, either increased basal

testicular E2 production and/or decreased LH-stimulated testicular E2 production in prepubertal male LE rats (Figure 7 and Figure 8). These results further imply that both DEHP-10 and DINP-10 exerted estrogenic effects in the male gonad (82,84,87,89,91,92). Together, results of overall 28-d PAE exposure studies demonstrated that these agents have the capacity to increase the estrogen load (burden) in aromatase-expressing tissues.

Experiments 14_B, 28_A, and 28_B: DINP potentially had an additive effect on DEHP-induced basal testicular T production in prepubertal LE rats.

To our knowledge, the present study is the first to investigate the effects of DINP/DEHP on the serum concentration and testicular production of T and E2 in prepubertal male rats. This claim is supported by how, among the peer-reviewed publications that were available at the time this thesis was written, the reproductive effects of DINP/DEHP exposure had only been investigated during the fetal and perinatal life stages of male rats (93,118). For example, two different studies conducted by the same principal investigator examined the steroidogenic effects of DINP/DEHP (350 mg DEHP/kg bodyweight/day + 750 mg DINP/kg bodyweight/day) in fetal rats from gestational day (GD) 7-21 (93,118). The authors reported that, although statistically insignificant, their data trended toward DINP having an additive effect on the steroidogenic effects of DEHP.

The present study investigated the steroidogenic effects of DINP/DEHP exposure in prepubertal male LE rats after 14- and 28-d periods of chemical exposure (experiments 28-d_A, 14-d_B, and 28-d_B). We aimed to determine whether DINP modulates, attenuates, enhances, or has no effect on the steroidogenic effects of DEHP (or vice versa) in the prepubertal male rat gonad. Compared to their respective controls, exposure to DINP/DEHP in drinking water decreased basal testicular T production in prepubertal rats during experiment 14-d_B and increased basal testicular T production during experiment 28-d_B.

Additionally, during experiment 28-d_B, the increase in basal testicular T production in rats treated with DINP/DEHP was significantly greater than in rats treated with DEHP-5 or DINP-5 (paired t test, $P = 0.0283$). These results indicated that exposure to DINP/DEHP had an

additive effect on testicular androgen secretion and are consistent with results from past studies of PAE mixture effects (93,118).

Chapter 4 – Summary and Conclusions

The results presented in our study indicate that the steroidogenic capacity of the prepubertal male rat gonad was variously affected by PAE exposures. DINP and DEHP individually regulate serum T and E2 in prepubertal male LE rats. Across all four experiments, DEHP elicited the greatest number of effects on the serum concentrations and testicular production of T and E2 in prepubertal male rats, implying that DINP may be less toxic than DEHP in the prepubertal male rat gonad.

Nevertheless, DINP-10 and DEHP-10 both increased basal testicular E2 and decreased LH-stimulated testicular E2 secretion. Consequently, to advance our understanding of the reproductive effects of 14- and 28-d DINP, DEHP, or DINP/DEHP exposures in prepubertal male rats, future studies will address the following objectives:

1. Determine whether the activation of a cAMP-independent pathway is responsible for the estrogenic effects of DINP and/or DEHP exposure;
2. Compare the exposure effects of DINP, DEHP, and DINP+DEHP on the expression of steroidogenic enzymes that are involved in testicular T (e.g., 3β- and 17β-HSD) and E2 (e.g., aromatase) production.
3. Investigate the effects of DINP, DEHP, and DINP+DEHP on SC function by identifying transcription factors that are targeted by PAEs to disrupt spermatogenesis (e.g., AR, ER, FSHR, LHR, ABP, and Ih-B).
4. Extend the current understanding of chemical mixture effects due to DEHP+DINP.

In order to advance public health, future studies will analyze the effects of low-dose PAE exposures on molecular targets that mediate testicular steroidogenesis and spermatogenesis (e.g., on LH receptors, androgen receptors, aromatase, 3β- and 17β-HSD, ERα, ERβ, and StAR), in

growing male rats (20,22,33,39,41,119). In this manner, additional DINP and DEHP exposure studies will provide greater insights into the reproductive toxicity of DINP and the implications for male fertility. The results will also help to determine whether DINP can be a suitable replacement for DEHP in consumer products.

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