Chemical regulation of steroidogenic capacity in the male rat gonad

by

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Abstract

The hypothalamic-pituitary-gonadal (HPG) axis is critical in the maturation and maintenance of reproductive organs, particularly the initiation of Sertoli and Leydig cell differentiation in males. Due to the presence of estrogen receptors (ER) throughout the HPG-axis, it is susceptible to disruption by a wide variety of endocrine disrupting chemicals (EDC), particularly those classified as estrogen mimicking compounds (EMC). This poses a great concern in male reproductive health. This dissertation describes the adverse effects of developmental exposure on testicular function in the rat model to environmental EDCs, such as the EMCs known commonly as forever chemicals, or per- and polyfluoroalkyl substances (PFAS), plasticizers Bisphenol A (BPA) and Bisphenol S (BPS), and the synthetic form of estrogen, 17α-ethynylestradiol (EE2), used in the manufacture of female oral contraceptives. Specifically, the second chapter of this dissertation compares the effects of legacy PFAS, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), to their emerging PFAS counterparts, perfluorobutanoic acid (PFBA) and perfluorobutanesulfonic acid (PFBS), while the third compares three common estrogen mimicking compounds, BPA, BPS, and EE2, all of which are of high prevalence in the environment.

Leydig cells are the primary producers of the male sex steroid testosterone. Disruption of this process is shown to alter sexual differentiation and development of secondary sexual characteristics, as well as delaying puberty, or impacting male fertility. Sertoli cells act to support spermatogenesis by facilitating the interactions between germ cells and their regulatory hormones. The overall aim of this dissertation is to

analyze the effect of EDCs in the rat testis. This aim is achieved using the following specific objectives:

1. Investigate comparative effects of prepubertal and pubertal legacy and emerging PFAS exposures on gonadal steroid hormone secretion
2. Investigate the effect of plasticizers BPA and BPA, and the synthetic estrogen, EE2, on steroid hormone secretion in the prepubertal male rat gonad

To achieve the first objective, 21 day-old male rats were provided drinking water containing 0, 1, 10, 100 and 1000 ng/L PFOA and PFBA for 14 days to investigate prepubertal effects. We repeated the experiment using the same exposure paradigm to assess effects due to a sulfonic acid-containing legacy and emerging PFAS (PFOS, PFBS). Then, 21 day-old male rats were provided drinking water containing 0, 10, and 100 ng/L of a carboxylic acid-containing legacy and emerging PFAS, i.e., PFOA and PFBA and a sulfonic acid-containing legacy and emerging PFAS, i.e., PFOS and PFBS for 28 days to determine pubertal effects. Additionally, two separate experiments were conducted in which prepubertal male rats at 21 days of age were provided drinking water containing 10 ng/L of each test chemical and their combination at 5 ng/L of individual chemicals: control, PFOA, PFOS, PFOA+PFOS in the first experiment and control, PFBA, PFBS, PFBA+PFBS in the second.

To achieve the second objective, three separate experiments were performed in which 21 day old prepubertal male rats were maintained on deionized water with graded doses, 0, 1, 5, 10, 15, or 20 µg/L, of BPA, BPS, or EE2 for 56 days.

The findings from these objectives on the effects of PFAS and the estrogen mimicking compounds (BPA, BPS, and EE2) on testicular cells support the view that exposure of the population to environmental EDCs have the potential to impair reproductive health. Additional studies are warranted to elucidate the mechanisms by which these compounds, both individually and in mixtures, impact testicular cells and overall HPG-axis function and regulation.

Artificial Intelligence (AI) Use Disclosure Statement

In the preparation of this dissertation, the following Artificial Intelligence (AI) tools were used: Grammarly. This tool was used primarily to check for grammar errors and improve sentence structure. The author acknowledges full responsibility for the intellectual content of this work and has ensured that all AI-assisted sections have been reviewed and revised for accuracy and appropriate academic style. All AI-generated content was reviewed and validated for relevance, appropriateness, and accuracy before incorporation into the final document to maintain scholarly integrity of this research.

Digital Accessibility Disclosure Statement

In the preparation of this dissertation, the following digital accessibility tools were used to ensure this document complies with federal requirements: Adobe Acrobat. The author acknowledges full responsibility for the intellectual content of this work and has made a good faith effort to comply with digital accessibility requirements in publishing, wherein the nature of the content does not significantly change in order to do so. Furthermore, all content has been reviewed and revised to meet these requirements prior to final publication.

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List of Abbreviations

ABP	Androgen binding protein
AP1	Activator protein 1
AR	Androgen receptor (AR in humans, Ar in rats)
ARC	Arcuate nucleus
BPA	Bisphenol A
BPS	Bisphenol S
BSA	Bovine serum albumin
cAMP	cyclic-adenosine monophosphate
CON	Control (normal deionized water)
CREB	cAMP response binding element
DAG	Diacylglycerol
DMEM	Dulbecco Modified Eagle Medium
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE2	17α-ethynylestradiol
EMC	Estrogen mimicking compound
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GRG	Groucho-related gene
HPG	Hypothalamic-pituitary-gonadal

IP3	Inositol 1,4,5-triphosphate
LH	Luteinizing hormone
MIS	Mullerian inhibiting substance
PBS	Phosphate-buffered saline
PFAS	Per- and polyfluoroalkyl substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PKA	Protein kinase A
PKC	Protein kinase C
PND	Postnatal day
RIA	Radioimmunoassay
StAR	Steroidogenic acute regulatory protein
T	Testosterone
US EPA	United States Environmental Protection Agency

Chapter 1 - Molecular Regulation of Male Reproductive Development and Function by the HPG-axis: A Literature Review

Abstract

The hypothalamic-pituitary-gonadal (HPG) axis is paramount in cellular differentiation and maturation of reproductive organs, particularly the initiation of Sertoli and Leydig cell differentiation. This review explores the HPG axis and its crucial roles in the regulation and maintenance of the male reproductive system by delving into the intricate regulatory mechanisms of the HPG axis, focusing on the hypothalamus and pituitary gland, and subsequently explores the role of the testis, including endocrine, exocrine, and paracrine functions.

Gonadotropin releasing hormone (GnRH) pulsatile release is identified as a driving force behind steroidogenesis and spermatogenesis, with kisspeptin playing a crucial role in this process. The pituitary's gonadotroph cells are the primary target of GnRH, which activate the intracellular pathways regulating the release of LH and FSH. Testicular regulation by gonadotropins includes synthesis of testosterone in Leydig cells and the support of germ cells through spermatogenesis by Sertoli cells. Paracrine relationships between Leydig, Sertoli, and germ cells are also critical in endocrine function of the HPG axis.

In summary, this chapter provides an overview of the HPG axis in the male reproductive system, offering insights into its development, regulation, and functions while highlighting the intricate intercellular relationships crucial for the process of reproduction.

Introduction

It is commonly known that the hypothalamic-pituitary-gonadal (HPG) axis is paramount in the cellular differentiation and maturation of reproductive organs, even if some of the exact mechanisms of development are yet to be elucidated[1]. In fact, the differentiation of Sertoli and Leydig cells begins the process of male reproductive development in a growing organism[2]. Inappropriate or disrupted differentiation could result in failed degradation of the female reproductive tract or incomplete formation of the male gonad. Appropriate differentiation is dependent on gonadotropin release[1], thus marking the beginning of HPG axis interactions during development.

Important to the function of the HPG axis in development is its role in reproductive function and maintenance. Indeed, the role of the HPG axis in reproductive function is its primary physiological requirement in mature animals[3] in order to ensure species survival. The release of sex steroid hormones and the process of spermatogenesis both rely on the proper activation from gonadotropins released by the pituitary[4]. As such, an effective relationship between the members of the HPG axis is crucial to reproduction. Infertility and reproductive defects may occur when the balance between the three levels (hypothalamus, pituitary, and gonads) of the HPG axis are dysregulated or interrupted[5].

The three levels of the HPG axis operate within the controls of its negative feedback loop (**Fig. 1**). At the first level, the hypothalamus provides a negative feedback activation of the pituitary gland through the release of gonadotropin releasing hormone (GnRH). The second level also operates by positive activation, in which the pituitary releases gonadotropins that act in the gonad. The gonad then completes the

relationship at the third level of the HPG axis with the release of sex steroid hormones and inhibins plus activins that provide negative feedback to the hypothalamus[6] and pituitary.

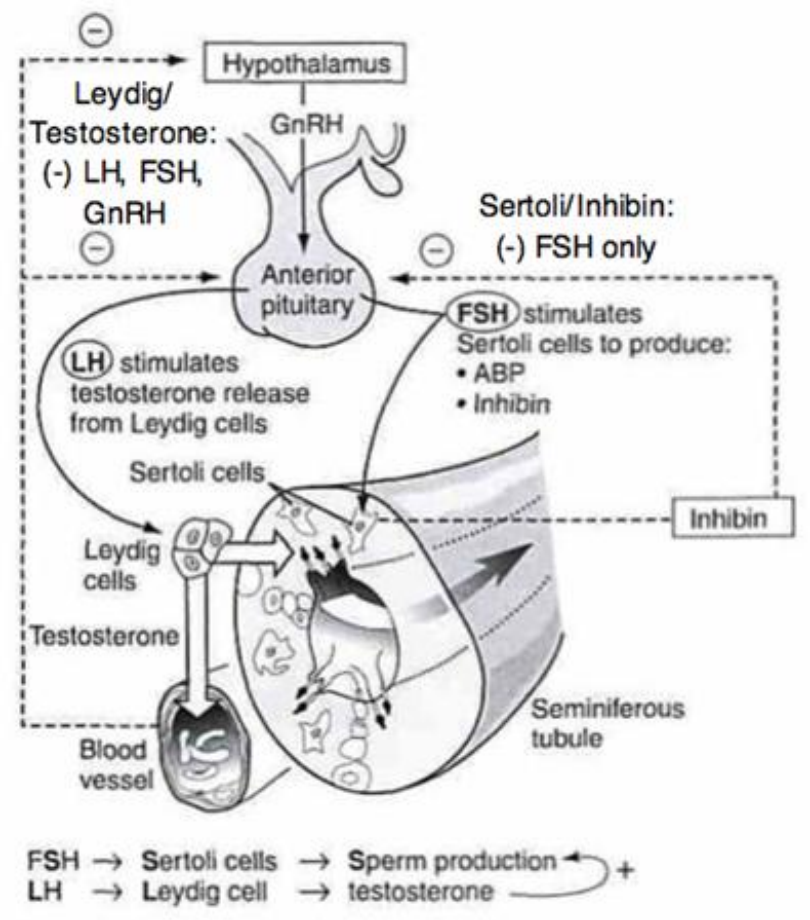

Figure 1: Representation of the HPG axis in males. Image from Rosenfield *et al.*[7]

For the purposes of this review, we will focus on the regulation of the HPG axis within the male reproductive system. We will start with the regulatory action of the hypothalamus and pituitary, then we will look at the role of the testis in the HPG axis, including its endocrine, exocrine, and paracrine functions.

Hypothalamic regulation

Function

GnRH is released in a pulsatile fashion that is responsible for the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary[8]. This pulsatile release is the driving force behind both steroidogenesis and spermatogenesis[9]. Several studies have shown that kisspeptin is the operating force behind this pulsatile release. Kisspeptin is found in the arcuate nucleus (ARC) of the hypothalamus[10] and when a light-induced stimulation is received[11], the pulsatile release of GnRH occurs[12]. This results in a pulsatile release of LH and FSH from the pituitary as well[13] (**Fig. 2**).

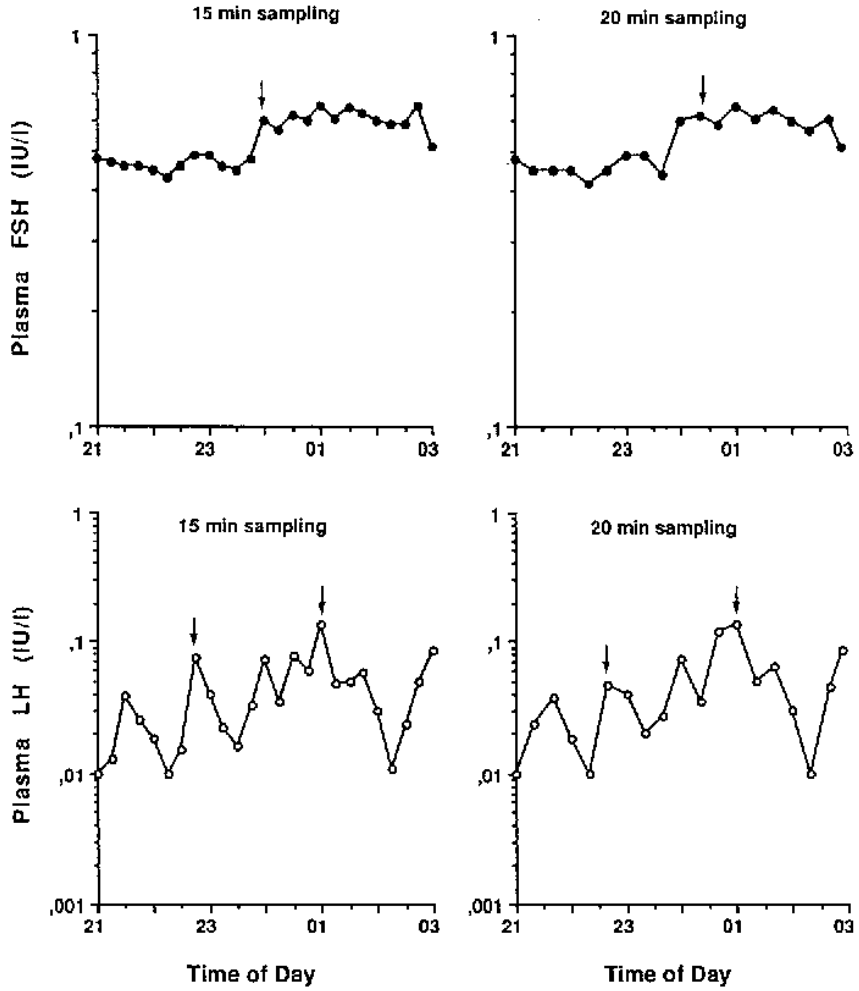

Figure 2: Time course of plasma LH (open circles) and FSH (solid circles) concentration in one pubertal boy indicating the effect of sampling interval on gonadotropin profiles. Figure from Dunkel *et al.* [14] Arrows denote significant pulses.

It is important to note that estrogen receptor α (ERα) and androgen receptor (AR) are not expressed in GnRH neurons[15]. As such, the steroid hormones are incapable of exerting the inhibitory action of the negative feedback look on GnRH directly. Instead, it is the kisspeptin neurons present within the ARC that express these steroid hormone

receptors[16], demonstrating the critical role that kisspeptin plays as the driving force of the HPG axis.

Regulation

The modulation of GnRH transcription is predominantly controlled by transcription factors Oct1, Msx1, and Dlx2. Oct1 acts as the primary gene promoter for GnRH transcription[17]. Therefore, Oct1 is a primary target of regulatory action by the negative feedback loop, necessary to maintain appropriate levels of GnRH transcriptional activity. This occurs through ARs present on GnRH's promoter region, inhibiting Oct1 action[18]. Msx1 and Dlx2, conversely, regulate each other by competing to bind the ATTA sites on GnRH transcription factors. In this relationship, Msx1 acts as the transcription repressor while Dlx2 acts as the transcription promoter[19] (**Fig. 3**).

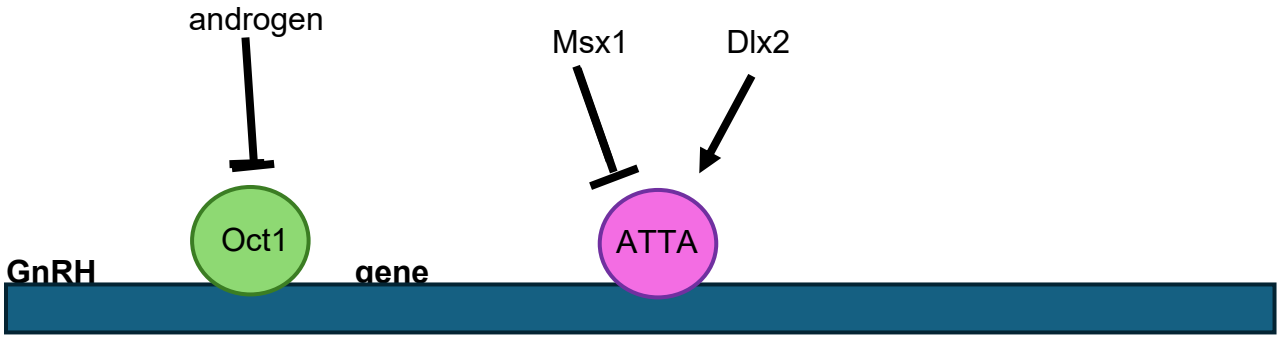

Figure 3: A simplified model of the regulation of GnRH transcription. Label → denotes an activating function, while ⊣ denotes an inhibiting function.

Further regulatory cofactors include, but are not limited to, Groucho-related gene (GRG) proteins, Pbx1, Otx2, GATA, PKC, c-Jun, c-Fos, CREB, and AP1. GRG proteins are co-repressors that bind and repress the functions of Oct1 and Msx1[20]. Pbx1 is a cofactor that binds near Oct1 binding sites to increase GnRH transcription[21]. Otx2 acts to increase both basal and enhancer modulated transcription of GnRH when bound to its gene receptor site[22]. A GRG protein regulates Otx2 activation by binding Otx2 and repressing its function[23]. When activated, GATA binding sites are crucial enhancers of *GnRH* gene activation[24]. On the other hand, the protein kinase C (PKC) pathway acts as a transcription repressor by phosphorylating Oct1, Dlx2, or Pbx1, and thus suppressing their activating functions[25]. Similarly, c-Jun, c-Fos, and CREB also act as repressors. Increased c-Jun and c-Fos gene levels are known to decrease levels of GnRH transcription[26], while the Jun and Fos proteins themselves can bind activator protein 1 (AP1) to reduce GnRH synthesis[27]. The cAMP response element-binding (CREB) proteins are known to inhibit the *GnRH* gene promotor site, though the mechanism of action is still unclear[28].

Pituital regulation

Regulation by GnRH

Gonadotroph cells within the pituitary gland are the primary and fundamental target of GnRH[29]. Gonadotrophs exhibit GnRH receptor expression on their plasma membrane and the release of LH and FSH[30]. When GnRH binds its receptor, Gαq/11 activates phospholipase activity, which in turn forms inositol 1,4,5-trisphosphate (IP3)

and diacylglycerol (DAG)[31]. The IP3 activation works to release calcium (Ca^{2+}) stores, and the increased intracellular calcium works in tandem with DAG to activate PKC[32]. PKC then works directly and indirectly, through the activation of the MEK/ERK pathway, to produce gonadotropins, LH and FSH[33] (**Fig. 4**).

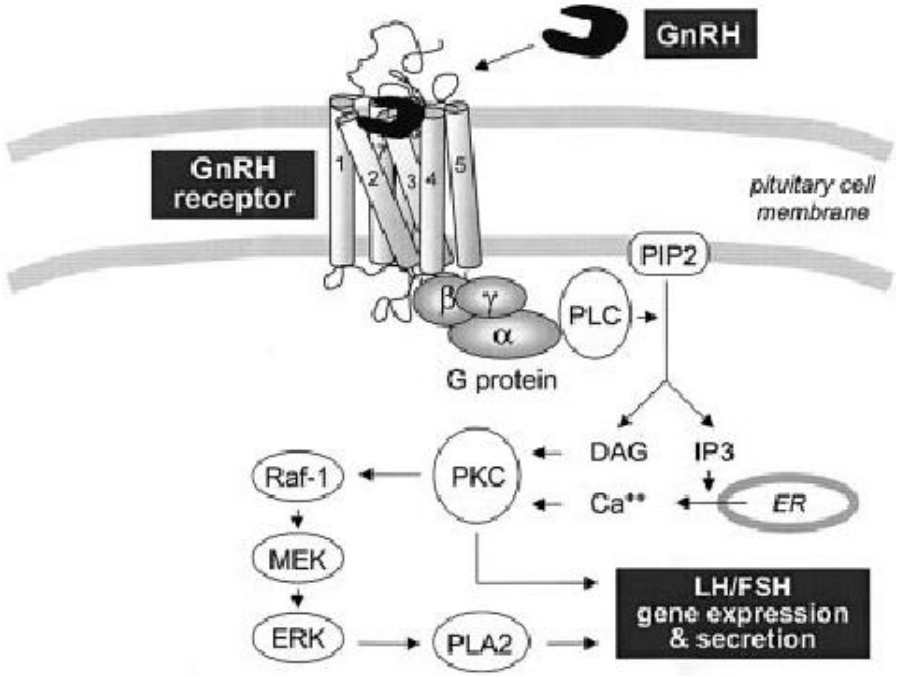

Figure 4: **Gonadotropin release is mediated by GnRH receptor activation. Image from Kaiser *et al.*[32]**

Though the exact mechanisms are not fully understood, the two gonadotropins specifically release at *opposite* pulsating frequencies, with LH transcription and translation increased at higher frequencies while FSH is increased at lower frequencies[34]. However, current data suggests that LH transcription is likely modulated by the Ca^{2+}-calmodulin kinase (CAMK) pathway[35], while FSH transcription is modulated by PKC activation from DAG and Ca^{2+} increases[36].

Regulation by the gonad

In opposition to the activation provided by GnRH, FSH is inhibited when receptors in the pituitary gland are bound by inhibin released from testicular Sertoli cells[37]. This plays an important role in maintaining homeostasis through the negative feedback loop. Furthermore, gonadotropins are also regulated by the sex steroid hormones released from the testes, which bind their receptors in the hypothalamus. This action, as previously discussed, inhibits the release of the GnRH responsible for gonadotropin activation. However, steroid hormones may also act within the pituitary to directly regulate the release of gonadotropins. For example, ERα has been shown to bind the *Lhb* gene estrogen response element directly in rat pituitary cells, activating the secretion of LH[38]. In rodents, Ar has been shown to bind hormone response elements in *FSHβ* to induce FSH synthesis[39]. Further, this same effect was still seen in GnRH-deficient rodents, suggesting a strong activation of FSH secretion by androgens, presenting a potential therapeutic target for cases of hypothalamic trauma. This effect is not conserved across species, however, because human data shows that androgens actually suppress *FSHβ* expression[40,41].

Testicular regulation

When gonadotropins arrive at the testis, LH binds its receptors present in Leydig cells to activate the synthesis of testosterone (T), while FSH binds its receptors in Sertoli cells to support germ cell development and spermatogenesis[42]. The gonadotropins also modulate a paracrine relationship between the Leydig, Sertoli, and

germ cells. In response to activation by the gonadotropins, Leydig cells produce androgen while Sertoli cells produce inhibin which are circulated back to the hypothalamus (androgen only) and pituitary to provide a negative-feedback inhibition.

LH regulation of the Leydig cell

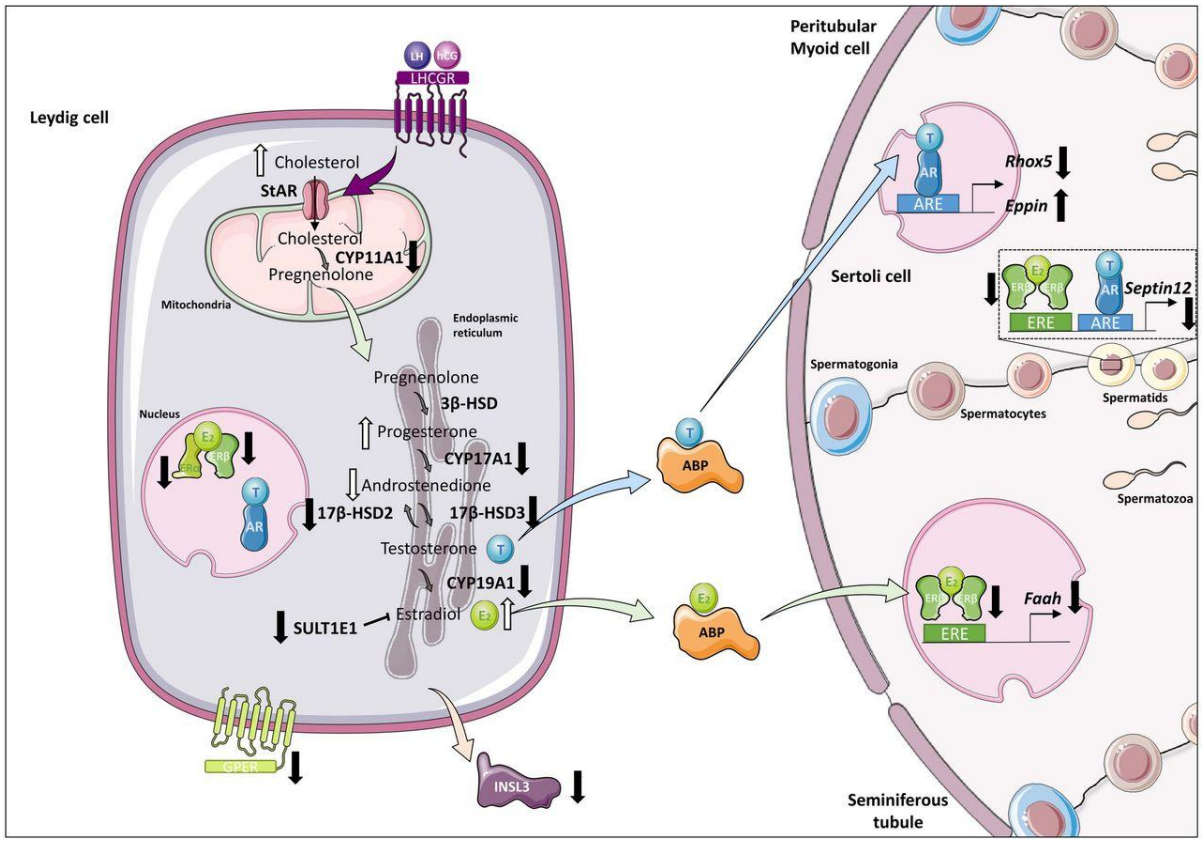

Figure 5: LH plays a crucial role in the production of steroid hormones within the Leydig cell. Image from Moutard *et al.*[43]

When LH binds its receptor on the Leydig cell, it activates the cyclic-adenosine monophosphate-protein kinase A (cAMP-PKA) pathway to release free cholesterol,

which is then recruited by steroidogenic acute regulatory protein (StAR) for transportation into mitochondria. There, it is cleaved into pregnenolone before being released into the cell matrix where it undergoes a series of enzymatic actions to form steroid hormones, T and estradiol (E2) (**Fig. 5**). Leydig cell nuclei contain both androgen and estrogen receptors that are suppressed when steroid hormone levels are in their basal state by autocrine regulation of the Leydig cell. The majority of the steroid hormones are released into the bloodstream or circulate to the hypothalamus and pituitary gland to perform negative feedback regulation. Some amount of androgens are bound by androgen binding protein (ABP) secreted from the Sertoli cell which functions to maintain appropriate levels of T and E2 within the testes[44]. ABP delivers bound T and E2 to the seminiferous tubules where they are then recruited by Sertoli cells. Germ cells also express E2 receptors, suggesting a paracrine relationship between germ cells and Leydig cells. Though the exact mechanism is not known, studies have shown that E2 also plays an important role in the maturation and function of spermatids[45].

FSH regulation of the Sertoli cell

When FSH binds its receptors in Sertoli cells, it activates the cAMP-PKA pathway[46], which allows for the development of a micro-atmosphere of specialized and self-contained matrix around the cell[42]. This micro-environment is capable of supporting 30-50 germ cells through various stages of spermatogenesis[47] by secreting various support factors.

Paracrine regulation of testicular cells

Paracrine relationship of Sertoli cells and germ cells

Androgen and FSH are the main regulators of germ cell spermatogenesis. However, germ cells have neither FSH receptors nor androgen receptors. We discussed previously how FSH binds Sertoli cells to facilitate the creation of a micro-environment capable of supporting a number of germ cells. Sertoli cells also mediate the use of androgen throughout spermatogenesis. Both androgen and FSH are absolutely necessary for spermatogenesis to occur[48], thus making the relationship between germ cells and Sertoli cells a critical one. In turn, germ cells maintain a cyclical control over signaling in Sertoli cells. Though the exact mechanism is unclear, factors released from germ cells are shown to modulate levels of protein secretion from Sertoli cells, depending on the germ cell's stage of development[49]. Sertoli cells are often called "nurse" cells, due to their support of germ cells. Androgen bound from Leydig cells and transported by ABP allows Sertoli cells to modulate the secretion of Eppin, which is involved in sperm motility[50], and Rhox5, which helps to regulate spermatogenesis and provide protection against apoptosis[51].

Paracrine relationship of Sertoli cells and Leydig cells

Androgen, specifically T, is absolutely necessary for spermatogenesis and is modulated by Sertoli cells. Because T is produced by Leydig cells, that interaction is critical to the paracrine relationship between these two cell types. Conversely, though Leydig cells don't have FSH receptors, studies have shown that FSH increases Leydig cell responsiveness to LH, and thus, increases spermatogenesis. It is hypothesized that

regulatory factors released by FSH-bound Sertoli cells modulate LH responsiveness in the Leydig cell[52]. Furthermore, the *Faah* gene found in Sertoli cells, which is involved in spermatogenesis, is thought to be the first direct testicular target of E2[53]. Interestingly, Leydig cells have receptors for inhibin produced by the Sertoli cells, but it is currently unknown if any physiological effect occurs from this interaction[54]. Leydig cells also have receptors for Sertoli cell-secreted Müllerian inhibiting substance (MIS). Studies show that MIS is a negative regulator of LH receptor expression in Leydig cells (**Fig. 6**)[55]. Expectedly, MIS reduced expression of several enzymes in the steroidogenic pathway, suggesting that MIS targeting of the LH receptor disrupts all downstream targets of steroidogenesis.

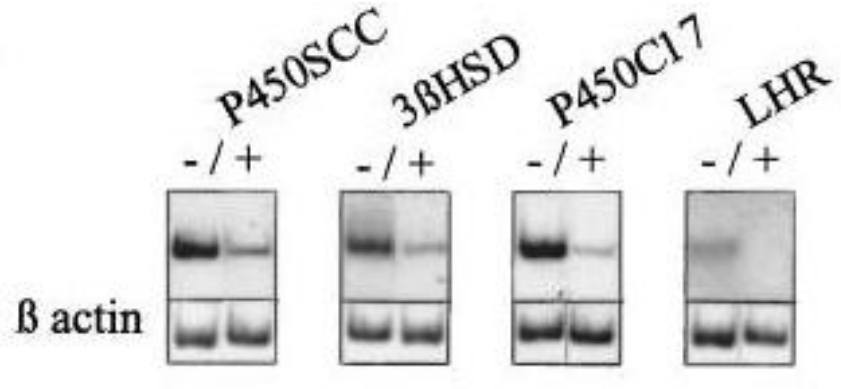

Figure 6: **Expression of various steroidogenic protein mRNAs in purified Leydig cells obtained by southern blot hybridization of RT-PCR products (30 cycles) of P450c17, 3β-HSD, P450scc, and LHR.** RNA was extracted from purified Leydig cells incubated 3 days in the presence (+) or absence (−) of MIS. Actin served as a control. Figure from Racine *et al.*[55]

Conclusion

In conclusion, current understanding of the HPG axis underscores its pivotal role in orchestrating the intricate processes of male reproductive development, regulation, and maintenance. The HPG axis emerges as a linchpin in cellular differentiation and maturation of reproductive organs, with a specific focus on the initiation of Sertoli and Leydig cells' differentiation. Interference with and/or disrupted differentiation is a potential source of reproductive anomalies, supporting the critical role of HPG axis function to maintain the integrity and function of the male and female reproductive tracts. The mechanisms of regulation of the HPG axis include the involvement of many molecular factors, including GnRH, kisspeptin, and intricate transcriptional regulatory networks mediated by Oct1, Msx1, and Dlx2. The dynamic interplay among the hypothalamus, pituitary gland, and gonads within the negative feedback loop emerges as a finely tuned mechanism crucial for maintaining the delicate balance required for reproductive success.

The role of the HPG axis in the development and maturation of the reproductive tract is underscored by its primary responsibility for sex steroid hormone release and the process of spermatogenesis. Moreover, the paracrine relationships among Leydig, Sertoli, and germ cells involve a complex communication network essential for orchestrating testicular functions. This intricate web of interactions involves the synthesis of T, the support of germ cells, and the maintenance of a negative-feedback inhibition loop, highlighting the reciprocal relationships of HPG axis components.

This review covers the basis for understanding the role of the HPG axis in the male reproductive system, and elucidating its multifaceted functions and regulatory

networks. The integration of developmental, regulatory, and maintenance aspects provides a holistic perspective of the underlying mechanisms governing male reproductive physiology. Our present understanding offers a foundation for further research that is designed to determine the complexities of this crucial physiological axis.

Chapter 2 - Legacy and emerging per- and polyfluoroalkyl substances (PFAS) regulate steroidogenesis in the male gonad

Abstract

Per- and polyfluoroalkyl substances (PFAS) are widely used in a variety of industrial processes and manufacturing of consumer products. Current efforts by the manufacturing industry will limit use of long-chain or legacy PFAS represented by perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) and replace with short-chain or emerging PFAS such as perfluorobutanoic acid (PFBA) and perfluorobutane sulfonic acid (PFBS). However, there is little to no information on the toxicity of new and emerging PFAS. Therefore, we performed experiments in growing Long-Evans male rats to investigate effects of low dose prepubertal and pubertal exposures to PFAS on gonadal steroid hormone secretion. The results demonstrated that both legacy and emerging PFAS have the capacity to regulate testicular steroidogenesis. For example, prepubertal exposures to PFOS, PFBA and PFBS increased serum and testicular T concentrations. Exposure to PFBA increased testicular E2 concentrations while PFOS and PFBS both decreased serum E2 concentrations while stimulating testicular E2 secretion. The data also demonstrated additive effects due to legacy and emerging PFAS mixtures compared to the individual chemicals. The gonadal effects due to PFAS exposures occurred at nanomolar concentrations, which approximate PFAS levels in the environment. Taken together, the present study support the need for development of cost-effective and sustainable filtration media for different processes to remove PFAS from water and other sources of exposure. Current action

by regulatory agencies such as the US Environmental Protection Agency to limit use of PFAS in the manufacture of consumer products will protect public health.

Introduction

Per- and polyfluoroalkyl substances (PFAS) represent a group of water- and grease-repellent chemicals that are widely used in a variety of industrial processes and consumer products, including cookware, food packaging, and textiles. These compounds are present in humans and wildlife, implying that exposure of the population to PFAS is significant[56-58]. The concentrations of perfluoroalkyl carboxylic acid (PFOA) in human sera range from 3-5 ng/mL although these levels have been decreasing lately[59-62]. However, studies of human populations exposed to contaminated drinking water showed exposures that are approximately 10 times greater than the average population, with blood PFAS concentrations reaching up to 22 000 ng/mL[63,64]. In response to growing public concern related to potential health effects, there have been efforts by the manufacturing industry to limit the use of long-chain and so-called legacy PFAS such as PFOA and perfluorooctane sulfonic acid (PFOS) and replace with short-chain PFAS, including perfluorobutanoic acid (PFBA) and perfluorobutane sulfonic acid (PFBS). The emerging compounds, e.g., PFBA and PFBS, have shorter half-lives and diminished potential for environmental persistence due to their shorter chain lengths[65]. Moreover, the European Commission and the United States Environmental Protection Agency (US EPA) have recently issued health advisory guidelines restricting the use of PFOA and PFOS[66]. The persistence of legacy PFAS in the environment implies that there is continuing exposure of all segments of the population and a need for monitoring and regulation to protect human health[67]. Infants and children are particularly vulnerable to environmental toxicants, including PFAS, due to higher body burdens than adults as related to differences in exposure sources, body size, and body surface

area[68]. It is also likely that changes affecting tissue and organ system differentiation during sensitive periods of development have implications for health later in life[69].

There are inconsistencies in epidemiological data in the literature but there appears to be a general agreement that legacy PFAS have the capacity to interfere with endocrine signaling pathways, regulate hormone-sensitive organs, and thereby affect reproductive health. For example, prenatal exposures to PFOA was related to decreased sperm concentration and total sperm counts, which were associated with elevated serum luteinizing hormone (LH) and follicle-stimulating hormones (FSH) in adult men[70]. A separate study showed that PFOS levels were negatively associated with testosterone (T) and serum gonadotropins in healthy men[71]. However, other reports failed to establish a link between PFOA and PFOS exposures and semen volume, sperm concentration and sperm motility, but chemical levels were positively correlated with plasma LH concentrations in the subjects[72,73]. Nevertheless, persistence of legacy PFAS such as PFOA and PFOS in the environment and increasing use of new and emerging replacements in the manufacturing sector make PFAS subjects for further investigation.

Testicular Leydig cells are a known target for PFAS because administration of ammonium perfluorooctanoate (C8) to adult male CD rats by gavage for 14 days decreased serum and testicular interstitial fluid T levels, increased serum E2 concentration, and induced Leydig cell hyperplasia. Incubation of isolated Leydig cells with C8 caused a dose-dependent decrease in T secretion, implying direct C8 action in Leydig cells. Also, exposure of pubertal male rats to PFOS was found to induce Leydg cell hyperplasia[74,75]. Prenatal exposures to PFOS affected development of

steroidogenic capacity in fetal Leydig cells while exposure to PFOA was associated with increased incidence of Leydig cell adenoma[76,77]. These observations imply that continued study of PFAS toxicity in the male gonad is required. To evaluate the potential risk associated with exposure to PFAS, we investigated testicular toxicity due to legacy PFAS, i.e., PFOA and PFOS, and emerging PFAS, i.e., PFBA and PFBS. In the present study, it was hypothesized that exposure of growing male rats to legacy and emerging PFAS both impact development of steroidogenic capacity in the male gonad. The average weaning age for the rat is 21 days and puberty is attained at 50 days of age; these times correspond to six months and 11.5 years in humans[78,79]. In this regard, there is a general agreement that the prepubertal and pubertal phases of development in the rat approximate infancy and early childhood in the population[80]. Based on our current interests in developmental toxicity, Long-Evans male rats were administered legacy (PFOA, PFOS) and emerging PFAS (i. e., PFBA, PFBS) during the prepubertal (PND 21-35) and pubertal (PND 21-49) periods.

Materials & Methods

Chemicals and Reagents

PFOA, PFOS, PFBA and PFBS were purchased from Sigma-Aldrich Company. (St. Louis, MO). Trypsin inhibitor, EDTA, HEPES, bovine serum albumin BSA), bovine lipoprotein, sodium bicarbonate ($NaHCO_3$), Dulbecco Modified Eagle Medium (DMEM) nutrient mixture (Ham's F-12 (DMEM/F-12; 1:1 mixture without phenol red]), albumin, Percoll, etiocholan-3β-ol-17-one, and gentamicin were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco phosphate-buffered saline (PBS), medium 199,

and 10X Hanks balanced salt solution were from purchased Life Technologies, Inc. (Grand Island, NY). Collagenase, dispase, and deoxyribonuclease (DNase) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Ovine LH was a gift from the National Hormone and Pituitary Program (NIDDK, Bethesda, MD).

Animal Studies

We performed animal and euthanasia procedures in accordance with a protocol approved by the Auburn University Institutional Animal Care and Use Committee and based on recommendations of the panel on Euthanasia of the American Veterinary Medical Association. Male Long–Evans rats were from Envigo (Madison, WI). We allowed animals three days to acclimatize at the Division of Laboratory Animal Health Housing Facility, College of Veterinary Medicine. We provided water in plastic water bottles *ad libitum*. Animals were housed in flexible, standardized, research-ready caging made of high viscosity PET (Innovive San Diego, CA) in order to minimize background exposure to other chemicals in the environment[81]. Animals were maintained under constant conditions of light (12 L: 12 D) and temperature (20–23.38 °C) with free access to pelleted food. All animals were fed the same diet with no or little soybean content (X2020, Harlan-Teklad, Madison, WI). PFOS and PFOA, the most common PFAS found in samples of human milk from mothers in parts of the United States and other countries, measure in the nanomolar range[82-85]. Furthermore, the US EPA has set the combined concentration of PFOA and PFOS in drinking water at 70 ng/L[86]. For these reasons, test chemicals were provided in drinking water within the dose range of 1-10^3 ng/L.

Experiment 1

Experiments were performed to assess effects due to prepubertal PFAS exposures on gonadal steroid hormone secretion by comparative assessment of a carboxylic acid-containing legacy and emerging PFAS (i. e., PFOA, PFBA) (**Fig. 7**). Thus, 21 day-old male rats (n=5/group) were randomized by weight into five groups and provided drinking water containing 0, 1, 10, 100 and 1000 ng/L PFOA and PFBA. Chemical exposures lasted 14 days (postnatal day, PND 21-35). We repeated the experiment using the same exposure paradigm to assess effects due to a sulfonic acid-containing legacy and emerging PFAS (i.e., PFOS, PFBS). At the end of both experiments, we sacrificed animals by cervical dislocation immediately upon terminating chemical exposure and collected blood and testes for steroid hormone measurements (T and 17β-estradiol (E2)).

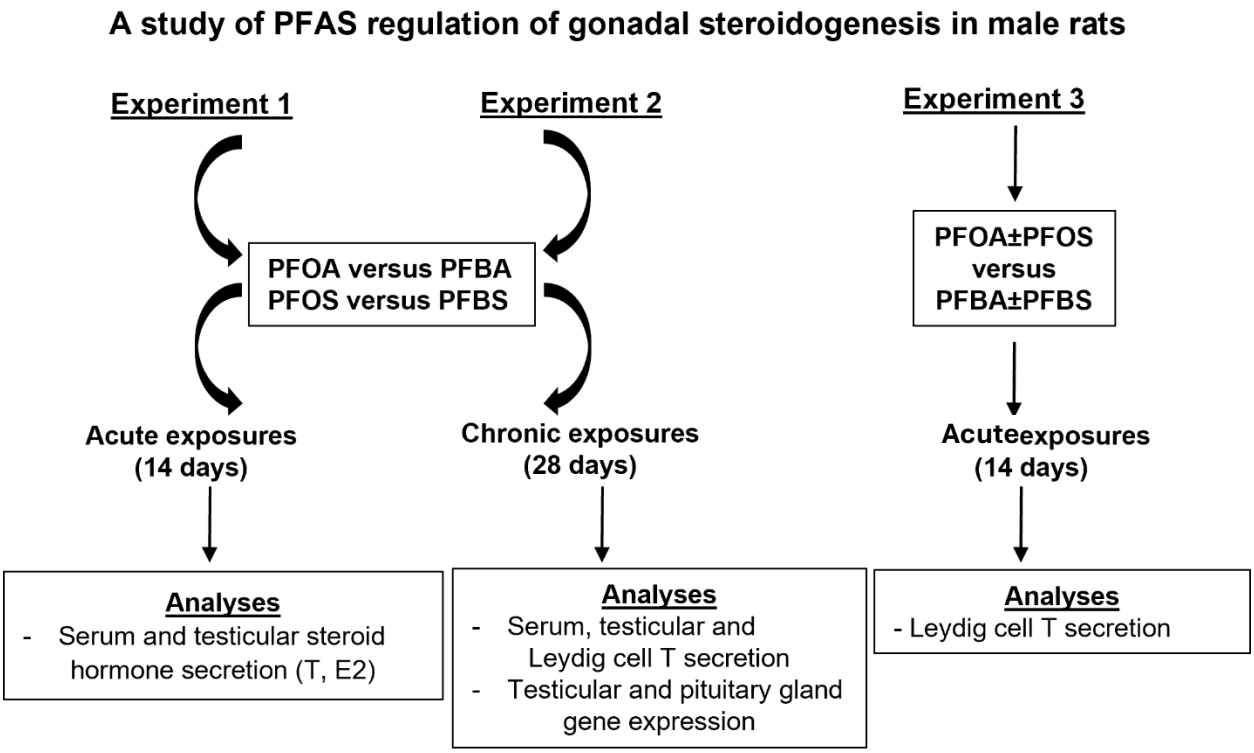

Figure 7: Experimental approach to study of per- and polyfluoro alkyl substances (PFAS) in the male gonad. T, testosterone; E2, 17β-estradiol; PFOA, perfluoroalkyl carboxylic acid; PFOS, perfluorooctane sulfonic acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid. In this study, carboxylic acid containing PFOA and sulfonic acid containing PFOS are legacy PFAS while PFBA and PFBS are their emerging PFAS replacements.

Experiment 2

Experiments were performed to determine effects due to pubertal PFAS exposures on gonadal steroid hormone secretion. Therefore, 21 day-old male rats randomly assigned to five groups (n=12/group) were provided drinking water containing 0, 10, and 100 ng/L of a carboxylic acid-containing legacy and emerging PFAS, i.e.,

PFOA and PFBA (first experiment) and a sulfonic acid-containing legacy and emerging PFAS, i.e., PFOS and PFBS (second experiment). Chemical exposures lastedfor 28 days. Animals were sacrificed at termination of chemical exposures to collect blood and testes for steroid hormone measurements (T, E2). Leydig cells were isolated from testes from each treatment group. In addition, testes and pituitary glands were obtained to measure gene protein expression.

Experiment 3

Experiments were performed to determine effects due to PFAS when acting alone and in combination. In the first experiment, we investigated two legacy compounds and their mixture. Thus, prepubertal male rats at 21 days of age were provided drinking water containing 10 ng/L of each test chemical and their combination at 5 ng/L of individual chemicals: control, PFOA, PFOS, PFOA+PFOS (four groups; n=10 animals/group). In a separate experiment, 21 day-old prepubertal male rats were fed drinking water containing 10 ng/L PFBA or PFBS and their combination at 5 ng/L of individual chemicals: control, PFBA, PFBS, PFBA+PFBS (four groups; n=10 animals/groups). In both experiments, chemical exposures were for a total of 14 days (PND 21-35, prepubertal exposure). At termination of chemical exposure, testes were obtained from animals at sacrifice for isolation of Leydig cells. We elected to limit assessment of mixture effects to Leydig cell T production because it was practicable to normalize Leydig cell numbers across treatment groups *ex vivo* to measure T production and facilitate data analysis.

Isolation of Leydig cells

At sacrifice, testes were obtained for digestion in a dissociation buffer containing 0.25 mg/mL collagenase, 46 µg/mL dispase and 6 µg/mL DNase for 1 h in a shaking water bath at 34 °C. Seminiferous tubules were removed by passing through a nylon mesh with a pore size of 0.2 µm (Spectrum Laboratories, New Brunswick, NJ) and supernatant centrifuged at 1000 rpm for 10 min at 4°C. Cell fractions were then loaded onto a Percoll gradient (Sigma-Aldrich, St. Louis, MO) and centrifuged at 13 500 rpm for 60 min at 4°C. Leydig cells were isolated from the Percoll gradient based on density at 1.069–1.073[87,88]. Leydig cell numbers were estimated with a hemocytometer. We assessed the purity of Leydig cell fractions by histochemical staining for 3βHSD using 0.4 mM etiocholan-3β-ol-17-one as the enzyme substrate (Sigma-Aldrich, St. Louis, MO).

Hormone Measurements

Serum was from trunk blood collected at sacrifice. Testicular explants (~100 mg) and aliquots of Leydig cells ($0.1–0.2 \times 10^6$) were incubated in microcentrifuge tubes containing DMEM/F-12 culture medium buffered with 14 mm $NaHCO_3$, 15 mm HEPES, 0.1% BSA, and 0.5 mg/mL bovine lipoprotein. Incubations were conducted without (basal) and with a maximally stimulating dose of ovine LH (100 ng/mL; LH-stimulated) for 3 h at 34 °C. Aliquots of serum and spent media were assayed for steroid hormone concentrations (T, 17β-estradiol [E2]) using a tritium-based radioimmunoassay with an inter-assay variation of 7–8%[89]. Hormone concentrations are in nanogram per testicular mass (ng/g) and ng/10^6 Leydig cells.

SDS-PAGE

We analyzed expression of several proteins in testes (Amh, inhibin Bβ, StAR,) and pituitary glands (LHβ, FSHβ). Tissues were homogenized in T-PER lysis buffer (Pierce Biotechnology, Rockford, IL) that was freshly supplemented with a protease inhibitor cocktail (Catalog #78410; Pierce Biotechnology, Rockford, IL). To remove cellular debris, samples were centrifuged at 3000 rpm for 14 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay with BSA as standard (Bio-Rad Laboratories, Hercules, CA). We added aliquots (50 µL) of whole-cell lysates to equal volumes of Laemmli buffer with 5% β-mercaptoethanol and boiled for 5 min at 95 °C. All samples were resolved on varying percentages of Tris-HCl acrylamide gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To reduce nonspecific binding by antibodies, proteins were transferred to nitrocellulose membranes (Catalog #1620147; Bio-Rad Laboratories) and incubated in blocking buffer (Intercept Blocking Buffer with PBS; LI-COR Biosciences) for 1 h at room temperature. Incubation of membranes were performed in blocking buffer containing appropriate primary antibodies overnight at 4 °C. Parameters of primary antibodies used in the present study are in **Table 1**. The following day, blots were washed once in distilled water and three times in 0.1% Tween-20 PBS to remove any unbound primary antibody before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing once in distilled water and at least four times in 0.1% Tween-20 PBS, membranes were scanned using a LI-COR Odyssey Infrared Scanner (Lincoln, NE). All measures of protein are normalized to β-actin.

Table 1: Primary antibodies nomenclature and dilutions.

Target	Antibody Sequence	Antibody Name/MW	Manufacturer, Catalog Number	Polyclonal/ Monoclonal	Dilution Used	Antibody Research Resource Identifier
Steroidogenic Acute Regulatory Protein	StAR (human) mapping to 8p11.23; StAR (mouse) mapping to 8 A2	StAR (D-2) MW (StAR) 30 kDa	Santa Cruz Biotechnologies, Sc166821	Mouse monoclonal IgG	1:250	AB_2197661
Anti-Mullerian Hormone	Genetic locus: AMH (human) mapping to 19 p13.3; AMH (mouse) mapping to 10C1	MIS (C-20) MW (AMH) 70/74 kDa	Santa Cruz Biotechnologies, Sc-6886	Mouse monoclonal IgG	1:500	AB_649207
InhibinB-β	Belongs to the TGFβ superfamily og growth and differentiation factors. Genetic locus: INHBB (human) mapping to 2q14.2; inhbb (mouse) mapping to 1 E2.3	Inhibin β-B (B-9) MW 45kDa	Santa Cruz Biotechnologies, sc-390959	Mouse monoclonal IgG	1:500	AB_2943028
Follicle Stimulating Hormone-β	Genetic locus: FSHB (human) mapping to 11q14.1; Fshb (mouse) mapping to 2E3	FSHβ (C-12) MW (FSHβ) 24 kDa	Santa Cruz Biotechnologies, Sc-374452	Mouse monoclonal IgG	1:200	AB_10990134
Luteinizing Hormone-β	Genetic locus: LHB (human) mapping to 19q13.33; Lhb(mouse) mapping to 7B4	Lutropin-β (C-6) MW (LHβ) 22 kDa	Santa Cruz Biotechnologies, sc-373941	Mouse monoclonal IgG	1:200	AB_10917935
Actin-β	Epitope mapping at the C\ terminus of actin of human origin	Beta Actin antibody (GT5512) MW 42 kDa	GeneTex, GTX629630	Mouse monoclonal IgG	1:2000	AB_2728646

Statistical analysis

Data are described as mean ± SEM. Analysis of data was by one-way ANOVA and the Dunnett test for multiple group comparisons (Graph Pad Prism software. San Diego, CA). Differences of ≤0.05 were significant.

Results

General observations

Body weights at the beginning and end of each experiment were similar in control and treated groups (data not shown). There were no signs of weakness or illness during the treatment period and no deaths occurred for any treatment group.

Effects of prepubertal exposures to PFAS on testicular steroid hormone production

Exposure of growing male rats to PFAS was in drinking water containing graded doses of test chemicals for two weeks (14 days). The results showed that PFOA did not affect ($P>0.05$) serum T concentrations (**Fig. 8A**), basal (**Fig. 8B**) and LH-stimulated testicular T production (**Fig. 8C**). On the other hand, exposure to 10 ng/L PFBA increased all three parameters, i.e., serum T ($P<0.01$) (**Fig. 8D**), basal ($P<0.0001$) (**Fig. 8E**) and LH-stimulated testicular T production ($P<0.05$; **Fig. 8F**). Also, serum T concentrations were increased ($P<0.05$) after exposure of male rats to 100 ng/L PFOS (**Fig. 9A**) while basal testicular T production was increased by the 1000 ng/L PFOS dose ($P<0.05$) (**Fig. 9B**). Interestingly, LH-stimulated T production was decreased after exposure to the 10 ng/L PFOS dose ($P<0.01$) (**Fig. 9C**). However, exposure to PFBA at

a lower dose (1 ng/L) increased (P<0.01) serum T (**Fig. 9D**) and basal testicular T production (**Fig. 9E**) but PFOS did not affect LH-stimulated testicular T production (P>0.05) at all test doses (**Fig. 9F**).

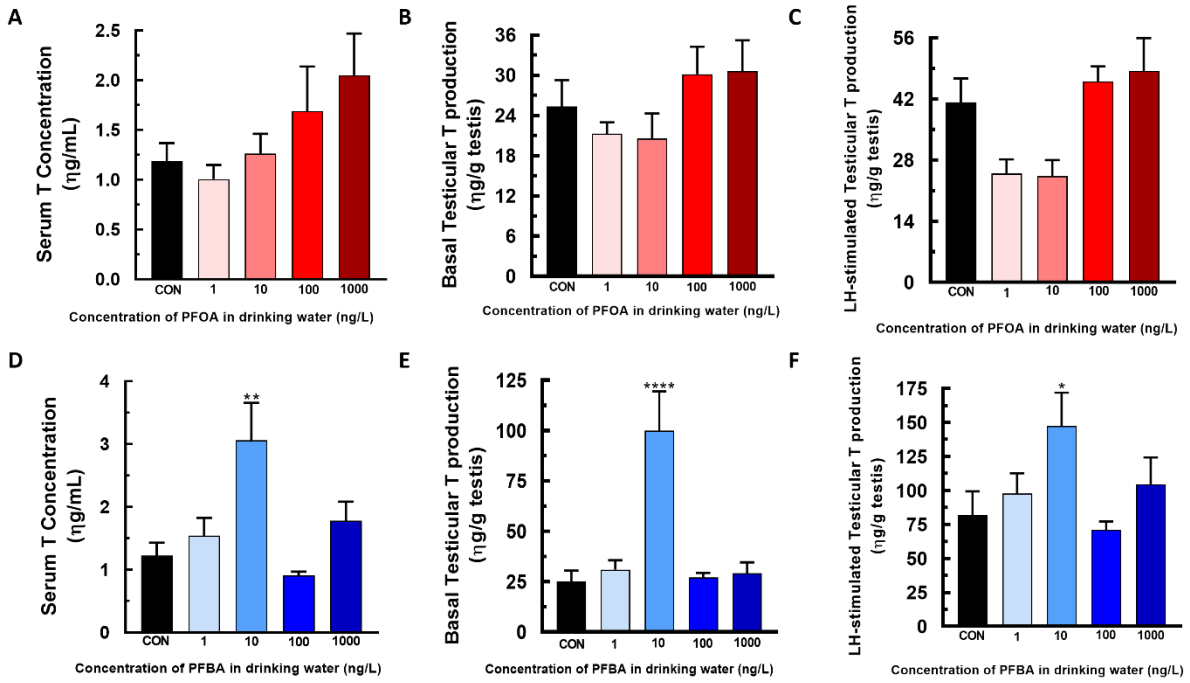

Figure 8: Effects of prepubertal exposure to PFOA and PFBA on serum and testicular testosterone (T) production. Male rats at 21 days of age (n=5/group) were provided drinking water or water containing 1, 10, 100, or 1,000 ng/L of carboxylic acid containing PFOA and PFBA for 14 days. Serum, basal and LH-stimulated testicular T production were measured by RIA as described in Materials and Methods. Color legend: black, control (CON); red, PFOA; blue, PFBA. *P < 0.05, **P < 0.01, ****P<0.0001 versus control.

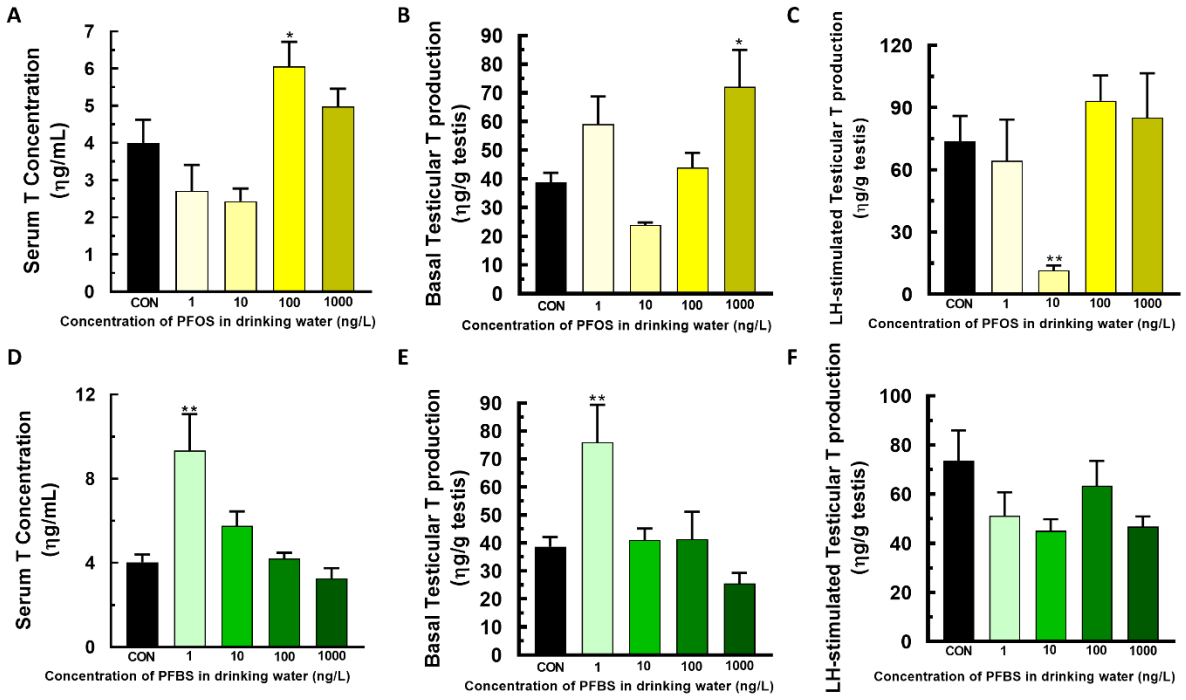

Figure 9: Effects of prepubertal exposure to PFOS and PFBS on serum and testicular testosterone (T) production. Male rats at 21 days of age (n=5/group) were provided drinking water or water containing 1, 10, 100, or 1,000 ng/L of sulfonic acid containing PFOS and PFBS for 14 days. Serum, basal and LH-stimulated testicular T production were measured by RIA as described in Materials and Methods. Color legend: black, control (CON); yellow, PFOS; green, PFBS. *P < 0.05, **P < 0.01 versus control.

Serum E2 concentrations were unaffected by exposure of male rats to PFOA (P>0.05; **Fig. 10A**). However, exposure to the 100 ng/L dose decreased (P<0.05) basal testicular E2 production (**Fig. 10B**) while PFOA did not affect LH stimulated T production at all doses (**Fig. 10C**). Serum E2 concentrations were also not affected by PFBA (P>0.05; **Fig. 10D**) but the highest PFBA dose (1000 ng/L) increased basal

(P<0.01; **Fig. 10E**) and LH-stimulated testicular E2 production (P<0.05; **Fig. 10F**). Interestingly, exposure to PFOS decreased serum E2 concentrations only at the 1000 ng/L dose (P<0.01; **Fig. 11A**), but did not affect basal testicular E2 production at all test doses (P>0.05; **Fig. 11B**) and increased LH-stimulated testicular E2 production at the 10 and 100 ng/L doses (P<0.01; **Fig. 11C**). In contrast, serum E2 concentrations were decreased (P<0.001) by exposure to PFBS at all test doses (**Fig. 11D**) while basal testicular E2 production was increased (P<0.05) by the 1000 ng/L dose (**Fig. 11E**) and exposure to the 10 and 1000 ng/L doses increased (<0.01) LH-stimulated testicular E2 production (**Fig. 11F**).

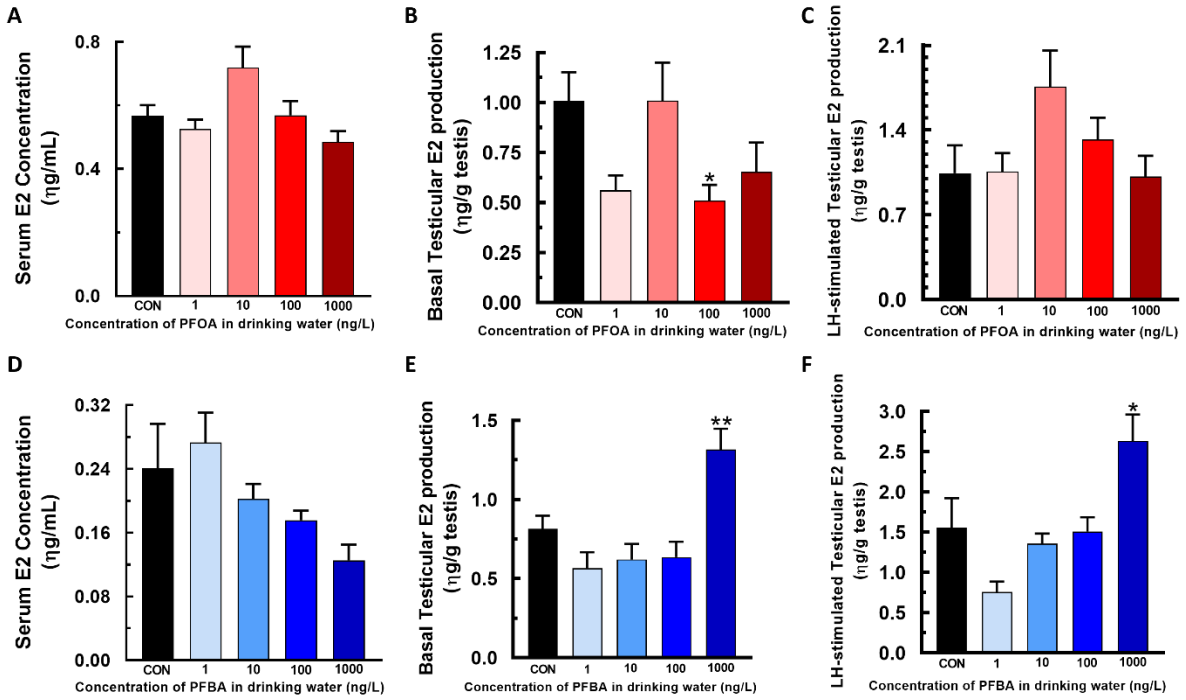

Figure 10: Effects of prepubertal exposure to PFOA and PFBA on serum and testicular 17β-estradiol (E2) production. Male rats at 21 days of age (n=5/group) were provided drinking water or water containing 1, 10, 100, or 1,000 ng/L of carboxylic

acid containing PFOA and PFBA for 14 days. Serum, basal and LH-stimulated testicular E2 production were measured by RIA as described in Materials and Methods. Color legend: black, control (CON), red, PFOA; blue, PFBA. *P < 0.05, **P < 0.01 versus control.

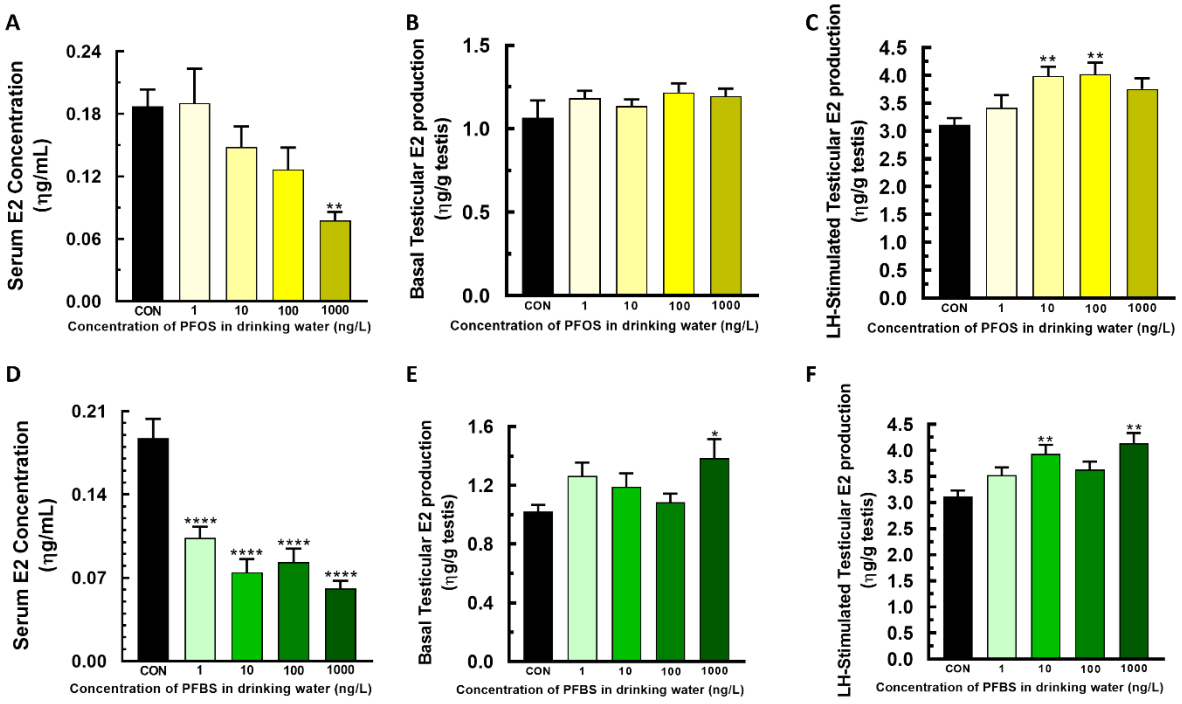

Figure 11: Effects of prepubertal exposure to PFOS and PFBS on serum and testicular 17β-estradiol (E2) production. Male rats at 21 days of age (5/group) were provided drinking water or water containing 1, 10, 100, or 1,000 ng/L of sulfonic acid containing PFOS and PFBS for 14 days. Serum (A, B), basal (C, D), and LH-stimulated testicular E2 production were measured by RIA as described in Materials and Methods. Color legend: black, control (CON); yellow, PFOS; green, PFBS. *P < 0.05, **P < 0.01, P<0.0001 versus control.

Effects of pubertal exposures to PFAS on testicular steroid hormone production

Exposure of male rats to PFAS was through drinking water containing 10 or 100 ng/L of test chemicals for 4 weeks (28 days). The results showed that exposure to 10 ng/L PFOA decreased but PFBA at the same dose increased serum T concentrations ($P<0.05$) (**Fig. 12A**). Basal testicular T production was unaffected by PFOA and PFBA ($P>0.05$; **Fig. 12B**) while exposure to 10 ng/L PFBS increased ($P<0.05$) LH-stimulated testicular T production (**Fig. 12C**). Similarly, exposure to 10 ng/L PFOA and PFBA both increased ($P<0.001$) basal and LH-stimulated Leydig cell T production but the 100 ng/L PFBA dose caused a decrease ($P<0.01$) in basal Leydig cell T production (**Fig. 12D, E**). On the other hand, exposure of male rats to the 10 and 100 ng/L doses of both PFOS and PFBS did not affect serum T concentrations ($P<0.05$; **Fig. 13A**), but these doses all decreased basal testicular T production ($P<0.01$) (**Fig. 13B**) with no effect on LH-stimulated testicular T production (**Fig. 13C**). Exposure to 10 and 100 ng/L doses of PFOS and PFBS decreased ($P<0.001$) basal Leydig cell T production (**Fig. 13D**). However, LH-stimulated Leydig cell T production was decreased ($P<0.001$) only by the 10 ng/L dose of PFOS and PFBS with no effect due to the larger 100 ng/L dose (**Fig. 13E**).

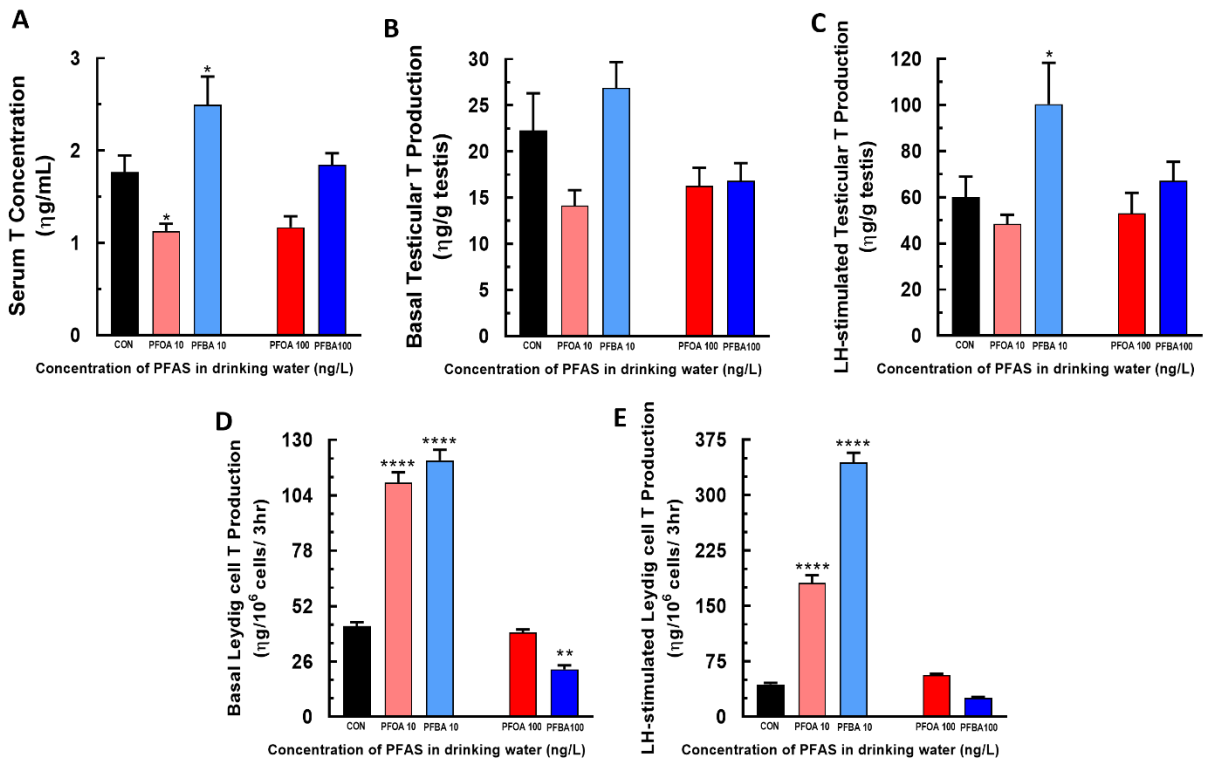

Figure 12: Effects of pubertal exposure to PFOA and PFBA on testicular testosterone (T) production. Male rats at 21 days of age (n=12/group) were provided with drinking water or water containing 10 or 100 ng/L of carboxylic acid containing PFOA and PFBA for 28 days. Serum (A), testicular explants (B, C) and Leydig cell T production (D, E) were measured by RIA as described in Materials and Methods. Color legend: black (CON); red, PFOA; blue, PFBS. *P<0.05, **P < 0.01, ****P < 0.0001 versus control.

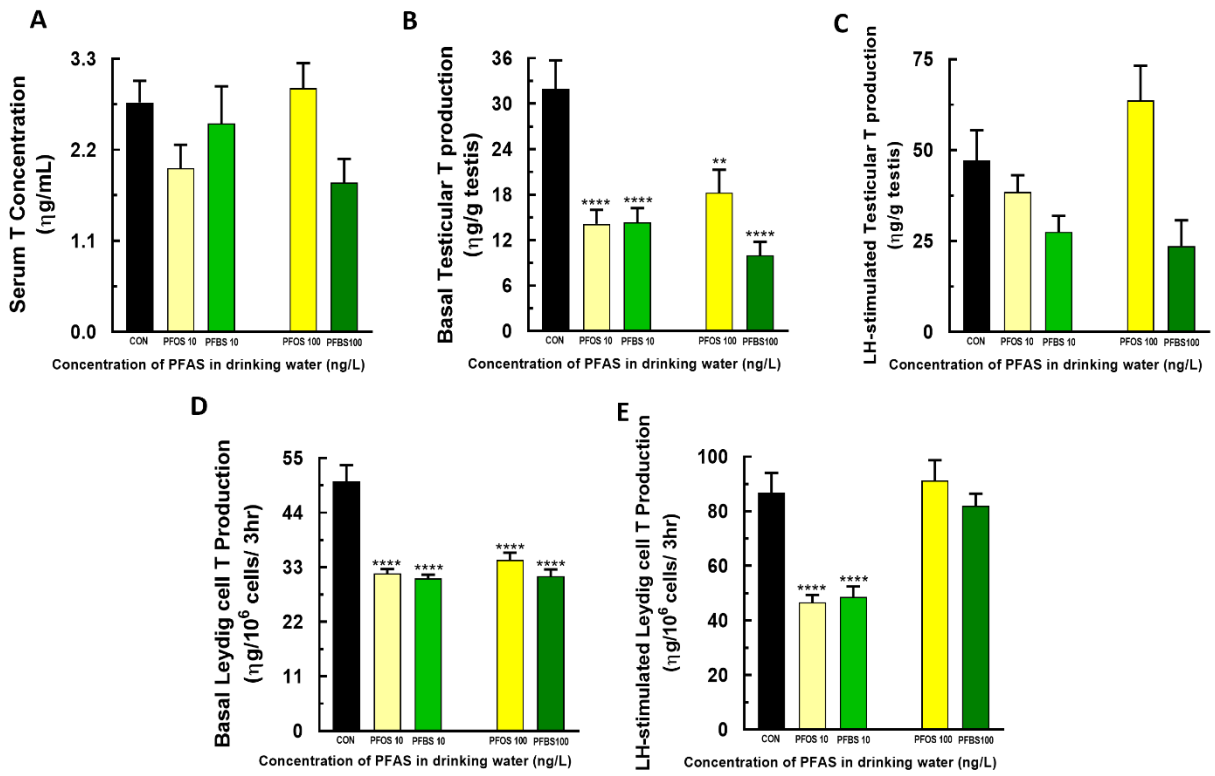

Figure 13: Effects of pubertal exposure to PFOS and PFBS on testicular testosterone (T) production. Male rats at 21 days of age (n=12/group) were provided with drinking water or water containing 10 or 100 ng/L of sulfonic acid containing PFOS and PFBS for 28 days. Serum (A), testicular explants (B, C) and Leydig cell T production (D, E) were measured by RIA as described in Materials and Methods. Color legend: black, control (CON); yellow, PFOS; green PFBS. **P < 0.01, ****P < 0.0001 versus control.

Effects of pubertal exposures to PFAS on gene expression in the testes and pituitary gland

Analysis of western blots showed that exposure of male rats to the 100 ng/L dose of PFBA and PFOS both increased ($P<0.05$) testicular StAR protein compared to control (**Fig. 14A, B**). Similarly, the MIS protein was increased by exposure to the 100 ng/L dose of PFOA ($P<0.05$) and PFBA ($P<0.01$) and 10 ng/L PFOS ($P<0.01$; **Fig. 14C, D**). However, testicular inhibin Bβ was decreased after exposure to 10 and 100 ng/L PFOA ($P<0.05$), 100 ng/L PFBA ($P<0.01$), and the 10 and 100 ng/L doses of PFOS and PFBS ($P<0.01$) (**Fig. 14E, F**).

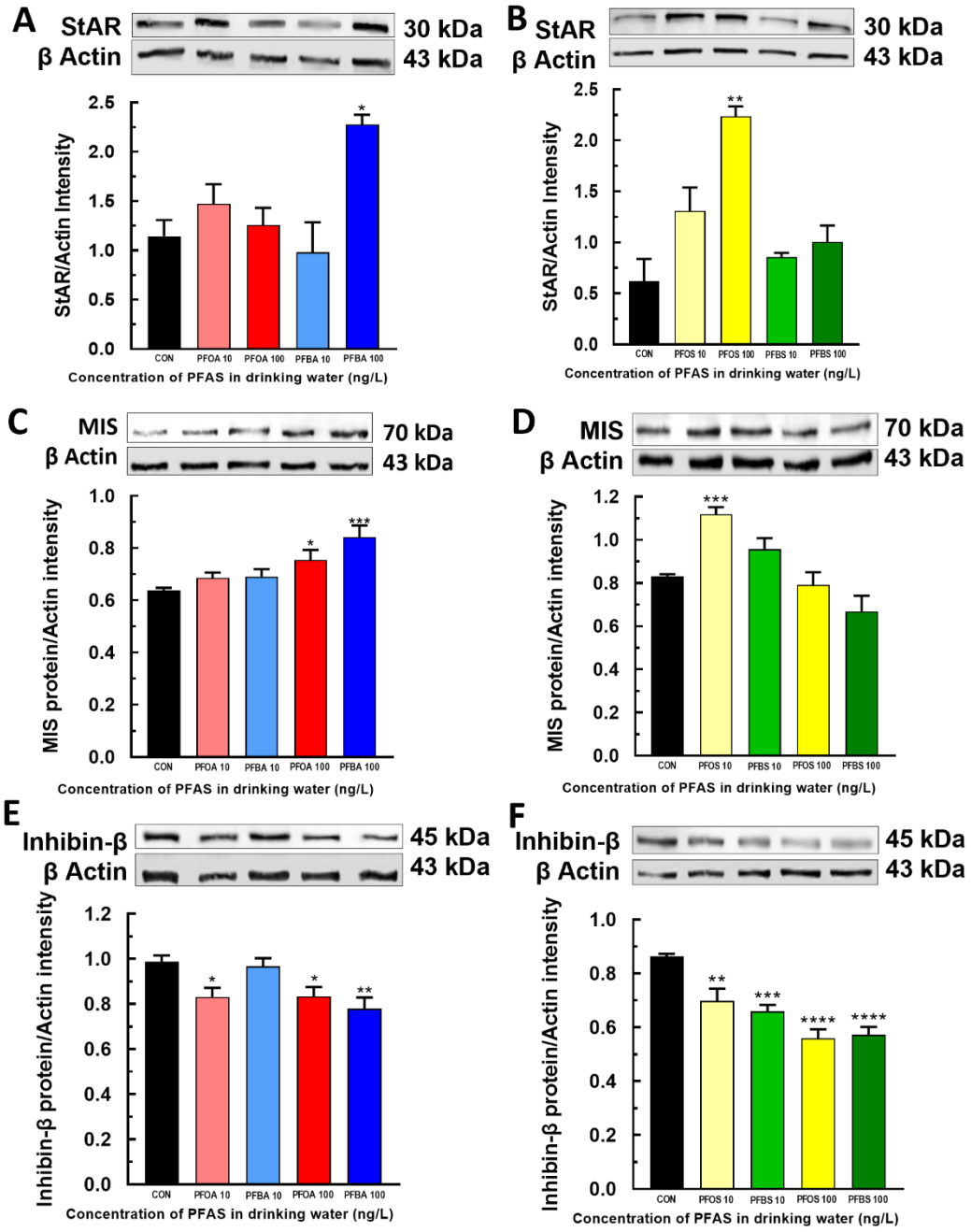

Figure 14: Effects of pubertal exposure to PFAS on testicular gene expression.

Male rats at 21 days of age (n=12/group) were provided with drinking water or water containing 10 or 100 ng/L of PFOA and PFBA or PFOS and PFBS for 28 days. The steroidogenic acute regulatory protein (StAR) (A, B), Müllerian inhibiting substance (MIS, C, D) and inhibin Bβ (E, F) were measured in western blots of testes from control

and PFAS-treated groups (n=3-4/group) and normalized to β-actin as described in Materials and Methods. Color legend: black, control (CON); red, PFOA; blue, PFBA; yellow, PFOS; green, PFBS. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001 versus control.

Furthermore, exposure of male rats to PFAS dysregulated gonadotropin β-subunit protein because the 10 ng/L PFBA dose (P<0.05) and 10 ng/L (P<0.01) and 100 ng/L PFOS doses decreased pituitary FSHβ subunit protein (**Fig. 15A, B**). However, the LHβ subunit protein was increased after exposure to 10 ng/L PFOA (P<0.01; **Fig. 15C**) and was decreased (P<0.05) by exposure to the 10 and 100 ng/L doses of PFOS (P<0.05) (**Fig. 15D**).

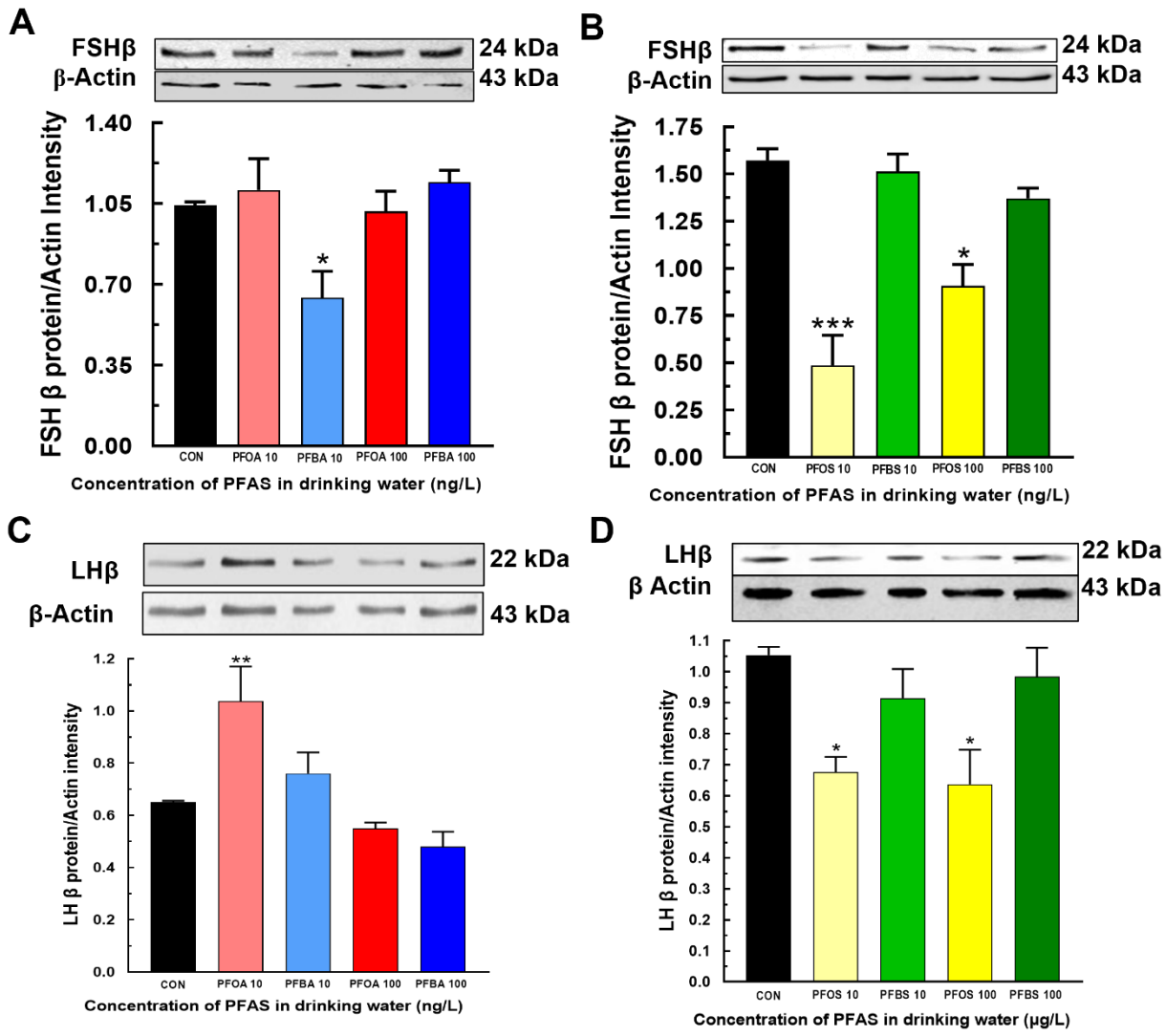

Figure 15: Effects of pubertal exposure to PFAS on gonadotropin-subunit protein expression. Male rats at 21 days of age were provided with drinking water or water containing 10 or 100 ng/L of PFOA and PFBA or PFOS and PFBS for 28 days (n=12/group). The follicle stimulating hormone β-subunit (FSHβ) (A, B) and lutrotopin β-subunit (LHβ) (C, D) were measured in western blots of pituitary glands from control and PFAS-treated groups (n=3-4/group) and normalized to β-actin as described in Materials and Methods. Color legend: black, control (CON); red, PFOA; blue, emerging PFAS PFBA; yellow, PFOS; green, PFBS.

*P < 0.05, **P<0.01, ***P<0.001 versus control.

Effects of prepubertal exposure to individual and PFAS mixtures on Leydig cell T production

Exposure to PFOA+PFOS, but not the individual compounds, i.e., PFOA and PFOS, decreased basal Leydig cell T production (**Fig. 16A**). Interestingly, exposure to PFOA, PFOS and PFOA+PFBS all decreased LH-stimulated Leydig cell T production to the same degree (P<0.001) (**Fig. 16B**). On the other hand, exposure to PFBA and/or PFBS did not affect basal Leydig cell T production (P>0.05) (**Fig. 16C**) but exposures to PFBS individually, as well as in combination with PFOA (i.e., PFBA+PFBS) decreased LH-stimulated Leydig cell T production (P<0.05) (**Fig. 16D**).

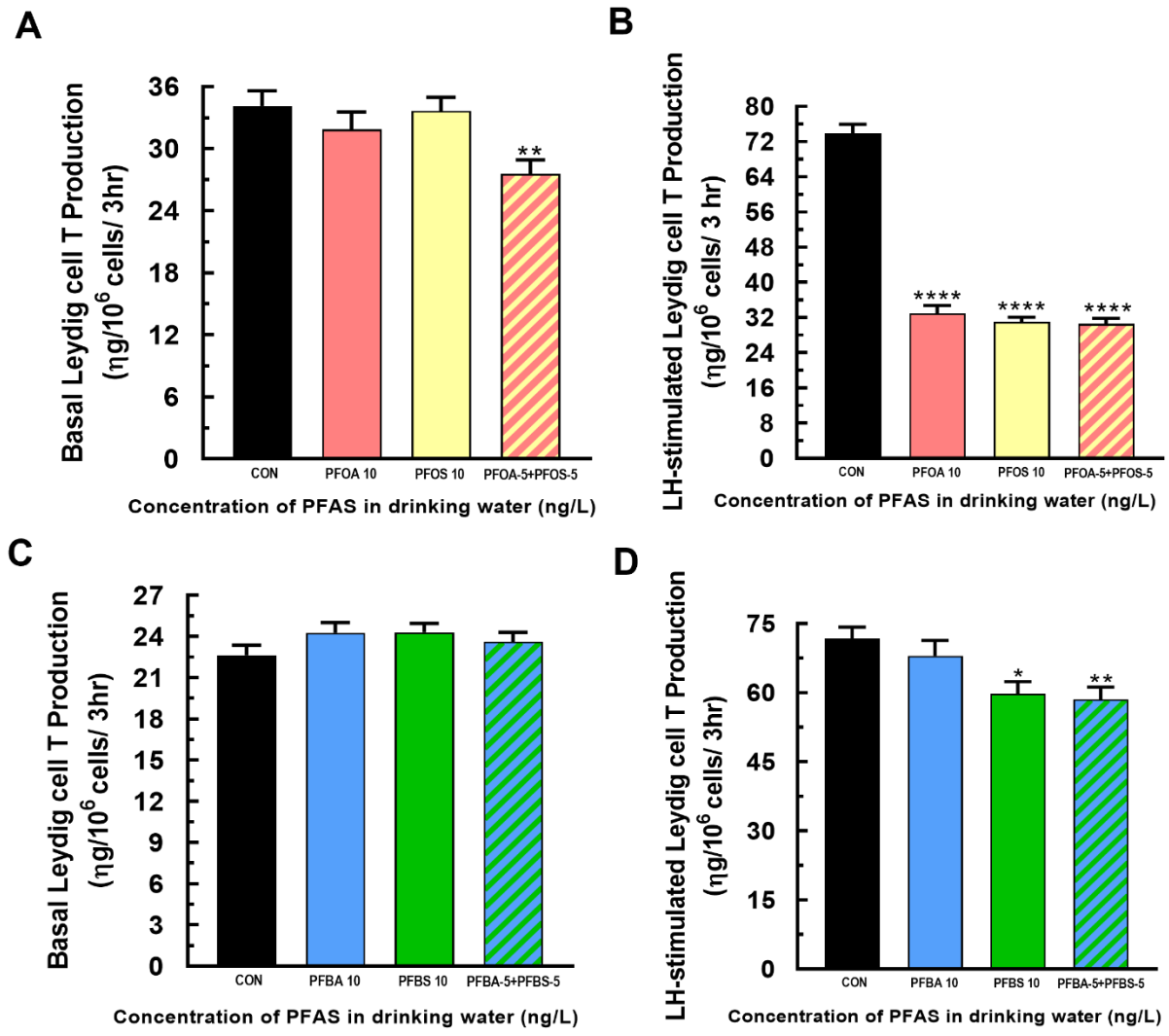

Figure 16: Effects of prepubertal exposure to single and combined PFAS on Leydig cell testosterone (T) production. Prepubertal male rats were provided drinking water containing 10 ng/L PFOA or PFOS and their combination: control (black), PFOA (red), PFOS (yellow) and PFOA+PFOS (yellow/red) (four groups; A, B). Experiment was repeated with PFBA (blue), PFBS (green) and PFBA+PFBS (blue/green) for 14 days (n=12/group; C, D). Basal and LH-stimulated Leydig cell T production was measured by RIA as described in Materials and Methods.*P <0.05, **P < 0.01, ****P < 0.0001 versus control.

Discussion

The present study demonstrated that PFAS have the capacity to regulate testicular steroidogenesis. Overall, prepubertal exposures to both PFOS, PFBA and PFBS increased serum and testicular T concentrations. Exposure PFBA increased testicular E2 concentrations while PFOS and PFBS both decreased serum E2 concentrations while stimulating testicular E2 secretion. On the other hand, pubertal low dose exposures to legacy and emerging PFAS regulated Leydig cell T production. Although direct PFAS effects in Leydig cells were not determined, past studies with human and rodent testicular cells showed that PFOA and PFOS decreased expression of steroidogenic proteins and enzymes[90-92]. Leydig cell androgen biosynthesis is under the primary control of pituitary LH, which binds to its receptors to stimulate mobilization and utilization of cholesterol for T biosynthesis in Leydig cells. Although Leydig cell numbers were equivalent in assays of T concentrations, we observed that pubertal exposures to both legacy and emerging PFAS increased or decreased Leydig cell T secretion. For example, low dose exposures to carboxylic acid containing PFAS tended to stimulate Leydig cell T secretion, whereas sulfonic acid containing PFOS and PFBS caused the opposite effect, i.e., inhibition of Leydig cell T secretion. These observations probably relate to PFAS modulation of Leydig cell sensitivity to LH stimulation[93]. Indeed, we observed that PFOS and PFBA both dysregulated expression of the StAR protein, which shuttles cholesterol substrate into Leydig cells. Thus, altered testicular T production due to PFAS exposures is related to disruption of steroidogenic capacity in Leydig cells. Analysis of the androgen biosynthetic pathway in Leydig cells in future

studies will identify specific steps targeted by PFAS. However, disparities between serum and testicular and Leydig cell T secretion may result in part from PFAS regulation of proliferative activity in Leydig cells. We did not investigate this possibility, but many EDCs are known to act as mitogens and stimulate Leydig cell proliferation during development in growing animals[94,95].

Moreover, both legacy and merging PFAS regulated serum E2 serum and testicular E2 secretion. The disparity between serum and testicular E2 concentrations may be related to PFAS regulation of steroidogenesis in adipose tissue, the largest source of circulating E2[96]. In this regard, EDCs are antiandrogenic if they suppressed gonadal androgen secretion and/or have the capacity to block androgen receptor (AR)-mediated activity. On the other hand, they are described as estrogenic if they regulated aromatase activity, blocked estrogen receptor (ER)-mediated signaling and/or dysregulated E2 production[97]. Analysis of AR and ER signaling were outside the focus of the present study, but PFAS-related changes in steroid hormone secretion (T and E2) suggest that PFAS possess both antiandrogenic and estrogenic properties. These findings have implications for reproductive activity because reproductive tract tissues and the neuroendocrine axis are sensitive to changes in their hormonal milieu related to T and E2 concentrations. Also, the present data showing that low dose pubertal exposures to PFOA and PFBA increased but PFOS and PFBS decreased Leydig cell T production is related to reports that EDCs can cause U- or inverted U-shaped nonmonotonic dose-response curves[98,99]. The nonmonotonic phenomenon is presumably receptor-mediated and likely to occur at low dose chemical exposures[100,101].

Reproductive development and function are subject to regulation by the interaction between steroid hormones, pituitary gonadotropins, and hypothalamic GnRH. These interactions are through paracrine factors and feedback mechanisms active at all levels of the HPG axis[102]. We evaluated the HPG axis by analysis of paracrine regulators, namely, MIS, inhibin Bβ and pituitary gonadotropin-subunit proteins (FSHβ, LHβ). Exposure to both legacy and emerging PFAS increased or decreased expression of testicular MIS and inhibin Bβ protein and affected FSHβ- and LHβ-subunit protein expression. Given our *in vivo* exposure model, it was not clear that PFAS changes were direct PFAS effects in testicular cells, pituitary gland and/or hypothalamus or were the result of changes in paracrine regulation of the HPG axis as mediated by T, E2, MIS and/or inhibin Bβ. Several lines of evidence showed previously that PFAS regulated the neuroendocrine axis. For example, exposure to PFOS suppressed ERα-induced activation of AVPV-kisspeptin neurons[103] while PFOA and PFOS both suppressed Kiss1, Kiss1r and ERα mRNA expression[104]. Similarly, PFAS effects on ovarian function were absent in *in vitro* studies compared to *in vivo* exposures, implying that PFOA is likely not acting directly in the ovary but disrupting the HPG axis[105]. Additional studies will identify the sites of PFAS action in the HPG axis. Nevertheless, PFAS-induced changes in paracrine regulators of the neuroendocrine axis are potentially detrimental to reproductive development and function.

In the present study, we assessed PFAS mixture effects on Leydig cell T secretion. The results showed that additive effects were due to PFAS mixtures compared to the individual chemicals. It is likely that mixture effects represent the sum of activity due to individual chemical constituents of the mixture. Thus, our observations

reinforce the view that studies of chemical mixtures, rather than single chemicals, offer a more holistic basis for risk assessment of the population. Indeed, a previous report demonstrated that combined maternal PFAS exposures were associated with lower sperm concentrations in young men compared to the action of individual chemicals[106]. Nevertheless, chemicals present within a mixture may act to counteract or enhance each other's action depending on whether they utilized similar mechanisms of action and/or were engaged in competitive binding to receptors. Also, chemical mixtures may generate metabolites with unknown or undetermined effects[58,67]. Together, these possibilities support development of strategies for studies of chemical mixture effects designed to quantify the totality of activity due to all chemicals acting together within a cocktail.

In summary, the present study provided evidence showing that both legacy and emerging PFAS have the capacity to regulate gonadal steroid hormone secretion. Interestingly, these effects occurred mostly at nanomolar concentrations, which approximate PFAS levels in the environment[107]. Both legacy and emerging PFAS also regulated testicular E2 concentrations with implications for androgen secretion and germ cell development. Although limited, our assessment of PFAS mixtures implies an additive effect, an important but concerning finding given the large number of PFAS present in the environment. Taken together, data from the present study support the need to develop cost-effective, adaptable, and sustainable filtration media for different processes to remove PFAS from water and other sources of exposure[65]. The results are also in tandem with action by regulatory agencies to limit use of PFAS in the manufacturing sector due to toxicity.

Chapter 2, Legacy and emerging per- and polyfluoroalkyl substances (PFAS) regulate steroidogenesis in the male gonad, is a published works.

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Chapter 3 - Regulation of gonadal steroidogenesis in male rats exposed to BPA, BPS, and EE2

Abstract

Bisphenol A (BPA) is a plasticizer commonly used in the production of household items. Its estrogenic characteristics have been linked to various human diseases, including diabetes, cardiovascular conditions, and reproductive issues in males. Due to ongoing concerns about BPA's safety, alternative bisphenols like Bisphenol S (BPS) have emerged, though their safety relative to BPA is still uncertain[108]. 17α-ethinyl estradiol (EE2), a synthetic form of the natural hormone 17β-estradiol (E2), is classified as a xenoestrogen and known to accumulate in the body. All three of these target chemicals are known endocrine disrupting chemicals (EDCs). Therefore, our research focuses on examining the effects of BPA, BPS, and EE2 on the male reproductive system. Experiments were conducted on Long-Evans male rats, exposing them to graded doses of BPA, BPS, or EE2, and impact on gonadal steroid hormone secretion was assessed. The findings indicated that these compounds can influence testicular steroidogenesis. For instance, BPA exposure over 56 days led to decreased serum testosterone (T) concentrations but increased basal testicular T across all doses. Leydig cells exhibited a dose-dependent response with both increases and decreases in T production. BPS exposure resulted in reduced serum and testicular T levels, while Leydig cells once again showed dose-dependent fluctuations. EE2 exposure at higher doses decreased serum and testicular T levels, while lower doses increased basal and

LH-stimulated Leydig cell T production, with the highest dose reducing it. Additionally, EE2 exposure reduced LH-stimulated testicular E2 production and increased basal Leydig cell E2 secretion at certain doses. Culturing Leydig cells with EE2 enhanced T production at most doses. Exposure to BPA increased the expression of testicular StAR and pituitary LHβ-subunit proteins. The study underscores the necessity for developing sustainable alternatives to BPA, BPS, and EE2, and improving filtration methods to eliminate these compounds from food and water, as their presence has significant implications for reproductive health. Further research is needed to understand the mechanisms by which these synthetic estrogens affect testicular function and the dysregulation of the HPG axis.

Introduction

Bisphenol A (BPA) is a high-production plasticizing agent used in manufacturing common household items like water bottles, food packaging, thermal receipts, and baby toys[109]. Discovered to have estrogenic properties in 1936, approximately 3.4 million tons of BPA are used annually in consumer products, leasing to widespread contamination of food and water sources[110]. BPA exposure has been linked to numerous human diseases, including diabetes, cardiovascular diseases, and reproductive abnormalities in both genders[111], and although the FDA has restricted the use of BPA in baby products, environmental contamination continues to expose infants. Consequently, further studies on BPA remain pertinent.

Concerns about BPA's safety have led to the introduction of bisphenol analogs like Bisphenol S (BPS), marketed as "BPA-free" in various products. Despite BPS's widespread use, its safety compared to BPA is still uncertain[108], with evidence suggesting possible toxicity, such as impaired sperm production in male rats[112]. Despite structural similarities to BPA, no regulations currently limit the use of BPS.

17α-ethinyl estradiol (EE2) is the primary synthetic form of E2 used in female oral contraceptives. In the human body, studies have shown that EE2 has a binding affinity of approximately twice that of E2[113] and is resistant to metabolism[114]. This results in significant unmetabolized excretion of approximately 80% of the administered dose into the environment via sewage, with 30% of EE2 remaining after wastewater treatment, significantly exceeding the lowest observed effect concentrations by about 61 fold[115]. Therefore, EE2 is one of the most potent and prevalent EDCs found in water systems[116], including surface water[117] used in agriculture and drinking water[118].

Altogether, EE2 is a prevalent EDC in water systems[119] impacting both ecological and public health[114]. For example, EE2 exposure disrupted reproductive health and caused morphological abnormalities in aquatic species[120-130], leading to increased mortality[131]. While extensive research exists on EE2's effects in aquatic species, its impact on mammalian male reproductive health are still widely unknown. Limited data suggest that EE2 exposures affects testosterone production in rats[115], possibly due to exposure to large amounts of various EDC exposures.

We hypothesized that graded doses of BPA, BPS, or EE2 impact steroidogenic development and capacity in the male gonad. The average weaning age for the rat is 21 days and puberty is attained at 50 days of age, which correspond to six months and 11.5 years in humans[78,79]. As such, the prepubertal and pubertal phases of development in the rat reflect infancy and early childhood in humans[80]. Given these parallels and our interests in developmental toxicity, prepubertal (PND 21-77) Long-Evans male rats were exposed to BPA, BPS, or EE2 for this study.

Materials & Methods

Chemicals and Reagents

Bisphenol A (Lot # 1065060 13706030), Bisphenol S (lot # BCBV2462), 17α- ethinylestradiol (lot # WXBC6630V), trypsin inhibitor, EDTA, HEPES, bovine serum albumin (BSA), bovine lipoprotein, sodium bicarbonate ($NaHCO_3$), Dulbecco Modified Eagle Medium (DMEM) nutrient mixture (Ham's F-12 (DMEM/F-12; 1:1 mixture without phenol red]), albumin, Percoll, etiocholan-3β-ol-17-one, and gentamicin were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco phosphate-buffered saline

(PBS), medium 199, and 10X Hanks balanced salt solution were obtained from Life Technologies, Inc. (Grand Island, NY). Collagenase, dispase, and deoxyribonuclease (DNase) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Ovine LH was a gift from the National Hormone and Pituitary Program (NIDDK, Bethesda, MD).

Animal Studies

Animal and euthanasia procedures were conducted following approved protocol by the Auburn University Institutional Animal Care and Use Committee and based on recommendations of the panel on Euthanasia of the American Veterinary Medical Association (protocol #2021-3977). Male Long–Evans rats were sourced from Envigo (Madison, WI). Animals were acclimated for two days at the Division of Laboratory Animal Health Housing Facility, College of Veterinary Medicine at Auburn University. Deionized water was provided in glass water bottles *ad libitum*. Animals were housed in plastic cages from Snyder Manufacturing Company (Centennial, CO) in order to minimize background exposure to EDCs that are present in resin-containing cages[132]. Animals were maintained under constant conditions of light (12 L: 12 D) and temperature (20–23.38 °C) with free access to pelleted food. All animals were fed the same diet with no or little soybean content (X2020, Harlan-Teklad, Madison, WI).

Experimental Approach

Experiments were performed to determine effects due to prepubertal BPA, BPS, or EE2 exposures on gonadal steroid hormone secretion. Therefore, 21 day-old male

rats randomly assigned to six groups (n=6/group) were provided drinking water containing 0, 1, 5, 10, 15, or 20 µg/L of BPA, BPS, or EE2. This exposure method mimics natural pathways[133]. Chemical exposures were for 56 days (**Fig 17**). Animals were sacrificed at termination of chemical exposures on PND 77 to collect blood and testes for steroid hormone measurements (T, E2). Leydig cells were isolated from testes from each treatment group to assess T production capacity.

Figure 17: Animal studies experimental timeline from birth to sacrifice.

Male rats at 19 days of age were received, grouped, and allowed two days of environmental acclimation. At weaning on 21 days of age, treatment commenced and continued for 56 days. Animals were sacrificed at 77 days of age.

Isolation of Leydig cells

Upon sacrifice, testes were digested in a dissociation buffer containing 0.25 mg/mL collagenase, 46 µg/mL dispase and 6 µg/mL DNase for 1 h in a shaking water bath at 34 °C. Seminiferous tubules were removed by passing through a nylon mesh with a pore size of 0.2 µm (Spectrum Laboratories, New Brunswick, NJ) and supernatant centrifuged at 1000 rpm for 10 min at 4°C. Cell fractions were then loaded onto a Percoll gradient (Sigma-Aldrich, St. Louis, MO) and centrifuged at 13 500 rpm for 60 min at 4°C. Leydig cells were isolated from the Percoll gradient based on density at 1.069–1.073[87,88]. Leydig cell numbers were estimated with a hemocytometer. The purity

of Leydig cell fractions was assessed by histochemical staining for 3βHSD using 0.4 mM etiocholan-3β-ol-17-one as the enzyme substrate (Sigma-Aldrich, St. Louis, MO).

Hormone Measurements

Serum was separated from trunk blood collected at sacrifice. Testicular explants (~100 mg) and Leydig cell aliquots (0.1–0.2 × 10^6) were incubated in microcentrifuge tubes containing DMEM/F-12 culture medium buffered with 14 mm NaHCO3, 15 mm HEPES, 0.1% BSA, and 0.5 mg/mL bovine lipoprotein. Incubations were conducted without (basal) and with a maximally stimulating dose of ovine LH (100 ng/mL; LH-stimulated) for 3 h at 34 °C. Aliquots of serum and spent media were assayed for steroid hormone concentrations (T, 17β-estradiol [E2]) using a tritium-based radioimmunoassay with an inter-assay variation of 7–8%[89]. Hormone concentrations are described in nanogram per mL for serum, ng/g for testes, and ng/10^6 Leydig cells.

In Vitro *Studies*

In vitro assays were performed with primary Leydig cell cultures isolated from 35-day-old male rats not previously exposed to BPA, BPS, or EE2. Leydig cells were incubated in DMEM/F-12 culture medium containing BPA, BPS, or EE2 at 0, 0.01, 0.1, 1, 10 μM in the presence of LH (10 ηg/mL ovine LH) for 18 h at a temperature of 34°C. After treatment, aliquots of spent media were analyzed for T concentrations and E2 production.

Statistical analysis

Data are mean ± SEM. Analysis of data was performed by one-way ANOVA followed by the Dunnett test for multiple group comparisons (Graph Pad Prism software. San Diego, CA). Differences of ≤0.05 were considered to be significant.

Results

General Observations

Body weights and testes weights at the beginning and end of chemical exposure remained consistent across control and treated groups for all compounds. Average body weights for EE2 are shown below **(Table 2).** Likewise, average daily food and water intake was similar in control and treatment groups for all compounds (data not shown). There were no signs of weakness or illness during the treatment period and no mortality occurred for any treatment group.

Table 2: Average change in EE2- treated animal weights (g) by group over time.

	Control	1	5	10	15	20
Starting weight	79.0 ± 2.63	81.3 ± 2.72	79.0 ± 3.97	80.8 ± 2.89	79.3 ± 1.76	80.6 ± 2.80
Week 2	129.5 ± 4.58	135.7 ± 5.41	135.5 ± 5.98	137.7 ± 4.33	132.0 ± 3.33	131.8 ± 4.41
Week 3	200.3 ± 9.00	208.8 ± 7.96	207.0 ± 8.15	208.3 ± 7.43	199.3 ± 4.81	196.7 ± 6.26
Week 4	278.7 ± 15.55	293.5 ± 12.42	299.2 ± 11.53	298.5 ± 12.19	274.8 ± 5.92	268.8 ± 5.95
Week 5	310.2 ± 16.56	327.0 ± 12.95	331.0 ± 11.91	325.8 ± 13.85	304.2 ± 7.76	295.2 ± 7.28
Week 6	340.5 ± 18.82	358.5 ± 14.67	364.5 ± 13.36	359.5 ± 16.30	334.5 ± 10.49	325.7 ± 6.84
Week 7	366.3 ± 20.62	390.5 ± 15.50	390.0 ± 14.62	381.0 ± 16.84	356.5 ± 10.64	344.7 ± 8.02
Ending weight	374.2 ± 19.81	394.5 ± 18.56	405.7 ± 13.51	393.3 ± 17.58	364.2 ± 10.96	354.3 ± 8.61

Male rats at 21 days of age (n=6/group) were maintained on normal deionized water (CON) or water with 1, 5, 10, 15, or 20 µg/L of EE2 for 56 days. Weights were recorded periodically throughout treatment.

Effects of prepubertal exposures to BPA, BPS, and EE2 on serum and testicular testosterone (T) secretion

Exposure of growing male rats to BPA, BPS, or EE2 was in drinking water containing graded doses of the compounds for 56 days (PND 21-77). The results showed that BPA did not affect (P>0.05) serum T concentrations or basal testicular T production (**Fig. 18A, B**). LH-stimulated testicular T production, however, was increased in a dose-dependent manner at the 10 (P<0.05) and 15 (P<0.01) µg/L doses (**Fig. 18C**). On the other hand, exposure to BPA at the higher doses of 10, 15, and 20 µg/L increased (P<0.0001) basal Leydig cell T production, with a smaller, but still significant increase (P<0.05) also seen at the smallest dose of 1 µg/L (**Fig. 18D**) while LH-stimulated (**Fig. 18E**) Leydig cell T production was increased (P<0.0001) by only the highest dose. Interestingly, the 5, 10, and 15 µg/L doses decreased (P<0.0001) LH-stimulated Leydig cell T production.

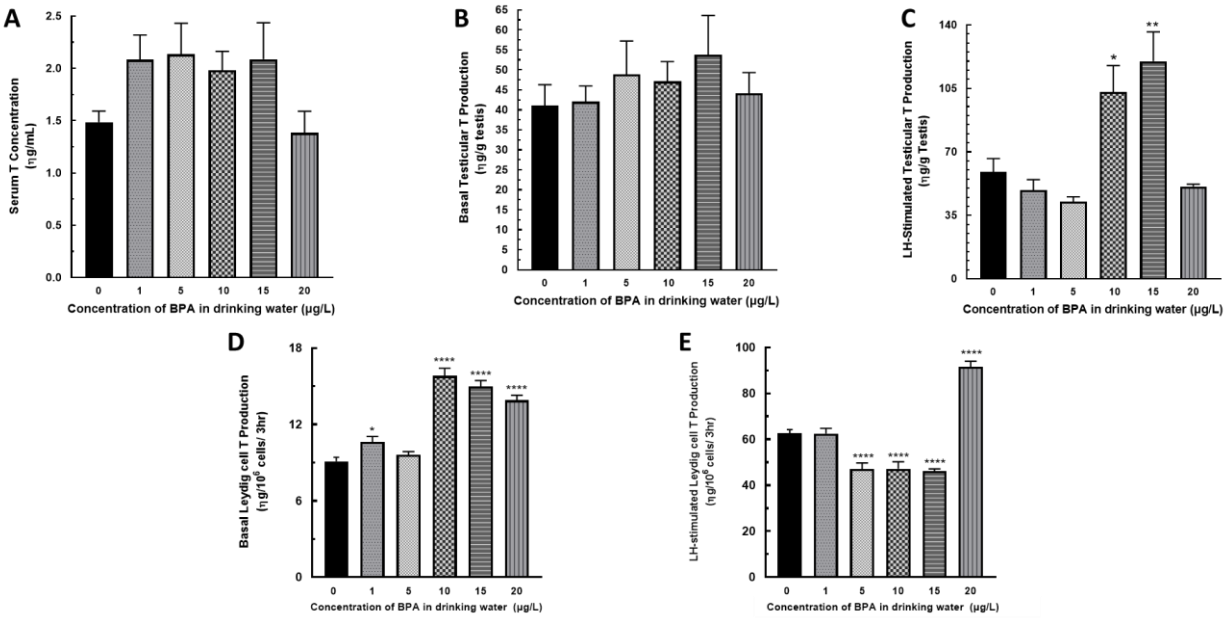

Figure 18: Effects of prepubertal exposure to BPA on testicular testosterone (T) production. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of BPA for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular explants (B, C) and Leydig cell T production (D, E) were measured by RIA. *P < 0.05, **P < 0.01, ****P < 0.0001 versus control.

Exposure to BPS decreased (P<0.05) serum T concentrations at all doses, but conversely increased (P<0.0001) basal testicular T production at all doses (**Fig. 19A, B**). LH-stimulated testicular T production (**Fig. 19C**) showed a decrease (P<0.01) only at the 5 µg/L dose. Exposure of basal Leydig cells to BPS resulted in a strong dose-dependent curve in T production, with an increase at the 1 (P<0.0001) and 5 (P<0.05)

µg/L doses, but a decrease at the 15 (P<0.01) and 20 (P<0.0001) µg/L doses (**Fig. 19D**). LH-stimulated (**Fig. 19E**) Leydig cell T production was decreased (P<0.05) only at the highest dose of BPS.

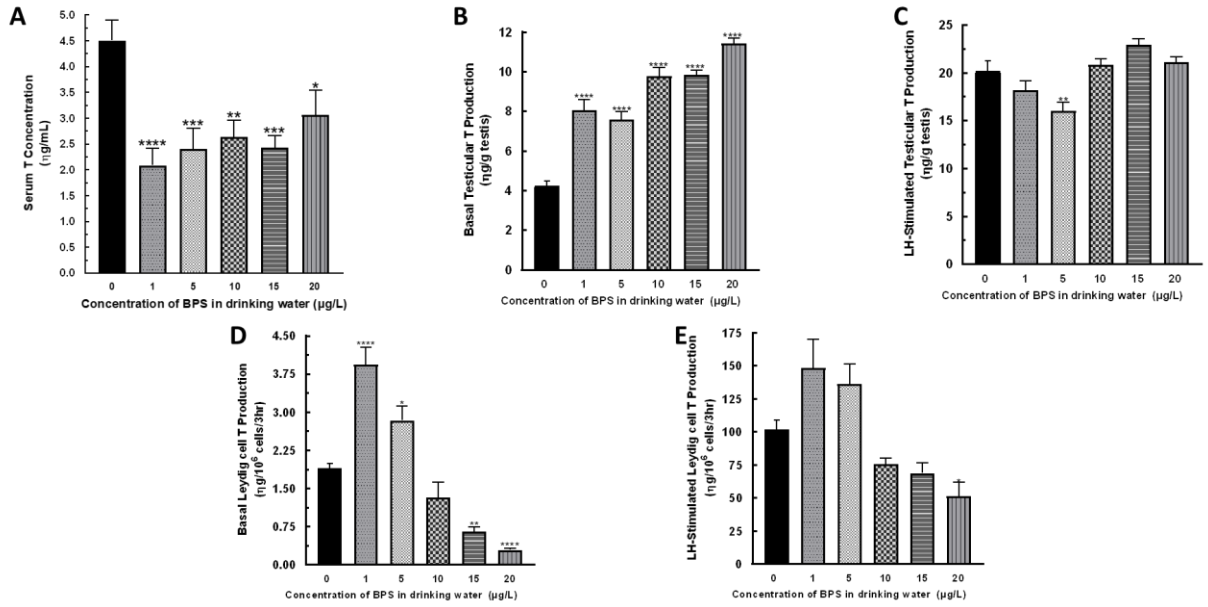

Figure 19: Effects of prepubertal exposure to BPS on testicular testosterone (T) production. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of BPS for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular explants (B, C) and Leydig cell T production (D, E) were measured by RIA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus control.

EE2 did not affect (P>0.05) serum T concentrations at the lower doses, but caused a decrease at the higher doses of 15 and 20 µg/L (P<0.01 and P<0.05, respectively) (**Fig. 20A**). Basal testicular T production was lower in all treatment groups

(**Fig. 20B**), but LH-stimulated testicular T production was similar (**Fig. 20C**). On the other hand, exposure to 1 and 5 µg/L doses of EE2 increased (P<0.05) basal Leydig cell T production while the higher doses caused a decreased (P<0.0001, **Fig. 20D**). LH-stimulated Leydig cell T production was increased by exposure to the 1, 5, and 15 µg /L doses, but was decreased by the 20 µg /L dose, while no effect was seen at the 10 µg /L dose (**Fig. 20E**).

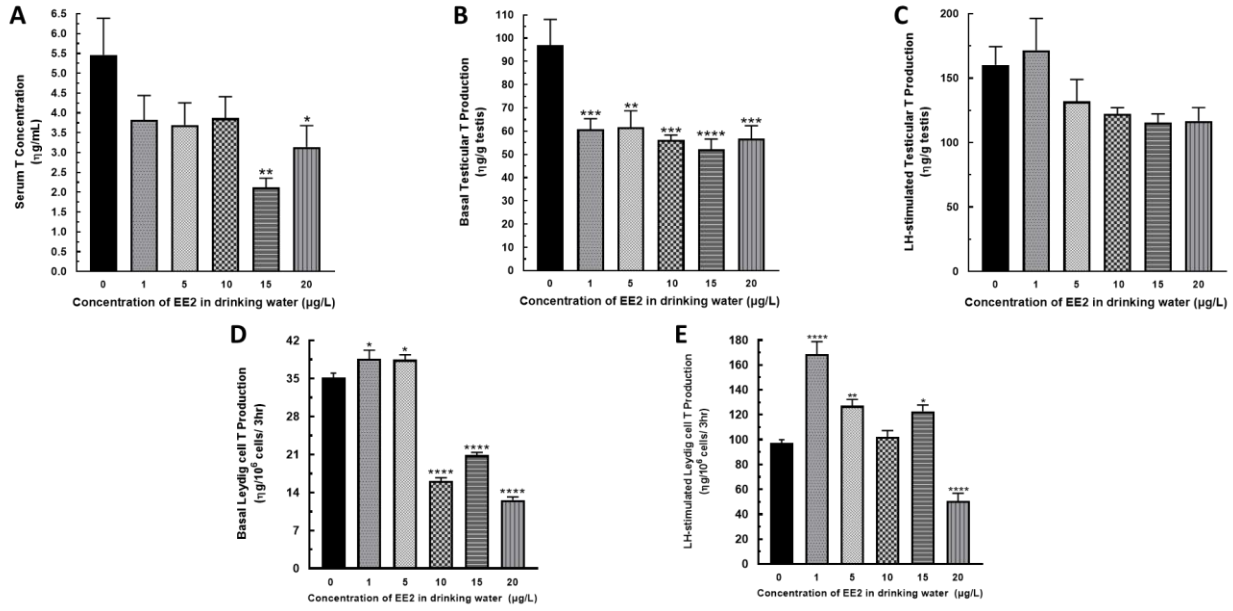

Figure 20: Effects of prepubertal exposure to EE2 on testicular testosterone (T) production. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of EE2 for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular explants (B, C) and Leydig cell (D, E) T production were measured by RIA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus control.

Effects of pubertal exposures to BPA on testicular and pituitary gene expression

Analysis of western blots showed that exposure of male rats to the 1 µg/L dose of BPA increased (P<0.05) testicular StAR protein compared to control (**Fig. 21A**). On the other hand, LHβ protein was increased (P<0.05) by exposure to the 15 µg/L dose of BPA (**Fig. 21B**). However, FSHβ showed no effect (P>0.05) at any dose (**Fig. 21C**).

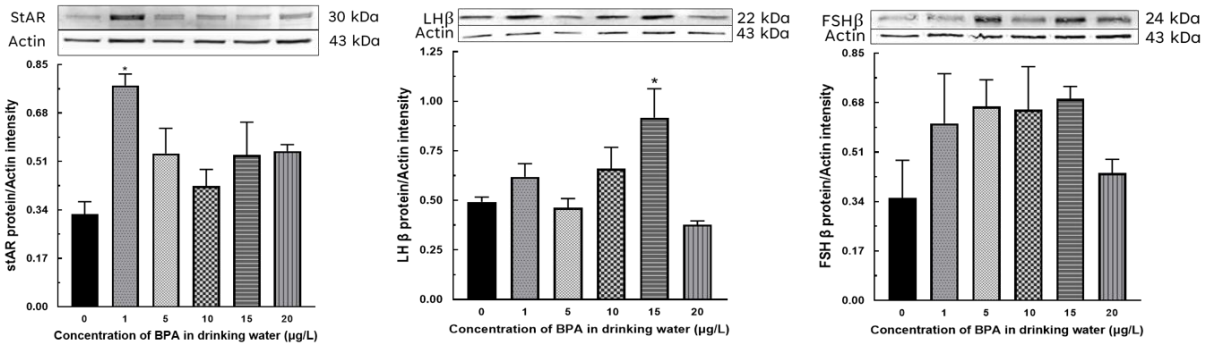

Figure 21: Effects of BPA on gene expression in the testis-pituitary gland axis. Male rats at 21 (n=6) days of age were provided water without and with graded doses of BPA (1 µg/L, 5 µg/L, 10 µg/L, 15 µg/L, or 20 µg/L) for 56 days. After sacrifice, tissues were processed by western blotting procedures to analyze steroidogenic acute regulatory protein (StAR) in testes (A) and pituitary gland LHβ (B) and FSHβ subunit proteins (C). Proteins were normalized to actin (StAR = 30 kDa, LHβ = 22 kDa, FSHβ = 21 kDa,, Actin = 43 kDa. *P < 0.05 versus control.

Effects of prepubertal exposures to BPA, BPS, and EE2 on serum and testicular 17β-estradiol (E2) secretion

Serum E2 concentrations were unaffected by exposure of male rats to BPA (P>0.05; **Fig. 22A**) while basal testicular E2 production was decreased (P<0.05) by the

15 and 20 µg/L doses (**Fig. 22B**). LH-stimulated testicular E2 production (**Fig. 22C**) and basal (**Fig. 22D**) Leydig cell E2 secretion were unaffected (P>0.05) by exposure to BPA at all doses. LH-stimulated (**Fig. 22E**) Leydig cell E2 secretion was substantially increased only at the 20 µg/L BPA dose (P<0.0001).

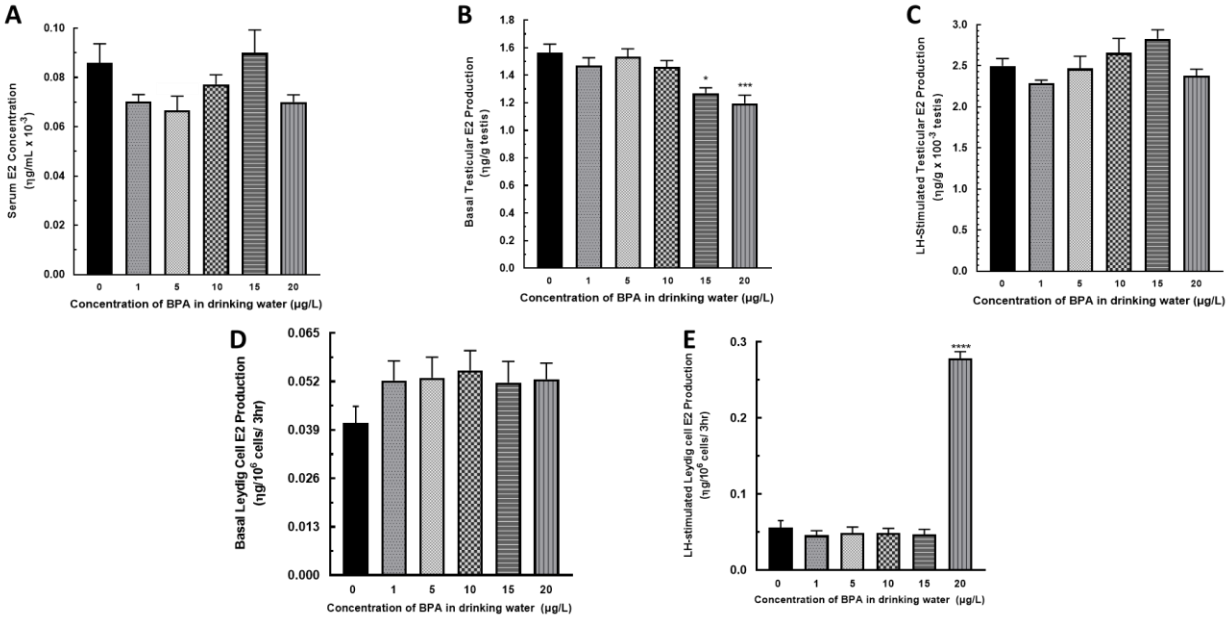

Figure 22: Effects of prepubertal exposure to BPA on testicular 17β-estradiol (E2) secretion. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of BPA for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular explants (B, C) and Leydig cell E2 production (D, E) were measured by RIA. *P < 0.05, ***P < 0.001, ****P < 0.0001 versus control.

Serum E2 concentrations were increased (P<0.05) by exposure of male rats to BPS only at the 10 µg/L dose (**Fig. 23A**) while basal testicular E2 production was increased (P<0.001) at the 15 and 20 µg/L doses (**Fig. 23B**). However, LH-stimulated testicular E2 production was unaffected at all doses (P>0.05, **Fig. 23C**). Basal (**Fig. 23D**) Leydig cell E2 secretion was similarly unaffected. On the other hand, LH-stimulated (**Fig. 23E**) Leydig cell E2 secretion was increased by the 1 and 10 µg/L doses (P<0.01) of BPS, and decreased by the 15 and 20 µg/L doses (P<0.05).

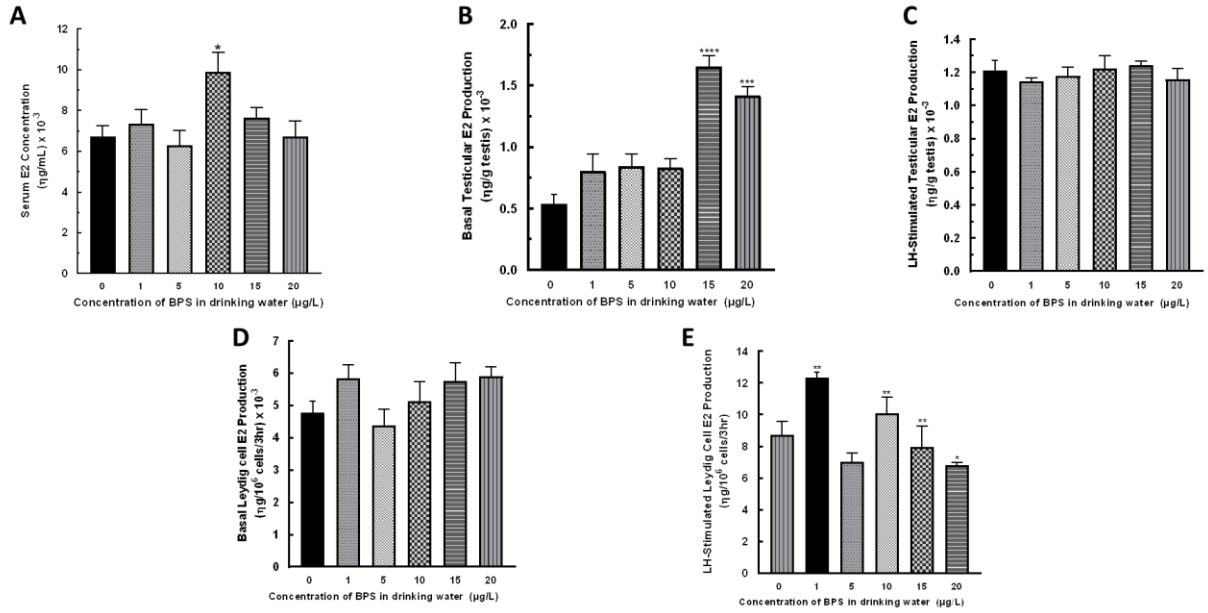

Figure 23: Effects of prepubertal exposure to BPS on testicular 17β-estradiol (E2) secretion. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of BPS for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular

explants (B, C) and Leydig cell E2 production (D, E) were measured by RIA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus control.

Serum E2 concentrations and basal testicular E2 were unaffected by exposure of male rats to EE2 (P>0.05; **Fig. 24A,B**). However, exposure to the 20 µg /L dose decreased (P<0.01) LH-stimulated testicular E2 production (**Fig. 24C**). On the other hand, basal Leydig cell E2 was increased (P<0.0001) at all doses except the lowest of 1 µg/L (**Fig. 24D**). However, LH-stimulated (**Fig. 24E**) Leydig cell E2 secretion was increased (P<0.01) only at the 20 µg/L dose compared to control.

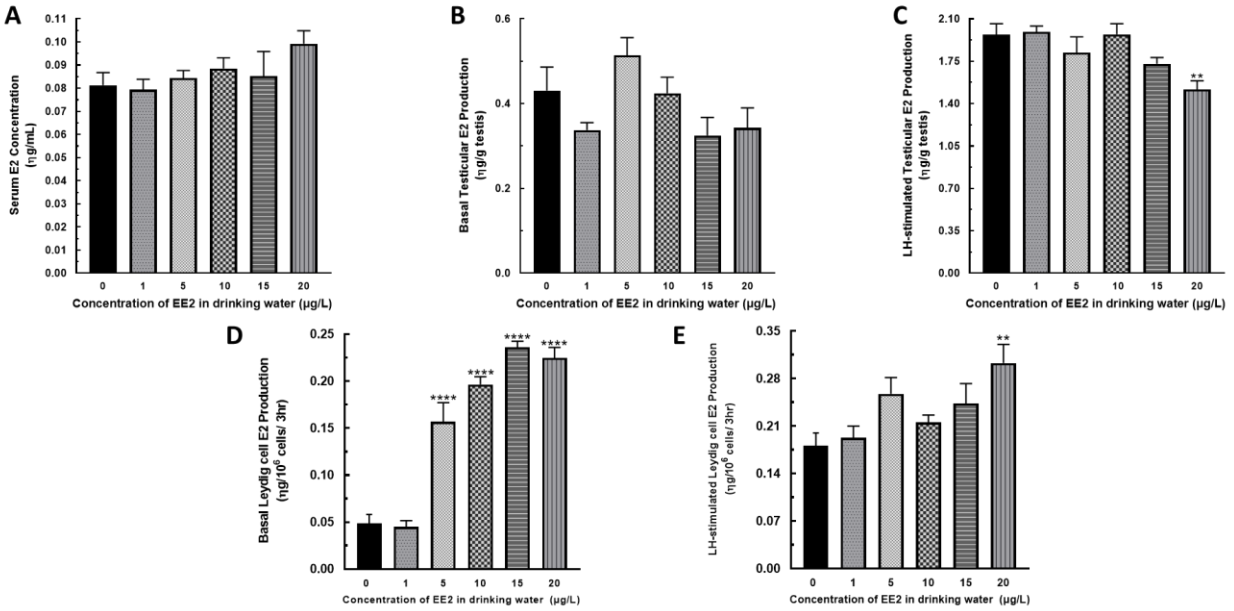

Figure 24: Effects of prepubertal exposure to EE2 on testicular 17β-estradiol (E2) secretion. Male rats at 21 days of age (n=6/group) were provided drinking water (CON) or water containing 1, 5, 10, 15, or 20 µg/L of EE2 for 56 days. After sacrifice, serum and testicular explants were obtained and testes were pooled to isolate Leydig cells. Testicular explants and Leydig cells were then incubated in DMEM/Ham's F-12 culture

medium without (basal) or with (LH-stimulated) ovine LH for 3 h. Serum (A), testicular explants (B, C) and Leydig cell E2 production (D, E) were measured by RIA. **P < 0.01, ****P < 0.0001 versus control.

Effects of in vitro exposure to BPA, BPS, and EE2 on Leydig cell steroid hormone secretion

Exposure of Leydig cells to BPA showed no effect (P>0.05) at any dose for T concentrations (**Fig. 25A**). Similarly, exposure of Leydig cells to BPS shows no effect in LH-stimulated T concentrations (**Fig. 25B**). Exposure of Leydig cells to EE2 increased (P<0.05) LH- stimulated Leydig cell T concentration at the 10, 100, and 1000 nM doses (**Fig. 25C**).

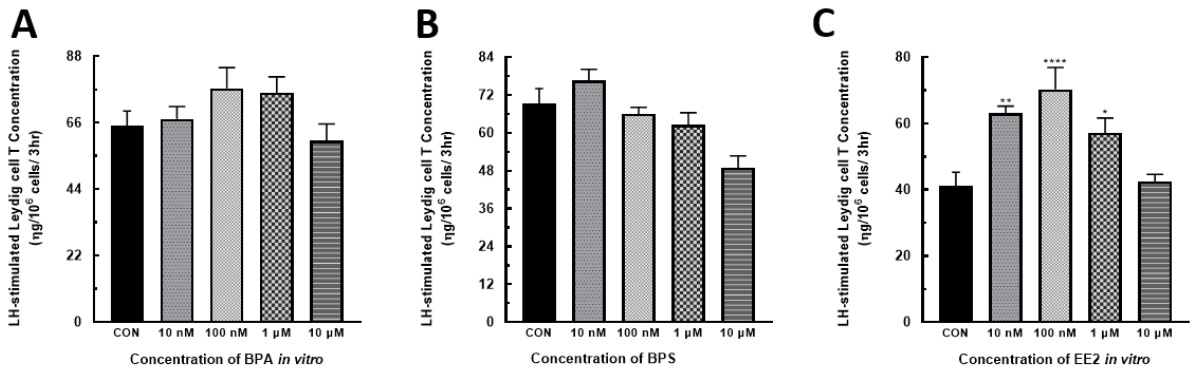

Figure 25: Effects of BPA, BPS, and EE2 on Leydig cell T production *in vitro*. Male rats at 35 days of age (n=10) not previously exposed to any chemicals were sacrificed and testes were pooled to isolate Leydig cells. Leydig cells were then plated at 50,000 cells/well in triplicate and incubated in DMEM/Ham's F-12 culture medium containing ovine LH and 0, 10, 100, 1,000, or 10,000 nM BPA, BPS, and EE2 for 3 h. T

concentrations were measured by RIA. *P < 0.05, **P < 0.01, ****P < 0.0001 versus control.

Exposure of Leydig cells to BPA showed no effect (P>0.05) at any dose for E2 production (**Fig. 26A**). Similarly, exposure of Leydig cells to BPS shows no effect in LH-stimulated E2 production (**Fig. 26B**). Interestingly, exposure to EE2 increased (P<0.0001) LH-stimulated Leydig cell E2 production only at the highest dose of 10 μM EE2 (**Fig. 26C**).

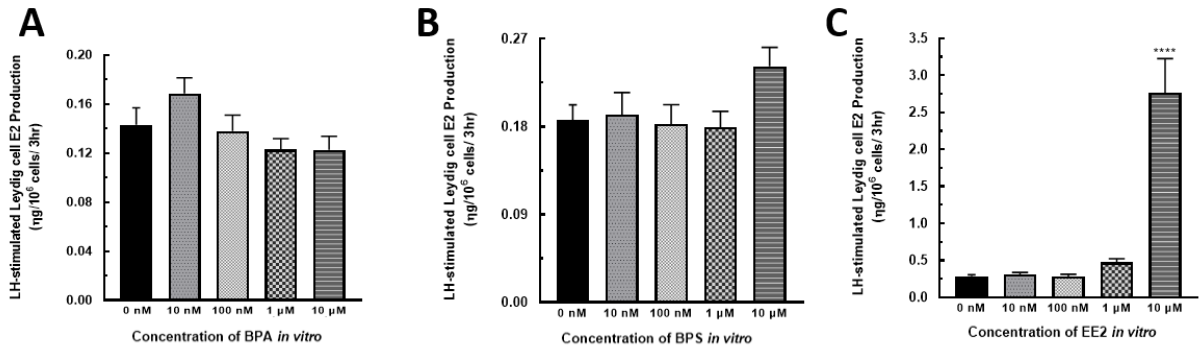

Figure 26: Effects of BPA, BPS, and EE2 on Leydig cell E2 production *in vitro*. Male rats at 35 days of age (n=10) not previously exposed to any chemicals were sacrificed and testes were pooled to isolate Leydig cells. Leydig cells were then plated at 50,000 cells/well in triplicate and incubated in DMEM/Ham's F-12 culture medium containing ovine LH and 0, 10, 100, 1,000, or 10,000 nM BPA, BPS, and EE2 for 3 h. E2 concentrations were measured by RIA. ****P < 0.0001 versus control.

Discussion

The results of the present study demonstrate that exposures of male rats to environmentally relevant concentrations of BPA, BPS, and EE2 altered sex steroid hormone production in a dose-dependent manner in the male rat gonad. We previously reported that prepubertal and pubertal exposures to BPA, BPS, or EE2 decreased testicular androgen secretion in an age-dependent manner in male rats[93]. On the other hand, the present study showed that androgen secretion is disrupted at all doses. While the previous findings only showed decreases in androgen secretion by BPA[134,135], BPS[136], and EE2[137], the present findings demonstrated both increased and decreased T secretion in a dose-dependent manner. For example, exposure to BPA decreased serum T concentrations at all doses, but increased basal testicular T at all doses, while in Leydig cells, a dose-dependent curve with both an increase and a decrease was observed. BPS exposures exhibited decreased serum and testicular T, consistent with the previous findings[93], but in Leydig cells, dose-dependent increases and decreases are again observed.

Interestingly, the two lower doses of EE2 increased androgen production in Leydig cells, while the higher doses caused a decrease, marking a notable parallel to a study showing dose-dependent inhibition of Leydig cell T production by EE2[137]. Basal testicular T production was decreased by all doses of EE2, as in previous reports[115,138].

These data are consistent with the known non-monotonic effects of estrogenic chemicals[139]. These inverted-U and U-shaped data may be the result of any number of effects. For example, some ER subtypes may be preferentially activated by a lower dose while another may be activated by the higher doses[139-141]. Receptor saturation

could also be a factor, with inhibitory pathways being activated beyond the saturation point[139,142]. The negative feedback loop discussed in chapter 1 may also be at play in these non-monotonic effects[142]. Further, gene expression could even change when exposed to different doses[141,143]. This is especially relevant in males whose natural estrogen levels are very low, and these curves seen in data trends are often a direct result of the HPG-axis at work to regulate the system.

Surprisingly, culture of Leydig cells with BPA and BPS showed no effect on T or E2 production, but EE2 increased T production at most doses. This could perhaps be due to EE2 competitively inhibiting CYP19A1 from synthesizing T into E2, allowing for accumulation or increased aromatase activity to occur[144].

We evaluated the effects of BPA on HPG axis by analysis of StAR, FSHβ, LHβ. Exposure to BPA increased expression of testicular StAR and pituitary LHβ-subunit protein expression but did not affect FSHβ. Previous studies investigated the mechanisms of chemical disruption of the male reproductive tract. For example, previous findings show that BPA and BPS decrease Cyp11a1 activity[136,145], the side-chain cleavage enzyme responsible for cleaving cholesterol for androgen biosynthesis. BPA is a known inhibitor of several steroidogenic enzymes, including Hsd17β, Cyp17a1, and Hsd3β in human testes[135]. In zebrafish testes, BPS exposure resulted in decreased Hsd17β mRNA levels[146]. EE2 has also been previously reported to decrease Cyp11a1 and Hsd17β enzyme activity[93]. One report demonstrated that men exposed to EE2 had suppression of LH secretion[147,148]. Another study showed a decrease in FSH secretion in men exposed to EE2[149]. Further, testicular expression of StAR, MIS, and inhibin-β

decreased after exposure of rats, zebrafish, and human cell lines to EE2, indicating a direct inhibition of androgen biosynthesis[115,150,151].

Furthermore, disparities between *in vivo* and *in vitro* data presented here can be explained by the absence of the HPG-axis in the *in vitro* assays. Changes in circulating steroid hormone concentrations are highly regulated through the negative feedback loop, as discussed in chapter 1. On the other hand, *in vitro* assays involve direct exposures of isolated cells to fixed quantities of endocrine disruptors without the whole of the endocrine system present to respond. As a result, treated cell cultures can reflect only the direct receptor-mediated effects without any compensatory mediation from the negative feedback loop, or any of its paracrine tissue interactions[142,152]. Further studies are warranted to elucidate the mechanisms of action by BPA, BPS, and EE2 in the HPG-axis and male reproductive development and function.

Together, the present findings support the need for development of cost-effective and sustainable filtration media to remove BPA, BPS, and EE2 from water sources because exposure of the population to these EDCs at even the smallest dose has implications for male reproductive health. In this regard, some algal systems have shown promise as effective EE2 filtration and degradation systems in aquatic environments[118]. These are potential alternatives to current removal methods. Absorption filtration methods, such as carbon filtration, are known to be highly effective at the removal of bisphenol analogs[153], and thus, efforts to increase their availability and use are warranted. Furthermore, effective filtration of our natural water sources directly improves public health by preventing waterborne illness via removal of bacteria, parasites, and viruses, and removing toxins such as heavy metals and industrial

pollutants while reducing exposure to microplastics. Future studies should focus on elucidating the primary points of disruption within the steroidogenic pathway.

Chapter 4 - Summary and Conclusions

The overall aim of this dissertation was to analyze endocrine disrupting chemical effects on testicular function. We have investigated this aim under the following specific objectives.

I. Investigate comparative effects of prepubertal and pubertal legacy and emerging PFAS exposures on gonadal steroid hormone secretion

II. Investigate the effect of plasticizers BPA and BPA, and the synthetic estrogen, EE2, on steroid hormone secretion in the prepubertal male rat gonad

The HPG-axis is a precisely controlled regulatory process for reproductive function. The male rat gonad contains multiple cell types that function together to maintain homeostasis of the testis endocrine, exocrine, and paracrine functions. Assessing the HPG-associated hormones responsible for the regulation of these cells, in particular Sertoli and Leydig cells, provides invaluable insight into testicular function. Analyzing Leydig cell steroid hormone production further supports these efforts, as Leydig cells are chiefly responsible to produce testosterone. Disruption of testosterone biosynthesis directly impacts fertility by regulating spermatogenesis. Thus, changes in testicular steroidogenesis measured in serum, testis, and Leydig cells are crucial to understanding the mechanisms by which endocrine disruptors impact male fertility and reproductive function. Sertoli cells are responsible for spermatogenesis and paracrine communication between testicular cell types. As such, measurable changes to MIS and inhibin Bβ provide invaluable insight into the impact of EDCs on Sertoli cell function.

This dissertation demonstrates that disruptions in both the prepubertal and pubertal rat gonad occur from PFAS exposure regardless of compound carbon-chain size, meaning both legacy and emerging PFAS exert effects on testicular function. For example, exposure to both legacy and emerging PFAS altered sex steroid concentrations i.e., estradiol and testosterone, in serum, the testis, and isolated Leydig cells from prepubertal and pubertal male rats. The PFAS-specific and dose-dependent changes in androgen secretion were related in part to disrupted expression of steroidogenic enzyme proteins and pituitary gland FSHβ and LHβ proteins expression. Importantly, these results indicate that PFAS altered sex steroid concentrations, suggesting that PFAS may impact spermatogenesis and male fertility. Sertoli cell expression of Mullerian inhibiting substance was increased while inhibin Bβ proteins were decreased as a result of exposure to both legacy and emerging PFAS. This study also demonstrated that different PFAS compounds acting in combination exert mixture effects in the testis, likely a result of the additive effect of the individual compounds. For example, combinations of legacy PFAS with their emerging counterparts decreased Leydig cell testosterone production, with carboxylic acid-containing mixtures impacting both basal and LH-stimulated T production while sulfonic acid-containing mixtures only impacting T production under LH stimulation. Overall, our data provided evidence showing that both legacy and emerging PFAS have the capacity to regulate gonadal steroid hormone secretion at varying doses and mixture states, showing the capacity to disrupt hypothalamic-pituitary-gonadal axis function at every level by acting directly in testes and pituitary or by interfering with negative feedback regulation. Data from the present study support the need to develop cost-effective, adaptable, and sustainable

filtration media for different processes to remove PFAS from water and other sources of exposure in tandem with action by regulatory agencies to limit use of PFAS in the manufacturing sector.

In addition to our report on PFAS effects on testicular function, we analyzed the effects of the synthetic estrogenic chemicals, BPS, BPS, and EE2, on the rat gonad. Our results demonstrated that environmentally relevant concentrations of BPA, BPS, and EE2 altered testicular steroid hormone production in prepubertal male rats. The mechanisms underlying these specific altered sex steroid secretions warrant further investigation as steroidogenic enzyme and pituitary gland protein expressions vastly contradicted previous studies, suggesting that BPA, BPS, and EE2 may act through numerous pathways to impact reproductive function. Regardless, our data indicate that environmentally relevant concentrations of these estrogen mimicking compounds are detrimental to testicular reproductive function. It is of note that exposure to xenoestrogens tends to occur in mixtures, and it is a limitation of this study that these test chemicals were used as single compound exposures. It is of note that most of the contradicting data found in other studies were the result of chemical mixtures, not BPA, BPS, or EE2 alone. Therefore, future studies should evaluate the mechanism of action on HPG regulation with BPA, BPS, and EE2 alone and in combination with other endocrine disrupting compounds.

Taken together, our observation on PFAS and xenoestrogens, BPA, BPS, and EE2, are relevant to public health due to:

1. The persistence and prevalence of these EDCs in the environment
2. Inadvertent exposure to these EDCs of the population and

3. The increased dysregulation of reproductive function in the population such as decreased testosterone[56,154], altered spermatogenesis[155,156], disruption of HPG signaling[157], and delayed puberty[56].

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