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Effects of endocrine disrupting chemicals on gonad development: Mechanistic insights from fish and mammals

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ABSTRACT

Over the past century, evidence has emerged that endocrine disrupting chemicals (EDCs) have an impact on reproductive health. An increased frequency of reproductive disorders has been observed worldwide in both wildlife and humans that is correlated with accidental exposures to EDCs and their increased production. Epidemiological and experimental studies have highlighted the consequences of early exposures and the existence of key windows of sensitivity during development. Such early in life exposures can have an immediate impact on gonadal and reproductive tract development, as well as on long-term reproductive health in both males and females. Traditionally, EDCs were thought to exert their effects by modifying the endocrine pathways controlling reproduction. Advances in knowledge of the mechanisms regulating sex determination, differentiation and gonadal development in fish and rodents have led to a better understanding of the molecular mechanisms underlying the effects of early exposure to EDCs on reproduction. In this manuscript, we review the key developmental stages sensitive to EDCs and the state of knowledge on the mechanisms by which model EDCs affect these processes, based on the roadmap of gonad development specific to fish and mammals.

Introduction

Concerns have arisen regarding the impact of endocrine disrupting chemicals (EDCs) on reproductive health. There is widespread exposure to populations when these chemicals, found in a large variety of consumer products, including electronic equipment, building materials, cosmetics, medical devices and food packaging, leach out into the environment (Metcalfe et al., this issue). Many of these chemicals are used as plasticizers, flame retardants, or pesticides, while others are pharmaceuticals or industrial by-products. Correlating with accidental exposures to EDCs and the rise in their production, an increased frequency of reproductive disorders has been observed worldwide in both wildlife and humans (Marlatt et al. this issue; WHO, 2002). Such disorders include feminization in fish, abnormal development of the reproductive tract, precocious puberty, testicular and ovarian cancer, low sperm count and poor gamete quality (Marlatt et al. this issue). A growing number of epidemiological and wildlife studies have highlighted the impact of early exposures and the existence of key windows of sensitivity during development. This is in line with the concept of the developmental origins of health and diseases (DOHaD) that early

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perinatal exposures to environmental influences affect long-term health and disease susceptibility (Gillman, 2005). According to this principle, immediate effects may be observed shortly after exposure, but it is also possible that the phenotypic consequences of molecular effects on the programming of a tissue may be visible only later, in the adult. In fact, it has been shown that, depending on the type of EDCs, the dose and the precise time of exposure, early exposure to EDCs can induce immediate effects on the establishment of the different cell types of the gonads or on the development of the reproductive tract; later effects may be revealed on hormonal homeostasis, somatic cell differentiation, gamete production and gamete quality (Johansson et al., 2017; Wohlfahrt-Veje et al., 2009).

EDCs exert their effects by altering endocrine pathways, including those that control reproduction. Traditionally, the mechanisms of action of EDCs are defined as affecting receptor binding, synthesis or transport of hormones. Thus, many studies are focussed on studying activation/inhibition of estrogen, androgen, and thyroid receptor signaling and effects on steroidogenesis (EATS-pathways) (Martyniuk et al. this issue; Robitaille et al. this issue; Amir et al., 2021). However, non-EATS pathways are now gaining importance and interest as a modality by which EDCs impact reproductive health (Martyniuk et al. this issue). A better understanding of the underlying physiology is required to anticipate the potential effects of environmental chemicals such as EDCs. In recent years, fundamental studies, primarily in fish and rodents, have led to a better understanding of sex determination, gonadal differentiation, and the mechanisms underlying the events during the embryo and early life stages that are critical to the development of the reproductive system and germ cell lineage. These advances in knowledge increase our understanding of the molecular mechanisms by which EDCs affect key events and induce long-term effects on reproduction.

In this manuscript, we will review the key events in the establishment of the gonads and the state of knowledge on the mechanisms by which EDCs affect these processes. This will be based on the roadmaps of development specific to fish and mammals, from gonad development and differentiation to the entry of germ cells into meiosis. The selection of EDCs reported here, is based on the prevalence of exposure in the study model and known reproductive effects; our focus is on EDCs for which we have some understanding of the mechanisms involved in gonadal dysregulation during development.

Part 1: evidence from Fish

An increasing number of chemicals coming from wastewater treatment plants, leaching from agricultural lands or present in industrial effluents or in urban runoff, among others, are contaminating aquatic ecosystems and producing adverse effects on fish populations (reviewed by Segner, 2011). In the past decades, the effects of EDCs on reproductive function in several fish species have been the focus of an increasing number of investigations (Marlatt et al. this issue). But, only a few studies have addressed the effect of EDCs in fish prior to pubertal onset and little is known about their precise mode of action. Yet, evidence demonstrates that in fish, exposure to EDCs is responsible for: 1) the disruption of gonad development and sex differentiation, inducing intersex; 2) abnormal gonad differentiation, affecting the number of germ cells, leading to episodes of sterility; and, 3) alterations in the timing of puberty. Here we summarize the physiology of these key stages of development and review how early exposures to EDCs affect fish reproduction from sex determination to entry into puberty (Figs. 1 and 2, Table 1).

1. *Gonad development*

1.1. *Development of germ cells*

The establishment of the germ line is a crucial process for the reproductive success of an individual. This includes several well-orchestrated steps, beginning with specification of the germ cell identity in the early stages of development (Ewen-Campen et al., 2010). These germ cell precursors of gametes, named primordial germ cells (PGCs), migrate actively through the body via the coelom, reaching their final location, the gonadal primordium (Nishimura and Tanaka, 2016; Weidinger et al., 2002). This primordium presents an exclusive characteristic in terms of organ development because it differentiates into an ovary or a testis, two dissimilar organs from the same origin (Capel, 2017). When PGCs reach the gonadal primordium, they are named embryonic germline stem cells (EGSCs) and are arrested at G1/G0 phase of the cell cycle. These germ cells become the precursors of spermatogonial stem cells (SSCs) in the testis and oogonial stem cells (OSCs) in the ovary (Nakamura et al., 2010). During migration and even after reaching the gonadal primordium, some of these EGSCs will start asynchronous proliferation in both sexes to give rise to the primary population of germ cells in the gonads (Chen and Liu, 2015c; Nakamura

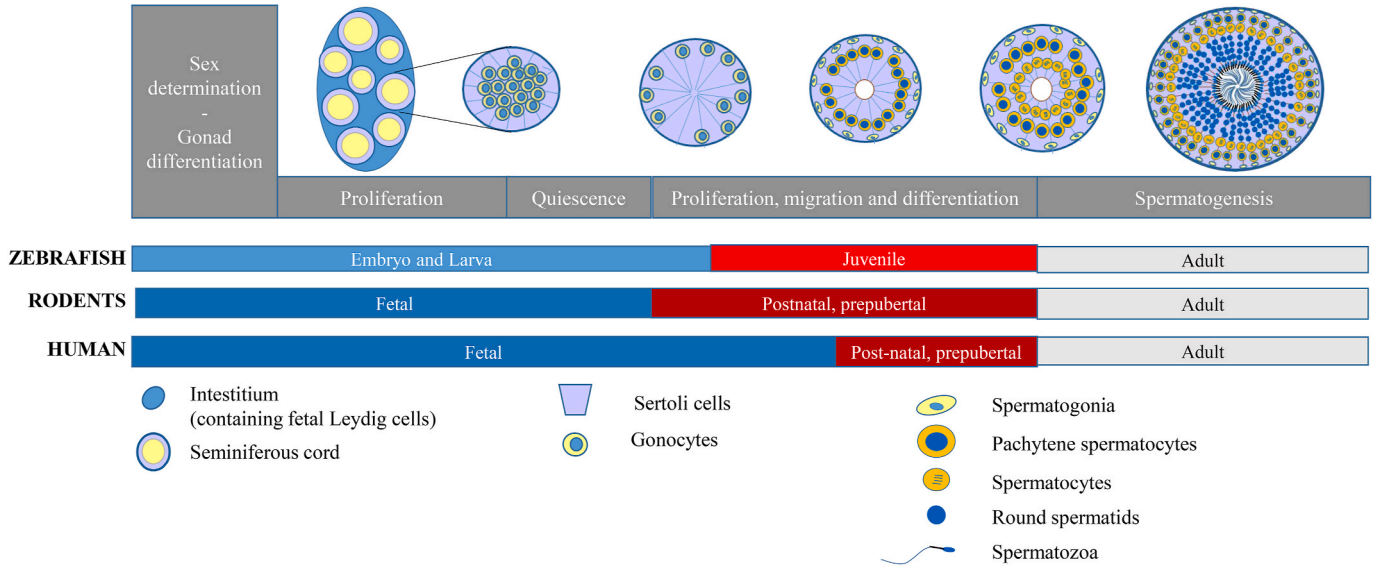

Fig. 1. Timing of testicular development in zebrafish, rodents and humans. The diagram illustrates the fate and development of germ cells in the testis, as they proliferate by mitosis, and organize to form tubules (cysts in zebrafish) and then enter spermatogenesis.

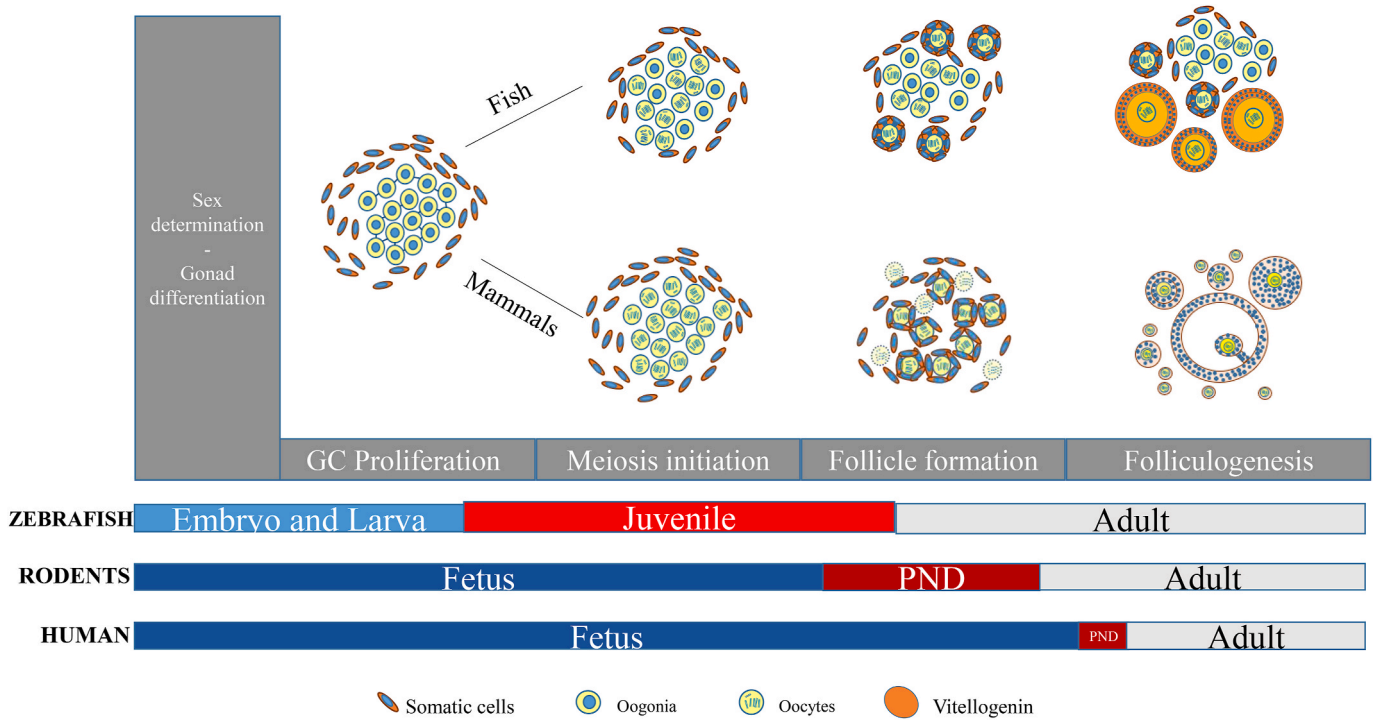

Fig. 2. Timing of ovarian development in zebrafish, rodents and humans. The diagram illustrates the fate and development of germ cells in the ovary as they proliferate by mitosis, organize to form ovarian follicles, and ultimately undergo folliculogenesis. One major difference between fish versus mammals is that fish maintain a population of germ stem cells in the adult ovary whereas in mammals at birth all germ cells are arrested in meiotic prophase, forming primordial follicles, and are incapable of dividing mitotically subsequently.

et al., 2010; Tanaka, 2019), while others remain in a quiescent state. This first phase of germline proliferation is regulated by the Anti--Müllerian hormone (Amh), as described by Morinaga et al. (2007). Any alterations of this proliferation and of the number of EGSCs are involved in gonadal sex reversal. For example, in Japanese medaka (*Oryzias latipes*) hyperproliferation of EGSCs induces male-to-female reversal, while in zebrafish (*Danio rerio*), the lack of this proliferation induces the female-to-male sex reversal (Kurokawa et al., 2007; Rodríguez-Marí et al., 2010). Before meiosis, EGSCs enter a second round of proliferation, this time synchronous, which generates a group of cells known as cysts or nests, surrounded by somatic cells (Saito et al., 2007, Figs. 1 and 2). Timing of when this second proliferation begins is specific to sex; mostly, it begins first in females and is an initial marker of sexual dimorphism (Tanaka, 2019). Although this second round of proliferation is not related to the establishment of gonadal sex, the early increase in the number of germ cells is a necessary step for reproductive success, as changes can alter the number of eggs in females at spawning, and the fertilization rate and mating behavior of males (Padilla et al., 2021).

1.2. Regulation and impacts of EDCs

The possibility that PGCs are a target of EDC action has been studied in zebrafish embryos (Hu et al., 2014; Lombó et al., 2019). Embryos were injected with the gfp-nanos-3′UTR mRNA and were immediately exposed to different concentrations of the potent synthetic estrogen 17α-ethynylestradiol (EE2). The migration and distribution of PGCs were affected by exposure to high doses of EE2 (500 ng/L) as the embryos displayed ectopic PGCs. Knocking down the estrogen receptor *esr2a* or application of a ESR2 inhibitor significantly reduced the percentage of embryos with ectopic PGCs after exposure to EE2, indicating that ESR2a may play an important role in EE2-related PGC phenotypes. In other studies, zebrafish embryos were exposed to Bisphenol A (BPA) during the first 24 h of development. Vasa immunostaining of PGCs revealed that exposure to BPA impaired their migration to the genital ridge and two pivotal genes of PGCs migration (C-X-C Motif Chemokine Receptor 4 (*cxcr4b*) and C-X-C Motif Chemokine Ligand 12 (*sdf1a*) were highly dysregulated (Lombó et al., 2019). Interestingly, once the embryos reached adulthood normal testes were developed. Collectively, these studies show that PGCs may be targeted by EDCs in fish.

2. Sex-determination and gonadal differentiation

2.1. Control of sex determination and gonadal differentiation

Fish are a group of vertebrates with a large range of mechanisms for sex determination, including genetic and environmental sex determination mechanisms (GSD and ESD, respectively; Devlin and Nagahama, 2002; Hattori et al., 2020). In species with GSD, sex is determined at fertilization, driven by a gene or chromosomal difference between sexes. Different heterogametic systems have been characterized in fish. However, the genes that initiate the development of males (XX/XY system) or females (ZZ/ZW system) vary. For example, male heterogametic systems have been identified that exhibit different sex determining genes in closely related families (Hattori et al., 2019; Maitre et al., 2017; Matsuda, 2005). Moreover, a similar range has been observed in female heterogametic systems. Many closely related species exhibit both types of heterogamety (Capel, 2017), showing that genes that direct gonadal fate have appeared several times, independently, in fish. In ESD strategies, gonadal fate is determined at embryo or juvenile stages, driven by the effects of changes in the environment, such as temperature, dissolved oxygen or pH (Hattori et al., 2020).

Interestingly, GSD and ESD can coexist under environmentally relevant conditions, exhibiting a continuum of genetic and environmental mechanisms (Xiong et al., 2020; Yamamoto et al., 2014). As an example, it was recently established that the brain, through the hypothalamus, detects changes in the environment by raising cortisol levels (Castañeda Cortés et al., 2019). This stress hormone produces, as a by-product, changes to the ratio of sex steroids. On one hand, it can increase androgen levels by the up-regulation of the *hsd11b2* gene that encodes for the 11β-hydroxysteroid dehydrogenase type 2 enzyme

Table 1
Impacts of early exposure to EDCs on gonad development and puberty in fish.

Species	Exposure Window	Compounds	Gonadal effects	Endocrine and Molecular effects	References
Zebrafish (*Danio rerio*)	1 hpf - 60dph	EE2	Delayed puberty in both males and females	Increase in VTG in both males and females	Baumann et al. (2014), 2013
		4-Tert-Pentylphenol	Delayed puberty in both males and females	Decrease in VTG levels in females, increase in males	
		DHT	Delayed puberty in females and acceleration in males	Decrease in VTG in both males and females	
		Tb	Delayed puberty in females and acceleration in males	Decrease in VTG in both males and females	
		Prochloraz	Hormesis-like effects with no clear results	Hormesis-like effects with increased VTG levels at intermediate concentrations	
	2 hpf-56 dph	EE2	Decreased testicular development	Decreased amh and decreased dmrt1	Schulz et al. (2007)
	1 - 60dph	EE2	Precocious puberty in females, determined by ovaries showing only pre-vitellogenic oocytes	Increased VTG levels at 38 dph	Örn et al. (2006)
		Tb	Precocious puberty in males, determined by increased testicular area and increased area with spermatozoa in the testis lumen	Decreased VTG levels at 38 dph	
	1 dpf - 60dph	Tb	Precocious puberty in males, determined by acceleration of spermatogenesis	No effects on VTG levels at 60 dph	Morthorst et al. (2010)
	6 dpf - 5 months	Natural mixture of POPs	Precocious puberty, determined by treated fish taking less time to first spawning	Transcriptomic alterations in testis and liver related to steroid and thyroid hormone functions, insulin signaling and metabolic homeostasis.	Nourizadeh-Lillabadi et al. (2009)
	2 - 60dph	NP + EE2	Delayed puberty in females, determined by additive effects (NP + EE2) on ovarian follicle development. Exposures increased the presence of oogonia and decreased previtellogenic follicles in females at 60 dph.	No differences in HSP70 expression, increasing NP concentration acted antagonistically to EE2 in terms of VTG induction at 60 dph	Lin and Janz (2006)
	20 - 80 dpf	Levonorgestrel	Sex reversal (all-male populations). Precocious puberty, determined by advanced gonadal maturation in males but not in females	Increase in the expression of *amh*, *cyp11b*, *fshb*, and *lhb* and decrease of *cyp19a1a* at 44 dpf	Svensson et al. (2016)
		Progesterone	No effect on sex ratios. Precocious puberty, determined by advanced gonadal maturation in males but not in females	No effects on gene expression levels	
	20-115 dpf	EE2 DBP DBP + EE2	EE2, DHP and the combination decreased testicular development (Reduced spermatozoa and increased spermatogonia and spermatocytes) EE2, DHP the combination reduced ovaraian development through reduction in vitellogenic oocytes and degenerative changes EE2 and EE2 plus DHP induced 100% females whereas DHP had no effect on sex ratio	Increased vtg protein at 45 d No effect on vtg protein at 45 d Increased vtg protein at 45d	Chen et al. (2015b)
	1-120dpf	triadimefon	Sex ratio of fish skewed to male and female exposed to 0.5 g/mL triadimefon had immature ovary. No obvious effects on males. Decreased spawning and fertility success	Reduced vtg expression but no effects on testosterone or estradiol levels	Liu et al. (2014)
	1-60dpf	prochloraz	Increased numbers of males and a higher proportion of intersex fish	Decreased vtg in females; in males low levels of prochloraz increased vtg in males whereas high levels decreased vtg	Kinnberg et al. (2007)
Wild-type and *cyp19a1a* and nER mutant zebrafish (*Danio rerio*)	1-60dpf	prochloraz	Increased numbers of males and a higher proportion of intersex fish	Decreased vtg in females; in males low levels of prochloraz increased vtg in males whereas high levels decreased vtg	Song et al. (2020)
Casper mutant transparent zebrafish (*Danio rerio*)	8 - 9wpf	DES	Delayed puberty in both males and females as assessed by follicle size and gonadal and vent morphology.		Lessman and Brantley (2020)
Japanese medaka (*Oryzias latipes*)	1 - 100 dph	Genistein	Delayed puberty, determined by delayed oocyte maturation, atresia, reduced oocyte number, larger ovarian lumen and higher incidence of PGCs in females. No effects on		Kiparissis et al. (2003)

(*continued on next page*)

Table 1 (*continued*)

Species	Exposure Window	Compounds	Gonadal effects	Endocrine and Molecular effects	References
			spermatogenesis but increase in testis connective tissue, lobular fibrosis and decreased spermatozoa densities		
		Equol	Delayed puberty in females, determined by delayed oocyte maturation, atresia, larger ovarian lumen and development of somatic stromal tissue in females. Delayed puberty in males, determined by retarded spermatogenesis		
	1 dph - 60dph		EE2	Precocious puberty, determined by higher proportion early vitellogenic oocytes	Increased VTG levels at 38 dph
Tb	Precocious puberty, determined by increased area with spermatozoa in the testis lumen	Örn et al. (2006)	Decreased VTG levels at 38 dph		
Rainbow trout (*Oncorhynchus mykiss*)	6 month to 18 mo (first gametogenesis and spawning)	Genistein	Delayed puberty in females, determined by delays in oocyte maturation. Acceleration of spermatogenesis and decrease in sperm density and motility	Induction of VTG, decrease in, 17a,20b-dihydroxyprogesterone, testosterone and *fshb* and *lhb* in both females and males.	Bennetau-Pelissero et al. (2001)
Roach (*Rutilus rutilus*)	1 year-old- 28 days exposure (first gametogenesis)	Levonorgestrel	No effect in ovaries Decrease in numbers of spermatogonia type B in testis	Increased *vtg* and *er1* expression in both sexes Increased *lhb* and suppression of *fshb* expression in both sexes Decrease of 11 KT and E2 and increase of T in females Decrease 11 KT in males	Kroupova et al. (2014)
White suckers (*Catostomus commersoni*)		Pulp mill effluents	Delayed puberty, determined by increased age to maturity	Decreased testosterone, 17a,20b-dihydroxyprogesterone in both sexes, 11-KT in males and E2 in females.	McMaster et al. (1991); Munkittrick et al. (1992)

11 KT, 11ketotestosterone; Amh, antimüllerian hormone, BPA, Bisphenol A; cyp11b, cytochrome P450 11 subfamily B; cyp19a1a, cytochrome P450 19 subfamily A member 1a (aromatase); DES, Diethylstilbestrol; dph, days post hatch, dpf, days post fertilization; DHT, Dihydrotestosterone; E2, 17β estradiol; EE2, 17α-Ethinylestradiol; fshb, Follicle stimulating hormone subunit beta; hpf, hours post fertilization; lhb, luteinizing hormone subunit beta; NP, Nonylphenol; POP, persistent organic pollutants; Tb, 17b-Trenbolone; VTG, vitellogenin; wpf, week post fertilization.
(Note that examples of studies are presented here; the list is not comprehensive).

(11β-HSD2) involved in the synthesis of 11-oxygenated androgen, which is the most potent androgen in fish (Castañeda-Cortés et al., 2020). On the other hand, it affects estrogen levels by the down-regulation of cytochrome P450 family 19 subfamily A member 1 (*cyp19a1a*), which is the gene encoding the aromatase enzyme involved in the biosynthesis of estradiol (Yamaguchi et al., 2010). This complex interaction between the environment, stress hormones and alterations of gene expression induces the development of testis.

The diversity of sex determination mechanisms, and subsequent gonadal differentiation, is possible in fish due to a high gonadal plasticity and to the similarity of extremely simple structures in both sexes - mainly the germline cells surrounded by somatic supporting-cells (Nishimura et al., 2016). Although the triggers of gonadal development vary enormously between fish, the molecular networks that promote the development of testes or ovaries are highly conserved, even with mammals (Capel, 2017). Depending on the way in which the differentiation of the gonads progresses, they are classified into two reproductive strategies, gonochorism and hermaphroditism (Devlin and Nagahama, 2002).

Gonochoristic species differentiate as males or females and remain the same sex throughout their lifespan, while hermaphroditic species develop both the male and female phenotype at some point in their life history (Devlin and Nagahama, 2002). In the gonochoristic group, the gonadal primordium activates the gonadal differentiation network, based on the sex determination system, establishing a balance between testis- and ovary-related genes. Within these genes, which include several transcription and growth factors, the male-related genes, such as *amh*, mab3-related transcription factor 1 (*dmrt1*), sry-box containing gene 3 (*sox 3*) and gonadal soma derived factor (*gsdf*) are up-regulated in the bipotential gonadal primordium and during gonadal differentiation to induce and maintain the development of testis. Alternately, the female-related genes, such as R-spondin 1 (*rspo1*), forkhead box protein L2 and L3 (*foxl2*, *foxl3*) and cytochrome P450 1A1 (*cyp1a1*), are up-regulated to induce the development of ovaries (Guiguen et al., 2018; Nishimura et al., 2015). Moreover, other hormones, receptors and enzymes that regulate gonadal steroidogenesis, such as follicle-stimulating hormone receptor (*fshr*), Cytochrome P450 Family 11 Subfamily B Member 1 (cyp11b1) and *hsd11b2*, are expressed downstream within the gonadal gene regulatory network to initiate and/or maintain testis development. The female-related gene *cyp19a1a* is necessary for ovary development and/or maintenance (Guiguen et al., 2018; Hattori et al., 2020). Once the gonad has differentiated into a testis or ovary, the gonadal fate in gonochoristic fish species cannot subsequently be reversed under normal conditions. However, in adult gonochoristic fish, intersex gonads have been observed upon hormonal manipulation; that is, ovaries that had varying amounts of male germline cells or testes that had varying amounts of primary oocytes (Devlin and Nagahama, 2002), establishing that the lability of the gonadal somatic and germline cells persists even in adulthood. For example, the transplantation of germ stem cells, such as SSCs or OSCs, into a host of the opposite sex results in the generation of oocytes or spermatocytes, respectively (Yoshizaki and Yazawa, 2019).

Gonadal differentiation in fish with hermaphrodite strategies can shift from one to another gonadal biological sex, depending mainly on social factors (Gemmell et al., 2019). Hermaphrodites that develop sequentially are either protandrous, if they first mature as males, or protogynous, if they first mature as females. This plasticity can occur in fish because, unlike mammals, gonads contain germ cells as stem cells during adulthood in both sexes (Nakamura et al., 2010). Like in gonochoristic fish, a conserved gonadal gene regulatory network has been

characterized in hermaphroditic fish during sex change (Ortega-Recalde et al., 2020). However, it is unusual among teleosts for the same gonad to display stages of both spermatogenesis and oogenesis; when this is observed, it is usually referred to as gonadal "intersex", or sometimes "testis-ova", or "ovotestes" among gonochoristic or hermaphroditic teleost (see Marlatt et al. this issue, Fig. 2).

2.2. Impact of EDCs

Intersex and other alterations to gonadal development have been observed in several populations of wild fish, and this is often viewed as evidence of exposure to EDCs (Metcalfe et al., 2010 and Marlatt et al. this issue). In a review of the literature on gonadal intersex in populations of teleosts in the wild, Bahamonde et al. (2013) tabulated reports of this condition in 37 fish species from field surveys conducted in 24 countries. However, there are questions concerning whether this condition is definitively linked to exposures of wild fish to EDCs and whether a "background" prevalence of gonadal intersex is a natural condition in some species of gonochoristic teleosts (Bahamonde et al., 2013).

With the high fecundity and the availability of large numbers of fertilized eggs and larvae, a common approach for studies of the effects of EDCs and environmental contaminants on reproductive development in fish involves their treatment, grow out and evaluation of subsequent effects on sexual development. Many studies have reported gender bias and effects on gonad developmental in fish treated with EDCs. Santos et al. (2017) summarized the studies testing the effects of more than 25 suspected EDCs on sex development and the sex ratio of zebrafish. These studies showed that treatment with compounds that mimic the action of estrogens [e.g. EE2, BPA] result in a shift in the sex ratio towards females. Exposure to androgenic EDCs, such as 17β-trenbolone and aromatase inhibitors [prochloraz and fadrozole], masculinize zebrafish, leading to male skewed sex ratios. The timing of exposure was critical in that complete masculinization occurred when zebrafish were exposed from hatch or from 20 to 60 days post-hatch (dph), which corresponds to the period of gonad differentiation.

Exposure occurring outside of the period of sexual differentiation leads to changes in secondary sex characteristics or effects on the reproductive system, such as inhibition of spermatogenesis, degenerative changes in gonads, reduced fecundity and alterations of the transcription of genes involved in reproductive development. Yet, there is evidence that sex reversal in fish can be induced outside the period of gonad differentiation if exposure to EDCs is longer and in higher doses. Takatsu Takatsu et al. (2013) exposed sexually mature adult female zebrafish to the aromatase inhibitor fadrozole (0.2 mg/g) for 5 months and observed an ovarian retraction followed by the development of testes-like organs, which contained sperm heads without tails. Moreover, even after discontinuing the treatment, normal and fertilization-competent sperm were produced by these females. This study suggested that undifferentiated PGCs may persist in adult zebrafish female which enables the differentiation to male germ cells and confer sexual plasticity (Takatsu Takatsu et al., 2013).

Increasingly studies of the effects of EDCs on fish undergoing sex differentiation involve monitoring changes in the expression of genes and measurement of sex steroid hormone levels related to sex differentiation. As one example, Yang et al. (2018) exposed zebrafish embryos/larvae to 1–1000 μg/L of Bisphenol F (BPF) for 0–60 days post-fertilization (dpf) and showed alterations in survival, growth, the balance of steroid hormones, and sex differentiation from 100 μg/L of BPF. This led to a skewed sex ratio in favor of females in the high BPF exposure groups and a higher frequency of fish that exhibited ovotestis. Testosterone levels decreased and 17β-estradiol levels increased in zebrafish in response to BPF. BPF exposure suppressed the expression of doublesex and mab-3 related transcription factor 1, (*dmrt1*), fushi tarazu factor 1d (*ff1d*), sry-box transcription factor 9a (*sox9a*) and *amh*; induced expression of *foxl2*, leading to increased expression of *cyp19a1a*, which promoted production of estrogens, and further caused phenotypic feminization of zebrafish. In other studies, various EDCs, including nonylphenol, EE2, BPA, phthalates and 17β-trenbolone or runoff from lands fertilized with animal manure from concentrated animal feeding operations (a source of EDCs), have been shown to influence sex differentiation in various fish species (Hill and Janz, 2003; Leet et al., 2015; Ye et al., 2014). Collectively, these results illustrate the sensitivity of fish to EDCs during sexual differentiation and identify effects on multiple target genes.

The permanency of EDC effects on gonad development varies depending on the compound and its mode of action, concentration, and the period of exposure (see Santos et al., 2017). Zebrafish often recover from estrogenic EDCs exposures whereas more permanent effects are induced by exposures to aromatase inhibitors. The basis of these differences is not fully understood but it has been proposed that estrogens only delay the normal sexual differentiation of males, rather than promoting real sex reversal. Alternately, androgens promote the loss of PGCs and this is significant in that PGCs are critical to ovarian development. In the absence of PGCs, development of females would not be possible. There are also concerns of possible multigenerational effects of EDCs in fish, such that the resulting progeny may have a higher sensitivity than the exposed parental generation (Santos et al., 2017; Robaire et al., this issue). For example, female-biased sex ratios were reported in F1 and F2 generation zebrafish following exposure to 1 nM (0.228 μg/L) BPA (Chen et al., 2015a); female-biased sex ratios were reported in the untreated F1 and F2 generations following TCDD treatment of just the parents (Baker et al., 2014). While the mechanisms responsible for such effects are not known, these may involve modification of the epigenome (Robaire et al., this issue).

3. Puberty

3.1. Physiology of puberty

Like other vertebrates, puberty in fish is a transitional period in the life history that includes all the processes by which an individual attains, for the first time, the capability to reproduce as an adult, integrating life history traits, environmental and internal cues, gene expression and signaling pathways (Carrillo et al., 2014; Okuzawa, 2002; Strüssmann and Nakamura, 2002). Pubertal development starts after sex differentiation and is completed by the time of the first gametogenesis, requiring the fine tuning of the hormonal system regulated by the hypothalamic-pituitary-gonad axis (Taranger et al., 2010). The activation of this axis involves the secretion of gonadotropin releasing hormone (GnRH) by hypothalamic neurons. GnRH neurons directly innervate the pituitary and stimulate the synthesis and secretion of two gonadotropins i.e., follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Both gonadotropins are secreted from the pituitary into the bloodstream, ultimately reaching the gonads to control gametogenesis and steroidogenesis (Carrillo et al., 2014; Schulz et al., 2010; Taranger et al., 2010). FSH is the first hormone to be secreted, suggesting that it is a key player in the initial stages of pubertal onset (Molés et al., 2012; Prat et al., 1996). Some studies show that the administration of FSH to prepubertal male Sea bass *(Sebastes* sp.) results in spermatogenesis progression (Mazón et al., 2014), reinforcing its role in the onset of puberty.

At the onset of puberty, SSCs and OSCs leave their quiescent stage and enter a process of proliferation that includes several cycles of mitosis followed by a differentiation into spermatogonia and oogonia, respectively (Lacerda et al., 2014; Lubzens et al., 2010, Figs. 1 and 2). These differentiated germ cells initiate a unique process in sexual reproduction, termed meiosis, considered as the starting event leading to puberty, during which their genetic content will be reduced to half (from 2n to n) for the necessary formation of gametes (n). At the same time, specialized Sertoli and Leydig cells in the testis, and follicular cells in the ovaries are activated to provide structural support, growth factors, and trigger the synthesis and secretion of sex steroids, thus providing the proper hormonal milieu needed for maturation of the gonad and gametes (Alix

et al., 2020; Kagawa, 2013; Lubzens et al., 2010; Schulz et al., 2010, Figs. 1 and 2). This process, in fish species with seasonal reproduction, is repeated cyclically throughout their life span and may be regulated by similar mechanisms (Blázquez et al., 2017).

In several fish species, the entry into meiosis is marked by significant increases in plasma levels of 11-ketotestosterone (Blázquez et al., 2017; Rolland et al., 2013; Schulz et al., 2010). Different genes also appear to be altered during pubertal onset, as is the case for *amh*, a potent inhibitor of FSH secretion, and, therefore an inhibitor of spermatogenesis (Blázquez et al., 2017; Crespo et al., 2016; Rolland et al., 2013). Moreover, expression profiling studies have revealed changes in the expression of several genes that control signaling pathways, cell proliferation-, cell cycle-, and meiosis progression at the start of pubertal development (Blázquez et al., 2017; Rolland et al., 2013). In this regard, the role of 17α,20β-dihydroxy-4-pregnen-3-one (*17,α20βP*), a natural progestin inducing spermatogonia entering into meiotic prophase, has been a topic of research in several fish species (Lubzens et al., 2010; Miura et al, 2006, 2007, 2006; Schulz et al., 2010). Moreover, retinoic acid (RA), an active derivative of vitamin A, has been reported to play a key role as a trigger of meiosis onset, not only for different fish species (Adolfi et al., 2016; Blázquez et al., 2017; Medina et al., 2019; Peng et al., 2020; Rodríguez-Marí et al., 2013), but also in rodents, birds (Bowles et al., 2008; Smith et al., 2008) and amphibians (Wallacides et al., 2009). RA homeostasis, regulated by *cyp26a1,* has an essential role in the maintenance of germ cell production. In tetrapods, the RA-signaling pathway activates the transcription of a meiosis specific gene, stimulated by retinoic acid gene 8 protein (*Stra8*) (Griswold et al., 2012). Although this gene is absent in the majority of fish species (Medina et al., 2019; Pasquier et al., 2016; Rodríguez-Marí et al., 2013), a *stra8* homolog has been identified in Southern catfish, *Silurus meridionalis* (Dong et al., 2013) and other fish species (Pasquier et al., 2016), suggesting that in fish two different mechanisms might regulate the RA-mediated entry into meiosis, one dependent and the other independent of the presence of *stra8* (Feng et al., 2015; M. Li et al., 2016b; Medina et al., 2019).

3.2. Impact of EDCs

Spermatogenesis in fish is highly dependent on steroid hormones with estrogens, androgens and progestins having distinct and critical roles (Chen et al., 2013; Melo et al., 2015; Schulz et al., 2010). Estrogens play a critical role in stem cell renewal, androgens are critical for spermatogonial differentiation, and progestins promote the proliferation of early spermatogonia and their differentiation into late spermatogonia and spermatocytes. Similarly, oogenesis and follicle development are highly dependent on steroids (Clelland and Peng, 2009; Devlin and Nagahama, 2002) with estrogens responsible for cell proliferation and vitellogenesis, androgens for feedback mechanisms in the hypothalamus and pituitary and progestins in oocyte maturation and ovulation. Given that many EDCs act by modulating steroid hormone synthesis or action, it is perhaps not surprising that there are many examples of alterations in sexual development in fish. Table 1 summarizes the available literature on the effects of EDCs on the onset of puberty in fish. Traditionally, attention has focussed on the study of the effects of natural and synthetic sex steroids, such as EE2, 17β-trenbolone, diethylstilbestrol (DES), and dihydrotestosterone (DHT). These studies have reported that sex steroids are capable of inducing precocious puberty and delays in puberty in both females and males in zebrafish and Japanese medaka (Baumann et al, 2013, 2014; Lessman and Brantley, 2020; Morthorst et al., 2010; Örn et al., 2006; Tokumoto et al, 2004, 2005).

Although a full understanding of mechanisms by which EDCs effect gonad development during puberty have yet to be realized, several studies have shown that EDCs affect folliculogenesis. For example, EE2 affected follicular development in the zebrafish, as evidenced by reduced ovarian size and a reduction in the proportion of cortical alveolus, vitellogenic and mature follicles compared to controls (Cosme et al., 2015). In addition, a more recent study reported that exposure to 1–10 μg/L of DES for 6 days in juvenile *Casper* zebrafish delayed puberty between 5 and 10 weeks in females and males, respectively (Lessman and Brantley, 2020). These effects were found to be reversible since after cessation of DES exposure animals were capable of reaching puberty and spawning. Long-term exposure to BPA in juvenile zebrafish during gonad differentiation and puberty maturation was found to induce adverse effects on reproduction as well as delays and failure of pubertal onset in male and female zebrafish (Song et al., 2020). In this regard, chronic exposure to 1 and 10 μM BPA induced a delay in the formation of previtellogenic follicles, considered as markers of female puberty onset; in BPA treated males only a few cysts of meiotic spermatocytes and the absence of mature spermatozoa were observed. Interestingly, both males and females exposed to 10 μM BPA showed little signs of post-pubertal folliculogenesis or spermatogenesis, leading the authors of the study to conclude that exposure to BPA blocked the first step in gonad maturation (Song et al., 2020). In the same study, transgenic zebrafish lines null for *cyp19a1a* and the nuclear form of the estrogen receptor (nER: esr1$^{-/-}$, esr2a$^{-/-}$ and esr2b$^{-/-}$) were used to elucidate the mechanism of action of BPA; these experiments indicated that nERs, were involved in the impairment of spermatogenesis induced by BPA. Thus, these recent studies suggest that delayed pubertal onset is due to estrogenic EDCs exerting actions prior to folliculogenesis; for some EDCs this appears to cause severe, irreversible effects on reproductive capacity. However, further investigations and the development of endpoints focussing on the effects of different EDC exposures on the early stages of testis and ovary development are warranted to identify the mechanisms of action and better understand the severity of such impacts.

Growing evidence from environmental epidemiological studies report an association between EDCs and alterations in pubertal timing (reviewed by Zawatski and Lee, 2013). In earlier studies focussing on the effects of pulp mill effluents in white suckers (*Catostomus commersoni*), alterations of gonadal development and delayed puberty were linked to reduced levels of sex steroids (McMaster et al., 1991; Munkittrick et al., 1992). Later studies determined the presence of vitellogenin (VTG) as a biomarker of alterations in gonadal maturation, particularly in male fish (Denslow et al., 1999; Garcia-Reyero et al., 2009; Sumpter and Jobling, 1995). For example, gonadal VTG protein levels and the degree of gonad maturation in juvenile zebrafish were found to be directly correlated in females and inversely correlated in males (Baumann et al., 2013), indicating that high levels of VTG in females and low levels in males are needed at the time of pubertal onset. For this reason, VTG levels have been monitored in many studies as a biomarker of the effects of EDCs on reproduction and some studies have reported VTG levels measured during puberty (Table 1). Synthetic progestagens and their derivatives, widely used for example in contraceptive pills, for pregnancy maintenance in humans or as growth promoters in livestock, are other EDCs with proven ecotoxicological effects that appear as compounds of highest environmental concern after EE2 (Fent, 2015; Kumar et al., 2015; Liu et al., 2012). For example, juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to effluents from sewage treatment plants in Sweden (Fick et al., 2010) and fish from marine aquaculture farms in China (Liu et al., 2017) had elevated levels of plasma progestagens, reinforcing the need to increase the focus of research on these compounds. Although no mechanistic studies on gonad development in rainbow trout are available, juvenile zebrafish exposed during sex differentiation and puberty to the androgenic progestin, levonorgestrel (5.5–834 ng/L) and the non-androgenic progestin, progesterone (3.7–1122 ng/L) showed clear signs of precocious puberty in males (Svensson et al., 2016). Moreover, a study on the effects of 20 different progestagens demonstrated reproductive impairment in fish exposed to environmentally relevant concentrations (reviewed by Kumar et al., 2015). Although, natural progestagens have a direct effect on the onset of meiosis and are considered key players in this process (reviewed by Regidor, 2018), the molecular mechanism by which they alter the first meiosis and onset of puberty in fish remains to be elucidated. Thus, to date eco-epidemiological field studies, combined with controlled

experimental studies, support the impacts of EDCs on gonad development during puberty.

Whether EDCs act exclusively by disrupting the neuroendocrine system and/or by altering gonadal development *per se* is still unclear. Possible modes of action of EDCs include genomic and non-genomic mechanisms, as well as estrogen, androgen and thyroid receptor-mediated effects (Faheem and Bhandari, 2021), yet other mechanisms could also play a major role in EDCs-induced gonadal disruption and puberty onset alteration. For example, RA a key player in the onset of meiosis and spermatogenesis in fish (Adolfi et al., 2016; Blázquez et al., 2017; Medina et al., 2019; Peng et al., 2020; Rodríguez-Marí et al., 2013), could be a target of EDCs. In fact, effluents from pulp and paper mills that are known to contribute to impaired reproduction and delayed puberty are a significant source of ligands that bind to the RA receptors and retinoid X receptors (Alsop et al., 2003). Toxicogenomic approaches, together with the development of new non-target molecular methodologies, have proven to be a valuable approach to elucidate new molecular key events related to responses to EDCs (Caballero-Gallardo et al., 2016). However, to the best of our knowledge, very few studies in the field of fish puberty research have used this approach. Nourizadeh-Lillabadi et al. (2009) reported that precocious puberty was related to alterations of transcriptomic key regulators of steroid and thyroid hormone functions, insulin signaling and metabolic homeostasis. Therefore, we suggest that further investigations using toxicogenomic tools are required to characterize the molecular mechanisms modulated by EDCs, and to elucidate if EDCs can directly trigger the gonad to enter (or not) into meiosis and proceed towards (or arrest) gonadal development and maturation in fish.

Part 2: evidence from Mammals

Exposure to a wide variety of EDCs has been associated with adverse effects on gonad development in mammals (Marlatt et al. this issue), but there is very little certainty about their molecular mechanism of action on perinatal gonads. Here, we aimed to highlight key sensitive events of gonad development mammals (Figs. 1 and 2), and targeted our review on 4 families of EDCs for which human exposure has been tested and for which we have some insight in to their mode of action, both in males (Table 2) and females (Table 3). These include: 1) estrogenic substances from therapeutic sources (i.e. DES or EE2) or epoxy resin-derived bisphenols (i.e. BPA); 2) phthalates, used to make plastics flexible, found in building materials, cosmetics, food packaging, toys and medical devices; 3) flame retardants, such as polybrominated diphenyl ethers (PBDE) and organophosphate esters (OPEs); and, 4) perfluoroalkyl and polyfluoroalkyl substances (PFAS) that comprise a large group of chemicals used for a variety of applications (i.e. stain and water repellants in clothing, carpets and paper; nonstick cookware; food packaging; firefighting foams; photographic and electronic equipment; and, industrial coatings on tiles, stones and versatile consumer products).

1. Gonad differentiation

1.1. Gonad formation

The early stages of gonadogenesis in mammals, understood primarily based on work in rodents, consist of the formation of genital crests on the ventromedial surface of the embryonic kidneys (mesonephros; Pelosi and Koopman, 2017). These crests arise from the accelerated proliferation of celiac epithelial cells, transforming the monolayer of celiac epithelium into a dense, pseudostratified form. This phenomenon occurs around gestation day (GD) 10.5 in mice, GD12.5 in rats and 4–5 weeks of gestation (GW4-5) in humans. The primordial gonads continue to develop through extensive cell proliferation, by ingression of cells from the coelomic epithelium and by recruitment of cells from the mesonephros. These cells will be the origin of the different somatic cells present in the gonads (reviewed Piprek et al., 2016). In parallel, PGCs, which have differentiated from the epiblast under the influence of bone morphogenic protein signals from the extra-embryonic ectoderm (Lawson et al., 1999; Ying et al., 2000), will migrate from the allantois to the genital crests via the posterior part of the endoderm while multiplying rapidly (reviewed in Saitou and Yamaji, 2012; Svingen and Koopman, 2013). PGCs arrive in the genital ridges at around GD11.5 in mice, 13.5 in rats and GW6-7 in humans (Pelosi and Koopman, 2017). At that stage, the DNA of PGCs is mostly demethylated (Hill et al., 2018).

1.2. Sex determination and gonad differentiation

The newly formed gonad is bipotential and can develop into a testis or an ovary. Sex determination in mammals mostly depends on the presence of the Y chromosome and the timely expression of the gene sex-determining region of Y chromosome (*Sry*), a transcription factor of the SOX family, in cells of the coelomic epithelium in the genital crests (Gubbay et al., 1990; Sinclair et al., 1990). If SRY is not expressed or is transcribed in sufficient quantities, the coelomic epithelium differentiates into pre-granulosa cells and the gonads develop into ovaries (Buaas et al., 2009; Kato et al., 2013; Larney et al., 2014; Wu et al., 2012). Expression of SRY triggers a cascade of gene expression leading to differentiation of Sertoli cells which will induce the differentiation of other testicular cell lines and form aggregates around PGCs, creating the characteristic structures of the testis: the seminiferous cords, surrounded by a basement membrane and the interstitium (reviewed in Cool et al., 2012, Fig. 1). In the interstitium, fetal Leydig cells differentiate shortly after seminiferous cord formation, which triggers androgen production, including testosterone (Habert and Picon, 1984). In males, PGCs are called gonocytes, while female PGCs are called oogonia (Pelosi and Koopman, 2017, Figs. 1 and 2).

1.3. Impact of EDCs

To date, cases of sex reversal or, very rarely, cases of ovotestes in mammalian species (including humans), are due to genetic mutations (Dewing et al., 2002). However, a study by Yasuda et al. (1985) showed that oral administration of 0.02–2 mg/kg/d of EE2, a strong estrogen agonist, to Jc1:ICR mice from GD11 to GD17 induced the appearance of ovotestes in male fetuses and ovarian hypoplasia in female fetuses (Yasuda et al., 1985). More recently, *in vitro* experiments in a human testis-derived cell line, NT2/D1, showed that exposure to EE2 significantly decreased the mRNA levels of key genes for Sertoli cell differentiation and male gonad differentiation; these include SOX9, SRY, AMH, Fibroblast Growth Factor 9 (FGF9) and Prostaglandin D2 Synthase (PTGD), and later, significantly increased the mRNA levels of key granulosa cell genes, such as FOXL2 and Wnt family member 4 (WNT4; Stewart et al., 2020). This suggests that an excess of estrogen may affect Sertoli cell differentiation. However, in rodents most *in utero* EDC exposures at the time, or including the time, of gonad differentiation, did not induce sex reversal in gonad differentiation. We can therefore conclude that there is no evidence to date that endocrine disruptors can reverse genetic sex determination, but there is evidence that they may have effects on early somatic cell differentiation. However, there is now evidence that epigenetic mechanisms play a role in sex determination (Garcia-Moreno et al., 2018), leading to new hypotheses on environmental influence, and possible molecular targets involved in the deregulation of this process.

2. Testis development

The testis differentiates at GD12.5 in mice, GD14.5 in rats and GW6-8 in humans (Pelosi and Koopman, 2017). At this stage of development, the testis consists of two compartments representing the two main functions of the testis, gametogenesis in the seminiferous cords and steroidogenesis in the interstitium (Fig. 1).

The seminiferous cords contain the gonocytes, surrounded by Sertoli cells, and are composed of an epithelium bordered by peritubular cells and a basement membrane. Sertoli cells proliferate and differentiate until after birth when the blood-testis barrier forms (Enders, 1993). They

Table 2
Early exposure to EDCs and testicular development in mammals.

Compound	Species	Dose	Exposure Window (Route)	Effects on Testis	Molecular effects	Reference
DES	CD-1 mice	10, 50, 100 μg/kg/d	GD10.5–17.5 (Sc. Injection)	Decrease in the intratesticular testosterone level of GD18.5 testes treated with 10 μg/kg/d.	Decrease in the expression of *P450c17*, *StAR* (mRNA and protein) in GD18.5 testes treated with 100 μg/kg/d.	Guyot et al. (2004)
DES or Zeranol	NMRI mice	150 μg/kg/d	GD9-10 (Sc. Injection)	Presence of multinucleated gonocytes in GD17-18 testes.		Perez-Martinez et al. (1996)
DES or 17β-estradiol	CD-1 mice	20 μg DES or 6 mg 17β-estradiol	GD11.5, 13.5 and 15.5 (Sc. Injection)	Cryptorchidism	Decrease in expression of *Insl3* at GD 17.5 and PND0	Nef et al. (2000)
DES	Sprague-Dawley Rats	1.5, 15 μg/kg/d	GD7-21 (Sc. Injection)	Decrease in blood testosterone levels at 6 weeks after birth.		Yamamoto et al. (2003)
		0.01–2 μg/kg/d	GD14 – birth (Gavage)		Increase in expression of *Pdgfrα* at PND3 Decrease in expression of *Pdgfrβ* at PND3 (2 μg/kg/d)	Thuillier et al. (2003)
		6 μg/kg/d	GD14 – birth (Gavage)	Decrease in sperm count, motility and altered morphology at PND75		Ahmad et al. (2014)
EE2	CF-1 mice	0.002–20 μg/kg/d	GD0-17 (micropipette)	Decrease in daily sperm production at PND50		Thayer et al. (2001)
	Jc1:ICR mice	0.02, 0.2 mg/kg/d	GD11-17 (Gavage)	Decrease in intratesticular testosterone level and LC hyperplasia in 20–22-month testes exposed *in utero* to 0.2 mg/kg/d.		Yasuda et al. (1988)
	Sprague-Dawley Rats	0.001–10 μg/kg/d	GD11-20 (Sc. Injection)	No morphological changes in the developing reproductive system	Decrease in expression of *Cyp11a1, StAR, Cyp17a1* at the highest dose on GD20	Naciff et al. (2005)
Bisphenol A	ICR mice	5, 50 mg/kg	GD7 and GD14 (Sc. Injection)	At 6 weeks: Decrease in sperm count, motility and morphology Decreased number of SC per seminiferous tube.	Decrease in expression of genes associated with Sertoli cell functions (*Msi1h*, *Ncoa1*, *Nid1*, *Hspb2*, *Gata6*) at the highest dose.	Tainaka et al. (2012)
	C57BL/6J mice	5, 50 μg/mL (1, 10 mg/kg/d)	GD1-birth (Drinking water)	Increase in gonocyte apoptosis at PND14 (both doses) and PND35 (50 μg/mL).	Decrease in expression of *StAR*, *Cyp11a1*, *3βhsd* in PND1 testes.	Yang et al. (2019)
	Kunming mice	2.5–40 mg/kg/day	GD0.5–17.5 (Gavage)	Decrease in post-natal testis weight with the high dose Decrease in serum T and E2 at PND21 and PND56	upregulated testicular expression of *Dnmt1*, *Esr1* and *Ar* and genes related to apoptosis at PND21 Decreased testicular expression of *Dnmt3A* and *Dnmt3B* at PND 21	Wei et al. (2019)
	Sprague-Dawley Rats	4, 40, 400 mg/kg/d	GD16-21 (Gavage)	Decrease in testosterone production and the number of LC at GD 21 (40 and 400 mg/kg/d)	Decrease in expression of *Insl3*, *Hsd17b3* (protein and mARN) at GD21.5 Decrease in expression of *Lhcgr*, *Cyp11a1*, *Cyp17a1*, *Amh* (protein and mARN) at GD21.5 (400 mg/kg/d)	Lv et al. (2019)
		0.002–400 mg/kg/d	GD11-20 (Sc. Injection)	No morphological changes in the developing reproductive system	Decrease in expression of *Cyp11a1*, *StAR*, *Cyp17a1* in GD20 testes treated with the highest dose at GD20	Naciff et al. (2005)
		0.2–200 μg/mL	GD1 -2 h after birth (Drinking Water)	Dose-dependant decrease in blood testosterone level		Tanaka et al. (2006)
		0.1–200 mg/kg/d	GD14- birth (Gavage)		Increase in expression of *Pdgfrα* and *Pdgfrβ* at PND3 (≥1 mg/kg/d)	Thuillier et al. (2003)
		200 mg/kg/d	GD14- birth (Gavage)	Decrease in the percentage of proliferating gonocytes at PND3.	Decrease in expression of *Erk1* (protein and mRNA)	Thuillier et al. (2009)
	Wistar Rats	25, 250 μg/kg/d	GD10-21 (Gavage)	Decrease in sperm count and viability at PND120		Olukole et al. (2019)
Dibutyl phthalate (DBP)	Wistar Rat Wistar Rat	500 mg/kg/d	GD13-21 (Gavage)	Decrease of 90% in testosterone secretion at GD19 associated with LC hyperplasia. High rate (>60%) of cryptorchidism, hypospadias, infertility, and testicular impairment. Presence of malformed tubules and LC inside seminiferous tubules. Presence of multinucleated gonocytes up to PND10 and immature SC.		Fisher et al. (2003)
		100–500 mg/kg/d	GD13.5–20.5 (Gavage)	Decrease in testosterone and changes in LC distribution at		Mahood et al. (2007)

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Table 2 (*continued*)

Compound	Species	Dose	Exposure Window (Route)	Effects on Testis	Molecular effects	Reference
				GD21.5 Increase in occurrence of multinucleated gonocytes at GD21.5		
		750 mg/kg/d	GD15.5–18.5 (Gavage)	Decrease in intratesticular testosterone level. Changes in LC distribution due to large LC aggregates in GD21.5 testes.		van den Driesche et al. (2012)
		500 mg/kg/d	GD13.5–20.5 (Gavage)	Germ cells are aggregate at GD21.5 Increased occurrence of multinucleated gonocytes at GD21.5 Decreased number of germ cells at GD21.5 when exposed from GD13.5–15.5		van den Driesche et al. (2015)
		750 mg/kg/d	GD15.5–18.5 (Gavage)	Decrease of 47–48% in intratesticular testosterone level at GD17.5 associated with aggregation of LC at GD 17.5 and 21.5 Focal dysgenesis characterized in ectopic SC scattered among the aggregated LC at GD21.5		van den Driesche et al. (2017)
Di (2-ethylhexyl) phthalate (DEHP)	Wistar Rats	405 mg/kg/d	GD6 –PND21 (Gavage)	Multinucleated gonocytes observed at PND1		Andrade et al. (2006)
		750 mg/kg/d	GD7 –PND17 (Gavage)	Decrease in intratesticular and circulating levels of testosterone at GD21.		Borch et al. (2004)
		5 or 50 μg/kg bw	GD12- 21 (Gavage)	No effect on post-natal testis weight Increased serum AMH in adults No change in Serum T to E2 ratios	dose-dependent increases in SOX9 and AMH at PND10 Increased CYP19A1, AR and DNMT3B in adult testis	Abdel-Maksoud et al. (2015)
	Sprague-Dawley Rats	234–1250 mg/kg/d	GD14.5–birth (Gavage)	Hyperplasia of LC No change in absolute volume of germ cells Significant decrease of fetal testosterone production and adult serum testosterone levels associated to	Decreased expression of *Cyp11a1*, *Cy17a1*, *Star*, and *Tspo* at GD19 Increased expression of *Cyp11a1*, *Cy17a1*, *Star*, and *Tspo* from PND3 to adults	Culty et al. (2008)
		30 or 300 mg/kg/day	GD8- PND21 (Gavage)	Decreased Anogenital index Increased incidence of hemorrhagic testis and multinucleated gonocytes No effect on steroid level or sperm quality in adult rats	55 DEG in adult testis, including some related to estrogen function and signaling (Nr5a2, Ltf and Runx 2)	Albert et al. (2018); Nardelli et al. (2017)
		10 mg/kg/day	GD14.5–birth (Gavage)	No change in anogenital distance No change in adult serum testosterone Increased adult testis weight	Altered expression of mRNA coding for steroidogenic enzymes, markers of Leydig progenitor, Sertoli cells and germ cells, proliferation, redox balance and xenobiotic transporter markers from PNd3 to adulthood	Jones et al. (2015), 2014
Diisononyl phthalate (DiNP)	Wistar Rats	750 mg/kg/d	GD7-21 (Gavage)	Decrease in testosterone production and intra-testicular testosterone level at GD21		Borch et al. (2004)
		1000 mg/kg/d	GD12-21 (Gavage)	Decrease in testosterone secretion LC hyperplasia and aggregation Presence of multinucleated gonocytes	Decrease in mRNA and protein levels of *Insl3* and *3βhsd*	Li et al. (2015)
2,2,4,4,5-pentabromodiphenyl ether (BDE-99)	Long-Evans Rat	1 or 10 mg/kg/d	GD10-18 (Sc. Injection)	Reduced anogenital distance Decrease serum testosterone		Lilienthal et al. (2006)
BDE-47	Wistar rats	0.2 mg/kg body weight/day	GD8-birth (micropipette)	Smaller testes, Decreased sperm production, Increased percentage of morphologically abnormal spermatozoa	Decreased expression of transition proteins and protamine genes Increased expression of TNFα and IL3 Changes in sperm DNA methylation	Khalil et al. (2017) Suvorov et al. (2018)
Hexafluoropropylene oxide dimer acid (HFPO-DA)	Sprague-Dawley rat	1–500 mg/kg-body	GD14-18 (Gavage)	No change in fetal testis testosterone production Decreased adult testis and epididymis weights	No effect on gene expression in fetal testis (genes tested are known to be affected by phthalates)	Conley et al. (2019)
Perfluorotridecanoic acid (PFTrDA)	Sprague-Dawley rat	1, 5, 10 mg/kg/d	GD14-21 (Gavage)	Reduced anogenital distance (high dose)	Decreased mRNA and protein of Insl3, Lhcgr, Scarb1, Star, Hsd3b1,	Li et al. (2021)

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Table 2 (*continued*)

Compound	Species	Dose	Exposure Window (Route)	Effects on Testis	Molecular effects	Reference
				Decreased serum testosterone levels Abnormal aggregation of fetal Leydig cells	Cyp17a1, Nr5a1, and Dhh Increased antioxidants (SOD1, CAT, and GPX1), induced autophagy (increased levels of LC3II and beclin 1, and reduced the phosphorylation of mTOR)	

DES: Diethylstilbestrol; EE2: Ethynylestradiol; GD: Gestation day; PND: Post-natal Day; LC: Leydig cells; SC: Sertoli cells; #: number; Sc. Injection: Subcutaneous injection; Amh, Anti Mullerian hormone; bw, body weight.
(Note that examples of studies are presented here; the list is not comprehensive).
AR, Androgen receptor; CAT, Catalase; Cyp11a1, cytochrome P450 11 subfamily A member 1; Cyp17a1, cytochrome P450 17 subfamily A member 1; Cyp19a1, cytochrome P450 19 subfamily A member 1; Dhh, Desert Hedgehog; Dnmt, DNA methyltransferase; Esr1: Estrogen receptor 1; Erk1, Extracellular Signal-Regulated Kinase 1; Gata6, GATA Binding Protein 6; GPX1, Glutathione Peroxidase 1; Hspb2, Heat Shock Protein Family B (Small) Member 2; Hsd17b3, Hydroxysteroid 17-Beta Dehydrogenase 3; Hsd3b1, Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 1; Insl3, Insulin like 3; Lhcgr, luteinizing hormone/choriogonadotropin receptor; LC3II, microtubule-associated protein light chain 3; Msi1h, Musashi RNA Binding Protein 1; mTOR, Mechanistic Target Of Rapamycin Kinase; Ncoa1, Nuclear Receptor Coactivator 1; Nid1, Nidogen 1; Nr5a1, Nuclear Receptor Subfamily 5 Group A Member 1; P450c17, Cytochrome P450 Family 17 Subfamily A Member 1; Pdgfrα, Pdgfrβ, Platelet Derived Growth Factor Receptor Alpha and beta; Scarb1, Scavenger Receptor Class B Member 1; SOD1, Superoxide Dismutase 1; StAR, steroidogenic acute regulatory protein; Tspo, Translocator Protein 18-kDa.

act as support and feeder cells for the germ cells, notably by contributing to gonocyte survival and preventing their entry into meiosis (Bowles et al., 2006; Koubova et al., 2006; Li et al., 1997). They synthesize and secrete the AMH responsible for the regression of the Müllerian ducts (Magre and Jost, 1984). Gonocytes continue to proliferate for a few days until they enter a quiescent phase characterized by a cell cycle arrest in the G0/G1 phase at about GD16.5 in mice, GD18.5 in rats and GW18-19 in humans; the cell cycle resumes after birth in rodents (Culty, 2009, Fig. 1). Around this time, gonocytes undergo the epigenetic reprogramming that corresponds to the establishment of new DNA methylation marks and chromatin remodelling (Ly et al., 2015; Rwigemera et al., 2021; Wu et al., 2015). This contributes to the establishment of the male germ cell transcriptome and the formation of the spermatogonial stem cell pool in the neonatal testis (Culty, 2009; Manku and Culty, 2015; Rwigemera et al., 2021).

In the interstitium, fetal Leydig cells differentiate and contribute to the production of androgens and insulin-like 3 (INSL3) that are key to the further development of the testis and differentiation of the reproductive tract, inducing the maturation of the Wolffian duct and testicular descent. In rodents, Sertoli cells also contribute to steroidogenesis by converting androstenedione (A4) to testosterone, as fetal Leydig cells do not express the enzyme catalyzing this conversion, namely 17β-HSD (17β-hydroxysteroid dehydrogenase; Shima et al., 2013). In primates, substantial A4 is also produced by the fetal adrenal; in contrast, no androgens are produced by fetal rodent adrenals. Leydig cell steroidogenesis is responsive to LH stimulation from the fetal pituitary but, in rodents, it is mostly regulated by paracrine and autocrine factors, while in primates, chorionic gonadotropins enhance fetal Leydig cell androgen production (reviewed by Sharpe, 2020).

2.1. *The male masculinization window – a key stage of sensitivity to EDCs*

After the testis is formed, further masculinization of the reproductive tract is driven mostly by testosterone, AMH and INSL3 (Sharpe, 2006). At this stage, all testicular cell types express both the estrogen and androgen receptors; data from murine null mutation studies for the various forms of these receptors have shown that the proper development of these cells is in part regulated by steroid hormones (Rouiller--Fabre et al., 2015). Given this, and the fact that the masculinization of the reproductive system is sensitive to androgens, it is not surprising that this period of testicular and male genital tract development is particularly sensitive to potential endocrine disruption. This hypothesis was proposed in 1993 by Sharpe and Skakkebaek, who suggested that an impairment of androgen production or an excess of estrogen during this window of sensitivity could be the origin of developmental abnormalities observed in the testicular dysgenesis syndrome (Sharpe and Skakkebaek, 1993). Since then, experimental studies using flutamide, an anti-androgen, have allowed a better definition of the window of development during which androgen action is key for the establishment of reproductive functions in the male. This window of masculinization occurs between GD 15.5 and 19.5 in rats (corresponding to GW8 - 14 in humans) (Welsh et al., 2008). Developmental changes in testicular sensitivity to estrogens have also been observed in experiments with organ culture of perinatal testis sampled at different times during fetal and neonatal life, identifying a similar period of sensitivity to both androgens and estrogens (Delbès et al., 2007).

2.2. *Impact of EDCs on testicular steroidogenesis*

The effects of EDCs on steroidogenesis have been reviewed recently (Walker et al., 2021). EDCs have been implicated in impairing steroid hormone synthesis by the fetal testis, thereby affecting testicular development and masculinization. Table 2 summarizes some of the available *in vivo* exposure studies in rodents that have been particularly useful in characterizing the effects of EDCs on testicular steroidogenesis. Since there are differences between rodent and human testicular development, endocrinology and metabolism, a xenograft model in which human fetal testis is grafted to mice exposed to EDCs is also of interest (Heger et al., 2012), although a limitation of this approach is the age range at which fetal human testicular tissue can be obtained. Other tools for screening the impact of EDCs on testicular steroidogenesis are available. These include organ cultures of fetal testis (rat, mouse, human (early stages)) (Habert et al., 2014) and testicular cultures of primary cells or cell lines. There are currently no human Leydig cell models or fetal Leydig cell models, but several rodent adult Leydig cell lines, have been used to characterize the effects of chemicals on steroidogenesis (mouse LTC-1, MA-10 and TM3 (Nikula et al., 1999; Schang et al., 2016; Walsh et al., 2000) and rat R2C (Balbuena et al., 2013; Heneweer et al., 2004).

Table 2 highlights several studies in rats showing the negative impact of *in utero* exposure to **estrogenic substances**, such as EE2, DES and BPA on testosterone secretion. Some of these studies, including transcriptomic analyses of the testis, have shown an association with decreased expression of genes encoding steroidogenesis-related transcripts, such as steroidogenic acute regulatory protein (StAR), cytochrome P450 family 11 subfamily A member 1 (Cyp11a1), cytochrome P450 family 17 subfamily A member 1 (Cyp17a), hydroxysteroid 17 beta dehydrogenase 3 (Hsd17b3) and cytochrome P450 family 17 subfamily A member 1 (P450c17; Guyot et al., 2004; Lv et al., 2019; Naciff et al., 2005; Yang et al., 2019). Interestingly, the deleterious effects on steroidogenesis observed in the fetal (GD18) and post-natal (PND1) testes also persist into adulthood suggesting that early exposure to EDCs may

Table 3
Early exposure to EDCs and ovarian development in mammals.

Compound	Species	Dose	Exposure Window (Route)	Effects on Ovary	Molecular Effects	Reference
DES	CD-1 mice	0.5 mg/kg/d	PND 1–5 (Sc. injection)	Breakdown of nests of oogonia; reduced number of small antral follicles; induced multioocyte follicle formation	Decreased activin β subunit expression	Iguchi et al. (1986); Kipp et al. (2007)
EE2	C57BL/6J mice	0.0067–0.067 mg/kg/d	GD 10–18 (Sc. injection)	Reduced survival of foetuses and newborns; polyovular follicles; ovary-independent vaginal epithelial stratification		Kirigaya et al. (2006)
Bisphenol A	ICR/Jcl mice	10 mg/kg/d	GD 10–18 (Sc. injection)	Reduced the number of ovarian corpora lutea		Suzuki et al. (2002)
Bisphenol A	ICR/Jcl mice	0.150 mg/kg/d	Birth-PND 5 (Sc. injection)	Ovary-independent vaginal epithelial stratification		Suzuki et al. (2002)
Diethylhexyl phthalate	CD-1 mice	0.04 mg/kg/d	GD 1-birth (oral)	Delayed meiotic progression of early germ cells (17.5 DPC); delayed follicle assembly in newborn ovary; decreased number of primordial and increased number of secondary follicles (PND21) in F1 and F2 offspring	Reduced expression of STRA 8 (gene and protein) at 13.5 DPC; significant downregulation of *Cyp17a1* and *Cyp19a1* gene expression in fetal ovaries	Zhang et al. (2015)
	CD-1 mice	0.02, 0.2, 200, 500, or 750 mg/kg/d	GD 10.5-birth (oral)	Decreased folliculogenesis and increased serum estradiol (F1); dysregulated folliculogenesis and disrupted serum progesterone (F2); accelerated folliculogenesis (F3)		Rattan et al. (2018)
	CD-1 mice	0.02, 0.2, 500, or 750 mg/kg/d	GD 10.5-birth (oral)	Transgenerational disruption of DNA methylation (F1, F2 and F3)	Transgenerational (F1, F2 and F3) suppression of gene expression pathways required for folliculogenesis and steroidogenesis	Rattan et al. (2019)
	BalB/C mice	0.0025, 0.005, 0.010 mg/kg/d	PND 0–4 (IP injection)	Impaired germ nest breakdown and primordial follicle assembly		Mu et al. (2015)
	BalB/C mice	0.6 mg/kg	PND 0–4 (IP injection)	Severe disruption of primordial follicle formation; premature ovarian senescence and reduced fertility	Enhanced autophagy: increased autophagy-related gene expression and recognizable autophagosomes	Zhang et al. (2018)
	CD-1 mice	0.02, 0.04 mg/kg/d	PND 7–14 (hypodermal injection) (treatment 1) PND 5,10,15, and 20 (hypodermal injection) (treatment 2)	Decreased number of primordial follicles and increased secondary (treatment 2) and antral follicles.	Reduced and/or delayed methylation of imprinted genes such as *Igf2* and *Peg3* in oocytes Increased abnormal metaphase II spindles in oocytes matured *in vitro* (treatment 2)	Zhang et al. (2013)
	CD-1 mice	0.02, 0.04 mg/kg/d	PND 5, 10, and 15 (IP injection)	Inhibited antral follicle enlargement process	Increased mRNA levels of the apoptosis related genes; disturbed oxidative status	L. Li et al. (2016)
Mono-2-ethylhexyl phthalate (MEHP)	C57/Bl6 mice	100, 500, or 1000 mg/kg	GD 17–19 (gavage)	Reduced reproductive lifespan in F1 (1000 mg/kg)	Altered mRNA for the LHCGR and steroidogenic genes (aromatase, StAR)	Moyer and Hixon (2012)
Pentabrominated PBDE-99	Wistar rats	0.06, 0.3 mg/kg	GD 6 (gavage)	Increased rate of resorptions		Talsness et al. (2005)
Pentabrominated PBDE-99	Long Evans rats	1, 10 mg/kg/d	GD 10–18 (Sc. Injection)	Decreased ovarian primordial and secondary follicles		Lilienthal et al. (2006)
Tetrabrominated PBDE-47	Wistar rats	0.14, 0.7 mg/kg	GD 6 (gavage)	Decreased numbers of secondary and antral follicles and decreased ovary weights; decreased serum estradiol		Talsness et al. (2008)
Perfluorononanoic acid (PFOA)	Mice	3 mg/kg/d	GD 1–18 (oral)	Decreased ovary size; decreased numbers of primary, secondary and antral follicles, and corpora lutea; delayed onset of sexual maturity		Zhang et al. (2020)
Perfluorobutane sulfonate (PFOS)	Mice	200, 500 mg/kg/d	GD 1–20 (oral)	Reductions in all stages of ovarian follicles; decreased ovary and body size and weight; decreased serum estradiol; delayed onset of sexual maturity		Feng et al. (2017)
	Sprague-Dawley rats	0.1, 1, 10 mg/kg/d	PND 1–5 or 26–30 (Sc. Injection)	Reduction in the number of primordial and growing follicles and corpora lutea		Du et al. (2019)

DES: Diethylstilbestrol; EE2: Ethynylestradiol; GD: Gestation day; PND: Post-natal Day; Sc. Injection: Subcutaneous injection; IP: intraperitoneal.
(Note that examples of studies are presented here; the list is not comprehensive).
Cyp17a1, cytochrome P450 17 subfamily A member 1; Cyp19a1, cytochrome P450 19 subfamily A member 1; Ifg2, insulin like growth factor 2 receptor; LHGCR, luteinizing hormone/choriogonadotropin receptor; Peg3, paternal expressed gene 3; StAR, steroidogenic acute regulatory protein; Stra8, stimulated by retinoic acid gene 8 protein.

impair the establishment and function of adult Leydig cells (Yamamoto et al., 2003; Yang et al., 2019; Yasuda et al., 1988). The inhibitory effect of estrogenic substances on testosterone production was also observed throughout fetal life in dispersed testicular cell cultures and in organ cultures of rat and mouse fetal testes (Delbès et al., 2007; N'Tumba-Byn et al., 2012). However, the effects on testosterone that were observed in early fetal stage whole rat testes were masked by GD20.5, due to local production of estrogens (Delbès et al., 2007).

The human fetal testis seems to have a different sensitivity than rodents since it has been reported to be insensitive to the effects of DES in both the organ culture (N'Tumba-Byn et al., 2012) and the xenograft models (Mitchell et al., 2013), but more sensitive to the effects of BPA, at least in organ culture (N'Tumba-Byn et al., 2012). Studies with estrogen receptor knock-out (ERKO) mice have revealed that the effect of DES on steroidogenesis is mediated by ESR1, while the impact of BPA is independent of estrogen receptor (Delbès et al., 2007; N'Tumba-Byn et al., 2012). These data strongly suggest that the human testis is insensitive to effects mediated by the ESR1 at these early stages (Rouiller-Fabre et al., 2015). Other estrogen mediated pathways activated by BPA, such as estrogen related receptor gamma (ERRγ) (Liu et al., 2014), may play important roles, but have not yet been fully characterized in fetal rodent or human testes.

Similarly, many studies have described the negative impact of **phthalates** on testicular steroidogenesis. Although the exact molecular mechanism of action of phthalates on steroidogenesis has yet to be resolved, there is a growing body of evidence that such suppression of steroidogenesis is mediated by the activation of peroxisome proliferator-activated receptor (PPAR) α and γ nuclear receptors (Martinez-Arguelles et al., 2013). Fetal exposure during the masculinization window to dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) or diisononyl phthalate (DiNP) leads to underdevelopment and/or malformations of the male reproductive tract and genitalia as a consequence of reduced testosterone secretion by fetal Leydig cells (Table 2; Borch et al., 2004; Chen et al., 2021; Culty et al., 2008; Fisher et al., 2003; Mahood et al., 2007; van den Driesche et al., 2017, 2015). Outcomes in adult male rats associated with fetal exposures to these phthalates include decreased weights of reproductive organs, reduced anogenital distance (AGD), disrupted seminiferous epithelium with reduced spermatogenesis and decreased numbers of Sertoli and germ cells; in addition, malformations such as prostatic or epididymal agenesis, hypospadias, cryptorchidism, retained thoracic areolas or nipples have been observed after fetal exposure to phthalates. This suite of effects has been labelled the Phthalate syndrome (NRC, 2008). These effects are often associated with hyperplasia and aggregation of fetal Leydig cells and decreased expression of steroidogenic enzymes. Among the earliest gene expression changes in the fetal rat testis following *in vivo* exposure to an effective dose of phthalate are transcripts and proteins involved in the synthesis and uptake of the steroid hormone precursor cholesterol and in steroid hormone synthesis (Johnson et al., 2011). The potency by which phthalate esters induce these effects is highly dependent on the structure and location of the ester side chains; the more potent phthalates have side chains between 4 and 9 carbons, with potency diminishing with shorter chains (Gray et al., 2000). In contrast with the effects in rats, DBP exposure of mice during the masculinization window does not result in reduced testis androgen production (Albert and Jégou, 2014; Johnson et al., 2012), or alter the expression of steroidogenesis associated genes (Johnson et al., 2011). Yet, DBP exposure results in aggregation of fetal Leydig cells and increased incidence of hypospadias in exposed mice (Albert and Jégou, 2014). Similarly, studies using organ culture and xenograft of human fetal testes suggest that phthalates have little impact on fetal human testis steroidogenesis (Hallmark et al., 2007; Heger et al., 2012; Mitchell et al., 2012).

Effects of phthalate exposures on a range of other endpoints of human male development are less clear but have received considerable attention; this issue has been extensively reviewed (Albert and Jégou, 2014; Arzuaga et al., 2020; Habert et al., 2014; Howdeshell et al., 2008; Johnson et al., 2012; National Toxicology Program, 2003). The ubiquitous presence of diverse phthalates means that the majority of fetuses through the world have measurable exposure to some combination of phthalates, mostly at doses below those observed to disrupt male reproductive tract development in rats. Multiple studies have examined the correlation between phthalate exposures during early pregnancy (corresponding to the human masculinization window) and AGD; the results of these studies have been inconsistent. While several studies revealed a robust correlation between human fetal exposures to phthalates and reduced AGD in male infants (Swan et al, 2005, 2015), others have not found such a relationship (Jensen et al., 2016) or found that exposure was correlated with longer AGD (Arbuckle et al., 2019). When these data were considered in a meta-analysis, fetal exposures to DEHP was significantly associated with reduced AGD in human male infants (Dorman et al., 2018; Zarean et al., 2019). These results suggest that *in utero* exposures to certain phthalate esters may be associated with impaired androgen-dependent male reproductive tract development although fetal testis androgen production may be unaffected.

The effects of **PBDEs and OPEs,** chemicals commonly used as flame retardants, on testicular development have been reviewed recently (Hales and Robaire, 2020). There is evidence from several epidemiological studies that exposure to brominated flame retardant (BFRs) during gestation may affect endpoints in male offspring such as cryptorchidism or anogenital distance. In a study from Sweden, an association between an elevated incidence of cryptorchidism and PBDE concentrations in breast milk was significant (Main et al., 2007). Similarly, a study in a Canadian population reported that every 10-fold increase in maternal hair 2,2,4,4,5-pentabromodiphenyl ether (BDE-99) or 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100) was associated with at least a doubling in the risk of having a child with cryptorchidism (Goodyer et al., 2017). Data from Shanghai have suggested that prenatal exposure to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and Σ4PBDEs, even at low environmental levels, may be associated with shorter AGD in boys (Luan et al., 2019). Studies in several experimental models have provided evidence that exposure to PBDEs may affect testicular steroidogenesis, suggesting altered steroidogenesis as a possible causal factor inducing the phenotypes observed in human cohorts. In fetal porcine Leydig cells, several BFRs reduced testosterone synthesis by inhibiting specific enzymes in the steroidogenesis pathway (Mankidy et al., 2014). Using the mouse LTC-1 Leydig cells, Han et al. (2019) demonstrated an inhibition of steroid production after exposure to deca-brominated diphenyl ether (BDE-209). Treatment of Long-Evans rat dams with a single dose of BDE-99 on gestation day 6 reduced AGD and decreased circulating sex steroids in male offspring (Lilienthal et al., 2006). The underlying mechanism(s) for such effects have yet to be elucidated.

The persistence, bioaccumulation and toxicity of PBDEs has led to regulations of their use and created a need for their replacement to meet the flammability standards in many jurisdictions; the OPEs are substances that are commonly used as replacement flame retardants. While studies of the mechanism of action of these chemicals during fetal development on steroidogenesis are scarce, a few have demonstrated that these chemicals do affect steroid production in cultured Leydig cells. Tris (methylphenyl) phosphate (TMPP) was found to suppress TM3 Leydig cell testosterone secretion (Liu et al., 2016). Another OPE, 2-(butoxyethyl) phosphate (TBEP), was shown to induce an increase in oxidative stress and a decrease in steroid production in TM3 cells (Jin et al., 2016). Schang et al. (2016) compared the effects of seven OPEs on MA10 Leydig cell functions with those of BDE-47. All the OPEs affected mitochondrial activity, cell survival, and superoxide production. Five of the seven OPEs caused an increase in basal or cAMP-stimulated steroid secretion and several OPEs affected the expression of genes involved in steroid biosynthesis. Thus, the limited data available to date suggest that both PBDEs and OPEs may affect Leydig cell functions.

The effects of **PFAS** on testicular steroidogenesis have been reviewed recently (Zhu et al., 2020), highlighting three *in utero* exposure studies of perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS) and

perfluorononanoic acid (PFNA) that reported negative effects on Leydig cell development, testosterone secretion, and expression of steroidogenic enzymes. Similarly, a recent study of gestational exposure to perfluorotridecanoic acid (PFTrDA) in rats reported inhibition of the differentiation of fetal Leydig cells mainly through increasing oxidative stress and inducing autophagy (Li et al., 2021). *In utero* exposure of rats from GD14-18 to another perfluoroalkyl substance, hexafluoropropylene oxide dimer acid (HFPO-DA, also known as GenX; 1–500 mg/kg/day) was not reported to affect fetal testis development but negative effects on pup birth weights and the liver were observed (Conley et al, 2019, 2021).

2.3. Impact of EDCs on gametogenesis in males

Table 2 summarizes some of the available *in vivo* exposure studies in rodent models. Evidence from in *vivo* and *in vitro* experimental models range from immediate effects on germ cell proliferation or death, the appearance of multinucleated germ cells, changes in gene expression, or effects on the epigenetic reprogramming that takes place prior to the establishment of SSCs in males, to long term impacts on sperm production.

Experiments of early exposure to **estrogenic substances** in rats have shown immediate and long-term impacts on male germ cells. Gonocyte proliferation, essential for germline development, has been shown to be sensitive to exogenous estrogens such as DES, with high sensitivity during the fetal proliferation phase (Delbès et al., 2007; Lassurguère et al., 2003). Long term effects of early exposure to BPA, DES and EE2 on the adult germline have also been observed; these included increased germ cell apoptosis, reduced sperm count and motility and increased sperm morphological abnormalities (Ahmad et al., 2014; Olukole et al., 2019; Tainaka et al., 2012; Thayer et al., 2001; Yang et al., 2019). Using both *in vitro* and *in vivo* models of exposure to estradiol, DES or BPA, Culty and collaborators have shown that estrogens can target PGDF signaling in gonocytes and alter germ cell proliferation and differentiation after birth (Li et al., 1997; Manku et al., 2015; Thuillier et al, 2003, 2009, 2003; Wang and Culty, 2007). Similarly, one study reported an impact of *in utero* low-dose exposure to EE2 or BPA on the testis transcriptome, suggesting these chemicals may affect the expression of genes important for germ cell differentiation, even at doses at which no immediate phenotypical or histological changes were observed (Naciff et al., 2005). Interestingly, multinucleated gonocytes were observed in murine testes at GD17-18 after exposure to DES or Zeranol (150 μg/kg/day) from GD9-10, yet the mechanism is unknown (Perez-Martinez et al., 1996). Organ culture and xenograft experiments have revealed that exposure to 10 μM BPA may have negative effects on germ cells density and differentiation, suggesting similar sensitivity in human and rodents (Eladak et al., 2018).

There are several reports of the negative impact of **phthalates** on the male germline with many observations of decreased numbers of germ cells and the presence of multinucleated gonocytes after birth, immature Sertoli cells and focal testicular dysgenesis (Table 2) (Andrade et al., 2006; Fisher et al., 2003; Mahood et al., 2007; Nardelli et al., 2017; van den Driesche et al., 2017, 2015, 2012). Early exposure to DEHP has been reported to have immediate and long-term effects on testicular gene expression, some of which being related to the differentiation of germ cells (Jones et al, 2014, 2015). Interestingly, after MEHP exposure similar effects were observed in both rodent and human fetal testes; these included an increase in germ cell death in the absence of an effect on the proliferation or apoptosis of Sertoli cells (Habert et al., 2014). As MEHP does not affect testosterone production in humans, this suggests that MEHP may act directly on germ cells. Exposure of ERKO mice or AR-deficient mice to MEHP revealed that MEHP-induced germ cell damage is not predominantly mediated by steroid receptors (Rouiller--Fabre et al., 2015). Instead, MEHP targets PPAR and liver X receptor LXR pathways in the fetal testis; these pathways are also linked to the effects of MEHP in adults (Rouiller-Fabre et al., 2015).

Exposures during gestation to **PBDEs** have also been found to affect male germ cells. In a study on Wistar rats exposed to BDE-47 during gestation from day 8 onward, Khalil et al. (2017) reported that offspring had significantly smaller testes, decreased sperm production, and an increased percentage of morphologically abnormal spermatozoa. The expression of genes essential for spermatogenesis, such as transition proteins and protamines, was suppressed and immune response genes, such as the tumor necrosis factor 3 (Tnf 3) and interleukin 3 (Il3), were increased; these authors suggested that this treatment might be producing an aberrant sperm epigenome. A subsequent study revealed that BDE-47 exposure altered sperm DNA methylation (Suvorov et al., 2018). Using a stem cell-based spermatogenesis model, Greeson et al. (2020) showed that exposure to 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-153) decreased *de novo* and maintenance DNA methylation at regulatory elements controlling imprinted genes. Furthermore, PBB-153 affected DNA methylation as well as the expression of genes critical to proper human development (Greeson et al., 2020). Studies of the effects of two other flame retardants, hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA), on a human stem cell-based model of spermatogenesis, revealed that mitochondrial membrane potential was perturbed and reactive oxygen species were increased, leading to the apoptosis of spermatogonia and primary spermatocytes (Steves et al., 2018).

A recent review of the potential impact of **PFAS** on male fertility has linked increased exposure to PFAS with effects on semen quality (Tarapore and Ouyang, 2021). Yet, observations in experimental rodent exposure studies showed inconsistency from no effect to some negative effects on sperm production. Such variation may be due to species- and strain-specific differences in PFAS metabolism, the rat being the least adequate to compare PFAS toxicity studies relative to human health.

Together, these studies suggest that there are deleterious effects induced by a variety of EDCs on germ cells. Whether all of these are entirely direct or indirect (i.e. mediated by effects on somatic cell impairment) that consequently affect germ cells, remains to be determined. With recent development of flow cytometry-based methods to purify mouse and human germ cells, a few investigations have reported molecular impacts specifically on germ cells. Most report little immediate impact on germ cells' transcriptome and DNA methylation (Iqbal et al., 2015; Muczynski et al., 2012), but many other studies reported long-term impacts of *in utero* exposure on the offspring sperm epigenome (Robaire et al., this issue; Ho et al., 2017). These observations support the original TDS hypothesis proposed by Niels E. Skakkebaek and colleagues that it is the immediate early effects on the testicular somatic environment that subsequently influence germ cell development (Skakkebaek et al., 2016).

3. Ovary development

3.1. Primordial follicle formation

When the ovary is formed, the female PGCs differentiate into oogonia undergoing extensive mitotic proliferation and forming clusters of interconnected cells known as germ cell cysts or nests (Pepling, 2012; Pepling et al., 1999; Wang et al., 2017, Fig. 2). Around GD13.5 in mice, GD17.5 in rats and GW12-13 in humans, the germ cells enter meiosis (Motta, 1997; Wang et al., 2017). Oogonia become oocytes and progress through meiotic prophase I until they become arrested at the diplotene stage. Approximately two thirds of the oocytes in the germ cell nests undergo programed cell death (involving both apoptosis and autophagy), germ cell cysts break apart and the remaining single oocytes become surrounded by a flattened (squamous) layer of intruding somatic pre-granulosa cells, forming the primordial follicles (Johansson et al., 2017; Pepling, 2012). Germ nest breakdown and primordial follicle formation are prerequisites for the establishment of the ovarian reserve; among the many factors implicated in this process are programmed cell death regulators (BCL2 associated X, apoptosis regulator and BCL2 apoptosis regulator), growth factors and signaling molecules (activin A, AMH, notch signaling pathway, the

phosphoinositide-3-kinase (PI3K) pathway), transcription factors (aryl hydrocarbon receptor), hormones (estradiol, progesterone, FSH), meiotic regulators (RA, activin A, WNT4) and cell adhesion molecules (Pepling, 2012; Sun et al., 2017; Wang et al., 2017). In humans, primordial follicle assembly is initiated around mid-gestation and completed during fetal life, while in rodents the process continues until PND6 (Johansson et al., 2017). Fetal and neonatal periods are, therefore, critical windows for the establishment of the primordial follicles and any disturbance of the process may result in premature ovarian senescence in the offspring.

3.2. Folliculogenesis

Folliculogenesis occurs after puberty (Fig. 2). Four major developmental events can be distinguished in this process: primordial follicle recruitment; preantral follicle development; selection and growth of the antral follicle; and follicle atresia (Williams and Erickson, 2012). Folliculogenesis is irreversible once it has begun; thus, after primordial follicle recruitment is initiated, the follicle is destined to either ovulate or undergo atresia (Johansson et al., 2020; Zama and Uzumcu, 2010). In fact, approximately 99% of follicles undergo atresia, with only a small number reaching the preovulatory stage. This process is controlled by a precise balance of pro- and anti-apoptotic factors (Williams and Erickson, 2012). The primordial-to-primary follicle transition is characterized by oocyte growth and granulosa cell differentiation from a squamous to cuboidal form; these cells start to express FSH receptors (Edson et al., 2009). In the secondary or pre-antral follicles, granulosa cells continue to proliferate and form two or more layers. It is at this stage that the thecal cells begin to emerge and form a layer around the granulosa–oocyte structure (Zama and Uzumcu, 2010); the main function of thecal cells is the production of androgens for neighboring granulosa cells to convert to estrogens (Edson et al., 2009). This phase is known as gonadotropin-independent growth (or the pre-antral phase) and is under the control of autocrine and paracrine regulatory factors (Edson et al., 2009; Hannon et al., 2015). Follicles then develop into antral follicles that are characterized by more than five layers of granulosa cells, a fluid filled cavity (antrum), and an oocyte enclosed by cumulus cells that are derived from the granulosa cells. Further, thecal cells develop into the theca externa and theca interna layers; the theca interna start to express receptors for LH. At this stage, follicles become gonadotropin-dependent (controlled mainly by pituitary FSH and LH) and continue to grow until they reach a preovulatory size (Edson et al., 2009). A surge of LH can result in maturation and completion of the first meiotic division and ensuing ovulation (Edson et al., 2009; Johansson et al., 2020).

Intraovarian triggers are responsible for primordial follicle activation (Mark-Kappeler et al., 2011). A number of growth factors, hormones, and cytokines have been shown to be important for primordial follicle recruitment and maintenance. PTEN (phosphatase and tensin homolog), Foxo3a (a transcription factor produced in both oocytes and granulosa cells), and SDF-1 (stromal derived factor-1) generated by primordial oocytes restrain their own activation (Williams and Erickson, 2012). Kit ligand (KL, also known as stem cell factor secreted by pre-granulosa cells) has been found to induce the transition of primordial to primary follicles (Oktem and Urman, 2010; Parrott and Skinner, 1999) exerting its effects through the PI3K pathway, a critical determinant of primordial follicle activation (Crain et al., 2008; Edson et al., 2009; Sarraj and Drummond, 2012, Fig. 2). Opposingly, AMH, produced by the granulosa cells of growing follicles, exerts an inhibitory influence on primordial oocytes, keeping them in a dormant state (Johansson et al., 2020; Zama and Uzumcu, 2010) and thus negatively regulating recruitment (Williams and Erickson, 2012). Androgens also have a clear and important physiological role in follicle development at all stages (preantral and antral follicle development), including the promotion of granulosa cell proliferation (Franks and Hardy, 2018).

3.3. Impact of EDCs on ovarian development and function

Reviews of the impact of EDCs on ovarian development and function have discussed specific time windows when the ovary is sensitive to disruption. These times/events, include gonadal sex determination, meiosis, follicle assembly and the first wave of follicle recruitment (Johansson et al., 2017). EDCs also affect folliculogenesis and steroidogenesis in the adult ovary (Patel et al., 2015).

Studies in rodents have revealed that exposures to **estrogenic substances** during early life influence the number of normal primordial follicles and increase the number of abnormal ovarian follicles containing multiple oocytes or multi-ovular follicles (MOF) (Table 3). MOF contain two or more oocytes, each with distinct zona pellucida, within the same granulosa cell layer, basement membrane and theca layer. Rodents exposed to DES, a pharmaceutical estrogen, early in life exhibit reduced primordial follicles (Kipp et al., 2007; Rodríguez et al., 2010). The frequency of MOF in the post pubertal or adult ovaries of mice was increased by DES exposure during late fetal development and prior to weaning (Iguchi et al, 1986, 1990; Iguchi and Takasugi, 1986; Kipp et al., 2007). The most dramatic increase in the frequency and relative number of MOF occurred in females exposed in the first 5 postnatal days (Iguchi et al., 1986), coinciding with the breakdown of nests of oogonia and early follicle formation (Fig. 2). The formation of MOF induced by DES exposure appeared to be independent of the effects of DES on the developing hypothalamic-pituitary-gonad axis, as DES caused a similar effect in neonatal ovaries implanted in ovariectomized adult females or cultured *ex vivo* (Iguchi et al., 1990). In addition, oocytes collected from super-ovulated juvenile mice treated as neonates with DES had substantially reduced capacity for fertilization *in vitro* (Iguchi et al., 1990) and *in vivo* (Iguchi et al., 2002) compared to those from untreated mice. No MOF were observed in ovaries of mice lacking functional nuclear ESR2 while ESR1 knockout mice remained vulnerable (Kirigaya et al., 2006) suggesting that MOF formation is mediated via DES signaling through ESR2. DES treatment of the neonatal ovary reduced activin mRNA and protein (Kipp et al., 2007) and increased the mRNA of inhibin, an activin antagonist (Oikawa et al., 2019). Like DES, many other estrogenic substances have similar impacts on follicular formation in rodents. Exposure of neonatal mice or rats to chemicals with estrogenic activity, such as BPA (Suzuki et al., 2002), EE2 (Kirigaya et al., 2006), genistein (Losa et al., 2011) and tamoxifen (Irisawa and Iguchi, 1990), all cause disruption of follicular formation with increased MOF in the ovaries after puberty.

Despite extensive study, no specific ovarian phenotype has been described for women exposed to DES as fetuses. In extrapolating from rodent studies to humans, it is important to consider that oocyte nest breakdown occurs mid-gestation in the human fetus when the fetal zone produces estrogens from adrenal androgens; while in rodents oogonial nest breakdown and early follicle formation occur very late in gestation and continue into the early post-natal period (Pepling and Spradling, 2001, Fig. 2). This process in mice is believed to be initiated by a reduction in estrogen levels in the late fetal ovary (Chen et al., 2007; Dutta et al., 2014). In a study in baboons, treatment with an aromatase inhibitor through mid gestation resulted in a reduction in follicle number, and surviving follicles were in a poor condition at puberty (Pepe et al., 2006). This suggests not only that estrogen has a critical role in follicle survival but also that the timing and presence of endogenous estrogens may render follicular formation in the primate ovary less sensitive than in the rodent, where nest breakdown is driven by germ cell apoptosis due to a precipitous decline in estrogen exposure perinatally.

A number of studies have shown that early life exposure to **phthalates** can affect ovarian development in female offspring, disrupting fundamental processes like meiotic initiation, primordial follicle assembly and folliculogenesis (Table 3, Fig. 2). One of the most studied phthalates is di (2-ethylhexyl) phthalate (DEHP) and its active metabolite, mono (2-ethylhexyl) phthalate (MEHP). In mice, the daily oral administration of DEHP (40 μg/kg) throughout gestation led to reduced serum estradiol levels in the dams and significantly downregulated expression of *Cyp17a1* and *Cyp19a1* in the fetal ovaries at GD12.5. A

delay in the meiotic progression of early germ cells was observed in the fetal ovaries on GD17.5. This effect was associated with considerably reduced mRNA and protein expression of *Stra8* at GD13.5, suggesting that DEHP exposure can deregulate meiosis related genes, although the exact molecular mechanisms remain to be determined (Johansson et al., 2020; Zhang et al., 2015). Large regions of germ-cell cysts and rare follicles were also observed in newborn ovaries, indicative of delayed follicle assembly (Zhang et al., 2015). As in rodents, meiotic progression and primordial follicle assembly are related; reduced *Stra8* expression can explain reduced numbers of assembled primordial follicles (Johansson et al., 2017). However, DEHP impaired primordial follicle formation might also be caused by oxidative stress in germ cells, as well as by disturbances in the interaction between germ cells and pre-granulosa cells mediated by transforming growth factor-beta (TGF-beta) signaling, as revealed recently by Wang et al. (2021). Further, gestational DEHP exposure significantly decreased the number of primordial follicles and increased the number of secondary follicles at PND21, indicating an accelerated rate of follicle recruitment (Zhang et al., 2015). Similarly, exposure to MEHP at 100–1000 mg/kg during a narrow but relevant window of exposure before parturition (GD17-19) resulted in an increase in preantral and antral follicles in F1 female mice at PND56. The premature reproductive senescence observed at the highest dose was attributed to accelerated folliculogenesis (Moyer and Hixon, 2012). It has been suggested that overactivation of PI3K signaling can most likely explain MEHP accelerated primordial follicle recruitment (Hannon et al., 2015). Exposure to DEHP (20 μg/kg/day-750 mg/kg/day) during the second half of gestation (GD10.5 – birth) resulted in decreased folliculogenesis in adult F1 ovaries (Rattan et al., 2018). In a subsequent study, numerous signaling pathways necessary for healthy ovarian function were analyzed in the F1; this study revealed disrupted expression of cell cycle regulators, PPARs, and increased *Pten* expression in the PI3K pathway (Rattan et al., 2019).

The early postnatal period of ovary development is also a window of susceptibility to DEHP. Indeed, newborn female mice injected intraperitoneally with DEHP (2.5–10 μg/g b. w./day) on PND0–4 displayed impaired germ nest breakdown and follicle assembly (Mu et al., 2015). This effect was shown to be mediated through both ER dependent and independent mechanisms (i.e., Notch 2 signaling components). Additionally, in a neonatal *in vivo* model and in an ovary culture model, DEHP exposure was shown to induce autophagy in the newborn mouse ovary and to up-regulate the expression of autophagy-related genes and the key components of AMPK-SKP2-CARM1 signaling, thus further reducing the primordial follicle pool and female fertility (Zhang et al., 2018). When the exposure took place from PND 7–14, DEHP (20 and 40 μg/kg body weight (bw)) significantly decreased the number of primordial and increased antral follicles at PND 15 in female mice (Zhang et al., 2013). Much stronger depletion of the primordial follicle pool and an increased number of secondary and antral follicles were observed at PND 21 after single day exposure to DEHP (20 and 40 μg/kg bw) on PND 5, 10, 15 and 20 (Zhang et al., 2013). The DEHP-induced acceleration in primordial follicle recruitment was proposed to be mediated by its active metabolite, MEHP, via overactivation of the PI3K signaling pathway (Hannon et al., 2015; Johansson et al., 2020). A significant increase in abnormal metaphase II spindles that may result in aneuploidy was observed in mature oocytes (Zhang et al., 2013). Further, DEHP was found to inhibit the antral follicle enlargement process in pre-pubertal mice exposed to 20 or 40 μg/kg DEHP at PND 5, 10 and 15 (Li et al., 2016a). This inhibition was linked to disturbed oxidative status and increased follicle somatic cell apoptosis caused by DEHP exposure.

Several studies provide evidence that *in utero* exposure to **PBDEs** may have an impact on development of the ovaries in progeny. After the treatment of Wistar rats with BDE-99 (60 or 300 μg/kg bw) on gestation day 6, the F1 female progeny that were mated to unexposed males had an increased rate of resorptions (Talsness et al., 2005). In another study, Long Evans rats exposed to BDE-99 (1 or 10 mg/kg body weight daily) from GD10 to18 had F1 female offspring with decreased ovarian primordial and secondary follicles (Lilienthal et al., 2006). A decrease in the numbers of secondary and antral follicles and a decrease in ovary weights was also observed in the F1 progeny of Wistar rats treated on GD6 with BDE-47 (140 or 700 μg/mL); this effect was accompanied by a reduction in serum estradiol concentrations, although ovarian aromatase activity was not affected (Talsness et al., 2008).

"Real life" exposure to environmental chemicals is to complex mixtures, yet the safety data that we have available are usually for individual compounds. When adult female Sprague-Dawley rats were exposed to a "house dust" mixture of brominated flame retardants before mating and during gestation, ovarian folliculogenesis and steroidogenesis were disrupted in the dams (F0; Lefèvre et al., 2016). Interestingly, multi-oocyte follicles were observed in the ovaries of their F1 progeny, and this was accompanied by advanced puberty (Allais et al., 2020). Postnatal PBDE exposures may also affect the ovary. The treatment of female Sprague Dawley rats on PND10 with BDE-47 (10 mg/kg) decreased ovary weights and altered expression of markers for the apoptosis/stress response in the adult F1 ovary (Wang et al., 2016).

The mechanisms by which exposure to PBDEs affect ovary development are not clear. Since PBDEs, and especially their hydroxylated metabolites, are potent ligands for PPARγ receptors, one possibility is that they disrupt PPARγ signaling in the developing ovary (Fang et al., 2015). There is also evidence from studies with cell lines and follicle cultures that exposure to PBDEs may affect steroidogenesis in ovarian granulosa cells. Experiments with a human granulosa cell line (KGN granulosa cells) have provided evidence that exposure to the mixture of PBDEs detected in follicular fluid affects steroidogenesis and induces oxidative stress (Lefevre et al., 2016). Other studies have provided evidence that exposure to PBDEs alters steroid secretion in cells from ovarian follicles (Gregoraszczuk et al., 2008; Karpeta et al., 2011; Karpeta and Gregoraszczuk, 2010). It has been proposed that PBDE exposures may disrupt epigenetic programming during oocyte development or by affecting mitochondria and inducing oxidative stress (Sun et al., 2020).

There is mounting evidence, based on laboratory studies in rodents, that early life stage exposure to **PFAS** impacts the developing ovary, causing ovarian dysfunction and leading to altered folliculogenesis, abnormal hormone levels and delayed puberty in the female rodent model (Table 3, Fig. 2). For example, Zhang et al. (2020) demonstrated that *in utero* exposure of mice to PFNA, from GD 1 to 18 via maternal oral dosing (3 mg/kg bw), led to a decrease in ovary size, in the numbers of primary, secondary and antral follicles, and corpora lutea, along with delayed vaginal opening and first estrus. In addition, in these same offspring, Zhang et al. (2020) reported increased liver weight and fibroblast growth factor 21 from postnatal day 1–21 (FGF21; a PPAR-mediated hepatokine), decreased pubertal activation of kisspeptin neurons and GnRH neurons, leading to the hypothesis that these effects of PFNA were key in suppressing puberty and altering ovarian folliculogenesis. Similarly, neonatal exposure of female rats to 0.1 and 1 mg/kg/day of PFOA or to 0.1 and 10 mg/kg/day of PFOS caused a significant reduction in the number of primordial, growing and corpora lutea (Du et al., 2019). Female mice exposed *in utero* to 200 or 500 mg/kg day of perfluorobutane sulfonate (PFBS) from GD 1–20 exhibited similar effects, with reductions in all stages of ovarian follicles, in addition to decreased ovary and body size and weight, delayed vaginal opening, onset of sexual maturity and decreased serum estradiol (Feng et al., 2017). Interestingly, Feng et al. (2017) also observed decreased thyroid hormones, triiodothyronine (T3) and thyroxine (T4), in the dams on gestation day 20, as well as in the female offspring exposed *in utero*.

The mechanistic evidence for ovarian development as a target of PFAS toxicity is not clear, but one hypothesis is that it is due to the induction of oxidative stress (Ding et al., 2020). Indeed, several *in vivo* and *in vitro* experimental models have demonstrated that PFAS induces oxidative stress in various tissues via increased production of reactive oxygen species (ROS) (Wielsøe et al., 2015; Chen et al., 2017; Lu et al.,

2016; Mashayekhi et al., 2015; Suh et al., 2017). Although ROS are produced by all living organisms as a result of normal cellular metabolism, ROS-induced injury is mitigated in cells by enzymatic and non-enzymatic antioxidant systems. However, when antioxidant system capacity is exceeded due to abnormally high ROS production, damage to various cellular components may ensue (i.e. carbohydrates, nucleic acids, proteins, lipids), resulting in many pathological conditions and diseases (Birben et al., 2012; Qian et al., 2010). Some recent studies have shown that PFAS induces ROS in early stages of ovarian development, particularly, during germ cell nest formation and mitosis. For example, fetal mouse ovaries explanted on GD17, cultured for 7 days *in vitro* and then exposed for 24 h to 28.2 or 112.86 μM PFOA, exhibited a concentration-dependent increase in ROS levels as well as apoptosis and necrosis (López-Arellano et al., 2019). There is also considerable evidence to indicate that PFAS may disrupt the developing ovary by disturbing lipid metabolism via PPAR signaling pathways (Ding et al., 2020). The three known PPARs, α, β/δ and ϒ, are found in the mammalian ovary with α, β/δ primarily expressed in the thecal and stromal cells, and ϒ at higher levels in the granulosa cells and corpora lutea (Braissant et al., 1996; Komar, 2005). The PPARα pathway plays a major role in maintaining lipid and glucose homeostasis, as well as regulating inflammatory response, cell proliferation, and differentiation (Escher and Wahli, 2000).

In summary, there is evidence that exposure to EDCs during ovarian development may delay meiotic progression and primordial follicle assembly, increase the numbers of abnormal ovarian follicles containing multiple oocytes, and alter the rate of follicle recruitment. There is a possibility that these exposures may affect the quality of the oocytes that are produced, resulting in a decrease in the rate on *in vitro* fertilisation. Steroidogenesis may also be affected. A number of signaling pathways have been implicated, including, disrupted expression of steroidogenic enzymes, disrupted PPAR signaling, disrupted PI3K pathway, cell cycle regulators, deregulation of meiosis related genes, apoptosis, and oxidative stress factors. This early life disruption of ovarian development can have long lasting effects on female reproductive health, lead to premature ovarian senescence and various comorbidities that can arise from deprivation of ovarian sex hormones, thus affecting women's overall health (Marlatt et al., this issue).

Conclusions

Based on experimental studies from different species, there is evidence that early exposure to EDCs can cause immediate change in gonad development with long-term consequences on reproductive functions. Effects range from impacts on sex determination, gonad differentiation and gonad development in fish. In mammals, *in utero* EDCs exposures do not alter sex determination or gonad differentiation; however, they can affect gonad development and steroidogenesis and may alter the programming of the germline. The discrepancy between effects observed in fish and mammals are largely due to the differences in the regulation of sex determination and timing of germline development outlined in Figs. 1 and 2. Taking into consideration that fish have many different reproductive strategies, from opportunistic (characterized by early maturation, frequent reproduction over an extended spawning season) to periodic (delayed maturation with large egg batches), while humans have a limited and relatively inefficient reproductive system (millions of spermatozoa for a single oocyte per ovulation cycle leading to a majority of singleton pregnancies), the impact of EDCs on human reproduction may carry a greater impact. Yet, the negative impact of EDCs on reproduction appears to be global in wildlife and humans (Marlatt et al. this issue).

Our review of the literature allows us to identify common mechanisms of endocrine disruption in fish and mammalian gonads; some of these are related to the importance of steroid hormone homeostasis in gonad development and function across all species. However, other signaling pathways have been shown to be important for gonadal development and appear to be altered by EDCs, inducing various short and long-term phenotypes. Pathways related to PPAR, ERR, ROS, PI3K, AHR, RA, among others, are demonstrated targets of EDCs and may play an important role. There is also some evidence that early exposures to EDCs affect DNA methylation in mature gametes but the molecular mechanism for this is not understood. Despite progress in improving our understanding of the molecular events leading to gonadal dysgenesis after exposure to EDCs, there are still many gaps in our knowledge. In addition, other associated challenges that need to be addressed include: 1) linking mechanisms identified in experimental models to actual exposure levels requires better screening of population exposure levels; 2) the consequences of cumulative effects of exposure to multiple EDCs is not well studied; and 3) many new alternative substances that are replacing regulated EDCs are already in our environment and we have limited knowledge of their potential effects. Together, these challenges highlight the pressing need to better identify the molecular targets of EDCs and understand their mode of action.

Recently much attention has been directed to developing adverse outcome pathways (AOPs) that describe the chain of causally linked events at different levels of biological organization that lead to an adverse health or ecotoxicological effect. In the future, more emphasis should be directed to this approach as it affords the opportunity to proactively support chemical risk assessment based on mechanistic understanding of the targets and pathways affected by chemical exposure. Many of the molecular pathways controlling gonadal development are conserved across species as illustrated by the common features underpinning sex determination and gonadal differentiation in fish and mammals, including humans. Furthermore, Marlatt et al. (this issue) report consistent trends in ovarian and testicular abnormalities across adult vertebrate taxa for several well studied estrogenic EDCs, strongly supporting direct effects on EDCs on gonads and a link to organism level adverse effects on reproductive capacity. Given the common features such as the roles of estrogens and androgens and downstream regulators in mediating these processes, it is not surprising that environmental chemicals that interact with these receptors have overlapping toxicological consequences across vertebrates. The AOP framework may provide insight into targets and pathways that should be considered in conducting hazard assessments.

Credit author statement

All authors participated in the conceptualization and organization of this review. All contributed equally to the data collection and writing. GD led the project, centralized the different sections and prepared the first draft for editing by VM first followed by all the authors for final approval.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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