Environmental Research 204 (2022) 112063

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

# A cross-species comparative approach to assessing multi- and transgenerational effects of endocrine disrupting chemicals

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ARTICLE INFO

*Keywords:*
Hormones
Epigenetics
Bisphenol A
Pesticides
Phthalates
Flame-retardants

ABSTRACT

A wide range of chemicals have been identified as endocrine disrupting chemicals (EDCs) in vertebrate species. Most studies of EDCs have focused on exposure of both male and female adults to these chemicals; however, there is clear evidence that EDCs have dramatic effects when mature or developing gametes are exposed, and consequently are associated with in multigenerational and transgenerational effects. Several publications have reviewed such actions of EDCs in subgroups of species, e.g., fish or rodents. In this review, we take a holistic approach synthesizing knowledge of the effects of EDCs across vertebrate species, including fish, anurans, birds, and mammals, and discuss the potential mechanism(s) mediating such multi- and transgenerational effects. We also propose a series of recommendations aimed at moving the field forward in a structured and coherent manner.

## Authors' contributions

All authors contributed to the planning and writing of the manuscript. Other than BR who was the lead in planning the manuscript, and JM who contributed extensively to the section on fish, all other authors are listed in alphabetical order.

## 1. Introduction

Over the past two decades, many studies have reported that the effects of exposure to endocrine disrupting chemicals (EDCs) are not limited to effects seen in adults (reviewed in Gore et al., 2015; and Lacouture et al.; Marlatt et al.; Martyniuk et al.; Thambirajah et al.; Vaudin et al., this issue) or when exposure to such chemicals occurs during fetal development with possible effects on adults (reviewed in Delbes et al.; Plante et al., this issue). Indeed, there is mounting evidence that, in a variety of taxa, effects of EDCs can be passed on to the next generation and even persist over several generations.

The term multigenerational is used to describe effects that are transmitted to one or more subsequent generations. Many mechanisms for these types of effects are possible, including maternal transfer of contaminants (e.g., from the mother to egg in the case of oviparous species) or direct exposure of germ cells (e.g., during gestation in mammals). For an effect to be considered 'transgenerational', it must appear in individuals that were never directly exposed to the EDC themselves. Such effects are often thought to occur through epigenetic marks, although other mechanisms such as genetics, altered metabolism, and parental behaviour are also possible. Transgenerational inheritance first appears in the F2 or F3 generation, depending on the exposure scenario, the sex, and the reproductive strategy of the species in question (Fig. 1). For example, in oviparous species (e.g., all birds, many fish and anurans), effects observed in the F2 generation are generally the first to be considered transgenerational. However, if the EDC is maternally transferred to the egg from an exposed adult female,

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https://doi.org/10.1016/j.envres.2021.112063
Received 31 May 2021; Received in revised form 9 September 2021; Accepted 10 September 2021
Available online 22 September 2021

the developing embryo as well as the germ cells within the embryo are both directly exposed to the contaminant. In this case, transgenerational effects would only be observed in the F3 generation. The life-stage of the individual exposed is also an important consideration. The experimental designs illustrated in Fig. 1 all consider the initial exposure to occur in adults; however, in many studies, particularly those involving oviparous species, experimental exposure occurs at the embryonic stage (e.g., waterborne exposure of fish embryos, or egg injection studies in birds). These mimic maternal deposition of the contaminant into the egg, and the potential appearance of transgenerational effects in the grandchildren of the exposed embryos. Ultimately the subset of multigenerational effects that are termed to be 'transgenerational' must be carefully considered for each experiment, considering the reproductive strategy of the species, the capacity of the chemical to be maternally transferred, and the experimental design.

While many excellent recent reviews have already been published on this topic (Brehm and Flaws, 2019; Navarro-Martin et al., 2020; Nilsson et al., 2018; Rattan and Flaws, 2019; Thompson et al., 2020; Van Cauwenbergh et al., 2020), the objective of this review it to provide a cross-species comparison of the multi- and transgenerational effects of EDCs in vertebrates. Our intention is to use a comparative approach to highlight differences among animals with disparate reproductive strategies. We will focus on the species most extensively studied, from vertebrate taxa that include fish, birds, anurans, and mammals. While this review is not intended to be comprehensive, we have endeavoured to highlight major advances in this field and point to several excellent reviews that cover different aspects of multi- and transgenerational effect of EDCs. We will also discuss the mechanisms that have been identified to explain how effects of ancestral exposure to EDCs can be transmitted to several subsequent generations. Indeed, epigenetic transgenerational inheritance has been proposed as the underlying mechanism for such transmission for a variety of diseases and abnormalities, targeting testis, ovary, prostate, kidney, mammary glands, the immune and metabolic systems as well as the central nervous system (Nilsson et al., 2018). Epigenetic inheritance can be driven by DNA methylation, histone post-translational modifications, and noncoding RNAs. Yet the precise mechanism by which EDCs affect such processes and the existence of windows of sensitivity remain largely unexplored.

## 2. Teleost fish as models for multi- and transgenerational effects of EDCs

Apart from mammals, there is no other vertebrate taxon that has been more widely studied for multi- and transgenerational effects of environmental chemical exposures than teleost fish. Small-bodied fish have significant advantages over their mammalian counterparts for such studies due to their small size, lower cost of husbandry, and the capacity to assess biological consequences of ancestral exposure with high throughput phenotyping approaches. These approaches are rapidly evolving both in scope and resolution, allowing unprecedented analysis of biological effects. Fuelled by increased availability of numerous characterized genomes, gene editing and other experimental techniques to investigate underlying epigenetic marks and their dynamics (reviewed in Best et al., 2018; Navarro-Martin et al., 2020), studies in zebrafish (*Danio rerio; Cypriniformes*) medaka (*Oryzias latipes and Oryzias melastigma; Beloniformes*), trout (*Oncorhynchus mykiss*; *Salmoniformes*), and different killifish (*Cyprinidontiformes*) have provided unequivocal evidence for the occurrence of multi-generational consequences of exposure to EDCs. A PubMed search (May 2021) of the scientific literature using each of the fish species with both "transgenerational" and "offspring" terms identified many of publications with zebrafish having the most at 495; manual screening and retention of primary studies using chemicals with known EDC properties and multigenerational or transgenerational experimental designs further narrows the number of publications, although zebrafish still had the most at 151 (Fig. 2). Despite this rapidly evolving knowledge, it is nevertheless important to note that the investigation of multigenerational effects of EDCs has largely been restricted to these few teleost species and families. This is mostly due to their genetic tractability and associated role as developmental model species (zebrafish, medaka) or their historical use in physiological and aquaculture research (rainbow trout). These models will continue to provide several advantages in the exploration of multi-

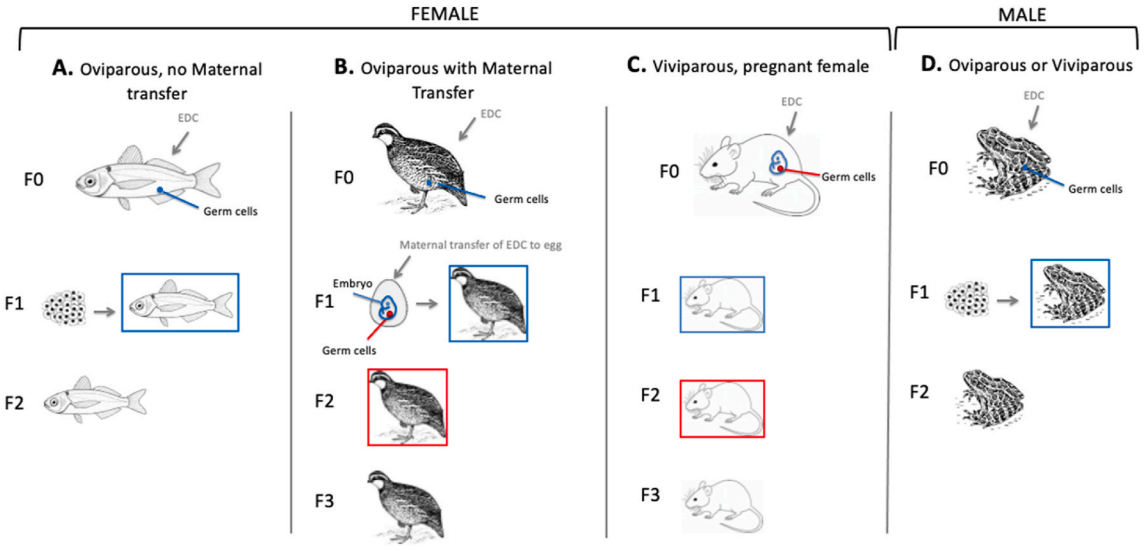


**Fig. 1.** Exposure scenarios for transgenerational inheritance in animals with different reproductive strategies. Effects of EDCs are considered to be transgenerationally inherited when they occur in an individual that was never directly exposed itself. For oviparous species, this would generally be the F2 generation, because the germ cells (F1, blue) were exposed in the F0 adult (panels A and D). However, if the EDC is maternally transferred into the egg from exposed females, both the embryo (F1, blue) and the germ cells within the embryo (F2, red) are exposed in the egg, and transgenerational inheritance would occur only in F3 (panel B). Similarly, when viviparous females carrying offspring are exposed to an EDC, transgenerational inheritance also occurs in F3 individuals (panel C). In both viviparous and oviparous species, transgenerational inheritance would occur in the F2 generation when adult males are exposed (panel D). This figure illustrates exposures to adults, but in many experiments involving fish, birds, and frogs, exposure to an EDC occurs at the embryonic stage. This scenario would be similar to the bottom part of the illustration shown in panel B; when eggs are exposed, effects are considered to be transgenerational when they occur in that embryo's grandchildren. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

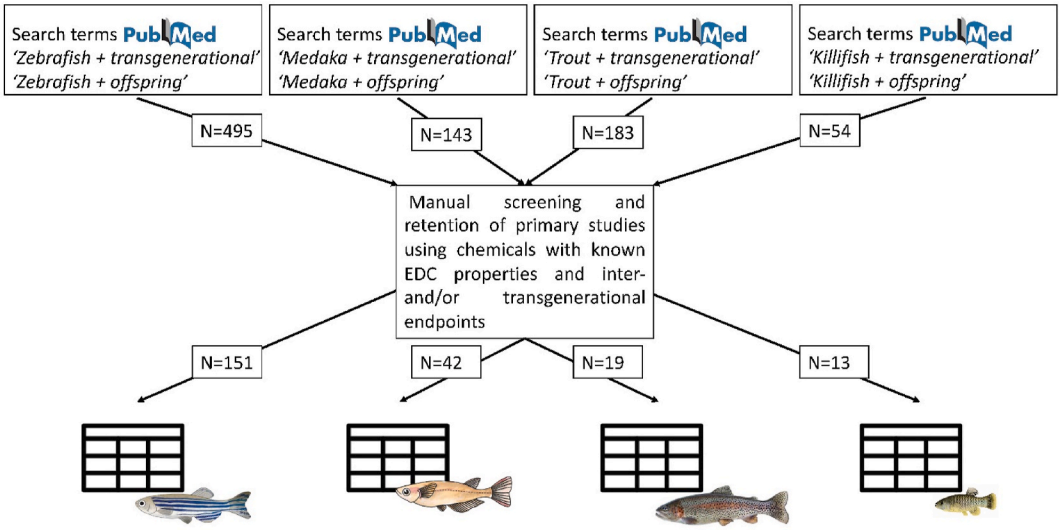


**Fig. 2.** Publications identified in PubMed (May 2021) for zebrafish, medaka, trout and killfish. Terms used for searching were "transgenerational" and "offspring".

and transgenerational effects of EDCs. In some of these species, profiling of DNA methylation, histone post-translational modifications, and noncoding RNAs already occurs with unprecedented resolution at the genome, as well as gene promoter-specific level allowing for descriptive analysis of molecular epigenetic changes in germ cells and somatic cells that coincide with observed multi- and transgenerational effects of EDC exposure (Navarro-Martin et al., 2020).

It is anticipated that rapidly evolving epigenome-editing approaches will allow for detailed functional mechanistic probing of multigenerational inheritance phenomena (Navarro-Martin et al., 2020). Nevertheless, the highly diverse infraclass of teleost fish, that comprises >26, 000 species and >400 families, clearly warrants the exertion of caution regarding the extrapolation of multi- and transgenerational effects reported for EDCs to fish in general. This is especially true given that reports of such effects of EDCs in fish are based on a limited number of fish species, many of which are inbred lab strains. Indeed, it is becoming clear that the well-described diversity in life-history and reproductive biology in teleost fishes also extends to a diversity of epigenetic mechanisms involved in multi- and transgenerational inheritance in fish (reviewed by Navarro-Martin et al., 2020). Rapid advances in genomic resources and increased resolution of phenotyping assays are therefore expected to facilitate investigations in diverse and environmentally relevant species for multi- and transgenerational effects of EDCs in fish; these advances will also likely reveal further complexity of the effects of EDCs in fish.

Using examples from a critical review of multi- and transgenerational effects in fish (Navarro-Martin et al., 2020), we highlight evidence for multi- and transgenerational effects of EDCs acting via estrogen, androgen, thyroid, and steroidogenesis (EATS). We will then focus on diversity between specific fish species in this field with emphasis on rapidly emerging evidence for diversity in molecular epigenetic dynamics relevant to multi- and transgenerational inheritance and discuss specific findings of effects of EDCs.

### *2.1. Three case studies for multi- and transgenerational consequences of EDC exposure in fish*

#### *2.1.1. Bisphenol A: an EDC that affects several endocrine axes in multiple fish species*

Perhaps no other EDC has been as comprehensively studied in fish from a multi- and transgenerational perspective than bisphenol A (BPA), a ubiquitous chemical used in polycarbonate and epoxy resins. The long-term effects of developmental and adult exposure to BPA within a generation has been studied in zebrafish, medaka, and rainbow trout. These studies revealed long-term consequences of BPA exposure during developmental on the adult endocrine reproductive-, stress-, and somatotropic axes, suggesting BPA acts via both EATS and non-EATS modes of action (reviewed by Canesi and Fabbri, 2015; Delbés et al., this issue). While the comparability of BPA effects between species is difficult due to the lack of dedicated comparative studies standardizing experimental parameters such as concentration and durations of BPA exposure as well as the assessment of (endocrine) endpoints, these results reveal consistent effects, especially regarding reproduction, in multiple fish species (Canesi and Fabbri, 2015). Importantly, several reported developmental effects of BPA on intra-generational endocrine endpoints were subsequently assessed in multi- and transgenerational experimental designs.

Reproductive effects of BPA initially reported in Japanese medaka (Ramakrishnan and Wayne, 2008) were assessed in a multi- and transgenerational context in the same species (Bhandari et al., 2015). While these studies reported a consistent, multi- and transgenerational decrease in fertilization and embryo survival in response to 100 μg/L BPA generally mimicked by the estrogenic control of 0.05 μg ethinylestradiol ($EE_2$), subsequent crossbreeding studies linked this effect to subfertility in males (Thayil et al., 2020). This study also revealed consistent increases in brain expression of kisspeptin (*kiss)* and gonadotropin releasing hormone (*gnrh)* as well as their receptor genes between $F_0$ and $F_2$ males, in addition to global hypomethylation in testes of both F0 and $F_2$ generation males. Future studies probing the role of epigenetic mechanisms in mediating these transgenerational effects on gene expression, their transmission via sperm or egg epimutations, as well as functional linkage of altered gene expression changes with respect to the observed reproductive phenotype represent exciting avenues for future studies in this model system.

Reproductive multi- and transgenerational effects of BPA have also been described in zebrafish (Akhter et al., 2018; Santangeli et al., 2019). Using different exposure routes (dietary delivery of ~13.5 μg/g $bw^{-1}$ $d^{-1}$) and developmental exposure windows (adults 3–9 months post fertilization (mpf) compared to the medaka studies, consistent reductions in fertility and embryo development were described in $F_1$, $F_2$ and $F_3$ offspring from both maternal and paternal exposure lineages (Akther et al., 2018). Taking advantage of the genetically tractable zebrafish model, the authors employed a transgenic line (β-actin: EGFP; roy), which selectively labels gills and oocytes in a translucent zebrafish. Using a combination of classical immunohistological approaches in conjunction with fluorescence microscopy, the authors reported

generation-dependent reduction on male and female gonadal mass and gamete differentiation, in line with reported effects in both parental lineages. Unfortunately, since neither an estrogenic control nor indices of the reproductive axis were profiled, the potential estrogenic action of BPA for multi- and transgenerational effects in the contribution of endocrine axis dysregulation were not resolved in this zebrafish study. Following the demonstration of intragenerational effects on oocyte growth and maturation in adult female zebrafish exposed to 20 μg/L BPA and the exploration of potentially underlying epigenetic mechanisms, specifically the description of the concurrent decreases in permissive H3K4me3 and H3K27me3 histone marks in the transcription start site (TSS) of the downregulated follicle stimulating hormone receptor gene (*fshr)* and steroidogenic acute regulatory protein (STAR) (Santangeli et al., 2016), the same group recently investigated potential transgenerational reproductive effects of waterborne BPA exposure in the same species (Santangeli et al., 2019). The authors exposed adult female zebrafish to 20 μg/L BPA for 4 weeks. In the $F_2$, but not $F_1$ generation, female fish exhibited a significant reduction in the gonadosomatic index (GSI) and bodyweight, suggesting transgenerational, maternal-lineage dependent effects of BPA on reproduction and somatic growth. Given that the sexually mature females were directly exposed as adults in this experimental design, it cannot be excluded that BPA may have been transferred to eggs, although this was not quantified in the study. This contrasts with early embryonic exposure designs in oviparous fish where investigation of biological effects in the F3 generation is necessary to ascertain true transgenerational effects, similar to viviparous animal models (Fig. 1).

While the expression of several transcripts involved in sex differentiation, the reproductive endocrine axis, and DNA methylation were differentially affected in ovaries and whole larvae between $F_0$–$F_3$ generations, consistent reductions in the expression of the anti-Müllerian hormone gene (*amh*) were detected in all larvae and ovaries of direct offspring as well as the subsequent lineage derived from ancestrally BPA exposed females (Santangeli et al., 2019). This pattern correlated with consistent DNA hypermethylation in the putative promotor region of *amh,* suggesting that the multigenerational suppression of *amh* is dependent on a heritable epigenetic control mechanism. However, the potential reproductive relevance of these epigenetically transmitted changes is not clear, since the authors report only measurements of GSI, and did not assess additional indices of reproductive performance or success in these females. In contrast, a recent study investigating intergenerational consequences following a waterborne exposure of male zebrafish to BPA at a concentration of 100 μg and 2000 μg/L reported decreased fertilization of eggs from unexposed female offspring at the higher exposure concentration and decreased embryo survival at both paternal BPA exposure concentrations (Gonzalez-Rojo et al., 2019). While global histone hyper-acetylation and dose-dependent alterations of specific posttranslational modifications of histones were reported in exposed male zebrafish testes, the relevance of these epigenetic changes is difficult to interpret, given that overt genotoxic effects at the DNA level were demonstrated by TUNEL assay at the higher concentration.

In rainbow trout, developmental BPA exposure via microinjection in eggs prior to fertilization results in endocrine disruption. One study showed that egg-injected BPA with as little as 1 ng per egg attenuates stress responsiveness and growth in larval and juvenile rainbow trout while reducing post-stress of both cortisol and growth hormone (GH) content (Aluru et al., 2010; Birceanu et al., 2015). Using the same experimental protocol, this group subsequently demonstrated that ancestral BPA exposure in eggs attenuates not only the F1, but also F2 cortisol response in one year old trout (Thomas et al., 2018). Similarly, deposition of BPA in trout eggs (4 and 40 ng per egg) led to a reduction of growth, albeit limited to juvenile stages, in both $F_1$ and $F_2$ generations (Sadoul et al., 2017). Together these studies demonstrate that aspects of the initially observed BPA mediated developmental effects on endocrine stress and somatic growth axes extend to multi- and transgenerational effects.

Overall, despite of experimental differences including exposure concentration, duration and timing on the one hand, and the choice of assessed endpoints on the other, studies in fish suggest BPA exerts significant transgenerational effects on reproduction, stress and somatic growth. Future standardized comparative studies are needed to determine the underpinning mechanism(s) of these phenotypes across generations.

#### *2.1.2. The selective serotonin reuptake inhibitor fluoxetine: transgenerational hypocortisolism linked to disrupted interrenal steroidogenesis*

The pharmaceutical class of selective serotonin reuptake inhibitors in general, and fluoxetine in particular, have been widely investigated with regard to endocrine disruption in fish. Following the detection of their presence in the aquatic environment downstream of waste-water treatment plant effluents (reviewed by Mole and Brooks, 2019) and the discovery of their potential for bioconcentration in fish tissues including brain, liver, and muscle tissues (Brooks et al., 2005; Schultz et al., 2010), concerns were raised regarding biological consequences of the exposure to fluoxetine in fish due to the high evolutionarily conserved serotonin transporter, the compound's molecular target (Kreke and Dietrich, 2008; Mennigen et al., 2011). Because the central serotonergic system in fish is involved in the regulation of several neuroendocrine pathways (Somoza et al., 1991; Winberg et al., 1997), the potential for fluoxetine to act as a neuroendocrine disrupting chemical in fish has been studied in goldfish (*Carassius auratus*), a long-standing model system for the investigation of neuroendocrine control of reproduction and energy balance (Blanco et al., 2018; Popesku et al., 2008). These studies revealed that fluoxetine, at environmentally relevant (0.54 and 54 μg/L) concentrations, could disrupt the reproductive axis in goldfish (Mennigen et al., 2008, 2010, 2017; Silva de Assis, 2013), a finding which extends to other fish species (Bain et al., 2016; Lister et al., 2009; Schultz et al., 2011), mammals (Müller et al., 2012; Pop et al., 2015) and, in some cases invertebrates (Lazarra et al., 2012). More recently, fluoxetine, at environmentally relevant concentrations (1–50 μg/L) was also demonstrated to inhibit the endocrine stress axis in zebrafish through attenuation of the total body cortisol response in response to a mechanical stressor in zebrafish (Abreu et al., 2014). Together, these studies revealed the potential for fluoxetine to disrupt several endocrine axes in fish.

A recent study investigating the potential multi- and transgenerational consequences of fluoxetine exposure in zebrafish (Vera-Chang et al., 2018) revealed a sex-specific, consistent multigenerational ($F_1$–$F_3$) endocrine disruption phenotype in response to developmental exposure to fluoxetine (0–6 dpf) at clinically and environmentally and clinically relevant concentrations (0.54 and 54 μg/L). Across multiple generations ($F_1$–$F_3$), a standardized handling stressor induced cortisol response was significantly attenuated in male fluoxetine exposed-lineage descendants. Mechanistically, the endocrine disruption of fluoxetine was localized to interrenal tissue, where the responsiveness to adrenocorticotropic hormone was reduced in $F_0$ and $F_3$ males. Transcriptomic analysis of $F_1$ and $F_3$ interrenal tissue supported these findings, revealing an enrichment of differentially expressed steroidogenesis transcripts. Persistent behavioural consequences of the multi- and transgenerational disruption of the endocrine stress axis following developmental fluoxetine exposure were equally observed and linked to transgenerational hypocortisolism, providing organismal relevance to the transgenerational phenotype. Importantly, follow-up studies also demonstrated that the fluoxetine-induced germ-line dependent transgenerational endocrine disruption has the potential to modulate responses to subsequent stressors exemplified by a generationally-separated exposure to another antidepressant aquatic contaminant, venlafaxine (5 μg/L, in the $F_4$ generation (Vera-Chang et al., 2019). The question of how context- and/or germ line dependent multigenerational and germ-line dependent transgenerational inheritance of the fluoxetine-induced endocrine disruption of the stress axis is mediated at the molecular level clearly

warrants further investigation.

In a study mimicking the developmental exposure protocol, the multigenerational inheritance of decreased whole body baseline cortisol in $F_1$ larvae has been shown to be maternally transmitted, to be dependent on parental age, and to coincide with non-coding RNA changes in the eggs (Martinez et al., 2019). While future studies should extend investigation of gamete epigenetic marks to transgenerational timescales and include organismal assessment of stressor-induced cortisol responses in offspring, the reported maternal transmission is interesting given that germline DNA methylation mark inheritance is reported to be almost exclusively paternal in zebrafish (Skvortsova et al., 2019). Thus, future studies of the germline exposome following developmental fluoxetine should focus on the study of maternally transmitted non-coding RNAs, which provide the benefit of functional interrogation via microinjection of miRNA mimics or antagonists in externally fertilized zebrafish eggs or embryos (Navarro-Martin et al., 2020).

Recent studies on zebrafish and turquoise killifish (*Nothobranchius furzeri*), an emerging fish model in ecotoxicological research, also investigated multi- and transgenerational effects of fluoxetine (Al Shuraiqi et al., 2021). $F_1$ offspring zebrafish exposed to a range of fluoxetine concentrations (5ng/L-5μg/L) as adults in different social settings for different periods of time (7 d, 14 d, 28 d) revealed complex, non-linear dose-response alterations in activity, with significant increases in larvae whose parents were exposed to 100 ng/L and significant decreases in larvae whose parents were exposed to 500 ng/L (Al Shuraiqi et al., 2021). However, a possible underlying disruption of the stress axis in general and cortisol responses in particular were not investigated in this study. Using the turquoise killifish, Thoré et al. (2020) first reported multigenerational effects of waterborne fluoxetine exposure in the μg/L range on reproductive, growth and behavioural endpoints. Using a life-cycle exposure set-up beginning at 9 dpf, the authors reported that exposure to 0.5 μg/L fluoxetine resulted in significant reductions in offspring body weight (Thoré et al., 2021). While demonstrating the utility of killifish in life cycle and intergenerational assessment of fluoxetine and other compounds of ecotoxicological concern, the study neither probed the involvement of endocrine disrupting mechanisms in the offspring phenotype nor assessed potentially (epigenetic) molecular underpinnings of transmission and manifestation of the fluoxetine-dependent phenotype in the subsequent generations.

#### 2.1.3. Hypoxia: an environmental factor condition linked to transgenerational disruption of the reproductive axis

In addition to chemical contaminants, pervasive environmental factors such as low concentration of oxygen (<2.8 mg/L) in aquatic habitats termed hypoxic zones (Diaz et al., 2008), have been linked to endocrine disruption in fish, particularly of the reproductive axis. Importantly, in spite of their characterization as environmental factor, hypoxic aquatic zones are often dependent on anthropogenic influences including contamination, albeit indirectly. For example, marine hypoxic dead zones on shallow continental shelves have been linked to eutrophication and global warming, which act to increase oxygen use and decrease its water solubility, respectively (Diaz et al., 2008). Initially characterized and coined as endocrine disruption in carp, *Cyprinus carpio* (Wu et al., 2003), hypoxia-induced intragenerational endocrine disruption of the reproductive axes has been confirmed in several complementary field and laboratory studies in the Atlantic croaker (*Micropogonias undulates;* Thomas et al., 2007; Thomas et al., 2012), and marine medaka, *Oryzias melastigma* (Cheung et al., 2014; Lai et al., 2016). Common characteristics across these fish studies reveal an EATS-based mode of action, characterized by often profound reductions in circulating sex steroids, a reduction in gonadosomatic indices, which at least in some cases, are associated with masculinization, reduced fertilization success and offspring development (Cheung et al., 2014; Thomas et al., 2009; Thomas et al., 2012; Wu et al., 2003). At least in the Atlantic croaker, mechanistic investigation further points to neuroendocrine mode of action, mediated at least in part via disruption of the serotonergic system exerting a positive control on the reproductive endocrine axis in this species (Rahman et al., 2011).

More recently, studies using the marine medaka have demonstrated that such hypoxia-induced endocrine disruption of the reproductive axis acts transgenerationally in both male (Wang et al., 2016) and female fish (Lai et al., 2019). Ancestral exposure of marine medaka to 1.4 mg/L $O_2$ across their lifecycle resulted in consistent intra- ($F_0$) multi- ($F_1$) and trans-generational ($F_2$) impairment of sperm quality and quantity in fish (Wang et al., 2016). These effects coincide with consistent transgenerational expression changes in testes of the ancestral hypoxia-lineage animals, which include the induction of euchromatic histone-lysine N-methyltransferase 2 (*ehmt2*) and (*pkt2b*) with vital roles in DNA methylation and chromatin modification during germ-cell development and spermatid maturation, respectively. While the $F_2$ sperm was globally hypermethylated, the promoter regions of both *ehmt2* and *ptk2B* genes were hypomethylated, providing a plausible mechanistic molecular epigenetic basis for the observed paternally-mediated transgenerational reduction of sperm development in the hypoxic lineage. Further evidence supporting this mode of action is the finding that this transgenerational upregulation of *ehmt2* in testis of the ancestral hypoxia exposure lineage was associated with the elevation in histone H3 lysine 9 dimethylation, an important epigenetic marker for gene-silencing induced by hypoxia. Thus, it appears likely that, at least in part, ancestral hypoxia exposure affects male reproductive function via sperm methylation of genes that include histone modifying enzymes to regulate spermatogenesis in unexposed $F_1$ and $F_2$ offspring.

In a more recent companion study, Lai et al. (2019) demonstrated that these transgenerational effects extend to females, for which increased follicular atresia in ovaries was observed across generations; this in turn was linked to decreased reproductive success of females as indicated by a reduction in egg hatching. Similar to the initial study exploring transgenerational effects on male gamete maturation (Wang et al., 2016), concurrent analysis of ovarian methylome and transcriptome resulted in the identification of candidate genes whose expression inversely correlated with gene-specific methylome signatures. Among these genes, *tp53* was identified as an induced hub, providing a possible mechanistic link to the observed phenotypic reproductive effect female gametogenesis following ancestral hypoxia exposure. While providing excellent examples of integration of epigenetic and transcriptomic approaches to further the mechanistic basis of the observed transgenerational consequences of ancestral exposure, it is unfortunate that neither study investigated potential causal or downstream effects on the reproductive endocrine system shown to be affected by hypoxia intra-generationally. Similarly, despite convincing demonstration of coherent epimutations in sperm, the experimental study neither followed maternal or paternal lineages, nor did it include an outbreeding design. Thus, a formal distinction of whether the observed changes are inherited via paternal or maternal lineages, or both, remains elusive.

Together, these three case studies provide clear evidence for EDC-induced multi- and transgenerational effects in fish. However, compared to earlier, more detailed intra-generational studies, the specific implication of endocrine axis disruption, the investigation of molecular mechanisms, including epigenetic marks, and especially, their multi- and transgenerational effects, remain a largely understudied area in the field. Furthermore, many reports of transgenerational effects of specific EDCs remain restricted to single research groups and consequently single fish species. Given the high diversity of fish, future comparative approaches are clearly warranted. The following section will briefly review species-specific characteristics relevant to future investigation of multi- and transgenerational effects of EDCs in fish.

### 2.2. The diversity of teleost models in multi- and transgenerational research: current knowledge in comparative epigenetics and opportunities and limitations for EDC research

#### 2.2.1. Zebrafish

Zebrafish have a long history as genetically tractable and developmental research model. The advantages this species include ease of maintenance, high fecundity, rapid development, and well-annotated genome (Howe et al., 2013). Recent advances in high throughput phenotyping in small fish across different life stages (Rafferty and Quinn, 2018; Tu et al., 2019) and increasingly efficient profiling methods of genome-level epigenetic marks at a high resolution (Navarro-Martin et al., 2020), have led to an increase in zebrafish studies reporting multi- and transgenerational effects (Fig. 3). Briefly, zebrafish embryos hatch 2–3 days post-fertilization (dpf) to enter the eleutheroembryonic stage that in turn lasts until exogenous feeding commences with the larval stage at 4 dpf. Zebrafish can reach sexual maturity at ~3 mpf. Advances in life-stage specific profiling of major epigenetic marks have provided unprecedented insight into the developmental dynamics of these marks in zebrafish. There is an almost complete inheritance of paternal DNA methylation patterns in zebrafish offspring somatic cells following zygotic gene activation (Jiang et al., 2013; Potok et al., 2013); this pattern holds true for zebrafish germ cells (Ortega Recalde et al., 2019; Skvortsova et al., 2019), revealing important implications for the role of this pathway in transgenerational inheritance. Similarly, following early developmental description and functional characterization of microRNAs in zebrafish (Chen et al., 2005; Mishima et al., 2012; Soares et al., 2009; Wienholds, 2005) recent studies reveal clear sex-specific profiles of miRNAomes in gamete (Presslauer et al., 2017), providing a basis for assessing and functionally probing roles of miRNAs in transgenerational inheritance in zebrafish (Navarro-Martin, 2020). Significant advances in the understanding of changes during development of histone modification marks have been recently made in this model (Cavalieri, 2020; Horsfield, 2019; Kaaij et al., 2018; Zhu et al., 2019), albeit. However, these remain largely restricted to somatic cells.

While the zebrafish is arguably the most utilised fish model in multi- and transgenerational EDC research in fish (Fig. 3), the field, in some respects, may be lagging behind compared to recent advances in mammalian epigenetics. For example, the detailed mapping of histone modifications and chromatin landscapes and the investigation of baseline dynamic changes of these marks across life-cycle stages have only recently been described in somatic cells (Baranasic et al., 2021) and are only beginning to be studied in germ cells (D'Orazio et al., 2021). Our understanding into the functional roles of specific molecular epigenetic marks, including microRNAs and other non-coding RNAs in fish, remains preliminary. In light of the notable differences in epigenetic mark dynamics compared to mammals (Mennigen et al., 2016; Navarro-Martin et al., 2020), functional studies of specific epigenetic marks are clearly warranted in zebrafish.

Additionally, in contrast to larger fish and mammals, detailed investigation of perturbations of the endocrine axis in zebrafish across generations remains limited to select transcripts or whole-body steroid hormone assays, impacting the resolution of the functional assessment of the EDC effects on endocrine axis function. The integration of these molecular epigenetic and endocrine endpoints is often lacking. Finally, a comprehensive review of the zebrafish literature reveals that while there is a variety of EDCs that have been studied, the large majority are those that disrupt the EATS modalities. Thus, non-EATS pathways should also be interrogated (Martyniuk et al., 2021; this issue), and investigations of multi- and transgenerational effects of EDCs that act primarily via other hormone signaling pathways should be prioritized in the zebrafish model.

#### 2.2.2. Medaka

Two species of medaka have been used in experiments assessing multigenerational effects of EDCs, the freshwater Japanese medaka (*O. latipes*) and the marine medaka (*O. melastigma*). The life-history of Japanese medaka, a genetically tractable lab model (Kasahara et al., 2007), has been well described (Iwamatsu et al., 2004). Of relevance to multi- and transgenerational studies of EDCs, sex differentiation (5–8 dpf) is completed before hatching at 9–10 dpf, and medaka are sexually mature at ~2mpf (Bhandari et al., 2016). Given its traditional use as a developmental model, extensive genomic resources, and its comparatively early sexual differentiation compared to zebrafish, Japanese medaka, and more specifically the inbred reference Hd-rR strain, have been proposed as a model for studying environmentally induced epigenetic transgenerational inheritance (Bhandari et al., 2016). Since then, significant progress has been made in identifying molecular epigenetic repertoires and dynamics. For example, primordial germ cell DNA methylation patterns have, in contrast to the situation in zebrafish, been shown to undergo mammalian-like de-methylation processes between 8 dpf-12dpf, and sex-specific remethylation at 15 dpf in males and 25 dpf in females (Wang and Bhandari, 2020a,b). At the chromatin and posttranscriptional levels, histone modifications and microRNA repertoire have also been characterized for Japanese medaka (Li et al., 2010, 2016; Qiu et al., 2018), thus providing the possibility of exploring their regulation in germ and somatic cells, and, ultimately, their functional contribution to multi- and transgenerational effects of EDCs. Targeted manipulation of both DNA epigenetic marks and noncoding RNA epigenetic marks have now been described in medaka (Cheung et al., 2017; Fukushima et al., 2019; Gay et al., 2018), providing molecular toolkits for such functional interrogations.

Similar advances in characterizing (epi)genetic mechanisms have been made in marine medaka. This is highlighted in the context of the previously described multi- and transgenerational effects on male and female medaka in response to ancestral hypoxia exposure (Lai et al., 2019; Wang et al., 2016). A detailed review of the reported literature of multi- and transgenerational effects of medaka largely confirms the current lack of integrative studies, which clearly link endocrine endpoints to epigenetic mechanisms across generations.

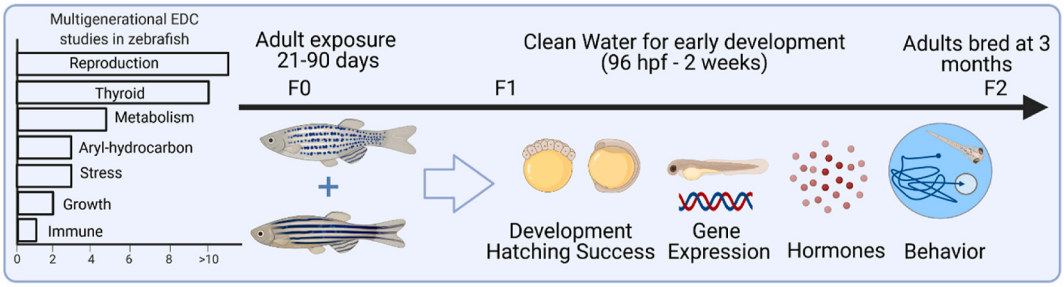


**Fig. 3. Multigenerational studies investigating the effects of endocrine disruptors in the zebrafish model.** EDCs that target sex steroid receptors are investigated most often followed by thyroid hormone receptor modulators. Also depicted is a typical experimental design for the zebrafish. Adults are often exposed to the chemical in water for 3 week or 3 months, and bred. Eggs are allowed to develop in clean water and at a given time, gene expression, hormones, and behaviour are assessed. Fewer studies on EDCs extend into the F2 and fewer than 5 have conducted multigenerational studies of EDCs beyond the F2 in zebrafish.

#### 2.2.3. Trout

The rainbow trout is native to lakes and rivers in North America west of the Rocky Mountains. However, it has been introduced to all continents except Antarctica. Rainbow trout are a relevant and long-standing model of comparative endocrinology, physiology, ecology and toxicology (Best et al., 2018). They have significant economic importance as a sport fish and aquaculture species and is also the first salmonid with a sequenced genome (Berthelot et al., 2014). This advantage has spurred significant advances for the characterization and targeted investigation of epigenetic marks in the complex rainbow trout genome across different stages of its lifecycle, including histone modifications, DNA methylation (Marandel et al., 2016), and microRNAs (Juanchich et al., 2016; Kostyniuk et al.,2019a, 2019b, 2020). While developmental stages are well characterized (Vernier et al., 1969), the long time to reach sexual maturation (1–2 years), coupled with elaborate animal housing demands have so far limited the number of multi- and transgenerational studies in rainbow trout and related salmonid species. In contrast to other fish model species, the work investigating multi- and in rare cases transgenerational effects of EDCs in rainbow trout often includes lower, environmentally relevant concentration ranges (ng/l) of mostly estrogenic EDCs. The investigation of endocrine disruption across generations derived from ancestrally exposed trout also employ a more targeted approach, largely owing to the long-standing availability of hormone quantification assays. This is exemplified by studies investigating the consequences of egg-deposited BPA on somatotropic and stress related parameters described previously (Sadoul et al., 2017; Thomas et al., 2018). There are only few reports of transgenerational compared to intragenerational penetrance of effects following ancestral EDC exposure, likely reflecting the experimental limitations of slowly maturing species. Furthermore, the investigation of underlying effects of EDCs on epigenetic marks are currently entirely lacking in a multi- or transgenerational context. While feasible, especially at the gene specific level (Marandel et al., 2016), global genome-wide profiling of epigenetic marks in these species will remain difficult to interpret in the future given the often-uncharacterized role of potentially sub- or neo-functionalized paralogues following the salmonid-specific genome duplication (Berthelot et al., 2014; Baerwald et al., 2016). Artificial clonal lines exist for rainbow trout (Sadoul et al., 2015), which allow for investigations of heritable epigenetic modes of action of EDCs in genetically homogenous backgrounds.

#### 2.2.4. Killifish

Wild killifish populations have traditionally been used in multigenerational studies to explore inheritance of resistance to chemical re-exposure in lab environments, providing insight into likely contribution of genetic as well as non-genetic inheritance mechanisms. The mangrove killifish (*Kryptolebias marmoratus*) is unique in its well-recognized plasticity in response to environmental and anthropogenic factors, which has made it a relevant ecotoxicology model (Whitehead et al., 2010). The lifecycle of several killifish species has been well described and notably includes unique alternative reproductive strategies of selfing to generate clonal offspring in addition to sexual reproduction under specific environmental conditions (Ellison et al., 2015). The homogenous genetic background, as well as the extremely short maturation and generation time of some killifish, exemplified by the short generation time of 2 weeks for turquoise killifishes, the shortest of any vertebrate, have led to increased popularity of this research model to probe multigenerational consequences of exposure to EDCs. Genome sequences (Valenzano et al., 2015) and epigenetic tools (Berbel-Filho et al., 2020; Ellison et al., 2015; Fellous et al., 2018; Romney and Podrabsky et al., 2018) are becoming increasingly available for EDC studies in killifish. While used to investigate multi- and transgenerational effects (Thoré et al., 2021), current studies lack mechanistic investigation of both endocrine and epigenetic endpoints.

### 2.3. General conclusions on teleost fish

While the case studies provide unequivocal evidence for the occurrence of multi- and transgenerational effects of ancestral EDC exposure, it is clear that many have focussed on a single EDC compound in a single species, limiting the general assessment of the effect of EDCs in the vast and diverse group of teleost fishes. It will be important to incorporate the emerging field of comparative epigenetics into future studies, since different fish species reveal often profound differences in epigenetic dynamics (Table 1). Thus, while species-specific differences exist, coordinated comparative experimental approaches are needed to determine whether multi- and transgenerational effects of EDCs in fish as well as their epigenetic modes of transmission apply widely or are restricted to specific models. In the reviewed literature there are widespread theoretical and experimental limitations, manifested through both imprecise language and/or lack of experimental rigor in the interpretation of germ-line dependent and context-dependent transmission of endocrine disruption in fish models.

## 3. Evidence from anurans

### 3.1. Within-generation impacts of early life-stage EDC exposures in anurans

During early stages, anuran gonads are undifferentiated and their development is sensitive to EDCs. Considerable data indicate that estrogens and androgens are respectively ovary- and testes-promoting steroids in frogs (Duarte-Guterman et al., 2009; Hayes, 1998; Roosenfeld et al., 2017). There are steroid-sensitive and steroid-insensitive periods for sex-inversion. Early exposures to the contraceptive steroid $EE_2$ (5 nM) leads to sex-inversion and a female bias sex ratio at metamorphosis in the leopard frog *Lithobates pipiens* (Hogan et al., 2008). In contrast, exposure in late metamorphic periods had no effects on the sex ratio. Neither of these treatments affected time to metamorphic climax, yet $EE_2$ exposure in a mid-metamorphic period delayed metamorphosis but had no effects on sex ratios (Hogan et al., 2008). These data indicate developmentally-dependent changes in steroid sensitivity of the gonads. In the case of the Western clawed frog *Silurana tropicalis*, there are also persistent abnormalities associated with $EE_2$ exposures. Following $EE_2$ exposures from hatching to metamorphosis, there was a dose-dependent increase in adult females with ovaries but without oviducts (Pettersson et al., 2006).

A lesser-known disruption involves the thyroid hormones (TH). Previously it was thought that THs played no role in sexual development (Buchholz et al., 2006; Hayes, 1998). Exposure to environmentally relevant doses of ammonium perchlorate (0.002–14 ppm) under laboratory conditions induce female-biased sex ratios in *Xenopus laevis* (Goleman et al., 2002). However, other data indicate that developing gonads are sensitive to THs. Triiodothyronine treatments increases the expression of genes associated with androgens, or suppress those associated with estrogens, depending on the species investigated (Duarte--Guterman et al., 2014; Flood et al., 2013). Perchlorate is a competitive inhibitor of iodine uptake and inhibits TH synthesis in the thyroid follicular cells. Life-cycle exposure to environmentally relevant doses of perchlorate in *S. tropicalis* masculinizes (decreases) female body size and morphometrics, indicating a possible loss of natural sexual dimorphism (Campbell et al., 2018).

These observations have important implications for our understanding of sexual development and its disruption by environmental pollutants. The concept of thyroid-gonad crosstalk has been elegantly reviewed previously (Castaneda Cortes et al., 2014, Flood et al., Flood et al., 2014).

**Table 1**
Relevant biological and methodological characteristics of principal fish models used in multi- and transgenerational EDC research.

| Species | Relevant life history and biological traits for multi- and transgenerational EDC studies | Major epigenetic mark dynamics characterized across or at specific life-cycle stages |
|---|---|---|
| **Salmoniformes**<br>**Rainbow trout**<br>*Oncorhynchus mykiss*<br>  <br>**Brown Trout**<br>Salmo Trutta<br>  <br>**Cutthroat trout**<br>Oncorhynchus clarkii<br>  | ● Generation time ~2 years<br>● Genome sequenced and genome underwent teleost and salmonid specific duplication events with possible neo- or sub-functionalization in epigenetic machinery<br>● Existence of isogenic lines<br>● Globally introduced freshwater species of economic and ecotoxicological importance<br>● Migratory and nonmigratory populations<br>● Availability of circulating hormone assays<br>● Availability of primary cell culture and cell-lines for mechanistic *in vitro* assays | ● Histone modifications<br>● DNA methylation<br>● microRNA |
| **Beloniformes**<br>**Japanese medaka**<br>*Oryzias latipes*<br>  <br>**Marine medaka**<br>Oryzias melastigma<br>  | ● Generation time of 7 weeks<br>● Genome sequenced lab model amenable to transgenesis (KO and reporter lines)<br>● human-like lifecycle DNA methylation dynamics<br>● High throuput with increasing phenotyping tools<br>● Limited circulating hormone assays | ● DNA methylation<br>● microRNAs<br>● Histone modifications |
| **Cypriniformes**<br>Zebrafish<br>*Danio rerio*<br>  | ● Generation time of 9 weeks<br>● Genome sequenced lab model amenable to transgenesis (KO and reporter lines)<br>● Characterized strains<br>● Paternal transmission of methylome in PGCs and somatic cells<br>● High throuput with increasing phenotyping tools<br>● Limited circulating hormone assays | ● DNA methylation<br>● microRNAs<br>● Histone modifications |
| **Cyprinidontiformes**<br>**Mummichog**<br>*Fundulus heteroclitus*<br>  <br>**Turquoise killifish**<br>*Nothobranchius furzeri*<br>  <br>**Mangrove killifish**<br>*Kryptolebias marmoratus* | ● Short lifecycle in turquoise killifish, sexually mature in 2 weeks<br>● clonal and sexual reproduction in mangrove killifish<br>● Limited circulating hormone assays | ● DNA methylation |

**Table 1** (*continued*)

| Species | Relevant life history and biological traits for multi- and transgenerational EDC studies | Major epigenetic mark dynamics characterized across or at specific life-cycle stages |
|---|---|---|
|  <br>**Sheepshead minnow**<br>*Cyprinodon variegatus*<br>  | | |

### 3.2. Perturbation of metabolism by EDCs may lead to transgenerational impairment of reproduction in anurans

It is widely accepted that transmission of EDC effects across generations may result from epigenetic mechanisms (Major et al., 2020; Perera et al., 2020). However, in many species, it has been suggested that healthy offspring are born by a metabolically healthy mother (Swain and Nayak, 2009) since offspring survival is dependent on female investment in the egg yolk production (Hedayatirad et al., 2020). Life-history theory states that animals must partition limited resources between growth and reproduction. Since resources are limited, physiological constraints at the adult stages cannot be modified without causing deleterious effects on reproduction and progeny survival (Lardner and Loman, 2003). In female anurans, a large component of liver metabolism is directed to the constitution of fat bodies associated with reproduction or overwintering survival (Fitzpatrick, 1976; Wright, 2003). Under the tight regulation of estrogens, cholesterol synthesis in liver serves for vitellogenin lipidation that is stored in fat bodies for oocyte yolk reserves (Di Croce et al., 1997). In sexually mature anurans, coelomic fat bodies largely support gonadal growth and maturation over somatic tissues and this is supported by an inverse relationship between coelomic fat body size and gonad size (Wright, 2003). In a healthy, sexually mature female anurans with a normal ovarian cycle, lipids are preferentially allocated to oocyte over deposition into fat depots. Females with large fat bodies therefore highlight a defect in oocyte maturation (Wright, 2003).

Disruption of metabolism by EDCs leading to egg reserve depletion has been suggested as a potential cause of reproduction defects in exposed animals and in their progeny suggesting a mechanism in addition to epigenetics for explaining multigenerational EDC effects. Initial evidence that EDCs can alter frog metabolism with possible reproductive consequence was documented in the Western clawed frog (*S. tropicalis)* after acute exposure to benzo [a]pyrene (BaP), a chemical that also has mutagenic activity Kummer et al., 2008). Transcriptomic changes observed in the livers of BaP-exposed females (10 μg/L) revealed an EDC-dependent gene dysregulation of important biochemical pathways involved in resource allocation to reproduction, such as the insulin signaling pathway, adipocytokine signaling pathway and glycolysis/gluconeogenesis pathway and in egg reserve constitution such as cholesterol biosynthesis (Regnault et al., 2014). Controlled exposures of *S. tropicalis* throughout their life cycle to BaP and triclosan, alone or in mixtures at environmentally relevant concentrations (50 ng/L) induced a metabolic syndrome featuring a pre-diabetic state. The treated frogs produced a F1 progeny with slower development, smaller adult size, delayed sexual maturity, metabolic impairment at the adult stage and reduced reproductive success (Regnault et al., 2018; Usal et al., 2019). In addition, animals exposed to BaP produced F2 progeny with delayed metamorphosis (50% reached metamorphosis 20 days later) and sexual maturity (80% reached maturity 80 days later). At the adult stage, F2–BaP females displayed a marked metabolic syndrome and laid eggs with metabolite contents significantly different from control, and these eggs did not produce viable progeny. Analysis of discriminative

metabolites in the F2–BaP group showed a decrease of total cholesterol, cholesterol ester and free cholesterol contents which are essential for oocyte maturation (Usal et al., 2021).

Field studies confirmed the involvement of metabolic disruption by EDCs in progenitors for multigenerational reproductive effects. Data obtained in toads (*Bufo bufo*) suggest that maternal exposure to EDCs had a much larger effect on growth, metamorphic development, and sexual differentiation of progeny than direct exposure (Orton and Routledge, 2011). In the same species, reduced performance was observed in the offspring originating from agricultural and urban ponds compared to those originating from natural ponds. Despite being raised in a contaminant-free environment that allowed for maximal investment into development and growth, individuals originating from anthropogenically influenced habitats took longer to complete metamorphosis and had smaller body mass both as larvae and as juveniles. These results suggest that the offspring of toads in anthropogenically influenced habitats have reduced chances of becoming successful reproducing adults perhaps due to multigenerational costs of tolerance to chemical contaminants in their genitor (Bókony et al., 2018).

Taken together, these data suggest that reduced maternal investment in reproduction might be a direct cause for explaining multigenerational impacts of EDCs. Deregulated egg reserves may lead to progeny that have reduced fitness. Research on egg nutrient content is needed to unravel the basis for multigenerational reproductive defects observed in EDC-exposed frogs.

## 4. Evidence from birds

Birds are known to be sensitive to the effects of EDCs (Ottinger et al., 2015), but multi- and transgenerational impacts have seldom been explored in avian species. This is an important knowledge gap, since birds differ fundamentally from the more studied vertebrates (i.e., fish and mammals) in basic physiological characteristics relevant to endocrine disruption. For example, Ottinger and Rochester (2013) highlight that in contrast to mammals, birds have a higher body temperature, which can accelerate metabolic capacity. Other notable differences are that female birds have a single ovary, and males are the homogametic sex (ZZ vs ZW for females). Unlike fish, which exhibit extensive reproductive plasticity, birds are universally oviparous with embryos that develop within the protective environment of a shelled egg. However, development strategy may be an important consideration for effects of EDCs in birds since the timing of sexual differentiation, i.e., pre- or post-hatch, appears to differ between precocial and altricial species.

In contrast to the extensive datasets available for fish and mammals, few studies have examined multi-generational effects of EDCs in birds. Japanese quail (*Coturnix japonica*) are the most frequently used model species for such studies, due to the ease of animal husbandry and relatively short generation time (3–4 generations per year). A standardized Japanese quail reproduction test is used to explore consequences of maternal transfer of a chemical to offspring via the egg (reviewed in Ottinger and Rochester, 2013), but this test does not extend beyond the F1 generation. Outside of a regulatory toxicity testing context, several groups have used similar study designs to assess effects of parental exposure to EDCs on offspring. For example, injection of adult female quail with 1 μg of $EE_2$ day for 5 days during the laying period had no effect on reproductive endpoints in male offspring (Maeda et al., 2002). A similar study design was used to investigate the effects of 10 μg nonylphenol/day. In this case, a significant decrease in the density of the tubular gland within the shell gland was observed in F1 females (Yoshimura et al., 2002). A comprehensive study on American kestrel (*Falco sparverius*) found that dietary exposure of adults to the polybrominated diphenylether (PBDE) technical mixture, DE-71, resulted in reduced reproductive success in F1 males, with both fertility and courtship behaviour affected (Marteinson et al., 2010).

More recently, the U.S. Environmental Protection Agency (EPA) spearheaded the development of a two-generation avian toxicity test, under the Endocrine Disruptor Screening Program (EDSP). This test captures outcomes through one parental and two filial generations. Individuals in the first generation (F0) are exposed to the test chemical as adults through their diet. F1 individuals are exposed through maternal deposition into the egg, as well as lifelong dietary intake. F2 individuals are fed a control diet and thus receive the chemical uniquely through maternal deposition (Ottinger and Rochester, 2013). This study design does not permit the detection of transgenerational effects, but it does differentiate between effects of dietary exposure alone (F0), developmental and dietary exposure (F1), and developmental exposure alone (F2).

To date, only a handful of studies have used the two-generation avian test to assess impacts of EDCs. An inter-laboratory validation exercise evaluated the two-generation test to assess the effects of the fungicide, vinclozolin, in Japanese quail. The F0 and F1 birds were exposed to dietary concentrations of up to 1000 ppm, but few effects on body weight, thyroid weight, feed consumption, reproduction, or mating behaviour were observed at any life stage through to the F2 generation (in one lab a no observed effect concentration (NOEC) of 1000 ppm was established) (Ottinger and Rochester, 2013). This contrasts with the many reports of multi- and transgenerational effects of vinclozolin in rodents and other mammals as highlighted in section 5 of this paper. In another application of the two-generation test, adult Japanese quail were exposed to the androgenic growth promoter, 17β-trenbolone. Estradiol and testosterone levels were altered in F0 and F1 individuals, with little impact observed on the F2 generation (Karouna-Renier et al., 2017). Similarly, exposure of Japanese quail and bobwhite quail (*Colinus virginianus*) to the organochlorine pesticide, methoxychlor, had a minimal effect on several endocrine and behavioural endpoints (Ottinger and Dean, 2011). Decreases in fecal sex hormone concentrations and delayed sexual maturation were observed in F1 females, but these changes were not consistently observed across individuals, and the average age to initiate laying was unaffected. Clearer impacts of treatment were observed in male Japanese quail; exposure to methoxychlor was associated with reduced reproductive success in both the F1 and F2 generations (Ottinger and Dean, 2011). We are aware of a single study reporting transgenerational impacts of EDCs in birds. Injection of Japanese quail eggs with the phytoestrogen, genistein (500 μg/egg), was associated with a highly significant delay in sexual maturity in the F3 generation (Leroux et al., 2017). Weight and behavioural endpoints were also affected by the treatment, but global methylation was not different between the offspring of genistein-treated and control eggs.

Available literature suggests that EDCs can cause both multi- and transgenerational effects in birds, but the scarcity of data makes it is difficult to draw broad conclusions about mechanisms involved, and the prevalence and type of effects in comparison with more studied taxa such as fish and mammals. Additional work is needed in this area. Recent research characterizing the avian epigenome (Guerrero-Boasagna et al., 2018) and providing roadmaps for studying transgenerational inheritance in birds in the context of genetic variation (Leroux et al., 2017) will help with this effort.

## 5. Evidence from mammalian species

### 5.1. Rodents

For several decades, it has been known that exposure of male or female rats and mice as adults to drugs and EDCs can alter their gametes and that these modified gametes may result in altered progeny outcome (reviewed in Gore et al., 2015; Downey et al., 2018). Many studies focussed primarily on the effects on male germ cells and demonstrated that multigeneration effects occur when spermatozoa are exposed to various toxicants, forming the basis for the field of male mediated adverse progeny outcome (Anderson and Brinkworth, 2006; Hales et al., 1992; Olshan and Mattison, 1994; Robaire and Hales, 2003). Beyond multigenerational effects, the concept that gametes of adult rodents

exposed during pregnancy may have transgenerational effects can be traced to studies from Skinner's group (Anway et al., 2005). Since this time his group has published over ninety articles describing both the range of EDCs and drugs that can have transgenerational effects and proposed that epigenetic mechanisms account for such effects (reviewed in Skinner, 2016). Several groups have presented evidence both to support and challenge this concept. It is important to note that most of the molecular studies have focussed on transmission through the paternal germline as it is easier to obtain large amounts of spermatozoa to run whole (epi)genome analyses. Evidence of transmission through the female germline exists, yet, as eggs can only be obtained in small quantity, little is known about equivalent molecular mechanisms.

Although the Organization for Economic Development (OECD, 2021) has developed guidelines to test chemicals for their potential multigenerational impact in mammals that include the TG443 (Extended One-Generation Reproductive Toxicity Study) and TG416 (Two-Generation Reproduction Toxicity Study), no standardized guidelines exist to investigate the transgenerational impact of chemicals in mammals. Since a search in PubMed (May 23, 2021) identified 2380 research articles were published using key words "endocrine disrupting chemicals AND rodents", and 622 for "rodents and transgenerational", we cannot be comprehensive in this review. Rather, we focus primarily on studies in which the experimental exposure of mice or rats occurred at some point during gestation/lactation in F0 dams and effects were observed in the reproductive system of the F3 generation or beyond (Fig. 1, panel C). We have specifically reviewed the three most studied families of chemical that underlie the current mechanistic understanding of transgenerational effects in mammals: pesticides, phthalates, and bisphenols.

#### 5.1.1. Pesticides

One of the first experimental study demonstrating transgenerational effects reported the impact of gestational intraperitoneal administration of the agricultural fungicide vinclozolin (100 mg/kg/day), during the period of gonadal sex determination in rats (gestation days (GD) 8–14) (Anway et al., 2005). In this and subsequent publications, the F3 generation animals derived through the paternal lineage demonstrated an increased risk of tumors, behavioural changes, testicular germ cell apoptosis, decreased sperm motility, and changes in sperm DNA methylation (Anway et al., 2005; Guillette et al., 2014; Skinner and Anway, 2007). This transgenerational effect was attributed to an altered DNA methylation pattern in sperm from the F0 generation. More specifically, approximately 50 differentially methylated regions (DMRs) were identified in gene promoters in vinclozolin lineage F3 generation sperm DNA that differed from the control lineage (Guerrero-Bosagna et al., 2010). Interestingly, these DMRs were different from the ones identified in the F1 generation (Skinner et al., 2014). More recently, Ben Mammar et al. (2018) demonstrated a transgenerational impact of ancestral exposure to vinclozolin (i.p. 100 mg/kg/day PND 8–14) on histone retention sites and noncoding RNAs in sperm from the F3 generation males. The potential transgenerational effects of other pesticides, such as atrazine, methoxychlor, permethrin and N, N-diethyl-meta-toluamide (DEET), have also been examined by the Skinner group (Thorson et al., 2020a,b). Using a similar high dose i. p. treatment of rats exposed for a defined period during gestation as that described for vinclozolin, a range of pathologies was found in the F3 males and evidence that these effects were transmitted via epigenetic modification of the male germ line was presented. Epigenetic transgenerational inheritance after prenatal exposure of female rats with an i. p. administration of vinclozolin (100 mg/kg/day) has also been suggested in the female-lineage as differentially expressed genes (DEG) and noncoding RNA changes have been identified in granulosa cells of the F3 generation (Nillson et al., 2018). In a study of the effect of prenatal exposure to vinclozolin in mice, Brieño-Enríquez et al. (2015) reported a decrease in the rate of fertility as well as a reduction in the number of embryonic primordial germ cells (PGCs), and dysregulation of some of their microRNAs (p.o. 1 and 100 mg/kg/day PND 1–13.5). However, there was no evidence of changes in the DNA methylation in either primordial germ cells or from spermatozoa from the F1 generation, suggesting that mechanism other than methylation were involved in the transgenerational effects observed.

It is important to note that several studies in rats (Schneider et al., 2008; Inawaka et al., 2009; Gray and Furr, 2008) and mice (Iqbal et al., 2015; Brieño-Enríquez et al., 2015) have not demonstrated similar transgenerational effects/mechanism of action of vinclozolin. While some groups did not observe transgenerational effects (Schneider et., 2008; Inawaka et al., 2009; Gray and Furr, 2008), others identified transgenerational effects but were unable to demonstrate changes in DNA methylation in the F3 generation (Inawaka et al., 2009; Iqbal et al., 2015; Brieño-Enríquez et al., 2015). Although Iqbal et al. (2015) identified altered DNA methylation in F1 germ cells after maternal exposure to vinclozolin, these changes were not maintained in the subsequent generation. This group thus hypothesized that epigenetic modifications caused by vinclozolin are corrected by reprogramming events in the next generation, but it should be borne in mind that these studies differed from those done by Skinner's group in that they were done in mice and after EDCs were administered orally. The important discrepancies between these studies have called into question whether such effects occur and how they are mediated. It is important to emphasize that different doses and routes of administration were used by the various research groups. There were also strain and species difference in the timing of exposure during gonad differentiation, and that there are differences in metabolism among strains and species. The functional significance of these findings from the perspective of toxicologists concerned about hazard of exposures to vinclozolin and other fungicides remains to be resolved.

#### 5.1.2. Phthalates

Di (2-ethylhexyl) phthalate (DEHP) is a well-established EDC that is used extensively as a plasticizer in consumer products and medical devices (Canadian Environmental Protection Act, 1994; Rowdhwal and Chen, 2018). Transgenerational effects of DEHP and/or DBP have been reported in the F3 male progeny of rodents after maternal exposure during pregnancy for a defined time period (oral administration of 500 mg/kg/day on GD 8–14 in rats (Yuan et al., 2017), and GD 7–14 in mice (Doyle et al., 2013)). In the rat study, DBP caused a decrease in sperm and Sertoli cell numbers up to the F3 generation, as well as a reduction in specific methylation sites in spermatozoa of the F1 generation; however, these methylation site changes were not seen in spermatozoa of the F2 and F3 generations. In the mouse study, DEHP administration resulted in a decrease in sperm count and motility down to the F4 generation; spermatogonial stem cell (SSC) transplantation studies using F3 generation SSCs indicated that a disorganization phenotype occurred at the level of the SSCs. In a recent study, Thorson et al. (2021) reported that intraperitoneal treatment of F0 female rats with a mixture of DEHP (375 mg/kg) or DBP (33 mg/kg) and BPA (BPA 25 mg/kg) from GD 8–14 altered the epigenetic profile of DMRs of the sperm from F3 generation males. Disease-specific DMRs were identified for testis and kidney, leading these researchers to speculate that ancestrally derived epimutations may be transmitted transgenerationally.

More extensive studies, using a wider range of doses, have been undertaken to determine the potential transgenerational effects of phthalates, alone or in mixtures, on female reproduction (reviewed in Brehm and Flaws (2019)). In rats, DEHP (oral administration at 20 mg/kg from GD14-birth) resulted in a decreased body weight, pregnancy rate, and increased litter size in the F3 generation of F0 ancestors (Meltzer et al., 2015). In mice, where more extensive studies have been done, it was shown that treatment over a wide range of doses from 20 μg/day to 750 mg/day administered in different time windows during gestation and lactation resulted in effects on F3 females that included decreased primordial follicle numbers and increased preantral follicles, indicating that there was an acceleration of folliculogenesis, accelerated

onset of puberty, disruption of estrous cyclicity, an increase in the number of female pups per litter, and a decrease in female pup anogenital indices (Pocar et al., 2017; Rattan et al., 2018a,b; Brehm et al., 2018). While transgenerational effects for phthalates on female reproduction have been described by several groups, little focus has been placed so far on identifying underlying molecular mechanisms for such effects.

### 5.1.3. Bisphenol A

In rodent models, transgenerational studies have evaluated the impact of xenoestrogens after exposure to both females and males (reviewed in Brehm and Flaws, 2019). In a mouse study, Berger et al. (2016) found that treatment of F0 pregnant females with BPA (0.5, 20, and 50 μg/kg/day) from GD 11 to birth resulted in altered gene expression in a selected set of transcripts but did not affect follicle numbers in the ovaries of the F3 progeny at PND 21. This treatment also delayed the age of vaginal opening, the timing of first estrus, and the fertility index in the F3 generation of these mice (Ziv-Gal et al., 2015). Consequences on the F3 generation of maternal exposure to BPA on male progeny have also been reported. Salian et al. (2009a) found that exposing pregnant rats to environmentally relevant doses of BPA (1.2, 2.4 μg/kg/day) or DES (10 μg/kg/day) reduced male fertility from the F1 to F3 generations. This fertility decline was characterized by a reduction in litter size, sperm count and sperm motility. The transmission of these effects to subsequent generations was through the sire because males were crossed with untreated females. Another group of researchers confirmed the negative impact of BPA (0.5, 50 μg/kg/day) on motility and sperm count in F3 generation rats (Shi et al., 2019). In addition to its germline action, BPA altered the expression of hormone receptors and their co-regulators. Indeed, Salian et al. (2009b) noted a

**Table 2**
Multigenerational effects of EDCs in non-rodent mammals.

| Species | Treatment (μg/kg/d) | Time of exposure | Time of observation | Multigenerational Effects (F1) | References |
|---|---|---|---|---|---|
| New Zealand rabbit | BPA (0.05) | 15-30 dpc | 17, 21, 26, 27, 28, 29, 30 and 31 dpc and at 1, 2, and 3 dpp | altered expression levels of essential genes involved in androgen paracrine signaling (SFI, CYP11A1, 3 b-HSD and AR genes)<br>modified proliferation and differentiation of the fetal Leydig cells<br>altered serum testosterone after birth | Ortega-García et al. (2020) |
| Rabbit | Vinclozolin (0.01) | GD 15 to pnd 24 | 42 dpp | brain: increased calbindin expression in the ventral POA/AH; significantly decreased number of GnRH neurons selectively in the region of the organum vasculosum of the lamina terminalis (OVLT) but not more caudally in the POA/AH | Bisenius et al. (2006) |
| Rhesus monkey | BPA (400) | GD 50 to 100 and LG 100 to term | GD 100 or term | altered fetal heart transcriptome: genes with role in cardiac pathophysiologies, e.g. myosin heavy chain, cardiac isoform alpha (Myh6) was down-regulated in the left ventricle, and 'A Disintegrin and Metalloprotease 12', long isoform (Adam12-l) was up-regulated in both ventricles, and the right atrium of the heart | Chapalamadugu et al. (2014) |
| Pig Landrace × Yorkshire crossbred sows | BPA (0.05) | GD 1 to 110 | at birth | exposure reduced the pH and redness value of meat, but increased the lightness value, lactate content, glycolytic potential and lactate dehydrogenase (LDH) enzyme activity in the LT muscle.<br>increased LDH mRNA levels in the LT muscle at birth and the finishing stage, and reduced methylation at the LDH promoter | Zhou et al., 2018 |
| Pig Landrace × Yorkshire crossbred sows | BPA (0.05) | GD 1 to birth | at birth | increased malondialdehyde protein concentration in liver<br>decreased total antioxidant activity in umbilical cord plasma | Mou et al. (2018) |
| Norwegian female goats | PCB 126 (0.049, three times weekly) | GD 60 to pnd 40 | 12–40 weeks of age | mean plasma testosterone concentrations higher before onset of puberty and lower from around puberty and for about 5 weeks of the subsequent breeding period | Oskam et al., 2005 |
| | PCB 153 (0.098, three times weekly) | GD 60 to pnd 40 | 12–40 weeks of age | smaller testis diameter<br>lower ratio of interstitium area to seminiferous tubules<br>area and proportion of diploid testis cells were observed for the PCB153 group<br>higher percentage of sperm with damaged DNA<br>mean plasma testosterone concentrations higher before onset of puberty and lower from around puberty and for about 5 weeks of the subsequent breeding period | |
| Ram | Lindane (0.001) | conception to 28 dpp | 28 dpp<br>28 dpp | decreased reproductive behaviour<br>reduced LH and estradiol concentrations during reproductive development, LH pulse frequency at 27 weeks and testosterone secretion after GnRH treatment | Beard et al. (1999) |
| Ram | Pentachlorophenol (0.001) | conception to 28 dpp | conception to 28 dpp | increased scrotal circumference<br>reduced thyroxine<br>seminiferous tubule atrophy and epididymal sperm density reduced | |
| Rhesus Monkey | BPA (400) | GD 100 - 165 | 1 to 3 dpp | fetal decrease in brain tyrosine hydroxylase-expressing (dopamine) neuron | Elsworth et al. (2013) |
| Rhesus Monkey | BPA (2.5 mL implant x 2 implants) | GD 100 to 155 | GD 155 | reduction in fetal spine synapses in the CA1 region of hippocampus | |
| Rhesus Monkey | BPA (400) | GD 100 to term | 1 dpp | increase in multioocyte follicles | Hunt et al. (2012) |
| Rhesus Monkey | BPA (2.2–3.3 ng/mL serum levels) | GD 50–100 | GD 100 | persistent unenclosed oocytes in the medullary region and small, nongrowing oocytes in secondary and antral follicles<br>- | |

dpc, days post-coitus; dpp, days post-parturition; GD, gestation days; pnd, postnatal day.

decrease in the expression of the androgen receptor and the estrogen receptor ESR2 in the testes of adult F3 rats (Salian et al., 2009b). Moreover, there was a change in the expression of co-regulators of the SRC family of steroid receptors that are believed to play a role in fertility (SRC-1), spermatogenesis (p/CIP) and spermiogenesis (GRIP-1) (Salian et al., 2009b; Saidur Rahman et al., 2020).

#### 5.1.4. General conclusions on rodents

Epigenetic reprograming has been proposed as the underlying mechanism to explain the observed transgenerational effects for the three families of EDCs discussed above. With genome-wide erasure of DNA methylation at the time of exposure during gestation, it is reasonable to hypothesize that this is indeed at the origin of this phenomena. Yet, no direct evidence of early impact on epigenetic marks or on the reproducibility of altered pattern between generations has been presented. The few studies that attempted to correlate DMRs, ncRNA or histone retention sites between generations or between EDC exposures have yet to demonstrate significant overlap. To better understand the mechanisms involved in mediating the effects of exposure to EDCs, it is fundamental to better understand the timing and dynamics of epigenetic reprogramming during gestational and perinatal life in mammals that are not restricted to DNA methylation. To date, most studies of when demethylation and *de novo* methylation occur in gametes have used the mouse as a model, and the findings have been extrapolated to rats, other mammals and even humans. However, it is known that the timing of germ cell development varies between these species (Delbés et al., this issue, Figs. 1 and 2). The limited evidence in rats suggests that the windows of demethylation and *de novo* methylation relative to steps of germ cell differentiation may differ in rats, a species of choice for toxicology studies, compared to mice (Rwigemera et al., 2017, 2021).

### 5.2. Non-rodent mammals

Studies of EDCs in some livestock (pigs, goats, sheep), small non-rodent mammals (rabbits), and primates have been reported (Table 2). For example, maternal exposure studies to PCBs, organochlorine pesticides and BPA have demonstrated adverse effects in these mammalian species on a variety of outcomes in offspring after *in utero* exposure scenarios. Several of the adverse effects observed impacted the endocrine and nervous systems, pointing to multigeneration effects. Nevertheless, there is a lack of multi- and transgenerational studies of EDCs in non-rodent mammalian models. Considering their ecological significance and importance for human consumption, there are significant data gaps for many mammalian families that warrants urgent attention.

While BPA is a well-established EDC with multi- and transgenerational effects, surprisingly, there is a paucity of studies of the toxicity of this chemical in non-rodent mammalian species. One study in rabbits (*Oryctolagus cuniculus*) and four studies in rhesus monkeys (*Macaca mulatta*) demonstrate effects on various health measures of F1 generation offspring (Table 2). Ortega-Garcia et al. (2020) demonstrated that male rabbits exposed *in utero* to BPA (0.05 mg/kg/d oral maternal exposure 15–30 days post-coitus) exhibited changes in the expression levels of essential genes involved in androgen paracrine signaling, modified proliferation and differentiation of fetal Leydig cells, and had altered the serum testosterone levels 3 days after birth. Thus, in male rabbits after *in utero* exposure, BPA appears to change postnatal levels of serum testosterone most likely due to the impaired fetal Leydig cells formed by the proliferating stem and non-proliferating fetal Leydig cells.

Multigenerational studies of BPA in female rhesus monkeys have revealed abnormal ovarian, brain and cardiovascular development. Hunt et al. (2012) showed that continuous maternal exposure to BPA via polydimethylsiloxane (Silastic) implants (maternal serum levels 2.2–3.3 ng/mL) from gestation day 50–100 increased meiotic aberrations (synaptic defects) and the number of recombination events between homologous chromosomes in the fetal developing ovary during meiotic prophase. In mammals, these events are critical for the segregation of homologous chromosomes at the first meiotic division. For example, Susiarjo et al. (2007) previously showed that in mice, subtle BPA-induced disturbances during prophase increase the incidence of chromosomally abnormal oocytes produced by the mature female mouse. Hunt et al. (2012) also reported an increase in multi-oocyte follicles in fetal rhesus monkeys exposed via maternal dietary exposure to BPA (400 μg/kg/d) during the third trimester (gestation day 100 to birth). Thus, these studies demonstrated that low level BPA exposure affects the developing fetal ovary in the rhesus monkey during the events surrounding germ cell differentiation and meiotic entry as well as the formation of follicles in the perinatal ovary. Elsworth et al. (2013) showed that low dose BPA exposure of rhesus monkeys during the final 2 months of gestation induced abnormalities in the fetal brain, particularly the ventral mesencephalon and hippocampus. Using light microscopy, BPA exposed fetuses exhibited fewer tyrosine hydroxylase-expressing (dopamine) neurons in the mid-brain as well as a reduction in spine synapses in the CA1 region of the hippocampus.

In addition to these effects on the female reproductive endocrine axis development after *in utero* BPA exposure in primates, Chapalamadugu et al. (2014) showed multigenerational effects on fetal heart development. Daily oral administered of 400 mg/kg body weight of BPA during early (50–100 ± 2 days post conception, dpc) or late (100 ± 2 dpc to term) gestation to pregnant rhesus monkeys. At the end of treatment, fetal heart tissues were collected, and chamber-specific transcriptome expression was assessed for selected genes and ventricular tissue glycogen content was quantified. BPA exposure altered transcription of genes that are recognized for their role in cardiac pathologies. For example, myosin heavy chain, cardiac isoform alpha (Myh6) was down-regulated in the left ventricle, and 'a disintegrin and metalloprotease 12' long isoform (Adam12-l) was up-regulated in both ventricles, and the right atrium of the heart in BPA exposed fetuses. Collectively these studies provide further evidence of the impact of BPA on the reproductive and endocrine systems, but also show multigenerational effects on fetal primate ovarian and cardiovascular system development and, in the male fetal rabbit model, on testicular development.

Although no other EDC has been as well characterized as BPA in primates, several studies using pesticides and polychlorinated biphenyls support the premise that such chemicals can have multigeneration effects. Pesticides are a diverse group of chemicals used globally, and several have adverse effects on the endocrine system (Jepson et al., 2020). Three studies in non-rodent mammalian models have described the multigenerational effects of pesticides identified as EDCs. Bisenius et al. (2006) exposed pregnant rabbits from gestation day 15 to postnatal week 4, i.e., until weaning, to vinclozolin. In both male and female offspring, vinclozolin exposure significantly increased calbindin expression in the ventral preoptic/anterior hypothalamic area and significantly decreased the number of GnRH neurons selectively in the region of the organum vasculosum of the lamina terminalis (Bisenius et al., 2006). Although this study did not report sex-specific differences in the rabbit brain as predicted based on its antiandrogenic nature, it did demonstrate that vinclozolin influenced brain development in regions known to be critical for neuroendocrine function in rabbits after fetal and postnatal exposure.

In ram lambs, Beard et al. (1999) reported multigenerational effects of the organochlorine pesticides, lindane and pentachlorophenol. In this study, these pesticide exposures were from conception to 28 weeks of age via maternal dietary exposure to 1 mg/kg body weight/day. Semen was collected from 19 to 27 weeks of age and serum was collected every two weeks for various hormone measurements and reproductive behaviour was tested at 26 weeks of age. Neither of these pesticides affected body weight, semen characteristics or caused overt toxicity in male offspring. In pentachlorophenol-treated rams, scrotal circumference increased, seminiferous tubules exhibited atrophy, epididymal sperm density and serum thyroxine decreased, but no effects were observed on reproductive behaviour. Reproductive behaviour was

reduced in lindane-treated rams compared with control rams along with decreased reproductive hormones, i.e., serum luteinizing hormone (LH) and estradiol concentrations during reproductive development, LH pulse frequency at 27 weeks and testosterone secretion after GnRH treatment. Based on these results, it was hypothesized that the effects of pentachlorophenol on the testis may be linked to a decrease in thyroxine concentrations, while reduced reproductive behaviour in lindane-treated rams may be related to decreased LH, estradiol, and testosterone concentrations. These studies demonstrate that three pesticides elicited adverse multigenerational effects on the endocrine system in non-rodent mammals.

Polychlorinated biphenyls are a group of organochlorine compounds (209 congeners) that were banned in the 1970s due to their persistent, toxic and bioaccumulative nature, and several are known EDCs (Breivik et al., 2007). PCBs are still produced as unintentional by-products during various industrial thermal processes (i.e. waste incineration, cement production, metallurgy) and remain contaminants of concern globally (Gong et al., 2017). The multigenerational effects of PCB 126 (0.05 μg/kg body weight per day) and PCB153 (98 μg/kg body weight per day) were tested after long-term maternal exposures in female Norwegian goats from gestational day 60 until delivery at approximately day 150, and through lactation until post-natal day 40 (Oskam et al., 2005). No effects on postnatal follicle stimulating hormone (FSH) levels, conventional sperm parameters or testis histology were observed. The PCB153-treated animals differed significantly from the control group with respect to plasma LH and testosterone levels, while PCB126-treated animals only differed from the controls in plasma testosterone concentrations. Furthermore, PCB153 caused a significantly lower ratio of interstitial to seminiferous tubule area, proportion of diploid testis cells were observed for the PCB153 group and a significantly higher percentage of sperm with damaged DNA. Oskam et al. (2005) showed that PCB153 induced alterations in reproductive endpoints related to the hypothalamic-pituitary-axis as well as to the testis. Ultimately, the effects observed in male kids after a long-term maternal exposure to PCB153 support the concept that exposure to a well-known EDC during fetal development may lead to adverse reproductive effects in adult life in small ruminants.

Although the underlying mechanism(s) of multigenerational effects of EDCs in the non-rodent mammalian species reported here are unknown, it is hypothesized that epigenetic processes are contributing to some extent. Despite the current lack of data available to support this hypothesis, studies of other stressors in livestock (reviewed by Wang et al., 2021) report intergenerational epigenetic alterations in response to nutritional stressors, heat stress, hypoxic conditions, transport stress and pathogen exposure. For example, heat-stressed pigs exhibited 57, 147 DMRs including 1422 differentially methylated genes associated with energy and lipid metabolism, cellular defense and stress responses in longissimus dorsi muscles, indicative of epigenetic regulation of pig muscle development, meat quality and heat stress processes (Hao et al., 2016). Based on the epigenetic effects in livestock reviewed by Wang et al. (2021) for non-chemical stressors and evidence in other species presented in this review, it is hypothesized that some types of chemical exposure in non-rodent mammals, such as EDCs, will elicit epigenetic effects causing multi- and transgenerational adverse effects. To address the inadequate data available for non-rodent mammals, more studies focussed on EDC-induced epigenetic process changes using multi- and transgenerational exposure study designs are needed in a wide variety of species with differing life histories.

## 6. Overall conclusions and the path forward

Many studies have clearly demonstrated that EDCs have multi- and transgenerational effects in a wide variety of species. The nature of the transmission of information across generations depends on whether we consider multi or transgenerational effects. Understanding the mechanisms underlying multigenerational effects will require not only (epi) genetic studies but also understanding the role of social, metabolic, and environmental stressors (temperature, stress, etc..). Although a range of epigenetic mechanism has been implicated in attempting to elucidate the underlying molecular mechanism for such transmission, we believe that studies in this field will have major implications for biodiversity and for the future of the environment and human health. Consequently, we should like to make the following recommendations for future investigations. These can provide the foundations for deep understanding of multi- and transgenerational effects and thus mitigation of adverse outcomes on reproductive and global health.

1. Comparative Approaches. We must take advantage of the power of comparison across taxa in order to understand multi- and transgenerational effects of EDCs. While studies have made great strides in elucidating molecular epigenetic underpinnings in select model species, remarkable differences and plasticity exists among some species with respect to their reproductive system and sensitivity to EDCs that ultimately reflect deeply rooted differences in endocrine physiology. Under a comparative framework, these differences can be exploited to help uncover mechanisms of action of EDCs in species with different life history and reproductive strategies. Moreover, a complete understanding of the potential impacts of multi- and transgenerational effects of EDCs on biodiversity will require knowledge about how a range of taxa are affected. This is of importance as reproductive strategies and control of epigenetic reprogramming appear to be diverse and highly species-specific.
2. Experimental design. Explicitly described experimental designs should be provided for all studies. A uniform and precise nomenclature should be used to clearly separate context-dependent from germ-line dependent, true transgenerational effects. For example, in fish studies the (maternal) transfer of (persistent) contaminants, as well as parental hormones to the egg needs to be addressed analytically and discussed appropriately. Future experimental designs should thus aim to dissociate potential ‘indirect’ effects from ‘direct’ chemical effects on the germline.
3. Use of environmentally relevant doses and routes of administration. Some important new concepts have emerged based on experiments with high doses of EDCs or routes of exposure that are not functionally relevant. However, in assessing the actual impact of multi- and transgenerational effects of EDCs on the environment, animal species and humans, it is important to include both doses and routes of exposure that reflect probable real-life situations.
4. Extend EDC multi- and transgenerational studies beyond EATS. Multi- and especially true transgenerational effects of many classes of EDCs remain uncharacterized in all taxa discussed in this review. The interactions of reproductive, developmental, metabolic and growth-regulating hormones must now be more carefully considered when attempting to interpret the mechanism(s) of action of EDCs. Most studies have focused on EDCs that exert effects via estrogen, androgen, or thyroid hormone receptor activation. In addition to the EATS modalities, limited attention has been given to EDCs that act through other mechanisms, referred to as the non-EATS. This includes activation of retinoic acid signaling, PPARs, and glucocorticoid/mineralocorticoid receptor signaling lead to metabolic disruption, effects on the immune system, and crosstalk with the EATS pathways. In this context, the extension of the concept of hormonal crosstalk across generations and the role of epigenetic signals warrant further exploration. This topic is addressed elsewhere (Martyniuk et al., 2021, this issue)
5. Integrative functional relevance. Emerging (epi)genome editing tools should be used to directly test the functional role of altered epigenetic marks in the transmission of ancestral EDC exposure dependent phenotypes. Such an approach may thus validate potentially predictive epigenetic marks in the germline in a controlled lab setting, although the relevance of individual epigenetic marks in a

sea of many, likely interacting and epigenetic mechanisms, remains another exiting field of future study.

6. Mechanism of action. While it is now clear from both multi-transgenerational studies that various epigenetic marks can be affected after exposure to EDCs. This descriptive evidence does not provide an explanation for the molecular action of EDCs. Indeed, no molecular mechanism has been put forward to explain how chemicals such as BPA can actually modify these epigenetic marks. Investigations into chromatin organization and remodelling after EDC treatment may shed light on this process.

## Credit author statement

All authors contributed to the conceptualization, writing and editing of the manuscript.

## 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.

## Acknowledgments

The authors are grateful to the Intersectoral Centre for Endocrine Disruptor Analysis (ICEDA)'s researcher network that facilitated this Special Issue.

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