Ecotoxicological Perspectives of Sex Determination

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Abstract

Sex determination or the commitment of the embryo to its sexual fate is a fundamental developmental process with paramount consequences in ecology and evolution. This process, whether triggered by environmental factors or genotypic constitution, can be derailed by environmental contaminants that alter the endocrine system, which is a key component of the regulatory network underlying vertebrate sex determination. Here, we review the molecular basis of sex determination, the endocrine components of its regulation, the maternal and endogenous sources of hormones to the developing embryo, and the routes through which endocrine disrupting chemicals (EDCs) affect gonadal development in reptiles, especially turtles. Among EDCs, we focus on PCBs, BPA, pesticides like atrazine, nitrates, and heavy metals. We also consider whether adaptation might be possible in the face of persistent chemical insult and conclude that, while plausible, contemporary environmental change may outpace adaptive evolution, particularly for many species that are already endangered and suffer from small population sizes.

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Sex determination is the biological process by which the sexual fate of an individual is decided, and thus, it is fundamental for the production of sex ratios. Disruptions of the natural process of sex determination can have broad consequences, from short-term effects on the dynamics of wild populations or human reproduction to long-term effects on species diversity. Therefore, illuminating the causes and consequences of such disruptions is important for our understanding of sexual development at ecological and evolutionary time scales.

Sex-determining mechanisms range between 2 extremes [Valenzuela et al., 2003; Sarre et al., 2004]. At one end is genotypic sex determination (GSD), where sex chromosomes inherited from the parents at fertilization decide gonadal fate and the ensuing sex differentiation [Barske and Capel, 2008]. At the other extreme of the sex determination spectrum lies environmental sex determination (ESD) where the sexual fate is decided based on environmental factors experienced after fertilization [Bull, 1983; Valenzuela and Lance, 2004]. Temperature is the most common environmental factor that affects sex determination in vertebrates (TSD), where the incubation temperature at a time called the thermosensitive period commits the embryo to the male or female fate [Valenzuela and Lance, 2004; Janzen and Phillips, 2006; Bachtrog et al., 2014] in the absence of sex chromosomes [Valenzuela et al., 2014].

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Importantly, environmental changes influence species whose sex-determining mechanism is susceptible to external factors and thus, a lot of attention in this respect has focused on amphibians (GSD) and ESD reptiles. Such changes may be natural as with the climatic changes that the earth has undergone over geological time and which may have mediated transitions in sex determination in turtles [Valenzuela and Adams, 2011]. Other changes are anthropogenic and encompass contemporary climate changes which could endanger many TSD species [Janzen, 1994; Neuwald and Valenzuela, 2011] or habitat degradation from chemical pollution that could alter sex determination by disrupting the genetic machinery responsible for gonadal formation [Hayes et al., 2002]. For instance, soil and water contamination by pesticides and other contaminants from agriculture, industry, and domestic effluents may directly affect the embryonic development of many species of reptiles and amphibians. Because some species lay eggs in agricultural areas or in natural areas such as lakes and ponds that receive effluents from sewage treatment plants, they are highly exposed to contaminants which can penetrate the eggshell dissolved in the aqueous phase in the substrate or through air exchange due the substrate's gaseous phase [De Solla and Martin, 2011].

The endocrine pathways are key during the processes of sex determination, differentiation, and reproduction in all sex-determining systems. Endocrine disruptor chemicals (EDCs) are generally anthropogenic, and as its name refers to, they disrupt natural processes. Therefore, the sex determination mechanism, whether driven by sex chromosomes or environmental factors, may be altered by EDCs. These compounds can act separately or in combination and affect diverse aspects of sex determination, including gonadal development [Matsumoto and Crews, 2012], testosterone levels, and number of germ cells [Hayes et al., 2010]. EDCs can also affect adult sexual behaviors, viability of offspring, as well as fertility and hatching rates, and are linked to reproductive disorders in humans including reproductive cancers [Fan et al., 2007a]. Such effects can negatively alter wild populations [Bergeron et al., 1994]. Furthermore, since these compounds have the ability to bioaccumulate due to their lipophilic characteristics [Lutz and Kloas, 1999], understanding the consequences of contaminants to the sex determination mechanism and embryonic development is essential for conservation and human health.

Reptiles are good models to study the effects of EDCs in sex determination and development because they vary in their mechanisms of sex determination (GSD and TSD), and in their reproductive mode (viviparity and oviparity) [Crain and Guillette, 1998]. Additionally, oviparous reptiles are good systems to study maternal effects, since it is possible to explore or alter maternal contribution to the offspring and observe the effects [Bowden et al., 2009]. Here, we review some basics about sex determination with special emphasis on reptiles in general and turtles in particular and the effects of EDCs in sex determination.

Vertebrate Sex Determination

The most common GSD mechanism involves sex chromosomes, that is, a pair of chromosomes harboring the master sex-determining gene(s) whose presence directs the embryo to the male or female fate. GSD mechanisms are developmentally canalized and thus resilient to environmental disturbance to a great degree. In a XY sex chromosome system males carry 2 different sex chromosomes (male heterogamety), and in a ZW system females are the heterogametic sex. Sex chromosomes may be morphologically different between males and females (heteromorphic) which permits relatively easy identification of XX/XY or ZZ/ZW systems. However, in some taxa the sex chromosomes are morphologically similar (homomorphic) such that the heterogametic sex cannot be identified by classic cytogenetic techniques [O'Meally et al., 2012] but by molecular methods with higher resolution [Ezaz et al., 2005, 2006; Martinez et al., 2008; Badenhorst et al., 2013]. Naturally occurring or abnormal sex reversal can be identified in these taxa when the phenotypic sex (testis or ovaries) and genotypic sex (e.g. XX, XY, ZZ, ZW) of an individual are not concordant. Among vertebrates, mammals, birds, amphibians, and snakes have sex determination virtually exclusively driven by genetic factors [Tree of Sex Consortium et al., 2014] such that consistent genotypic differences exist between the sexes [Brelsford et al., 2016].

In TSD systems that lack sex chromosomes [Valenzuela et al., 2014] no consistent genotypic difference distinguishes males and females in contrast to sex chromosomal systems. TSD is present in many vertebrates such as crocodilians, tuatara, most turtles, some lizards, and some fish [Valenzuela and Lance, 2004; Tree of Sex Consortium et al., 2014]. Besides temperature, other environmental factors, such as nutrition, pH, and oxygen levels, can also affect sex determination in some vertebrates (e.g. fish) and invertebrates (e.g. some crustaceans, insects,
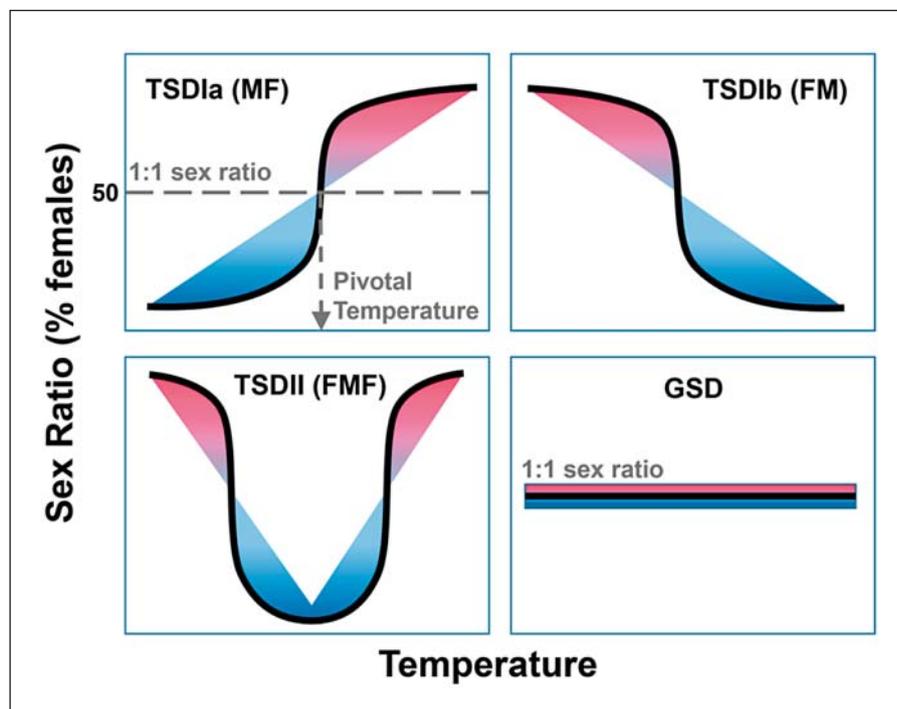
Fig. 1. TSD and GSD reaction norms of sex ratio as a function of exposure to environmental temperature during sexual development. Red = female production; blue = male production.

and nematodes), but such effects are unknown in reptiles [Korpelainen, 1990]. Between the GSD and TSD extremes we find vertebrates with intermediate mechanisms where a strong genotypic component exists but which may be overridden by environmental factors such as temperature [Valenzuela et al., 2003; Sarre et al., 2004]. In some cases, sex chromosomes exist and temperatures at some extreme values encountered in nature cause a sex reversal [Shine et al., 2002; Holleley et al., 2015]. In other cases, the environmental factor may simply prevent the fertilization by one of the sex chromosomes in an otherwise GSD taxa or induce the death of embryos of a specific sex, resulting in biased sex ratios [reviewed in Valenzuela et al., 2003]. In yet other cases, a key genotypic component is expressed at a limited range of temperatures in what would appear as a pure TSD system [Yamamoto et al., 2014]. Thus, a full continuum of mechanisms is expected to range from systems where sex determination is virtually completely canalized to systems of virtually complete phenotypic plasticity. Whether this theoretical expectation is realized in nature remains an open question of active research.

TSD is an example of phenotypic plasticity in which individuals of identical genotypes become male or female, depending on the environmental temperature which initiates the cascade of molecular events that favor the de-velopment of one sex or the other by ultimately altering and controlling gene expression and cellular signaling of steroid hormones, hormone receptors, and steroidogenic enzymes during development [Crews et al., 1995; Crain et al., 1997; Gale et al., 2002]. TSD follows 3 patterns described by the reaction-norm of sex ratios produced by temperature (fig. 1) [Valenzuela and Lance, 2004; Inamdar et al., 2012]: (1) TSDIa in which males are produced at lower temperatures and females at higher values (also termed MF), as found in most turtles; (2) TSDIb in which females are produced at low temperatures and males at higher values (also called FM), as found in many lizards and some crocodilians, and (3) TSDII in which females are produced at both low and high temperatures and males at intermediate values (also called FMF), as found in some lizards and crocodilians [Valenzuela and Lance, 2004]. The pivotal temperature is the constant temperature value that produces a 1:1 ratio of males and females, which can vary by species and populations [Valenzuela and Lance, 2004; Warner, 2011]. Sex ratios produced at the pivotal temperature may vary by clutch of origin, perhaps reflecting some genetic predisposition towards one sex or the other, or perhaps as the result of non-genetic maternal effects such as yolk hormones allocated by females [Lance, 2009].
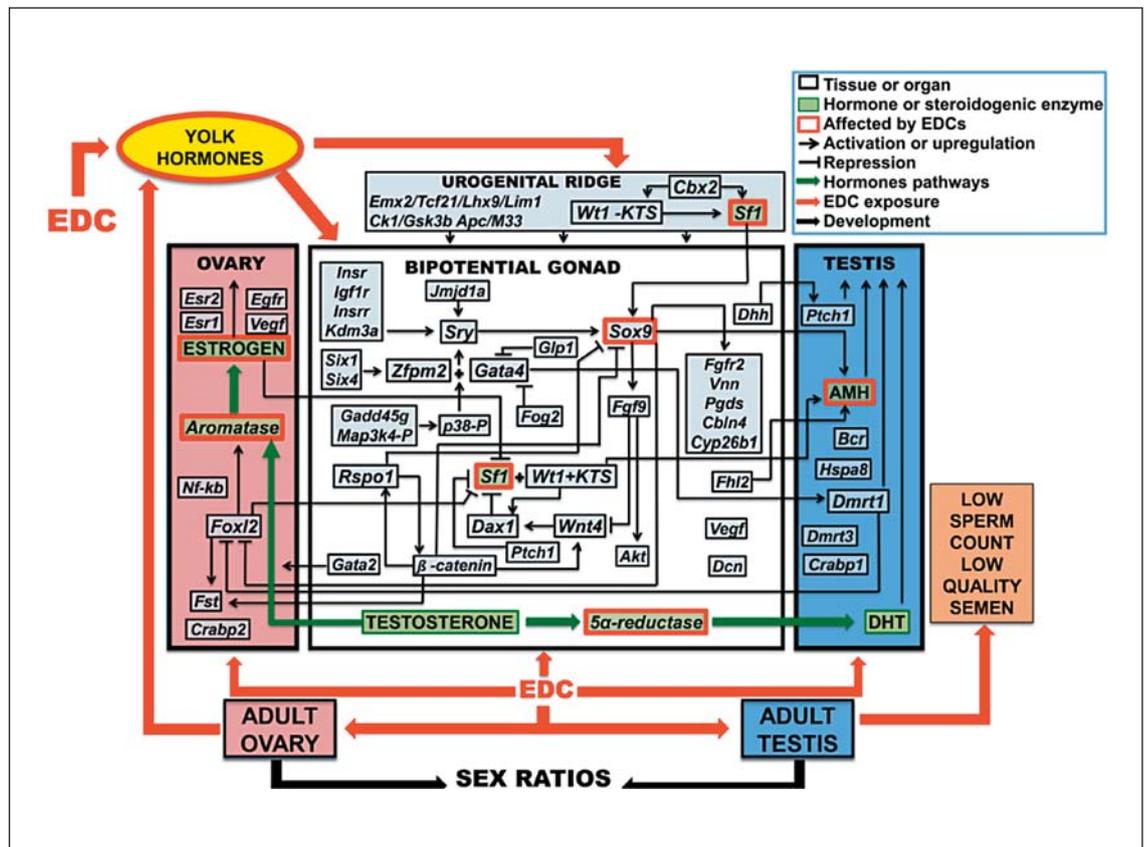
Fig. 2. Partial GSD gene-regulatory network and some documented routes of disruption of sex determination by EDCs. GSD network based on Valenzuela [2008] and Eggers et al. [2014].

During the thermosensitive period, the sex of the TSD embryo is reversibly determined, and any variation of temperature in this time can initiate or suppress the development of one sex or the other [Lance, 2009]. Gonads may still be sex-reversed after the thermosensitive period when exposed to exogenous hormones or inhibitors that affect the maintenance of gonadal differentiation [Dorizzi et al., 1996; Belaid et al., 2001]. The duration of the thermosensitive period may vary by species, by temperature, and even by population [Yntema, 1979; Pieau and Dorizzi, 1981; Wibbels et al., 1991; Valenzuela, 2001; Hewavisenthi and Parmenter, 2002; Bonach et al., 2011; Rhen et al., 2015]. Under fluctuating temperature regimes, as occur in natural nests, sex is determined by the cumulative effect of the temperatures above the pivotal temperature (or above the value that causes development to halt) experienced during the thermosensitive period [Georges et al., 2005; Neuwald and Valenzuela, 2011].

Molecular Machinery of Sex Determination and the Endocrine Regulatory Axes

The molecular machinery that regulates gonadal formation, other than the top-most elements, shares essentially the same components between GSD and TSD species (fig. 2). The best characterization of this gene regulatory network derives from mammalian and avian models [Eggers et al., 2014]. The endocrine system is a key component of this regulatory network, and the influence of endogenous and exogenous hormones, inhibitors, competitors, and mimics has been illuminated by extensive research [reviewed in Place and Lance, 2004; Merchant-Larios and Diaz-Hernandez, 2013]. Furthermore, the endocrine signals from hormones not only affect sex differentiation but also the general development of the embryo, generating phenotypic variation among individuals that could affect their fitness in particular environments [Paitz and Casto, 2012].

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Briefly, the formation of the bipotential gonad during mammalian embryogenesis is mediated by the action of multiple transcription factors [Eggers et al., 2014], of which *Wt1, Lhx9*, and *Sf-1* (steroidogenic factor 1) have been studied in TSD taxa [Barske and Capel, 2010; Rhen and Schroeder, 2010; Valenzuela et al., 2013; Bieser and Wibbels, 2014]. The bipotential gonad of males and females possess Wolffian ducts (paramesonephric ducts) and Müllerian ducts (mesonephric ducts), respectively [Place and Lance, 2004; Warner, 2011]. As embryonic testes develop, the production of anti-Müllerian hormone (AMH) induces the regression of the Müllerian ducts while testosterone stabilizes the Wolffian ducts that will eventually form the seminal vesicles, epididymis, and vas deferens [Place and Lance, 2004; Warner, 2011]. *Lhx9* mediates the production of both AMH and testosterone [Eggers et al., 2014]. Reptiles do not possess *Sry*, the trigger of male development present in eutherian mammals, but they do have other downstream genes such as *Sox9*, *Wt1,* and *Dmrt1*, which are responsible for male gonad development [Shoemaker and Crews, 2009; Eggers et al., 2014]. If the Müllerian ducts do not regress, they form the oviducts and other organs of the female reproductive tract [Eggers et al., 2014]. Genes such as the transcription factor *Foxl2* and the signaling molecule *Rspo1* that regulate ovarian formation [Eggers et al., 2014] have also been studied in reptiles [Rhen and Schroeder, 2010]. Estrogen (E2) is a key component of the endocrine system necessary for this female differentiation process in reptiles [Ramsey and Crews, 2009], and its exogenous application can downregulate *Sox9* expression [Barske and Capel, 2010]. Consequently, aromatase, the steroidogenic enzyme that converts testosterone into estrogen [Simpson et al., 2002], is also essential for ovarian formation. Aromatase expression and activity in TSD turtles is higher at female-producing temperatures, but it starts during or after the thermosensitive period [Desvages and Pieau, 1992; Simpson et al., 2002; Valenzuela et al., 2013] indicating that aromatase is important for female differentiation although it does not initiate sex determination. Aromatase is also upregulated at female-producing temperatures in the brain of TSD turtles when the thermosensitive period starts, and it can mediate sexually dimorphic behaviors [Willingham et al., 2000]. On the other hand, 5α-reductase, the enzyme responsible for 5α-dihydrotestosterone (DHT) synthesis from testosterone, is a required enzyme for male development in vertebrates [Andersson et al., 1989; Langlois et al., 2010]. Inhibition of 5α-reductase in rats can cause prostate regression and underdeveloped genitalia [Russell and Wilson, 1994].

Yolk Hormones and Sex Determination

An initial source of hormones to the developing embryo is of maternal origin. Indeed, hormones present in yolk, such as progesterone, estrogen, and testosterone, are maternally derived and play important roles in sex determination, sometimes overriding the effect of temperature in TSD taxa particularly at the pivotal temperature. Further, yolk hormones may alter the production of steroidogenic enzymes such as aromatase and 5α-reductase, thus affecting the capacity of the gonads to differentiate [Bowden et al., 2002]. The allocation of hormones to the yolk varies among females as observed in turtles where yolk levels reflect circulating levels found in females at vitellogenesis [Bowden et al., 2000; Elf et al., 2002]. The dynamics of yolk hormone levels after oviposition depends on the incubation temperature. For instance, snapping turtles, *Chelydra serpentina,* exhibit higher E2 levels at female-producing temperatures, intermediate levels at the pivotal temperature, and lower levels at male-producing temperatures [Elf et al., 2002]. Painted turtles, *Chrysemys picta,* clutches incubated at the pivotal temperature responded differently according to seasonal variation of endogenous hormone levels [Bowden et al., 2000]. Namely, estrogen levels increased seasonally while testosterone decreased, resulting in a higher estrogen:testosterone ratio over time, which correlated with early-season clutches being male biased and late-season clutches being female biased [Bowden et al., 2000]. In slider turtles, *Trachemys scripta*, the levels of progesterone, estradiol-17β, and testosterone are high during oviposition and decline as the embryo develops, with estradiol-17β declining faster and reaching low levels before the start of the thermosensitive period [Bowden et al., 2002]. In *C. serpentina*, levels of estradiol-17β and testosterone are also high and decline differently by temperature. Thus, temperature influences egg yolk hormonal levels and could affect the sex determination of the developing embryo [Elf et al., 2002]. Consequently, modification of female hormonal levels normally found in natural populations could disrupt this process.

Metabolism of maternally-derived yolk steroid hormones usually occurs in 2 phases, the first is oxidation or reduction (phase I) and the second is sulfonation or glucoronidation (phase II) [Paitz and Bowden, 2015]. The sulfotransferase/sulfatase (SULTS/STS) pathway may drive the sulfonation of steroid hormones in the yolk [Paitz and Bowden, 2013]. Estrogen and other steroids, such as progesterone and testosterone, are converted to an inactive water-soluble form by sulfotransferases, which are more

easily transferred from the yolk to the embryo, and when necessary, they are converted to the bioactivated form by sulfate enzymes [Paitz and Bowden, 2013]. Exogenous estrogen when applied to *T. scripta* eggs was more concentrated in the yolk than in the albumen and declined throughout development as sulfotransferase concentration increased and the exogenous estrogen was metabolized to a bioactive form [Paitz and Bowden, 2008]. Exogenous estrone sulfate injected to eggs feminized *T. scripta* embryos incubated at male-producing temperature, linking this sulfonated compound to sex determination [Paitz and Bowden, 2013].

Estrogen levels can affect the expression of *Sf-1*, which is an activator of AMH [Elf, 2003]. In turtles, E2 levels may be maintained at high levels at female-producing temperatures decreasing SF-1 expression, which in turn would inhibit AMH expression, leading to female development [Elf, 2003]. A threshold of E2 levels may exist to activate or repress SF-1 synthesis with temperature, maintaining these levels at female-producing temperatures or degrading them at male-producing temperatures [Elf, 2003]. At pivotal temperatures, yolk hormones could tip this process such that eggs with higher E2 concentrations would surpass the threshold and develop as females, and eggs with lower concentrations would develop as males [Elf, 2003]. Thus, the interplay between temperature and steroid hormones may affect differential activation of sex genes during the thermosensitive period, which could lead to ovarian determination [Ramsey and Crews, 2009].

Effect of Contaminants in Sex Determination

The endocrine system, responsible for regulating hormones that affect the sex determination pathway as described above, works in a dosage manner. Thus, disruptions of hormonal signals will cause the unbalanced expression of certain products, resulting in a deviation from the original sex fate [Milnes et al., 2006] irrespective of whether this fate is determined by the genotype (GSD) or environmental factors (e.g. TSD). Contaminants differ in their endocrine action, and the consequent alteration of the endocrine system that overcomes the temperature effects disrupts sexual development [Crain et al., 1997]. Contaminants may act as aromatase inducers [Hayes et al., 2010], hormone mimics [Bergeron et al., 1994], hormone antagonists, or they may alter the synthesis or degradation process of hormones [Guillette et al., 2000].

Namely, EDCs are compounds present in the environment that have the potential to mimic or block the action of hormones [Crews and McLachlan, 2006]. EDCs compounds may present estrogenicity, the ability to induce cellular proliferation of estrogen-sensitive cells or the ability to bind to estrogen receptors. These effects are caused in various ways, such as by altering the estrogen/androgen ratio via the increase of gonadal estrogen production, or by decreasing gonadal androgen production via the decrease in cytosolic binding proteins (CBPs), or via the agonistic binding of the compound to an estrogen receptor [Guillette et al., 2000]. Because in non-mammalian vertebrates sex steroids act in gonadal development, EDCs will alter this process by affecting the endocrine system [Guillette and Gunderson, 2001], with the potential of having an enormous biasing effect on sex ratios and the future reproduction of many species inhabiting polluted areas [Basile et al., 2011]. Embryos are very sensitive to any disruption that can change the structure and function of developing organs, because they are phenotypically flexible and susceptible to the compounds present in the environment [Guillette and Gunderson, 2001]. Also, embryos may respond differently than adults to endocrine signals [Willingham and Crews, 2000]. EDCs that act as hormone mimics may interact synergistically with naturally present hormones and cause the system to surpass thresholds that induce a developmental cascade at the wrong time or in the wrong context, causing an adverse effect [Willingham and Crews, 2000].

The negative effects of EDCs are not limited to species with environmental sex determination. Indeed, the power of EDCs in GSD species is evident in mammals, where some contaminants cause male reproductive dysfunction, such as reduced sperm count and/or low semen quality, due to alterations in the endocrine system, gene expression, steroidogenesis, and epigenetic effects, including oxidative stress in the testis which disrupts cell junctions, increasing epithelial and endothelial permeability, which could cause damage to the testis [Wong and Cheng, 2011]. Furthermore, frogs living in suburban areas with higher exposure to different kinds of EDCs tend to have increased feminized sex ratios compared to populations inhabiting forested areas [Lambert et al., 2015]. Thus, it should be expected that EDCs put at risk GSD and TSD turtles alike, despite their differences in sex determination.

Exposure to EDCs can also be detrimental to adults, and these effects are sex-specific due to behavioral and physiological differences between males and females. For instance, exposure to EDCs may be higher in oviparous females than males if they eat more to support the higher energy demand of egg production, and in so doing they ingest more contaminants. Further, physiological and

hormonal differences exist in detoxification between the sexes [Fowler et al., 2012]. Namely, the hepatic biotransformation of many steroids and toxins can have a sex-specific pattern, especially because contaminants alter the metabolic effect of cytochrome P450 enzymes, which are important in the hormone conversion process [Guillette and Gunderson, 2001].

As mentioned above, exogenous estradiol-17β can override sex determination by temperature in TSD taxa, producing females at temperature that normally produce only males [Crews, 1996] and, similarly but in the opposite direction, the application of an aromatase inhibitor induces male development at female-producing temperatures [Jeyasuria and Place, 1998]. The important role of aromatase in TSD is evident from the failure of exogenous testosterone to induce male development at female-producing temperatures, because aromatase converts this testosterone into estradiol, which has a feminizing effect [Crews and Bergeron, 1994]. EDCs could disrupt the steroidogenic enzyme pathways by altering SF-1 expression, as SF-1 is an upstream element of the aromatase pathway, thus changing aromatase activity [Willingham and Crews, 2000]. For instance, alligator eggs treated with different contaminant EDCs exhibited altered aromatase activity and experienced sex reversal. Therefore, disrupting aromatase enzymatic activity might be one way of disrupting steroid hormones [Crain et al., 1997]. Individuals are susceptible to these effects not only during the thermosensitive period of embryonic development. In fact, exposure to an aromatase inhibitor after the thermosensitive period sex-reversed developing embryos of the European pond turtle, *Emys orbicularis*, incubated at a female-producing temperature, revealing that sex differentiation was still labile, albeit harder to override, since the gonadal aromatase and estrogen levels are already well established at this developmental stage [Belaid et al., 2001]. Yet, a high dose of estradiol-17β in *C. picta* and *C. serpentina* yielded, opposite to expectation, a high percentage of males at both male- and female-producing temperatures, perhaps because a high concentration of estradiol permitted the interaction of estrogen with androgen receptors or caused aromatase inhibition [Warner et al., 2014].

Routes of Disruption and Specific EDCs

EDCs alter phenotypes, and it is unlikely that these effects are due to modifications in the genetic code. Instead, EDCs are more likely to induce epigenetics changes, perhaps by altering DNA methylation and chromatin remodeling [Crews and McLachlan, 2006] as reported in amphibians and reptiles, where DNA methylation was modified by contaminants and the effect persisted over generations [Head, 2014]. Early-life exposure to EDCs could strongly alter the expression of neuroendocrine genes and DNA methylation, which, together, can cause reproductive dysfunction in mice [Collotta et al., 2013]. Nonetheless, little is known about how exactly contaminants affect DNA methylation in reptiles [Head, 2014].

One important EDC is PCB (polychlorinated biphenyl), an environmental chemical that is no longer commercially produced since 1979 but is found in many products and materials such as electrical components, plastics, and adhesives [Basile et al., 2011]. PCBs are chemically resistant and take long periods to degrade once released into the environment where they bioaccumulate in fatty tissues of many animals and thus are present in many food webs [McKinney and Waller, 1994]. Diamonback turtles, *Malaclemys terrapin,* from Barnegat Bay (New Jersey), a highly industrialized region which receives ~70% of water effluents, show high concentrations of PBCs and other contaminants in fat and plasma in both sexes [Basile et al., 2011]. PCBs are potentially carcinogenic [McKinney and Waller, 1994] and are of high concern regarding sex determination, especially for TSD taxa, as they are estrogen mimics [Bergeron et al., 1994].

For instance, *T. scripta* turtles incubated at male-producing temperature are feminized when exposed to different types of PCB [Bergeron et al., 1994]. Two out of 11 PCBs tested were the most effective: 2′,4′,6′-trichloro-4-biphenylol and 2′,3′,4′,5′-tetrachloro-4-biphenylol, which show high affinity with estrogen receptors and have a synergistic effect [Bergeron et al., 1994]. Additionally, some compounds, such as aroclor 1242, p,p′-DDE, and chlordanes, induce sex reversal when applied individually to *T. scripta* eggs, whereas only chlordane is synergistic with estradiol and increases sex reversal when both are applied in combination [Willingham and Crews, 1999]. A similar result was observed in *T. scripta* incubated at male-producing temperatures with different doses and combinations of mixtures with *trans*-nonachlor, chlordane, and p,p′-DDE. Chlordane and p,p′-DDE in their lowest doses induced female-biased sex ratios, whereas higher doses had little effect [Willingham, 2004]. These discrepancies occur because compounds differ in their modes of action, affinity to different receptors, or the signaling pathways with which they interact [Willingham and Crews, 1999; Willingham, 2004]. However, p,p′-DDE by itself was unable to sex-reverse *C. ser-*

pentina embryos incubated at the male-producing temperature, either because the applied dose did not reach the embryo or because this chemical cannot bind to estrogen receptors [Portelli et al., 1999].

Females living in contaminated areas can potentially transfer EDCs to the eggs in a similar manner as they transfer hormones to the yolk, affecting the embryonic endocrine environment and the development and sex of their offspring. Thus, EDCs could reach the offspring via these maternal effects even though the offspring was never exposed to contaminants directly. For instance, a significant correlation in PCB levels was detected between mother and eggs of the snapping turtle, *C. serpentina* [Pagano et al., 1999]. Maternal transfer of contaminants was confirmed in the leatherback turtle, *Dermochelys coriacea*, where organochlorine compounds in female blood were correlated with levels detected in yolks, and the transfer decreased between clutches [Guirlet et al., 2010]. Therefore, studies looking at the effects of contaminants in reptile embryo development must take these maternal effects into account, since the environment or the mother may be the proximate source of contamination.

A second important type of EDCs has the ability of interfering with steroid metabolic pathways, such as bisphenol-A (BPA), a plastic softener broadly used and commonly found in the environment. BPA can disrupt estrogen metabolism by acting on sulfotransferase enzymes whose role is to decrease steroid binding to its receptor and reduce its hydrophobicity, thus lowering the concentration of estrogen in the tissue. Yolk of *T. scripta* eggs treated with BPA contained higher levels of estrogen and estrone (a sulfonation metabolite) but lower levels of sulfate estrone (the second metabolite). This indicates that BPA alters sulfonation by inhibiting the conversion of estrone to estrogen sulfate, increasing the availability of active steroids during embryogenesis [Clairardin et al., 2013; Paitz and Bowden, 2015], which could directly affect the development and sex determination of the embryo, independently of the temperature at which the eggs are exposed. Moreover, BPA can interact with the estrogen receptor (ER) as it is structurally similar to estrogen [Levy et al., 2004; Viñas et al., 2013]. ER transcription in *Xenopus laevis* tadpoles exposed to BPA increased compared to controls, revealing the uptake of BPA and its estrogenic effects [Levy et al., 2004]. Further, *C. picta* eggs incubated at male-producing temperatures and exposed to BPA at the beginning of the thermosensitive period produced individuals with ovary-like cortical tissue and disorganized testicular tubules, and an associat-

ed SOX9 signaling disruption proportional to the concentration of BPA [Jandegian et al., 2015].

A third category of EDCs are pesticides, chemical products used to eliminate or control unwanted organisms, such as plants, insects, and fungi. Exposure to these contaminants can cause diverse health effects from skin irritation to neurodegenerative effects. Moreover, reproductive function and other traits can be affected, causing birth defects and infertility [Collotta et al., 2013]. Atrazine, an EDC pesticide, is a herbicide widely used in agriculture and present in ground and surface water, such that most water sources in the United States contain higher levels than the effective doses determined in the laboratory [Hayes et al., 2002]. Atrazine is estrogenic but it does not seem to bind estrogen receptors. Instead, atrazine and its metabolites inhibit the activity of phosphodiesterase (PDE) enzymes, responsible to hydrolyze cyclic adenosine monophosphate (cAMP) to 5′-AMP, inducing aromatase expression [Roberge et al., 2004]. This induction can also be due to the interaction of atrazine with SF-1, leading to the activation of the aromatase promoter, ArPII [Fan et al., 2007b] and increasing the production of estrogens above normal levels in exposed males [Hayes et al., 2002]. This disruption by atrazine has 'demasculinizing' effects, reducing male gonadal characteristics, such as testicular size, number of Sertoli cells and sperm production [Hayes et al., 2011]. *C. serpentina* hatchlings whose eggs were exposed to atrazine in laboratory and in agricultural fields developed oocytes in the testis, demonstrating that atrazine can affect gonadal development even without causing complete sex reversal [De Solla et al., 2006]. *Alligator mississippiensis* embryos exposed to atrazine showed a tendency, albeit non-significant, to produce higher than normal levels of aromatase in the developing testis, suggesting that this chemical might alter embryonic steroidogenesis [Crain et al., 1997].

Exposure to atrazine affects sex differentiation (lower levels of testosterone, fertility, and number of germ cells compared to control males) and can cause sex reversal, directly impacting reproduction in male African clawed frogs, *X. laevis*, a GSD species [Hayes et al., 2010]. Furthermore, atrazine may lower the activity of 5α-reductase and affect dihydrotestosterone binding to its receptor in rats [Kniewald et al., 2000; Trentacoste et al., 2001]. However, further research is needed to fully elucidate the mode of action of this pesticide. Atrazine can also affect viviparous organisms, where the placenta may serve as a vehicle of exposure of EDCs since it transfers hormones to the embryo. Females of the viviparous lizard *Niveoscin-*

cus metallicus exposed to a single dose of atrazine during the initiation of sexual differentiation produced female offspring with delayed ovarian development and male offspring with demasculinized gonads likely due to the increased estrogen signaling and atrazine's ability to inhibit 5α-reductase [Parsley et al., 2015].

A fourth category of EDCs of increasing concern is nitrates in the environment, especially in freshwater habitats, estuaries, and agricultural lands. Although nitrates are naturally found in the environment as a results of atmospheric deposition, dilution of geological nitrogen deposits, and N_2 fixation by plants, anthropogenic actions have increased the deposit of inorganic nitrogen through animal farming, industrial, agricultural and urban wastes, and sewage effluents [Camargo et al., 2005]. Nitrates can not only be toxic but may also alter steroidogenic levels in some animals. For instance, testosterone levels in plasma of juvenile alligators correlate negatively with total nitrate concentration in their aquatic habitat, suggesting that some components could affect the level of androgen steroids [Guillette and Edwards, 2005]. The endocrine disrupting action of inorganic nitrates and nitrites may be due to their conversion in NO (nitric oxide) in steroidogenic tissues. NO has the ability to bind to the heme group of cytochrome P450 enzymes, important for different steroidogenic pathways [Hamlin et al., 2008]. Exposure of Siberian sturgeon, *Acipenser baeri*, to high concentrations of nitrate caused an increase of testosterone, 17β estradiol, and 11-ketotestosterone levels in plasma [Hamlin et al., 2008]. Gonadal development and morphology of *Rana pipiens* tadpoles treated with nitrate or atrazine was affected such that treated groups showed lower percentage of spermatocytes and a higher percentage of spermatids in testes. The feminizing effect of atrazine and nitrate was synergistic and not simply additive, suggesting that these 2 compounds combined may influence sex ratios in *R. pipiens* population [Orton et al., 2006].

A final group of EDCs that will be considered here is heavy metals whose toxicity and ability to accumulate through food webs has raised concern. Heavy metals are introduced in the environment via fuel combustion, atmospheric fallout from industrialization, waste dumping, accidental leaks, and industrial and domestic effluents [Al-Yousuf et al., 2000]. Metal ions are considered EDCs, because they interfere with estrogen action [Simoniello et al., 2010] and thyroid hormones secretion [Guirlet et al., 2008]. Heavy metals can be maternally transferred to eggs, as occurs for selenium and cadmium whose concentration in egg yolk correlates with their concentration in maternal blood in leatherback sea turtles [Guirlet et al., 2008]. However, maternal transfer of cadmium was not observed in *T. scripta* despite the accumulation in females [Nagle et al., 2001], indicating that maternal transfer can be species-specific. Cadmium exposure during early development can affect primordial germ cell migration, leading to lower fertility [Kitana and Callard, 2008]. Cadmium levels were higher in *C. picta* eggs from a contaminated site where oocyte apoptosis was also higher compared to a reference site [Kitana and Callard, 2008]. Also, topical applications of cadmium on *T. scripta* eggs decreased the number of germ cells and increased oocyte apoptosis compared to controls, suggesting that cadmium may interfere with the proliferation and migration of germ cells to the genital ridge, decreasing the number of viable oocytes during ovarian development [Kitana and Callard, 2008]. Cadmium mimics the oogonial proliferation and oocyte recruitment-stimulating effect of FSH (follicle-stimulating hormone) in the lizard *Podarcis sicula*, but these oocytes degenerate, ultimately reducing female reproductive output [Simoniello et al., 2010]. Cadmium, in addition to zinc, affects the stress-response mechanisms in the brown trout, *Salmo trutta*, stimulating the release of corticotropin (ACTH) and the proliferation of corticotropin-releasing hormone (CRH)-immunoreactive cells that are responsible for releasing the stress response hormone cortisol in the hypothalamus [Norris, 2000]. Therefore, individuals under chronical exposure of heavy metals suffer reduced fitness due to the cost imposed by the hypothalamus-pituitary-adrenocortical axis while maintaining homeostasis during the stress response [Norris, 2000].

Temperature and EDCs

Temperature and EDCs can have a synergistic effect on sex determination [Crews, 1996]. For instance, atrazine had a feminizing effect on *T. scripta* eggs incubated at male-producing and pivotal temperatures compared to controls [Willingham, 2005]. Furthermore, temperature can increase the capacity of contaminants to induce sex reversal. Indeed, exogenous estradiol applied in a lower dose to alligator eggs incubated at male-producing temperature induces a more extreme female bias than a higher dose. Similarly, p,p′-DDE failed to induce sex reversal at male-producing temperatures, whereas at intermediate temperatures the sex ratio was affected significantly [Milnes et al., 2005].

EDCs, Phenotypic Plasticity, and Evolution by Genetic Accommodation

What happens if EDCs become persistent and unavoidable environmental components? Phenotypic plasticity allows individuals and populations to live in changing habitats even though fitness may be reduced [Grether, 2014], thus making it possible for adaptation to take place and extinction to be avoided. Given enough time and a persistent environmental disturbance, the plastic phenotype may be produced even without exposure to the environmental factor through a process called genetic accommodation [Braendle and Flatt, 2006; Pigliucci et al., 2006; Pfennig et al., 2010]. For instance, if an organism lives in an area contaminated with EDCs, a change in gene frequency due to selection could theoretically evolutionarily accommodate the 'new' phenotype as a consequence of EDC exposure [Grether, 2014], rendering it a stable inherited character instead of a plastic character induced by environmental conditions [Pigliucci and Murren, 2003]. This evolutionary process would occur in steps: (1) *trait origin* where the environment induces a new phenotype, (2) *phenotypic accommodation* to the new trait via plasticity, (3) *initial spread* of the new phenotype as it is induced by the environment, and which could increase its frequency in the population, and, at last, (4) *genetic accommodation* in which selection drives the fixation of an allelic substitution that codes for the novel phenotype [Braendle and Flatt, 2006; Pigliucci et al., 2006]. Under this scenario, plasticity is reduced overtime as genetic evolution proceeds to maintain the optimal phenotype in the new environment [Lande, 2009]. This way, a population could theoretically adapt to live in contaminated areas with a new optimized fitness [Grether, 2014], perhaps even undergoing diversification triggered by genetic accommodation which could produce genetic differences between populations induced by the environmental shift [Pfennig et al., 2010]. Of the many ways to increase fitness in an EDC-contaminated environment, one is the evolution of resistance to contaminant chemicals, as would be the case if hormone receptors evolved higher specificity for their ligands, narrowing their ability to bind only to endogenous hormones instead of binding structurally similar molecules [Grether, 2014]. Whether genetic accommodation will facilitate phenotypic diversification of populations living in disturbed habitats [Braendle and Flatt, 2006] promoting adaptive radiation or allowing the colonization of novel environments [Pfennig et al., 2010] remains uncertain.

Conclusion

Advancing our knowledge about ecotoxicological aspects of sex determination is imperative, as the world is undergoing dramatic environmental changes that can be detrimental to species in the full spectrum of sex-determining systems, including humans. Reptiles have emerged as good models to study the proximate mechanisms and evolutionary forces behind sex determination, and they also serve as environmental sentinels to warn about the effects of environmental pollutants that can derail sexual development. While adaptation to such contaminants may be plausible, the rapid pace of some environmental changes today greatly exceeds the pace of adaptive evolution, particularly for species that are already endangered and have small population sizes. Thus, further research is warranted and is accelerating, thanks to the development of genomic technologies that enable the collection of high throughput data and comparative approaches that contrast diverse species with alternative mechanisms. Such continued and comprehensive efforts will help us decipher the mysteries of sex determination.

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