Sexual Development

Review Article

Sex Dev
DOI: 10.1159/000526009

Received: February 1, 2022
Accepted: July 8, 2022

Sex Determination and Ovarian Development in Reptiles and Amphibians: From Genetic Pathways to Environmental Influences

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Keywords
Contaminants · Gonad · Temperature-dependent sex determination · Ovary · Differentiation

Abstract

Background: Reptiles and amphibians provide untapped potential for discovering how a diversity of genetic pathways and environmental conditions are incorporated into developmental processes that can lead to similar functional outcomes. These groups display a multitude of reproductive strategies, and whereas many attributes are conserved within groups and even across vertebrates, several aspects of sexual development show considerable variation. ***Summary:*** In this review, we focus our attention on the development of the reptilian and amphibian ovary. First, we review and describe the events leading to ovarian development, including sex determination and ovarian maturation, through a comparative lens. We then describe how these events are influenced by environmental factors, focusing on temperature and exposure to anthropogenic chemicals. Lastly, we identify critical knowledge gaps and future research directions that will be crucial to moving forward in our understanding of ovarian development and the influences of the environment in reptiles and amphibians. ***Key Messages:*** Reptiles and amphibians provide excellent models for understanding the diversity of sex determination strategies and reproductive development. However, a greater understanding of the basic biology of these systems is necessary for deciphering the adaptive and potentially disruptive implications of embryo-by-environment interactions in a rapidly changing world.

Introduction

The environment experienced by an organism during development can have profound organizational and ecological effects, some of which persist throughout life [West-Eberhard, 1989; Miner et al., 2005]. This ability to alter phenotype in response to environmental cues (i.e., phenotypic plasticity) can be adaptive in several contexts, but also leaves organisms sensitive and vulnerable to changing or altered environmental conditions [DeWitt et al., 1998]. Reptiles and amphibians display a broad range of reproductive strategies, spanning oviparous to viviparous, lecithotrophic to matrotrophic, and environmental

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to genotypic sex determination [Thompson and Speake, 2002; Bachtrog et al., 2014; Blackburn, 2015]. Relative to other vertebrate groups, this diversity is unparalleled and provides fertile ground for comparative developmental studies. Yet, the potential for understanding how environmental cues are incorporated into diverse developmental programs in adaptive and disruptive contexts remains relatively untapped in these groups. Arguably, one of the most important developmental processes for adult reproductive fitness is the proper organization of the germ cell-containing gonad. The decision to develop a testis or ovary requires precise coordination of genetic and endocrine signaling pathways in response to intrinsic and extrinsic cues [Capel, 2017], and sexual development in reptiles and amphibians provides unique opportunities for disentangling how divergent pathways arising from conserved genes can lead to similar functional outcomes under varied environments. However, understanding this diversity from both proximate and ultimate perspectives requires extensive knowledge of the basic biological events that occur within an organism in an adaptive context, which is lagging in these groups, especially relative to mammals.

Basic Comparative Development

Mechanisms of Sex Determination

Ovaries and testes emerge during development from common bipotential primordia. A remarkable diversity of mechanisms exists to initially steer the bipotential gonad toward the ovarian versus testicular fate, in a process known as primary sex determination. Reptiles and amphibians uniquely exemplify this diversity with every major sex-determining mechanism (SDM) represented in these groups, including female heterogametic (ZW) sex chromosomes (e.g., majority of snakes [Matsubara et al., 2006], African clawed frog [Yoshimoto et al., 2010]), male heterogametic (XY) sex chromosomes (e.g., some lizards [Gamble et al., 2014, 2015], boas and pythons [Gamble et al., 2017]), polygenic sex determination (e.g., some amphibians [Nakamura, 2009; Miura, 2017; Ruiz-Garciá et al., 2021]), and environmental sex determination (e.g., some squamates [Charnier, 1966; Holleley et al., 2015], many turtles [Bull, 1980], all crocodilians [Lang and Andrews, 1994], and tuatara [Mitchell et al., 2006]) [reviewed extensively elsewhere; for example, see Valenzuela and Lance, 2004; Bachtrog et al., 2014; Capel, 2017]. The distribution of different SDMs across the phylogeny of reptiles and amphibians suggests that evolutionary transitions between SDMs occur frequently and sometimes rapidly [Janzen and Phillips, 2006; Pokorná and Kratochvíl, 2009; Bachtrog et al., 2014; Jeffries et al., 2018]. For example, a study of the SDMs of 12 gecko species found evidence for 17–25 transitions between XX/XY, ZZ/ZW, and temperature-dependent sex determination (TSD) systems [Gamble et al., 2015]. Transitions between sex determination systems also occur within species, as is shown in the frog species *Rana rugosa* which exhibits different SDMs (XX/XY and ZZ/ZW) across different populations in Japan [Miura, 2007]. Thus, this group provides opportunities to gain novel insights into variation in the transcriptional networks and cellular processes underlying early ovarian development as well as the evolutionary forces shaping this variation.

The lens through which mechanisms of sex determination in reptiles and amphibians are examined has largely been shaped by studies of the mammalian XY and avian ZW systems. Both of these SDMs are evolutionarily stable, highly canalized, and under the control of a single "master" sex-determining locus (e.g., *SRY* in mammals and *DMRT1* in birds) [Sinclair et al., 1990; Koopman et al., 1991; Smith et al., 2009; Graves, 2016; Capel, 2017]. This contributed to the idea that vertebrate sex determination broadly invokes the same transcriptional hierarchy, and variation in SDMs across species is limited to the initial upstream "switch" operating at the top of this hierarchy [McLaren, 1988; Wilkins, 1995; Crews and Bull, 2009]. However, subsequent work in reptiles and amphibians is revealing a much more complex picture [Shoemaker and Crews, 2009; Czerwinski et al., 2016; Yatsu et al., 2016; Deveson et al., 2017]. In fact, sex determination often involves nuanced interactions between multiple loci and environmental signals in these taxa [Barske and Capel, 2008; Pen et al., 2010; Bachtrog et al., 2014; Holleley et al., 2015; Deveson et al., 2017; Lambert et al., 2018, 2019]. Variation exists at multiple points in the transcriptional networks underlying sexual fate commitment and gonadal differentiation, and the key unifying property of these networks is not their hierarchical nature, but rather a robust mutual antagonism (i.e., genes promoting ovarian fate simultaneously inhibit those promoting testis fate, and vice versa) which permits network flexibility [Crews and Bull, 2009; Herpin and Schartl, 2015; Capel, 2017; Adolfi et al., 2021].

Despite the profound differences in SDMs between mammals, birds, reptiles, and amphibians, many of the same genes or gene families have been recruited to serve in the transcriptional networks underlying sex determination across taxa, albeit in varying configurations. Doublesex and mab-3 (DM) domain genes, named for their association with sex determination in *Drosophila melanogaster* and *Caenorhabditis elegans* and characterized by a zinc-finger DNA-binding motif, are perhaps the best examples of this phenomenon [Matson and Zarkower, 2012]. A paralogue of the DM-domain gene *DMRT1*, which determines sex in birds through a Z-linked dosage-sensitive mechanism [Smith et al., 2009], resides on the W-chromosome in the African clawed frog (*Xenopus laevis*) and serves as the decisive regulatory signal in this species directing gonadal development toward the ovarian fate [Yoshimoto et al., 2008]. *X. laevis* also possesses an autosomal copy of *DMRT1*, which directs testis development in the absence of the W-linked copy (DM-W). The DM-W locus encodes a truncated protein lacking key functional domains and is thought to antagonize the masculinizing function of the autosomal copy of *DMRT1* through an underlying dominant negative mechanism [Okada et al., 2009; Matson and Zarkower, 2012]. In the red-eared slider turtle (*Trachemys scripta*), a species relying solely on temperature to determine gonadal fate, *DMRT1* expression responds to incubation temperature early in the thermosensitive period and is necessary for testis development. Interruption of *DMRT1* expression in *T. scripta* embryos at male-promoting temperatures results in the bipotential gonad proceeding towards the ovarian fate [Ge et al., 2017]. Even among mammals, including humans and mice, *DMRT1* serves a critical role in promoting testis differentiation and maintaining testicular fate into adulthood through its antagonism of the ovarian-specific transcription factor forkhead box L2 (*FOXL2*) [Raymond et al., 2000; Krentz et al., 2009; Matson et al., 2011; Capel, 2017].

Many of the genes that show evidence of conserved upstream roles in sex determination across vertebrates are involved in promoting testis fate (e.g., DM-domain genes, Sox genes, *AMH*), and in their absence ovarian development proceeds [Herpin and Schartl, 2015]. There are currently no well-substantiated explanations for this pattern, but what is clear is that development of an ovary requires robust repression of the transcriptional networks promoting testis fate, and vice versa. The interaction of DM-domain genes and Sox genes with *FOXL2* and Wnt signaling (particularly the WNT4/R-spondin 1/β-catenin pathway) typifies this mutual antagonism. During mammalian sex determination, *WNT4* and *RSPO1* (R-spondin 1) are expressed in the somatic progenitors of the gonad (which will eventually take on the fate of either granulosa cells or Sertoli cells) and trigger stabilization and translocation of cytoplasmic β-catenin into the nucleus [Rotgers et al., 2018]. Following this initiation of the WNT4/R-spondin 1/β-catenin pathway, expression of *FOXL2* is upregulated leading somatic progenitors to take on the fate of granulosa cells and ultimately promoting ovarian differentiation [Rotgers et al., 2018]. Interruption of *Wnt4, Rspo1,* or *Ctnnb1* (β-catenin) function in mice leads to upregulation of *Sox9* and partial ovarian to testicular fate reversal [Vainio et al., 1999; Jeays-Ward et al., 2003; Chassot et al., 2008; Rotgers et al., 2018]. Further, deletion of *Foxl2* in adult mouse ovaries leads to upregulation of *Dmrt1* and *Sox9* and transdifferentiation of the ovary to testicular morphology, implicating *Foxl2* in the active maintenance of ovarian fate into adulthood (similar to the previously discussed role of *Dmrt1* in testis maintenance) [Uhlenhaut et al., 2009].

The role of *FOXL2* and the WNT4/R-spondin 1/β-catenin pathway in promoting ovarian development and antagonizing the masculinizing actions of *DMRT1* and *SOX9* appears to be at least partially conserved among reptiles and amphibians. In several reptiles with TSD, including *T. scripta* and *Chelydra serpentina* [Rhen et al., 2007, 2021], *RSPO1* and *FOXL2* exhibit upregulation at female-promoting temperatures during the thermosensitive period. Further, in *T. scripta*, ectopic activation of canonical Wnt signaling at male-promoting temperatures results in partial sex reversal of the gonadal medulla, however inhibition of Wnt signaling does not sex-reverse differentiating ovaries suggesting this signaling pathway is not necessary for ovarian development in this species [Mork and Capel, 2013]. In the American alligator (*Alligator mississippiensis*), another species with TSD, *RSPO1* does not exhibit differential expression during the thermosensitive period, though both *WNT4* and *FOXL2* are upregulated at female-promoting temperatures [Yatsu et al., 2016; Rice et al., 2017]. In 6 anuran species (*X. laevis, Bombina bombina*, *Bufo viridis*, *Hyla arborea*, *Rana arvalis*, and *Rana temporaria*) with genetic sex determination (GSD), *RSPO1* expression is upregulated in females during sex determination [Piprek et al., 2013]. In the common Indian garden lizard (*Calotes versicolor*), a species with GSD but for which the mechanism is poorly understood, both *FOXL2* and *RSPO1* are expressed in the developing ovary, and proteins appear to be localized in pre-granulosa cells [Priyanka et al., 2018]. The transcriptional networks underlying sex determination in reptiles and amphibians appear to rely on genes from conserved gene families with known roles in vertebrate sex determination, particularly through the antagonistic actions of testis-promoting DM-domain genes/Sox genes and ovary-promoting FOXL2 and the WNT4/R-spondin 1/

β-catenin pathway. It should be noted, however, that our view remains limited. There is substantial evidence that genes with conserved roles in sex determination do not necessarily follow the same temporal trajectory during development in these taxa as they do in the well-characterized systems of model organisms (i.e., SOX9 expression in mammals vs. reptiles with TSD) [Western et al., 1999]. This suggests that as we increasingly move away from candidate-gene approaches and instead apply more unbiased sequencing approaches to examine the reproductive development of reptile and amphibian species, we are likely to uncover a more varied and nuanced perspective on the diversity of vertebrate sex determination.

Dual Functions of Transcriptional Pathways in Sex Determination and Gonadal Differentiation

The connections between variation in transcriptional networks underlying sex determination and variation in processes governing the morphological differentiation of the ovary and testis across species are far from clear, especially in reptiles and amphibians. One complication that clouds understanding of these connections is the fact that many of the same genes that have been co-opted to serve as regulators of sex determination have dual functions in the morphological differentiation and/or gonadal function of both sexes. Further, the roles of these genes in morphological differentiation are not necessarily dependent upon their roles in sex determination. For example, *SOX9* does not appear to play an upstream role in sex determination in *Xenopus tropicalis*, as it does in mammals, but *SOX9* does appear to serve important roles in gonadal function. In this species, *SOX9* is upregulated after gonadal differentiation in both sexes, however in the testis, *SOX9* is restricted to the nuclei of Sertoli cells, while in the ovary, *SOX9* is first localized in the nuclei of previtellogenic oocytes and then later is localized to vitellogenic oocytes [el Jamil et al., 2008; Vining et al., 2021]. In *X. laevis*, *DMRT1* contains 2 promoters, one of which controls germ cell numbers in both sexes, and when knocked down, leads to female-to-male sex reversal in a subset of frogs [Mawaribuchi et al., 2017]. Further, closely related species with very little variation in adult ovarian and testicular morphology/function can rely on vastly different systems of sex determination (e.g., clades of turtles with both TSD and GSD species) [Valenzuela and Lance, 2004]. Few studies in reptiles and amphibians have simultaneously characterized the time-course of transcriptional and cellular events during sex determination [Yao and Capel, 2005], yet research of this nature is likely to yield critical insights into ways in which transcriptional variation during sex determination may relate to variation in cellular processes underlying gonadogenesis and differentiation. For example, the somatic precursors to granulosa or Sertoli cells in the bipotential gonad are largely considered to be the site of primary sex determination in many vertebrates, yet the role of germ cells in this process has been relatively neglected, though germ cell-specific genes have been implicated in contributing to sex determination in some species, such as the TSD species, *Mauremys mutica* [Liu et al., 2021]. How might reptiles and amphibians differ in the site of primary sex determination within the bipotential gonad, and how might this influence the behavior of different cell populations during gonadogenesis? Are there patterns of cellular organization or gonadal structure that link species relying on similar SDMs? These and many other related questions await further inquiry.

The Role of Estrogen Signaling in the Developing Ovary

Development of the ovary in reptiles and amphibians largely appears to converge on the production of estrogen, which generally serves as a conserved readout for commitment to the ovarian fate. One of the earliest pieces of evidence for this came from the demonstration of reptile and amphibian embryos' sensitivity to the influence of exogenous estrogen exposure [Bull et al., 1988; Hayes, 1998]. Reptiles with TSD provide some of the clearest examples of this sensitivity. Early experiments in *T. scripta*, *A. mississippiensis*, *C. serpentina, Eublepharis macularius,* and *Trionyx spiniferus* revealed that embryos incubated at male-promoting temperatures and exposed to exogenous 17β-estradiol (E2) during the thermosensitive period developed ovaries [Bull et al., 1988; Crews et al., 1989; Wibbels et al., 1991; Rhen and Lang, 1994; Kohno et al., 2015]. These findings were followed by the discovery that expression of the gene *CYP19A1* which encodes aromatase, the enzyme that converts testosterone to estrogen, is upregulated at female-promoting temperatures in these species, though the timing of this upregulation varies. In *C. serpentina*, *CYP19A1* exhibits delayed upregulation in response to female-promoting temperatures suggesting the influence of estrogen may be limited to ovarian differentiation rather than initial sex determination [Rhen et al., 2007; Rhen and Schroeder, 2010]. Similarly, *CYP19A1* expression and aromatase activity is not upregulated until late in the thermosensitive period (stage 23–24) in *A. mississippiensis* [Milnes et al., 2002; Parrott et al., 2014]. In *T. scripta*, *CYP19A1* expression is upregulated in the middle of the thermosensitive period (stage 18), though in some studies its expression appears to precede that of *FOXL2* [Ramsey et al., 2007; Shoemaker et al., 2007; Bieser and Wibbels, 2014; Czerwinski et al., 2016]. Given this evidence, it remains unclear whether aromatase and, in turn estrogen production, plays an upstream role in sex determination in some reptile and amphibian species initiating the ovarian transcriptional network, or if it is primarily involved in canalizing ovarian fate and differentiation.

Estrogen is clearly a powerful suppressor of the testicular transcriptional network [Barske and Capel, 2010], yet *CYP19A1* expression tends to lag temporally in its upregulation at female-promoting temperatures relative to the upregulation of testis-promoting genes such as *DMRT1* at male-promoting temperatures during TSD [Czerwinski et al., 2016; Ge et al., 2017]. Further, in *T. scripta,* if *DMRT1* expression is interrupted at male-promoting temperatures, *CYP19A1* expression increases and ovarian development proceeds [Ge et al., 2017, 2018], yet it remains unclear what upregulates *CYP19A1* expression in the absence of a female-promoting temperature cue. Clearly, there is much more to be learned about the role of estrogen in regulating sex determination and ovarian differentiation in reptiles and amphibians. As it relates to the structure and function of the ovary, sex determination systems in reptiles and amphibians seem to be divergent means to a similar end, though subtle species differences in ovarian development and cellular architecture undoubtedly harbor interesting comparative insights.

Differentiation and Maturation of the Ovary

After primary sex is determined, many of the morphological aspects of reptilian and amphibian ovarian development are broadly conserved across groups. Similar to mammals, the ovary differentiates into 2 parts: a cortex and medulla. The cortex contains actively proliferating germ cells that become organized into nests that are connected by intercellular bridges, while the medulla contains dense connective tissue that regresses during maturation. Nests of germ cells proliferate in the cortex until they enter meiosis, becoming oocytes. Oocytes in the diplotene stage become surrounded by somatic (pre-granulosa) cells, break from nests, and become fully enveloped by granulosa cells, followed by the theca layer. The resulting follicles continue to enlarge and mature, bulging into the medulla and becoming vacuolated. Vitellogenesis proceeds as the follicles fill with yolk platelets prior to ovulation and await fertilization. Several papers have described these events in detail for specific species in each taxon: Crocodilia [Uribe and Guillette, 2000; Calderón et al., 2004; Moore et al., 2008, 2010a, c], Testudines [Callebaut et al., 1997; Hei et al., 2010; Pérez-Bermúdez et al., 2012], Squamata [Doddamani, 1994; Hernández-Franyutti et al., 2005; Vieira et al., 2010; Aldokhi et al., 2019; Delssin et al., 2019], Anura [Dumont, 1972; Ogielska and Kotusz, 2004; Piprek et al., 2017], and Caudata [Chardard and Dournon, 1999; Chardard et al., 2003; Mendoza-Cruz et al., 2017]. For the purposes of this review, the remainder of this section will focus primarily on the major differences that exist across groups and important gaps that must be addressed to gain a comprehensive understanding of ovarian development in reptiles and amphibians.

Histological differences in both the cortex and medulla are evident when looking comparatively across groups. For example, unlike reptiles in which the entire ovary is a single unit, anuran and caudate ovaries contain several sacs, each of which consists of a cortex and medulla [Uribe, 2003; Ogielska and Kotusz, 2004]. While the presence of germinal nests in the cortex is ubiquitous across groups, the number of nests varies substantially. Crocodilians, turtles, and frogs generally have several [Callebaut et al., 1997; Uribe and Guillette, 2000; Ogielska and Kotusz, 2004; Moore et al., 2008; Pérez-Bermúdez et al., 2012], while most squamates have 1 or 2, but as many as 6 nests [Jones et al., 1982; Aldokhi et al., 2019]. It has been hypothesized that germinal nest number is related to clutch size and frequency of reproduction [Guraya, 1989]. This was examined across several lizard species, in which Radder et al. [2008a] found a significant relationship between germinal bed number and clutch size, but not reproductive mode, brood frequency, or number of clutches per year. Whether variation exists in other groups is unknown, but additional comparative studies have the potential to further link aspects of ovarian development (e.g., germinal nest number) to variation in reproductive ecology and life history.

The ovarian medulla in crocodilians and turtles develops a system of large lacunae that continually increase in size during ovarian maturation [Callebaut et al., 1997; Uribe and Guillette, 2000; Moore et al., 2010c; Pérez-Bermúdez et al., 2012]. Anurans and caudates also develop a similar central cavity within each ovarian sac, termed a lumen [Uribe, 2003; Ogielska and Kotusz, 2004], while squamates lack such a structure [Aldokhi et al., 2019; Delssin et al., 2019]. Ovarian lacunae are also evident in birds, although their size and number vary both within and across taxonomic groups [Uribe and Guillette, 2000; Hei et al., 2010; Pérez-Bermúdez et al., 2012]. Their function is unknown, but proposed hypotheses include allowing space for follicular growth, providing a supportive structure for follicles, and nourishing growing oocytes [Pérez-Bermúdez et al., 2012]. More work is needed to understand the consequences and function of ovarian lacunae and why these structures are present in some but not all groups.

Other intriguing, histological differences also exist during later stage follicular maturation. For example, in crocodilians [Uribe and Guillette, 2000; Moore et al., 2008], testudines [Callebaut et al., 1997; Hei et al., 2010; Pérez-Bermúdez et al., 2012], anurans [Dumont, 1972], and caudates [Uribe, 2009] a single or few layers of homogenous granulosa cells surround the oocyte and remain relatively unchanged throughout, whereas lizards [Delssin et al., 2019; Aldokhi et al., 2019] and snakes [Tumkiratiwong et al., 2012] begin with a single layer of cells termed small cells that develop into pyriform cells, with an intermediate cell stage, giving the appearance of 3 layers that eventually regress back to a single layer. In squamates, intercellular bridges between small cells and the growing oocyte have been observed that are believed to play a nutritive role in nourishing the oocyte [Andreuiccetti, 1992]. Such bridges have not been observed in other groups, but abundant microvilli reaching from follicular cells to the oocyte suggest material transfer also occurs in turtles [Hei et al., 2010; Pérez-Bermúdez et al., 2012], anurans [Dumont, 1972; Konduktorova and Luchinskaya, 2013], and caudates [Uribe, 2003].

At the onset of vitellogenesis, yolk deposition occurs within the oocyte and the animal and vegetal poles become distinguishable. Details have been described in crocodilians [Uribe and Guillette, 2000; Calderón et al., 2004], turtles [Callebaut et al., 1997; Hei et al., 2010], anurans [Dumont, 1972], and caudates [Uribe, 2003]. Generally, vacuoles appear at the periphery of the oocyte and yolk spheres accumulate, mostly within the vacuoles. As yolk deposition continues, yolk platelets form and distribute nonrandomly within the oocyte, appearing smaller at the periphery and center and larger in the intermediary region. In crocodilians [Uribe and Guillette, 2000; Calderón et al., 2004] and turtles [Hei et al., 2010], yolk deposition has been compared to birds, in which it occurs in 3 successive stages (primordial, intermediate, and late yolk). However, it appears that differences exist even within groups and no such comparisons have been made in amphibians or squamates. Unsurprisingly, in matrotrophic-like squamates harboring varying degrees of placental development, yolk deposition is reduced, with fewer yolk droplets [Hernández-Franyutti et al., 2005; Vieira et al., 2010].

A defining feature of the ovary in vertebrates is the early transition of germ cells from a mitotic to meiotic state as opposed to at sexual maturation in testes. Interestingly, the details of this transition in reptiles and amphibians remain largely unknown. The timing of oogonia-to-oocyte transition appears to be species-specific in squamates, where it can be initiated either during embryonic development [Doddamani, 1994] or early in juvenile life [Antonio-Rubio et al., 2015; Delssin et al., 2019]. On the other hand, turtles and crocodilians show early-stage oocytes at birth [Moore et al., 2008; Rhen et al., 2015], suggesting it begins during late embryonic stages. In anurans, the oogonia-to-oocyte transition has been observed during hindfoot development (Gosner stages 36–40) and during late larval development [Ogielska and Kotusz, 2004; Wallacides et al., 2009]. However, as this process has only been studied in a few species, drawing overarching conclusions with respect to taxonomic diversity is difficult. Further, the proximate mechanisms underlying the mitotic-to-meiotic transition in reptiles and amphibians are largely unknown. The current consensus in mammals is that retinoic acid signaling from somatic cells upregulates *STRA8* expression, leading to meiotic entry [Bowles et al., 2006; Wang et al., 2017]. This idea was recently challenged by Vernet et al. [2020] who showed that *STRA8* expression is reduced in mice lacking retinoic acid receptors, but meiosis progresses normally, suggesting other factors also play a role. Nonetheless, the role of retinoic acid is conserved in chicken and fish [Smith et al., 2008; Feng et al., 2015; Li et al., 2016], with both *STRA8*-dependent and -independent pathways existing in the latter [Feng et al., 2015]. As for reptiles and amphibians, the role of retinoic acid has only been examined in the salamander *Pleurodeles waltl* in which exogenous retinoic acid triggers meiotic transition [Wallacides et al., 2009]. While this indicates that retinoic acid performs a conserved role in the mitotic-to-meiotic transition across vertebrates, a time-series comparison examining retinoic acid, *STRA8* expression, and germ cell behavior during embryonic and early post-natal life across several species would be an intriguing new direction of inquiry into this critical aspect of ovarian development.

Unlike mammals, in which all oogonia enter meiosis early in life, in most reptiles and amphibians, the adult ovary retains nests of mitotic oogonia which can serve as an active supply of new oocytes. This includes crocodilians, turtles, squamates, and caudates [Callebaut et al., 1997; Uribe and Guillette, 2000; Uribe, 2009; Hei et al., 2010; Aldokhi et al., 2019]. This unique feature opens interesting questions about reproductive senescence in these groups, since it appears the oocyte pool has the potential to evade depletion. How new oogonia are selected to enter meiosis throughout life is unknown, as studies specifically examining this aspect of germ cells in adult animals are nonexistent. Interestingly, anurans are the exception and, like mammals, form a definitive pool of diplotene oocytes during the juvenile stages, a subset of which are recruited each breeding cycle [Callen et al., 1986; Ogielska et al., 2013]. Primary oogonia become restricted to germ patches after the juvenile period and no longer contribute to the pool of oocytes, degenerating thereafter [Ogielska et al., 2013]. Once again, due to the small number of species examined, it is difficult to say if this is a defining feature of anurans or if variation exists within groups, which would raise interesting evolutionary questions about the costs and benefits of a regenerating oocyte pool.

Another important aspect of ovarian maturation, the recruitment of primary follicles from the primordial follicle pool of oocytes, is an area of active research in mammals and also almost completely unknown in reptiles and amphibians. After entering meiosis, oocytes break from nests and enter a quiescent state until they are recruited as primary follicles. This involves complex interactions between many factors, including several members of the TGF-β superfamily, such as *AMH*, inhibins, activins, BMPs, and GDFs. Excellent reviews have been published on this process in mammals [Findlay et al., 2002; Skinner, 2005; Trombly et al., 2009; Wang et al., 2017], and it appears a multitude of coordinated interactions are required for proper nest breakdown and follicle recruitment. Very few data exist in reptiles and none in amphibians. In *A. mississippiensis*, activin, follistatin, and aromatase follow similar expression profiles in ovaries during the first 5 months of life, starting with elevated expression during early post-natal life as germ cells leave nests and form primary follicles. This is followed by a decrease in expression coinciding with later-stage follicle formation [Moore et al., 2008, 2010a]. In contrast, *GDF9* and *PCNA* expression increases during this time and correlates with follicle maturation and somatic cell proliferation [Moore et al., 2010a]. While these patterns are largely consistent with what is observed in mammals, in which proper ratios of activin and estrogen are required for normal follicle formation [Trombly et al., 2009], only a few of the key signaling components have been examined in a single species and our understanding of the molecular mechanisms driving such processes are far from resolved. Once primary follicles are formed, in several reptile species, administration of mammalian FSH leads to increased growth and maturation in sexually immature animals [e.g., Jones et al., 1975; Moore et al., 2012a; Hale et al., 2019], again suggesting conserved mechanisms of late-stage follicle maturation. Early work showed that the granulosa layer is likely responsible for ovarian responses to gonadotropins, as isolated granulosa, but not thecal cells, produced progesterone under FSH stimulation [Crews and Licht, 1975]. However, the technique used to separate cells in the latter study prevented complete separation of theca and granulosa cell layers. Future work should focus on linking molecular mechanisms to the histological events characterizing reptilian and amphibian folliculogenesis, including the role of different cell types in this process.

Environmental Influences on the Development of the Ovary

Temperature and Climate Influence Sex Determination and Ovarian Development

In the context of ongoing shifts in global thermal regimes, an understanding of the intricate connections between temperature, ovarian development, and reproductive performance in reptiles and amphibians will provide critical information toward efforts to predict and potentially mitigate adverse population-level consequences of rapid environmental change [Janzen, 1994; Benard, 2015; Jensen et al., 2018; Bock et al., 2020a]. In addition, interactions between thermal cues and reproductive development in reptiles and amphibians provide models in which to investigate how environmental variation is integrated into the biology of the ovary in adaptive contexts. Temperature can influence the ovarian function of reptiles and amphibians across the entire ontogeny of an organism – from the initial decision to develop an ovary during sex determination in embryos [Bull, 1980; Capel, 2017] to the initiation of folliculogenesis and oogenesis during the breeding season in adults [Marion, 1970; Licht, 1973; James and Shine, 1985; Lance, 1989; Sarkar et al., 1996; Pancharatna and Patil, 1997]. The molecular mechanisms by which thermal cues are translated into biological responses which direct ovarian determination, maturation, and function remain poorly understood, however emerging evidence, particularly from studies of species with TSD, continues to shed light on these fundamental processes [McCoy et al., 2016; Carter et al., 2018; Ge et al., 2018; Bock et al., 2020b; Weber et al., 2020].

Thermal plasticity of reptile and amphibian sex determination exists along a continuum between TSD systems, in which temperature cues dictate whether to initiate ovarian or testis development, and GSD systems, in which genetic signals are the sole determinant of gonadal fate [Sarre et al., 2004; Barske and Capel, 2008]. Growing evidence suggests many species likely fall somewhere in between these two extremes, with gene-by-environment interactions shaping the developmental trajectory of the bipotential gonad [Radder et al., 2008b; Pen et al., 2010; Mork et al., 2014; Holleley et al., 2015, 2016]. For example, in TSD species, it is possible that cryptic genetic influences may be present that bias sex determination towards a male or female fate. When gonads from embryos incubated at a pivotal temperature (producing both sexes) in red-eared slider are removed and cultured separately at the pivotal temperature, matched gonads show a predisposition towards the same sexual fate [Mork et al., 2014]. Additionally, sex ratios from incubation at identical temperatures vary across clutches and populations along latitudinal clines, suggesting factors other than just temperature contribute to sex determination in some species [Rhen and Lang, 1998; Ewert et al., 2005]. However, whether these results are due to genetic variation, cryptic GSD influences, or other maternal effects such as yolk steroid hormones [Bowden et al., 2000] requires further inquiry. On the other hand, temperature can exert major influences on GSD systems. In the central bearded dragon, *Pogona vitticeps*, sex is generally determined by sex micro-chromosomes in a ZZ/ZW system [Ezaz et al., 2005], however incubation temperatures above ~32°C can override this genetic system to produce phenotypic females with a ZZ chromosomal complement [Quinn et al., 2007]. Sex-reversed ZZ females possess functional ovaries with similar transcriptional profiles to those of normal ZW females [Deveson et al., 2017] and can reproduce with ZZ males to produce offspring whose sex is determined solely by temperature [Holleley et al., 2015]. Accordingly, high temperature-induced sex reversal can trigger a rapid transition from GSD to TSD, and this has been demonstrated in wild populations of *P. vitticeps* [Holleley et al., 2015, 2016]. Temperature-induced sex reversal also occurs in the scincid lizard, *Bassiana duperreyi*, though in this case, low temperatures override an XX/XY system [Radder et al., 2008b]. Intriguingly, there is even evidence for gene-by-environment interactions driving offspring sex in a live-bearing lizard, the snow skink (*Niveoscincus ocellatus*), which inhabits an elevational gradient conferring vastly different microclimates [Pen et al., 2010]. In highland populations of *N. ocellatus*, offspring sex is determined by a genetic system, while in lowland populations offspring sex ratio depends on maternal gestation temperature [Wapstra et al., 2004; Pen et al., 2010]. This population variation in sex determination system is hypothesized to have arisen via evolution of the threshold for temperature-induced sex reversal resulting from climate-related selective pressures [Pen et al., 2010]. The specific mechanisms by which temperature interacts with genotype to direct the sexual trajectory of an embryo in these species remain largely unresolved, however increased application of high-throughput sequencing approaches in these systems [Deveson et al., 2017; Whiteley et al., 2021] points to unique discoveries on the horizon.

Evidence for temperature-induced sex reversal has also been found in certain amphibian species including 2 newts (*Pleurodeles poireti* and *P. waltl*) [Dournon et al., 1990], wood frog (*Rana sylvatica*) [Lambert et al., 2018], and green frog (*Rana clamitans*) [Lambert et al., 2019]. When exposed to different temperature treatments between 19°C and 34°C, *R. sylvatica* tadpoles exhibited increasingly male-biased sex ratios at higher temperatures, with only males produced at the highest temperature treatments [Lambert et al., 2018]. Interestingly, offspring sex ratio exhibited a linear relationship with rearing temperature in this species [Lambert et al., 2018]. This pattern stands in sharp contrast to the sigmoidal pattern of most reptile temperature-by-sex ratio reaction norms in which mixed sex ratios only tend to result from a narrow range of temperatures (termed the transitional range of temperatures) [Valenzuela and Lance, 2004]. Such differences in temperature-by-sex ratio reaction norms between reptiles and amphibians raise the possibility that fundamentally different mechanisms mediate temperature influences on sexual development in these taxa. The linear thermal reaction norm of *R. sylvatica* resembles the reaction norms of several fish species with temperature-induced sex reversal, some of which have implicated germ cell-related mechanisms in mediating temperature effects on sex ratio [Slanchev et al., 2005; Ospina-Alvarez and Piferrer, 2008; Siegfried and Nüsslein-Volhard, 2008; Nakamura et al., 2012; Adolfi et al., 2019]. Further experiments are needed to understand the mechanisms underlying amphibian temperature-induced sex reversal and their relationship to variation in thermal reaction norms across species.

Among reptiles with TSD, comparisons of transcriptional, post-transcriptional, and epigenetic patterns across species have shed light on the underlying mechanisms by which temperature is translated into a sex-determining signal during development. Current models suggest chromatin modifiers and epigenetic modifications function at the interface between ancient cellular thermo-sensors that translate temperature into cellular signals and conserved transcriptional networks regulating sex determination [Weber and Capel, 2021]. This includes interactions between calcium ion flux, redox status, and epigenetic mechanisms regulating gene expression [Castelli et al., 2020]. Two genes encoding chromatin modifiers in the *Jumonji* family, *KDM6B* and *JARID2*, are among the first to respond to temperature during the thermosensitive period in both *T. scripta* and *A. mississippiensis* [Czerwinski et al., 2016; Yatsu et al., 2016]. *KDM6B* encodes a histone demethylase which removes the repressive histone 3 lysine 27 trimethylation (H3K27me3) mark to activate the transcription of its targets [Agger et al., 2007; Lan et al., 2007], while *JARID2* encodes a component of the polycomb repressive complex 2 (PRC2) which functions in the localization of this complex to its target genes for silencing via the addition of H3K27me3 [Peng et al., 2009; Landeira and Fisher, 2011; da Rocha et al., 2014; Kaneko et al., 2014; Sanulli et al., 2015]. In *T. scripta*, when *KDM6B* function is interrupted via RNA interference, embryos incubated at male-promoting temperatures develop ovaries and exhibit increased H3K27me3 at the promoter of *DMRT1*, a key regulator of testis development [Ge et al., 2018]. This provided the first demonstration of a functional role for higher order epigenetic processes in regulating the expression of genes with conserved roles in vertebrate sex determination in response to temperature [Georges and Holleley, 2018]. Though the expression of *KDM6B* and *JARID2* is upregulated in both *A. mississippiensis* and *T. scripta* embryos at "low" temperatures (~26–30°C), in *A. mississippiensis* these temperatures promote ovarian development, while in *T. scripta* they promote testis development [Yatsu et al., 2016; Bock et al., 2020b]. This suggests that the function and localization of these chromatin modifiers likely depends upon the genomic context in which they operate, though the target loci of JARID2 and KDM6B have yet to be elucidated across different TSD species. Other epigenetic processes, including DNA methylation, have also been implicated in TSD [Navarro-Martín et al., 2011; Matsumoto et al., 2013; Parrott et al., 2014], and it is likely these mechanisms operate in a coordinated manner to shape the dynamic epigenome during sex determination, as has been demonstrated in other key developmental processes [DiGiacomo et al., 2013; Potok et al., 2013].

Beyond sex determination, developmental temperature can exert lasting influences on other phenotypic traits in reptiles and amphibians with persistent consequences for adult reproductive function [Singh et al., 2020]. At the transcriptional level, evidence for temperature-related intersexual and intrasexual variation in reptiles and amphibians is sparse. However, in *A. mississippiensis*, incubation temperature was shown to influence the degree of sexually dimorphic gonadal gene expression observed in hatchlings [McCoy et al., 2016]. In particular, the magnitude of sexually dimorphic expression of *SOX9* and *AMH* tended to be greater in hatchlings resulting from higher incubation temperatures (34°C) compared to those derived from lower incubation temperatures (32°C) [McCoy et al., 2016]. It is unclear whether these temperature-related differences in intersexual transcriptional variation persist into adulthood and, if so, whether they have consequences for the functioning of the adult ovary and testis. Even so, it is intriguing to consider that the early developmental thermal plasticity characteristic of many reptile and amphibian species may shape patterns of adult reproductive function. In *E. macularius*, incubation temperature has been shown to not only determine offspring sex, but also influence intrasexual variation in growth, female fertility (as indicated by the proportion of infertile eggs laid), and the ratio of circulating plasma androgens to estrogens [Crews et al., 1998]. Further, in the jacky dragon (*Amphibolurus muricatus*), both females and males reared in a seminatural field enclosure exhibited intrasexual differences in lifetime reproductive success related to incubation temperature [Warner and Shine, 2008]. The mechanisms underlying this persistent temperature-related variation in reproductive endpoints remain unclear, and future studies examining the contributions of both direct effects of developmental temperature on organizational processes shaping gonadal physiology as well as indirect effects mediated by temperature effects on correlated phenotypic traits (e.g., body size) will be highly informative.

Anthropogenic Contaminants Alter Normal Ovarian Trajectories

Exposure to anthropogenic contaminants is now a common experience for nearly all life on earth [Gore et al., 2015], and the environmental sensitivity of reptile and amphibian reproductive development appears to convey a distinct vulnerability to many of these compounds [Crews et al., 1995; Guillette, 2006; Orton and Tyler, 2015]. Specific impacts of exposure to environmental contaminants on ovarian biology in these taxa range from outright sex reversal to more nuanced effects on ovarian function detected at the molecular and cellular levels. For example, alterations to gene expression networks and disrupted folliculogenesis are connected to systemic effects on circulating steroid hormone levels and reduced fertility in exposed individuals [Sifakis et al., 2017]. Perhaps the most common and widely studied of these contaminants are endocrine-disrupting compounds (EDCs), which are capable of interacting with steroid hormone receptors, especially the estrogen receptors [Kuiper et al., 1997; Bolger et al., 1998; Kiyama and Wada-Kiyama, 2015]. Mirroring the underlying complexity of the endocrine system, EDCs, in addition to interacting with hormone receptors, have also been shown to affect hepatic biotransformation of steroid hormones and inhibit steroid hormone synthesis [Fisher, 2004].

Given the central role of estrogen signaling in sex determination, a commonly assessed impact of EDC exposure on reptiles and amphibians is their ability to cause sex reversal. Yet, research has shown that sex reversal resulting from exposure to exogenous hormones and their mimics may not invoke the same mechanisms that determine sex in their absence. For example, the natural hormone (E2) is commonly used as a positive control for EDCs and sex reversal [Matsumoto et al., 2014; Jandegian et al., 2015], which has been shown in alligators to occur via stimulation of ESR1 [Kohno et al., 2015; Doheny et al., 2016]. In *Caiman latirostris*, embryos sex-reversed by E2 exhibit altered timing and expression levels of aromatase, *ESR1*, and progesterone receptor, as well as changes in apoptosis and proliferation dynamics during later stages of development compared to normal females [Canesini et al., 2018]. In the same species, sex-reversal by E2 and by bisphenol A (BPA) results in females with reduced late-stage follicles [Stoker et al., 2008]. In the sea turtle *Lepidochelys olivacea*, sex reversal by E2 at male-producing temperatures leads to small, underdeveloped ovaries with decreased cellular proliferation, delayed downregulation of *SOX9* and upregulation of aromatase, and precocious upregulation of *FOXL2* [Díaz-Hernández et al., 2015]. In *T. scripta*, exposure to high doses of PCBs leads to sex reversal. While sex-reversed animals show similar expression of key ovarian genes, including *FOXL2*, *CYP19A1*, and *RSPO1*, the response is delayed and less robust, and methylation patterns established at the aromatase promoter do not follow a normal female pattern, but remain similar to control males [Matsumoto et al., 2014]. These results suggest sex-reversed females have ovaries with altered morphology and function compared to normal females. Thus, population-level impacts of contaminant-induced sex reversal in nature may extend beyond skewed sex ratios, and also encompass impaired fertility and reproductive success. A list of environmentally relevant compounds known to cause complete sex reversal with references can be found in Table 1. The remainder of this section will focus on instances of altered ovarian development from laboratory experimental studies and well-developed natural systems.

Laboratory Exposures

Laboratory studies evaluating reptiles and amphibians treated with EDCs are useful for determining potential impacts of anthropogenic chemicals but are difficult to draw overarching conclusions from because of variation in the route, developmental timing, and duration of exposure. Further, exposures in controlled settings often lack the environmental and ecological complexity present in nature. Despite these limitations, such studies tend to be favored for amphibian ecotoxicology and have shown that, at both a histological and gross morphological scale, contaminants generally negatively impact oogenesis and reproductive capability. Estrogenic compounds have been found to slow development in *X. laevis*, *Anaxyrus terrestris*, *Hyla versicolor*, *Rana pipiens*, and *Rana sphenocephalus* and have negative downstream impacts on female reproductive health [Tavera-Mendoza et al., 2002; Hayes et al., 2003; Storrs and Semlitsch, 2008]. A wealth of research (and controversy) has focused on atrazine, one of the most used pesticides in the United States [Rohr, 2021]. Exposure to this compound has been reported to induce complete feminization, testicular oocytes in males, delayed gonadal development, as well as extraneous gonads in *R. pipiens* [Hayes et al., 2002, 2003, 2010]. Atrazine exposure during sexual differentiation in *X. laevis* also leads to higher rates of primary and secondary oocyte atresia [Tavera-Mendoza et al., 2002]. It is suspected that atrazine induces aromatase gene expression and increases conversion of androgens to estrogens, driving such abnormalities [Hayes et al., 2002]. Exposure to other pesticides such as triadimefon (and its metabolites) and methoxychlor can cause increased oocyte abscission and inhibit oogenesis altogether [Pickford and Morris, 1999; Zhang et al., 2020]. Additional alterations include increased oocyte atresia in *R. sylvatica* exposed to flavone [Mackenzie et al., 2003] and inhibited oviduct development in *X. tropicalis* exposed to progestin or ethynylestradiol [Gyllenhammar et al., 2009b; Kvarnryd et al., 2011]. BPA and 17α-ethinyl estradiol (EE2) also generally cause feminization in a number of amphibian species as well as upregulation of vitellogenin, a biomarker of estrogen exposure (for an in-depth review on EE2 and BPA's effects, see Bhandari et al. [2015]).

Similar to these cases in amphibians, laboratory studies have also been instrumental in gauging the potential impact of environmental contaminants on ovarian devel-

Table 1. Environmentally relevant compounds inducing sex reversal in reptiles and amphibians

Taxa	Species	Compound	Type	Study
Crocodilia	*Alligator mississippiensis*	17β-estradiol	Hormone	Bull et al., 1988
	Alligator mississippiensis	Corexit 9500	Oil dispersant	Williams et al., 2018
	Alligator mississippiensis	Dicofol	Pesticide	Rooney, 1998
	Alligator mississippiensis	*trans*-Nonachlor	Pesticide	Rooney, 1998
	Alligator mississippiensis	*p,p′*-DDD	Pesticide	Rooney, 1998
	Alligator mississippiensis	*p,p′*-DDE	Pesticide	Matter et al., 1998
	Alligator mississippiensis	2,3,7,8-tetrachlorodibenzo-p-dioxin	Polychlorinated biphenol	Matter et al., 1998
	Alligator mississippiensis	Ethynylestradiol	Synthetic hormone	Matter et al., 1998
	*Alligator mississippiensis**	17α-methyltestosterone	Synthetic androgen	Murray et al., 2016
	Caiman latirostris	Bisphenol A	BPA	Stoker et al., 2008
Squamata	*Calotes versicolor**	Testosterone	Hormone	Ganesh and Raman, 1995
	Eublepharis mecularius	17β-estradiol	Hormone	Bull et al., 1988
Testudines	*Chrysemys picta*	Bisphenol A	BPA	Jandegian et al., 2015
	*Emys orbicularis**	Letrozole	Aromatase inhibitor	Richard-Mercier et al., 1995
	Trachemys scripta	2′,4′,6′-trichloro-4-biphenylol	PCB	Crews et al., 1995
	Trachemys scripta	2′,3′,4′,5′-tetrachloro-4-biphenylol	PCB	Crews et al., 1995
	Trachemys scripta	*cis*-Nonachlor	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	*p,p′*-DDE	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	Chlordane	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	*trans*-Nonachlor	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	Aroclor	PCB	Willingham and Crews, 1999
	Trachemys scripta	4-hydroxy-2,4,6-trichlorobiphenyl	PCB	Matsumoto et al., 2014
	Trachemys scripta	4MA	Reductase inhibitor	Crews and Bergeron, 1994
	Trachemys scripta	MK906	Reductase inhibitor	Crews and Bergeron, 1994
	Trionyx spiniferus	17β-estradiol	Hormone	Bull et al., 1988
Anura	*Acris crepitans**	PCB/PCDF (general)	PCB/PCDF	Reeder et al., 1998
	*Pelophylax nigromaculatus**	17β-trenbolone	Hormone	Li et al., 2015
	Rana pipiens	17α-ethinylestradiol	Hormone	Mackenzie et al., 200; 3Hogan et al., 2008
	Rana pipiens	Atrazine	Pesticide	Hayes et al., 2003; Orton et al., 2006; Langlois et al., 2010
	Rana pipiens	DE-71	PBDE	Van Schmidt et al., 2012
	*Rana pipiens**	ICI 182780	Anti-estrogen	Mackenzie et al., 2003
	Rana pipiens	Nonlyphenol	Surfactant	Mackenzie et al., 2003
	Rana pipiens	PCB-70	PCB	Jofré and Karasov, 2008
	Rana pipiens	PCB-101	PCB	Jofré and Karasov, 2008

Table 1 (continued)

Taxa	Species	Compound	Type	Study
Anura	*Rana pipiens*	Sodium nitrate	Fertilizer	Orton et al., 2006
	Rana rugosa	Dibutyl phthalate	Plasticizer	Ohtani et al., 2000
	Rana sylvatica	17α-ethinylestradiol	Synthetic hormone	Tompsett et al., 2013
	*Rana sylvatica**	Clover root exudate	Phytoestrogen	Lambert et al., 2015
	Rana temporaria	17α-ethinylestradiol	Synthetic hormone	Pettersson and Berg, 2007
	Xenopus laevis	3-*t*-butyl-4-hydroxyanisol	Preservative	Kloas et al., 1999
	Xenopus laevis	4-octylphenol	Surfactant	Kloas et al., 1999
	Xenopus laevis	Atrazine	Pesticide	Hayes et al., 2002, 2010
	Xenopus laevis	Bisphenol A	BPA	Kloas et al., 1999; Levy et al., 2004
	Xenopus laevis	Nonlyphenol	Surfactant	Kloas et al., 1999
	Xenopus [Silurana] tropicalis	17α-ethinylestradiol	Synthetic hormone	Pettersson et al., 2006; Pettersson and Berg, 2007; Berg et al., 2009; Gyllenhammar et al., 2009a,b; Hirakawa et al., 2013
	*Xenopus [Silurana] tropicalis**	17β-trenbolone	Hormone	Olmstead et al., 2012

* Female-to-male sex reversal.

opment and function in reptiles. For example, *C. latirostris* exposed to atrazine or BPA as embryos, or injected with the same dose of BPA as juveniles, show advanced stages of ovarian and oviductal development, determined via histological examination or gene expression [Stoker et al., 2008; Galoppo et al., 2017]. Both studies used E2 as a positive control with many of the same effects, including sex-reversal at higher doses with opposite effects at the low dose treatments. This suggests that disrupted estrogen signaling is likely responsible for the altered phenotypes and that the effects are dose-dependent and nonmonotonic. Mechanistic reasons for the opposite effects of low versus high doses are unknown. Other alterations to ovarian morphology have been observed in embryonic lizards exposed to maternal atrazine [Parsley et al., 2015a], diethylbestrol [Parsley et al., 2015b], or injected with cadmium [Simoniello et al., 2010]. These exposures often induced altered cellular structure of the ovary and disrupted oogenesis and folliculogenesis. In one of these cases [Simoniello et al., 2010], the effects were more similar to FSH exposure than E2, which were used as controls.

Altogether, while these laboratory studies provide evidence of the ability of EDCs to disrupt ovarian development, results are complicated by differences in dosage, timing of exposure, and organismal variation. While including controls such as FSH and E2 has provided important insights into mechanisms of action, we are still lacking an understanding of which specific pathways are impacted, largely due to a gap in our understanding of how such processes operate in the context of normal ovarian development and function. Moving forward, resolving the multifaceted roles estrogen signaling plays in ovarian development in reptiles and amphibians is needed. Mechanistic studies examining the molecular and cellular dynamics, rather than observational exposure experiments, are likely to provide key insights into how EDCs interact with ovarian development to affect downstream reproductive function.

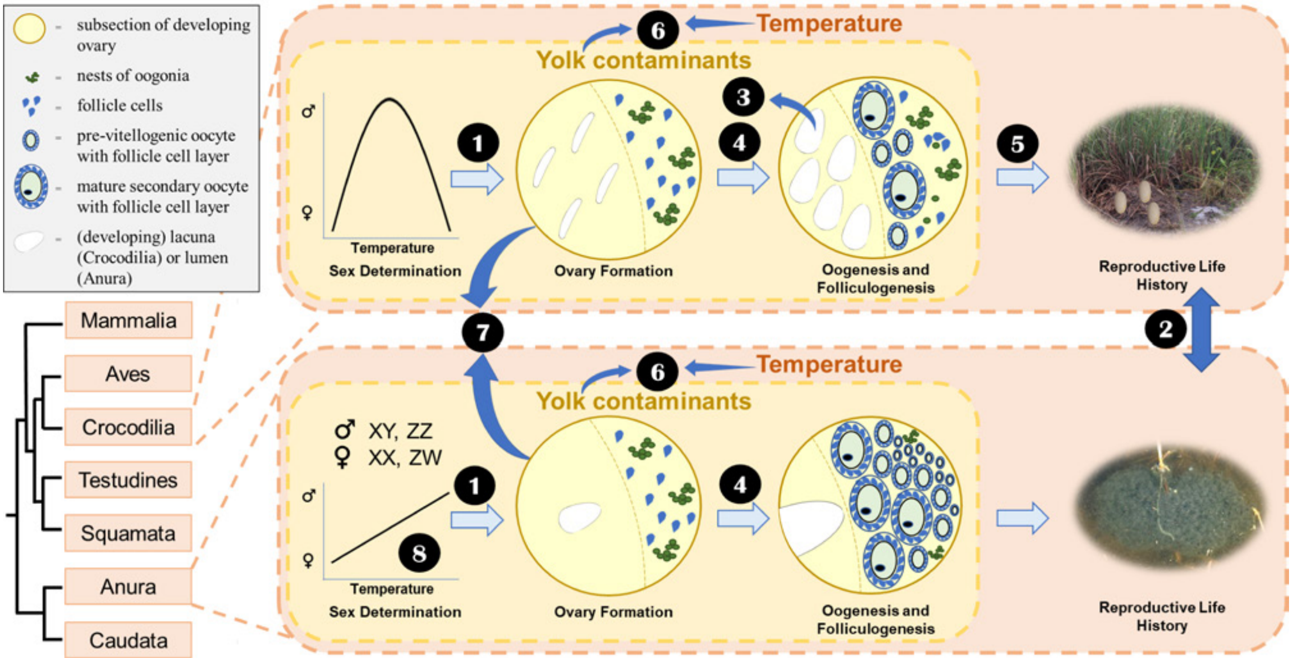
Natural Exposures

Wild populations exposed to EDCs provide a unique opportunity to understand their consequences in natural settings, which is more directly applicable to discovering the ecological impacts of these compounds. One of the earliest observations of altered ovarian development in polluted environments is the *A. mississippiensis* population at Lake Apopka, FL, USA. Extensive inputs of organochlorine pesticides from agricultural practices and an industrial spill event has led to sustained exposure detectable not only in plasma of alligators but also in egg yolks [Heinz et al., 1991]. Following observations of reduced juvenile recruitment at the population level, abnormalities to ovarian follicles, such as multi-oocytic follicles and polyovular follicles, were first reported in juvenile alligators inhabiting the lake [Guillette et al., 1994]. Subsequent studies using a combination of field collections and lab incubations revealed that disruptions in steroidogenic and TGF-β signaling pathways along with impeded folliculogenesis occurring in juvenile ovaries likely stemmed from earlier embryonic exposure due to the maternal deposition of these contaminants in yolks [Moore et al., 2010b, 2011, 2012b; Hale et al., 2019; Hale and Parrott., 2020]. Altogether, these and other reports from Lake Apopka suggest that embryonic exposure to EDCs alters transcriptional networks involved in early oogenesis and folliculogenesis and that these perturbations persist into juvenile life and perhaps beyond.

The mechanisms by which maternally deposited EDCs interact with ovarian development to affect reproductive function are not well resolved in natural populations. Based on the proclivity of many EDCs, including those at Lake Apopka, to activate estrogen receptors in vitro, it is suggested that disruptions to estrogen signaling during gonadal differentiation are responsible. This idea is supported by observations that EDCs and their metabolites are capable of competitively binding estrogen and progesterone receptors in alligator oviducts [Vonier et al., 1996]. However, in this study and others, EDCs typically exhibit weak agonism of nuclear estrogen receptors, with activation on par with E2 only observed at high concentrations [Vonier et al., 1996; Bolger et al., 1998; Guillette et al., 2002]. Interestingly, when alligator eggs collected from a reference lake are treated with E2 prior to the start of ovarian estrogen synthesis (measured by *CYP19A1* expression), the ovarian transcriptome in resulting juvenile alligators broadly mirrors those measured in alligators from Lake Apopka [Hale et al., 2019; Hale and Parrott, 2020]. Additionally, impediments to folliculogenesis observed in ovaries of Lake Apopka alligators, including severe decreases in stage III follicles and increases in germ cell nests were also induced by embryonic treatment with E2 [Hale and Parrott, 2020]. These findings support an alternative hypothesis explaining disrupted ovarian development in Lake Apopka alligators in which EDCs induce weak, but developmentally precocious estrogen signaling to impact germ cell behavior in the early differentiation of the ovary. This hypothesis is also consistent with previous reports demonstrating that key pathways involved in germ cell nest breakdown and folliculogenesis (e.g., activin, inhibin, follistatin) are altered in Lake Apopka ovaries [Moore et al., 2010b, 2012b]. In this model, the precocious timing of estrogen signaling, rather than a simple increase in estrogenic activity, is the precipitating event. However, further investigations examining the influence of precocious estrogen signaling on early germ cell behavior, including their initial proliferation within the gonad, the mitotic to meiotic transition, primordial follicle formation and recruitment, and follicle maturation as well as the genomic changes occurring during each of these events are needed.

Another example of a wild population with ovarian defects in reptiles occurs at Moody Pond, MA, USA, where painted turtles (*Chrysemys picta*) are exposed to various heavy metals and organic compounds during development. When compared to a control site, ovaries in Moody Pond turtles harbor fewer small and large follicles [Rie et al., 2005], and adult females have lower vitellogenin and an abated response to gonadotropin stimulation [Kitana et al., 2006]. When eggs collected from this same site are raised in the lab, they have higher levels of oocyte apoptosis than the control population [Kitana and Callard, 2008]. This same study measured cadmium content in eggs from Moody Pond and exposed control *T. scripta* eggs to relevant doses, which resulted in elevated oocyte apoptosis, suggesting embryonically exposed turtles originating from Moody Pond have reduced reproductive ability via reductions in oocyte number and abated response to FSH.

Complementary studies of contaminants' effects on ovarian development in wild amphibian populations are less detailed, but have been reported. For example, Hayes et al. [2003] observed slowed gonadal development, testicular oogenesis, and even oocyte growth in male leopard frogs (*R. pipiens*) in multiple populations across several states exposed to the pesticide atrazine. In suburban ponds with increased levels of EDC contamination, a greater proportion of females in populations of *R. clamitans* were observed, suggestive of skews in population sex ratios [Lambert et al., 2015]. Nemesházi et al. [2020] also observed that agricultural areas harbored an increased prevalence of female-to-male sex reversal in agile frog (*Rana dalmatina*) populations in north-central Hungary. Mink frog (*Rana septentrionalis*) populations exposed to E2 at an experimental lake in Ontario, Canada, exhibited higher rates of individuals with intersex gonads whereas no intersex individuals were observed in green frogs (*R. clamitans)* occupying the same habitat [Park and Kidd, 2005]. These studies have focused on gonadal aberrations and sex reversal, and reveal the potential impact of environmental contaminants occurring at population scales. Whereas our understanding of the underlying proximate mechanisms in these systems are generally lacking, they have great potential to reveal the extent to



① In which cell types does primary sex determination occur across reptiles and amphibians? Are patterns of cellular organization and/or gonadal structure linked to SDMs across species?

② Is variation in ovarian development (e.g., germinal nest number) linked to inter-specific differences in reproductive ecology and life-history?

③ What is the function(s) of ovarian lacunae and why are these structures present in some but not all groups?

④ What are the pathways that mediate the mitotic to meiotic transition in reptilian and amphibian germ cells?

⑤ What are the evolutionary costs and benefits of a regenerating oocyte pool?

⑥ How are transient environmental cues integrated into robust developmental responses and what are the downstream consequences on intrasexual variation in reproductive function?

⑦ What is the role of estrogen signaling on germ cell biology within the differentiating ovary and how important is the developmental timing of these events?

⑧ What are mechanisms responsible for environmental sex determination in amphibians?

Fig. 1. Illustration of differences in sex determination and ovarian development in the context of emerging and outstanding questions in the field.

which contaminant-mediated impacts on reproduction affect populations and ecological communities.

Summary and Future Directions

Reptiles and amphibians offer excellent models in which perspectives and approaches from biological, ecological, and evolutionary fields can be applied to understand the diversity of sex determination strategies and reproductive development. Whereas many facets of ovarian development appear broadly conserved, we identify here several characteristics in which differences are observed both within these taxa and/or in comparison to other vertebrate clades. In many cases, simple descriptions of fundamental biological processes involved in sex determination, ovarian differentiation, and germ cell biology are lacking, despite their necessity to better gauge the extent of existing variation and to resolve the attendant taxonomic relationships. In other cases, especially species in which ovarian development is relatively well studied, connecting ovarian biology to taxon-specific aspects of life history and ecology has the potential to reveal key ecological factors and evolutionary pressures that drive diversity in reproductive development. Recent work in TSD reptiles is leading to exciting breakthroughs regarding the molecular mechanisms translating thermal cues into biological responses, but how these pathways respond to and operate within complex and dynamic environments is a critical question moving forward. In addition, whether the pathways identified in TSD reptiles also mediate the environmental sensitivity of sex determination and ovarian development in amphibians and across other SDMs remains unknown. Further, the extent to which TSD is represented across amphibians more broadly is an important question for conservation efforts in a rapidly changing world. Similarly, well-developed model systems for elucidating the influence of anthropogenic compounds on reproductive development are present in these groups and offer great potential for deciphering the impact of contaminants in natural settings. Lastly, the returns of past advances in unbiased sequencing approaches are reflected by new discoveries in comparative reproductive biology that would never have occurred relying on candidate gene/pathway approaches. This is especially true in reptiles and amphibians, in which a lack of technological and funding resources historically constrained approaches to comparisons of genetic pathways to mammalian models. The increasing availability of such approaches in these species opens up new, intriguing opportunities for answering fundamental questions connecting basic biology with environmental factors. In closing, we propose a set of outstanding and critical questions, which subjectively represent some of the most pressing issues that the field might consider moving forward (Fig. 1).

Acknowledgements

The authors would like to thank members of the Parrott Laboratory for helpful conversations during the preparation of the manuscript.

Conflict of Interest Statement

The authors have no conflict of interest to declare.

Funding Sources

This work was supported by the National Science Foundation under Award Number 1754903 (B.B.P.) and the Department of Energy Office of Environmental Management and National Nuclear Security Administration under award number DE-EM0005228 to the University of Georgia Research Foundation. This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the USA.

Author Contributions

C.R. Smaga, S.L. Bock, J.M. Johnson, and B.B. Parrott all contributed to the writing of the review.

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