

Genetic & Environmental Sex Determination in Cold-Blooded Vertebrates: Fishes, Amphibians, and Reptiles

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Abstract

This article focuses on sex determination in cold-blooded vertebrates, which comprise over half of all living vertebrate species and display a dizzying array of sex determination systems and regulatory mechanisms. Evolution of these sex determination systems has been driven by millions of years of radiation and adaptation to extreme aquatic and terrestrial environments. Recent findings support a greater role of the genome and epigenome in sex determination in species previously thought to exhibit strict environmental sex determination. This exciting area of research truly stands at the intersection where genetics and the environment meet.

Nomenclature

Nomenclature for genes and proteins, and their associated symbols, for various animal taxa follows the conventions described below:

Mammals and birds: Gene symbols are italicized and in all capital letters (*GENE1*); protein symbols are non-italicized and in all capital letters (PROTEIN1).

Fishes, amphibians, and reptiles: Gene symbols are italicized and in all lowercase letters (*gene1*); protein symbols are non-italicized with only the first letter capitalized (Protein1).

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Glossary

Endotherms Animals that, while active, draw upon external sources and sinks of heat to maintain their body temperature within narrow limits.

Epigenetics Processes involved in the translation of a common genotype to multiple phenotypes ranging from cellular differentiation to whole organism phenotype, which may be heritable. Examples are DNA methylation and histone modification.

Genetic sex determination Sex determination driven by sex chromosomes that bear a master sex-determining gene or, more rarely, by the interaction of multiple genes on different chromosomes in one individual.

Gonadal primordium The region of the body, ultimately consisting of bipotential germ and somatic cell lines, from which the gonads ultimately develop.

Gonochoristic species Species in which individuals develop as either male or female and remain the same sex throughout life (i.e., non-sex changing species); it is in contrast with hermaphroditic, sex-changing species.

Heterogamety Refers to the sex of a species that possesses different sex chromosomes (e.g., XY in human males), as opposed to homogamety where the sex chromosomes are the same (e.g., XX in human females).

Poikilotherms Animals that cannot regulate their own body temperature and, thus, whose body temperatures vary in close agreement with ambient/environmental temperatures.

Sex reversal Occurs when an underlying genetic propensity to be one sex or the other (e.g., sex chromosomes) is overridden by environmental influences via hormonal or epigenetic pathways, as in thermal sex reversal.

Temperature-dependent sex determination (TSD) A type of environmental sex determination in which the temperature to which an animal is exposed during embryonic or juvenile development determines its sex hormonally or epigenetically in the absence of a definitive genetic influence (e.g., sex chromosomes).

Thermosensitive period The developmental period during which the germ and somatic cells of the gonads are sensitive to and may be influenced by temperature; also known as the thermolabile period.

Key Points

- Poikilothermic and ectothermic vertebrates (collectively referred to as “cold-blooded vertebrates”) exhibit a broad array of sex determination systems and regulatory mechanisms that reflect a close association and responsiveness to their external environment.
- Two major genetic sex determination (GSD) systems, XX/XY and ZZ/ZW, are found in cold-blooded vertebrates and coexist with environmental sex determination (ESD) in reptiles and fishes. The major GSD systems have independently evolved numerous times in closely related species, and in some instances even co-occur within the same species.
- Once considered distinct modes of sex determination, GSD and ESD are now viewed as much more intertwined, bringing the prevalence of pure ESD, without underlying genetic influence, into question.
- GSD in some fishes, amphibians, and reptiles can be influenced or overridden by environmental factors, such as rearing temperature, and reflect sex reversals that have now been documented not only in captive studies, but also in nature.
- Recently, numerous master sex-determining genes have been identified in fishes, most of which are transcription factors or transforming growth factor-associated genes. Only one master sex-determining gene, a transcription factor, has been demonstrated in amphibians and none in reptiles.
- Regardless of the mechanism of sex determination, downstream signaling associated with gonadal sex differentiation appears to be highly conserved among vertebrates and to reflect antagonistic pathways leading to the formation of either ovaries or testes.
- At a time when climate change and global warming are accelerating and environments that organisms inhabit are changing beyond their ability to adapt, the survival of cold-blooded vertebrates with ESD or those that are susceptible to thermal sex reversal may be in jeopardy.

Introduction

Sex determination is the process by which the sexual fate of an organism is set into motion. This pivotal event subsequently dictates numerous aspects of the organism’s life history, potentially including its morphology, growth, behavior, and lifespan. In vertebrates, sex may be determined by the presence and activation of a single gene, a combination of genes, exposure to an environmental cue(s), or a combination of both genetic and environmental cues. This triggers sexual differentiation, a relatively conserved molecular signaling cascade and cellular and anatomical changes, leading to the formation of either ovaries or testes from an undifferentiated gonadal primordium.

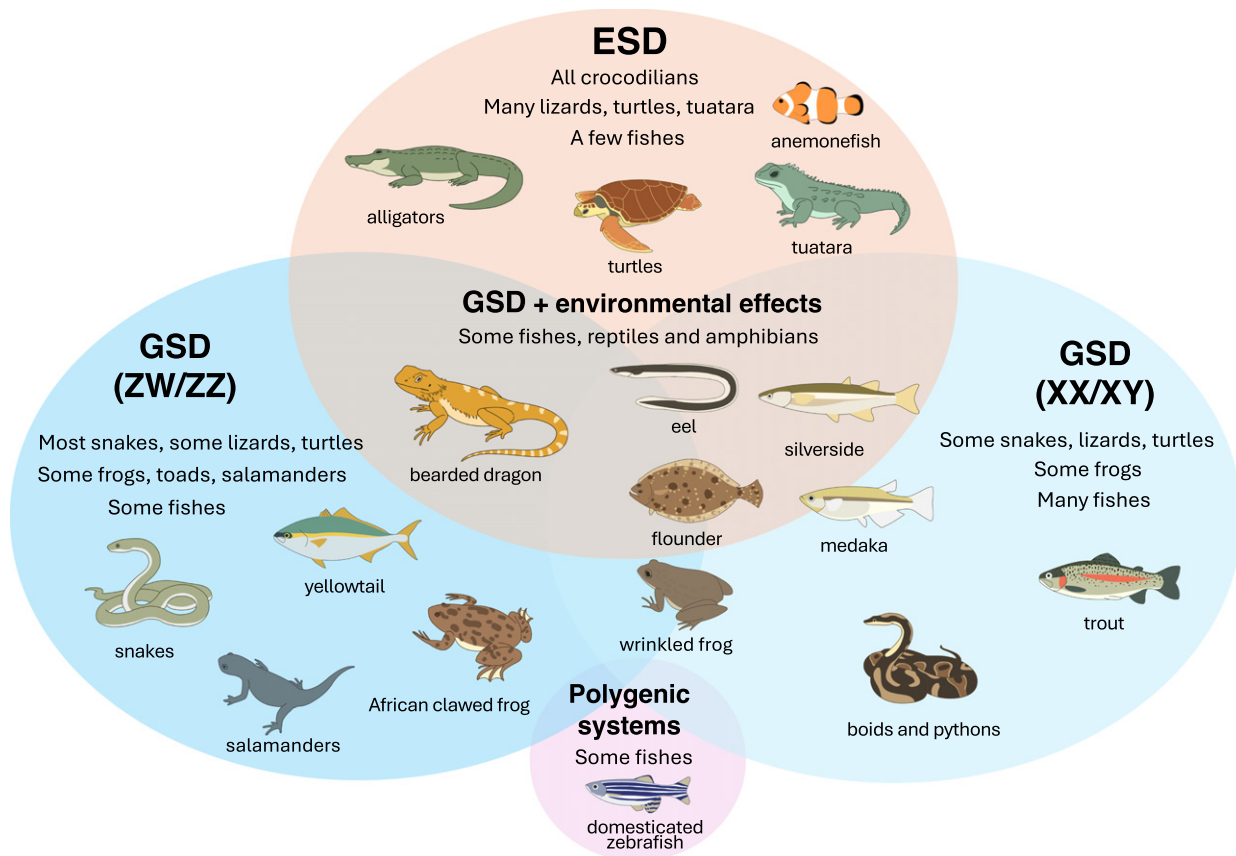


Fig. 1 The variety of sex determination systems in cold-blooded vertebrates. Environmental sex determination (ESD) is common in reptiles and observed in a few fishes (hermaphroditic species). The major genetic sex determination (GSD) systems, ZZ/ZW and XX/XY, are found in all classes of cold-blooded vertebrates, although some species are sensitive to environmental factors and GSD can be influenced/overridden (GSD + environmental effects). Complex polygenic systems are observed in some fishes but considered rare. Clip art of animals by Chinatsu Yamamoto.

Although sexual differentiation and, indeed, sexual reproduction (mating of a female and male) are highly conserved through vertebrate evolution, the process of sex determination exhibits a much higher degree of plasticity. This is especially true for poikilothermic and ectothermic vertebrates (fishes, amphibians and reptiles, collectively referred to hereafter as “cold-blooded”), which make up over half of all living vertebrates. Species from these classes exhibit a dizzying array of sex determination systems that have traditionally been categorized as either genetic (GSD) or environmental sex determination (ESD) (Fig. 1). However, an ever-growing number of species previously thought to exhibit strict GSD or ESD demonstrate interplay between genetic and environmental cues leading to the ultimate female or male sexual phenotype.

The diversity of sex determination mechanisms in cold-blooded vertebrates has been driven by millions of years of radiation and adaptation to extreme aquatic and terrestrial environments. This exciting research area truly stands at the intersection where genetics meets environment and is important to our understanding of the evolution of sex determination mechanisms and sexual reproduction. The goal of this review is to provide a brief summary of sex determination in cold-blooded vertebrates, including recent developments in the field and vital questions that remain unanswered. Although we were limited to primarily citing relevant reviews, we encourage the reader to explore the excellent basic literature on this topic.

Genetic Sex Determination

In nearly all mammals, including humans, sex is irreversibly determined at conception by the presence or absence of the *sex-determining region of the Y chromosome (SRY)* gene. If *SRY* is paternally inherited and properly expressed, testicular development is triggered, whereas in the absence of *SRY*, ovarian development is initiated. This type of system is referred to as monogenic because it is controlled by a single sex-determining locus or ‘master switch’ that initiates the gonadal sex differentiation cascade. This relatively simple and highly conserved system in mammals is in stark contrast to what is seen in many cold-blooded vertebrates. Indeed, these animals exhibit a multitude of GSD systems ranging from monogenic to complex multifactorial (polygenic) systems that are still not well understood (Fig. 1).

There are numerous examples of monogenic GSD in cold-blooded vertebrates. So-called XX/XY and ZZ/ZW systems are most common and appear to have evolved independently numerous times in some taxa. In XX/XY systems, like that of humans, males are the heterogametic sex (XY) and females the homogametic sex (XX). The opposite holds true for ZZ/ZW systems; females are heterogametic and males are homogametic. This basically means that sex is determined by the genetic contribution of the male parent in XX/XY species and the female parent in ZZ/ZW species. It does not mean however that the same gene or genes on the sex chromosomes determines sex for all XX/XY or ZZ/ZW species. In fact, in some taxa there appears to have been high turnover of master sex-determining genes as we discuss later.

GSD in Fishes

Many gonochoristic fish species are in the process of what is considered initial sex chromosome differentiation and possess homomorphic sex chromosomes, which do not noticeably differ from each other in cytological appearance like autosomes (Penman and Piferrer, 2008). In contrast, some species have heteromorphic sex chromosomes, which differ in appearance. For example, the human Y chromosome has undergone significant reduction in the number of genes it possesses and its overall size during evolution, making it easily distinguishable from the X, and the XY pair easily distinguishable from autosomes by karyotype.

Systems of GSD have primarily been characterized in model fishes (e.g., medaka *Oryzias latipes*) and species of importance for aquaculture (e.g., Nile tilapia *Oreochromis niloticus*; rainbow trout *Oncorhynchus mykiss*). Identification of a species' sex determination system is often achieved through chromosome set manipulation (e.g., gynogenesis) and/or sex reversal and evaluation of progeny sex ratios from subsequent breeding crosses (Devlin and Nagahama, 2002; Luckenbach and Guzmán, 2022). Unlike in mammals, the process of sex determination and gonadal differentiation in most cold-blooded vertebrates can be easily overridden by exogenous treatments during what is referred to as the sexually labile period, which varies by species. Sex reversal can be intentionally induced, typically through immersion and/or dietary treatment of animals with androgens, estrogens, inhibitors of steroid synthesis (e.g., aromatase inhibitors), or environmental exposures (see ESD section below). These approaches can be used directly or indirectly to produce monosex stocks of fish for aquaculture, which is not only important from an applied perspective (e.g., one sex exhibiting superior growth in aquaculture) but can also reveal the underlying GSD system (Luckenbach and Guzmán, 2022). However, unintended sex reversals, among other impacts to reproductive physiology, are a growing concern for wild fish and amphibian populations due to the release of pollutants containing endocrine-disrupting chemicals into aquatic ecosystems.

XX/XY and ZZ/ZW GSD systems are most common in fishes, with XX/XY being predominant (Fig. 1; Nagahama et al., 2021). There are also cases in which the system of sex determination differs among species of the same genera and even within a species. Thus, there appears to be no strong genetic selection for male versus female heterogamety in fishes.

Variation on major GSD systems above has also been documented, as some fishes exhibit systems (XX/XO and ZW/ZO) in which one sex chromosome has been lost in males (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). Females thus inherit two sex chromosomes (XX or ZW) while males inherit a single sex chromosome (X or Z). In these cases, "O" in the nomenclature denotes the lack of a second sex chromosome. Additionally, there are cases of multiple sex chromosome systems (e.g., XX/X₁X₂Y; ZW₁W₂/ZZ) in which chromosomal fusions have given rise to females and males having a different diploid number of chromosomes.

Observation of sex ratios that do not fit any of the above patterns often indicates influence of environmental factors such as temperature, polymorphic variation in the sex determining system among individuals of a species or, more rarely, a polygenic system (polygenic sex determination; PSD) operating within individuals of a species. True polygenic systems are considerably more rare than monogenic ones and considered transient or intermediate states toward establishment of a monogenic system (Penman and Piferrer, 2008; Schartl et al., 2023). Well-studied examples of PSD are live-bearing species of platyfishes and swordtails (genus *Xiphophorus*); however, in at least one species (*X. maculatus*), homologous X, Y and W chromosomes are present in one population, but only two in any one individual, which is not strictly polygenic. In the widely used model species, zebrafish (*Danio rerio*), sex determination differs among wild and domesticated strains. Wild populations of zebrafish have an unequivocal ZZ/ZW system, whereas in captive colonies the Z chromosome was lost, leaving only WW genotypes and a PSD system, the latter of which is considered a transitory state.

GSD in Amphibians

Similar to fishes, GSD systems in amphibians have largely been identified through gynogenesis and/or induced sex reversal and progeny testing. Research has shown that most amphibians have homomorphic sex chromosomes and either an XX/XY or ZZ/ZW system (Fig. 1). Most urodeles (salamanders) of families Salamandridae and Plethodontidae and anurans (frogs) of Ranidae have an XX/XY system, whereas the tropical clawed frogs of family Pipidae, including the well-known model frog species *Xenopus laevis*, and urodeles of Ambystomatidae and Cryptobranchioidea have a ZZ/ZW system (Hillis and Green, 1990; Ma and Veltsos, 2021). Studies indicate that sex chromosomes have evolved numerous times in each lineage. Also, as in fishes, within-species differences in the sex determination system have been documented. The wrinkled frog (*Glandirana rugosa*) for instance exhibits either an XX/XY or ZZ/ZW system, all variants of the same chromosome, depending on its local population in Japan (Miura, 2018). On one large island, a hybrid zone between the XX/XY and ZZ/ZW forms occurs, with various combinations of these four sex chromosomes observed; thus, there may be a single sex-determining locus that is polyallelic (most likely variants of the *sox3* gene). This

variability within and among species again underscores the lack of selection for one particular GSD system in cold-blooded vertebrates.

A new category of sex determination, “random sex determination” was proposed by Perrin (2016). Similar to classical definitions of PSD, his theory states that developmental noise, including environmental factors, determines phenotypic sex instead of GSD alone. For example, in the northern Sweden population of *Rana temporaria*, *dmrt1* on chromosome 1 is slightly linked to sex, suggesting a GSD system, although many XY females and XX males are found in the wild, indicating that sex reversals are occurring (see “Environmental Sex Determination” section below).

Kinship is another proposed cue for sex determination in frogs. The Japanese rice frog (*Fejervarya kawamurai*) shows sex-ratio variation depending on kinship: Interbreeding between parents and/or sisters promotes male-skewed sex ratios, whereas outbreeding between non-relatives within a population produces female-skewed sex ratios. Outbreeding across different populations nearly balances sex ratios, suggesting that heterozygosity of multi-genes is involved in female sex determination, similar to complementary sex determination in bees and ants of Hymenoptera (Miura *et al.*, 2015). No sex chromosome has yet been identified in frogs.

GSD in Reptiles

Many reptile species exhibit ESD; however, many snakes and some lizards and turtles exhibit GSD (Fig. 1; Janzen and Krenz, 2004). Consistent with fishes and amphibians, there are examples of both XX/XY and ZZ/ZW systems in reptiles. All snakes investigated to date in the clade Caenophidia are ZZ/ZW, some boids and pythons are XX/XY, and the poorly studied blind snakes appear variable. Turtles and lizards with GSD have either a ZZ/ZW or XX/XY system.

Ancestral reptiles are generally thought to have exhibited ESD, but there is still debate regarding whether ESD or GSD came first. Given the propensity for rapid transitions between these modes of sex determination and numerous reversals, as indicated by their haphazard distribution across the reptile phylogeny, the question of which came first, ESD or GSD, may be unanswerable. Sister clades at all taxonomic levels have ESD versus GSD in the reptile phylogeny, and this is likely to also occur even among populations of single species. There are some generalizations, however: ESD seems to have evolved more commonly in ZZ/ZW lineages, and some lineages appear locked into one form of sex determination or the other. For example, chelid turtles and iguanas have XX/XY systems, Caenophidian snakes all have ZZ/ZW systems, and all crocodylians and marine turtles have ESD.

Environmental Sex Determination

ESD is non-genetic sex determination driven by environmental cues that serve as the master determinants of sex. In fishes, the stress-endocrine axis is thought to be fundamental to the mechanism of ESD. The primary glucocorticoid, cortisol, has been identified as a key mediator between temperature and sexual development. On the other hand, there are increasing reports that epigenetics underlie the ESD mechanism in reptiles as well as some fishes. As a phenotypic response, ESD necessarily invokes epigenetic processes at some point in the regulatory pathways. Capturing the environmental signal does not necessarily fall in the class of epigenetic processes, nor do the signaling pathways of the cell, but ultimately an epigenetic process is involved to modify gene expression. These epigenetic processes, central to the mechanisms by which temperature affects sexual outcomes in both ESD and sex reversal, appear to involve a set of highly conserved and ancient components of cellular epigenetic machinery as outlined later in this review.

As noted above, ESD is generally considered a precursor sex determination mechanism, which was largely replaced by the evolution of sex chromosomes and establishment of GSD. Yet, ESD has persisted (or recurred) in some reptiles and fishes and offers a high degree of plasticity that may hold fitness advantages. Prevailing theory proposed by Charnov and Bull (1977) indicates that ESD is advantageous when environmental conditions are patchy and different fitness levels exist for each sex (examples below).

Conventionally, GSD and ESD have been considered mutually exclusive systems. However, based on recent discoveries, revealed in some cases via genomics technologies, there are likely to be few if any cases of ESD that do not involve an underlying genetic predisposition to be one sex or the other. This new information is shifting the field’s view to question whether classically defined ESD (i.e., that directed only by non-genetic factors) actually exists.

In recent years there has also been accumulating evidence for what has been referred to as thermal sex reversals or anomalous GSD, among other terms, which applies to cases where environmental factors influence sex determination and/or differentiation, and ultimate sexual phenotype, but may not be relevant in the natural environment (Conover, 2004; Ospina-Álvarez and Piferrer, 2008). An example of this would be exposure of a species to extremely low or high temperatures that result in skewed sex ratios (Fig. 2). This is still debated by researchers and has received more attention in light of climate change (Ospina-Álvarez and Piferrer, 2008; Lema *et al.*, 2024).

ESD in Fishes

To date, hermaphroditism has been reported in about 2% of teleost fishes. In hermaphroditic species, sex change is often triggered by social factors or individual body size/age. The remaining 98% of teleost fishes exhibit gonochorism, and numerous

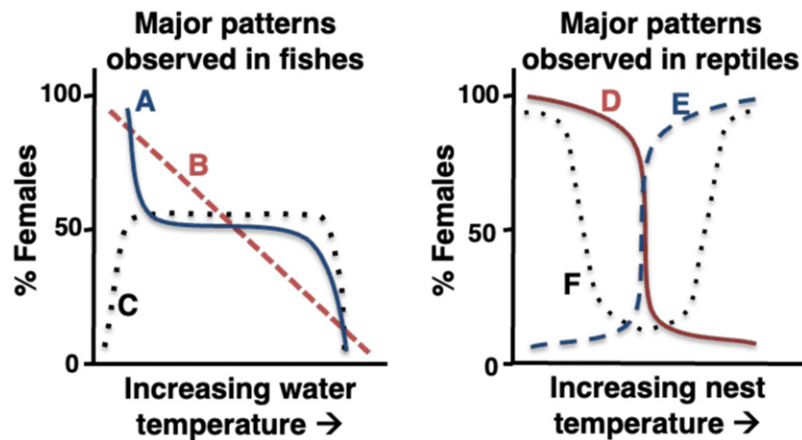


Fig. 2 Major observed patterns of temperature effects on sex determination in fishes and reptiles. Most commonly in fishes, increasing water temperatures significantly reduce female proportions (A–B). In some flatfishes, exposure to low or high temperatures reduces female and increases male proportions (C). In reptiles, low temperatures may induce high (D) or low female proportions (E) with the patterns flipping at higher temperatures. Other reptiles exhibit a female-male-female pattern (F) with high female proportions at low and high temperatures and high male proportions at intermediate temperatures. Based on Conover (2004).

environmental factors have been shown to influence their sex determination/differentiation (Baroiller *et al.*, 2009). By far the most prevalent environmental factor shown to influence sex is temperature (Ospina-Álvarez and Piferrer, 2008; Kitano *et al.*, 2024). The first fish in which a thermal influence on sex was documented was the Atlantic silverside (*Menidia menidia*, family Atherinopsidae). Atlantic silversides have an “annual” life cycle and attain maturity within one year of hatching. Sex ratios in this species are female-biased at cooler temperatures at the beginning of the breeding season; however, warmer temperatures later in the breeding season induce male-biased sex ratios (Fig. 2B). This is because reproductive fitness (e.g., body size, fecundity) is higher in females than males, and thus being born earlier and having a longer breeding season is more beneficial to females than males (Conover, 2004).

There is also geographical variation in the thermal response in Atlantic silversides (Conover, 2004; Duffy *et al.*, 2015). Interestingly, no populations display pure ESD or GSD, but the response to temperature is uniformly high in their southern range and then declines rapidly approaching their northern range, where GSD dominates. The thermal response then increases to a moderate level in northern-most populations. The length of the growing season and high gene flow among populations is what likely drives this latitudinal variation in the thermal response. As mentioned above, longer growing seasons are more beneficial to females than males. In lower latitude areas, cooler water temperatures during larval development signal a longer growing season compared to higher latitude areas. Following the Chamov and Bull model, female reproductive fitness would therefore be enhanced in southern populations by a response to temperature yielding more females, whereas fitness is enhanced for individuals born late in the season by developing as males, which have a better chance of reproducing at a smaller body size than do females.

Another atherinid species, pejerrey (*Odontesthes bonariensis*), native to South America presents one of the strongest known thermal responses in fishes (Strüssmann *et al.*, 1997). Monosex female or male stocks of pejerrey can consistently be produced when larvae from different broods are exposed to environmentally-relevant temperatures of 17°C or 29°C, respectively. The master sex-determining gene, *Y-chromosome specific anti-Müllerian hormone (amhy)*, was discovered in *O. hatcheri*, a congeneric species that exhibits GSD. An *amhy* homolog was then identified in pejerrey and its presence or absence was demonstrated to favor male or female development, respectively, when fish were reared at a ‘sexually neutral temperature’ (Yamamoto *et al.*, 2014). These results in atherinids indicated that the presence of a strong thermal effect on sex does not exclude the possibility of underlying GSD mechanisms.

In addition to temperature, several other environmental factors may influence fish sex ratios (Heule *et al.*, 2014). An effect of pH on sex determination has been observed in swordtail and some cichlid species. For instance, in *X. helleri*, acidic water induces all-male populations, while a neutral pH produces mostly females. Density is also known as an environmental factor that can modulate sex ratios. In eels, both field and laboratory observations revealed that low densities favor female development, while high densities favor males. Even the color of the rearing enclosure can influence sex. In southern flounder (*Paralichthys lethostigma*), rearing fry in blue-colored tanks induced significantly male-skewed sex ratios relative to black or gray tanks. This is in addition to documented temperature effects on sex ratios in this and other flounder species (Luckenbach *et al.*, 2009).

While modulation of sex determination by environmental factors has been clearly demonstrated for many fishes under laboratory conditions, there are few reports on the existence of ESD in the wild. However, the availability of molecular markers of genotypic sex (or of the sex-determining gene itself) in some species with ESD facilitates studies of its occurrence in the wild. For example, the absence or presence of the *amhy* gene in cobaltcap silverside (*Hypoatherina tsurugae*) can distinguish XX and XY/YY fish, and the concordance between genotypic and phenotypic sex in wild fish can thus be easily evaluated (Miyoshi *et al.*, 2020). A three-year field study (2014–2016) in Tokyo Bay, Japan, revealed that the number of male sex reversals (XX males) increased as

larvae experienced higher temperatures during early development, which in turn led to an increasingly male-biased sex ratio in the wild population, the long-term effects of which are yet to be determined (Miyoshi *et al.*, 2020; Lema *et al.*, 2024).

ESD in Amphibians

ESD species have not yet been described in Amphibia (Ma and Veltsos, 2021; Eggert, 2004). However, temperature can change the phenotypic sex of individuals via sex reversal. For example, European and north African salamanders of genus *Pleurodeles* have ZZ/ZW-type GSD with heteromorphic sex chromosomes (Dourmon *et al.*, 1990). In *P. waltl*, rearing larvae at 16°C produces balanced sex ratios, 30°C promotes male-biased sex ratios, and 32°C produces almost all males, indicating that ZW females are sex-reversed to phenotypic males. Interestingly, the thermal sex reversal is opposite in the sister species *P. poireti*: rearing at 30°C or 32°C induces female-biased sex ratios, indicating that the ZZ males are sex-reversed to phenotypic females. In frogs, the brown frog species can be easily sex-reversed in the lab and wild: XX males are discovered in the wild in *Rana japonica* (Miura, 1994), *R. temporaria* (Rodrigues *et al.*, 2017), and *R. dybowskii* (Xu *et al.*, 2022). However, the factors that induce sex reversal are unknown. In some 'true frogs' (family Ranidae), in addition to temperature, genomic cues such as parthenogenesis and gynogenesis change the phenotypic sex of genetic females, producing XX males (Kawamura, 1939; Kawamura and Nishioka, 1977).

ESD in Reptiles

All crocodylians, many lizards and turtles, and the tuatara exhibit ESD (Fig. 1; Lang and Andrews, 1994). These egg-laying species bury their eggs below ground or in nests comprised of dead vegetation where the embryos develop until they hatch and depart the nest. Studies indicate that the depth and position of eggs within a nest can influence the temperature exposure. This effect occurs not because of a trend in average temperatures with depth (they remain constant below the first centimeter or so) but because sex is influenced by the daily variance in temperature in addition to the mean. The variance is dampened with depth in the nest, with a corresponding impact on offspring sex ratios. Temperature exerts this effect during the thermosensitive period, which is typically around the mid-trimester of embryonic development (not necessarily the middle third in time).

TSD responses in reptiles vary considerably by species. In crocodylians, a female-male-female pattern is ubiquitous (Fig. 2(F)). For example, in American alligator (*Alligator mississippiensis*), if embryos are incubated below 31.5°C or above 35°C during the thermosensitive period, monosex female populations can be obtained, while the percentage of males increases and can reach 100% at 32.5–33.0°C (Lang and Andrews, 1994). On the other hand, red-ear slider turtles (*Trachemys scripta*) exhibit a male-female pattern of TSD where incubation of embryos at 26 or 31°C produce monosex female or male populations, respectively (Fig. 2(E); Wibbels *et al.*, 1991). Likewise, tuatara have a male-female TSD pattern. The range of temperatures at which both sexes are produced varies with species, thought to arise from inter-individual variability in the temperature that defines the boundary between male and female.

Another fascinating case that highlights the interplay between environmental and genetic mechanisms is the Australian central bearded dragon (*Pogona vitticeps*). This species has a ZZ/ZW system, however high temperature may override this GSD system and induce female sex reversal (ZZ females) under experimental conditions (Quinn *et al.*, 2007). It was recently reported that such sex reversal can also occur naturally in wild dragon populations and that sex-reversed females are viable and fertile (Holleley *et al.*, 2015). Further, levels of GSD versus sex reversal were compared among progeny from ZW and sex-reversed ZZ mothers, in both cases mated with normal ZZ males. Progeny from normal ZW mothers were dominant in the range of 22–32°C (phenotypic sex ratio of 1:1), but temperature began to interact with and override GSD above 32°C. Conversely, in progeny from sex-reversed ZZ mothers, sex was determined solely by temperature, with no genetic influence observed. The female W-chromosome was thus eliminated from the lineage in just a single generation. This demonstrates a rapid transition under experimental conditions from a predominantly GSD system (with heteromorphic sex chromosomes) to an ESD system (Holleley *et al.*, 2015). Such a transition in the wild can be driven entirely by Fisher's frequency-dependent selection, without the need to invoke conventional selection. As environmental temperatures rise and the frequency of sex reversal from male ZZ to female ZZ increases, the overproduction of females invokes frequency-dependent selection. Individuals that produce more of the rarer sex, in this case males, have a massive selective advantage in that they will automatically contribute more grandchildren to forthcoming generations. This can drive down the frequency of the W chromosome in the wild populations and, ultimately, is thought to lead to its complete loss. Offspring sex is then entirely determined by temperature – that is, TSD (Holleley *et al.*, 2015). This rapid transition provides an alternative mechanism to the prevailing view that ESD evolves in response to a sex-specific fitness advantage, the role of conventional selection being to consolidate and maintain the transition by optimizing the response of sex ratios to temperature. A parallel case occurs in the Australian three-lined skink (*Bassiana duperreyi*) which has an XX/XY system, with XX individuals reprogrammed to become males at low incubation temperatures (Dissanayake *et al.*, 2021).

Sex Determination Mechanisms and Key Players

Mechanisms in Fishes

Advancement of genomics technologies has led to significant progress in the identification of master sex-determining genes in cold-blooded vertebrates, particularly in fishes (Table 1, Nagahama *et al.*, 2021). Interestingly, warm-blooded vertebrates, like

Table 1 The diversity of proposed master sex-determining genes in cold-blooded vertebrates, their associated proteins and functional categories. For further information, see reviews by Nagahama *et al.* (2021) and Kitano *et al.* (2024)

<i>Taxon</i>	<i>Gene</i>	<i>Associated protein</i>	<i>Functional category</i>	<i>Example species</i>	
Fishes	<i>amhy / amhby</i>	Anti-Mullerian hormone	Transforming growth factor beta	Patagonian pejerrey <i>Odontesthes hatcheri</i>	
	<i>amhr2 / amhr2y / amhr2by</i>	Anti-Mullerian hormone receptor 2	Transforming growth factor beta	Tiger pufferfish <i>Takifugu rubripes</i>	
	<i>bcar1</i>	Breast cancer antiresistance 1	Scaffold protein	Channel catfish <i>Ictalurus punctatus</i>	
	<i>bmpr1bby / bmp1ba</i>	Bone morphogenetic protein receptor type 1	Transforming growth factor beta	Atlantic herring <i>Clupea harengus</i>	
	<i>dmrt1 / dmrt1by / dmy</i>	Doublesex and mab-3 domain	Transcription factor	Medaka <i>Oryzias latipes</i>	
	<i>fshry</i>	Follicle-stimulating hormone receptor	Gonadotropin receptor	Senegalese sole <i>Solea senegalensis</i>	
	<i>gdf6 / gdf6b</i>	Growth differentiation factor 6	Transforming growth factor beta	Turquoise killifish <i>Nothobranchius furzeri</i>	
	<i>gsdfy</i>	Gonadal soma-derived factor	Transforming growth factor beta	Luzon medaka <i>Oryzias luzonensis</i>	
	<i>id2bby</i>	Inhibitor of DNA binding 2b	Transcription factor	Arapaima <i>Arapaima gigas</i>	
	<i>hsd17b</i>	Hydroxysteroid dehydrogenase 17 beta	Steroidogenic enzyme	Yellowtail <i>Seriola quinqueradiata</i>	
	<i>pfpdz1</i>	PDZ domain-containing protein	Signal transduction regulation	Yellow catfish <i>Pelteobagrus fulvidraco</i>	
	<i>sdv</i>	Sexually dimorphic on the Y chromosome	Interferon regulatory factor	Rainbow trout <i>Oncorhynchus mykiss</i>	
	<i>sox2</i>	Sex-determining region Y-related HMG box 2	Transcription factor	Turbot <i>Scophthalmus maximus</i>	
	<i>sox3y</i>	Sex-determining region Y-related HMG box 3	Transcription factor	Indian medaka <i>Oryzias dancena</i>	
	<i>sult1st6y</i>	Sulfotransferase family 1, cytosolic sulfotransferase 6	Inhibition of estrogens	Pacific bluefin tuna <i>Thunnus orientalis</i>	
	<i>zky</i>	Zinc knuckle on the Y chromosome	Zinc binding motif	Atlantic cod <i>Gadus morhua</i>	
	Amphibians	<i>dmw</i>	Doublesex and mab-3 domain	Transcription factor	African clawed frog <i>Xenopus laevis</i>
		<i>zar</i>	Androgen receptor on the Z chromosome	Steroid receptor	Japanese wrinkled frog <i>Glandirana rugosa</i>

mammals and birds, show a high degree of conservation in sex-determining genes; nearly all mammals use *SRY* and most or all birds use the transcription factor *DMRT1*. With some exceptions, fishes have experienced high turnover of sex-determining genes. For example, just within the genus *Oryzias*, four unique sex-determining genes have been identified, two transcription factors (*dmrt1by* and *sox3y*) and two transforming growth factors (*gsdfy* and *amhy*) (Kitano *et al.*, 2024). High gene turnover among these closely related species contrasts with conservation of *sdv*, a former immune-associated gene, as the master sex-determining gene in all salmonid fishes (genera *Salmo*, *Oncorhynchus*, and *Salvelinus*). The reason for the evolution of such a high number of sex-determining genes in fishes and variability among genera is unknown, although with identification of more master genes, a pattern based on life history or phylogeny may ultimately emerge.

The mechanisms of ESD, particularly high temperature-induced masculinization in some fishes, are exerted, in part by the stress-endocrine (or hypothalamic-pituitary-interrenal gland; HPI) axis (Hattori *et al.*, 2020; Lema *et al.*, 2024). Broadly, the primary response to the perception of a stressor involves a neuroendocrine cascade involving the secretion and synthesis of catecholamines and glucocorticoids. As noted above, cortisol in particular has been identified as an important mediator between temperature and sexual development. The first response of the neuroendocrine system to environmental stress is an increase in hypothalamic corticotropin-releasing hormone (Crh). Crh then stimulates the secretion of adrenocorticotropic hormone (Acth) from the pituitary gland, which in turn regulates cortisol levels from the interrenal glands. In medaka and pejerrey, for instance, transcripts for *crh* and its receptor *crhr* were upregulated at high water temperature during the sexually labile period and were associated with elevated whole-body cortisol. In addition, larvae treated with exogenous cortisol exhibited elevated levels of androgen, testosterone, and 11-ketotestosterone (11-KT) concomitant with up- and down-regulation of genes associated with testicular (e.g., *dmrt1*, *amh*) and ovarian development (e.g., *cyp19a1a*), respectively. A key player in the crosstalk between the stress and reproductive endocrine axes is the steroidogenic enzyme, 11 β -hydroxysteroid dehydrogenase (Hsd11 β 2). In fishes, Hsd11 β 2 is responsible for the inactivation of glucocorticoids and the synthesis of androgens, like 11-KT. In pejerrey, 11-KT production is induced by the cortisol-mediated activation of *hsd11b2* gene expression. These observations support a key role for stress and cortisol in high temperature-induced masculinization. There is also evidence for a direct inhibitory effect of cortisol on the feminization process in fishes. Gonadal E2 production is regulated by *Cyp19a1a* activity, which is regulated by follicle-stimulating hormone (Fsh) from the pituitary via activation of the cAMP second messenger pathway. In olive flounder (*Paralichthys olivaceus*), exposure to high temperatures induces cortisol production. Cortisol binds to its receptor and the hormone-receptor complex suppresses *cyp19a1a* expression (Yamaguchi *et al.*, 2010). The consequent reduction in E2 production impairs ovarian differentiation in XX fish and induces masculinization.

Recently, an interaction between the HPI and hypothalamic-pituitary-thyroid (HPT) axes during high temperature-induced masculinization has been reported (Castañeda-Cortés *et al.*, 2023). In medaka, high temperature-induced elevations of *crh* expression upregulate thyroid-stimulating hormone beta subunit (*tshb*) mRNA levels and triiodothyronine (T3) hormone, which in turn induces masculinization of XX fish by concomitant up- and down-regulation of gonadal soma-derived factor (*gsdf*, a gene influencing testicular differentiation) and *cyp19a1a*, respectively. Thus, high temperature-induced masculinization of medaka is orchestrated by changes in HPI, HPT, and HPG axis signaling.

More direct evidence of epigenetic regulation of genes involved in sex determination/differentiation has also been documented in fishes. For example, in European seabass (*Dicentrarchus labrax*), high temperature can increase methylation of the *cyp19a1* promoter, inactivating its expression and leading to masculinization (Navarro-Martín *et al.*, 2011). In contrast, in the half-smooth tongue sole (*Cynoglossus semilaevis*), exposure to high temperatures reduced methylation of the *dmrt1* promoter (hypomethylation), resulting in masculinization of ZW females. Notably, hypomethylation in these ZW males was heritable to ZW-genotype progeny that later developed as phenotypic males without high temperature exposure (Shao *et al.*, 2014). Thus, high temperature-induced sex reversal in the parental line may result in a less stable GSD system in the progeny (Piferrer, 2021).

Mechanisms in Amphibians

The GSD mechanism in amphibians is best characterized in *Xenopus laevis*, which as mentioned above, has a ZZ/ZW system. The master sex-determining gene in this species is *dmw*, a paralog of *dmrt1*, which was identified on the female W chromosome (Yoshimoto *et al.*, 2008); *dmrt1* is a key upstream inducer of male development in *X. laevis* and other vertebrates. In ZW individuals, *dmw* inhibits transcription of *dmrt1*, which in turn inhibits the male pathway and induces female development. So far, *dmw* is the only fully verified sex-determining gene identified in amphibians, and only “W-linked,” dominant female sex-determining gene identified in any vertebrate.

Although the master sex-determining gene has not yet been identified in any other amphibians, autosomes that are anticipated to ultimately become sex chromosomes have been identified in true frogs; they are chromosomes 1, 2, 3, 4, 7 and 9 in 13 complements of the basic karyotype ($2n=26$), and are referred to as “potential sex chromosomes” (Miura, 2018). They include the orthologous genes for sex determination identified in vertebrates, such as *dmrt1*, *amh*, *amhr*, and *sox3*, among other genes involved in gonadal sex differentiation. Moreover, sex chromosome turnover (a transition between sex chromosomes and autosomes) is repeated between the six members without any chromosomal rearrangements. Recently, chromosome 13 was added to the six members (Kuwana *et al.*, 2021).

Mechanisms in Reptiles

No master sex determining gene has yet been confirmed for reptiles, though there are some promising candidates. In dragon lizards, the gene *nr5a1* encoding steroidogenic factor 1 (Sf1) is a promising sex determining candidate. The gene differs little in sequence between the Z and W chromosomes, but there is a major difference in the transcript isoform composition between male ZZ and female ZW individuals. ZZ individuals have three transcript isoforms of *nr5a1*, whereas ZW individuals have those three plus an additional 13 isoforms, many of which are abbreviated. This opens the possibility that the abbreviated isoforms can compete with the canonical isoform to decrease the efficacy of *nr5a1* in the ZW individuals (Zhang *et al.*, 2022). As Sf1 is integral to the male-producing regulatory pathway of vertebrates, the male pathway would be impeded by the abbreviated transcripts in the ZW individuals, and a female phenotype is produced.

For decades, understanding of the mechanisms of TSD in reptiles has eluded science. The epigenetic regulation of sex determination by environmental temperature does not need to involve genes that reside on sex chromosomes, even if they existed (as in cases of thermal sex reversal). Indeed, any autosomal gene that comes to influence a key gene or gene product early in sex differentiation could be a candidate. Thus, for a time, the problem of identifying a temperature-sensitive sex determining gene appeared intractable, leading to the proposition of a system-wide displacement of regulatory pathways determining sex (the consensus or parliamentary model; Georges *et al.*, 2010). However, recent studies have led to several important discoveries. In the American alligator it was recently revealed that thermosensitive transient receptor potential (TRPV) channels can act as molecular environmental sensors and are involved in the masculinization process during TSD (Yatsu *et al.*, 2016). These TRPV calcium channels are thermosensitive in their action *in vitro*, and led to the proposition that cellular calcium ion concentration coupled with reactive oxygen species (calcium-redox balance) is the means by which the cell captures the signal of environmental temperature (Fig. 3; Castelli *et al.*, 2020). This is thought to trigger epigenetic responses via ubiquitous cellular signaling pathways that influence the action of highly conserved chromatin modifier genes, those responsible for opening or closing opportunities for particular genes to express in the cell. These ideas are most well developed in red-eared slider turtles (Weber and Capel, 2021), in which the chromosome modifier gene *kdm6b*, under the influence of these signaling pathways, associates with the promoter of *dmrt1*. Experiments showed that *dmrt1* is both necessary and sufficient for masculinization of slider turtles and, importantly, that environmental temperature, via the products of *kdm6b* and *stat3*, affects the DNA methylation (epigenetic) pattern in a sex-specific manner in the promoter region of *dmrt1*.

Another element of the epigenetic mechanism of chromatin modification (Fig. 3) has broad implications for our understanding of reptile TSD. It involves thermosensitive intron retention in chromatin-modifying genes, including histone demethylase *kdm6b* and histone methylase *jarid2* (Deveson *et al.*, 2017). These intron retention mechanisms are ancient, as the same intron retention occurs in turtles, crocodiles and dragon lizards. This study revealed that intron retention is favored by extreme rearing temperatures in dragon lizards, affecting gene function and altering the epigenetic landscape (Deveson *et al.*, 2017). This work on turtles and bearded dragons is exciting because it suggests that, far from a consensus or parliamentary system of thermal influence, a set of highly conserved and ancient components of cellular epigenetic machinery are central to the mechanisms by which temperature affects sexual outcomes in both TSD and sex reversal.

Conservation of Downstream Players

Regardless of the sex determination mechanism, downstream signaling associated with gonadal sex differentiation appears to be highly conserved among vertebrates and reflects antagonistic pathways leading to either ovarian or testicular differentiation. In cold-blooded vertebrates, sex-specific downstream patterns of gene expression related to steroid biosynthesis and differences in steroid production are ubiquitous (Nagahama *et al.*, 2021). A now classic example of this is *cyp19a1* encoding Cyp19a1/aromatase, which appears to have a highly conserved role in ovarian development and maintenance, and is thought to be a major player in sexual differentiation.

Conversely, androgen production appears to be an important component of testicular differentiation and development in some cold-blooded vertebrates. Treatment with androgens can generally masculinize genotypic female individuals. However, interestingly, no studies to our knowledge have demonstrated a masculinizing effect of androgens in reptiles. Environmental effects on sex ratios have also been linked to changes in gonadal production of estrogens and androgens.

Conclusions and Future Considerations

Mechanisms of sex determination are highly variable in cold-blooded vertebrates; much more so than in warm-blooded vertebrates. Two major systems of GSD (XX/XY and ZZ/ZW) are found in cold-blooded vertebrates and coexist with ESD in reptiles and fishes. In addition, there are examples of slight variation on these major systems, as well as complex, polygenic systems. Among cold-blooded vertebrates, GSD systems appear to show no clear phylogenetic segregation, suggesting there is little or no evolutionary pressure toward a unified system. This is further supported by the fact that the major GSD systems have independently evolved numerous times in closely related species, and in some cases even co-occur within a species. Additionally, a growing number of unique sex-determining genes from a variety of functional categories have been identified in fishes.

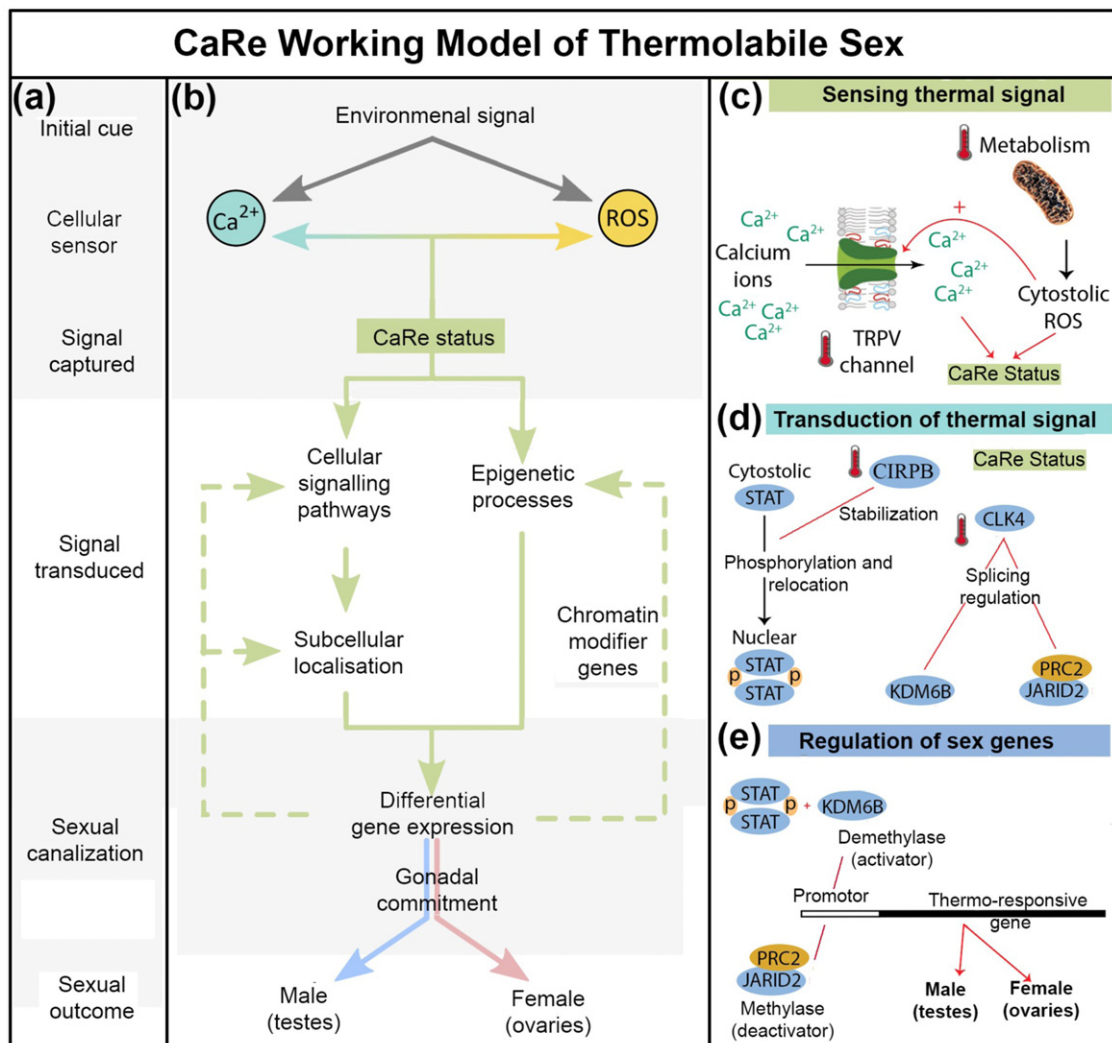


Fig. 3 The mechanisms of temperature-dependent sex determination (A) are thought to involve ancient and ubiquitous elements of cellular machinery (B). Under this working model, the cell senses temperature via calcium channels (TRPV) in the cell membrane which govern the influx and efflux of calcium ions (C). Temperature also affects metabolic rate and the production of oxidants (reactive oxygen species, ROS). ROS feeds back to the TRPV calcium channels to influence their activity. The balance between calcium ions and ROS is referred to as calcium-redox (CaRe) status. The external thermal signal is captured by the cell as CaRe status (C); the cell “knows” how hot its environment is. CaRe status governs the cellular environment for the phosphorylation of effector molecules (e.g., Stat3 in turtle) to allow them to migrate to the nucleus (D) and for their cytoplasmic stabilization in complexes called stress granules. This stabilization may involve thermosensitive genes like *cirbp* (D). Stat3 in turtle associates with chromatin modifier *Kdm6b* to allow it to do its work as a demethylase in activating the target sex gene *dmt1* leading to male development (E). A second chromatin modifier gene, *jarid2*, is a methylase that works in opposition to *kdm6b*. It forms a complex with polycomb repressive complex 2 (PRC2, panel D), the primary complex of the histone methylation of histone marker H23k27; *jarid2* gives this complex its target specificity. Temperature-dependent splicing regulation of RNA transcripts of the two chromatin modifier genes *kdm6b* and *jarid2* has been found. Transcripts of both genes do their work in the context of truncated, potentially competitive isoforms, the production of which is temperature sensitive (D). Thus, programming (TSD) or reprogramming (sex reversal) of sex is governed by complex pathways that involve several steps thought to be temperature sensitive – a caucous of influences in a context of highly conserved and ubiquitous epigenetic machinery.

ESD is generally considered a highly plastic, ancestral mechanism, yet this form of sex determination has stood the test of time. Reptilian species with ESD are numerous, whereas environmentally-relevant ESD has been ascribed to relatively few fishes and no amphibians. Still, there are numerous reports of environmental factors (e.g., temperature, pH) that affect sex ratios. Some of these cases are considered anomalous, thermal sex ratio distortions. However, more field-based experiments are needed to conclude on the prevalence of ESD. Mounting evidence also indicates that GSD and ESD are not mutually exclusive mechanisms, as once believed, and that even in classical ESD species, the genome and/or epigenome play important roles. As noted by Bull (2015), GSD and ESD mechanisms can each be highly functional and adaptive, and may, in theory, evolve back-and-forth along neutral pathways in an evolutionary progression.

The high degree of variance in sex determination among cold-blooded vertebrates may leave one with the impression that there is little semblance of order in this biological event. However, downstream processes, such as those associated with steroid biosynthesis, appear to be highly conserved. This suggests that there are several paths toward the same outcome, and that upstream mechanisms can change/shift, as long as critical connections to downstream factors that drive development of the two sexes are maintained.

We also touched on several cases of stability in sex determination in this article. A prime example of this is snakes of Caenophidia, which all exhibit GSD and a ZZ/ZW system, despite the fact that their fellow reptilians have diverse sex determination mechanisms. While there are several theories as to why snakes of this clade display this remarkable stability (e.g., shorter lifespan), the answer remains unknown.

Given the intimate association of cold-blooded vertebrates with their environment, especially with regard to temperature, it is not altogether surprising that they have maintained a higher level of plasticity in some physiological mechanisms relative to warm-blooded vertebrates. The fact that TSD has not been replaced in some extremely old vertebrate taxa (e.g., crocodylians) is intriguing. One might imagine that millions of years of environmental change and evolutionary pressure would have forced such change. Yet, TSD persists. One possibility is that natural environmental change typically occurs very slowly, allowing time for adaptation of ESD mechanisms. This is not the case however with climate change currently being driven by anthropogenic causes. There are limits to the abilities of TSD and thermally-sensitive species to accommodate climate change via latitudinal and elevational shifts in distribution, timing of nesting, and/or nest site selection. The question now is whether species with TSD will be able to survive in our rapidly changing world, and if more species with GSD will begin to display well-documented thermal sex ratio distortions observed in the laboratory, and more recently in the field. We do not currently have evidence of widespread occurrence of thermal sex reversals in nature, however it is imperative to continue research in this area and model long-term consequences of such effects.

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