# The ends of a continuum: genetic and temperature-dependent sex determination in reptiles

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**Summary**

**Two prevailing paradigms explain the diversity of sex-determining modes in reptiles. Many researchers, particularly those who study reptiles, consider genetic and environmental sex-determining mechanisms to be fundamentally different, and that one can be demonstrated experimentally to the exclusion of the other. Other researchers, principally those who take a broader taxonomic perspective, argue that no clear boundaries exist between them. Indeed, we argue that genetic and environmental sex determination in reptiles should be seen as a continuum of states represented by species whose sex is determined primarily by genotype, species where genetic and environmental mechanisms coexist and interact in lesser or greater measure to bring about sex phenotypes, and species where sex is determined primarily by environment. To do otherwise limits the scope of investigations into the transition between the two and reduces opportunities to use studies of reptiles to advance understanding of vertebrate sex determination generally. *BioEssays* 26:639–645, 2004. © 2004 Wiley Periodicals, Inc.**

## Introduction

Sex determination is a fundamental biological process that is of profound importance for the development of individuals and the formation of sex ratios in natural populations.(1) It is therefore a phenomenon of significance in biological evolution. Although considerable understanding has been developed through comparisons of the mammalian sex-determination systems,(2) there is much to be learned from the many organisms with other forms of sex determination. Here, we argue that reptiles with their high lability in sex-determining systems can provide important models for investigating the evolution of sex-determination systems in vertebrates. In particular, the intrageneric distribution within some reptile families of environmental and genetic modes of sex determination and the apparent interaction of both modes within some species provide the opportunity for considerable insight into this important process.

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Sex differentiation is the development of the testes or ovaries from indifferent or undifferentiated gonads.(3) This is not to be confused with sex determination, the focus of this essay, which is the process that directs differentiation to proceed down one or the other pathway, male or female. Mammals show a genetic or chromosomal form of sex determination, referred to as genotypic sex determination or GSD, with all but mole voles and a spiny rat(4,5) having an XY male/XX female system. Birds too have a stable chromosomal sex-determining system—all taxa have female heterogamety.(6) Perhaps because of these inflexible patterns in birds and mammals, we are accustomed to thinking of sex determination as under genetic control and fixed at conception.

In contrast to both birds and mammals, reptiles have an impressive array of sex-determining modes, comparable to the variety observed in fish(7,8) and frogs.(9) Male heterogamety (XY or XXY) is known in turtles,(10) female heterogamety (ZW, ZZW, or ZWW) is known in snakes(11–13) and both are known in lizards.(14) Many species have GSD in the absence of any gross heteromorphy in the sex chromosomes.(15) Many others have temperature-dependent sex determination (TSD),(16) a form of environmental sex determination.

In species with GSD, sex is considered to be determined by genetic factors that operate largely independently of the environment, whereas in reptiles with temperature-dependent sex determination, sex is determined after fertilization by the environmental conditions that prevail during embryonic incubation, and largely independent of direct genetic influences.(17) The conventional view, which emerged from the early work on sex determination in reptiles, is that these two mechanisms are mutually exclusive(18,19) and can therefore be viewed as discrete and fundamentally different.(20,21) Implicit in this perspective is that differences in the mechanisms between the two modes are complex,(22) that they constitute a discrete dichotomous process, and that through appropriate experimental approaches, one can be demonstrated to the exclusion of the other.(21) Pieau,(23) reflecting that proposed for insects,(24) offered an alternative view by suggesting that a common underlying sex-differentiation pathway implied that there were no clear boundaries between TSD and GSD and empirical evidence(25) and a broader taxonomic perspective led Wilkins(26–28) to suggest that it is probable that all sex-determining systems have some genetic component. Recent research on the genes involved in sex differentiation in alligators and turtles with TSD,(29–32) which demonstrates remarkable homology in structure, function and expression of the sex-differentiation genes of mammals and reptiles, lends considerable support to that view.(33)

In this essay, we argue the case that GSD and TSD in reptiles represent the ends of a continuum of states where, in many instances, genetic and environmental influences on sex determination co-exist and interact to produce sexual phenotypes. Rather than a dichotomy between two fundamentally different and complex mechanisms, current evidence suggests that there is a common underlying mechanism of sex differentiation in reptiles and that because of that commonality, variations in sex-determination mechanisms may be effected at several points in the sexual differentiation pathway. Such a scenario suggests that transitions between GSD and TSD mechanisms in reptiles may require only relatively small changes at the molecular level and that environmental and genetic influences on sex determination may co-occur frequently in nature.

## Genetic and temperature-dependent modes of sex determination in reptiles

Perhaps because of their novelty when compared with mammals and birds, most of the work on sex determination in reptiles has focussed on species with TSD. In the original typical model case of TSD in reptiles established for turtles, only one sex is produced at high temperatures, and the other sex at low temperatures(34) (but see also Ref. 35). A very narrow range of temperatures (referred to as the threshold temperature or pivotal temperature, but more properly as the pivotal range) produces both males and females and separates male-producing temperatures from female-producing temperatures. The extent of the pivotal range varies greatly among species, and many species have upper and lower pivotal ranges, with females produced at both extremes of temperature and males at intermediate temperatures.(36) The thermosensitive period, during which sex is irreversibly determined by temperature, is generally considered to lie in the middle third of development.(37)

There is general consensus that temperature exerts its influence in species with TSD by acting upon the genetic mechanisms that govern steroidogenic enzymes or steroid hormone receptors, thus altering the hormone environment of the sexually indifferent embryo and directing development in either a male or a female direction.(22,38) Administration of exogenous oestrogen in turtles will override the effect of a male-producing temperature to yield female hatchlings(39,40) and the period of sensitivity to exogenous oestrogen coincides with the thermosensitive period.(41) In reptiles, synthesis of oestrogens depends on the aromatization of testosterone and androstenedione to the oestrogens estrone and estradiol-17β. Administration of aromatase inhibitors to eggs incubated at female-producing temperatures will yield male hatchlings. In TSD reptiles, the inhibitors have a progressively more potent effect as the pivotal range is approached. Mechanisms by which sexual differentiation is influenced by the hormonal environment, involving androgens, oestrogens and aromatase, are conserved across birds (GSD),(42) reptiles (TSD),(40) amphibians(43) and fish.(8) In TSD reptile species, temperature exerts an influence at some point in the otherwise highly conserved and complex sequence of steps that leads to differentiation of the gonad.

Most understanding of GSD in reptiles comes through analogy to mammals and birds because there has been only limited investigation of GSD mechanisms in reptiles.(44,45) In most eutherian mammals, sex is determined by the presence of the *SRY* gene on the Y chromosome.(46) *SRY* is present on the Y chromosome of marsupials(47) but does not control all facets of sex determination in this group.(48) It appears to be absent from all non-mammalian vertebrates, and is probably evolved from a *SOX* gene.(49) These observations suggest that the *SRY* gene has evolved relatively recently.(50) The exact mechanism of sex determination in birds has thus far eluded investigators. The *DMRT1* gene on the Z chromosome has been suggested as a sex-determining gene(6) acting via a dosage mechanism, but this is yet to be confirmed. With the complex patterns of genetic sex determination involving both XY and ZW systems, it is unlikely that a single GSD mechanism or sex-determining gene is conserved among reptiles. We may expect sex-determining genes equivalent to *SRY* and *DMRT1* in reptiles with XY and ZW systems but, given that GSD has probably evolved multiple times, numerous forms of such sex-determining genes may be expected.

## Sex-related gene expression in reptiles and mammals

Conservatism in the hormonal environment in which the gonad develops is reflected in the presence and expression of sex-related genes. Recent molecular studies have shown that, with the notable exception of *SRY*, many genes involved in gonadal differentiation in mammals discovered thus far (*SF1*, *DMRT1*, *SOX9*, *AMH*, *DAX1* and *WT-1*) have homologues in reptiles with TSD. A number of these, including *DMRT1*, *SOX9*, *SF1*, *DAX1*, *AMH* and *WT-1* in alligators(31,51) and *DMRT1*, *SOX9* and *WT-1* in turtles(29,30) are expressed during gonadogenesis. This represents extraordinary conservatism in the gonadal developmental pathways among vertebrates.

Some of the sex-differentiation genes conserved across vertebrates are good candidates for involvement in temperature-dependent sex determination. In two reptiles with TSD, the red-eared slider turtle(29) and the American alligator,(31) *DMRT1* is upregulated in the indifferent gonad during the temperature-sensitive period when incubated at male-producing temperatures but downregulated at female-producing temperatures. These studies and others in humans(52) suggest that *DMRT1* has a central function in the development of the testes. In birds and reptiles, the conversion of androgenic steroids to oestrogenic steroids is regulated by the aromatase gene, the transcription of which may be activated by the *SF1* gene or repressed by the *AMH* gene.(53) This process acts to shift the endocrine balance between male and female differentiation in the developing embryo.(39–41)

Extraordinary conservatism across vertebrate orders in the genes involved in sexual differentiation, and the clear potential for some of these conserved genes to be involved in sexual determination, suggests that differences at the molecular level among reptiles with different sex-determining mechanisms might be small, potentially involving a few or perhaps only one gene.

## Transition between GSD and TSD

The changes necessary to bring about a transition between GSD and TSD in reptiles are not well understood. The predominant direction of change is not known, and neither GSD nor TSD can be regarded confidently as the derived state in a related group of organisms with both modes of sex determination. There is an almost haphazard distribution of TSD across the reptile phylogeny, with sister taxa at all levels exhibiting alternate modes of sex determination. At the level of order, crocodilians have TSD,(54) whereas their sister taxon, the birds, do not. At the level of suborder, snakes have GSD with a ZW chromosome sex-determining system whereas lizards exhibit many forms of sex determination including TSD.(19) At the level of family, the pig-nosed turtle *Carettochelys insculpta* has TSD(55) whereas their sister taxon, the Trionychidae, do not,(56) a pattern reproduced in the Pelomedusidae-Chelidae sister pair.(57–60) At the level of genus, *Clemmys guttata* has TSD whereas *C. insculpta* does not.(56) With the exception of the genera *Platemys* (XY, Chelidae), *Staurotypus* (XY, Kinosternidae), *Siebenrockiella* (XY) and *Kachuga smithii* (ZW, Bataguridae), turtles lack heteromorphic sex chromosomes.(10,18,61) Recent work on the Australian dragon lizards (Agamidae) shows a complex distribution of species with TSD versus those with GSD, with even closely related species exhibiting alternate modes of sex determination.(62,63) Similar patterns of differing sex-determining mechanisms among closely related taxa are found across lower vertebrate and invertebrate taxa where the sex chromosomes are not highly differentiated.(26)

It would appear that both GSD and TSD have evolved a number of times in the Reptilia. The question at issue here is whether transitions between the two modes have been affected by the evolution of independent and fundamentally unique genetic mechanisms or by more subtle and possibly reversible modifications of some conserved underlying mechanism of sex determination.

A hypothetical example of the transition from a GSD to a TSD state is instructive (Fig. 1) as it shows how sex determination may be "captured" by an environmental influence such as temperature at any number of points in the pathway leading to male or female differentiation. Let us suppose that the ancestral state is an XY GSD system with a sex-determining gene or gene complex located on the Y chromosome only. Expression of this gene or genes in XY individuals leads ultimately, through a pathway or network(27) of influential

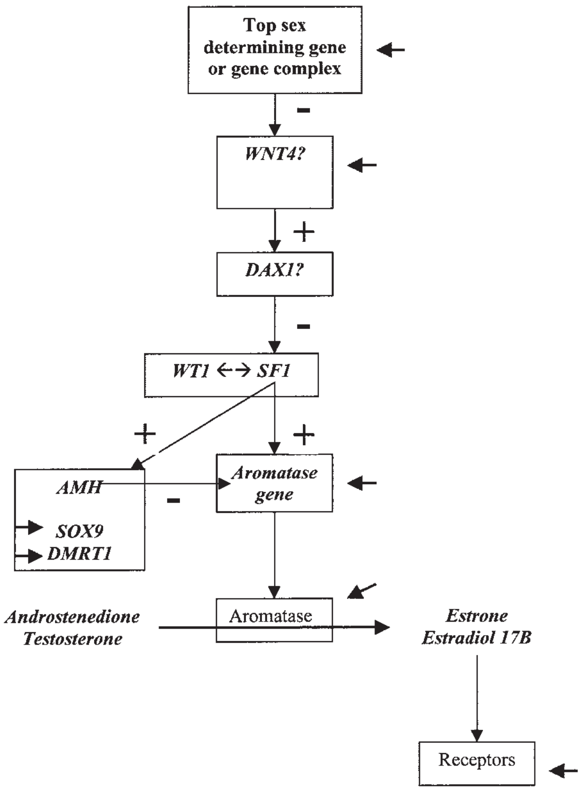

**Figure 1.** Hypothetical pathway for sexual differentiation in a GSD reptile illustrating that there are many points where temperature sensitivity could be effected to bring about TSD (short arrows). Though present in reptiles, the action of some of these genes (e.g. *WNT4*, *DAX1*) has been demonstrated in mammals, not reptiles, and they are included for the sake of illustration. Candidate genes identified by the short arrows are known to be widespread in vertebrates and to cause sex reversal in mammals (e.g. *WNT4*) or reptiles (e.g. *Aromatase*) when they or their products are manipulated. The degree to which the elements of the GSD mechanism are retained will determine the degree to which sex can potentially be influenced by the interaction of genetic and environmental influences.

sex genes (Fig. 1), to downregulation of the aromatase gene, reduction in aromatase activity and, consequently, the production of males. Absence of the sex-determining gene in the XX individuals leads ultimately to higher aromatase activity and the production of females. In this hypothetical example, sex determination could be captured by mutational change giving effect to environmental influences at a number of places in the sex-differentiation cascade. Good candidates might emerge from among the genes common to all vertebrates and known to cause sex reversal in mammals. The sex-determining gene itself could become temperature sensitive in its expression. Temperature sensitivity would be expressed only in XY individuals, not in XX individuals. A base level of female offspring at all temperatures is observed in some lizards and crocodilians,(64–66) but is not universal across all species with TSD. Under this scenario, XY females would be produced at some temperatures leading to the possibility of YY individuals in subsequent generations.

Alternatively, an autosomal sex gene that is influential downstream in the sex-differentiation cascade, could become temperature sensitive in its expression. A tendency for increasing conservatism in the genes as we progress down the differentiation cascade(27,28) suggests that candidate genes higher up in the chain of influence are more likely to be involved in the sex-determining switch mechanism. Genes conserved across vertebrates that have been implicated in sex reversal, or that exhibit dose dependency, are obvious candidates. Dose deficiency in *DMRT1* activity causes male-to-female sex reversal in humans(67) as does inactivation of *SOX9*.(68) Inactivation of *WNT4* causes female-to-male sex reversal and its duplication causes male-to-female sex reversal,(69) though a similar function has not yet been demonstrated in reptiles. Further downstream, in reptiles, the aromatase gene could become temperature sensitive in its expression, though this is now thought to be unlikely in turtles based on work on the gonad/adrenal/mesonephros (GAM) complex (but not in the gonad alone),(70) or a mutation may afford temperature sensitivity in the efficacy of the aromatase protein. In each of these scenarios, sex determination would have been captured by mutational change in a gene downstream in the sex-differentiation cascade. Several other points in the sexual differentiation mechanism have been identified as possible candidates for temperature sensitivity.(33) In each of these cases, sex could potentially be determined by the interplay of the temperature sensitivity of the downstream gene or its products and the differential effect of the presence or absence of the former sex-determination gene(s) carried by the Y. Indeed, if sex determination is captured by mutational change well down the sex-differentiation cascade, the upstream genetic machinery may lead to a predisposition toward one sex or the other, only to be over-ridden by environmental influences. If this were the case, there would be considerable scope for interaction between genetic and environmental influences. Either way, reversal would be relatively simple, involving loss or diminution of temperature sensitivity through a mutation of the gene affording temperature sensitivity.

The above scenario may be an intermediate, and potentially brief, stage in the transition between GSD and TSD. If the YY combination is lethal or leads to less fit individuals, this will favour selection for the elimination of the Y chromosome and with it the genetic mechanism of sex determination. If the Y is eliminated, all individuals would be (autosomal) XX with sex determined by the temperature sensitivity of the new sex-determining gene or its products. Reversal to a GSD condition will require the evolution of independent and probably fundamentally unique genetic mechanisms that may occur on any autosome.

The relative brevity of the coexistence of the genetic and thermosensitive mechanisms of sex determination would depend on the relative fitness of the XX, XY and YY chromosomal combinations. In GSD species where the sex chromosomes are homomorphic (at least grossly), the YY disadvantage may not be great, allowing the genotypic and temperature-dependent sex-determining mechanisms to coexist. Temperature would determine sex as sex reversal, leading to four states: XY males with concordant phenotypic sex, XX females with concordant phenotypic sex, XX males and XY females whose genetic and phenotypic sex are discordant. Relative fitness of each of these states in the social and ecological context of the species would lead to the maintenance of TSD as an interplay between genetic and environmental influences (Y retained), maintenance of TSD as a strictly environmental influence (Y lost), reversal to a GSD system as an evolutionary reversal (Y retained, temperature sensitivity lost) or convergent evolution (Y lost, then regained in another form, perhaps involving a completely different chromosome pair, temperature sensitivity lost).

## GSD and TSD extremes in a continuum

We argue that viewing GSD and TSD as alternate and fundamentally distinct modes of sex determination in reptiles is overly myopic. Such a view is not well supported by studies of other organisms with environmental sex determination.(8,26–28,43) It limits the scope of investigation into the transition between GSD and TSD in reptile studies and so reduces opportunity for such studies to improve our understanding of vertebrate sex determination generally.

Several lines of evidence suggest an interaction between genetic and environmental influences in sex determination of reptiles. The first recorded instance of the coexistence of genetic and environmental elements to sex determination, and the potential for interaction between the two, was in the gecko, *Gekko japonicus*. This species was shown to have heteromorphic sex chromosomes(71) and later demonstrated to exhibit TSD.(72) Unfortunately, the two phenomena were not investigated in a simultaneous study, so the possibility remains that the two investigations were of cryptic variants of the one species. In another example, the minisatellite DNA sequence Bkm, originally isolated from the W-chromosome of the banded krait, was shown by Demas et al.(73) to exhibit sex-specific bands when hybridized to genomic DNA of wild-caught adult green sea turtles (*Chelonia mydas*) and Kemp's Ridley sea turtles (*Lepidochelys kempi*). Both species have TSD when incubated at constant temperatures.(74) The authors suggested that temperature-regulated enzyme activity may cause the sex-specific excision of Bkm-related sequences leading to genetic differentiation between temperature-induced sexes. In this way, temperature may have imposed sex-reversal on an underlying genetic mechanism of sex determination. Demas et al.(73) did not consider the alternatives that these two species may exhibit GSD when incubated under natural conditions or that TSD and GSD may interact to produce less-viable sex-reversed hatchlings that are selected against under natural conditions. Additional investigations are required to fully explain this intriguing observation.

In an important series of laboratory experiments on the European pond turtle (*Emys orbicularis*), a species with TSD, Zaborski et al.(75,76) examined serologically defined H-Y antigen expression in the gonads and in the blood. These two tissue types showed important differences in their expression of this antigen. In the gonads, expression is closely associated with ovarian development whereas expression in the blood is independent of sexual phenotype when eggs are incubated at either male- or female-producing temperatures. However, when eggs were incubated within the pivotal range, H-Y antigen expression in the blood was highly correlated with sexual phenotype. The authors argued that H-Y antigen expression in the blood was indicative of an underlying sexual genotype that was over-ridden by the influence of temperature at temperatures outside the pivotal range. In a subsequent examination of a field population of *Emys orbicularis*, Girondot et al.(77) found that H-Y antigen expression in the gonads and blood was correlated, suggesting that either incubation in the field typically occurred within the pivotal range or that individuals for which genotypic sex was reversed by incubation temperature were less able to persist in the population.

More recent work by Shine et al.(78) provides the strongest evidence yet that TSD and GSD may coexist in reptiles. They incubated eggs from the montane and chromosomally heteromorphic three-lined skink, *Bassiana duperreyi*, under temperature regimes that mimicked natural temperature variation. When temperatures were similar to those in the field at high altitudes in cool summers, sex ratios were significantly skewed. Approximately 70% of eggs produced males, a result that could not be explained by differential mortality. This suggests that, at the lower extremes of the natural range of temperatures experienced during incubation, sex in this species is temperature sensitive resulting in temperature over-riding the underlying genotypic sex that prevails at other temperatures.

## Conclusion

It is now well established that there is great conservatism of the genes involved in sexual differentiation and the hormonal processes that govern differentiation, and the mechanisms by which genetic or environmental factors determine sex can be expected to draw upon elements of this common machinery. The underlying mechanisms governing sex determination in reptiles with TSD and those with GSD may not be as fundamentally different as once thought. Furthermore, there is little doubt that the transition between GSD and TSD has occurred independently several times in the evolutionary history of reptiles. The almost haphazard distribution of TSD and GSD across the reptile phylogeny suggests that the transition between the two is relatively easy to achieve. We believe that the diversity of sex-determining mechanisms observed in reptiles may be an expression of even greater diversity of transitional stages between GSD and TSD among extant species, with at least some species retaining coincident and potentially influential elements of both.

There will be species whose ancestors have moved from GSD to TSD where the transition to TSD is complete with the loss of the GSD mechanism and any genetic predisposition to be one sex or the other, say for example, when the Y chromosome is lost through YY genotype lethality. There will be species that have passed through a TSD state, or emerged from an ancestral TSD state, to express independently derived and novel solutions to achieving GSD. In this, for example, we see species with male heterogamety (XY or XXY),(10,14) female heterogamety (ZW or ZZW)(11,14) and GSD in the absence of gross heteromorphy in the chromosomes. Further study is likely to reveal more cases of the independent evolution of GSD among reptiles. Only one of these is likely to represent retention of an ancestral state for reptiles, if indeed GSD is ancestral for reptiles. There may well be species where elements of the GSD mechanisms have been retained so that there is the potential for coincident influences of both environment and genotype on sexual outcomes. We are seeing evidence of this in *Bassiana duperryii* and *Emys orbicularis*, and it may be quite widespread in reptiles where cytologically homomorphic chromosomes are common in both GSD and TSD species and the YY genotype is not particularly disadvantaged. Some species where the GSD mechanisms have remained largely intact in the presence of an over-riding environmental influence may have since reverted to GSD.

In this context, it is important to see GSD and TSD not as a dichotomy, but rather as a continuum of states represented by species whose sex is determined primarily by genotype, species where genetic and temperature mechanisms are coexisting and which interact in smaller or greater measure to bring about sex phenotypes, and species where sex is determined primarily by temperature. ''Sex reversal'' will often be a transitional step in the continuum between the extreme endpoints of GSD and TSD. Greater attention should be paid to looking for subtle environmental influences of environment on sex in species where sex determination is regarded as genotypic. It should be possible to find molecular markers that segregate with genotypic sex in species with TSD that have an underlying genetic mechanism.

We are exploring these possibilities in our laboratory using sister taxa where one species in the pair has GSD and the other TSD—markers that segregate with sex in the GSD species may also segregate with genotypic sex in the TSD species when incubation is within the pivotal range. This would allow the identification of individuals with concordant and discordant sexes, and the exploration of a range of hypotheses on the relative fitness of such individuals and selective forces likely to maintain or remove TSD.

Reptiles provide tractable approaches to studying sex determination through the provision of eggs before sex differentiation occurs and numerous examples of comparative GSD/TSD models for analysis that may be expected to shed considerable insight on sex determination and its evolution in all vertebrates. Our expectations are that sex-determining genes in GSD taxa have evolved multiple times and at multiple points in the sex-differentiation pathway.

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