

Reptile new insights into the molecular mechanisms underlying temperature-dependent sex determination

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Sex determination, which initiates undifferentiated gonads develop into either a testis or an ovary, is an important process for sexual reproduction to maintain genetic variation and population persistence in almost all vertebrates. Sex determination mechanisms, however, vary greatly among taxa. In contrast to birds and mammals that a genetic factor directs the gonadal fate as genetic sex determination (GSD), many reptiles including all crocodilians, many turtles and some lizards determine offspring sex by environmental temperature cues and genes interact during a critical period of embryogenesis as temperature-dependent sex determination (TSD). As climate change increases temperatures across the globe, the impact on species with TSD is a growing concern. Because warming conditions can excessively feminize populations, the extinction risk for these species is drastically amplified. Evidence of this lies in the leatherback sea turtle, where elevated temperatures skew sex ratios toward females. Exploring thermal influences on genetic processes and gene regulatory networks allow us to uncover the molecular and genetic mechanisms by which temperature can modulate sex determination, thereby supporting the conservation and survival of these species.^{1,2}

Conserved sex-determination regulatory cascades in TSD reptiles

Indeed, TSD has become one of the most captivating research hotspots that have drawn widespread interest among biologists over the past several decades. Since the testes and ovaries anatomical structures show high conservation across vertebrates, it is reasonable to infer that the genes and regulatory cascades controlling gonadal development and cell differentiation are also evolutionarily conserved. Therefore, to uncover the molecular basis of sex determination in reptiles with TSD, researchers have largely focused on homologs of sex-determining genes initially identified in mice, with turtles serving as a primary research model. During the temperature-sensitive period (TSP), three pivotal transcription factors (*Sox9*, *Dmrt1*, and *Foxl2*) and the *Cyp19a1* gene encoding aromatase present distinct expression profiles linked to sex dimorphism and ambient temperature in TSD turtles, establishing them as vital candidate sex-determining genes. Further research indicates that female-producing temperature (FPT) induce *Foxl2* to regulate *Cyp19a1* expression, and elevated aromatase forms a positive loop driving ovarian differentiation in turtle embryos.³ Recent findings have drastically redirected research emphasis: instead of investigating whether core sex-determining genes respond to temperature directly, scientists now prioritize upstream temperature-sensitive epigenetic events that selectively turn on key sex-regulatory genes.¹⁻⁴ However, as temperature may modify any related gene involved in gonadal development, even secondary regulatory factors, provided transcriptional fluctuations redirect embryonic sexual fate. For this reason, it thus comes as no surprise the molecular machinery enabling temperature to drastically reshape sex-determining developmental pathways has long remained elusive.

Upstream temperature sensors initiating TSD cascades

Even so, the proximal sensor of temperature that steers undifferentiated gonads to develop into either testes or ovaries has not been identified. According to the properties of the potential biochemical thermosensors mediating TSD, ideal candidate thermosensors need to satisfy the following three criteria: (1) possess intrinsic thermal responsiveness specifically during TSP of gonadal development; (2) required to interact with core molecular

regulators of confirmed sex differentiation pathways as well as upstream regulatory signaling molecules; and (3) exert conserved biological functions, meanwhile retaining sufficient regulatory flexibility to receive thermal stimuli and transduce thermal signals into sexual outcome across diverse TSD vertebrates. Transient receptor potential (TRP) channels, notably TRPV4, are candidate effectors for the initial heat-sensing step. Experimental evidence from *Alligator mississippiensis* demonstrates that pharmacological inhibition of TRPV4 alters the expression of male pathway genes (*Sox9* and *Amh*) and induces testis-like gonad development. In the red-eared slider turtle (*Trachemys scripta*), TRPV4 is up-regulated at FPT; however, this association remains correlative, and the direct functional link in turtles awaits experimental confirmation.¹ Two-pore domain potassium channels (KCNK) govern membrane potential and tightly regulate thermally induced calcium influx in gonadal thermosensitive steroidogenic cells (TSSCs), marking them as the primary thermal sensors.² Additionally, heat-shock proteins (HSPs) serve as another thermal sensors candidate. In *T. scripta*, several HSPs exhibit sexually dimorphic expression, acting as molecular chaperones to preserve the structural stability of sex-related proteins under different thermal conditions. Alternative temperature-sensitive sensors, such as RNA thermometers, coupled calcium and redox (CaRe) status or other ion channel families, may also play a role as intracellular sensors for external environmental stimuli in TSD organisms but have not been investigated in this system.⁵

Molecular mechanisms of TSD in reptiles: From thermal sensing to gonadal fate

In addition to upstream thermosensors molecules, it remains elusive how ambient thermal signals crosstalk with canonical sex determination pathways, restructure temporary gene regulatory networks in undifferentiated bipotential gonads, and eventually drive gonadal primordia toward ovarian or testicular differentiation. Rather than functioning as isolated events, recent findings suggest these mechanisms interact within a hierarchical cascade: Temperature signal → Thermosensor → Calcium signaling → Epigenetic remodeling → Alternative splicing → Sex-determining genes → Sex differentiation (Figure 1). Thermosensors perceive ambient temperature stimuli to initiate calcium signaling, which modulates protein stability and epigenetic modifications. Those changes drive alternative splicing and sex determining gene expression, ultimately directing the embryo toward female or male developmental pathways (Figure 1).

From thermal sensing to epigenetic remodeling

Epigenetics focuses on regulatory mechanisms that modulate gene transcriptional activity without changing an intrinsic genomic DNA sequence. In TSD species, the epigenetic modification status of genes involved in sex determination cascades is pivotal for triggering testicular or ovarian developmental signaling pathways. DNA methylation and histone modifications can act as transient epigenetic markers, maintaining the activation or silencing pattern of core sex-determining genes and governing male and female developmental program in TSD regulatory systems. Temperature can affect the expression level or functional activity of proteins, for instance by inducing structural alterations and post-translational modifications, and thereby participates in the regulation of sex determination. For *T. scripta* embryos, at MPT, KCNK closure induces calcium influx to aldosterone increase to activate GR, directly upregulating the male gene *Dmrt1*.^{2,4} Conversely, FPT-trig-

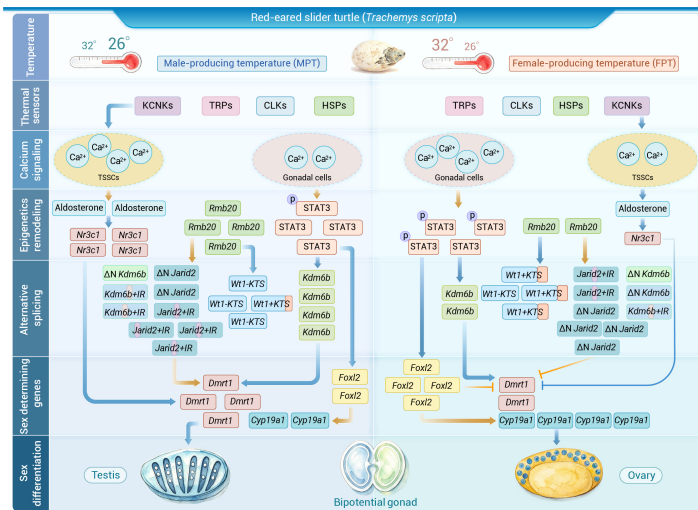


Figure 1. Molecular mechanisms regulating temperature-dependent sex determination in reptiles Gonadal cells can sense ambient temperature signals via thermal sensing molecules, which further trigger widespread intracellular signaling pathway or modify epigenetic regulatory events, ultimately leading to differential expression of sex-determining genes. At female-producing temperatures (FPT), substantial calcium inflow occurs in gonadal cells, inducing the phosphorylation and activation of signal transducer and activator of transcription 3 (pSTAT3). The activated pSTAT3 protein suppresses the expression of *Kdm6b*, thereby altering the methylation status at the promoter region of the male sex-determining gene *Dmrt1* and inhibiting the male developmental program. Meanwhile, pSTAT3 can directly bind to the genomic locus of the female key gene *Foxl2* and facilitate its transcription, which activates the female differentiation pathway. At male-producing temperatures (MPT), KCNK two-pore domain potassium channels closure induces calcium influx to trigger aldosterone production in thermosensitive steroidogenic cells (TSSCs). Aldosterone then signals in a paracrine fashion through glucocorticoid receptors (NR3C1/GR), directly upregulating transcription of the key male-determining gene *Dmrt1*. Additionally, RNA-binding motif protein 20 (RBM20) displays temperature-dependent sexually divergent expression in embryonic early gonadal tissues. This protein suppresses alternative splicing at the KTS sites of the Wilms tumor protein WT1, dynamically regulating the ratio between +KTS and -KTS WT1 isoforms under different thermal conditions. Such temperature-mediated splicing balance further directs gonadal cells toward male or female differentiation fates. Furthermore, both *Kdm6b* and *Jarid2* undergo sex-biased alternative splicing, generating multiple mRNA isoforms with significant sexual dimorphism. To date, the exact biological functions and underlying regulatory mechanisms of these differentially spliced isoforms remain poorly understood in the TSD regulatory network, requiring further systematic exploration.

gered robust calcium influx in gonad cell induce STAT3 phosphorylation (pSTAT3), which represses *Kdm6b* expression to alter the methylation status of the *Dmrt1* promoter and inhibit male development, while directly active *Foxl2* transcription to initiate female development.^{1,3} Furthermore, histone modification levels of JARID2 and KDM6B are strongly correlated with incubation temperature across multiple reptile species, confirming that epigenetic modulation serves as a core link between temperature and the sex-determining pathway.¹

From epigenetic remodeling to alternative splicing

Epigenetic changes can alter chromatin accessibility and recruit splicing factors, which expands genomic complexity by generating diverse transcripts. In TSD, AS may establish an initial molecular bias to trigger specific gonadal pathways and reinforce chromatin modifications to stabilize sexual fate. Sexually dimorphic AS patterns have been identified in TSD reptiles, among which *Kdm6b* and *Jarid2* are particularly representative. In *T. scripta*, the RNA binding motif protein RBM20 acts as a key splicing regulator during gonadal development by controlling the KTS splicing events of *Wt1*.⁵ Notably, the catalytic activity of CDC-like kinases (CLKs) is also sensitive to physiological temperature changes. Lower ambient temperature enhances the phosphorylation of splicing activator serine/arginine-rich protein (SR proteins), further initiating temperature-dependent alternative splicing. Among these kinases, CLK4 is considered to be involved in the regulation of alternative splicing in reptiles employing TSD.¹

Integration upon sex-determining genes to sex differentiation

Through this sequential cascade—spanning protein phosphorylation, chro-

matin remodeling, and transcript variant processing—ambient thermal cues are systematically translated into a molecular bias. The concerted action of STAT3-mediated transcriptional control, KCNK-aldosterone-GR-facilitated transcriptional change, KDM6B-directed histone demethylation, and CLK4/RBM20-regulated alternative splicing, ultimately converges on the sex-determining gene network, primarily centering on the pivotal sex-determining transcription factors (*Dmrt1* and *Foxl2*). This integrated regulatory output resolves the bipotential gonadal primordia differentiate into a committed testicular or ovarian developmental trajectory during embryogenesis.¹⁻⁴

Open challenges and perspectives

Temperature dominates the regulation of sex determination in species with TSD. Accordingly, mechanistic studies ought to focus on cellular processes that respond to various external stimuli, integrate multiple upstream signals, and trigger unified cellular reactions. Looking forward, key mechanistic questions remain regarding whether calcium activates STAT3 directly via CAMK or indirectly through calcium reservoir homeostasis. Evolutionarily ancient responsive pathways, including NF- κ B, heat shock, and antioxidant cascades, are hypothesized to participate in TSD regulation but require urgent functional validation. Additionally, identifying the specific ion channels initiating calcium signaling at FPT is essential to complete the TSD regulatory blueprint. Beyond this, key challenges lie in understanding the spatiotemporal integration of post-transcriptional and metabolic pathways. Deciphering how transient thermal signals are transduced into stable chromatin memory and elucidating the role of alternative splicing remain major frontiers. With expanding genomic resources and the growing accessible functional assays across diverse organisms, we expect to further clarify the intracellular signaling pathways that connect environmental thermal cues to gene transcription networks in TSD. By elucidating the transduction of temperature signals into sexual phenotypes will aid in establishing targeted conservation approaches to mitigate the negative impacts of ongoing climate change. Specifically, integrating these physiological insights with adaptive management strategies, including optimizing artificial incubation protocols, implementing nest thermal manipulation to restore balanced sex ratios, and prioritizing the protection of male-producing thermal refugia, will be crucial for safeguarding the demographic stability of TSD species under future climate scenarios.

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AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

No new data were created or analyzed in this study.