Received: 25 June 2022 | Revised: 14 October 2022 | Accepted: 5 December 2022

DOI: 10.1002/bies.202200123

BioEssays

IDEAS & SPECULATIONS

Insights & Perspectives

Three dimensions of thermolabile sex determination

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Abstract

The molecular mechanism of temperature-dependent sex determination (TSD) is a long-standing mystery. How is the thermal signal sensed, captured and transduced to regulate key sex genes? Although there is compelling evidence for pathways via which cells capture the temperature signal, there is no known mechanism by which cells transduce those thermal signals to affect gene expression. Here we propose a novel hypothesis we call 3D-TSD (the three dimensions of thermolabile sex determination). We postulate that the genome has capacity to remodel in response to temperature by changing 3D chromatin conformation, perhaps via temperature-sensitive transcriptional condensates. This could rewire enhancer–promoter interactions to alter the expression of key sex-determining genes. This hypothesis can accommodate monogenic or multigenic thermolabile sex-determining systems, and could be combined with upstream thermal sensing and transduction to the epigenome to commit gonadal fate.

INTRODUCTION

Sex determination is the process by which a bipotential gonad is directed down a male (testis) or female (ovary) developmental pathway. In mammals, and many other vertebrates, sex is controlled by sex chromosomes, defined by bearing sex-determining genes that trigger conserved testis or ovary developmental pathways. The master switch that triggers male or female development is strikingly different in different lineages, but gonad development is similar across vertebrates and the complex molecular pathways of sexual differentiation are relatively conserved.[1,2]

In all XY therian mammals, the Y-borne *Sry* is testis determining,[3] and in all ZW birds, the Z-borne *Dmrt1* determines sex in a dosage-dependent manner.[4] However, reptiles present a truly impressive array of different sex-determining systems. Among reptiles with genetic sex determination (GSD), turtles have male or female heterogamety (XY and ZW) (reviewed in Bista and Valenzuela[5]), most snakes have female heterogamety (ZW or ZZW)[6] and both male and

Abbreviations: 3C, chromosome conformation capture; 3D, three-dimensional; 3D-TSD, three dimensions of thermolabile sex determination; ESD, environmental sex determination; TSD, temperature-dependent sex determination; GSD, genetic sex determination; Hi-C, high-throughput chromosome conformation capture; TAD, topologically associated domain; Pg, progesterone; PRC, polycomb repressive complex.

Paul D. Waters and Aurora Ruiz-Herrera contributed equally to this study.

female heterogamety are observed in lizards (as well as XXY).[7] Sex chromosomes may be more or less differentiated.

In contrast to these systems of GSD, some vertebrates use environmental triggers, such as temperature, to determine sex (environmental sex determination, ESD). The most common ESD mechanism, temperature-dependent sex determination (TSD), was discovered in reptiles over 50 years ago,[8] challenging the orthodoxy of GSD that then prevailed. However, there are other systems of ESD involving different cues, including (but not exclusive to) social structure and resource availability (reviewed in Nagahama et al.[9]). ESD raises major questions about how continuous variation in an external environmental signal can shift cellular fate and commit development to either the ovary or testis molecular pathways.

Decades of research have yielded limited insight to the mechanisms by which temperature controls sex.[10,11] The problem of discovering epigenetic modification to an unknown differentially expressed gene (or genes) seems intractable. It is not clear whether focus should be on one candidate sex-determining gene, or a set of about 60 conserved sex genes (not necessarily on sex chromosomes in any species). Any gene or gene product that promotes or shifts the trajectory of male and female development, even if it acts indirectly, is a candidate for thermal influence. There is also the possibility of a consensus or parliamentary system, whereby the regulatory actions of many – perhaps all – sex differentiation genes are collectively displaced by temperature to influence sexual outcomes.[12,13] Even if a common thread was established by studies of traditional thermal sensing (e.g., cytosolic Ca^{2+} and reactive oxygen species balance, alternative intron retention, etc.[11,14–16]), this still leaves many candidate chromatin remodellers (epigenetic writers, readers or erasers) that can influence sex gene activation or suppression through differential transcription and isoform composition. Thus, identifying the mechanism of TSD has been, and remains, a difficult problem.

The fundamental questions underpinning TSD are (1) how is the thermal signal sensed? and (2) once captured, how is this signal transduced into epigenetic change that releases or represses expression of genes in sex-determining pathways? There have been several discoveries of changes in the expression, or transcripts, of downstream genes, but little progress in identifying how the embryo senses temperature.[11,17]

Recent research has shown that changes in the distribution of structural proteins coupled with epigenetic modifications (loading of active/inactive marks) can have profound impact on gene expression via a change to three-dimensional (3D) genome conformation. Indeed, such changes are an important part of cell differentiation during development.[18–20]

What is not clear, however, is how directly or indirectly temperature might affect the 3D structure of chromatin. For instance, temperature has been suggested to have its effect indirectly via alterations in a temperature-sensitive ion channel that alters the balance of cytosolic Ca^{2+} and reactive oxygen species and affects epigenetic modifiers via phosphorylation of a control gene.[11] However, there remains the possibility that increased temperature might alter 3D structures directly by disrupting existing promoter–enhancer interactions, or establishing new ones. Here, we outline a novel hypothesis that proposes that higher-order chromatin organization itself is thermosensitive, and changes in 3D structure result in modulated expression of key sex genes that impact gonadal fate. We have called this hypothesis the three dimensions of thermolabile sex determination (3D-TSD for short).

HIGHER-ORDER CHROMATIN ORGANIZATION

Genomes are packaged into a chromatin structure, the regulation of which depends on different levels of organization, including (i) chemical modifications of the DNA, (ii) modifications to the four core histones (H2A, H2B, H3 and H4) that comprise the nucleosomes around which the DNA wraps and (iii) the 3D high-order organization of chromatin inside the nucleus that can change during the cell cycle and cell differentiation (Figure 1).

Chromatin structure is maintained by DNA binding to histones, two each of H2A, H2B, H3 and H4, stabilised with H1. This structure is modulated by the addition of different chemical groups to histone tails (active or repressive marks), as well as by DNA methylation, and by their interactions with a host of other architectural factors, enzymes, modifiers and transcription factors. For instance, dramatic change of chromatin conformation associated with histone modification and DNA methylation mediates global transcriptional silencing of the inactive X chromosome in mammals.[21]

The combination of high-resolution microscopy and chromosome conformation capture (3C)-based methods (3C; 4C, 5C and Hi-C) has revealed that the 3D chromatin structure is complex and dynamic. It includes chromosome territories in the interphase nucleus, 'open/active' and 'closed/inactive' compartments (A and B), topologically associated domains (TADs), and looping interactions, which are established and maintained by structural proteins (i.e., cohesins and CTCF)[20,22–25] (Figure 1). The compartmentalization of the genome in this manner partitions genomes into 'regulatory neighbourhoods' by confining the activity of cis-regulatory elements to genes that fall within the same TAD.[26] It has been suggested that TAD boundaries can act as barriers between epigenetic states and that TADs harbour-specific epigenetic signatures.[27]

Changes in the distribution of structural proteins and transcription factors, coupled with histone modifications associated with the remodelling of high-order chromatin organization that impact on gene expression, occur during development and in the germline.[20,28–31] This is highlighted by the knockout of epigenetic machinery (e.g., histone deacetylases), which results in a changed epigenetic landscape that correlates with altered genomic contacts at promoters and enhances that change gene expression.[32] In fact, many features of 3D genome configuration of germ cells are highly dynamic, with cyclical transient chromatin–chromatin interactions that are established rapidly (reviewed in Refs. [24, 31]). The outcome is genomic plasticity that is poorly understood.

ENVIRONMENTAL 3D CHROMATIN REGULATION

Of the environmental stimuli that can influence chromatin regulation, temperature is the most common. All organisms can respond

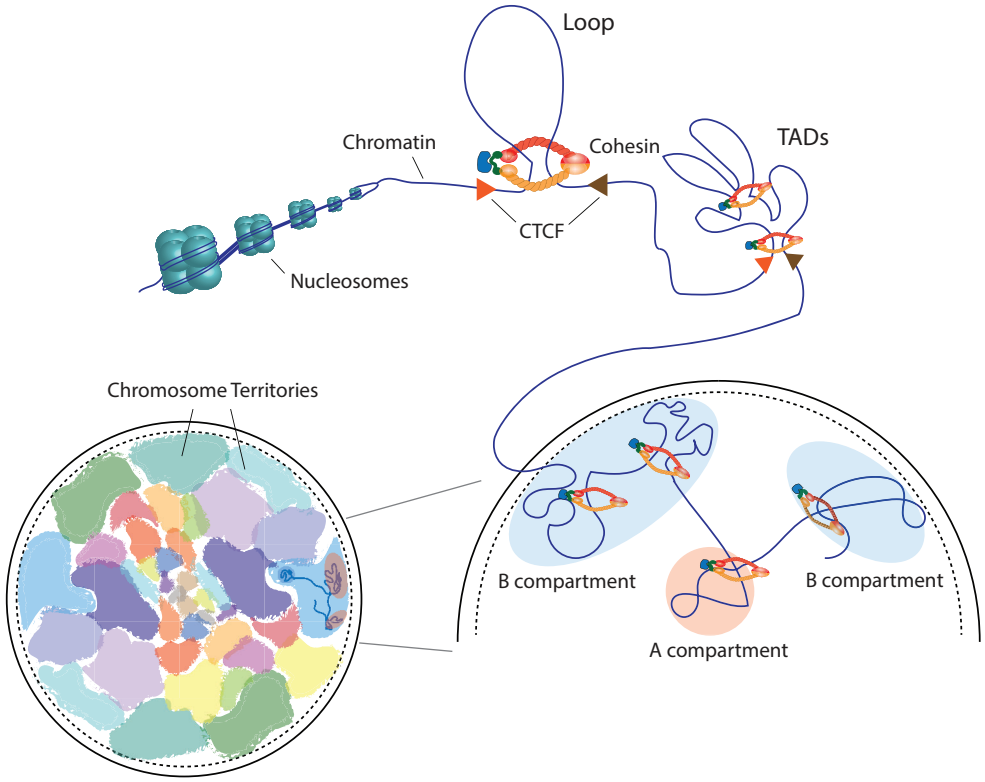

FIGURE 1 3D chromatin structure. The DNA wraps around histones forming nucleosomes, and constitutes the chromatin fibre. Chromatin fibres fold into chromatin loops forming topologically associated domains (TADs) with boundaries determined by cohesin complexes between CTCF convergent motifs. TADs are organised into A or B compartments, according to chromatin accessibility and transcriptional activity. Compartments are found within chromosome territories in nuclei.

to temperature by activating a common transcriptional programme. This heat shock response is well known to induce global changes to gene regulation, as revealed in studies from human, mouse and *Drosophila*.[33–35]

Recently, the implications for direct 3D genome remodelling by temperature have also been considered. New 3C-based methods that permit the study of how genomes remodel in response to the environment at a fine scale (reviewed in Kumar et al.[36]) have revealed that plant genomes can remodel in response to salicylic acid[37] and probably light (reviewed in Perrella et al.[38]). In mammals (e.g., mouse liver), TADs that harbour circadian genes switch between active and inactive compartments at different times of the day, resulting in cycles of transcription modulation.[39] Thus, the response of 3D structure of the genome to environmental stimuli is nothing if not dynamic.

While our understanding of genome-level responses to environmental changes is still limited, there is considerable variation in response to temperature across different organisms. Heat shock to cultured human and *Drosophila* cells caused dramatic transcriptional alteration without major changes in global chromatin architecture.[40] However, in plants and yeast, gene expression changes induced by heat stress were coupled with modification to 3D genome structure.[37,41,42] In different *Drosophila* cells, heat stress induces a redistribution of architectural proteins that modulate TAD boundaries.[43] This chromatin remodelling, coupled with covalent histone modifications, promoted new long-range interactions that formed new enhancer–promoter contacts that affected gene expression.[43]

Subsequent studies of human embryonic stem cells showed that response to temperature resulted in changed enhancer–promoter interactions that correlated with a redistribution of RAD21 cohesin and CTCF.[44] Studies of hormone-induced changes show that spatial structure of TADs plays a role in regulating the rapid transient response to external signals. In vitro studies using T47D breast cancer cells revealed that TAD border structures and their epigenetic modifications can be rapidly modified (1 h) upon hormone (i.e., progesterone, Pg) stimulation, and this can occur over large genomic domains.[27] In this case, the Pg-activated receptor interacts with kinase signalling networks that regulate the expression of thousands of genes.[45]

Collectively, this evidence suggests that the thermoregulation of gene expression is tightly linked to chromatin remodelling, via changes in structural proteins and transcription factors that can rapidly alter a host of interactions in response to environment (review in Kainth et al.[46]).

THE THREE DIMENSIONS OF SEX

This relationship between thermoregulation and chromatin remodelling suggests that the genome senses the thermal signal via

temperature-induced chromatin remodelling, triggering either testis or ovary determination at early stages of development.

Here we propose that 3D genome conformation can respond directly to temperature, resulting in chromatin remodelling in bipotential gonad precursor cells (Figure 2A). This could be reached by disrupting specific chromatin interactions, resulting in new genomic contacts that change sex gene expression. The plethora of chromatin binding proteins, such as architectural (cohesins and CTCF), remodellers (epigenetic writers, readers and erasers) and transcription factors (review in Misteli[24]), might be subjected to temperature-induced structural change. Altered folding of these proteins could promote changes in specific regulatory contacts. Significantly, recent studies in yeast have proposed that transcriptional condensates can rapidly and reversibly reconfigure the 3D genome in response to environmental conditions.[42]

Given the importance of the higher-order chromatin structure in demarcating the limits of gene-regulatory domains, disturbances of this architecture would represent a means for rapid change in gene expression. Shifting TADs or compartment boundaries in response to temperature would expose multiple genes to novel regulatory environments. This could break existing promotor enhancer contacts to turn genes off or establish new contacts to turn genes on (Figure 2A). Relevant to our hypothesis is recent evidence of the role of chromatin remodelling during sex determination in mouse.[47] By integrating Hi-C and ChIP-seq data, the authors uncover rewiring of 3D enhancer hubs during sex differentiation. In the light of this, we predict that the study of the structural and functional features that demarcate these dynamic boundaries in different vertebrate lineages (i.e., reptiles with TSD) will elucidate the mechanisms that govern higher-order genomic structure and function.

This thermal sensing would induce genome remodelling in somatic secretory gonadal cells (e.g., Sertoli, Leydig or granulosa) prior to sex differentiation, which would be maintained until the commitment of the gonad phenotype. Such thermosensitive chromatin interactions could bring key enhancers and promotors from distant locations into close proximity (as recently proposed for transcriptional condensates in yeast[42]) to alter gene expression in the gonad developmental pathways. Genes in newly formed compartments would then be directly regulated by this thermosensitive pathway.

Alternatively, the temperature may have a less direct effect on chromatin structure. For instance, polycomb repressive complexes (PRC) can alter chromatin structure (i.e., by H3K27me3 deposition) resulting in changes of both *cis* and *trans* enhancer–promoter interactions (reviewed in Illingworth[48]), ultimately regulating potential sex-determining genes[49,50] (Figure 2B). Therefore, non-canonical isoforms of chromatin modifiers (i.e., ΔN-*JARID2*[15]) with different affinity to PRC2 might act as a remodelling sensor, rather than a direct regulator of sex genes.

Another indirect effect of temperature on chromatin might also explain sex reversal in the half-smooth tongue sole. This fish has a ZZ male:ZW female sex-determining system, in which a higher dosage of the Z-borne *Dmrt1* directs male development.[51] However, higher temperature disrupts DNA methylation of the *Dmrt1* locus in ZW embryos, resulting in the expression of this gene and pseudomale development.[52] It is unknown how demethylation is mediated, but DNA methylation has profound effects on chromatin conformation[53] so its removal is likely to alter the 3D conformation and reactivate *Dmrt1*.

TESTING THERMOSENSITIVE 3D CONFORMATION AND SEXUAL FATE

Examining the potential role of 3D genome remodelling in developing and/or sex-reversing embryonic gonads is now possible through the implementation of an integrative approach that includes analysis of the epigenome (histone modifications and DNA methylation) with genome structure (Hi-C) at different developmental stages and at different temperatures, coupled with functional analysis (single cell RNA-seq) of key sex genes.

For example, an excellent study system is brumation (akin to hibernation) in the bearded dragon, during which thousands of genes are differentially expressed compared to individuals at non-brumating temperatures.[54,55] Comparing genome structure in cold-brumating individuals and warm individuals will reveal if the reptile genome has capacity to restructure in response to temperature. To determine if the genome is remodelled in direct response to temperature, or whether change to the underlaying epigenetic code is responsible, profiling the epigenome (ChIP-seq/CUT&RUN/bisulfite sequencing), including the detection of structural proteins such as cohesins and CTCF, in combination with Hi-C experiments in the developing reptile gonad (at different temperatures) will be key. Both epigenome and Hi-C approaches could be conducted at different developmental time points, from undifferentiated gonads through to developing ovaries and testes after sexual fate is decided. This would reveal if the genome remodelled before the occurrence of changes in the epigenome or the distribution of structural proteins, which would indicate capacity for direct sensing of the thermal signal. Alternatively, if key genomic interactions were remodelled upon epigenetic change, this would be indicative of an upstream sensing mechanism that results in epigenome change that subsequently alters the high-order 3D genome structure.

Research in reptile species with sex reversal might provide further insights into the mechanisms involved. We propose that as well as acting in strictly TSD species, 3D TSD acts also in sex reversal systems when temperature overrides a genetic sex-determining gene. Particularly instructive might be two sex-reversing reptile species with opposite temperature-induced sex reversal. *Pogona vitticeps* has a ZZ male:ZW female system in which ZZ develop as males at higher temperatures. *Bassiana duperreyi* has the opposite system whereby XX individuals reverse to male at low temperatures.[56,57] In both cases, we hypothesize that temperature acts directly to alter thermosensitive 3D conformation, and affect the expression of influential genes in the sex differentiation pathway.

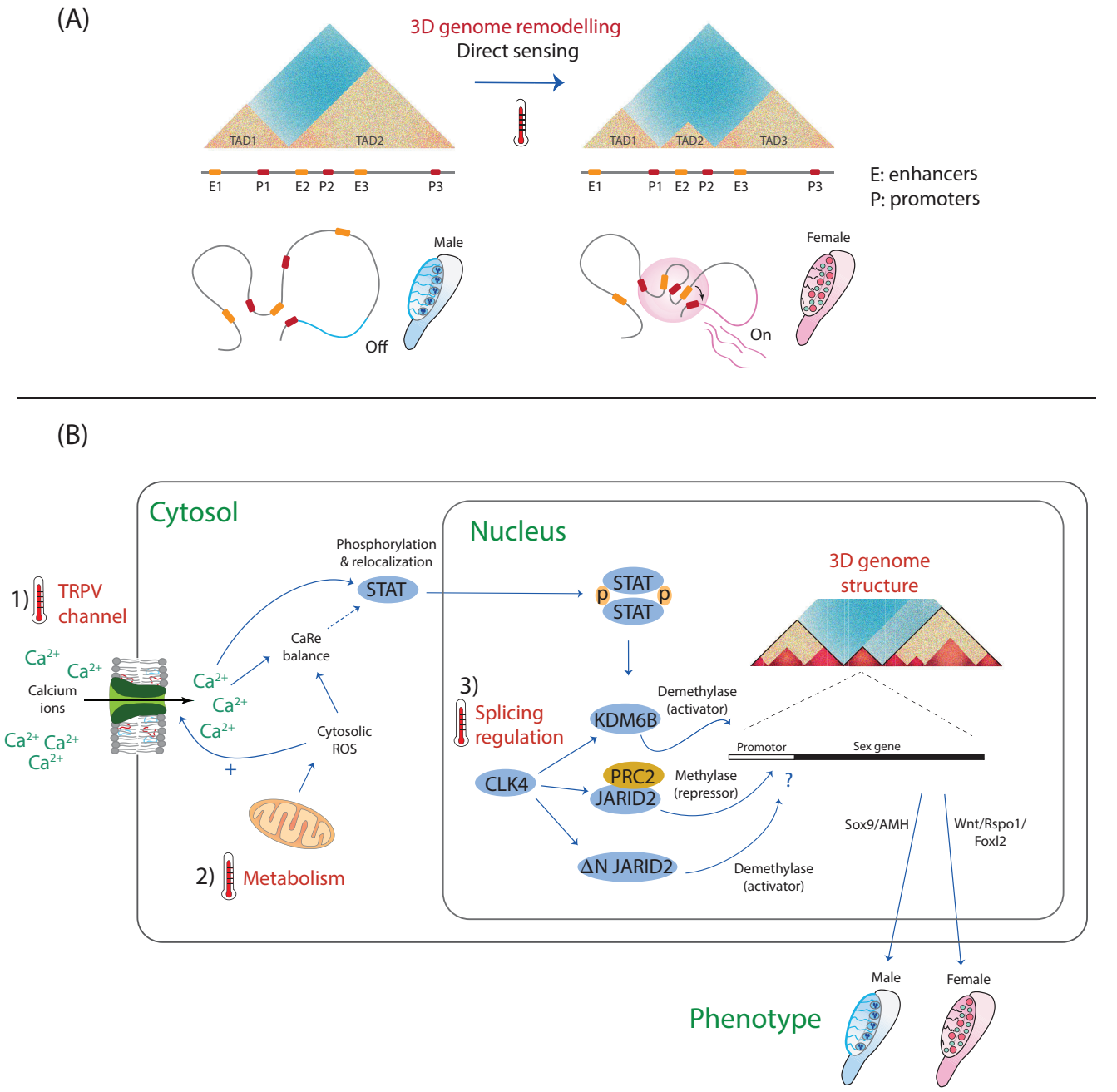

FIGURE 2 Sensing the thermal signal. (A) The genome could respond directly to temperature, resulting in sex genes being turned on or off. Three-dimensional genome (3D) remodelling could affect gene expression directly by disrupting establish (or establishing new) enhancer (E)–promoter (P) interactions within thermosensitive transcriptional condensates (pink circle). (B) A proposed route for how upstream thermal signals can be indirectly detected by the cell. ***Thermometer 1)*** TRPV channel activity is increased in response to temperature, increasing Ca^{2+} concentration in the cytosol. ***Thermometer 2)*** Higher temperature in reptiles increases metabolic rate and, therefore, ROS, which further activates TRPV channel activity. The thermosensitive CaRe balance results in phosphorylation (orange circles with p inside) of STAT, which subsequently relocates to the nucleus to affect epigenetic modifiers (e.g., KDM6B) and sex gene expression. ***Thermometer 3)*** Thermosensitive alternative splicing of epigenetic modifiers could directly alter the epigenetic landscapes that control to change sex gene expression. The thermo-sensitive CLK4 might regulate splicing.

CONCLUSION

We propose that temperature can directly influence sex reversal by chromatin remodelling without invoking intermediate signal transduction. We hypothesize that the thermal signal could be directly sensed by transcriptional condensates in the genome, resulting in altered enhancer–promoter interactions in the same TAD after compartment switching. If a critical gene (or genes) in the sex-determining pathway switched compartments, the result could be to turn off (or on) testis/ovary development, and ultimately reassign gonadal fate. This fascinating possibility envisages a direct genomic thermal sensor that skips intermediate signalling.

AUTHOR CONTRIBUTIONS

Aurora Ruiz-Herrera and Paul D. Waters wrote the first draft. Aurora Ruiz-Herrera, Paul D. Waters, Arthur Georges and Jennifer A. Marshall Graves conceived the idea and commented on manuscript drafts. Sarah L. Whiteley helped conceive ideas and prepared text for the supporting grant application, which we drew upon for this paper. Sarah L. Whiteley also commented on manuscript drafts.

ACKNOWLEDGEMENTS

This research has received support from the Australian Research Council [DP170101147 (A.G., P.D.W., J.A.M.G.), DP180100931 (P.D.W.), DP210103512 (P.D.W., J.A.M.G.), DP220101429 (A.G., P.D.W., J.A.M.G., A.R.-H.)]. P.D.W. is supported by an NHMRC Project Grant (APP1156598). S.L.W. was funded by DP170101147 and CSIRO. A.R.-H. was supported by the Spanish Ministry of Science and Innovation (PID2020-112557GB-I00). The authors are grateful to C. Vara for providing the first draft of Figure 1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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How to cite this article: Waters, P. D., Graves, J. A. M., Whiteley, S. L., Georges, A., & Ruiz-Herrera, A. (2023). Three Dimensions Of Thermolabile Sex Determination. *BioEssays*, *45*, e2200123. https://doi.org/10.1002/bies.202200123