# Gene Dose Indicates Presence of Sex Chromosomes in Collared Lizards (Crotaphytus collaris), a Species with Temperature-Influenced Sex Determination

Authors: Wiggins, Jodie M., Santoyo-Brito, Enrique, Scales, Jon B., and Fox, Stanley F.

Source: Herpetologica, 76(1) : 27-30

Published By: The Herpetologists' League

URL: https://doi.org/10.1655/Herpetologica-D-19-00036

*Herpetologica*, 76(1), 2020, 27–30


# Gene Dose Indicates Presence of Sex Chromosomes in Collared Lizards (*Crotaphytus collaris*), a Species with Temperature-Influenced Sex Determination

Jodie M. Wiggins[1,3], Enrique Santoyo-Brito[1], Jon B. Scales[2], and Stanley F. Fox[1]

[1] Oklahoma State University, Stillwater, OK 74074, USA
[2] Midwestern State University, Wichita Falls, TX 76308, USA

Abstract: The characteristics of a species' evolution can be influenced by its mode of sex determination and, indeed, sex determination mechanisms vary widely among eukaryotes. In nonavian reptiles, the sex determination mechanism has historically thought to be binary, determined either by temperature or genetics. In this study, we add to the growing evidence that sex determining mechanisms in reptiles fall along a continuum rather than existing as a mutually exclusive dichotomy. Using qPCR, we demonstrate that a lizard species, *Crotaphytus collaris*, possesses sex-based gene dosage consistent with the presence of sex microchromosomes, despite the fact that extreme incubation temperatures can influence hatchling sex ratio. Our results indicate that *C. collaris* might be the first non-Australian species of lizard having a temperature override of genotypic sex determination, and the first species in which sex switches at both high and low temperatures.

**Key words:** Crotaphytidae; Gene by environment; Genotypic sex determination; Iguania; Reptiles; Sex reversal

Long held has been the understanding that, within reptiles, there existed a single dichotomy of mutually exclusive sex determination mechanisms (SDM; Bull 1983; Janzen and Paukstis 1991). Either a species' sex was determined by the environment (ESD) or by sex chromosomes (GSD; Bull 1983; Janzen and Paukstis 1991; Bachtrog et al. 2014). In fact, reptilian species do utilize both male and female heterogamety (GSD; King 1977) and temperature-dependent sex determination (TSD, a specific type of ESD; Ewert and Nelson 1991). In recent years, however, the distinction between ESD and GSD has blurred, and an increasingly complex picture is emerging in which GSD and TSD are the ends of a continuum of SDMs in reptiles (Sarre et al. 2004; Holleley et al. 2015, 2016). Examples of intermediate SDMs include species in which different populations utilize different SDMs (Pen et al. 2010), temperature-dependent sex reversal of a lizard species with a ZZ/ZW GSD system (Quinn et al. 2007; Holleley et al. 2015), revelations of the genetic underpinnings of TSD in alligators and turtles (Spotila et al. 1998; Smith et al. 1999; Kettlewell et al. 2000; Western and Sinclair 2001), and the epigenetic mechanisms controlling temperature override of GSD in lizards and turtles (Deveson et al. 2017; Ge et al. 2018). This shifting landscape provides an exceptional study system for better understanding sex determination in vertebrates (Sarre et al. 2004; Holleley et al. 2016).

Collared Lizards, *Crotaphytus collaris*, are a widespread, oviparous species in which sex chromosomes have not been identified (Gorman 1973; De Smet 1981). Nevertheless, *C. collaris* has been classified as a GSD species based on phylogenetic analyses (Pokorná and Kratochvíl 2009). However, even members of the same species can utilize different SDMs (Pen et al. 2010). Thus, classifying *C. collaris* solely based on phylogeny might provide an incomplete picture of its SDM (Viets et al. 1994). When determining if *C. collaris* utilizes TSD, Santoyo-Brito et al. (2017) described an inverse TSD Type II pattern in which the percentage of female offspring declined as constant incubation temperatures or treatments approached high and low extremes. Although the authors did not report ratios of either sex nearing 100% at low and high treatments, their results indicate a temperature influence on sex determination in *C. collaris*. Their findings hint at a more complex SDM than pure TSD or pure GSD in *C. collaris* (Santoyo-Brito et al. 2017), as suggested by the absence of sharp inflection points at which sex ratios change in a few other lizard species (e.g., *Amphibolurus muricatus*; Warner and Shine 2008, 2010).

In spite of Santoyo-Brito et al. (2017) reporting a temperature influence on sex determination in *Crotaphytus collaris*, the species has previously been phylogenetically assigned as having GSD. In species that possess XY sex chromosomes (and thus, GSD), the heterogametic sex is expected to have half the dosage of X-linked genes (Rovatsos et al. 2014a). Indeed, sex-specific gene dosage at two loci in the closely related *C. insularis* points to heterogamety (with males being heterogametic) and, thus, to the possibility of GSD in crotaphytids (Rovatsos et al. 2014b). Thus, we sought to determine if *C. collaris* demonstrates gene dosage consistent with XY sex chromosomes which, taken together with the findings of Santoyo-Brito et al. (2017), suggest temperature-induced sex reversal in this species.

## Materials and Methods

### DNA Isolation and PCR

Upon capture, we collected blood samples from the toes of 20 wild-caught lizards (10 male, 10 female) at Sooner Lake Dam, Pawnee County, Oklahoma, in the summers of 2014–2016 and immediately preserved those samples on Whatman FTA classic cards (GE Healthcare). We later extracted DNA from the cards by excising a 3-mm square of blood-saturated card using sterile scissors then following the GE Healthcare extraction protocol using Chelex® 100 resin (Sigma-Aldrich Corporation). We tested for gene dosage in three genes linked to the X chromosome in *Anolis carolinensis*: sarcoplasmic reticulum calcium ATPase 2 (ATP2A2), transmembrane protein 123D (TMEM ), and phosphatidylethanolamine binding protein 1 (PEBP1; Table 1). We used

[3] Correspondence: e-mail, jodiewiggins18@gmail.com

Table 1.—Genes and primer sequences used to determine relative gene dosage through qPCR in *Crotaphytus collaris*. EF1a, present in only two copies/genome, was used for gene dosage normalization. TMEM, ATP2A2, and PEPBP1 each map to the *Anolis carolinensis* X chromosome and were used to identify heterogamety in *C. collaris*.

| Gene short name | Gene name | Forward primer | Reverse primer | Amplicon size (bp) |
|---|---|---|---|---|
| EF1a | Elongation factor 1 | CCTTATTGTTGCTGCTGGTGTT | GTGCTAACTTCTTTGACGATTTCC | 189 |
| TMEM | Transmembrane protein 132D | TATCCGAGCAGACCCAAAGTCC | AAGGAGACCCAACTCAGCCAC | 183 |
| ATP2A2 | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 | CAAAGCAGCGGGCATTTAGG | ATCACTGGGGACAACAAGGG | 160 |
| PEPBP1 | Phosphatidylethanolamine-binding protein 1 | GACAGGGCTCCATCGCTAC | CATAGTCATCCCACTCCGCC | 188 |

primer sequences for these three genes obtained from Rovatsos et al. (2014b; Table 1). The elongation factor 1α gene (EF1α), present in only two copies/genome, was used for gene dosage normalization. We assembled polymerase chain reactions (PCRs) in 25-μL final volumes containing 12.5 μL 2× Bullseye EvaGreen qPCR master mix buffer (MidSci), 25 ng genomic DNA, and 200 pmol each forward and reverse primers. The thermal profile was an initial denaturation of 10 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72° C for 45 s. Amplification via qPCR was executed in a Stratagene MX3005 thermocycler.

### Gene Dosage Calculations

We calculated final gene dosage ratios for the five male and five female lizards whose DNA reliably amplified across three replicates, calculating crossing-point values in MaxPro (Stratagene) then normalizing to EF1α. We determined gene dosage as in Rovatsos et al. (2014b) with: $R = [2^{Cp\ gene}/2^{Cp\ EF1\alpha}]^{-1}$ and $r$ (relative gene dosage ratio) = $R_{male}/R_{female}$ (Cp is crossing point). We expected that *C. collaris* males are the heterogametic sex based on results for the closely related *C. insularis* (Rovatsos et al. 2014b). Thus, an $r = 0.5$ is expected for single-copy X-linked genes while $r = 1.0$ is expected for double-copy X-linked genes.

## Results

Sex-linked differences were apparent for all three analyzed genes (ATP2A2, TMEM, and PEBP1; Fig 1). In each case, the average female $r$ value is exactly 1.0 (ATP2A2: 1.0 ± 0.13 SE, TMEM: 1.0 ± 0.08 SE, PEBP: 1.0 ± 0.17 SE). This result is consistent with females having two copies of the gene of interest and, thus, being the homogametic sex. For each gene, an average $r$ value near 0.5 was obtained in the males: $\bar{X}$ ATP2A2 = 0.59 ± 0.09 SE, TMEM = 0.61 ± 0.06 SE, PEBP1 = 0.50 ± 0.10 SE. This result is consistent with males having a single gene copy and being heterogametic.

## Discussion

Although sex chromosomes have not been detected in crotaphytid lizards (Gorman 1973; De Smet 1981), our results are consistent with heterogamety and point to the existence of GSD in *C. collaris*, as in *C. insularis* (Rovatsos et al. 2014b). While GSD is common among lizards, *C. collaris* has been shown to experience changes in sex ratios at different temperatures that are held constant throughout incubation (Santoyo-Brito et al. 2017). More investigation is warranted to determine if *C. collaris* is another example of a reptilian species with GSD that can be overridden by temperature extremes (Shine et al. 2002; Holleley et al. 2015). We agree with Santoyo-Brito et al. (2017) that *C. collaris* likely possesses sex microchromosomes in an XX/XY pattern (females/males), and that extreme incubation temperatures alter the sex-determining pathway such that XX individuals develop as phenotypic males. We further hypothesize that gravid *C. collaris* females select nest sites such that GSD will function without temperature interference as evidenced by field hatchling ratios of 50.9% male and 49.1% female ($n = 528$ individuals 2011–2016) as expected in GSD populations.

Evidence continues to emerge that some extant reptile species employ multimodal SDMs (Shine et al. 2002; Valenzuela et al. 2003; Sarre et al. 2004; Quinn et al. 2007; Radder et al. 2008; Holleley et al. 2015, 2016). These species might be in a transition from GSD to TSD. Our study species, *C. collaris*, demonstrates gene dosage consistent with that of 28 species of iguanian lizards (Pleurodonta) spanning 11 genera and including *A. carolinensis* and *C.*

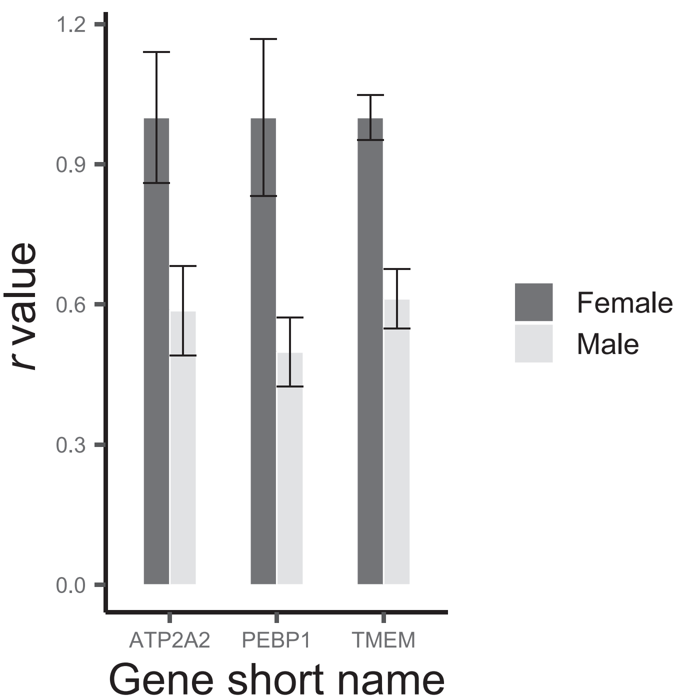


Fig. 1.—Relative gene dosage (reported as $\bar{X} \pm 1$ SE) at three X-linked genes that map to the *Anolis carolinensis* X-chromosome in wild-caught adult male and female *Crotaphytus collaris* ($n = 5$ for each sex). ATP2A2 = sarcoplasmic reticulum calcium ATPase 2; TMEM = transmembrane protein 123D; PEBP1 = phosphatidylethanolamine binding protein 1.

*insularis* (Rovatsos et al. 2014a,b). The absence of genes on the Y chromosome that are present on the X, coupled with the chromosomal looping in the *A. carolinensis* XY synaptonemal complex, points to a degenerate Y microchromosome in this species and, likely, those exhibiting gene dosage in the same genes (Alföldi et al. 2011; Bachtrog et al. 2014; Rovatsos et al. 2014a,b; Lisachov et al. 2017). Thus, the discovery of sex-based gene dosage for three *C. collaris* genes that are the same as those present on the *A. carolinensis* X chromosome indicates the presence of sex chromosomes in *C. collaris*. Yet, results from Santoyo-Brito et al. (2017) show a decline in number of females hatched at extreme high and low constant incubation temperatures, pointing to a temperature override that converts XX individuals into phenotypic males (XXm). If these XXm individuals are viable, they might mate and reproduce as males. Whereas this scenario will lead to an increase in the proportion of genetic females, the possibility of producing offspring who possess both degenerate chromosomes (YY) would be avoided, as in *Acritoscincus* (*Bassiana*) *duperreyi* (Shine et al. 2002). Ongoing climate change that can bring about increases in global temperatures might eliminate cooler nest site options and induce increasing shifts of XXf to XXm, potentially eliminating the presumptive Y microchromosome (Mitchell and Janzen 2010).

In summary, our data point to the presence of sex-based gene dosage and indicate the presence of as-yet-unidentified sex microchromosomes in *C. collaris*, as suggested by Santoyo-Brito et al. (2017). These findings, coupled with previous work documenting the influence of incubation temperature in this species (Santoyo-Brito et al. 2017), add to the growing evidence that SDMs in nonavian reptiles are not bimodal (Shine et al. 2002; Holleley et al. 2015) and call for investigation of other species in which distinct sex transition inflection points are absent (e.g., Harlow and Taylor 2000). Further inquiry into crotaphytid genomics and the effects of extreme temperature incubation on *C. collaris* sex determination is warranted; specifically, investigating whether some of the individuals incubated at either high or low temperature extremes are genotypically female but phenotypically male as suggested by Santoyo-Brito et al. (2017), and as shown in free-ranging *Pogona vitticeps* (Holleley et al. 2015). If this is the case, *C. collaris* will be both the first non-Australian lizard species in which such reversal has been demonstrated and the first in which reversal occurs at both high and low incubation temperatures (Holleley et al. 2016). The genotype–phenotype discordant individuals produce unique variation on which selection can act, potentially altering both the rate and direction of a species' evolution.

**Acknowledgments.**—We thank C. Holleley for manuscript advice and A. Kratochvil for qPCR troubleshooting. The project was supported by National Science Foundation grant IOS-1257881 to SFF and followed an animal care and use protocol ACUP-12-14 approved by Oklahoma State University.

## LITERATURE CITED

Alföldi, J., F. Di Palma, M. Grabherr, … K. Lindblad-Toh. 2011. The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 477:587–591.

Bachtrog, D., J.E. Mank, C.L. Peichel, … J.C. Vamosi. 2014. Sex determination: Why so many ways of doing it? PLoS Biology 12:1–13. DOI: https://doi.org/10.1371/journal.pbio.1001899

Bull, J.J. 1983. Evolution of Sex Determining Mechanisms. Benjamin/Cummings Publishing Company, USA.

De Smet, W.H.O. 1981. Description of the orcein stained karyotypes of 27 lizard species (Lacertilia, Reptilia) belonging to the families Iguanidae, Agamidae, Chamaeleontidae and Gekkonidae (Ascalabota). Acta Zoologica et Pathologica Antverpiensia 76:35–72.

Deveson, I.W., C.E. Holleley, J. Blackburn, J.A. Marshall Graves, J.S. Mattick, P.D. Waters, and A. Georges. 2017. Differential intron retention in Jumonji chromatin modifier genes is implicated in reptile temperature-dependent sex determination. Scientific Advances 3:e1700731. DOI: https://doi.org/10.1126/sciadv.1700731

Ewert, M.A., and C.E. Nelson. 1991. Sex determination in turtles: Patterns and some possible adaptive values. Copeia 1991:50–69.

Ge, C., C. Weber, C. Cai, G. Qian, Y. Zhou, H. Zhang, B. Capel, W. Sun, and J. Ye. 2018. The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. Science 360:645–648.

Gorman, G.C. 1973. The chromosomes of the Reptilia, a cytotaxonomic interpretation. Pp. 349–424 in Cytotaxonomy and Vertebrate Evolution (A.B. Chiarelli and E. Capanna, eds.). Academic Press, USA.

Harlow, P.S., and J.E. Taylor. 2000. Reproductive ecology of the jacky dragon (*Amphibolurus muricatus*): An agamid lizard with temperature-dependent sex determination. Austral Ecology 25:640–652.

Holleley, C.E., D. O'Meally, S.D. Sarre, J.A. Marshall-Graves, T. Ezaz, K. Matsubara, B. Azad, X. Zhang, and A. Georges. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523:79–82.

Holleley, C.E., S.D. Sarre, D. O'Meally, and A. Georges. 2016. Sex reversal in reptiles: Reproductive oddity or powerful driver of evolutionary change? Sexual Development 10:279–287. DOI: https://doi.org/10.1159/000450972

Janzen, F.J., and G. Paukstis. 1991. Environmental sex determination in reptiles: Ecology, evolution, and experimental design. Quarterly Review of Biology 66:149–179.

Kettlewell, J.R., C.S. Raymond, and D. Zarkower. 2000. Temperature-dependent expression of turtle Dmrt1 prior to sexual differentiation. Genesis 26:174–178.

King, M. 1977. Evolution and reproduction. Pp. 55–60 in Reproduction and Evolution (J.H. Calaby and C.H. Tyndale-Biscoe, eds.). Australian Academy of Science, Australia.

Lisachov, A.P., V.A. Trifonov, M. Giovannotti, M.A. Ferguson-Smith, and P.M. Borodin. 2017. Heteromorphism of "homomorphic" sex chromosomes in two anole species (Squamata, Dactyloidae) revealed by synaptonemal complex analysis. Cytogenetic Genome Research 151:89–95.

Mitchell, N.J., and F.J. Janzen. 2010. Temperature-dependent sex determination and contemporary climate change. Sexual Development 4:129–140. DOI: https://doi.org/10.1159/000282494

Pen, I., T. Uller, B. Feldmeyer, A. Harts, G.M. While, and E. Wapstra. 2010. Climate-driven population divergence in sex-determining systems. Nature 468:436–438.

Pokorná, M., and L. Kratochvíl. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? Zoolological Journal of the Linnean Society 156:168–183.

Quinn, A.E., A. Georges, S.D. Sarre, F. Guarino, T. Ezaz, and J.A.M. Graves. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316:411.

Radder, R.S., A.E. Quinn, A. Georges, S.D. Sarre, and R. Shine. 2008. Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. Biology Letters 4:176–178. DOI: https://doi.org/10.1098/rsbl.2007.0583

Rovatsos, M., M. Altmanová, M. Pokorná, and L. Kratochvíl. 2014a. Conserved sex chromosomes across adaptively radiated anolis lizards. Evolution 68:2079–2085. DOI: https://doi.org/10.1111/evo.12357

Rovatsos, M., M. Pokorná, M. Altmanová, and L. Kratochvíl. 2014b. Cretaceous park of sex determination: Sex chromosomes are conserved across iguanas. Biology Letters 10:20131093.

Santoyo-Brito, E., M. Anderson, and S.F. Fox. 2017. Incubation temperature modifies sex ratio of hatchlings in collared lizards, *Crotaphytus collaris*. Journal of Herpetology 51:197–201.

Sarre, S.D., A. Georges, and A.E. Quinn. 2004. The ends of a continuum: Genetic and temperature-dependent sex determination in reptiles. BioEssays 26:639–645. DOI: https://doi.org/10.1002/bies.20050

Shine, R., M.J. Elphick, and S. Donnellan. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecology Letters 5:486–489.

Smith, C.A., P.J. McClive, P.S. Western, K.J. Reed, and A.H. Sinclair. 1999. Conservation of a sex-determining gene. Nature 402:601–602.

Spotila, L.D., J.R. Spotila, and S.E. Hall. 1998. Sequence and expression analysis of Wt1 and Sox9 in the Red-Eared Slider turtle, *Trachemys scripta*. Journal of Experimental Zoology 281:417–427.

Valenzuela, M.N., D.C. Adams, and F.J. Janzen. 2003. Pattern does not equal process: Exactly when is sex environmentally determined? American Naturalist 161:676–683.

Viets, B.E., M.A. Ewert, L.G. Talent, and C.E. Nelson. 1994. Sex-determining mechanisms in squamate reptiles. Journal of Experimental Zoology 56:45–56. DOI: https://doi.org/10.1002/jez.1402700106

Warner, D.A., and R. Shine. 2008. The adaptive significance of temperature-dependent sex determination in a reptile. Nature 451:566–569.

Warner, D.A., and R. Shine. 2010. Interactions among thermal parameters determine offspring sex under temperature-dependent sex determination. Proceedings of the Royal Society of London B: Biological Sciences 278:256–265.

Western, P., and A. Sinclair. 2001. Sex, genes, and heat: Triggers of diversity. Journal of Experimental Zoology 290:624–631.

**Accepted on** 12 November 2019
**Associate Editor:** Matthew Gifford