Evidence for sex microchromosomes in a species with temperature-influenced sex determination (*Crotaphytus collaris*)

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Keywords

Sex-determining mechanism, genotypic sex determination, temperature-dependent sex determination, gene-dosage, sex microchromosome, reptile, *Crotaphytus collaris*

Abstract

The characteristics of a species' evolution can be powerfully influenced by its mode of sex determination and, indeed, sex determination mechanisms vary widely among eukaryotes. In non-avian reptiles, sex was long thought to be determined bimodally, either by temperature or genetics. Here 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 the lizard *Crotaphytus collaris* possesses sex-based gene dosage consistent with the presence of sex michrochromosomes, despite that extreme incubation temperatures can influence hatchling sex ratio. Our results suggest a temperature override that switches genotypic females to phenotypic males at high and low temperatures.

Introduction

Mode of sex determination has far reaching consequences affecting evolutionary processes such as speciation (Haldane 1922), adaptive capability and heterozygosity (Bull 1983; Shine et al. 2002; Burt and Trivers 2006), maternal capacity for sex ratio manipulation (Kratochvíl et al. 2008), extent of secondary sexual characteristics (Reeve and Pfennig 2003) and capacity to respond to climate change (Mitchell and Janzen 2010). Among eukaryotes, the array of sex determination mechanisms (SDMs) is diverse (Bull 1983; Charlsworth 1996; Bachtrog et al. 2014). Environmental sex determination (ESD) is characterized by a mode of sex determination entirely dependent on environmental factors such as temperature encountered during embryogenesis (Merchant-Larios and Díaz-Hernández 2013), photoperiod (Walker 2005; Guler et al. 2012), or social cues during subsequent development (Bull 1983; Janzen and Paukstis 1991; Valenzuela and Lance 2004; Bachtrog et al. 2014). Conversely, sex in GSD species is determined at conception by genes, and is uninfluenced by environment (Bull 1983; Sarre et al. 2004).

Long held was the belief that within reptilian lineages there existed a single dichotomy of mutually exclusive SDMs (Bull 1983; Janzen and Paukstis 1991). Either a species' sex was thought to be determined by the environment (environmental sex determination; ESD) or by sex chromosomes (genotypic sex determination; 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). However, in recent years, the line between ESD and GSD has become decidedly blurred, and an increasingly complex picture is emerging in which GSD and TSD are the ends of a continuum of SDMs in reptiles. Examples of intermediate SDMs include species in

which different populations utilize different sex determining mechanisms (Pen et al. 2010), temperature-dependent sex reversal of a species with a ZZ/ZW GSD system in the wild (Quinn et al. 2007; Holleley et al. 2015), and 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). This shifting landscape provides an exceptional study system for better understanding sex determination in vertebrates (Sarre et al. 2004).

The collared lizard, *Crotaphytus collaris*, is a widespread, oviparous species in which sex chromosomes have not been identified (Gorman 1973; De Smet 1981). Yet, *C. collaris* has been classified as a GSD species based on phylogenetic analyses (Pokorna and Kratochvíl 2009). However, as mentioned above, even members of the same species can utilize different sex determining mechanisms (Pen et al. 2010). Thus, classifying *C. collaris* solely based on phylogeny may provide an incorrect or incomplete picture (Viets et al. 1994). Compellingly, in an investigation seeking to determine if *C. collaris* utilizes TSD, an inverse TSD type II pattern was identified in which the percentage of female offspring declined as constant incubation temperatures or treatments approached high and low extremes (Santoyo-Brito et al. 2017). While the authors of this study point out that their sample size was low, this indicates a temperature influence on sex determination in *C. collaris*. However, even at low and high treatments this study did not find ratios of either sex nearing 100%. These findings hint at a more complex mechanism for sex determination than pure TSD or pure GSD in *C. collaris,* as suggested in other reptile species (Quinn et al. 2007; Radder et al. 2008; Holleley et al. 2015). In species that possess XY sex chromosomes, 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 *Crotaphytus insularis* points to heterogamety (males are heterogametic) and,

thus, to the possibility of GSD in Crotaphytids (Rovatsos et al. 2014b). The apparent conflict between the findings of the above studies compels us to determine if *C. collaris* demonstrates gene dosage akin to that identified in *C. insularis*.

Methods

DNA isolation and PCR

Blood was collected from the toes of twenty wild caught lizards (10 male, 10 female) upon capture at Sooner Lake Dam, Pawnee Co., Oklahoma. Blood was immediately preserved on Whatman FTA classic cards. DNA was later extracted 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. We tested for gene dosage in the genes ATP2A2 (sarcoplasmic reticulum calcium ATPase 2), TMEM (transmembrane protein 123D), and PEBP1 (phosphatidylethanolamine binding protein 1) (Table 1). Primer sequences for the genes TMEM, PEBP1, and ATP2A2 were obtained from Rosavatos et al (2014; Table 1). The single copy gene EF1α was used for gene dosage normalization. PCRs were assembled in 25-µl final volumes containing 12.5 µl 2X Bullseye EvaGreen qPCR master mix buffer (MidSci, St. Louis, MO), 25 ng genomic DNA, and 200 pMoles 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 sec, 55˚C for 30 sec, 72˚C for 45 sec. Amplification via qPCR was executed in a Stratagene MX3005 thermocycler.

Gene Dosage Calculations

Final gene dosage ratios were calculated for the two male and two female lizards whose DNA consistently and reliably amplified across three replicates. Crossing point values were

95 calculated in MaxPro (Stratagene) then normalized to EF1α. Gene dosage was calculated as in
96 Rosavotas et al. (2014b) with: $R = [2^{Cp\ gene}/2^{Cp\ EF1\alpha}]^{-1}$ and r (relative gene dosage ratio) =
97 R_{male}/R_{female}. It was expected that male *C. collaris* are the heterogametic sex based on results in
98 the closely related *Crotaphytus insularis* (Rovatsos et al. 2014b), thus an r = 0.5 is expected for
99 single copy, X-linked genes while r = 1.0 is expected for autosomal genes.
100

101 ## Results

102 Results demonstrate sex-linked differences for all three analyzed genes (ATP2A2,
103 TMEM, and PEBP1; Fig 1). In each case, the average female r value is exactly one. This result is
104 consistent with females having two copies of the gene of interest and, thus, being the
105 homogametic sex. For each gene an average value near 0.5 was obtained in the males (ATP2A2
106 = 0.59, TMEM = 0.61, PEBP1 = 0.59). This result is consistent with males having a single gene
107 copy and being heterogametic.
108

109 ## Discussion

110 Though sex chromosomes have not been detected in Crotaphytids (Gorman 1973; De
111 Smet 1981) our results are consistent with heterogamety and point to the existence of GSD in *C.*
112 *collaris* as in *C. insularis* (Rovatsos et al. 2014b). While GSD is common among lizards, *C.*
113 *collaris* has been shown to experience changes in sex ratios with varying incubation
114 temperatures (Santoyo-Brito et al. 2017). *Crotaphytus collaris* may be another in the emerging
115 examples of a reptilian species with GSD that can be overridden by temperature extremes (Shine
116 et al. 2002; Holleley et al. 2015). We agree with Santoyo-Brito et al. (2017) that *C. collaris*
117 likely possess sex microchromosomes in an XX/XY pattern and that extreme incubation

118 temperatures alter the sex determining pathway such that XX individuals develop as phenotypic
119 males. We further hypothesize that gravid *C. collaris* females select nest sites such that GSD will
120 function without temperature interference as evidenced by field hatchling ratios near 50/50 as
121 expected in GSD populations (Wiggins unpublished data).

122 Evidence continues to emerge that some extant reptile species employ multimodal sex
123 determining mechanisms (Shine et al. 2002; Valenzuela et al. 2003; Sarre et al. 2004; Quinn et
124 al. 2007; Radder et al. 2008; Holleley et al. 2015). These species may be at a transition point
125 from GSD to TSD. *Crotaphytus collaris* demonstrates gene dosage consistent with that of 28
126 species of Pleurodont iguanian lizards (Pleurodonta) spanning 11 genera and including *Anolis*
127 *carolinensis* and *C. insularis* (Rovatsos et al. 2014a,b). The absence of genes on the Y that are
128 present on the X, coupled with the chromosomal looping in the *A. carolinensis* XY synaptonemal
129 complex, points to a degenerate Y microchromosome in this species and, subsequently, those
130 with the same sex-based gene dosage (Alföldi et al. 2011; Bachtrog et al. 2014; Rovatsos et al.
131 2014a,b; Lisachov et al. 2017). Thus, there exists the strong possibility that *C. collaris* also
132 possesses a degenerate Y microchromosome. Results from Santoyo-Brito et al. (2017) show a
133 decline in number of females hatched at extreme high and low temperatures, pointing to a
134 temperature override that converts XX individuals into phenotypic males (XXm). If these XXm
135 are viable, they may mate and reproduce (as males). While this scenario will lead to an increase
136 in the proportion of genetic females, the possibility of producing offspring who possess both
137 degenerate chromosomes (YY) would be avoided as in *Bassiana duperreyi* (Shine et al. 2002).
138 As a result, GSD and TSD could conceivably co-exist in *C. collaris* without a definitive
139 transition to one or the other. However, as global temperatures rise, frequent shifts of XXf to
140 XXm could eliminate the Y microchromosome, driving this species to pure TSD and increasing

its vulnerability to extinction by fixation of homogamety and absence of XY individuals (Mitchell and Janzen 2010).

In summary, our data convincingly point to the presence of as-yet unidentified sex microchromosomes in *C. collaris* as suggested by Santoyo-Brito et al. (2017) and add to the growing evidence that SDMs in non-avian reptiles are not bimodal (Shine et al. 2002; Holleley et al. 2015). Further inquiry into the effects of extreme temperature incubations on *C. collaris* sex determination is warranted (Santoyo-Brito et al. 2017); 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 *P. vitticeps* (Holleley et al. 2015), if such individuals are reproductively viable, and if they exist in natural populations.

Literature Cited

Alföldi, J., F. Di Palma, M. Grabherr, C. Williams, L. Kong, E. Mauceli, P. Russell, C. B. Lowe, R. E. Glor, J. D. Jaffe, D. A. Ray, S. Boissinot, A. M. Shedlock, C. Botka, T. A. Castoe, J. K. Colbourne, M. K. Fujita, R. G. Moreno, B. F. ten Hallers, D. Haussler, A. Heger, D. Heiman, D. E. Janes, J. Johnson, P. J. de Jong, M. Y. Koriabine, M. Lara, P. A. Novick, C. L. Organ, S. E. Peach, S. Poe, D. D. Pollock, K. de Queiroz, T. Sanger, S. Searle, J. D. Smith, Z. Smith, R. Swofford, J. Turner-Maier, J. Wade, S. Young, A. Zadissa, S. V Edwards, T. C. Glenn, C. J. Schneider, J. B. Losos, E. S. Lander, M. Breen, C. P. Ponting, and K. Lindblad-Toh. 2011. The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 477:587–91.

Bachtrog, D., J. E. Mank, C. L. Peichel, M. Kirkpatrick, S. P. Otto, T.-L. Ashman, M. W. Hahn, J. Kitano, I. Mayrose, R. Ming, N. Perrin, L. Ross, M. N. Valenzuela, and J. C. Vamosi. 2014. Sex Determination: Why So Many Ways of Doing It? PLoS Biol. 12:1–13.

Bull, J. J. 1983. Evolution of sex determining mechanisms. Benjamin/Cummings Publishing Company, Menlo Park, California.

Burt, A., and R. Trivers. 2006. Genes in Conflict. Harvard University Press, Cambridge, Massachusetts.

Charlsworth, B. 1996. The evolution of chromosomal sex determination and dosage compensation. Curr. Biol. 6:149–162.

De Smet, W. 1981. Description of the orcein stained karyotypes of 27 lizard species (Lacertilia, Reptilia) belonging to the families Iguanidae, Agamidae, Chamaeleontidae and Gekkonidae (Ascala- bota). Acta Zool. Pathol. Antverp. 76:35–72.

Ewert, M. A., and C. E. Nelson. 1991. Sex determination in turtles: patterns and some possible

187 adaptive values. Copeia 1:50–69.

188 Gorman, G. 1973. The chromosomes of the Reptilia, a cytotaxonomic interpretation. Pp. 349–

189 424 *in* A. Chiarelle and E. Cpanna, eds. Cytotaxonomic and Vertebrate Evolution.

190 Academic Press, Inc., USA.

191 Guler, Y., S. Short, P. Kile, and A. Fort. 2012. Integrating field and laboratory evidence for

192 environmental sex determination in the amphipod, Echinogammarus marinus. Mar. Biol.

193 159:2885–2890.

194 Haldane, J. B. S. 1922. Sex ratio and unisexual sterility in hybrid animals. J. Genet. 12:101–109.

195 Holleley, C. E., D. O'Meally, S. D. Sarre, J. A. Marshall-Graves, T. Ezaz, K. Matsubara, B.

196 Azad, X. Zhang, and A. Georges. 2015. Sex reversal triggers the rapid transition from

197 genetic to temperature-dependent sex. Nature 523:79–82.

198 Janzen, F. J., and G. Paukstis. 1991. Environmental sex determination in reptiles: ecology,

199 evolution, and experimental design. Q. Rev. Biol. 66:149–179.

200 Kettlewell, J. R., C. S. Raymond, and D. Zarkower. 2000. Temperature-dependent expression of

201 turtle Dmrt1 prior to sexual differentiation. Genesis 26:174–178.

202 King, M. 1977. Evolution and Reproduction. Pp. 55–60 *in* J. Calaby and H. Tyndale-Biscoe, eds.

203 Evolution and Reproduction. Australian Academy of Science, Canberra.

204 Kratochvíl, L., L. Kubicka, and E. Landova. 2008. Does the mechanism of sex determination

205 constrain the potential for sex manipulation? A test in geckos with contrasting sex-

206 determining systems. Naturwissenschaften 95:209–215.

207 Lisachov, A. P., V. A. Trifonov, M. Giovannotti, M. A. Ferguson-Smith, and P. M. Borodin.

208 2017. Heteromorphism of "Homomorphic" Sex Chromosomes in Two Anole Species

209 (Squamata, Dactyloidae) Revealed by Synaptonemal Complex Analysis. Cytogenet.

Genome Res. 151:89–95.

Merchant-Larios, H., and V. Díaz-Hernández. 2013. Environmental sex determination mechanisms in reptiles. Sex Dev. 7:95–103.

Mitchell, N. J., and F. J. Janzen. 2010. Temperature-Dependent Sex Determination and Contemporary Climate Change. Sex. Dev. 4:129–140.

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. Nature Research.

Pokorna, M., and L. Kratochvíl. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool. J. Linn. Soc. 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. Biol. Lett. 4:176–8.

Reeve, H. K., and D. W. Pfennig. 2003. Genetic biases for showy males: Are some genetic systems especially conducive to sexual selection? PNAS 100:1089–1094.

Rovatsos, M., M. Altmanová, M. Pokorná, and L. Kratochvíl. 2014a. Conserved sex chromosomes across adaptively radiated anolis lizards. Evolution (N. Y). 68:2079–2085.

Rovatsos, M., M. Pokorná, M. Altmanová, L. Kratochvíl, and M. Pokorna. 2014b. Cretaceous park of sex determination : sex chromosomes are conserved across iguanas. Biol. Lett. 10:1–4.

Santoyo-Brito, E., M. Anderson, and S. F. Fox. 2017. Incubation Temperature Modifies Sex Ratio of Hatchlings in Collared Lizards, *Crotaphytus collaris*. J. Herpetol. 51:197–201.

233 Sarre, S. D., A. Georges, and A. E. Quinn. 2004. The ends of a continuum: Genetic and
234 temperature-dependent sex determination in reptiles. BioEssays 26:639–645.
235 Shine, R., M. J. Elphick, and S. Donnellan. 2002. Co-occurrence of multiple, supposedly
236 incompatible modes of sex determination in a lizard population. Ecol. Lett. 5:486–489.
237 Smith, C., P. McClive, P. Western, K. Reed, and A. Sinclair. 1999. Evolution- Conservation of a
238 sex-determining gene. Nature 402:601–602.
239 Spotila, L. D., J. R. Spotila, and S. E. Hall. 1998. Sequence and Expression Analysisof Wt1 and
240 Sox9 in the Red-Eared Slider Turtle, Trachemys Scripta. J. Exp. Zool. 281:417–427.
241 Valenzuela, M. N., D. C. Adams, and F. J. Janzen. 2003. Pattern Does Not Equal Process:
242 Exactly When Is Sex Environmentally Determined? Am. Nat. 161:676–683.
243 Valenzuela, M. N., and V. Lance. 2004. Temperature Dependent Sex Determination in
244 Vertebrates. Smithsonian Books, Washington, DC.
245 Viets, B. E., M. A. Ewert, L. G. Talent, and C. E. Nelson. 1994. Sex-Determining Mechanisms
246 in Squamate Reptiles. J. Exp. Zool. 56:45–56.
247 Walker, G. 2005. Sex determination in the larvae of the parasitic barnacle Heterosaccus lunatus:
248 an experimental approach. J. Exp. Mar. Bio. Ecol. 318:31–38.
249 Western, P., and A. Sinclair. 2001. Sex, genes, and heat: Triggers of diversity. J. Exp. Zool.
250 290:624–631.
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256 **Table 1. Genes and primer sequences used to determine relative gene dosage through**

257 **qPCR in *Crotaphytus collaris***

Gene short name	Gene name	Forward Primer	Reverse Primer	Amplicon size
EF1a	Elongation factor 1	CCTTATTGTTGCTGCTGGTGTT	GTGCTAACTTCTTTGACGATTTCC	189
TME	Transmembrane protein 132D	TATCCGAGCAGACCCAAAGTCC	AAGGAGACCCAACTCAGCCAC	183
ATP	Sarcoplasmic/ endoplasmic reticulum calcium ATPase 2	CAAAGCAGCGGGCATTTAGG	ATCACTGGGGACAACAAGGG	160
PEPB	Phosphatidylethoanolamine-binding protein 1	GACAGGGCTCCATCGCTAC	CATAGTCATCCCACTCCGCC	188

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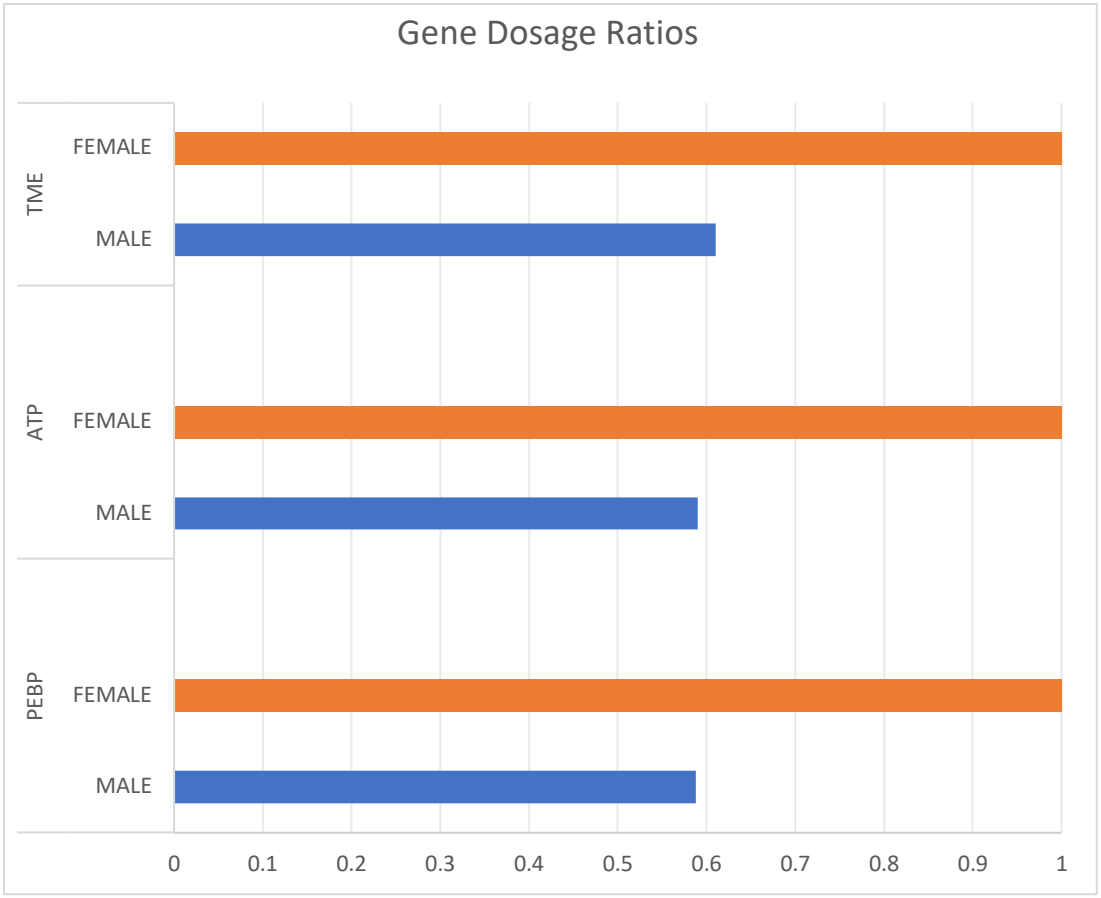

Figure 1. Relative gene dosage at genes PEBP, ATP, and TME for the two males and two females whose DNA amplified consistently and reliably across three replicates. For each gene, the female gene dosage is exactly 1 and the male gene dosage is near 0.5. Each of these genes maps to the *Anolis carolinensis* X microchromosome, thus, this pattern is consistent with male heterogamety.