EVOLUTION OF SEX-DETERMINING MECHANISMS IN REPTILES

by

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Male Central bearded dragon (*Pogona vitticeps*) at twilight, southwest Queensland.

"The three evolutionary questions – what, how, and why – may be applied separately to the study of sex determination, but a perspective combining all three levels can lead to a particularly enriched paradigm of study."

J.J. Bull (1985)

Statement of Originality

This thesis is my original work and has not been submitted, in whole or in part, for a degree at this or any other university. Nor does it contain, to the best of my knowledge and belief, any material published or written by another person, except as acknowledged in the text.

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Abstract

Reptiles exhibit marked diversity in sex-determining mechanisms. Many species exhibit genotypic sex determination (GSD) with male heterogamety (XX females/XY males), others have GSD with female heterogamety (ZW females/ZZ males), and still others exhibit temperature-dependent sex determination (TSD). The distribution of these mechanisms throughout the reptile phylogeny implies evolutionary lability in sex determination, and in some lineages there has been a number of transitions between GSD and TSD. Despite this diversity, GSD and TSD have traditionally been viewed as mutually-exclusive mechanisms of sex determination in reptiles, since there is little evidence for their co-occurrence. Considerable empirical and theoretical effort has been directed towards understanding the adaptive significance of TSD in reptiles. In comparison, there has been little focus on understanding how evolutionary transitions between GSD and TSD occur at a genetic and mechanistic level. I addressed this question by applying both empirical and theoretical approaches to investigate interaction of genotypic and temperature influences in the sex determination of two endemic species of Australian lizards.

The three-lined skink, *Bassiana duperreyi*, has XX/XY chromosomal sex determination, yet a previous investigation reported a significant male bias in the sex ratio of eggs incubated at low temperatures. To enable an explicit test for temperature-induced sex reversal in this species, a 185 bp Y chromosome marker was isolated by Amplified Fragment Length Polymorphism (AFLP) analysis. The marker was subsequently converted into a duplex PCR assay that co-amplified a 185 bp (or 92 bp) Y chromosome fragment and a 356 bp fragment of the single-copy nuclear gene C-*mos* (from both sexes) as a positive control. The accuracy of the PCR sex assay was tested on 78 individuals for which sex reversal was not expected. PCR genotype and sex phenotype were concordant for 96% of the animals. This is one of the very few sex tests developed for a reptile, and the first report of Y chromosome sequence from a reptile. The PCR assay was subsequently applied to genotype hatchlings from both cool (16±7.5°C) and warm (22±7.5°C) cyclical incubation temperature treatments, and identified sex reversal in 15% of genotypically female (XX) embryos (n=26) from the cool treatment, but no sex reversal in eggs from the warmer treatment (n=35). Thus, low incubation temperatures can over–ride genotypic sex determination in *B. duperreyi*, indicating that GSD and TSD co-occur in this species.

The Central bearded dragon, *Pogona vitticeps* (Agamidae), has ZZ/ZW chromosomal sex determination, and is a member of a lizard family in which GSD and TSD are both widespread, indicating evolutionary lability in sex determination. AFLP analysis was applied to isolate homologous Z and W chromosome-linked markers (71 bp and 72 bp, respectively) from this species. The AFLP sequences were subsequently extended into larger genomic fragments by a reiterated genome walking procedure, producing three non-overlapping contigs of 1.7 kb, 2.2 kb and 4.5 kb. The latter two fragments were verified as distinct, homologous Z/W chromosome fragments by PCR analyses. An amplified 3 kb fragment of the 4.5 kb contig was physically mapped to metaphase spreads, identifying the W microchromosome, and for the first time in this species, the Z microchromosome. PCR analyses indicated the presence of homologous sequences in other Australian agamid species, including both GSD and TSD species. The isolated sequences should therefore prove useful as a comparative genomic tool for investigating the genomic changes that have occurred in evolutionary transitions between sex-determining mechanisms in agamids, by enabling the identification of chromosomes in TSD species that are homologous to the sex chromosomes of *P. vitticeps.* The isolated sequences were further converted into a duplex DNA sex assay that co-amplified a 224 bp W chromosome fragment and a 963 bp positive control fragment in both sexes. This PCR assay diagnosed chromosomal sex in three *Pogona* species, but was not effective outside the genus.

Incubation treatment of *P. vitticeps* eggs revealed a strong and increasing female bias at high constant temperatures (34-36°C), but an unbiased sex ratio between 22-32°C. Hatchlings from three clutches split between 28°C and 34 or 36°C incubation treatments were genotyped with the W chromosome AFLP marker. At 28°C, the sex ratio was 1:1 but the high temperature treatments produced 2 males and 33 females. All but one of the 30 lizards (97%) incubated at 28°C had concordant sex phenotype and genotype, but only 18 of 35 animals (51%) from the high temperature treatment were concordant. All discordant animals were genotypic males (ZZ) that developed as females. Thus, temperature and genotypic influences can interact to determine sex in *P. vitticeps*.

These empirical findings for *B. duperreyi* and *P. vitticeps* were extended into a novel theory for the evolution of sex-determining mechanisms in reptiles, working within the framework that species with temperature-induced reversal of chromosomal sex determination are a window to transitional stages of evolution between GSD and TSD. A model was derived from the observation that in both lizards, an extreme of incubation temperature causes sex reversal of the homogametic genotype. In this model, the strength of a genetic regulatory signal for sex determination must exceed a threshold for development of the homogametic sex to occur (male in *Pogona*, female in *Bassiana*). The strength of this signal is also temperature-sensitive, so diminishes at extremes of temperature. Simulation modelling demonstrated that increasing the relative magnitude of the threshold for sexual development can cause evolutionary transitions between GSD and TSD. Even more remarkably, decreasing the relative magnitude of the threshold value causes an evolutionary transition between female and male heterogametic GSD. Quantitative adjustment of a single model parameter (the threshold value) thus charts a continuous evolutionary pathway between the three principal mechanisms of sex determination in reptiles (XX/XY—ZZ/ZW—TSD), which were previously considered to be qualitatively distinct mechanisms.

The experimental demonstration of temperature-induced reversal of chromosomal sex determination in both *B. duperreyi* and *P. vitticeps* presents a challenge to the traditional view that reptilian sex determination is strictly dichotomous (GSD *or* TSD), and suggests instead that sex determination in reptiles consists of a continuum of systems of interaction between genotypic and temperature influences. Simulation modelling provided solid theoretical support for this proposition, demonstrating that transitions along this continuum are effected simply through shifts in the mean population value for the sex-determining threshold, without requiring substantial genotypic innovation. An important implication of this theory is that transitions between XX/XY and ZZ/ZW modes of GSD may retain the same sex chromosome pair, and the same primary sex-determining gene, in contrast to previous models for heterogametic transitions. A more immediate implication of these findings is that many reptile species believed to have strict TSD (in particular, lizards and crocodilians), may in fact have a sex-determining system of GSD-TSD interaction, where there is an equilibrium between GSD and TSD individuals within the population.

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I thank my *Bassiana* collaborators at the University of Sydney - Raju Radder, Rick Shine and Mel Elphick - for their important contributions to my PhD research. I appreciated the opportunity to be involved in developing further an already well-established model reptile species. Again, I look forward to any future opportunities for collaboration.

I am very grateful to Jacqui Richardson for all her incredible work in maintaining the dragon colony at UC, and to Enzo Guarino for his work in the dragon incubation experiments, which included sexing and tissue sampling hundreds of hatchlings. Thank you also to Jill Bartlett, Shona Hodgetts, and Cameron Lockey for their involvement in animal husbandry and egg incubations. Thank you to Martha Rees, Kellee Parkes, Niccy Aitken, Megan McCann, Dave Bourne, Jill Bartlett, Cindy Chan, Cameron Lockey, Steve Sarre, Enzo Guarino and Scott Thomson for their willing assistance on field trips to NSW, Victoria, and Queensland to collect dragons. I would also like to thank the staff at Wyperfeld National Park in Victoria for providing the use of the depot building during our visits, and for helpfully allowing us access to the entire park. Thank you very much to Denis O'Meally for his dragon and tissue-acquiring efforts, and also to Deb Bower.

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Statement of Contribution

This thesis includes papers for which I am senior but not sole author. I took the lead in this research in that I designed the research, conceived and developed the novel ideas, undertook the fieldwork, analysed the data and wrote the manuscripts. I was, however, assisted by my co-authors. Manuscripts for which I am senior author have been included as central chapters, and papers for which I am a co-author are included as appendices. My contributions to the papers included as Appendices, were as follows:

Appendix 1 – I contributed as an equal partner in both the generation of the ideas and in the writing of the manuscript.

Appendix 2 – I was involved in the animal and laboratory work leading to the generation of the results secondary only to the senior author, and made a major contribution to the writing of the manuscript.

Appendix 3 – This paper depended on the development and application of the DNA sex test, both of which were my contribution, and I had input into writing the paper.

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Statement of Co-authorship

Core manuscripts:

Chapter 2: Quinn AE, Radder RS, Georges A, Sarre SD, Ezaz T, Shine R.
Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal. *To be submitted.*

Chapter 3: Quinn AE, Ezaz T, Sarre SD, Georges A, Graves JAM.
From AFLP to Z: Isolation, conversion, and physical mapping of sex chromosome sequence in a dragon lizard. *To be submitted.*

Chapter 4: Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JAM (2007) Temperature sex reversal implies sex gene dosage in a reptile. *Science* 316:411, plus supplementary online material.

Chapter 5: Quinn AE, Georges A, Sarre SD, Ezaz T, Graves JAM.
The evolutionary dynamics of sex as a threshold trait. *To be submitted.*

Appendix 3: Radder RS, Quinn AE, Georges A, Sarre SD, Shine R (2008)
Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biology Letters* 4: 176-178

Other associated manuscripts:

Appendix 1: Sarre SD, Georges A, Quinn AE (2004)
The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. *Bioessays* 26: 639-645.

Appendix 2: Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A (2005)
The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Research* 13: 763-776.

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