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Mechanisms underlying intraspecific divergence in sex determination in *Carinascincus ocellatus*.

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Declarations

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Statement of co-authorship

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Most people know I am a mad Dr Who fan, and my favourite incarnation is that of the 10th doctor played by David Tennant. As I regenerate into post-PhD life (eventually as Dr Hill) I frequently hear his catchphrase in my head: "*Allons-y!!!*" but, even though I am proud of my achievements and ready for the next step in my career, I can also relate to his last words before regenerating into the 11th Doctor: "I don't want to go".

Abstract

The division of individuals into separate sexes is ancient and near-ubiquitous in sexually reproducing organisms. However, sex determination, the switch that controls this division is diverse and subject to ongoing research. Sex can be determined by genes on sex chromosomes, known as genetic sex determination, GSD; or by the environment, often by temperature, known as temperature dependent sex determination, TSD. GSD is either male heterogametic, i.e. XX females and XY males such as in mammals, or female heterogametic, ZZ males and ZW females such as in birds. Mammals and birds possess ancient, conserved systems of genetic sex determination. However, sex determination is both more diverse and more labile in reptiles, with multiple and often recent evolutionary transitions and this is epitomised in lizards. Within lizards there are species with GSD – both XY and ZW heterogamety – and TSD. However, many lizard groups also combine genes and temperature to determine sex and the mechanisms of sex determination occur along a continuum. Understanding how transitions between TSD and GSD, and within GSD how transitions between XY and ZW heterogamety occur remains a major challenge in evolutionary biology.

In this thesis I use the viviparous Tasmanian spotted snow skink, *Carinascincus ocellatus* (formerly *Niveoscincus*), an extraordinary example of a species exhibiting incipient divergence in sex determination to understand the mechanisms that underpin evolutionary transitions in sex determination. Long term data on this species shows that in a high elevation population, sex ratios do not deviate from parity regardless of temperature, suggesting GSD. In a low elevation population, sex ratios correlate with developmental temperature and males are favoured in cool temperatures while females are favoured in warm temperatures implicating a strong role of temperature in sex determination. Warmer temperatures at low

elevation result in an extended activity season which benefits females. Females that are born early in this population mature early and therefore have a higher lifetime reproductive output. Cooler temperatures and short activity seasons at high elevation means there are no sex-specific benefits associated with birthdate. Building on this work by combining long-term field data, next generation sequencing, traditional cytogenetic and population genetics approaches and experimental manipulation I explored the mechanisms operating during early stages of within species incipient transitions in sex determination.

Specifically, I a) examined similarities and differences in sex-linked genetic sequences between the populations, b) identified the sex chromosomes in *C. ocellatus* and described both population-level and species level differences, c) investigated the role of temperature in determining sex in both populations and d) estimated the age of the divergence between the two populations and the amount of gene flow occurring since the divergence. I found that a) *C. ocellatus* has XY (male) heterogamety with sex-linked genetic sequence common to both populations in addition to population-specific sex-linked sequence, and evidence that recombination among sex chromosomes has been more disrupted in the high elevation population, b) the homomorphic X and Y chromosomes in both populations of *C. ocellatus* are chromosome pair seven, there are small differences between the Y chromosomes of each population, and sex chromosomes in some Scincid lineages likely evolved independently c) temperature influences sex determination in both populations of *C. ocellatus* by overriding the genetic signal to produce individuals with a sexual phenotype / genotype mismatch despite population-specific response of the sex ratio to temperature and d) high and low elevation populations of *C. ocellatus* diverged less than 900,000 years ago during the glacial cycles of the Pleistocene with no gene flow occurring between these populations since.

My thesis builds and expands on existing knowledge of sex determination transitions and provides a solid basis for understanding the mechanisms involved. In addition, this thesis provides a novel interpretation of the *C. ocellatus* system and the incipient transition in sex determination, and highlights that such transitions occur frequently because the changes to the genome that are required are minor and very little evolutionary time is needed for these changes to become apparent on the sex chromosomes and in the phenotype.

Contents

Chapter One

General Introduction

Sexual phenotype is important. Development into separate sexes represents one of the oldest evolutionary innovations accounting for the greatest amount of within-species variation. An individual's sex is a fundamental component of their phenotype that impacts survival, mating success, and fecundity. Sex is a major factor in all aspects of life history from birth to senescence. Decisions surrounding growth, reproduction, acquisition of resources, predation-risk and mating frequency often have sex-specific trade-offs (Orr 2009; Tarka et al. 2018; Zajitschek and Connallon 2018) with consequences that feed into population dynamics. At a population level, there is strong selection for 50:50 sex ratios (Fisher 1930) because sex ratios that become heavily biased towards either sex can have consequences for population growth and persistence if one sex becomes limiting (Boyle et al. 2014; Le Galliard et al. 2005; Valenzuela et al. 2019; Wedekind 2017). Selection can also favour biased sex ratios when sex-specific fitness benefits occur (e.g., Charnov and Bull 1977), however, populations with sex ratios that are consistently biased towards one sex can experience deterioration of genetic diversity and therefore have reduced adaptive potential, with consequences for species distributions (Mitchell and Janzen 2010).

The considerable scientific interest in understanding how sex is determined has led to substantial advances in our knowledge of the control of sexual phenotype. Development as male or female is the outcome of a highly conserved pathway initiated by diverse mechanisms. Sex can be determined by either genes on sex chromosomes (genetic sex determination, GSD), the environment (ESD, the most prominent form being temperature

dependent sex determination, TSD) or a combination of genes and environmental effects such as temperature (GSD+EE or thermosensitive GSD). Therian mammals (marsupial and eutherian) and birds have well-described, conserved systems of GSD (Bachtrog et al. 2014; Graves 2014, 2006). Therian mammals have male heterogametic GSD where males are XY and females are XX. Male phenotype is determined by the presence of the *Sry* gene on the Y chromosome, female phenotype is determined by the absence of *Sry* (Koopman et al. 1990). Birds have female heterogametic GSD which differs from therian mammals in both the identity and mode of action of the sex determining gene. Sex in birds is determined by the dosage of *DMRT1* located on the Z chromosome. Two copies (ZZ genotype) result in male phenotype and one copy (ZW) results in female phenotype (Smith et al. 2009). Amongst the remaining vertebrate classes, sex determination is highly varied. Amphibians have XY and ZW GSD while fish and reptiles have XY and ZW GSD along with several systems of ESD (Figure 1; Adkins-Regan and Reeve 2014; Bachtrog et al. 2014; Capel 2017; Quinn et al. 2011). TSD is the most prominent form of ESD found in reptiles and reptiles can also combine genes and temperature to determine offspring sex (Quinn et al. 2007; Shine et al. 2002). Reptiles show a predisposition to evolve TSD from GSD due to their sensitivity to temperature and the variable thermal environments experienced by offspring during development (Georges et al. 2010). Temperature sensitive sex determination is not limited to egg-laying reptiles as once proposed (Bull 1980), but also occurs in live-bearing taxa (Robert and Thompson 2010; Wapstra et al. 2004). Squamate reptiles are emerging as exceptional models for sex determination research because the phylogenetic diversity and evolutionary lability of sex determination within this group suggests independent and often recent evolutionary transitions (Figure 1; Pennell et al. 2018; Sarre et al. 2011).

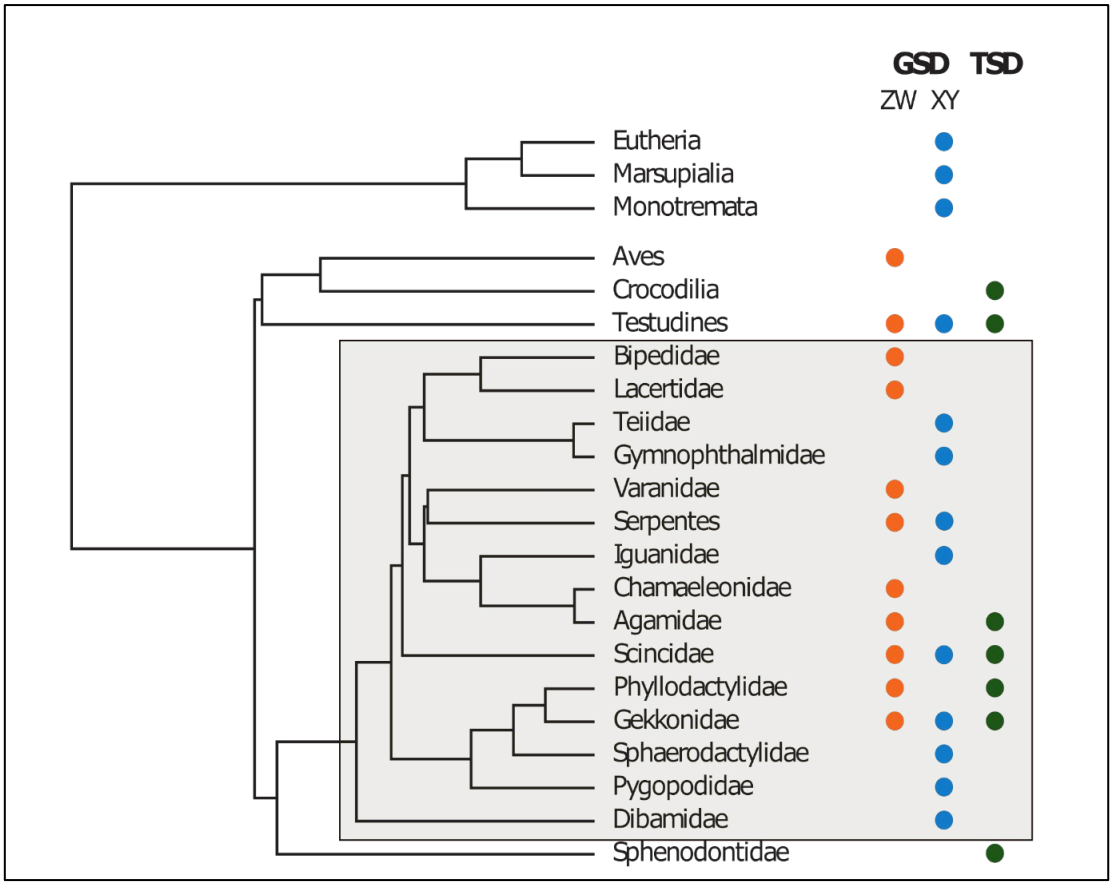

Figure 1. Evolutionary transitions in sex determination across mammals (XY GSD), birds (ZW GSD) and reptiles (XY and ZW GSD plus TSD) highlighting the evolutionary lability of sex determination in Squamates (grey shaded region) compared to other taxa. Image adapted from Sarre et al. (2011).

Sex chromosomes and genetic sex determination

Genetic sex determination occurs at fertilisation via the inheritance of sex determining genes on sex chromosomes. The sex chromosomes of therian mammals and birds are highly conserved within each group and their evolutionary origins are old (approximately 140 and 160 mya ago in birds and therian mammals, repsectively; Bachtrog et al. 2014; Vicoso et al. 2013). However, conserved, ancient sex chromosomes are the exception rather than the rule within vertebrates. Sex chromosomes are a dynamic component of the genome that experiences different selection than autosomes because they are unequally represented in males and females. For example, in XY taxa, the Y chromosome only occurs in males, and 2/3 of the X chromosome population occurs in females and 1/3 in males, whereas autosomes

occur equally in each sex (Charlesworth 2009; Ezaz and Deakin 2014; Johnson and Lachance 2012; Wilson Sayres 2018). Sex chromosomes therefore undergo specific evolutionary processes implicating them in lineage divergence and speciation (Haldane 1922; Johnson and Lachance 2012; Mank 2012). Indeed, sex determination and sex chromosomes are diverse in highly speciose reptile groups (Gamble et al. 2015; Srikulnath et al. 2019), reinforcing the evolutionary links between speciation and sex determination and sex chromosome evolution. Sex chromosomes in reptiles differ in morphology and evolutionary origin, displaying homology with avian sex chromosomes in some lineages (Kawai et al. 2009), and mammalian and avian autosomes in others (Ezaz et al. 2009a; Matsubara et al. 2006), yet can exhibit little homology between closely related reptile lineages (Matsubara et al. 2014).

Sex chromosomes evolve when one member of an autosomal pair (Figure 2a) acquires a sex determining locus (Figure 2b; blue 'allele'). This locus defines the proto-sex chromosomes. Alleles that are beneficial to one sex but detrimental to the other are thus sexually antagonistic and then accumulate near the sex determining locus. Recombination is suppressed between the proto-sex chromosomes in this region ensuring these alleles segregate into the sex they benefit (Figure 2c; yellow 'allele', grey hatching represents region of recombination suppression). Chromosomal inversions can expand the non-recombining region and play an important role in suppressing recombination between sex chromosomes (Charlesworth et al. 2005). Because recombination is suppressed around the sex determining locus, mutations, deletions and insertions such as repeat motifs and retrotransposable elements accumulate in the non-recombining region (Figure 2d; black 'allele'). This process can eventuate in loss of Y (or W) chromosome content and a morphologically and genetically differentiated pair of sex chromosomes (ZW or XY; Figure 2e) that can be distinguished readily using standard microscopy. However, sex chromosome evolution does not always progress to a state of heteromorphy and evolutionarily old sex chromosomes are not

necessarily highly differentiated (e.g., boid snakes and ratite birds; Bachtrog et al. 2014; Vicoso et al. 2013).

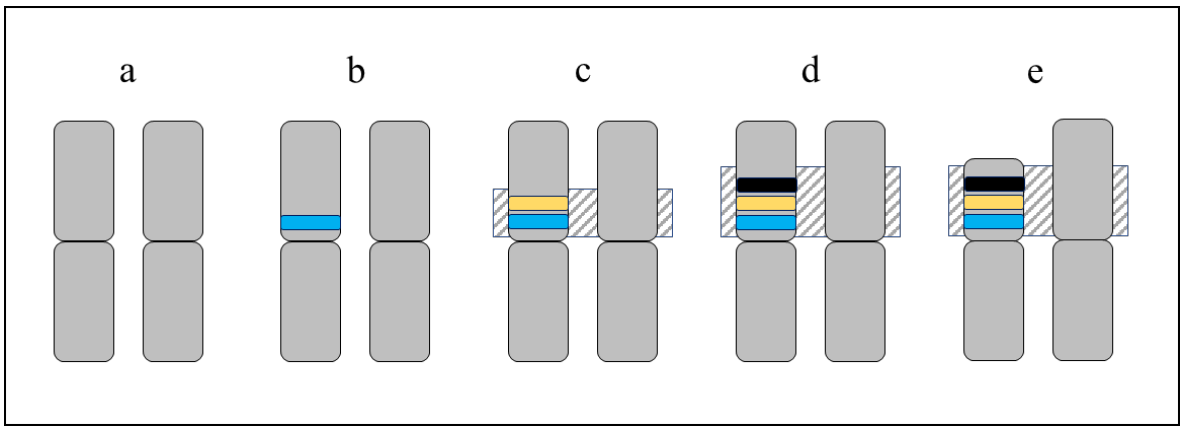

Figure 2. Sex chromosome evolution. An autosome (a) acquires a sex determining locus (b, blue allele) defining a proto sex chromosome pair. The accumulation of sexually antagonistic alleles (c, yellow) is favoured as is recombination suppression (grey hatching) to keep these alleles in the sex they benefit. Repeat elements and non-functional genes accumulate (d, black) as the region of recombination suppression spreads. Morphologically and genetically distinct sex chromosomes (e) sometimes result because recombination suppression prevents purging of deleterious mutations and deletions.

The environment and sex determination

Sex determination in species that lack sex chromosomes typically have a strong environmental component and sex is determined after fertilisation. In species with TSD, the temperature at which embryos develop determines sex. TSD occurs in Crocodilia (Lang and Andrews 1994), Sphenodontia (Nelson et al. 2004), and several species of turtle, lizard and fish (Adkins-Regan and Reeve 2014; Ewert et al. 2005; Janzen and Phillips 2006). In Crocodilia and Sphenodontia, the effect of temperature on sex is acute, with mixed sex clutches occurring at very narrow pivotal temperature ranges of 1–2°C (Figure 3).

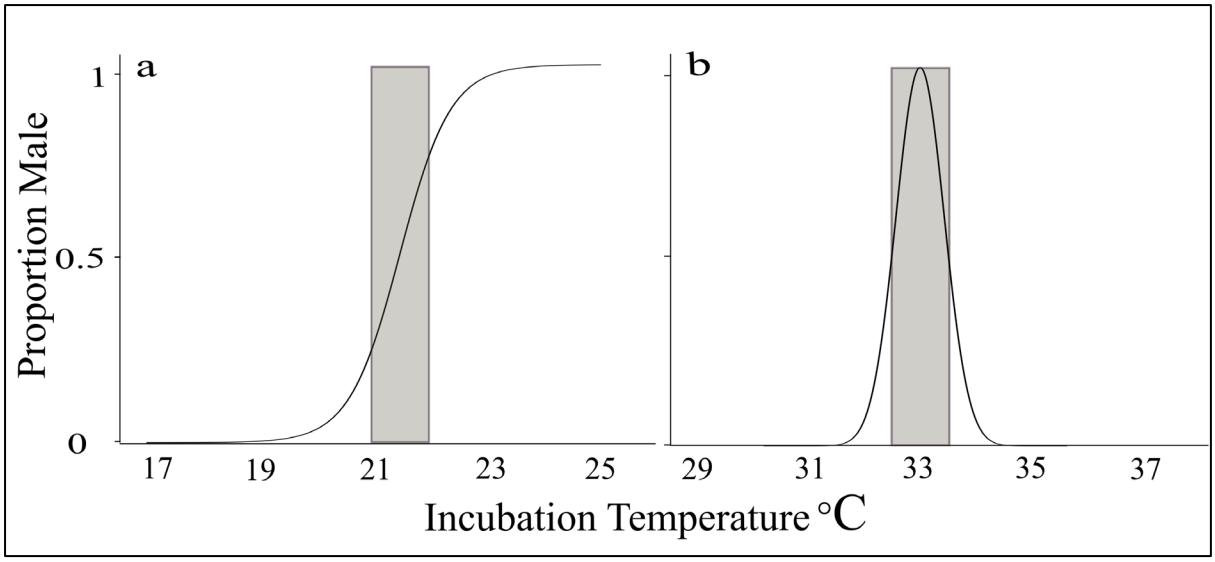

Figure 3. Relationship between clutch sex ratios (proportion male) and incubation temperature is acute for a) *Sphenodon punctatus* (Sphenodontia; Mitchell et al. 2006) and b) *Alligator mississippiensis* (Crocodilia; Lang and Andrews 1994), with 50:50 sex ratios at a narrow pivotal range (1–2°C; grey shading). In Crocodilia, the pivotal temperature can produce 100% males.

Crocodilian clutches are predominantly female at incubation temperatures either side of the pivotal range. Within the pivotal range, clutch sex ratios are between 50% and 100% male (Figure 3b; Lance et al. 2000; Lang and Andrews 1994), whereas in Sphenodontia, females are produced at low temperatures and males at high temperatures either side of the pivotal range (Figure 3a; Mitchell et al. 2006). Recent investigations into sex determination in reptiles has revealed that temperature can also impact sex determination in species that possess sex chromosomes by overriding the genetic signal (Holleley et al. 2015; Quinn et al. 2007; Shine et al. 2002). For example, in the central bearded dragon, *Pogona vitticeps* (Agamidae), mixed sex clutches are common across a broad temperature range with biased sex ratios occurring at warm extremes of the species' viable temperature range (Figure 4; Quinn et al. 2007). Sex determination is therefore considered along a continuum from environmental to genetic systems, between which genes and the environment interact to determine sex (Sarre et al. 2004).

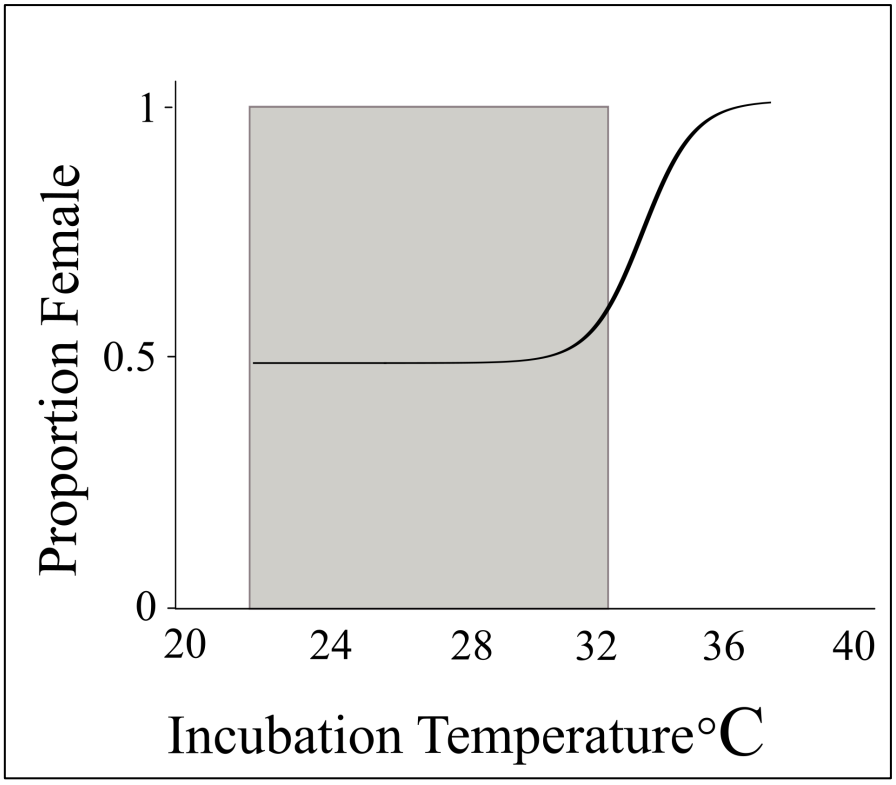

Figure 4. Relationship between clutch sex ratios (proportion female) and incubation temperature in *Pogona vitticeps*. 50:50 sex ratios occur across a broad temperature range (grey shading) and in *P. vitticeps* are female biased at warm temperatures. Figure adapted from Quinn et al. (2007).

When genes and temperature interact to determine sex, and developmental temperature overrides the genetic sex inherited at fertilisation, offspring with a phenotype / genotype mismatch result and this is known as sex reversal (Quinn et al. 2007; Radder et al. 2008). Sex reversal can occur during a temperature sensitive period that extends into the middle third of development in reptiles (Georges 1989; Shine et al. 2007). Gonadal and genital development may be asynchronous in reptiles (Neaves et al. 2006; Whiteley et al. 2018) and ovotestis (a gonadal phenotype consisting of a mix of male and female structures), documented in one species of agamid and common in teleost fish, suggests a period of antagonism between the male and female developmental pathways that may not be resolved until late in development in thermosensitive taxa compared to mammals and birds (Whiteley et al. 2018; Mank et al. 2006).

Models of gene dosage describe the interaction between temperature and the thermosensitive products of sex determining genes that leads to sex reversal (Figure 5; Quinn et al. 2011). In this dosage model, under normal developmental conditions, a double copy of a sex determining gene in the homogametic sex (XX or ZZ) exceeds a product threshold for sexual phenotype, thus deciding the developmental pathway. A single copy of the gene in the heterogametic sex (XY or ZW) does not reach this product threshold and the embryo is diverted down the opposite developmental pathway (Figure 5a). This suggests sex is determined via dosage of a gene on the homogametic sex chromosome (X or Z) as found in birds (Smith et al. 2009). If, in thermosensitive taxa, the thermal threshold for sexual phenotype is evolving, and gene dosage is regulated by developmental temperature, the sex determining signal in the homogametic sex may not reach the threshold for sexual phenotype (Figure 5b). Alternatively, the sex determining signal in the heterogametic sex may reach the threshold for sexual phenotype (Figure 5c). In both these scenarios, offspring sexual development is diverted along the alternative pathway resulting in sex reversal. Sex reversal results in sex ratio shifts away from the parity expected under strict GSD, as observed in the central bearded dragon *Pogona vitticeps* with a ZZ/ZW system (Quinn et al. 2007). Warm nest temperatures result in females (normally ZW) with a male genotype (ZZ) and a female biased sex ratio (Holleley et al. 2015). This suggests a male determining gene on the Z chromosome, which under warm developmental temperatures fails to reach the threshold for male phenotype, thus reversing the ZZ phenotype to female. Similarly, in the three-lined skink *Bassiana duperreyi* with XY/XX system (Radder et al. 2008; Shine et al. 2002), cool nest temperatures result in an excess of males due to the presence of XX sex reversed individuals (Holleley et al. 2016; Radder et al. 2008). No sex determining genes have been identified in reptiles although candidates will likely emerge from the vast network of genes involved in sexual differentiation across vertebrates (Graves 2013; Pan et al. 2016; Uller and

Helantera 2011). Sex reversal of the homogametic sex in both a ZZ/ZW and XX/XY reptile is evidence that dosage - dependent sex determination is common to reptiles and birds even if the sex determining genes are not. Sex reversal of the heterogametic sex, while theoretically possible (Quinn et al. 2011), has yet to be documented in reptiles (Capel 2017; Holleley et al. 2016).

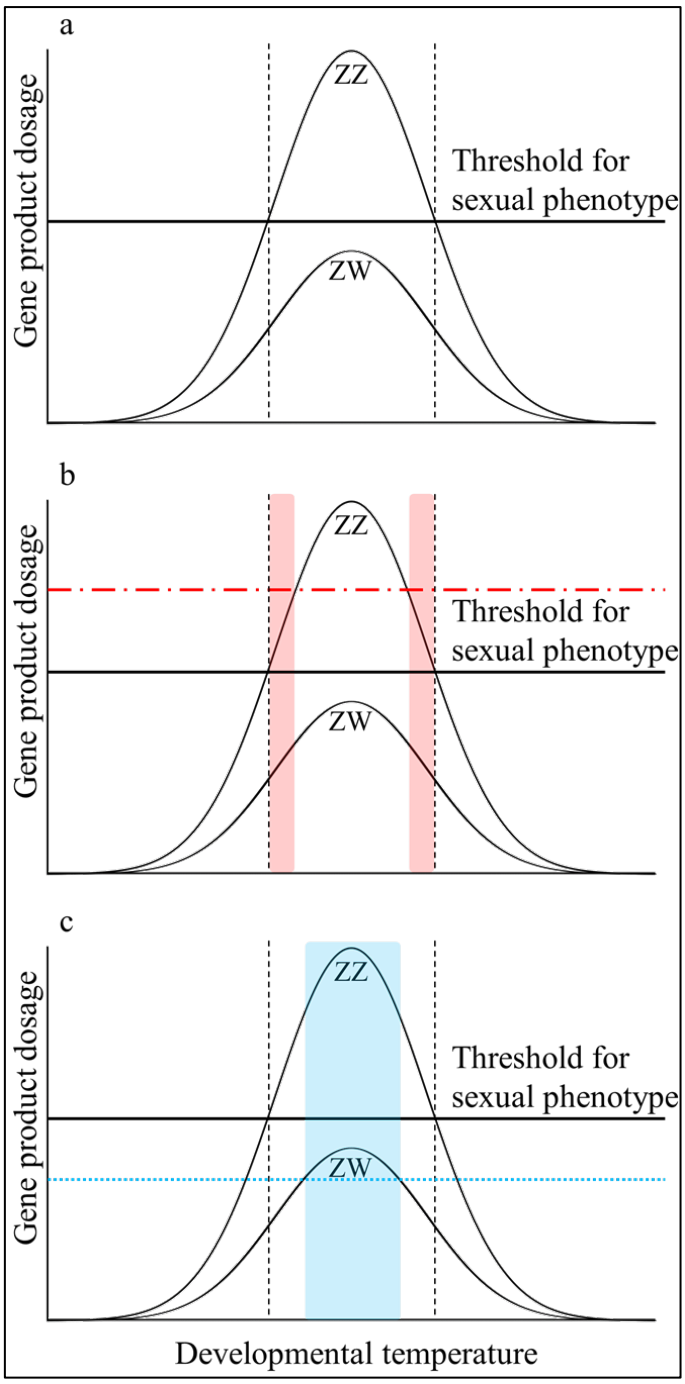

Figure 5. Sex determination as a dosage system, with evolving thresholds resulting in sex reversal, adapted from Quinn et al. (2007) and Quinn et al. (2011); examples given are for a ZZ ZW system of heterogamety but can be applied to an XX XY system. Double dosage (ZZ) of a sex determining gene product (a) reaches a threshold for sexual phenotype (black horizontal line), within species optimal viability range (dotted vertical lines). A single dose of in the ZW genotype does not achieve this threshold. If the threshold for sexual phenotype increases (b, red line), ZZ offspring will not reach the threshold depending on incubation temperature (red shaded areas). If this threshold decreases (c, blue line), ZW offspring may achieve the threshold for sexual phenotype depending on developmental temperature (blue shaded area). In both b and c, sex reversed offspring result from development at temperatures within the shaded regions.

Evolutionary transitions in sex determination

Transitions in sex determination can occur via several mechanisms (reviewed in Vicoso 2019). Sex chromosome evolution via acquisition of a novel sex determining allele on an autosome (Figure 2) can occur even if there are already functioning sex chromosomes in place if the newly evolved sex determining locus confers a stronger fitness advantage than the existing sex chromosomes (Bachtrog et al. 2014; Meisel 2020). This can result in a transition in heterogametic system (e.g., ZW to XY) or a transition to different sex chromosomes within the existing system of heterogamety (Figure 6; Bachtrog et al. 2014; Pennell et al. 2018) with the original sex chromosomes reverting back to autosomes (Roberts et al. 2009; Vicoso and Bachtrog 2013). Alternatively, one of the existing sex chromosome pair can fuse to an autosome thus creating a new sex chromosome system (Bracewell et al. 2017; Meisel 2020; Pennell et al. 2015).

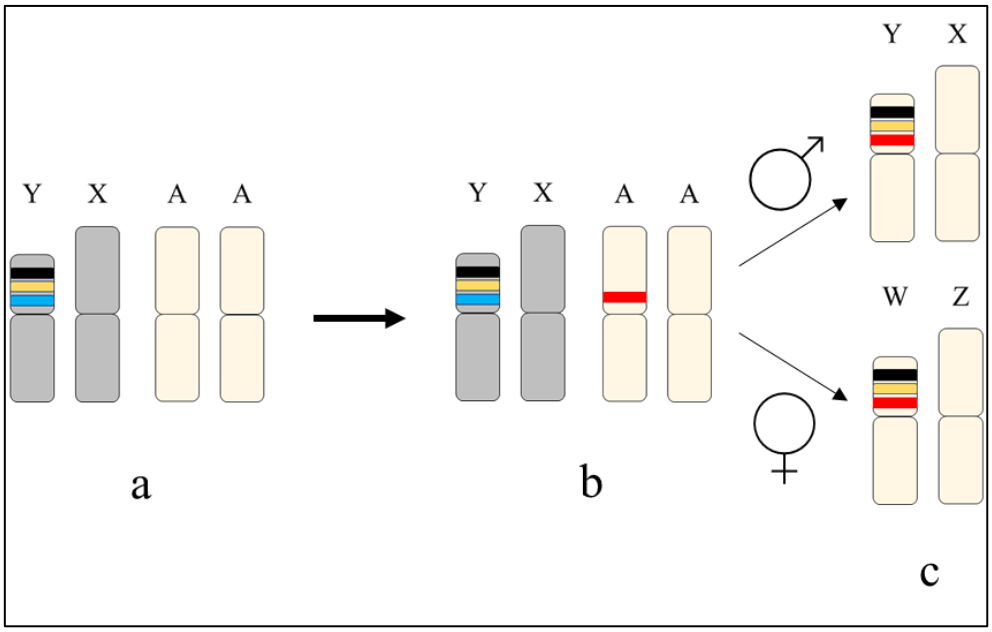

Figure 6. Sex chromosome turnover via acquisition of a novel sex determining locus on an autosome; a) original karyotype consisting of XY sex chromosomes (grey) and a pair of autosomes (A, yellow); b) A new sex determining allele (red) arises on an autosome conferring a greater sex-specific fitness advantage than the existing XY pair; c) If this new locus is female determining, it defines a new ZZ ZW system; if it is male determining, it defines a new XX XY system. Examples given are for turnover of sex chromosomes from a pre-existing XX XY system but can be applied to ZZ ZW systems.

Temperature sensitive sex determining genes combined with an evolving threshold for sexual phenotype (Figure 5) can lead to transitions in sex determination between GSD and TSD and between XY and ZW heterogamety if sex reversed offspring are viable and contribute to subsequent generations (Figure 7; Holleley et al. 2015; Quinn et al. 2011). Here I describe transitions from a ZZ ZW system of heterogamety, but the same outcomes are achieved with XX XY heterogamety. If ZZ sex reversed females mate with ZZ normal males, all offspring will be ZZ (Figure 7a); phenotypic sex will be determined by temperature and the threshold for sexual phenotype in these individuals. If the threshold for phenotypic sex continues to evolve, the W chromosome can reduce in frequency in the population and if lost, the system will transition from GSD to TSD (Figure 7a; Quinn et al. 2011). ZW sex reversed males that mate with ZW females will produce ZZ, ZW and WW individuals. The phenotypic sex of the ZW genotypes depends on the thermosensitivity of sex determination and the threshold for sexual phenotype in those individuals. If the WW genotype is viable, its phenotypic sex will be female. This can eventually lead to a system where all males are ZW and all females are WW if the threshold for sexual phenotype continues to evolve – essentially XY XX heterogamety (Figure 7b). However, several factors including heritable variation in the threshold for sex reversal, environmental fluctuations and gene flow are likely to attenuate transitions between sex determination systems (Schwanz et al. 2020). It is therefore important to understand the role of these factors to form a complete picture of the mechanics of transitions in sex determination and accurately predict the consequences of sex reversal for populations and species.

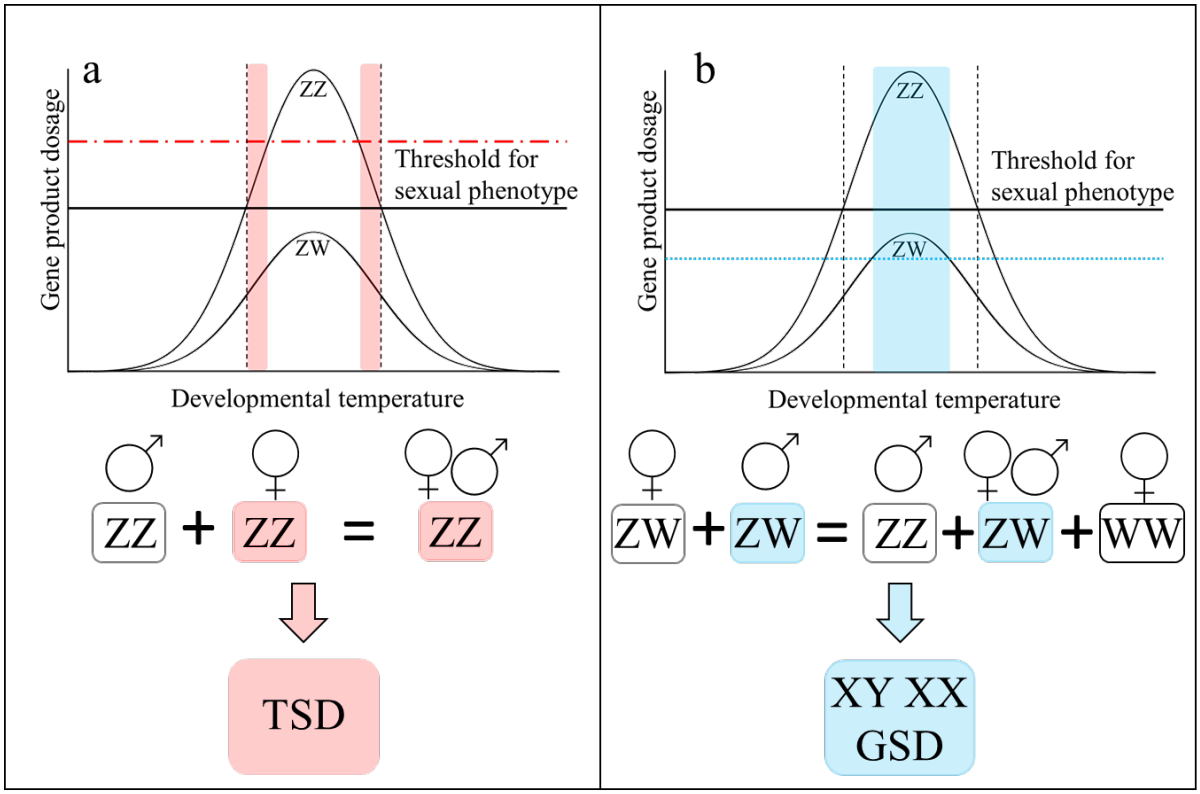

Figure 7. When sex determination is thermosensitive and the threshold for sexual phenotype is evolving, transitions between GSD and TSD (a) or between systems of heterogamety (b) can occur. Beginning with a system of ZZ ZW GSD, if the threshold for sexual phenotype increases (a), sex reversal of the ZZ (red) genotype can result. Mating between sex reversed females (ZZ) and normal males (ZZ) will produce all ZZ offspring and sex of these offspring is determined by temperature. The frequency of the W chromosome decreases, and it is eventually lost, resulting in pure TSD. If the threshold for sexual phenotype decreases (b), sex reversal of the ZW genotype can occur (blue). Mating between sex reversed males (ZW) and normal ZW females will produce ZZ and ZW males and ZW and WW females (assuming WW genotype is viable). The frequency of ZZ males and ZW females decreases; all males are ZW and all females are WW, essentially XY XX GSD. The outcomes when beginning with XX XY GSD are a) TSD and b) ZZ ZW heterogamety.

The evolutionary history of transitions between forms of sex determination raises the fundamental question of why and how the control of a highly conserved phenotypic outcome exhibits extreme lability. The study of reptiles has been advocated for improving our understanding of the evolutionary transitions in sex determination and sex chromosomes because of the diversity of systems represented. In Squamate reptiles, the phylogenetic distribution of sex determination suggests multiple, recent evolutionary transitions (Ezaz et al. 2009b; Gamble et al. 2015; Janzen and Phillips 2006; Pennell et al. 2018; Pokorna and

Kratochvil 2009). Because recent transitions will have components of both ancestral and derived mechanisms, detailed reconstructions of a transition are possible. Reptiles are also well-suited to experiments designed to reveal molecular and chromosomal components of sex determination and to uncover the links between genes, sex and temperature. For example, an ongoing natural population study of *Carinascincus ocellatus* (Scincidae) documents intraspecific divergence in sex ratio response to temperature driven by sex-specific climate effects on birthdate (Cunningham et al. 2017; Pen et al. 2010). Describing the mechanism of sex determination in each population (XY or ZW GSD or TSD) will allow the differences that facilitate population-specific sex ratio response to temperature to be explored.

Study system - *Carinascincus ocellatus*

Carinascincus ocellatus (formerly *Niveoscincus ocellatus*) is a small (3–10 g; 60–80 mm SVL) viviparous, widespread Tasmanian lizard with a broad altitudinal and climatic distribution. Variation in mitochondrial haplotypes suggest geographic differentiation among three regions during the last 2 million years (Figure 8a; Cliff et al. 2015). Two populations at the two altitudinal extremes in one of these regions are the subject of long-term study. Extensive field and laboratory experiments have documented divergent systems of sex determination between these populations (Cunningham et al. 2017; Pen et al. 2010). In a cool high elevation population (41 51'S, 146 34'E; elevation 1200 m; Figure 8a) sex ratios are not correlated with developmental temperature and do not deviate from parity, suggesting sex is determined genetically. In a warm low elevation population (42 34'S, 147 52' E; elevation 50 m; Figure 8a) sex ratios are male biased in cool seasons and female biased in warm seasons, suggesting sex determination has a temperature component.

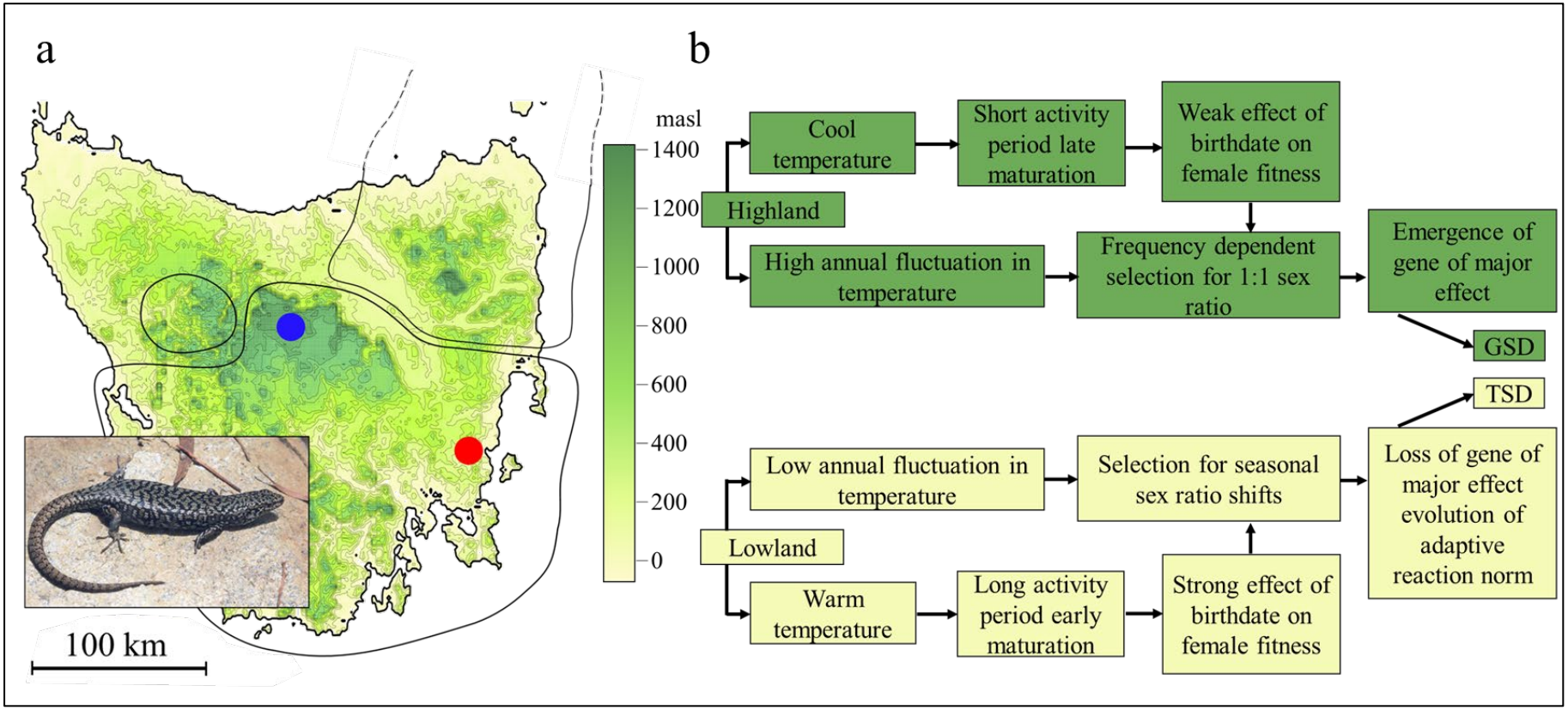

Figure 8. a) Location of high elevation (blue circle) and low elevation (red circle) populations of *Carinascincus ocellatus* (inset) with divergent systems of sex determination. Mitochondrial clades indicated (black lines), adapted from Cliff et al. (2015). The North-eastern clade extends to include Flinders island (not shown). b) Model depicting relationship between climate and population divergence in sex determination in these two populations. At low elevation, warm temperatures result in a long activity period and provide the opportunity for early birth which is beneficial for females because it results in earlier maturation and higher lifetime reproductive output. Combined with low interannual temperature fluctuations this selects for TSD. At high elevation, cool temperatures and a short activity period means no sex-specific benefits of early birth. In addition, high interannual fluctuation at high altitude selects for GSD as it avoids maladaptive sex ratio skews. Adapted from Pen et al. (2010).

The two *C. ocellatus* study populations appear genetically homogenous with respect to mitochondrial DNA, with evidence of demographic expansion during Pleistocene glacial cycles (Figure 8a). The ancestors of these populations likely occupied shared lowland refugia during Pleistocene glaciations, including the Last Glacial Maximum, and were potentially interbreeding (Cliff et al. 2015). In addition, *C. ocellatus* is more-or-less continuously distributed between the high and low elevation populations with no obvious large-scale barriers to movement, suggesting the possibility of contemporary gene flow. Models parameterised with field data suggest temperature sensitive sex determination is adaptive at low elevation because warm developmental temperature results in early birth (Figure 8b). Early birth benefits females because it results in earlier maturity and a higher lifetime reproductive output (Pen et al. 2010). At high elevation, cooler temperature and the resultant short activity season preclude variation in fitness with respect to birthdate for either sex. In addition, high interannual temperature fluctuations at high elevation favour GSD which prevents maladaptive sex ratio skews (this is theoretically modelled in Pen et al. 2010). Such patterns are consistent with theoretical models of adaptive temperature sensitive sex determination which predict sex ratio skews towards the sex that benefits from the prevailing environmental conditions (Charnov and Bull 1977; e.g., *Pogona vitticeps*, Holleley et al. 2015; *Bassiana duperreyi*, Shine et al. 1995; *Amphibolurus muricatus*, Warner and Shine 2008). What is less well understood are the mechanisms by which evolutionary transitions in sex determination occur in this species. Because sex determination systems in *C. ocellatus* have recently or are incipiently diverging, this presents a rare opportunity to expose the mechanisms responsible for transitions in sex determination that can be applied to inform our understanding of more ancient evolutionary transitions across vertebrates.

To test for sex chromosome homology amongst the subfamily Lygosominae, I also used another viviparous scincid, *Liopholis whitii*. *L. whitii* occurs at the same low elevation location as *C. ocellatus* and was chosen because it has a chromosome complement of 2n=32 with homomorphic sex chromosomes (Donnellan 1985) and is phylogenetically nested amongst other skinks that have heteromorphic sex chromosomes or have had their sex determining system described (Pyron et al. 2013; Donnellan 1985).

Research objectives

The overall aim of my thesis was to understand the mechanistic underpinnings of an evolutionary transition in sex determination. Using the recent divergence in *Carinascincus ocellatus*, I aimed to understand ancient transitions in sex determination that have led to the diversity in systems observed in contemporary vertebrates. To address this aim, I first resolved key knowledge gaps surrounding the i) molecular and ii) the chromosomal characteristics of sex determination in *C. ocellatus* populations, iii) I evaluated the role of sex reversal as a mechanism for sex ratio bias and iv) I investigated the evolutionary time required for a divergence in sex determination to become apparent and fixed in populations as a phenotype. This included understanding whether gene flow between these populations has occurred since divergence because of potential sympatry during Pleistocene glacial cycles (Cliff et al. 2015) and the presently continuous geographic distribution of the species. I specifically examined similarities and differences in population-specific sex determination to understand the retained and derived components of this transition. I chose an approach that leveraged long-term observations of sex ratios from these populations and combined next generation sequencing, traditional cytogenetic techniques, experimental manipulation in the laboratory and traditional and contemporary population genetics analyses.

By using this approach and focussing on a recent and potentially ongoing transition, my thesis answers several questions regarding the molecular, chromosomal, physiological and historical components of evolutionary transitions in sex determination. Reptiles are exceptional models for understanding evolutionary transitions in sex determination and my thesis addresses fundamental questions about the processes involved in these transitions during the earliest stages that cannot be addressed using traditional model organisms such as birds and mammals.

Thesis presentation

This thesis comprises four data chapters associated with the research objectives outlined above. All chapters have either been accepted for publication in relevant scientific journals or are in preparation for publication and therefore, each chapter is written as a stand-alone piece of work. Consequently, some repetition occurs among chapters, particularly in terms of methods describing animal collection, husbandry and population and species distribution. Each chapter may also vary slightly in terms of formatting because of the specific requirements for the journal in which it was submitted (or will be submitted).

During my candidature, the taxonomic name of my study species was changed from *Niveoscincus ocellatus* to *Carinascincus ocellatus*. In chapter 2 it appears as *Niveoscincus*, however, in all subsequent chapters and the general introduction and discussion I refer to it as *Carinascincus*. Sex determination in the high elevation population of *C. ocellatus* is called genetic sex determination (GSD) throughout this thesis, however, chapters may vary slightly in the terminology used to describe the sex determining system in the low elevation populations of *C. ocellatus*. Initially described by Wapstra et al. (2004) and Pen et al. (2010) as "TSD-like" (TSD: temperature dependent sex determination), in Chapter 2 I discuss the use of GSD plus environmental effects (GSD+EE, *sensu* Valenzuela et al. 2003) and in

subsequent chapters this term appears, however, I also use "thermosensitivity in sex determination" in places, particularly in chapter 4 and in the broad introduction and discussion.

Chapter 2 uses a molecular dataset generated by next generation sequencing (NGS) to describe molecular similarities and differences between the two *C. ocellatus* populations both in terms of the identity of common and population-specific sex-linked genetic sequence and in the degree of linkage between the sex-linked regions of the genome. I further expand on this in **Chapter 3** and identify the sex chromosomes in *C. ocellatus* using traditional cytogenetic techniques and a custom Y chromosome probe designed from NGS genotypes obtained in **Chapter 2**. This chapter evaluates differences between the populations in gross chromosomal morphology and addresses sex chromosome homology between *C. ocellatus* and a close relative *Liopholis whitii*. The presence of sex reversal (when phenotypic and genetic sex do not match) in high and low elevation populations of *C. ocellatus* is evaluated in **Chapter 4** and the degree to which sex reversal correlates with temperature during gestation and the resultant sex ratio is assessed. This chapter utilises targeted genotyping using NGS loci developed in **Chapter 2** of offspring from several developmental temperature regimes to test the association between developmental temperature, sex reversal and the sex ratio. Using coalescent analysis and complementing this with traditional population genetics, **Chapter 5** explores the relationship between sex determination, gene flow and time since divergence between *C. ocellatus* high and low elevation populations and the future potential consequences of secondary contact due to climate change. In **Chapter 6**, the General Discussion, I summarise and discuss my main findings and further questions emerging from my research.

References

Adkins-Regan E, Reeve HK. 2014. Sexual dimorphism in body size and the origin of sex-determination systems. Am Nat 183: 519-536. doi: 10.1086/675303

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, Tree of Sex Consortium. 2014. Sex determination: why so many ways of doing it? PLoS Biology 12: e1001899. doi: 10.1371/journal.pbio.1001899

Boyle M, Hone J, Schwanz LE, Georges A. 2014. Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? Ecol Evol 4: 4522-4533. doi: 10.1002/ece3.1316

Bracewell RR, Bentz BJ, Sullivan BT, Good JM. 2017. Rapid neo-sex chromosome evolution and incipient speciation in a major forest pest. Nat Commun 8: 1593. doi: 10.1038/s41467-017-01761-4

Bull JJ. 1980. Sex determination in reptiles. Q Rev Biol 55: 3-21. doi: 10.2307/2826077

Capel B. 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat Rev Genet 18: 675-689. doi: 10.1038/nrg.2017.60

Charlesworth B. 2009. Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. Nat Rev Genet 10: 195-205. doi: 10.1038/nrg2526

Charlesworth D, Charlesworth B, Marais G. 2005. Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128. doi: 10.1038/sj.hdy.6800697

Charnov EL, Bull J. 1977. When is sex environmentally determined? Nature 266: 828-830. doi: 10.1038/266828a0

Cliff HB, Wapstra E, Burridge CP. 2015. Persistence and dispersal in a Southern Hemisphere glaciated landscape: the phylogeography of the spotted snow skink (*Niveoscincus ocellatus*) in Tasmania. BMC Evol Biol 15: 121. doi: 10.1186/s12862-015-0397-y

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol Lett 13. doi: 10.1098/rsbl.2017.0218

Donnellan S 1985. The evolution of sex chromosomes in scincid lizards. [PhD]. [NSW]: Macquarie University

Ewert MA, Lang JW, Nelson CE. 2005. Geographic variation in the pattern of temperature-dependent sex determination in the American snapping turtle (*Chelydra serpentina*). J Zool 265: 81-95. doi: 10.1017/s0952836904006120

Ezaz T, Deakin JE. 2014. Repetitive sequence and sex chromosome evolution in vertebrates. Adv Evol Biol2014: 9. doi: 10.1155/2014/104683

Ezaz T, Moritz B, Waters P, Graves JA, Georges A, Sarre SD. 2009a. The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res 17: 965-973. doi: 10.1007/s10577-009-9102-6

Ezaz T, Sarre SD, O'Meally D, Graves JA, Georges A. 2009b. Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127: 249-260. doi: 10.1159/000300507

Fisher RA. 1930. The genetical theory of natural selection. New York: Dover Publications, Inc.

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D. 2015. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Mol Biol Evol 32: 1296-1309. doi: 10.1093/molbev/msv023

Georges A. 1989. Female turtles from hot nests: is it duration of incubation or proportion of development at high temperatures that matters? Oecologia 81: 323-328. doi: 10.1007/BF00377078

Georges A, Ezaz T, Quinn AE, Sarre SD. 2010. Are reptiles predisposed to temperature-dependent sex determination? Sex Dev 4: 7-15. doi: 10.1159/000279441

Graves JA. 2014. Avian sex, sex chromosomes, and dosage compensation in the age of genomics. Chromosome Res 22: 45-57. doi: 10.1007/s10577-014-9409-9

Graves JA. 2006. Sex chromosome specialization and degeneration in mammals. Cell 124: 901-914. doi: 10.1016/j.cell.2006.02.024

Haldane JBS. 1922. Sex ratio and unisexual sterility in hybrid animals. J Genet 12: 101-109. doi: 10.1007/bf02983075

Holleley CE, O'Meally D, Sarre SD, Graves JA, Ezaz T, Matsubara K, Azad B, Zhang X, Georges A. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Holleley CE, Sarre SD, O'Meally D, Georges A. 2016. Sex reversal in reptiles: reproductive oddity or powerful driver of evolutionary change? Sex Dev10: 279-287. doi: 10.1159/000450972

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J Evol Biol 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Johnson NA, Lachance J. 2012. The genetics of sex chromosomes: evolution and implications for hybrid incompatibility. Ann N.Y. Acad Sci 1256: E1-E22. doi: 10.1111/j.1749-6632.2012.06748.x

Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S, Matsuda Y. 2009. The ZW sex chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 118: 43-51. doi: 10.1007/s00412-008-0176-2

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348: 450-452. doi: 10.1038/348450a0

Lance VA, Elsey RM, Lang JW. 2000. Sex ratios of American alligators (Crocodylidae): male or female biased? J Zool 252: 71-78. doi: 10.1111/j.1469-7998.2000.tb00821.x

Lang JW, Andrews HV. 1994. Temperature-dependent sex determination in crocodilians. J Exp Zool 270: 28-44. doi: 10.1002/jez.1402700105

Le Galliard JF, Fitze PS, Ferriere R, Clobert J. 2005. Sex ratio bias, male aggression, and population collapse in lizards. Proc Natl Acad Sci U. S. A. 102: 18231-18236. doi: 10.1073/pnas.0505172102

Mank JE, Promislow DE, Avise JC 2006. Evolution of alternative sex-determining mechanisms in teleost fishes. Biological Journal of the Linnean Society 87: 83-93.

Mank JE. 2012. Small but mighty: the evolutionary dynamics of W and Y sex chromosomes. Chromosome Res 20: 21-33. doi: 10.1007/s10577-011-9251-2

Matsubara K, Gamble T, Matsuda Y, Zarkower D, Sarre SD, Georges A, Graves JA, Ezaz T. 2014. Non-homologous sex chromosomes in two geckos (Gekkonidae: Gekkota) with female heterogamety. Cytogenet Genome Res 143: 251-258. doi: 10.1159/000366172

Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara C, Agata K, Matsuda Y. 2006. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. Proc Natl Acad Sci U. S. A. 103: 18190-18195. doi: 10.1073/pnas.0605274103

Meisel RP. 2020. Evolution of sex determination and sex chromosomes: a novel alternative paradigm. BioEssays 42: 1900212. doi: 10.1002/bies.201900212

Mitchell NJ, Janzen FJ. 2010. Temperature-dependent sex determination and contemporary climate change. Sex Dev 4: 129-140. doi: 10.1159/000282494

Mitchell NJ, Nelson NJ, Cree A, Pledger S, Keall SN, Daugherty CH. 2006. Support for a rare pattern of temperature-dependent sex determination in archaic reptiles: evidence from two species of tuatara (*Sphenodon*). Front Zool 3: 9. doi: 10.1186/1742-9994-3-9

Neaves L, Wapstra E, Birch D, Girling JE, Joss JMP 2006. Embryonic gonadal and sexual organ development in a small viviparous skink, *Niveoscincus ocellatus*. Journal of

Experimental Zoology Part A: Comparative Experimental Biology 305A: 74-82. doi: 10.1002/jez.a.249

Nelson NJ, Cree A, Thompson MB, Keall SN, Daugherty CH. 2004. Temperature-dependent sex determination in tuatara. In: Valenzuela N, Lance VA, editors. Temperature-dependent sex determination in vertebrates. Washington D.C.: Smithsonian Institution Press.

Orr HA. 2009. Fitness and its role in evolutionary genetics. Nat Rev Genet 10: 531-539. doi: 10.1038/nrg2603

Pen I, Uller T, Feldmeyer B, Harts A, While GM, Wapstra E. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Pennell MW, Kirkpatrick M, Otto SP, Vamosi JC, Peichel CL, Valenzuela N, Kitano J. 2015. Y fuse? Sex chromosome fusions in fishes and reptiles. PLoS Genet 11. doi: 10.1371/journal.pgen.1005237

Pennell MW, Mank JE, Peichel CL. 2018. Transitions in sex determination and sex chromosomes across vertebrate species. Mol Ecol 27: 3950-3963. doi: 10.1111/mec.14540

Pokorna M, Kratochvil L. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool. J. Linn. Soc. 156: 168-183.

Pyron RA, Burbrink FT, Wiens JJ 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol Biol 13: 93. doi: 10.1186/1471-2148-13-93

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JA. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, Sarre SD, Ezaz T, Graves JA, Georges A. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol Lett 7: 443-448. doi: 10.1098/rsbl.2010.1126

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. Biol Lett 4: 176-178. doi: 10.1098/rsbl.2007.0583

Robert KA, Thompson MB. 2010. Viviparity and demperature-dependent sex determination. Sex Dev 4: 119-128. doi: 10.1159/000260373

Roberts RB, Ser JR, Kocher TD. 2009. Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. Science 326: 998-1001. doi: 10.1126/science.1174705

Sarre SD, Ezaz T, Georges A. 2011. Transitions between sex-determining systems in reptiles and amphibians. Annu. Rev. Genomics Hum. Genet. 12: 391-406. doi: 10.1146/annurev-genom-082410-101518

Sarre SD, Georges A, Quinn A. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645. doi: 10.1002/bies.20050

Schwanz LE, Georges A, Holleley CE, Sarre SD. 2020. Climate change, sex reversal and lability of sex-determining systems. J. Evol. Biol. 33: 270-281. doi: 10.1111/jeb.13587

Shine R, Elphick MJ, Donnellan S. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol. Lett. 5: 486-489. doi: 10.1046/j.1461-0248.2002.00351.x

Shine R, Elphick MJ, Harlow PS. 1995. Sisters like it hot. Nature 378: 451-452. doi: DOI 10.1038/378451a0

Shine R, Warner DA, Radder R. 2007. Windows of embryonic sexual lability in two lizard species with environmental sex determination. Ecology 88: 1781-1788. doi: 10.1890/06-2024.1

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH. 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461: 267-271. doi: 10.1038/nature08298

Srikulnath K, Azad B, Singchat W, Ezaz T. 2019. Distribution and amplification of interstitial telomeric sequences (ITSs) in Australian dragon lizards support frequent chromosome fusions in Iguania. PLOS One 14. doi: 10.1371/journal.pone.0212683

Tarka M, Guenther A, Niemelä PT, Nakagawa S, Noble DWA. 2018. Sex differences in life history, behavior, and physiology along a slow-fast continuum: a meta-analysis. Behav. Ecol. Sociobiol. 72: 132-132. doi: 10.1007/s00265-018-2534-2

Valenzuela N, Adams DC, Janzen FJ. 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am Nat 161: 676-683. doi: 10.1086/368292

Valenzuela N, Literman R, Neuwald JL, Mizoguchi B, Iverson JB, Riley JL, Litzgus JD. 2019. Extreme thermal fluctuations from climate change unexpectedly accelerate demographic collapse of vertebrates with temperature-dependent sex determination. Sci. Rep. 9. doi: 10.1038/s41598-019-40597-4

Vicoso B, Bachtrog D. 2013. Reversal of an ancient sex chromosome to an autosome in Drosophila. Nature 499: 332-335. doi: 10.1038/nature12235

Vicoso B, Kaiser VB, Bachtrog D. 2013. Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. Proc Natl Acad Sci U. S. A. 110: 6453-6458. doi: 10.1073/pnas.1217027110

Vicoso B 2019. Molecular and evolutionary dynamics of animal sex-chromosome turnover. Nature Ecology & Evolution 3: 1632-1641. doi: 10.1038/s41559-019-1050-8

Wapstra E, Olsson M, Shine R, Edwards A, Swain R, Joss JM. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. Biol. Lett. 271 Suppl 4: S230-232. doi: 10.1098/rsbl.2003.0152

Warner DA, Shine R. 2008. The adaptive significance of temperature-dependent sex determination in a reptile. Nature 451: 566-568. doi: 10.1038/nature06519

Wedekind C. 2017. Demographic and genetic consequences of disturbed sex determination. Phil. Trans. R. Soc. B 372. doi: 10.1098/rstb.2016.0326

Wilson Sayres MA. 2018. Genetic diversity on the sex chromosomes. Genome Biol. Evol. 10: 1064-1078. doi: 10.1093/gbe/evy039

Whiteley SL, Weisbecker V, Georges A, Gauthier ARG, Whitehead DL, Holleley CE 2018. Developmental asynchrony and antagonism of sex determination pathways in a lizard with temperature-induced sex reversal. Sci Rep 8: 14892. doi: 10.1038/s41598-018-33170-y

Zajitschek F, Connallon T. 2018. Antagonistic pleiotropy in species with separate sexes, and the maintenance of genetic variation in life-history traits and fitness. Evolution 72: 1306-1316. doi: 10.1111/evo.13493

Chapter Two

Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature dependent sex determination.

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Abstract

Sex determination systems are exceptionally diverse and have undergone multiple and independent evolutionary transitions among species, particularly reptiles. However, the mechanisms underlying these transitions have not been established. Here we tested for differences in sex-linked markers in the only known reptile that is polymorphic for sex determination system, the spotted snow skink, *Niveoscincus ocellatus*, to quantify the genomic differences that have accompanied this transition. In a highland population, sex is determined genetically, whilst in a lowland population, offspring sex ratio is influenced by temperature. We found a similar number of sex-linked loci in each population, including shared loci, with genotypes consistent with male heterogamety (XY). However, population-specific linkage disequilibrium suggests greater differentiation of sex chromosomes in the highland population. Our results suggest that transitions between sex determination systems can be facilitated by subtle genetic differences.

Keywords: GSD; TSD; heterogamety; population divergence; sex chromosomes; reptiles.

Introduction

Sex determination controls whether the embryonic gonads develop into testes or ovaries. Central to the development of sexually reproducing organisms, sex determination should be under strong purifying selection with highly conserved processes and limited evolutionary lability (Uller, et al. 2007). Contrary to these expectations, systems of sex determination are surprisingly diverse, and therefore there is substantial interest in their evolution (Capel 2017; Ezaz, et al. 2006; O'Meally, et al. 2012). Vertebrate sex can be determined by genes (genetic sex determination; GSD), the environment (environmental sex determination; ESD), or via interactions between the two (Sarre, et al. 2004; Shine, et al. 2002; Valenzuela, et al. 2003). Furthermore, an extraordinary number of evolutionary transitions between these modes have occurred unpredictably across vertebrate evolution (Bachtrog, et al. 2014; Janzen, et al. 2006; Pokorna, et al. 2016). Sex determination also directs population sex ratio, an important demographic parameter that has implications for population persistence (Boyle, et al. 2014).

The mechanisms underlying sex determination systems are diverse. While a master genetic "switch" directs gonadogenesis in GSD species, it can manifest as a single nucleotide polymorphism (SNP; e.g. Takifugu rubripes; Kamiya, et al. 2012), a dominant single gene system (e.g. Mammalian SRY; Koopman, et al. 1990), a single gene dosage system (e.g. Avian DMRT1; Smith, et al. 2004), or methylation status of genes or their promoters (e.g. half-smooth tongue sole; Chen, et al. 2014). GSD is ubiquitous in endotherms and amphibians, and is found throughout lineages of reptiles and fish (Ezaz, et al. 2006; Quinn, et al. 2011; Sarre, et al. 2011). Environmental control of sex occurs in many ectotherms (Adkins-Regan, et al. 2014; Bull 1980; Ewert, et al. 2005), with temperature determining sex in many reptiles (temperature dependent sex determination, TSD). The environment can also act to over-ride the genetic influence of sex determination in a predominantly GSD species

(GSD plus environmental effects, GSD+EE: Valenzuela, et al. 2003) with a temperature over-ride described in reptiles (Holleley, et al. 2015; Radder, et al. 2008; Shine, et al. 2002). Many reptiles may possess an environmental over-ride rather than strict GSD because sex determination is a continuous rather than dichotomous trait (Sarre, et al. 2004).

In pure TSD taxa sex ratios are close to all male or all female at sex-specific developmental temperatures, whilst at a very narrow pivotal temperature range they can be a mix of male and female (Ewert, et al. 2005; Lang, et al. 1994). A temperature over-ride of sex determination presents as sex ratios at 50:50 across a broad pivotal temperature range with deviations occurring outside this range (Holleley, et al. 2015; Shine, et al. 2002). Uncovering sex-linked genetic sequence in a species previously shown to have TSD places this species on the continuum between GSD and TSD (Sarre, et al. 2004; Valenzuela, et al. 2014). TSD has been extensively studied in oviparous reptiles (Georges 1989; Harlow, et al. 2000; Lang, et al. 1994; Valenzuela, et al. 2014), where offspring sex is labile until after the middle third of embryonic development (Shine, et al. 2007), and is mediated by nest temperature. Viviparity was traditionally considered incompatible with any form of temperature influence on sex determination (Bull 1980), yet it has recently been described in a handful of reptiles (Robert, et al. 2001; Wapstra, et al. 2004; Zhang, et al. 2010), with the temperature signal mediated by maternal basking behaviour.

The correlation between sex determination system and the presence or absence of differentiated sex chromosomes—chromosomes that differ morphologically between males and females—is surprisingly weak (Sarre, et al. 2004; Vicoso, et al. 2013b; Wright, et al. 2016). When present, vertebrate sex chromosomes are remarkably diverse, even between closely related taxa (Bachtrog, et al. 2014; Ezaz, et al. 2017; Georges, et al. 2010), suggesting that contemporary sex chromosomes have multiple evolutionary origins (Ezaz, et al. 2009a;

Kawai, et al. 2009; Matsubara, et al. 2006). Heterogamety for sex chromosomes can occur in males (XY, e.g., mammals) or females (ZW, e.g., birds), and sex chromosomes can be hetero- or homomorphic, regardless of sex determination mechanism (GSD, TSD or GSD+EE). Understanding how these multiple evolutionary transitions in sex determination have occurred requires exposing the mechanisms that underpin them at a molecular level; the degree to which sex chromosomes participate in, or are a product of, transitions between sex determination systems remains a key knowledge gap.

Dosage models have been used to explain both environmental influence on sex, and transitions in sex determination systems and sex chromosomes (Ezaz, et al. 2009b; Quinn, et al. 2007; Quinn, et al. 2011). Under a dosage model, one sex is determined when the product of a homogametic genotype reaches a certain threshold. If the gene product that determines sex possesses thermal sensitivity, it is possible for a heterogametic genotype to reach the same threshold, or a homogametic genotype to not, resulting in the reversal of genotypic sex and the bias of sex ratio towards the sex most likely to benefit from the environment experienced (Charnov, et al. 1977). Dosage models can also explain transitions in sex determination; selection on the threshold for sex can result in transitions between GSD and TSD, and between ZW and XY heterogamety if sex determination acquires temperature sensitivity (Quinn, et al. 2011). A transition in heterogamety can also occur via the invasion of a novel sex determining locus when existing sex chromosomes are undifferentiated (Bachtrog, et al. 2014; Schartl 2004). Sex chromosomes can also be lost during transitions from GSD to TSD (Holleley, et al. 2015).

Reptiles exhibit high diversity in sex determination systems and sex chromosome morphology and homology (Ezaz, et al. 2009b; Giovannotti, et al. 2010; Matsubara, et al. 2014; Norris 2003; Shine, et al. 2002), and therefore represent a valuable group for the study of transitions

in sex determination and sex chromosome systems. However, incipient transition in sex determination has been documented only within one reptile, the viviparous spotted snow skink *Niveoscincus ocellatus* (Cunningham, et al. 2017; Pen, et al. 2010), representing a powerful study system. A highland population has GSD, whilst in a lowland population, temperature subtly influences offspring sex ratio. This population has been previously described as "TSD-like" (Cunningham, et al. 2017; Pen, et al. 2010), which we retain here, but equally, GSD+EE could apply (sensu Valenzuela, et al. 2003), and it exists on the continuum between TSD and GSD (Sarre, et al. 2004). These populations diverged recently, within the last million years (Cliff, et al. 2015). Divergent natural selection on sex determination caused by climatic effects on lizard life history and variation in the size of inter-annual temperature fluctuations appears to be driving this transition (Pen, et al. 2010). Warmer years result in early birth in both populations but sex ratios respond to temperature only in the lowland (Cunningham, et al. 2017). Sex ratios in the lowland are female biased in warm years and male biased in cold years. Lowland females, but not males derive a selective advantage from being born early because birth date influences the onset of maturity and this is important for females (Wapstra, et al. 2004). In the highland sex ratios do not vary from parity regardless of temperature as birth date does not predict the onset of maturity in this population (Pen, et al. 2010). In addition, higher inter-annual variation in climate in the highland is thought to favour GSD because it prevents extreme sex ratios (Pen, et al. 2010). This establishes an adaptive explanation for intra-specific divergence in sex determination systems. However, knowledge gaps exist surrounding the mechanism of this transition and the background with respect to sex chromosome evolution. Modelling suggests divergence among populations in genes that control sex determination: loss of function in the lowland population, attainment of function in the highland population, or a combination of both (Pen, et al. 2010).

The aim of this study was to quantify the divergence of genomic regions associated with sex (sex-linked markers) in populations of *N. ocellatus* that have recently diverged in sex determination system. Explicitly, we test whether the two populations differ in the numbers of sex-linked markers and the levels of linkage disequilibrium around them. We discuss our findings with regard to sex determination and sex chromosome evolution.

Materials and Methods

Study species

Niveoscincus ocellatus is a small (60 to 80 mm snout-vent length, 3–10g) viviparous skink endemic to Tasmania, with a broad altitudinal distribution from sea level to 1200m (Wapstra, et al. 1999). Two study populations represent the climatic extremes of this species' range: a cool temperate lowland population (42 34'S, 147 52' E; elevation 50m; hereafter 'lowland population') and a cold temperate, sub-alpine population (41 51'S, 146 34'E; elevation 1200m; hereafter 'highland population'). Reproduction follows a similar pattern in both populations; females reproduce annually, and the reproductive cycle is completed in one season (Wapstra, et al. 1999). Gestation occurs in spring and parturition in summer. Long term data on these populations consistently documents their divergent sex determination systems (Cunningham, et al. 2017; Wapstra, et al. 2004; Wapstra, et al. 2001).

Genotyping by sequencing

Approximately 2–4mm of tail tip was sampled from 44 highland individuals (23 males, 21 females) and 44 lowland individuals (24 males and 20 females) during the 2014–15 season. Males were sexed in the field by hemipene eversion, and all females were observed to later give birth. DNA extractions and sequencing were performed using DArTseq™ (Diversity Arrays Technology PTY, LTD), a high-throughput genotyping by sequencing method (Kilian,

et al. 2012) that employs genomic complexity reduction using restriction enzyme pairs. This technology successfully developed a series of sex-linked markers in the frog *Rana clamitans* (Lambert, et al. 2016). DNA was digested using *PstI* and *SphI*. Ligation reactions were then performed using two adaptors: a *PstI* compatible adaptor consisting of Illumina flow-cell attachment sequence, sequencing primer sequence and a unique barcode sequence, and a *SphI* compatible adaptor consisting of an Illumina flow-cell attachment region. Ligated fragments were then PCR amplified using an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 45 sec, with a final extension step at 72°C for 7 min. Equimolar amounts of amplification products from each individual were pooled and subjected to Illumina's proprietary cBot (http://www.illumina.com/products/cbot.html) bridge PCR followed by sequencing on an Illumina Hiseq2000. Single read sequencing was run for 77 cycles.

Sequences were processed using proprietary DArTseq analytical pipelines (Ren et al. (2015). Initially, the Hiseq2000 output (FASTQ file) was processed to filter poor quality sequences. Two different thresholds of quality were applied. For the barcode region (allowing parsing of sequences into specific sample libraries), we applied more stringent selection (minimum phred pass score of 30, minimum pass percentage 75). For the remaining part of the sequence more relaxed thresholds were applied (minimum phred pass score 10, minimum pass percentage 50). Approximately 2,000,000 sequences per individual were identified and used in marker calling. Finally, identical sequences were collapsed into "fastqcoll" files. The fastqcoll files were used in the secondary proprietary pipeline (DArTsoft14) for SNP and *in silico* DArT (presence/absence of restriction fragments in the representation; PA loci) calling. DArTsoft14 implements a "reference-free" algorithm. All unique sequences from the set of FASTQCOL files are identified, and clustered by sequence similarity at a distance threshold

of 3 base variations using an optimised (fast) clustering algorithm (in many cases over 1 billion sequences are clustered within minutes). The sequence clusters are then parsed into SNP and *in silico* DArT markers utilising a range of metadata parameters derived from the quantity and distribution of each sequence across all samples in the analysis. Additionally, a high level of technical replication is included in the DArTseq genotyping process, which enables reproducibility scores to be calculated for each candidate marker. The candidate markers output by DArTsoft14 are further filtered on the basis of the reproducibility values, average count for each sequence (sequencing depth), the balance of average counts for each SNP allele, and the call-rate (proportion of samples for which the marker is scored).

Sex-linked loci selection

We assessed sex-linkage for both dominant (presence / absence of restriction fragments) and co-dominant (single nucleotide polymorphism, SNP) markers. Each population was analysed separately. Genotypes from the presence / absence (PA) dataset consist of either '0', '1' or '-', representing fragment absence, presence or putative heterozygosity, respectively. Genotypes from the SNP dataset consist of either '0','1','2' or '-' representing genotypes homozygous for the reference allele (the most common allele), homozygous for the SNP allele, heterozygous, and homozygous for a null allele (absence of the fragment in the genomic representation), respectively. To evaluate sex linkage, homogeneity of genotypes for all loci between males and females within each population was assessed by Fisher's exact test using 'fisher.test' in R (R Development Core Team 2017) from the 'stats' package. *P* values were corrected for false discovery rate by Benjamini and Yekutieli method (Benjamini and Yekutieli, 2001). We assessed sex-linkage amongst the SNP loci under two models. The null exclusive model was conducted with SNP homozygous null genotypes removed. Under this model, sex-linked genotypes that present as homozygous in one sex and heterozygous in the

other are expected. Subsequently, we conducted a null inclusive model with SNP homozygous null genotypes included. Under this model, additional sex-linked genotypes that present as null in one sex, and exhibiting only a single allele in the other, are expected. The genotypes of all individuals for the sex-linked loci were examined for association with XY and ZW heterogamety. Specifically, XY heterogamety is characterised by PA loci with restriction fragments present in males and absent in females. SNP loci homozygous in females (for either the reference or SNP allele) and heterozygous in males under the null exclusive model, or homozygous null in females and exhibiting only one allele among males under the null inclusive model, would support an XY system. The reciprocal is true for ZW heterogamety. The PA and SNP markers fitting the null exclusive model were assessed on their ability to discriminate between the sexes of both populations using a Hamming distance matrix calculated using a custom R script with null genotypes removed. The sex-linked loci within each population were compared to identify those common to both populations.

Comparative linkage disequilibrium (LD) analysis

We used linkage disequilibrium network analysis on a subset of sex-linked SNP loci to examine linkage disequilibrium (LD) within the two populations. LD between two loci occurs when recombination is suppressed along the length of DNA that separates them, and is a hallmark of sex chromosome development (Marshall-Graves 2006). Thus, the number and identity of SNPs in LD with sex-linked SNPs in each population will provide a comparative representation of the sex-determining regions in each population. For this analysis, only SNPs polymorphic in both populations (minor allele frequency > 0.05) were considered. A perfectly sex-linked SNP locus (all females homozygous and all males heterozygous) was chosen from each population, along with 100 randomly selected non-sex-linked loci. LD between each of these 101 SNPs and all other (12,893) SNPs in the dataset was calculated for each population

using Genepop V4 (Rousset 2008). SNPs in significant LD (Benjamini and Yekutieli adjusted p value <0.05) were taken for linkage disequilibrium network analysis within their respective population (highland n = 576, lowland n = 618) using the genetics (Warnes, et al. 2013) and LDna (Kemppainen, et al. 2015) packages in R. Parameters for cluster emergence were |E| (the minimum edges or number of connections between loci) set at 20 and phi (factor used to determine the minimum observed change in R^2 allowed when adding new loci to a cluster) set at 2. Resulting clusters were plotted using the igraph package in R (Csardi, et al. 2006).

Results

Sex-linked loci

After DArTsoft14 filtering, DArTseq returned 20,813 presence / absence (PA) loci and 32,663 SNP loci for *Niveoscincus ocellatus*. After correction for false discovery, Fisher's exact test revealed loci with a non-homogeneous distribution of genotypes between the sexes common to both populations; 152 PA and 54 SNP ($p < 0.001$ to 0.003; supplementary tables S2.1, S2.2 and S2.3). Of the 152 PA loci, three are perfectly sex-linked across both populations with the remainder having less than 16% of individuals with genotypes deviating from perfect sex-linkage.

Of the 54 sex-linked SNP loci, 21 (supplementary table S2.2) emerged from the null exclusive model and are homozygous in females and heterozygous in males. Seven of these SNPs are perfectly sex-linked across both populations with the remainder sex-linked in at least 77% of individuals. The remaining 33 (supplementary table S2.3) emerged from the null inclusive model. These loci appear more like presence / absence loci because the majority of females possess a homozygous null genotype and the majority of males exhibit only the same allele at that locus. One of these loci is perfectly sex-linked across both populations, with the

remainder having less than 17% of individuals with genotypes deviating from perfect sex-linkage (some females with non-null genotypes; some males with null genotype; some loci polymorphic for males).

Fisher's exact test revealed PA and SNP loci that are sex-linked in one population only ($p<0.001$ to 0.012; supplementary tables S1, S2 and S3). In the highland population there were 16 PA and 12 SNP loci from the null exclusive model (three and five loci perfectly sex-linked, respectively), and eight SNP loci from the null inclusive model (zero perfectly sex-linked). In the lowland population there were 20 PA and 16 SNP loci from the null exclusive model (zero and three loci perfectly sex-linked, respectively), and five from the null inclusive model (one perfectly sex-linked). Proportional pairwise Hamming's distances between males and females (Figure 1) using the population-specific PA and SNP loci (null exclusive model), demonstrate that they reliably reveal an organism's phenotypic sex within that population only. Highland males and females are on average 89.7% and 92.8% dissimilar from one another (Highland SNP and PA loci, respectively). Lowland males and females are on average 89.4% and 89.2% dissimilar from one another (Lowland SNP and PA loci, respectively).

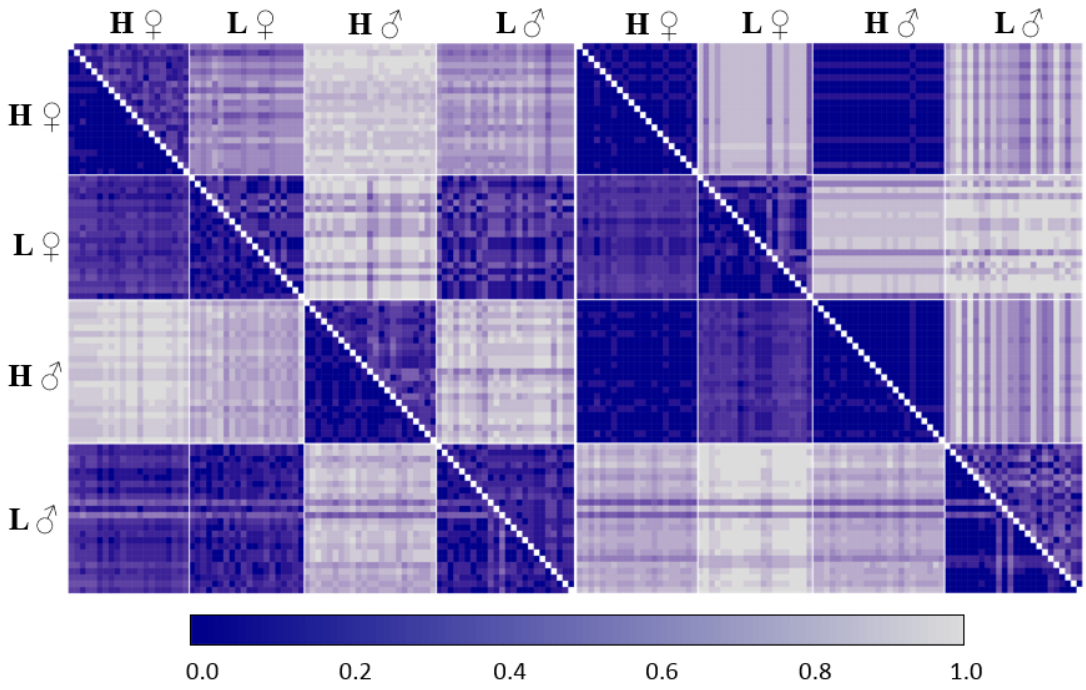

Figure 1. Hamming's proportional distance among *Niveoscincus ocellatus* individuals of highland (H) and lowland (L) populations for sex-linked loci unique to the highland (left panel) and lowland (right panel) populations. Presence Absence (PA; lower segment) and SNP (upper segment). Highland PA n=16, lowland PA n=20, highland SNPs n=12, lowland SNPs n=16.

All sex-linked SNP loci specific to a population (hereafter the "source population") were also genotyped in the other population (hereafter the "reciprocal population"). Genotypes in the reciprocal population for the majority of source population loci were not sex-linked, either because the population was fixed for an allele, or both alleles were homogenously distributed among the sexes. In several cases (four highland loci and two lowland loci), loci sex-linked in the source population under the null exclusive model presented as sex-linked in the reciprocal population under the null inclusive model. In all of these cases, females were predominantly homozygous null and males predominantly exhibited only a single allele at that locus. Apart from one highland locus, the source population X allele is missing from the reciprocal population, and males only exhibit the source population Y allele. In the one exception to this,

reciprocal population males only exhibit the source population X allele. For the population-specific sex-linked PA loci, in the reciprocal population the restriction fragment in question was either absent in all individuals, present in all individuals, or present at homogeneous frequencies between the sexes. Males and females are more dissimilar in the lowland (21.8% and 16.3%, SNP and PA loci respectively) than highland (2.6% and 4.4%) population based on sex-linked loci from the reciprocal population (Figure 1).

Sex-linked genotypes assort in a manner consistent with XY heterogamety: PA loci present in males, absent in females; SNPs are either heterozygous in males and homozygous in females, or males exhibit only a single allele and females are homozygous null. Exceptions are five loci in the lowland population. One lowland PA locus is absent from all males and 45% of females (homogeneity of genotypes, $p = 0.002$). Four SNP loci are homozygous for every male individual, but for both alleles at each locus, while most females are heterozygous, but with homozygotes also observed for both alleles at each locus (homogeneity of genotypes $p<0.001$). These five loci were recovered in the highland population, but the genotypes of the PA locus are homogeneous between the sexes ($p>0.05$) and those of the SNPs are homozygous for the reference allele in all individuals.

Comparative LD analysis

Linkage disequilibrium network analysis (LDNa) resolved a sex-linked cluster consisting of 32 SNP loci connected via 411 edges in the highland population (12.8 edges per locus; Figure 2). 175 non-sex-linked loci connected by 213 edges described a non-sex-linked cluster in this population (1.2 edges per locus). In the lowland population, LDNa resolved a sex-linked cluster with 34 SNP loci connected via 235 edges (6.9 edges per locus; Figure 2) and a non-sex-linked cluster containing 17 loci connected via 22 edges (1.3 edges per locus). The 21 common sex-linked SNP loci (Figure 2 - **a** to **u**; supplementary table S2.2) appear in both the

highland and lowland sex-linked clusters but vary in the degree to which they associate with the perfectly sex-linked locus for that population and each other. The non-sex-linked clusters from each population have no loci in common.

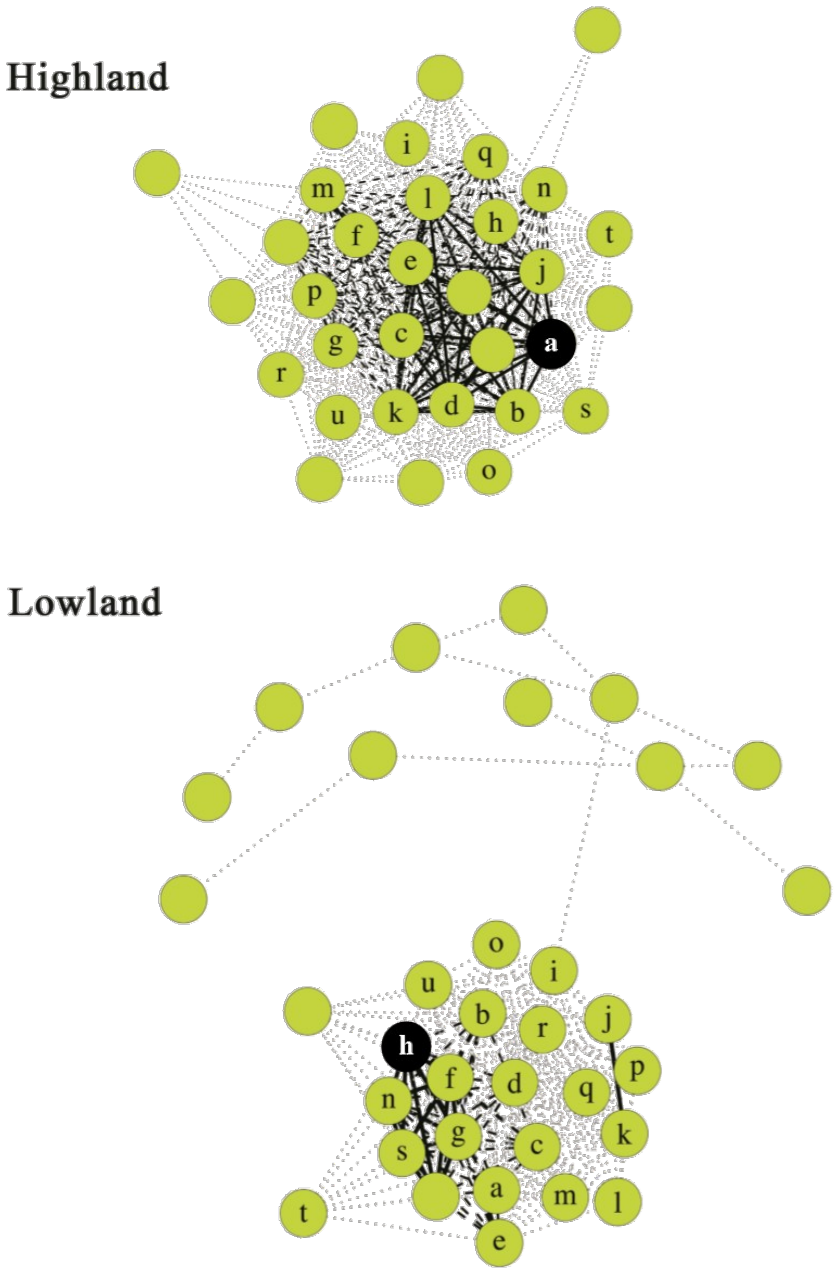

Figure 2. Linkage disequilibrium network plot of sex-linked clusters from the highland and lowland populations of *Niveoscincus ocellatus*. Green circles indicate sex-linked SNPs (n = 32 in the highland, n = 34 in the lowland populations); 'a' to 'u' denote 21 loci sex-linked in both the highland and lowland population. The perfectly sex-linked locus for each cluster is in black. Black solid edges have an R2 > 0.99, black dashed 0.99>R2>0.80, grey dotted R2<0.80.

Discussion

The subtle molecular differences in sex-linked markers between highland and lowland populations indicate that few changes are required for transitions between sex determination modes, and is also compatible with the short timeframe (<1 Myr) across which these populations have diverged (Cliff, et al. 2015). We identified a similar number of loci associated with phenotypic sex in both populations of *N. ocellatus*, despite the divergence in temperature effects on the sex ratio between these populations. This was surprising because lowland offspring sex ratios are correlated with temperature (Wapstra, et al. 2004), and models predict the loss of genes surrounding sex determination in this population, or the gain of such genes in the highland population (Pen, et al. 2010). In addition to a conserved set of sex-linked loci, population-specific sex-linked loci are also present in each population, highlighting the importance of a genetic contribution to sex in the lowland as well as the highland. Sex ratios in the lowland are 50:50 across a pivotal temperature range (Wapstra, et al. 2009), likely facilitated by random assortment of sex determining genes at meiosis and maintained due to frequency-dependent selection (Fisher 1958). Developmental temperatures outside this pivotal range provide sex-specific fitness advantages (Pen, et al. 2010), and there has been selection for a temperature mediated dosage component to sex determination both above and below the pivotal temperature in this population. *N. ocellatus* is an XY GSD species with the lowland population possessing a temperature over-ride in sex determination (GSD+EE); the maintenance of this mixed system in the lowland likely representing an adaptive optimum.

The presence of a temperature influence on sex in a population possessing sex-linked genetic markers can be explained by mechanisms that only occasionally over-ride GSD, and this is consistent with the low but significantly temperature-related deviations in sex ratio observed

in the lowland population (Cunningham, et al. 2017; Wapstra, et al. 2004). Both differential mortality and differential fertilization via cryptic female choice have been implicated in other taxa (Burger, et al. 1988; Eiby, et al. 2008; Olsson, et al. 2011), but have been ruled out in this species (Wapstra, et al. 2004). This leaves sex reversal as the most likely explanation for the sex ratio biases observed.

Sex reversals can occur via temperature sensitive gene dosage and in reptiles usually occurs in the homogametic sex (Ezaz, et al. 2009b; Holleley, et al. 2015; Quinn, et al. 2007; Quinn, et al. 2011). Explanations for this centre around a gene or gene product present on the homogametic chromosome and therefore present as one copy in one sex and two in the other. Temperature-sensitive, dosage-dependent expression of this gene or activity of its product can result in the homogametic genotype not reaching the threshold for sexual phenotype and becoming sex reversed. Male biased sex ratios in *N. ocellatus*, as observed in colder conditions, fit this pattern if sex determination in this species occurs via a feminizing gene on the X chromosome with sex reversed males (XX genotype) resulting from temperature sensitivity of this gene. When gene product fails to reach the required threshold to produce a female, a male is instead produced from this genotype. Female biased sex ratios, as observed in warmer conditions, could result from the over-expression of this feminizing allele in the XY genotype. Sex reversal in the heterogametic sex is thought to be unfavourable when sex chromosomes are highly heteromorphic; mating between sex reversed XY females and XY males producing YY progeny - unviable if there are necessary developmental genes on the X chromosome (Quinn, et al. 2011). However, sex reversal of the XY genotype to female in systems with homomorphic sex chromosomes is theoretically possible (Sarre, et al. 2004) and could explain the observed differences in recombination suppression in the two populations of *N. ocellatus*.

The ratio of female to male recombination rate varies considerably across taxa (Berset-Brändli, et al. 2008; Coimbra, et al. 2003; Perrin 2009) even in taxa without sex chromosomes (Isberg, et al. 2006), and is a function of phenotypic rather than genetic sex. Recombination between sex chromosomes can therefore occur in individuals that have been sex reversed (Perrin 2009). In an XY system the X and Y chromosome can undergo recombination at meiosis in sex reversed XY females, resulting in reduced associations between alleles on the Y. This interrupts the progressive degeneration of the Y chromosome because recombination suppression is necessary to keep alleles beneficial for one sex together. Sex reversals are described in reptiles, amphibians and fish (Alho, et al. 2010; Holleley, et al. 2015; Shao, et al. 2014), but have yet to be described in *N. ocellatus*. The putative existence of sex reversed females in the lowland population would explain lower linkage disequilibrium between the sex-linked SNP loci in this population.

Although the number of sex-linked SNPs and PA loci is similar in both populations, the presence of population-specific sex-linked variation nevertheless supports population divergence in the molecular mechanism surrounding sex determination. The degree of recombination suppression occurring amongst the 21 shared sex-linked markers also differs among populations, indicating sex chromosomes at different developmental stages. Sex chromosomes in the highland population are likely more differentiated than those in the lowland because of the lower independence of genotypes between sex-linked loci in this population. This lower independence manifests as both higher LD between loci and a greater number of connections among the 21 shared loci, suggesting a region that is tightly linked to sex determining locus (or loci) and more often travelling as a complete unit during meiosis because of higher recombination suppression. Many taxa (e.g., Ratite birds and Boid snakes) maintain recombination along much of the length of their sex chromosomes (Vicoso, et al.

2013a; Vicoso, et al. 2013b). Recombining sex chromosomes are advantageous as deleterious alleles are purged from the Y (or W) chromosome (Bachtrog, et al. 2014; van Doorn, et al. 2010). Recombination between the X and Y may contribute to the maintenance of a mixed system in the lowland population where temperature and genetics interact to determine sex (Sarre, et al. 2004) via the presence of sex reversed females.

Here we describe sex-linked genetic sequence in *Niveoscincus ocellatus*. The majority of sex-linked markers observed in this study were shared between populations, indicating inheritance from a common ancestor; those not shared may indicate independent gain or loss in a population. A thorough examination of sex determination across this genus using these loci will reveal the ancestral state of sex determination in *N. ocellatus* and whether population divergence in sex determination occurs elsewhere in the genus. Further, these loci can be used to assess the role of sex reversal in the transition in sex determination mode in this species, for cytological examination of the karyotype of this genus and to uncover the sex determining locus in Scincidae. Screening our archival samples, collected over more than a decade, with these sex-linked markers will be invaluable in capturing the tempo and mechanism of evolutionary transitions between modes of sex determination in reptiles.

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References

Adkins-Regan E, Hudson KR. 2014. Sexual dimorphism in body size and the origin of sex-determination systems. Am Nat 183: 519-536. doi: 10.1086/675303

Alho JS, Matsuba C, Merila J. 2010. Sex reversal and primary sex ratios in the common frog (*Rana temporaria*). Mol Ecol 19: 1763-1773. doi: 10.1111/j.1365-294X.2010.04607.x

Bachtrog D, et al. 2014. Sex determination: why so many ways of doing it? PloS Biol 12: e1001899. doi: 10.1371/journal.pbio.1001899

Berset-Brändli L, et al. 2008. Extreme heterochiasmy and nascent sex chromosomes in European tree frogs. P R Soc B 275: 1577-1585. doi: 10.1098/rspb.2008.0298

Boyle M, Hone J, Schwanz LE, Georges A. 2014. Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? Ecol Evol 4: 4522-4533. doi: 10.1002/ece3.1316

Bull JJ. 1980. Sex determination in reptiles. Q Rev Biol 55: 3-21. doi: 10.2307/2826077

Burger J, Zappalorti RT. 1988. Effects of incubation temperature on sex ratios in pine snakes: differential vulnerability of males and females. Am Nat 132: 492-505.

Capel B. 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat Rev Genet advance online publication. doi: 10.1038/nrg.2017.60

Charnov EL, Bull J. 1977. When is sex environmentally determined? Nature 266: 828-830. doi: 10.1038/266828a0

Chen S, et al. 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. Nat Genet 46: 253-260. doi: 10.1038/ng.2890

Cliff HB, Wapstra E, Burridge CP. 2015. Persistence and dispersal in a southern hemisphere glaciated landscape: the phylogeography of the spotted snow skink (*Niveoscincus ocellatus*) in Tasmania. BMC Evol Biol 15. doi: 10.1186/s12862-015-0397-y

Coimbra MRM, et al. 2003. A genetic linkage map of the Japanese flounder, *Paralichthys olivaceus*. Aquaculture 220: 203-218. doi: 10.1016/S0044-8486(02)00353-8

Csardi G, Nepusz T. 2006. The igraph software package for complex network research. InterJournal: Complex Systems: 1695. http://igraph.org

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol Letters 13. doi: 10.1098/rsbl.2017.0218

Eiby YA, Wilmer JW, Booth DT. 2008. Temperature-dependent sex-biased embryo mortality in a bird. P R Soc B 275: 2703-2706. doi: 10.1098/rspb.2008.0954

Ewert MA, Lang JW, Nelson CE. 2005. Geographic variation in the pattern of temperature-dependent sex determination in the American snapping turtle (*Chelydra serpentina*). J Zool 265: 81-95. doi: 10.1017/s0952836904006120

Ezaz T, et al. 2009a. The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res 17: 965-973. doi: 10.1007/s10577-009-9102-6

Ezaz T, et al. 2009b. Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet Genome Res 127: 249-260. doi: 10.1159/000300507

Ezaz T, Srikulnath K, Marshall Graves JA. 2017. Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? J Hered 108: 94-105. doi: 10.1093/jhered/esw053

Ezaz T, Stiglec R, Veyrunes F, Marshall Graves JA. 2006. Relationships between vertebrate ZW and XY sex chromosome systems. Curr Biol 16: 736-743. doi: 10.1016/j.cub.2006.08.021

Fisher RA. 1958. The genetical theory of natural selection. New York: Dover Publications, Inc.

Georges A. 1989. Female turtles from hot nests: is it duration of incubation or proportion of development at high temperatures that matters? Oecologia 81: 323-328. doi: 10.1007/BF00377078

Georges A, Ezaz T, Quinn AE, Sarre SD. 2010. Are reptiles predisposed to temperature-dependent sex determination? Sex Dev 4: 7-15. doi: 10.1159/000279441

Giovannotti M, et al. 2010. Skinks (Reptilia: Scincidae) have highly conserved karyotypes as revealed by chromosome painting. Cytogenet Genome Res 127: 224-231. doi: 10.1159/000295002

Harlow PS, Taylor JE. 2000. Reproductive ecology of the jacky dragon (*Amphibolourus muricatus*): an agamid lizard with temperature-dependent sex determination. Austral Ecol 25. doi: 10.1111/j.1442-9993.2000.tb00070.x

Holleley CE, et al. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Isberg SR, Johnston SM, Chen Y, Moran C. 2006. First evidence of higher female recombination in a species with temperature-dependent sex determination: the saltwater crocodile. J Hered 97: 599-602. doi: 10.1093/jhered/esl035

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J Evolution Biol 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Kamiya T, et al. 2012. A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (Fugu). PLoS Genet 8: e1002798. doi: 10.1371/journal.pgen.1002798

Kawai A, et al. 2009. The ZW sex chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 118: 43-51. doi: 10.1007/s00412-008-0176-2

Kemppainen P, et al. 2015. Linkage disequilibrium network analysis (LDna) gives a global view of chromosomal inversions, local adaptation and geographic structure. Mol Ecol Resour 15: 1031-1045. doi: 10.1111/1755-0998.12369

Kilian A, et al. 2012. Diversity Arrays Technology: a generic genome profiling technology on open platforms. In: Pompanon F, Bonin A, editors. *Data Production and Analysis in Population Genomics: Methods and Protocols*. Totowa, NJ: Humana Press. p. 67-89.

Koopman P, et al. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348: 450-452. doi: 10.1038/348450a0

Lambert MR, Skelly DK, Ezaz T. 2016. Sex-linked markers in the North American green frog (*Rana clamitans*) developed using DArTseq provide early insight into sex chromosome evolution. BMC Genom. 17. doi: 10.1186/s12864-016-3209-x

Lang JW, Andrews HV. 1994. Temperature-dependent sex determination in Crocodilians. J Exp Zool 270: 28-44. doi: 10.1002/jez.1402700105

Marshall-Graves JA. 2006. Sex chromosome specialization and degeneration in mammals. Cell 124: 901-914. doi: 10.1016/j.cell.2006.02.024

Matsubara K, et al. 2014. Non-homologous sex chromosomes in two geckos (*Gekkonidae: Gekkota*) with female heterogamety. Cytogenet Genome Res 143: 251-258. doi: 10.1159/000366172

Matsubara K, et al. 2006. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. P Natl Acad Sci USA 103: 18190-18195. doi: 10.1073/pnas.0605274103

Norris TB. 2003. Chromosomes of New Zealand skinks, genus Oligosoma. J Herpetol 37: 368-370. doi: 10.1670/0022-1511(2003)037[0368:conzsg]2.0.co;2

O'Meally D, et al. 2012. Are some chromosomes particularly good at sex? Insights from amniotes. Chromosome Res 20: 7-19. doi: 10.1007/s10577-011-9266-8

Olsson M, et al. 2011. In hot pursuit: fluctuating mating system and sexual selection in sand lizards. Evolution 65: 574-583. doi: 10.1111/j.1558-5646.2010.01152.x

Pen I, et al. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Perrin N. 2009. Sex reversal: A fountain of youth for sex chromosomes? Evolution 63: 3043-3049. doi: 10.1111/j.1558-5646.2009.00837.x

Pokorna MJ, Kratochvil L. 2016. What was the ancestral sex-determining mechanism in amniote vertebrates? Biol Rev Camb Philos Soc 91: 1-12. doi: 10.1111/brv.12156

Quinn AE, et al. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, et al. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol Letters 7: 443-448. doi: 10.1098/rsbl.2010.1126

R Development Core Team. 2017. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Radder RS, et al. 2008. Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. Biol Letters 4: 176-178. doi: 10.1098/rsbl.2007.0583

Robert KA, Thompson MB. 2001. Viviparous lizard selects sex of embryos. Nature 412: 698. doi: 10.1038/35089135

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour 8: 103-106. doi: 10.1111/j.1471-8286.2007.01931.x

Sarre SD, Ezaz T, Georges A. 2011. Transitions between sex-determining systems in reptiles and amphibians. Annu Rev Genom Hum G 12: 391-406. doi: 10.1146/annurev-genom-082410-101518

Sarre SD, Georges A, Quinn A. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645. doi: 10.1002/bies.20050

Schartl M. 2004. Sex chromosome evolution in non-mammalian vertebrates. Curr Opin Genet Dev 14: 634-641. doi: 10.1016/j.gde.2004.09.005

Shao C, et al. 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res 24: 604-615. doi: 10.1101/gr.162172.113

Shine R, Elphick MJ, Donnellan S. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol Lett 5: 486-489. doi: 10.1046/j.1461-0248.2002.00351.x

Shine R, Warner DA, Radder RS. 2007. Windows of embryonic sexual lability in two lizard species with environmental sex determination. Ecology 88: 1781-1788. doi: 10.1890/06-2024.1

Smith CA, Sinclair AH. 2004. Sex determination: insights from the chicken. Bioessays 26: 120-132. doi: 10.1002/bies.10400

Uller T, et al. 2007. The evolution of sex ratios and sex-determining systems. Trends Ecol Evol 22: 292-297. doi: 10.1016/j.tree.2007.03.008

Valenzuela N, Adams D, Janzen FJ. 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am Nat 161: 676-683. doi: 10.1086/368292

Valenzuela N, Badenhorst D, Montiel EE, Literman R. 2014. Molecular cytogenetic search for cryptic sex chromosomes in painted turtles *Chrysemys picta*. Cytogenet Genome Res 144: 39-46. doi: 10.1159/000366076

van Doorn GS, Kirkpatrick M. 2010. Transitions between male and female heterogamety caused by sex-antagonistic selection. Genetics 186: 629-645. doi: 10.1534/genetics.110.118596

Vicoso B, et al. 2013a. Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. PloS Biol 11: e1001643. doi: 10.1371/journal.pbio.1001643

Vicoso B, Kaiser VB, Bachtrog D. 2013b. Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. Proc Natl Acad Sci USA 110: 6453-6458. doi: 10.1073/pnas.1217027110

Wapstra E, et al. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. P R Soc B 271: S230-S232. doi: 10.1098/rsbl.2003.0152

Wapstra E, Swain R. 2001. Geographic and annual variation in life-history traits in a temperate zone Australian skink. J Herpetol 35: 194-203. doi: 10.2307/1566108

Wapstra E, Swain R, Jones SM, O'Reilly J. 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata : Scincidae). Aust J Zoo 47: 539-550. doi: 10.1071/ZO99038

Wapstra E, et al. 2009. Climate effects on offspring sex ratio in a viviparous lizard. J Anim Ecol 78: 84-90. doi: 10.1111/j.1365-2656.2008.01470.x

Warnes G, with contributions from Gorjanc G, Leisch F, and Man M. 2013. genetics: Population Genetics. R package version 1.3.8.1. https://CRAN.R-project.org/package=genetics.

Wright AE, Dean R, Zimmer F, Mank JE. 2016. How to make a sex chromosome. Nat Commun 7: 12087. doi: 10.1038/ncomms12087

Zhang D, et al. 2010. Effect of gestation temperature on sexual and morphological phenotypes of offspring in a viviparous lizard, *Eremias multiocellata*. J Therm Biol 35: 129-133. doi: 10.1016/j.jtherbio.2010.01.003

Supplementary data

Table S2.1. Sex linked Presence / Absence loci in *Niveoscincus ocellatus*. Proportion of presence / absence genotypes in males and females common to both highland and lowland populations and unique to each population of *Niveoscincus ocellatus*. Score of 1 denotes presence of the fragment, 0 denotes absence of the fragment and '-' denotes a putative heterozygous score for the fragment. All loci denote XY heterogamety with the exception of locus marked *.

Common loci	Males			Females			Fishers p value
	Prop. '1'	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. '0'	
NO27479	1.00	0.00	0.00	0.07	0.00	0.93	<0.001
NO25981	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO17412	0.96	0.04	0.00	0.00	0.02	0.98	<0.001
NO13113	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO11735	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO09026	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO06180	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO06174	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO06148	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO04112	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO04111	0.81	0.17	0.02	0.00	0.00	1.00	<0.001
NO04106	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO01619	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO01528	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO00798	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO00674	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO99977	0.94	0.06	0.00	0.00	0.02	0.98	<0.001
NO99347	0.96	0.02	0.02	0.00	0.00	1.00	<0.001
NO98718	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO98519	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO98495	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO95026	0.98	0.02	0.00	0.00	0.02	0.98	<0.001
NO95024	0.98	0.02	0.00	0.00	0.02	0.98	<0.001

Common loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO95017	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94951	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO94946	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO94938	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO94918	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO94907	0.98	0.02	0.00	0.00	0.02	0.98	<0.001
NO94905	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94890	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO94882	0.83	0.04	0.13	0.00	0.00	1.00	<0.001
NO94872	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO94858	0.94	0.04	0.02	0.00	0.00	1.00	<0.001
NO94833	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO94820	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94818	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO94817	0.89	0.09	0.02	0.00	0.00	1.00	<0.001
NO94803	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94785	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO94771	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94762	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94757	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94726	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94717	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO94701	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94680	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94670	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94660	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94656	0.94	0.06	0.00	0.00	0.00	1.00	<0.001

Common loci	Males			Females			Fishers
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	p value
NO94646	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94601	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO94543	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94449	0.98	0.02	0.00	0.00	0.00	1.00	<0.001
NO94291	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO95025	0.94	0.06	0.00	0.00	0.02	0.98	<0.001
NO94980	1.00	0.00	0.00	0.00	0.05	0.95	<0.001
NO94948	0.96	0.04	0.00	0.00	0.02	0.98	<0.001
NO94939	0.96	0.04	0.00	0.00	0.02	0.98	<0.001
NO27293	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO27141	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO25915	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO22758	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO20991	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO18137	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO15686	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO08370	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO07276	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO06426	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO04817	0.85	0.15	0.00	0.00	0.02	0.98	<0.001
NO04179	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO04137	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO04128	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO04085	0.94	0.06	0.00	0.00	0.02	0.98	<0.001
NO04068	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO01662	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO00349	0.89	0.11	0.00	0.00	0.00	1.00	<0.001

Common loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO99580	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO95016	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94991	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94975	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94972	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94965	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94947	0.94	0.06	0.00	0.00	0.02	0.98	<0.001
NO94943	0.96	0.04	0.00	0.00	0.02	0.98	<0.001
NO94934	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94928	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94921	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94902	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94880	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94865	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94860	0.81	0.19	0.00	0.00	0.00	1.00	<0.001
NO94847	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94828	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94825	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94824	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94815	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94806	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO94799	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94791	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94759	0.94	0.06	0.00	0.00	0.02	0.98	<0.001
NO94758	0.89	0.11	0.00	0.00	0.02	0.98	<0.001
NO94756	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94739	0.85	0.15	0.00	0.00	0.00	1.00	<0.001

Common loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO94721	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94702	0.85	0.13	0.02	0.00	0.00	1.00	<0.001
NO94699	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94694	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94675	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94667	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94640	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94627	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94605	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO94585	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94564	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94528	0.94	0.06	0.00	0.00	0.00	1.00	<0.001
NO94526	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO26343	0.91	0.09	0.00	0.00	0.02	0.98	<0.001
NO23032	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO10616	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO08457	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO04193	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO94976	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94810	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94794	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO94779	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO94695	0.87	0.13	0.00	0.00	0.02	0.98	<0.001
NO94665	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94599	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO94566	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO20597	0.85	0.15	0.00	0.00	0.02	0.98	<0.001

Common loci	Males			Females			Fishers
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	p value
NO01905	0.85	0.15	0.00	0.00	0.00	1.00	<0.001
NO95027	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94987	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94689	0.81	0.19	0.00	0.00	0.00	1.00	<0.001
NO94527	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94518	0.89	0.11	0.00	0.00	0.00	1.00	<0.001
NO94766	0.89	0.06	0.04	0.00	0.00	1.00	<0.001
NO94749	0.89	0.09	0.02	0.00	0.00	1.00	<0.001
NO27615	0.98	0.02	0.00	0.12	0.02	0.85	<0.001
NO94903	0.81	0.19	0.00	0.00	0.00	1.00	<0.001
NO94881	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO92667	0.98	0.02	0.00	0.02	0.15	0.83	<0.001
NO94732	0.87	0.11	0.02	0.00	0.00	1.00	<0.001
NO94530	0.87	0.11	0.02	0.00	0.00	1.00	<0.001
NO94666	0.83	0.15	0.02	0.00	0.00	1.00	<0.001
NO94941	0.79	0.19	0.02	0.00	0.00	1.00	<0.001
NO94539	0.79	0.15	0.06	0.00	0.00	1.00	<0.001
NO01037	0.98	0.02	0.00	0.05	0.20	0.76	<0.001
NO17728	1.00	0.00	0.00	0.10	0.02	0.88	<0.001
NO94843	0.91	0.00	0.09	0.20	0.00	0.80	<0.001
NO18471	0.94	0.04	0.02	0.22	0.05	0.73	0.002
Highland loci	Males			Females			Fishers
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	p value
NO26788	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO12766	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO95824	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO95537	0.96	0.04	0.00	0.00	0.00	1.00	<0.001

Highland loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO95785	1.00	0.00	0.00	0.00	0.05	0.95	<0.001
NO04320	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO95729	0.87	0.13	0.00	0.00	0.00	1.00	<0.001
NO95674	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO95621	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO92926	0.96	0.00	0.04	0.00	0.00	1.00	<0.001
NO25902	1.00	0.00	0.00	0.05	0.05	0.90	<0.001
NO13170	0.35	0.65	0.00	0.00	0.00	1.00	<0.001
NO09552	0.61	0.00	0.39	0.00	0.00	1.00	0.001
NO00517	0.74	0.26	0.00	0.19	0.29	0.52	0.001
NO04304	1.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO25722	1.00	0.00	0.00	0.38	0.10	0.52	0.001
Lowland loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO94625	0.96	0.04	0.00	0.00	0.00	1.00	<0.001
NO95018	1.00	0.00	0.00	0.00	0.05	0.95	<0.001
NO94661	0.96	0.04	0.00	0.00	0.05	0.95	<0.001
NO24878	0.96	0.00	0.04	0.00	0.00	1.00	<0.001
NO26368	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO94893	0.83	0.17	0.00	0.00	0.00	1.00	<0.001
NO27480	0.92	0.04	0.04	0.00	0.00	1.00	<0.001
NO12765	0.92	0.04	0.04	0.00	0.00	1.00	<0.001
NO27095	0.88	0.04	0.08	0.00	0.00	1.00	<0.001
NO23045	0.88	0.04	0.08	0.00	0.00	1.00	<0.001
NO94900	0.92	0.00	0.08	0.00	0.00	1.00	<0.001
NO94542	0.92	0.00	0.08	0.00	0.00	1.00	<0.001
NO94648	0.83	0.08	0.08	0.00	0.00	1.00	<0.001

Lowland loci	Males			Females			Fishers p value
	Prop. ‘1’	Prop. '-'	Prop. '0'	Prop. '1'	Prop. '-'	Prop. ‘0’	
NO24877	0.83	0.00	0.17	0.00	0.00	1.00	<0.001
NO01508	0.58	0.38	0.04	0.00	0.00	1.00	<0.001
NO17727	1.00	0.00	0.00	0.20	0.05	0.75	<0.001
NO00397	0.79	0.21	0.00	0.20	0.15	0.65	<0.001
NO95000	0.75	0.00	0.25	0.05	0.05	0.90	<0.001
NO90328	0.63	0.38	0.00	0.20	0.15	0.65	0.001
NO93463*	0.00	0.00	1.00	0.55	0.00	0.45	0.002

Table S2.2 Proportion of genotypes in males and females for SNPs with alleles that segregate with sex according to the 'null exclusive' sex-linked model in populations of *Niveoscincus ocellatus*. Score of '2' indicates heterozygous for the reference and SNP allele, '1' homozygous for the SNP allele, '0' homozygous for the reference allele,'-' homozygous for a null genotype. Alleles of all loci assort according to XY heterogamety. Fig 2 refers to labelling of these loci in figure 2 of manuscript.

Common Loci	Fig 2	REF > SNP	Males				Females				p value
			Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	
NO02579	a	G>T	0.98	0.02	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO17128	b	G>A	0.94	0.06	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO02507	c	C>A	0.96	0.04	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO02972	d	T>G	0.94	0.06	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO27498	e	C>T	0.96	0.04	0.00	0.00	0.02	0.00	0.98	0.00	<0.001
NO20596	f	T>C	0.98	0.02	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO18136	g	C>T	0.98	0.02	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO00135	h	C>T	0.98	0.02	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO05744	i	C>T	0.62	0.36	0.00	0.02	0.00	0.00	0.93	0.07	<0.001
NO08456	j	A>G	0.89	0.11	0.00	0.00	0.00	0.00	0.98	0.02	<0.001
NO10615	k	G>A	0.89	0.11	0.00	0.00	0.00	0.00	0.98	0.02	<0.001
NO18978	l	A>G	0.83	0.15	0.02	0.00	0.02	0.00	0.98	0.00	<0.001
NO25979	m	G>A	0.83	0.17	0.00	0.00	0.00	0.00	0.98	0.02	<0.001
NO07275	n	C>A	0.94	0.06	0.00	0.00	0.00	0.00	0.98	0.02	<0.001
NO25980	o	C>T	0.57	0.43	0.00	0.00	0.00	0.00	0.76	0.24	<0.001
NO98433	p	G>A	0.94	0.02	0.04	0.00	0.05	0.00	0.95	0.00	<0.001
NO06949	q	T>C	0.91	0.04	0.04	0.00	0.05	0.00	0.95	0.00	<0.001
NO27225	r	G>C	0.77	0.21	0.02	0.00	0.00	0.00	0.90	0.10	<0.001
NO09041	s	G>A	0.72	0.28	0.00	0.00	0.00	0.00	0.88	0.12	<0.001
NO01515	t	A>G	0.47	0.51	0.00	0.02	0.00	0.02	0.56	0.41	<0.001
NO98768	u	T>A	0.72	0.09	0.17	0.02	0.00	0.00	0.93	0.07	<0.001
Highland Loci	Fig 2	REF > SNP	Males				Females				p value
			Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	
NO12147	na	C>T	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	<0.001
NO12353	na	A>G	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO10335	na	G>A	0.91	0.09	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO27290	na	G>A	0.57	0.43	0.00	0.00	0.00	0.00	0.95	0.05	<0.001

NO08127	na	A>G	0.70	0.00	0.30	0.00	0.43	0.57	0.00	0.00	0.002
NO11944	na	T>C	0.74	0.26	0.00	0.00	0.19	0.10	0.71	0.00	0.002
NO02387	na	C>A	0.91	0.09	0.00	0.00	0.29	0.00	0.71	0.00	0.002
NO98731	na	C>A	0.78	0.00	0.22	0.00	0.29	0.67	0.05	0.00	0.001
NO01508	na	A>G	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO01904	na	A>T	0.96	0.04	0.00	0.00	0.00	0.00	1.00	0.00	<0.001
NO12765	na	A>G	0.17	0.83	0.00	0.00	0.00	0.00	0.62	0.38	<0.001
NO05305	na	G>A	0.87	0.09	0.04	0.00	0.24	0.05	0.71	0.00	0.002

Lowland Loci	Fig 2	REF > SNP	Males				Females				p value
			Prop. ‘2’	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. ‘0’	Prop. ‘-’	
NO98539	na	G>T	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	<0.001
NO14859	na	G>A	0.46	0.33	0.17	0.04	0.00	0.00	0.80	0.20	<0.001
NO17302	na	C>T	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	<0.001
NO05269	na	G>A	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	<0.001
NO13601	na	A>C	0.58	0.42	0.00	0.00	0.00	0.00	0.90	0.10	<0.001
NO07509	na	G>A	0.92	0.04	0.04	0.00	0.00	0.00	1.00	0.00	<0.001
NO16130	na	C>T	0.83	0.08	0.08	0.00	0.00	0.00	1.00	0.00	<0.001
NO00420	na	G>T	0.88	0.08	0.04	0.00	0.00	1.00	0.00	0.00	<0.001
NO02053	na	G>A	0.88	0.08	0.04	0.00	0.00	1.00	0.00	0.00	<0.001
NO98391	na	C>T	0.88	0.00	0.13	0.00	0.25	0.70	0.05	0.00	<0.001
NO00397	na	C>G	0.79	0.00	0.21	0.00	0.30	0.65	0.05	0.00	0.002
NO11068	na	A>G	0.08	0.79	0.00	0.13	0.00	0.00	0.35	0.65	<0.001

Table S2.3. Proportion of genotypes in males and females for SNPs with alleles that segregate with sex according to the 'null inclusive' sex-linked model in populations of *Niveoscincus ocellatus*. Score of '2' indicates heterozygous for the reference and SNP allele, '1' homozygous for the SNP allele[+], '0' homozygous for the reference allele[#],'-' homozygous for a null genotype. All loci denote XY heterogamety. Male genotypes of loci marked with * are '0' in one population and '1' in the other.

Common Loci	REF > SNP	Males				Females				p value
		Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	
NO04145*	A>T	0.00	0.36	0.43	0.21	0.00	0.00	0.00	1.00	<0.001
NO04127*	G>A	0.00	0.49	0.51	0.00	0.00	0.00	0.00	1.00	<0.001
NO04162	G>T	0.00	0.96	0.04	0.00	0.00	0.00	0.00	1.00	<0.001
NO07509*	G>T	0.00	0.45	0.49	0.06	0.00	0.00	0.00	1.00	<0.001
NO07552*	G>C	0.00	0.40	0.45	0.15	0.00	0.00	0.00	1.00	<0.001
NO11008*	C>G	0.00	0.45	0.45	0.11	0.00	0.00	0.00	1.00	<0.001
NO18978	C>G	0.00	0.87	0.00	0.13	0.00	0.00	0.02	0.98	<0.001
NO24879	G>A	0.04	0.96	0.00	0.00	0.00	0.02	0.05	0.93	<0.001
NO25173	A>C	0.02	0.98	0.00	0.00	0.00	0.07	0.15	0.78	<0.001
NO25258	G>C	0.04	0.96	0.00	0.00	0.00	0.00	0.12	0.88	<0.001
NO26563	T>C	0.02	0.94	0.04	0.00	0.00	0.00	0.00	1.00	<0.001
NO26593	C>A	0.00	0.77	0.00	0.23	0.00	0.00	0.05	0.95	<0.001
NO27294	T>G	0.02	0.98	0.00	0.00	0.00	0.00	0.12	0.88	<0.001
NO27500	T>C	0.00	1.00	0.00	0.00	0.00	0.02	0.02	0.95	<0.001
NO27612	A>G	0.00	1.00	0.00	0.00	0.00	0.02	0.07	0.90	<0.001
NO04199	T>C	0.00	0.89	0.04	0.06	0.00	0.00	0.00	1.00	<0.001
NO04080	G>C	0.00	0.04	0.96	0.00	0.00	0.00	0.00	1.00	<0.001
NO04084*	A>G	0.00	0.49	0.43	0.09	0.00	0.00	0.00	1.00	<0.001
NO04117	C>T	0.00	0.06	0.83	0.11	0.00	0.00	0.00	1.00	<0.001
NO04124*	G>A	0.00	0.43	0.45	0.13	0.00	0.00	0.00	1.00	<0.001
NO07495	G>A	0.00	0.04	0.85	0.11	0.00	0.00	0.02	0.98	0.003
NO04100	C>T	0.00	0.04	0.94	0.02	0.00	0.00	0.00	1.00	<0.001
NO08908	C>T	0.06	0.02	0.77	0.15	0.00	0.10	0.00	0.90	<0.001
NO09550	C>T	0.00	0.04	0.96	0.00	0.00	0.00	0.02	0.98	<0.001
NO12829	T>G	0.00	0.00	1.00	0.00	0.00	0.05	0.07	0.88	<0.001
NO13113	G>A	0.11	0.00	0.89	0.00	0.00	0.12	0.00	0.88	<0.001
NO13978	G>A	0.00	0.02	0.96	0.02	0.00	0.00	0.00	1.00	<0.001

NO17128	T>A	0.02	0.00	0.98	0.00	0.00	0.07	0.05	0.88	<0.001
NO21421	C>A	0.02	0.06	0.91	0.00	0.00	0.00	0.00	1.00	<0.001
NO22921	C>A	0.00	0.00	0.98	0.02	0.00	0.02	0.05	0.93	<0.001
NO25881	A>G	0.00	0.02	0.91	0.06	0.00	0.02	0.00	0.98	<0.001
NO25172	C>A	0.02	0.00	0.98	0.00	0.00	0.07	0.07	0.85	<0.001
NO27502	G>A	0.00	0.00	1.00	0.00	0.00	0.05	0.02	0.93	<0.001

Highland Loci	REF > SNP	Males				Females				p value
		Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	
NO10275	C>A	0.00	0.70	0.00	0.30	0.00	0.05	0.00	0.95	0.026
NO26341	G>A	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	<0.001
NO27242	G>C	0.13	0.70	0.04	0.13	0.00	0.00	0.29	0.71	<0.001
NO19405	C>A	0.00	0.00	0.91	0.09	0.00	0.00	0.00	1.00	<0.001
NO26343	T>A	0.04	0.00	0.96	0.00	0.00	0.24	0.00	0.76	<0.001
NO26549	C>G	0.00	0.00	1.00	0.00	0.00	0.00	0.05	0.95	<0.001

Lowland Loci	REF > SNP	Males				Females				p value
		Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	Prop. '2'	Prop. '1'	Prop. '0'	Prop. '-'	
NO07823	C>T	0.00	0.67	0.04	0.29	0.00	0.00	0.00	1.00	0.003
NO15685	T>C	0.00	0.92	0.00	0.08	0.00	0.00	0.00	1.00	<0.001
NO16129	G>A	0.00	0.92	0.00	0.08	0.00	0.00	0.05	0.95	<0.001
NO25994	A>T	0.00	0.67	0.04	0.29	0.00	0.00	0.15	0.85	0.012
NO26367	A>G	0.00	1.00	0.00	0.00	0.00	0.00	0.05	0.95	<0.001

[#+] Given the prevalence of null alleles in females it is accepted that individuals with a '0' or '1' genotype are in fact heterozygous for a null allele and the reference or SNP allele respectively.

Chapter Three

Differences in homomorphic sex chromosomes is associated with population divergence in sex determination in *Carinascincus ocellatus* (Scincidae: Lygosominae)

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Abstract

Sex determination directs development as male or female in sexually reproducing organisms. Evolutionary transitions in sex determination have occurred frequently, suggesting simple mechanisms behind the transitions, yet their detail remains elusive. Here we explore the links between mechanisms of transitions in sex determination and sex chromosome evolution at both recent and deeper temporal scales (<1 Myr; ~79 Myr). We studied a rare example of a species with intraspecific variation in sex determination, *Carinascincus ocellatus*, and a relative, *Liopholis whitii*, using c-banding and mapping of repeat motifs and a custom Y chromosome probe set to identify the sex chromosomes. We identified both unique and conserved regions of the Y chromosome among *C. ocellatus* populations differing in sex determination. There was no evidence for homology of sex chromosomes between *C. ocellatus* and *L. whitii*, suggesting independent evolutionary origins. We discuss sex chromosome homology between members of the subfamily Lygosominae and propose links between sex chromosome evolution, sex determination transitions, and karyotype evolution.

Keywords: cryptic sex chromosomes; karyotype; GSD; TSD; Niveoscincus.

Introduction

Development as male or female is central to sexual reproduction (Bachtrog et al. 2014). Sex determination decides the sexual fate of the developing gonad, and because the outcome is highly conserved, the underlying mechanism is also expected to be conserved (Uller et al. 2007). However, sex determination is surprisingly labile in vertebrates, which has generated considerable scientific interest (Bachtrog et al. 2014; Capel 2017; Ezaz et al. 2006; Janzen and Phillips 2006; Pennell et al. 2018; Pokorna and Kratochvil 2009). The diversity in sex determination in vertebrates is also accompanied by morphological and genetic diversity in sex chromosomes (Charlesworth 1991; Charlesworth and Mank 2010; Ezaz and Deakin 2014; Pennell et al. 2018; Sigeman et al. 2019). Because sex chromosomes also play a central role in postzygotic isolation and speciation (Coyne 2018; Haldane 1922; Hooper et al. 2019; Payseur et al. 2018), they may simultaneously reinforce any ongoing divergence in sex determination, raising fundamental questions about the links between sex chromosome evolution, sex determination transitions, and lineage divergence (Johnson and Lachance 2012; Mank 2012).

Our understanding of the potential contribution of sex chromosomes to transitions in sex determination relies on basic knowledge of sex chromosome evolution. The classic theory of sex chromosome evolution describes how they first arise when recombination around a sex determining locus is suppressed (Bachtrog et al. 2014; Charlesworth 1991). Recombination suppression on sex chromosomes is marked by the accumulation of inversions (Charlesworth et al. 2005), heterochromatinisation (Bachtrog 2013), transposable elements and other repetitive sequences such as microsatellite motifs (Reichwald et al. 2015), but it is unknown whether this is a cause or consequence of recombination suppression (Furman et al. 2020). The evolutionary trajectory of sex chromosomes in all but a few bird and mammal species has resulted in sex chromosomes progressing from homomorphy to heteromorphy (Ezaz et al.

2009; Graves 2014, 2006) and the origin of sex chromosomes in these lineages is ancient (approximately 140 and 180 mya ago in birds and mammals, repsectively; Bachtrog et al. 2014; Vicoso et al. 2013). In contrast, sex chromosomes are diverse in plants, invertebrates, fish and reptiles and several mechanisms contribute to this diversity (Bracewell et al. 2017; Lande et al. 2001; Ming et al. 2011; Pennell et al. 2015; Waters et al. 2001). For example, a pair of autosomes may usurp existing sex chromosomes via the acquisition of a new sex determining locus, or sex chromosome-autosome fusions may result in a new sex chromosome system (Meisel 2020). Further, thermosensitivity in sex determination can lead to transitions between alternative systems of heterogamety (XY male, ZW female) or from genetic to environmental sex determination, where sex chromosomes are lost (Holleley et al. 2015; Quinn et al. 2011). Sex chromosome evolution therefore follows diverse pathways, the dynamics of which are poorly understood.

Lizards provide an opportunity to understand sex chromosome evolution, and possible links to transitions in sex determination and speciation. However, the mechanisms that underpin transitions in sex determination and how they relate to sex chromosomes and speciation remain unclear because information regarding sex determination and sex chromosomes is lacking for the vast majority of lineages (Gamble et al. 2015). Among lizards, despite evidence of homology of sex-linked chromosomal regions among some species (Cornejo-Paramo et al. 2020; Dissanayake et al. 2020; Iannucci et al. 2019; Rovatsos et al. 2014; Rovatsos et al. 2016), sex chromosomes display extraordinary variation in morphology. Within the family Scincidae, XY heterogamety is prevalent, however, the recent discovery of a skink with ZW heterogamety (Patawang et al. 2018) highlights at least one transition between these systems. In addition, heteromorphic and homomorphic sex chromosomes are broadly distributed throughout skinks (Donnellan 1991a, b; Olmo and Signorino 2005) and skink species with sex chromosomes also exhibit temperature sensitivity of sex determination

(Cornejo-Paramo et al. 2020; Hill et al. 2018; Quinn et al. 2007; Radder et al. 2008; Robert and Thompson 2001; Shine et al. 2002; Wapstra et al. 2004). Sex determination transitions and sex chromosome evolution have occurred frequently in this group and the diploid chromosome complement is varied (Olmo and Signorino 2005).

Carinascincus ocellatus (Scincidae) is a rare example of a species exhibiting population divergence in sex determination; a high elevation population (1200 masl) has genetic sex determination (GSD) with XY heterogamety while a low elevation population (50 masl) has temperature sensitive XY GSD (Cunningham et al. 2017; Hill et al. 2018; Pen et al. 2010; Wapstra et al. 2009). Herein, we describe the low elevation population as having GSD+EE (Environmental Effects; *sensu* Valenzuela et al. 2003). Shared sex-linked markers in these populations define conserved sex chromosome sequence. However, these populations differ in the linkage disequilibrium among these shared sex-linked markers, and they also possess population-specific sex-linked markers, suggesting sex chromosome divergence (Hill et al. 2018). Recombination is more disrupted in the high elevation GSD population, representing molecular evidence of more progressed sex chromosome evolution. However, a karyotype analysis did not reveal differentiated sex chromosomes in *C. ocellatus* (Donnellan 1985).

Here we examine the karyotype of *C. ocellatus* (2n=30) and compare it to that of *Liopholis whitii* (2n=32, sex chromosomes homomorphic; Donnellan 1991a) from the subfamily Lygosominae (Figure 1) to identify sex chromosomes. Specifically, we examined the high and low elevation populations of *C. ocellatus* which diverged less than 1 Mya (Cliff et al. 2015; Chapter 5) to test whether intraspecific divergence in sex determination is reflected by gross sex chromosome variation. In addition, we provide a phylogenetic assessment of sex chromosome evolution through comparison between *C. ocellatus* and *L. whitii* which diverged approximately 79 Mya (Figure 1, divergence data retrieved from TimeTree; Kumar

et al. 2017) to test for homology of sex chromosomes in these lineages and to understand the mechanisms of sex chromosome evolution across deeper temporal scales. We examine metaphase spreads of males and females and used standard c-banding plus a custom probe set designed from *C. ocellatus* Y-linked sequence. In addition, we mapped repeats (AGAT and telomere) to the karyotypes of both species because of their association with sex chromosomes in a broad range of reptiles (Matsubara et al. 2016; Matsubara et al. 2015). We discuss sex chromosome evolution in the context of sex determination transitions and karyotype evolution.

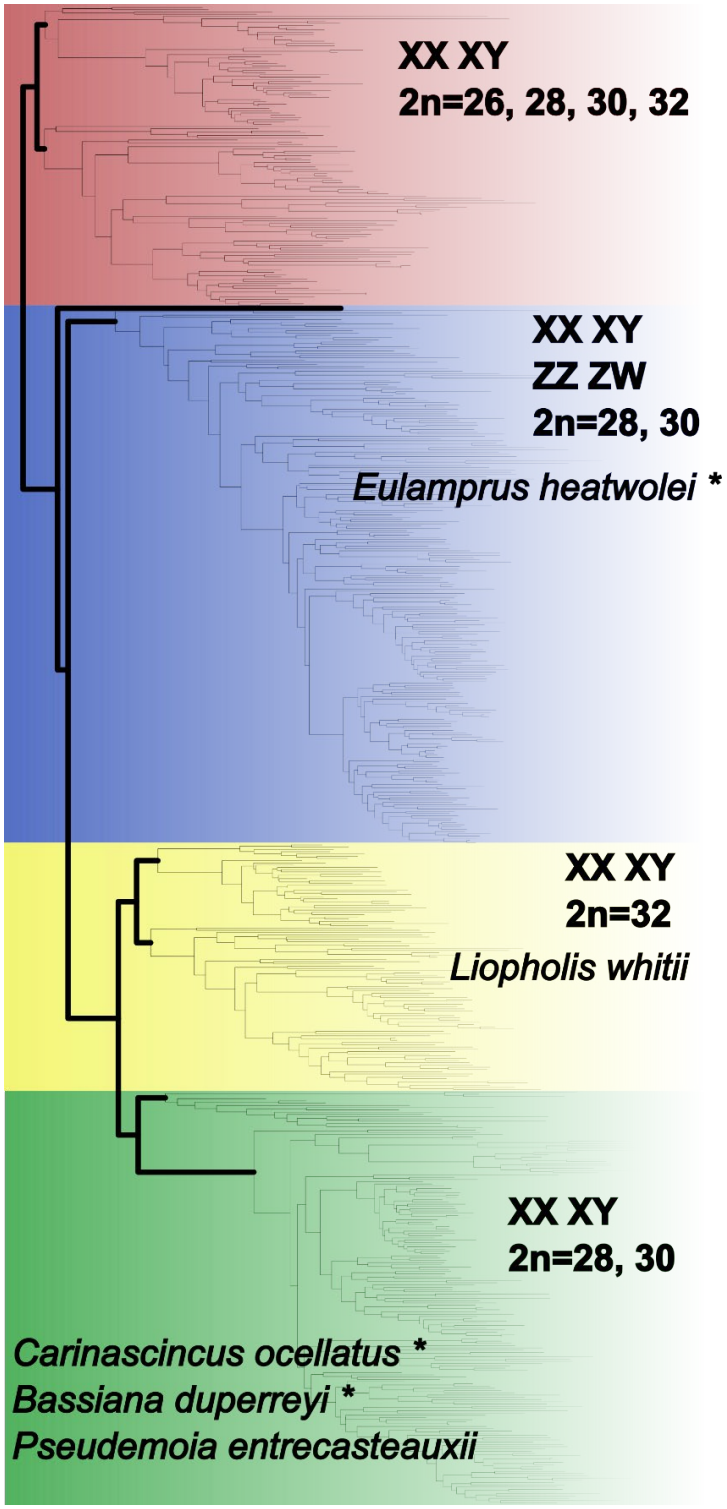

Figure 1. Variation in diploid chromosome complement and sex determination in Scincid subfamilies Scincinae (red) and Lygosominae (blue, yellow and green). The clades to which our study species (*Carinascincus ocellatus* and *Liopholis whitii*) belong and other species of interest discussed herein are included. The age of the split between clades containing *C. ocellatus*, *L. whitii* and *E. heatwolei* is estimated as 79 mya (Kumar et al. 2017). Adapted from Pyron et al. (2013) and Olmo and Signorino (2005). * denotes species where thermosensitive sex determination has been reported (Cornejo-Paramo et al. 2020; Pen et al. 2010; Radder et al. 2008).

Materials and Methods

Study species

Carinascincus ocellatus is a small (60 to 80 mm snout-vent length, 3 to10g) viviparous skink endemic to Tasmania, with a broad altitudinal distribution from sea level to 1200 m (Wapstra et al. 1999). We collected three males and three females from populations representing the climatic extremes of this species' range: a cool temperate low elevation population with GSD+EE (42 34'S, 147 52' E; elevation 50 m, Table 1, Figure 2) and a high elevation, cold temperate, population with GSD (41 51'S, 146 34'E; elevation 1200 m, Table 1, Figure 2). Long term data on these populations consistently documents their divergent sex determination (Cunningham et al. 2017; Pen et al. 2010; Wapstra et al. 2009).

Liopholis whitii is a medium viviparous skink (snout-vent length <100 mm) found throughout south eastern Australia. We examined three males and three females from the same low elevation location as *C. ocellatus* (Table 1, Figure 2).

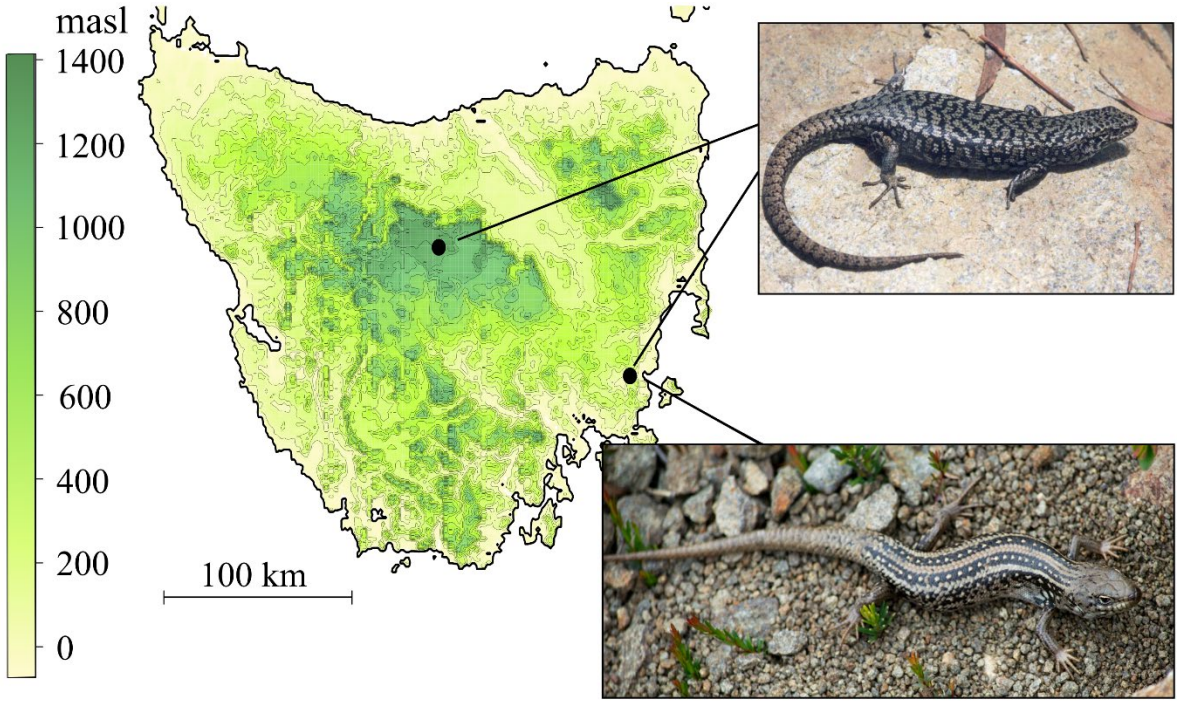

Figure 2. Sampling locations of *Carinascincus ocellatus* (inset, upper) and *Liopholis whitii* (inset, lower). *C. ocellatus* were sampled from high and low elevation populations exhibiting GSD and GSD+EE, respectively.

Blood culture and metaphase chromosome preparations

Metaphase spreads were obtained from whole blood using a modified version of the protocol described in Ezaz et al. (2005). Briefly, 50 µL of whole blood was taken via heparinised capillary tube from the sub-orbital sinus to set up 2 mL cultures in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (Gibco), 4% antibiotic-antimycotic solution (Gibco) and 8% Phytohemagglutinin from *Phaseolus vulgaris* (PHA-M, Sigma). Cells were cultured for 3 days at 28°C and 5% CO_2. Cell division was arrested 3.5 h prior to harvesting, using 0.05 µg/mL colcemid (Roche). Cells were treated with hypotonic solution (75 mM KCl) at 37°C and fixed in 3:1 methanol-acetic acid. The cell suspension was dropped onto glass slides, dehydrated through an ethanol series of 70%, 90%, 100%, air dried and stored at -80°C.

Development of sex-linked probe set for chromosome mapping

32 loci with sex-linked genotypes from double-digest, restriction-site associated DNA sequencing (RAD-seq) of *C. ocellatus* (Hill et al. 2018; Kilian et al. 2012) were chosen for inclusion in our custom probe set design. Of these, 26 represent Y-linked sequence (only present in males) common to both populations. Six loci were on both X and Y chromosomes, but with single nucleotide polymorphisms (SNPs) segregating chromosomes (males heterozygous, females homozygous). Oligonucleotides were synthesized from these Y-linked sequences and SNP RAD-tag sequences (38–69 bp) and each fragment was end-labelled with 3X ATTO594 dye (Arbor Biosciences; https://arborbiosci.com).

C-banding

We analysed 1–2 males and females from *L. whitii* and each *C. ocellatus* population and examined 3–31 cells per individual (Table 1). C-banding was performed as described by Sumner (1972), Ezaz et al. (2005) and Shams et al. (2019) with slight modification. Briefly, 10–15 µL of cell suspension was dropped on glass slide, air dried and aged for 60 min on a 60°C hot plate. Aged slides were treated with 0.2 N HCl at room temperature for 30 min, then rinsed in distilled water and subsequently treated with 5% $Ba(OH)_2$ at 50°C for 6 min. Slides were again rinsed in distilled water and then incubated at 60°C in 2× SSC (Saline sodium citrate) for 1 h. Finally, for DAPI (4',6-diamidino-2-phenylindole) staining, the slides were mounted with antifade medium Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 1.5 mg/mL DAPI.

Fluorescence in situ hybridisation (FISH) with microsatellite motif, telomere and Y-linked probe set

We analysed up to three males and three females from *L. whitii* and each *C. ocellatus* population and examined 15–43 cells per individual (Table 1) to determine distributions of the $(AGAT)_8$ microsatellite motif, $(TTAGGG)_7$ telomeric repeats and the custom Y-linked probe set in males and females. The microsatellite motif $(AGAT)_8$ was chosen because of its

association with sex chromosomes in multiple reptilian groups including the Y chromosome in a closely related skink, *Bassiana duperreyi* (Figure 1; Matsubara et al. 2016). Telomere probe and microsatellite motif probes were Cy3-labelled (GeneWorks, Hindmarsh, South Australia, Australia). FISH was performed as described in Ezaz et al. (2005) and Matsubara et al. (2013) with slight modifications. Briefly, 500 ng of microsatellite and telomere oligonucleotides and 135 ng of Y-linked probe set were[1] added to 12.5 μL hybridisation buffer (50% formamide, 10% dextran sulphate, 2× SSC, 40 mM sodium phosphate pH 7.0 and 1× Denhardt's solution) and warmed to 37°C. The hybridisation mixture was placed onto the slide and sealed with a coverslip and rubber cement. Probe and chromosome DNA were denatured at 68.5°C for 5 min followed by incubation in a moist hybridisation chamber at 37°C for 24–48 hr. Slides were then washed in 0.4x SSC, 0.3% IGEPAL (Sigma-Aldrich, St. Louis, Missouri, USA) at 60°C for 2 min followed by 2x SSC, 0.1% IGEPAL at room temperature for 1 min. Slides were dehydrated by ethanol series and air dried. Finally, the slides were mounted with antifade medium Vectashield containing 1.5 mg/mL DAPI.

In addition to the individual FISH experiments, we performed sequential FISH initially with the custom Y-linked probe set followed by the $(AGAT)_8$ probe on male metaphase from both *C. ocellatus* populations. Hybridisation signals from the Y-linked probe set were photographed, metaphase positions recorded then slides were washed in 0.4x SSC at 60°C for 1 min, followed by 2x SSC at room temperature for 2 min before hybridisation with the $(AGAT)_8$ probe. Because both probes were labelled using fluorophores with similar excitation-emission wavelengths, it was not possible to resolve these signals using our existing microscope filter systems. Therefore, photos were merged in Photoshop (CS6) with

[1] Briefly, 500 ng of microsatellite and telomere oligonucleotides and 135 ng of Y-linked probe set were each added to 12.5 μL of hybridisation buffer

the colour of the Y-linked signal altered digitally so its association with repeat motifs could be examined.

Table 1. Number of male and female individuals and cells examined from whole blood culture of GSD (high elevation) and GSD+EE (low elevation) populations of *Carinascincus ocellatus* and a single population of *Liopholis whitii*. 'Sequential' FISH represents Y-linked and microsatellite FISH in series.

	GSD+EE *C. ocellatus*				**GSD *C. ocellatus***				***L. whitii***			
	male		**female**		**male**		**female**		**male**		**female**	
	ind.	**cells**	**ind.**	**cells**	**ind.**	**cells**	**ind.**	**cells**	**ind.**	**cells**	**ind.**	**cells**
Karyotyping	3	73	3	50	3	129	3	64	3	43	3	67
c-banding	1	4	2	6	2	18	2	31	1	3	1	7
FISH												
Telomere	3	15	2	20	2	36	2	18	3	17	3	24
AGAT	2	28	2	14	2	43	2	28	2	14	2	24
Y-linked	3	19	2	16	2	33	3	18	3	12	3	19
Sequential	1	11			1	17						

Microscopy and image analysis

Chromosome images were captured using a Zeiss Axio Scope A1 epifluorescence microscope fitted with a high-resolution microscopy camera AxioCam MRm Rev. 3 (Carl Zeiss Ltd. Oberkochen, Germany) and a Leica DM6 B (Leica microsystems). Images were analysed using Metasystems Isis FISH Imaging System V 5.5.10 software (Metasystems, Altlussheim, Germany) as well as Thunder Imager 3D (Leica microsystems).

Marker homology

We used NCBI BLAST (Madden 2013) to identify homologs of our markers from publicly available sequences from vertebrates. Specifically, we searched the "nr" database with the default settings in NCBI's blastn suite and report matches related to sex determination or sexual development with e-values <0.001.

Results

DAPI karyotypes

The karyotype of GSD and GSD+EE populations of *C. ocellatus* is 2n=30, represented by eight pairs of macrochromosomes and seven pairs of microchromosomes (Figure 3a-h), while the karyotype of *L. whitii* is 2n=32, represented by nine pairs of macrochromosomes and seven pairs of microchromosomes (Figure 3i-l). These are consistent with karyotypes described in Donnellan (1985) and Donnellan (1991a). Sex chromosomes are homomorphic (Figure 3a-l).

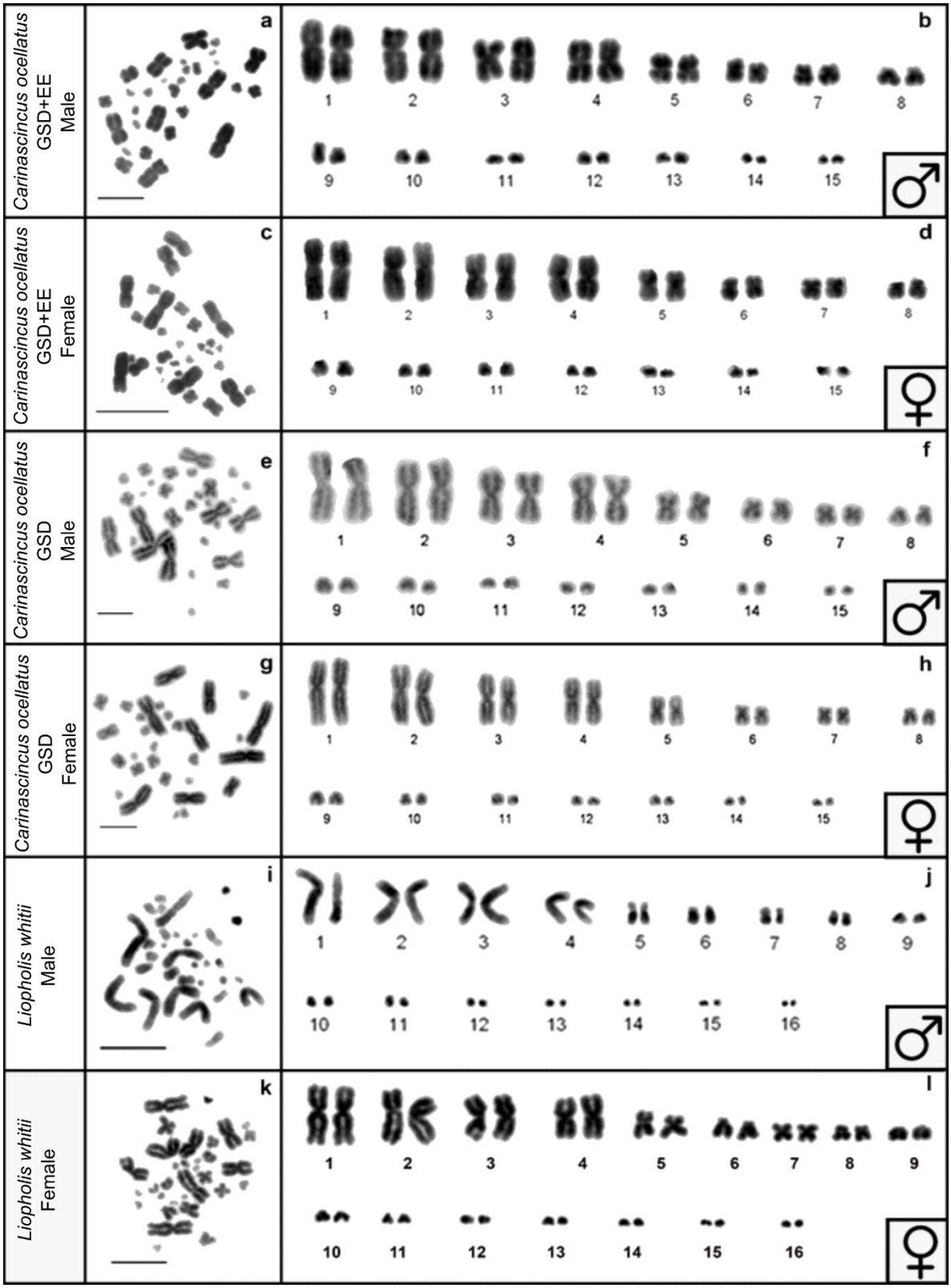

Figure 3. DAPI stained (inverted) metaphase spread and karyotypes in GSD+EE (low elevation) *Carinascincus ocellatus* male (a, b) and female (c, d), GSD (high elevation) *C. ocellatus* male (e, f) and female (g, h) and *Liopholis whitii* male (i, j) and female (k, l). Scale bar represents 10 μm.

Custom Y-linked C. ocellatus probe set

Our Y-linked probe set hybridised adjacent to the centromere on a single chromosome in males of both populations of *C. ocellatus*, and therefore identified the Y chromosome in both populations (Figure 4a, c). We did not detect any signal on females of either population, evidence that this probe set is specific to Y-linked sequences and does not contain sequences that are present on the X chromosome in quantities large enough to detect with FISH (Figure 4b, d). We did not detect any hybridisation signals on *L. whitii* (Figure 4e, f).

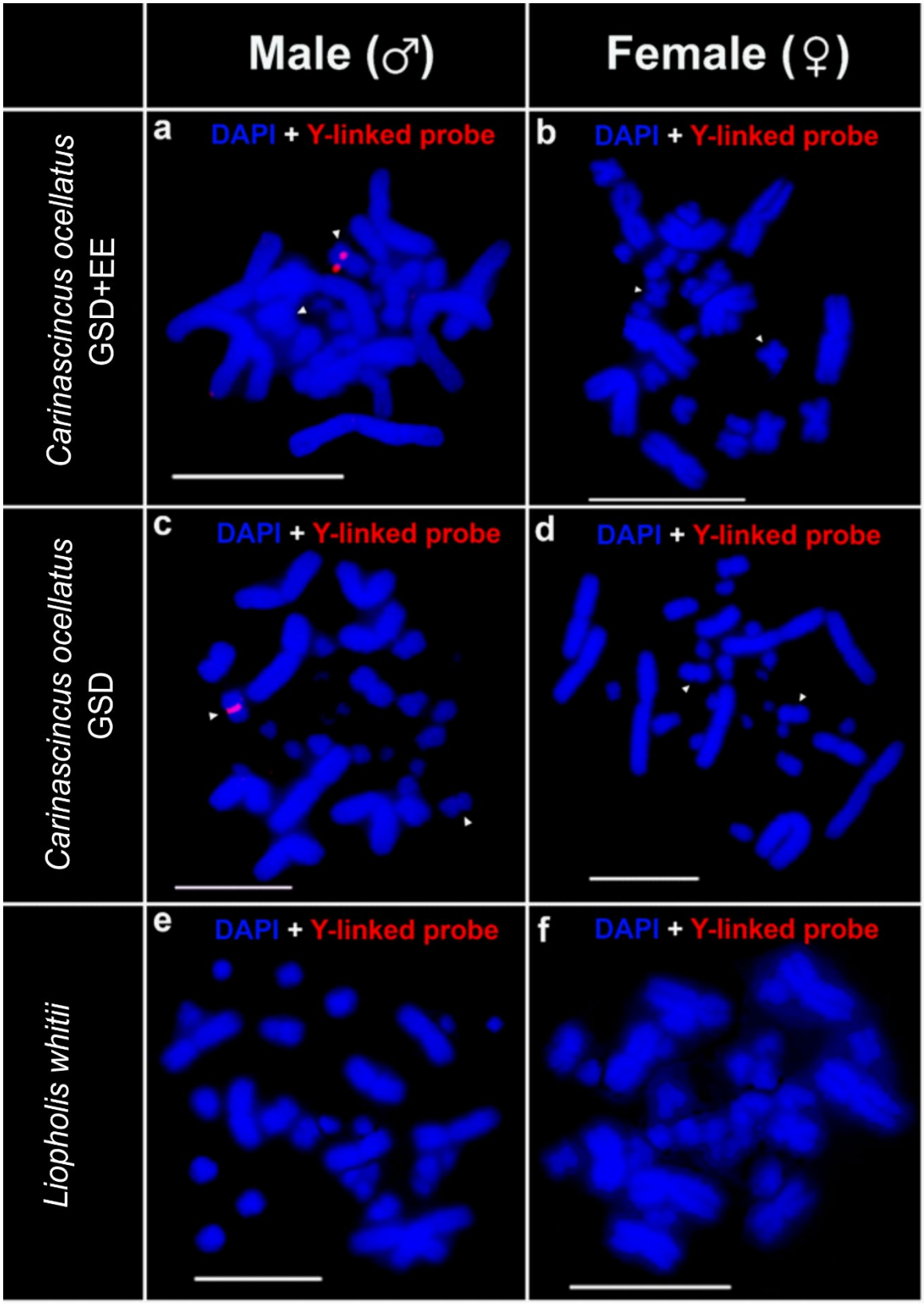

Figure 4. Chromosomal locations of *Carinascincus ocellatus* Y-linked FISH probe set in GSD+EE (low elevation) *C. ocellatus* male (a) and female (b), GSD (high elevation) *C. ocellatus* male (c) and female (d), and *Liopholis whitii* male (e) and female (f). Arrowhead indicates X and Y (the homologous pair) chromosomes in *C. ocellatus*. Scale bar represents 10 μm.

C-banding

Accumulation of multiple heterochromatic bands are consistently observed across all macrochromosomes in both populations of *C. ocellatus*, while only one major band was observed in all microchromosomes. At least four microchromosomes are highly heterochromatic (Figure 5a-h). Comparisons of C-banded karyotypes between males and females of both populations of *C. ocellatus* identified one of the homologs of chromosome pair 7 as highly heterochromatic in males but not females from the GSD (high elevation) population (Figure 5e-h); no sex specific heterochromatinisation was observed at low elevation (Figure 5a-d). This corroborates the signal from[2] the Y-specific probe and confirms the Y chromosome has a region of heterochromatinisation in the GSD population. C-banding in *L. whitii* revealed similar patterns to *C. ocellatus*, although we did not detect any sex specific heterochromatinisation between male and female *L. whitii* (Figure 5i-l). Multiple c-bands are observed in macrochromosomes and fewer in microchromosomes, and one macrochromosome pair and one microchromosome pair are highly heterochromatic (pairs 9 and 10; Figure 5i-l).

[2] The heterochromatin accumulation observed on *C. ocellatus* sex chromosomes only corroborates the Y-specific probe signal from the GSD population.

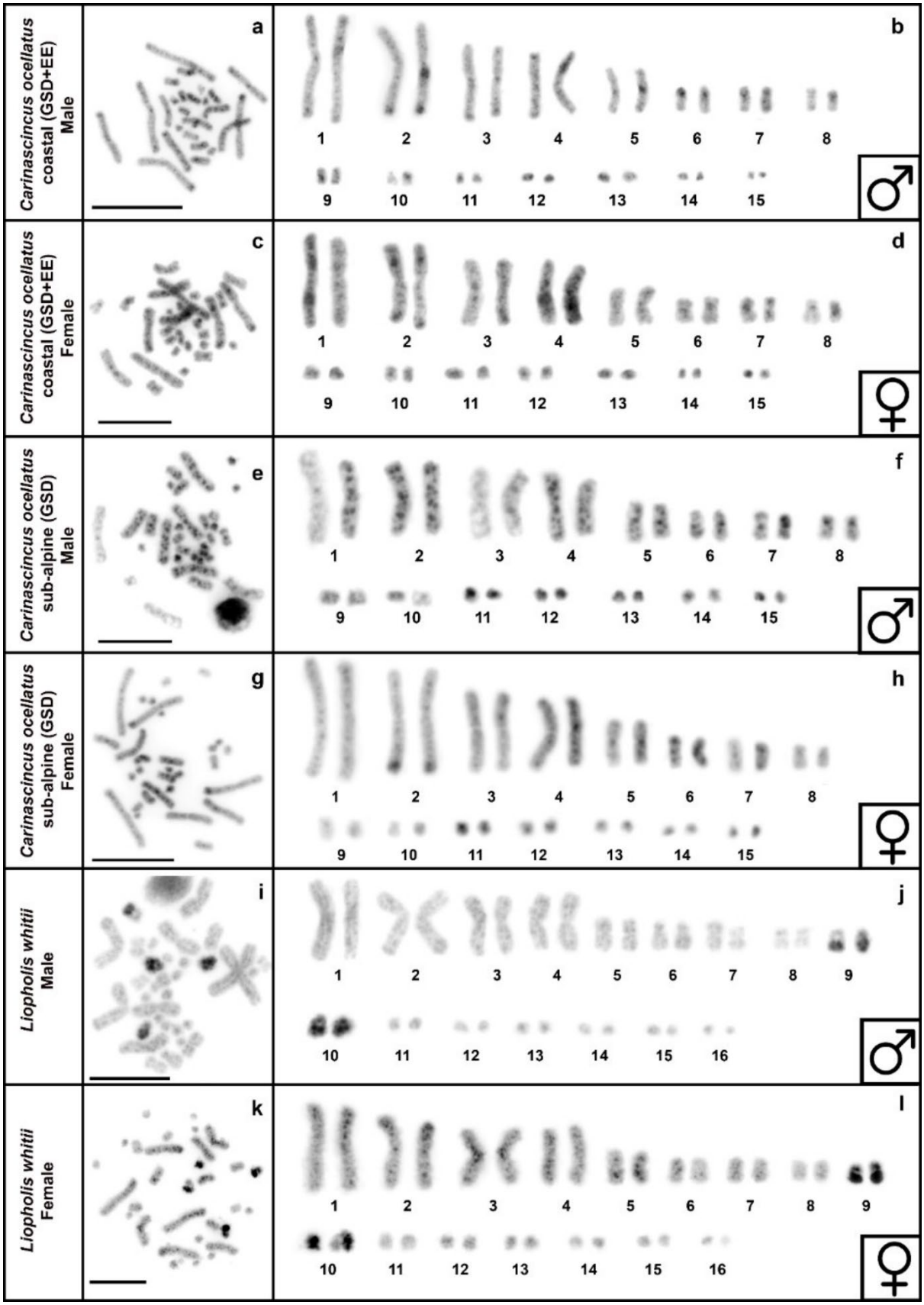

Figure 5. C-banded spread and karyotypes in GSD+EE (low elevation) *Carinascincus ocellatus* male (a, b) and female (c, d), GSD (high elevation) *C. ocellatus* male (e, f) and female (g, h) and *Liopholis whitii* male (i, j) and female (k, l). Scale bar represents 10 µm.

Telomere repeats

Telomeric repeats were observed to hybridise onto the distal regions of both micro and macrochromosomes of males and females of all individuals (Figure 6a-f). Telomeric repeats are also observed at the centromeres of the two largest pairs of macrochromosomes in *C. ocellatus* (Figure 6a-d). We did not detect any sex specificity of any distal or interstitial telomeric sequences in either species (Figure 6a-f).

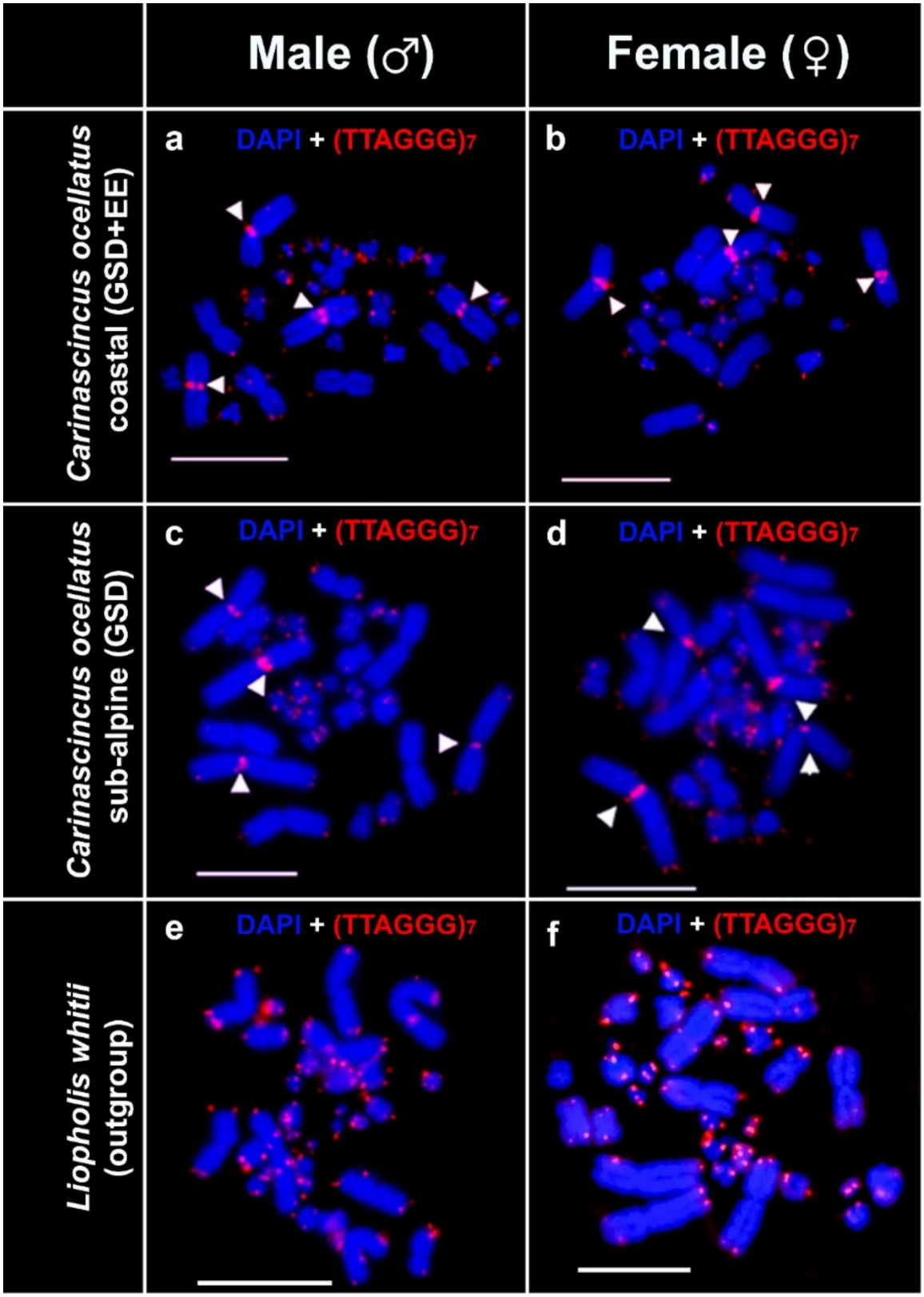

Figure 6. Chromosomal locations of the telomeric repeat $(TTAGGG)_7$ sequence in *Carinascincus ocellatus* GSD+EE (low elevation) male (a) and female (b), GSD (high elevation) *C. ocellatus* male (c) and female (d) and *Liopholis whitii* male (e) and female (f). Arrowhead indicates centromeric telomeres. Scale bars represent 10 μm.

Microsatellite motif (AGAT)$_8$ mapping

The (AGAT)$_8$ probe hybridised onto telomeric regions of most of the macro and microchromosomes in males and females of both populations of *C. ocellatus* (Figure 7a-d). In addition, amplified hybridisation signals were observed in several microchromosomes in some males and females of both populations of *C. ocellatus* (Figure 7a-d). We also detected sex specific amplification of (AGAT)$_8$ on chromosome pair seven of the GSD (high elevation) population of *C. ocellatus*. Hybridisation was observed at the distal ends of both arms of both members of this pair, however, in one member of the pair this signal was amplified on the p arm in males and identifies the Y chromosome (Figure 7c, d). This sex-specificity of the (AGAT)$_8$ signal was not observed in the GSD+EE (low elevation) population. We did not detect any hybridisation signals of the (AGAT)$_8$ probe in *L. whitii* (Figure 7e, f).

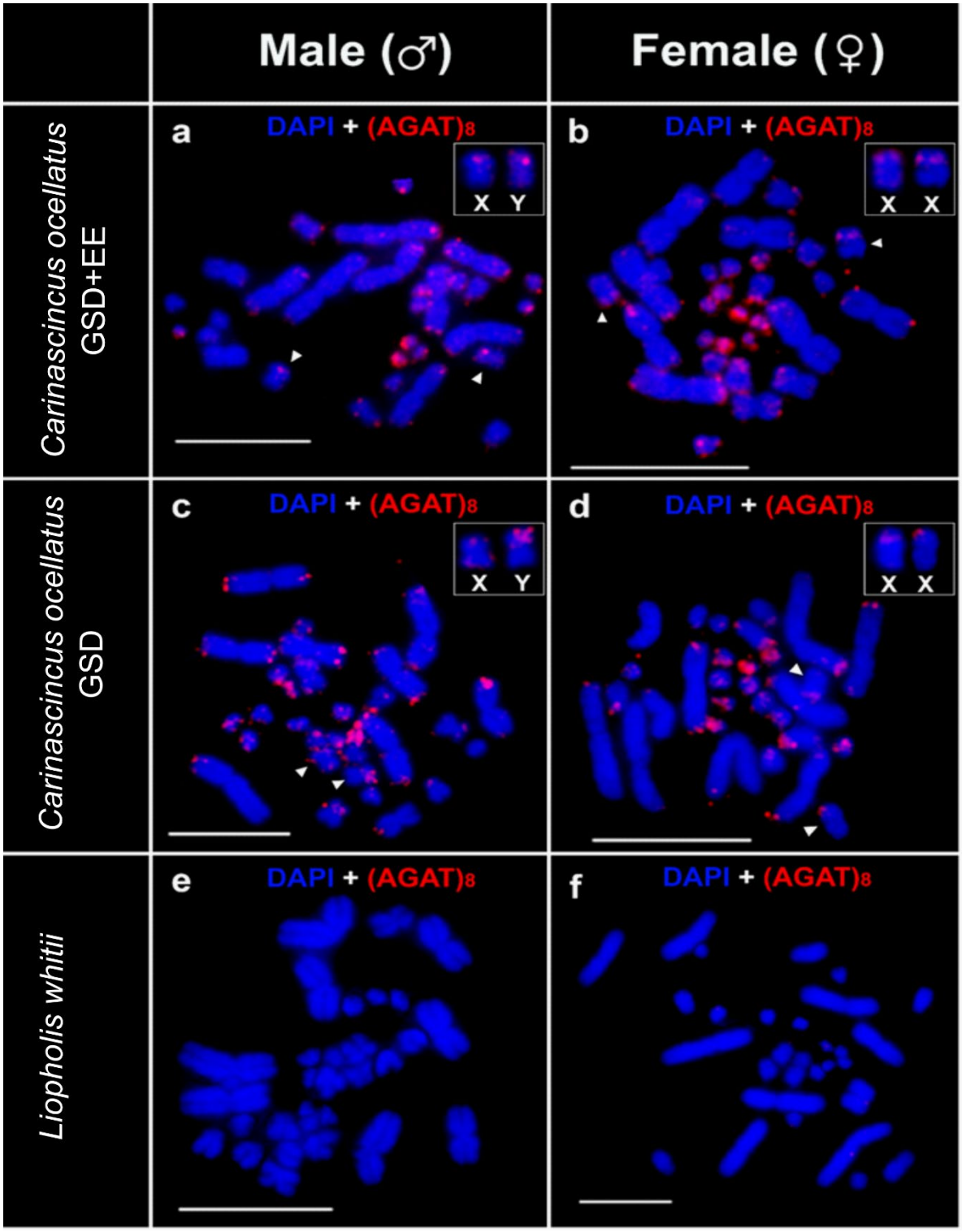

Figure 7. Chromosomal locations of the $(AGAT)_8$ repeat on *Carinascincus ocellatus* GSD+EE (low elevation) male (a) and female (b), GSD (high elevation) *C. ocellatus* male (c) and female (d) and *Liopholis whitii* male (e) and female (f). *C. ocellatus* sex chromosomes inset. Scale bars represent 10 μm.

Sequential FISH of $(AGAT)_8$ and custom Y-linked Probe set

The identity of the X and Y chromosome pair in both GSD and GSD+EE populations of *C. ocellatus* was confirmed as macrochromosome pair seven (Figure 8) based on sequential FISH, which also confirmed population-specific $(AGAT)_8$ signals on the Y chromosomes (Figure 8b, c, supplementary figure S1).

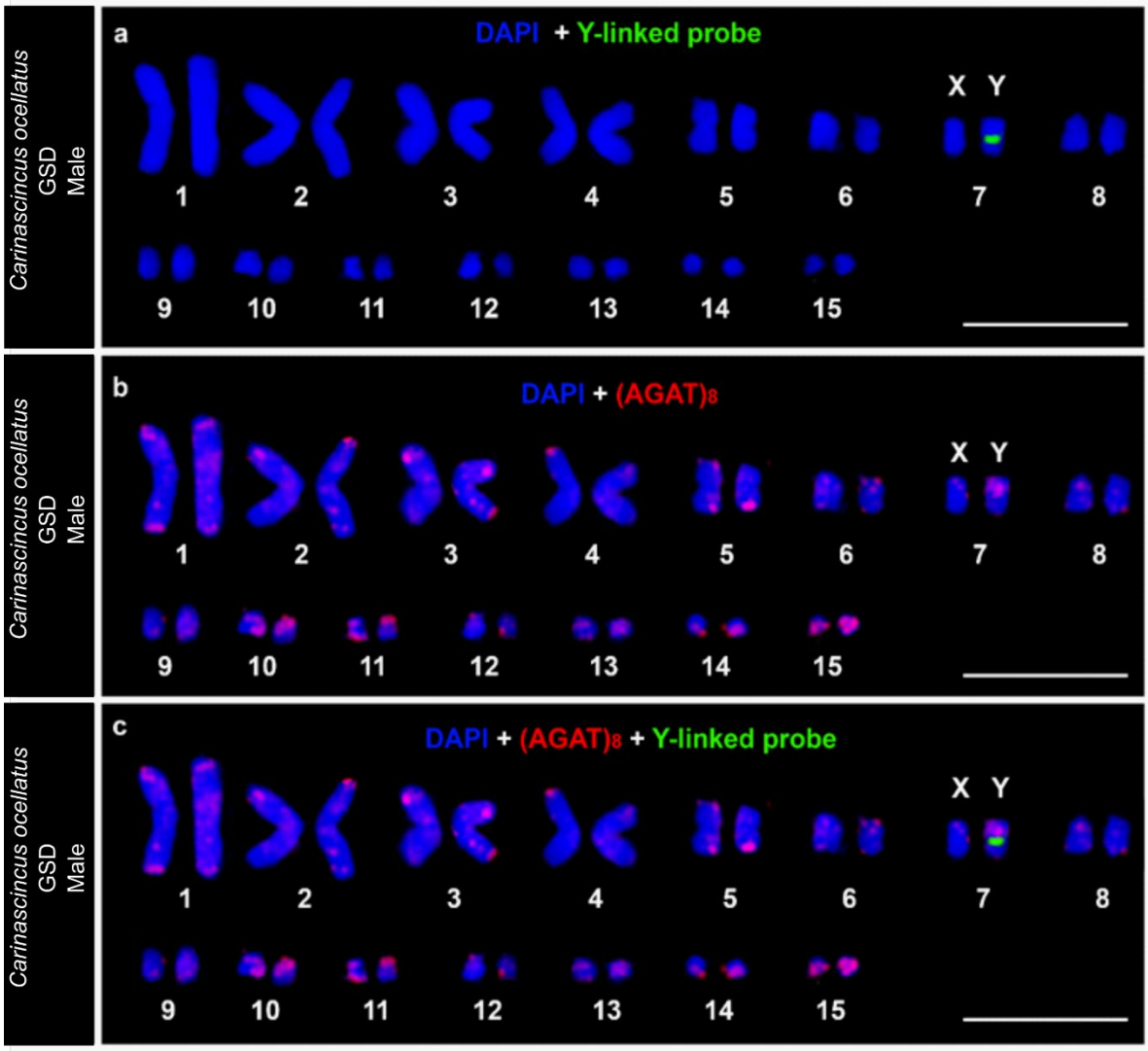

Figure 8. Sequential FISH and karyotyping in GSD (high elevation) population of *Carinascincus ocellatus* to confirm chromosome seven as the sex chromosome pair. a) DAPI stained karyotype with FISH signals from Y-linked probe set (Pseudocoloured image) on *C. ocellatus* male; b) DAPI stained karyotype with FISH signals from $(AGAT)_8$ microsatellite probe on the same metaphase; c) Superimposed image of both layers indicates $(AGAT)_8$ rich Y chromosome.

Marker homology

The only sequence with homology (e-value $2.34x10^{-4}$) to our *C. ocellatus* Y-linked probe set sequences that is relevant to sex determination or sex chromosome differentiation is a Sauria short interspersed nuclear element (SINE; Piskurek et al. 2006).

Discussion

Carinascincus ocellatus is a rare example of a species exhibiting population divergence in sex determination; GSD and GSD+EE occur in high and low elevation populations, respectively (Cunningham et al. 2017; Pen et al. 2010). Here we also report divergence between the same populations in sex chromosome evolution. Given divergence between our *C. ocellatus* study populations occurred less than 1 million years ago (Cliff et al. 2015; Chapter 5), the mechanisms underpinning the transition in sex determination and those governing early sex chromosome evolution are potentially linked.

The high elevation, GSD Y chromosome is more heterochromatic and the p arm is AGAT rich compared to the low elevation, GSD+EE Y chromosome. This is consistent with observations of greater linkage disequilibrium of sex-linked DNA markers in the high elevation population (Hill et al. 2018), and that reduced recombination between the sex chromosomes is associated with repeat and heterochromatin accumulation on the Y chromosome during early differentiation from the X chromosome (Bachtrog 2013; Ponnikas et al. 2018; Reichwald et al. 2015). The differences in sex chromosomes with elevation can be explained by population-specific selection that drives the divergence in sex determination (Pen et al. 2010). GSD is adaptive at high elevation because large interannual temperature fluctuations would produce maladaptive sex ratio skews if sex determination was thermosensitive (Cunningham et al. 2017). Therefore, sex chromosomes with lower recombination around a sex determining locus are the result of selection against skewed sex

ratios and thus selection towards balanced sex ratios and GSD. Selection for GSD+EE at low elevation occurs because of the sex-specific fitness benefits of climate-mediated birthdate variation in this population (Pen et al. 2010). Considering the lower selective advantage of GSD at low elevation, there is less selection against recombination around the sex determining locus in this population, and hence lower divergence between X and Y. Population-specific repeat and heterochromatin accumulation (this study), and recombination between the X and Y chromosomes (Hill et al. 2018), therefore likely reflect differences in the size of the pseudoautosomal region (the region of the sex chromosomes that continues to recombine) of the Y chromosome in each population resulting from differential selection for GSD. One alternative is that population size differences have led to different rates of accumulation of mutations on the Y chromosome, however, the high and low elevation populations are of similar size (Hill et al. 2021) making this unlikely.

Observations of heterochromatin and AGAT repeat accumulation in the high elevation GSD population suggest that the ancestral sex chromosomes and sex determination was closer to the current situation in the low elevation GSD+EE population. An alternative hypothesis is that selection favouring recombination between sex chromosomes has driven the evolution of GSD+EE at low elevation from a GSD ancestor that possessed sex chromosomes similar to those in the high elevation population. However, this alternative hypothesis is unlikely because transitions from environmental to genetic sex determination in squamates are higher than the reverse, suggesting GSD is more stable (Pennell et al. 2018). Because both populations are characterised by a Y-specific region with significant homology to a class of retrotransposable element implicated in recombination suppression (Ezaz et al. 2013; Ezaz and Deakin 2014; Harvey et al. 2003), selection in the ancestral population, and indeed the low elevation population currently, may have favoured subtle variations in sex ratio with climate, rather than a strict TSD system. Population divergence in sex determination and sex

chromosomes in *C. ocellatus* occurred without gene flow (Chapter 5) and our results show that the accumulation of changes that accompany transitions can occur over short evolutionary time scales.

While divergence in *C. ocellatus* sex determination has arisen recently, it is super-imposed over a deep evolutionary conservation of sex-linked sequences. Comparisons against *Eulamprus heatwolei* (Scincidae) suggests conservation of Y chromosome sequences in *C. ocellatus* for ~79 million years (Figure 1; Cornejo-Paramo et al. 2020). Likewise, heteromorphism of chromosome pair seven has been reported in two species of skink, *Bassiana duperreyi* (Shine et al. 2002) and *Pseudemoia entrecasteauxii* (Hutchinson and Donnellan 1992), both close relatives of *C. ocellatus* (Figure 1, divergence time 36-37 Mya, retrieved from TimeTree; Kumar et al. 2017; Pyron et al. 2013), suggesting a conserved role for pair seven as sex chromosomes (but requiring confirmation of sequence homology). The historical conservation of sex-linked sequence, combined with the recency of transition in sex determination in *C. ocellatus*, is compatible with only a small number of genetic changes potentially underlying this transition (Hill et al. 2018).

Observations from *L. whitii* provide a contrasting perspective on sex chromosome evolution among Lygosomine skinks. Sex-specific chromosomes (Y or W) usually display increased heterochromatinisation (Bachtrog 2013; Charlesworth 1991). Further, microsatellite and telomere repeat accumulation characterizes the sex chromosomes (Y and W) in a broad range of reptiles (Matsubara et al. 2016). We found no sex-specific patterns of telomere accumulation in the karyotype of *C. ocellatus* or *L. whitii*. In addition, we found no evidence to suggest the AGAT repeat is involved in sex chromosome evolution in *L. whitii*. Our custom Y-linked probe set also lacked accumulation on any *L. whitii* chromosome. These absences suggest independent evolution of sex chromosomes in *L. whitii*. Independent

evolution may include processes resulting from the loss of the Y chromosome region containing our *C. ocellatus* probe sequence and may also include accumulation of lineage-specific repeats. Sex chromosome evolution in subfamily Lygosominae may be associated with changes in chromosome number, because *C. ocellatus*, *B. duperreyi*, *P. entrecasteauxii* and *E. heatwolei* all possess a diploid chromosome compliment of 30, while *L. whitii*, belonging to a clade nested within these species' clades, possesses 32 (Figure 1; Donnellan 1991a, 1985; Hutchinson and Donnellan 1992; Shine et al. 2002). Differences in the number of acrocentric chromosomes (*C. ocellatus* =1; *L. whitii* =2) suggest chromosome fission or fusion events during speciation and karyotype evolution (Ezaz et al. 2017; Holmquist and Dancis 1984). Therefore, karyotype changes may favour the independent evolution of sex chromosomes and this may be one mechanism acting at the time of the split between the ancestor of *L. whitii* and other Lygosomine lineages with a diploid complement of 30. Sex chromosome origin coinciding with chromosome fission / fusion events is evident in Iguanids (Alam et al. 2020; Srikulnath et al. 2019), and may represent a common mechanism throughout squamates. This can be confirmed in Scincidae via further experiments designed to identify sex chromosome homology and identity among closely and distantly related species, alongside karyotype analysis.

Conclusion

By examining the karyotypes of *C. ocellatus* with intraspecific variation in sex determination, we reveal that structural changes in sex chromosomes such as heterochromatinisation and repeat accumulation could be associated with such transitions. This is evidence of links between sex determination transitions and sex chromosome evolution. Through broader taxonomic comparisons, we also reveal a potential association between sex chromosome

origin and karyotype evolution. Scincidae represents a valuable taxon for our understanding of diversity in sex determination, sex chromosomes and karyotype.

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References

Alam SMI, Altmanová M, Prasongmaneerut T, Georges A, Sarre SD, Nielsen SV, Gamble T, Srikulnath K, Rovatsos M, Kratochvíl L, Ezaz T. 2020. Cross-species BAC mapping highlights conservation of chromosome synteny across dragon lizards (Squamata: Agamidae). Genes 11: 698.

Bachtrog D. 2013. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. Nat Rev Genet 14: 113-124. doi: 10.1038/nrg3366

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, Tree of Sex Consortium. 2014. Sex determination: why so many ways of doing it? PLoS Biology 12: e1001899. doi: 10.1371/journal.pbio.1001899

Bracewell RR, Bentz BJ, Sullivan BT, Good JM. 2017. Rapid neo-sex chromosome evolution and incipient speciation in a major forest pest. Nat. Commun. 8: 1593. doi: 10.1038/s41467-017-01761-4

Capel B. 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat. Rev. Genet. 18: 675-689. doi: 10.1038/nrg.2017.60

Charlesworth B. 1991. The evolution of sex chromosomes. Science 251: 1030-1033.

Charlesworth D, Charlesworth B, Marais G. 2005. Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128. doi: 10.1038/sj.hdy.6800697

Charlesworth D, Mank JE. 2010. The birds and the bees and the flowers and the trees: lessons from genetic mapping of sex determination in plants and animals. Genetics 186: 9-31. doi: 10.1534/genetics.110.117697

Cliff HB, Wapstra E, Burridge CP. 2015. Persistence and dispersal in a Southern Hemisphere glaciated landscape: the phylogeography of the spotted snow skink (*Niveoscincus ocellatus*) in Tasmania. BMC Evol. Biol. 15: 121. doi: 10.1186/s12862-015-0397-y

Cornejo-Paramo P, Dissanayake DSB, Lira-Noriega A, Martinez-Pacheco ML, Acosta A, Ramirez-Suastegui C, Mendez-de-la-Cruz FR, Szekely T, Urrutia AO, Georges A, Cortez D. 2020. Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome Biol. Evol. 12: 924 - 930. doi: 10.1093/gbe/evaa104

Coyne JA. 2018. “Two Rules of Speciation” revisited. Mol. Ecol. 27: 3749-3752. doi: 10.1111/mec.14790

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol. Lett. 13. doi: 10.1098/rsbl.2017.0218

Dissanayake DSB, Holleley CE, Hill L, O'Meally D, Deakin J, Georges A. 2020. Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in-silico whole genome subtraction. BMC Genom. 21: 667.

Donnellan SC. 1991a. Chromosomes of Australian lygosomine skinks (Lacertilia: Scincidae) I the Egernia group. Genetica 83: 207-222. doi: 10.1007/bf00126227

Donnellan SC. 1991b. Chromosomes of Australian lygosomine skinks (Lacertilia: Scincidae) II The genus *Lampropholis*. Genetica 83: 223-234.

Donnellan SC 1985. The evolution of sex chromosomes in scincid lizards. [PhD]. [NSW]: Macquarie University.

Ezaz T, Azad B, O'Meally D, Young MJ, Matsubara K, Edwards MJ, Zhang X, Holleley CE, Deakin JE, Graves JA, Georges A, Edwards SV, Sarre SD. 2013. Sequence and gene content of a large fragment of a lizard sex chromosome and evaluation of candidate sex differentiating gene R-spondin 1. BMC Genom. 14: 899. doi: 10.1186/1471-2164-14-899

Ezaz T, Deakin JE. 2014. Repetitive sequence and sex chromosome evolution in vertebrates. Adv. Evol. Biol. 2014: 9. doi: 10.1155/2014/104683

Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Graves JA. 2005. The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. Chromosome Res. 13: 763-776. doi: 10.1007/s10577-005-1010-9

Ezaz T, Sarre SD, O'Meally D, Graves JA, Georges A. 2009. Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet. Genome Res. 127: 249-260. doi: 10.1159/000300507

Ezaz T, Srikulnath K, Graves JA. 2017. Origin of Amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? J. Hered. 108: 94-105. doi: 10.1093/jhered/esw053

Ezaz T, Stiglec R, Veyrunes F, Graves JA. 2006. Relationships between vertebrate ZW and XY sex chromosome systems. Curr. Biol. 16: R736-743. doi: 10.1016/j.cub.2006.08.021

Furman BLS, Metzger DCH, Darolti I, Wright AE, Sandkam BA, Almeida P, Shu JJ, Mank JE. 2020. Sex chromosome evolution: So many exceptions to the rules. Genome Biology and Evolution. doi: 10.1093/gbe/evaa081

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D. 2015. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Mol Biol Evol 32: 1296-1309. doi: 10.1093/molbev/msv023

Graves JA. 2014. Avian sex, sex chromosomes, and dosage compensation in the age of genomics. Chromosome Res. 22: 45-57. doi: 10.1007/s10577-014-9409-9

Graves JA. 2006. Sex chromosome specialization and degeneration in mammals. Cell 124: 901-914. doi: 10.1016/j.cell.2006.02.024

Haldane JBS. 1922. Sex ratio and unisexual sterility in hybrid animals. J. Genet. 12: 101-109. doi: 10.1007/bf02983075

Harvey SC, Boonphakdee C, Campos-Ramos R, Ezaz MT, Griffin DK, Bromage NR, Penman P. 2003. Analysis of repetitive DNA sequences in the sex chromosomes of *Oreochromis niloticus*. Cytogenet. Genome Res. 101: 314-319. doi: 10.1159/000074355

Hill PL, Burridge CP, Ezaz T, Wapstra E. 2018. Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. Genome Biol. Evol. 10: 1079-1087. doi: 10.1093/gbe/evy042

Hill PL, Wapstra E, Ezaz T, Burridge CP. 2021. Pleistocene divergence in the absence of gene flow among populations of a viviparous reptile with intraspecific variation in sex determination. Ecology and Evolution 00: 1-9. doi: DOI: 10.1002/ece3.7458

Holleley CE, O'Meally D, Sarre SD, Graves JA, Ezaz T, Matsubara K, Azad B, Zhang X, Georges A. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Holmquist G, Dancis BM. 1984. A general model of karyotype evolution. Genetica 52: 151-163.

Hooper DM, Griffith SC, Price TD. 2019. Sex chromosome inversions enforce reproductive isolation across an avian hybrid zone. Mol. Ecol. 28: 1246-1262. doi: 10.1111/mec.14874

Hutchinson MN, Donnellan SC. 1992. Taxonomy and genetic variation in the Australian lizards of the genus *Pseudemoia* (Scincidae: Lygosominae). J. Nat. Hist. 26: 215-264. doi: 10.1080/00222939200770091

Iannucci A, Altmanova M, Ciofi C, Ferguson-Smith M, Milan M, Pereira JC, Pether J, Rehak I, Rovatsos M, Stanyon R, Velensky P, Rab P, Kratochvil L, Pokorna MJ. 2019. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). Heredity 123: 215-227. doi: 10.1038/s41437-018-0179-6

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J. Evol. Biol. 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Johnson NA, Lachance J. 2012. The genetics of sex chromosomes: evolution and implications for hybrid incompatibility. Ann. N.Y. Acad. Sci. 1256: E1-E22. doi: 10.1111/j.1749-6632.2012.06748.x

Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H, Caig V, Heller-Uszynska K, Jaccoud D, Hopper C, Aschenbrenner-Kilian M, Evers M, Peng K, Cayla C, Hok P, Uszynski G. 2012. Diversity Arrays Technology: a generic genome profiling technology on open platforms. In: Pompanon F, Bonin A, editors. Data Production and Analysis in Population Genomics: Methods and Protocols. Totowa, NJ: Humana Press. p. 67-89 http://dx.doi.org/10.1007/978-1-61779-870-2_5.

Kumar S, Stecher G, Suleski M, Hedges SB. 2017. TimeTree: a resource for timelines, timetrees, and divergence times. Mol. Biol. Evol. 34: 1812-1819. doi: 10.1093/molbev/msx116.

Lande R, Seehausen O, van Alphen JJ. 2001. Mechanisms of rapid sympatric speciation by sex reversal and sexual selection in cichlid fish. Genetica 112-113: 435-443. doi: 10.1023/a:1013379521338

Madden T. 2013. The BLAST sequence analysis tool. In: McEntyre J, Ostell J, editors. The NCBI Handbook. Bethesda (MD): National Center for Biotechnology Information (US) https://www.ncbi.nlm.nih.gov/books/.

Mank JE. 2012. Small but mighty: the evolutionary dynamics of W and Y sex chromosomes. Chromosome Res. 20: 21-33. doi: 10.1007/s10577-011-9251-2

Matsubara K, Knopp T, Sarre SD, Georges A, Ezaz T. 2013. Karyotypic analysis and FISH mapping of microsatellite motifs reveal highly differentiated XX/XY sex chromosomes in the pink-tailed worm-lizard (*Aprasia parapulchella*, Pygopodidae, Squamata). Mol. Cytogenet. 6: 60. doi: 10.1186/1755-8166-6-60

Matsubara K, O'Meally D, Azad B, Georges A, Sarre SD, Graves JA, Matsuda Y, Ezaz T. 2016. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. Chromosoma 125: 111-123. doi: 10.1007/s00412-015-0531-z

Matsubara K, Uno Y, Srikulnath K, Matsuda Y, Miller E, Olsson M. 2015. No interstitial telomeres on autosomes but remarkable amplification of telomeric repeats on the W sex chromosome in the sand lizard (*Lacerta agilis*). J. Hered. 106: 753-757. doi: 10.1093/jhered/esv083

Meisel RP. 2020. Evolution of sex determination and sex chromosomes: a novel alternative paradigm. BioEssays 42: 1900212. doi: 10.1002/bies.201900212

Ming R, Bendahmane A, Renner SS. 2011. Sex chromosomes in land plants. Annu. Rev. Plant Biol. 62: 485-514. doi: 10.1146/annurev-arplant-042110-103914

Chromorep: A reptiles chromosomes database [Internet]. 2005. date accessed October 2020]. Available from http://chromorep.univpm.it.

Patawang I, Chuaynkern Y, Supanuam P, Maneechot N, Pinthong K, Tanomtong A. 2018. Cytogenetics of the skinks (Reptilia, Scincidae) from Thailand; IV: newly investigated karyotypic features of *Lygosoma quadrupes* and *Scincella melanosticta*. Caryologia 71: 29-34. doi: 10.1080/00087114.2017.1402249

Payseur BA, Presgraves DC, Filatov DA. 2018. Sex chromosomes and speciation. Mol. Ecol. 27: 3745-3748. doi: 10.1111/mec.14828

Pen I, Uller T, Feldmeyer B, Harts A, While GM, Wapstra E. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Pennell MW, Kirkpatrick M, Otto SP, Vamosi JC, Peichel CL, Valenzuela N, Kitano J. 2015. Y fuse? Sex chromosome fusions in fishes and reptiles. PLoS Genet. 11. doi: 10.1371/journal.pgen.1005237

Pennell MW, Mank JE, Peichel CL. 2018. Transitions in sex determination and sex chromosomes across vertebrate species. Mol. Ecol. 27: 3950-3963. doi: 10.1111/mec.14540

Piskurek O, Austin CC, Okada N. 2006. Sauria SINEs: Novel short interspersed retroposable elements that are widespread in reptile genomes. J. Mol. Evol. 62: 630-644. doi: 10.1007/s00239-005-0201-5

Pokorna M, Kratochvil L. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool. J. Linn. Soc. 156: 168-183.

Ponnikas S, Sigeman H, Abbott JK, Hansson B. 2018. Why do sex chromosomes stop recombining? Trends Genet. 34: 492-503. doi: https://doi.org/10.1016/j.tig.2018.04.001

Pyron RA, Burbrink FT, Wiens JJ. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol. Biol. 13: 93. doi: 10.1186/1471-2148-13-93

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JA. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, Sarre SD, Ezaz T, Graves JA, Georges A. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol. Lett. 7: 443-448. doi: 10.1098/rsbl.2010.1126

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. Biol. Lett. 4: 176-178. doi: 10.1098/rsbl.2007.0583

Reichwald K, Petzold A, Koch P, Downie BR, Hartmann N, Pietsch S, Baumgart M, Chalopin D, Felder M, Bens M, Sahm A, Szafranski K, Taudien S, Groth M, Arisi I, Weise A, Bhatt SS, Sharma V, Kraus JM, Schmid F, Priebe S, Liehr T, Görlach M, Than ME, Hiller M, Kestler HA, Volff J, Schartl M, Cellerino A, Englert C, Platzer M. 2015. Insights into sex chromosome evolution and aging from the genome of a short-lived fish. Cell 163: 1527-1538. doi: https://doi.org/10.1016/j.cell.2015.10.071

Robert KA, Thompson MB. 2001. Sex determination. Viviparous lizard selects sex of embryos. Nature 412: 698-699. doi: 10.1038/35089135

Rovatsos M, Altmanova M, Pokorna M, Kratochvil L. 2014. Conserved sex chromosomes across adaptively radiated *Anolis* lizards. Evolution 68: 2079-2085. doi: 10.1111/evo.12357

Rovatsos M, Vukic J, Kratochvil L. 2016. Mammalian X homolog acts as sex chromosome in lacertid lizards. Heredity 117: 8-13. doi: 10.1038/hdy.2016.18

Shams F, Dyer F, Thompson R, Duncan RP, Thiem JD, Majtánová Z, Ezaz T. 2019. Karyotypes and sex chromosomes in two Australian native freshwater fishes, golden perch (*Macquaria ambigua*) and Murray cod (*Maccullochella peelii*) (Percichthyidae). Int. J. Mol. Sci. 20: 4244.

Shine R, Elphick MJ, Donnellan S. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol. Lett. 5: 486-489. doi: 10.1046/j.1461-0248.2002.00351.x

Sigeman H, Ponnikas S, Chauhan P, Dierickx E, Brooke MD, Hansson B. 2019. Repeated sex chromosome evolution in vertebrates supported by expanded avian sex chromosomes. Proc. Royal Soc. 286. doi: 10.1098/rspb.2019.2051

Srikulnath K, Azad B, Singchat W, Ezaz T. 2019. Distribution and amplification of interstitial telomeric sequences (ITSs) in Australian dragon lizards support frequent chromosome fusions in Iguania. PLOS One 14. doi: 10.1371/journal.pone.0212683

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75: 304-306.

Uller T, Pen I, Wapstra E, Beukeboom LW, Komdeur J. 2007. The evolution of sex ratios and sex-determining systems. Trends Ecol. Evol. 22: 292-297. doi: 10.1016/j.tree.2007.03.008

Valenzuela N, Adams DC, Janzen FJ. 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am. Nat. 161: 676-683. doi: 10.1086/368292

Vicoso B, Kaiser VB, Bachtrog D. 2013. Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. Proc. Natl. Acad. Sci. U. S. A. 110: 6453-6458. doi: 10.1073/pnas.1217027110

Wapstra E, Olsson M, Shine R, Edwards A, Swain R, Joss JM. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. Biol. Lett. 271 Suppl 4: S230-232. doi: 10.1098/rsbl.2003.0152

Wapstra E, Swain R, Jones SM, O'Reilly J. 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata : Scincidae). Aust. J. Zool. 47: 539-550. doi: 10.1071/zo99038

Wapstra E, Uller T, Sinn DL, Olsson M, Mazurek K, Joss J, Shine R. 2009. Climate effects on offspring sex ratio in a viviparous lizard. J. Anim. Ecol. 78: 84-90. doi: 10.1111/j.1365-2656.2008.01470.x

Waters PD, Duffy B, Frost CJ, Delbridge ML, Graves JA. 2001. The human Y chromosome derives largely from a single autosomal region added to the sex chromosomes 80-130 million years ago. Cytogenet. Genome Res. 92: 74-79. doi: 10.1159/000056872

Supplementary data

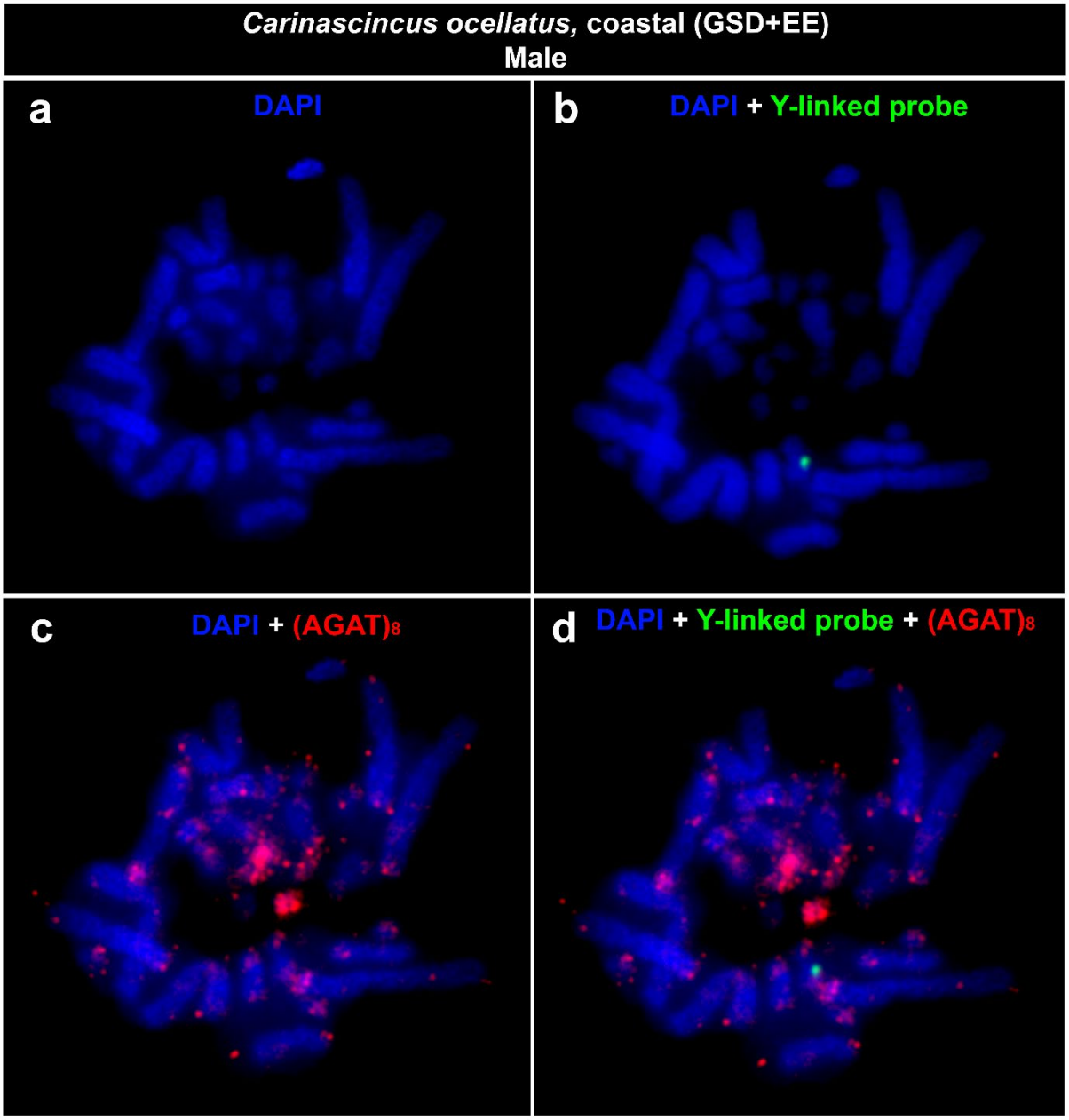

Figure S1: Sequential FISH with Y-linked probe set and $(AGAT)_8$ probe in low elevation (GSD+EE) population of *C. ocellatus*. a) DAPI stained metaphase spread of male *C. ocellatus*; b) FISH with Y-linked probe set (Pseudocoloured image) on same metaphase; c) FISH with $(AGAT)_8$ microsatellite probe on same metaphase; d) superimposed image of all layers (DAPI, signals from Y-linked probe set and signals from $(AGAT)_8$).

Supplementary Table 1. Custom Y-linked probe set sequences for *Carinascincus ocellatus* GSD and GSD+EE populations and homologs of our markers with publicly available sequences from vertebrates from NCBI BLAST (Madden 2013).

Locus ID	Sequence	homology	E value
15694756	TGCAGTCATGTGGCCGGTATGCCACATATATGCCAAG GTGAACAAAACGCTGTTGCCTTCCAACCAAAG	*Elaphe dione* clone ela2 Squam-1 SINE repeat sequence.	2.34×10^{-4}
15694756	TGCAGTCATGTGGCCGGTATGCCACATATATGCCAAG GTGAACAAAACGCTGTTGCCTTCCAACCAAAG	*Azemiops feae* clone AFE-4 SINE, complete sequence	8.17×10^{-4}
15705269	TGCAGGACACTGGCAGGGCCAGAGGGTGCCCCAGCA TCCCTCACCTGGG	NA	NA
15698539	TGCAGAAAAAGGGGTTGTGGCTATCAATTAAATTTTT ACCTATATAAATTGCTTACCCACTTTG	NA	NA
15701508	TGCAGGTAAGTCTCACAGTGGGTGGCAGAGCTAGAG GGGGACACAGTAGAGAACGTTGGAGAGGGG	NA	NA
15727479	TGCAGCCGCTACAGGGAGACTGAGGGGGGATCATTTC CAGCCAGG	NA	NA
15704137	TGCAGGAAACTTATGTCAACTCTACAGGAAATGGAAG GCGAGACCACAGTTGATTTGG	NA	NA
15694656	TGCAGCTGTTACTGGTTGTCAGGGACTCCAGGCACCT CAGCTGTTTAGTGTGATCTGAGGCAGGTCTC	NA	NA
15698519	TGCAGTTATGCAGGAAGCAGCCATTCCTGTGTCTGGC TCTTGGTTCACCAGGCCCACTTTGTCTGCACT	NA	NA
15704111	TGCAGCATTTCTGAGCCCAGCTCTGGGCGTGCGCACT GGGGACAATGAACGCGCCAGTGCTGAGTGCA	NA	NA
15712147	TGCAGCAAAAGCCTCAAACTTGTGCTGGATATAGCGC AAGCGG	NA	NA
15694951	TGCAGGCTGCTCTGGAAATGGCTCTTCTCGGTGTGCA GAGCTCCTGTG	NA	NA
15706148	TGCAGAAAGGGTGAAAACGTTCCTTTATCCATTCAGT G	NA	NA

15694660	TGCAGCTTCCTTCTCCCCATTGCTGAAGATCAAGGGATGCTCTGTAGCATCAT CCCATGCAGCACAGAG	NA	NA
15694518	TGCAGCTTCAAATCTCCAGGCCACTTGGCCTAGTTTTTTTAACAGCTTCCAGCTGCATCTAAAAGGGGA	NA	NA
15694646	TGCAGCTCAAGAACTCTGCCCAGTTTTCCCCCTTAAACAGTGCGGTCTGAGCATAGTTTCCCCTTTTAG	NA	NA
15694818	TGCAGTTTTGGACTGTTGCGAGGAGGGGTTTACAATAGTGGAAGAAACTTCTGCTATTGTAAATTGGCT	NA	NA
15700798	TGCAGGACTTAAAAGCTGCATATGAAGTGTGCATCTTAGGAAGTAAATTTTGCTGAACCTCAGCAGGG	NA	NA
15704112	TGCAGCCAAGGCTGTAGCCATGAGGTGGGGGAGGGACTTCGTCCTAGTCCCGCCTCCATGTAGATTCC	NA	NA
15725981	TGCAGGGAGGGAACAGGGATTGGGCTGCAATCCTGTTGTAAGAGAGCACTATGAACACAATCCTAAG	NA	NA
15694991	TGCAGTACTTTCCTGGAAAGAAGCCCTATGTCTAAGTGCATAGTG	NA	NA
15694717	TGCAGGGTTTGGCTCTACTGGGGTGAATGGCAAGTGGGGCTCTAGAGTGATGAAAGGAAGTTCTTTGTA	NA	NA
15694757	TGCAGTCCAGATGAAAGCTGGGCAGGCAATGGAAGAGCCTTATCCTCTAGGCCAGGTG	NA	NA
15694779	TGCAGTGACCAGCAGTGGCGTAGCTAGATGAGGGTGCAAAGCACTGTTTTGCAGGAAACTTCACCGCAG	NA	NA
15694858	TGCAGATCTAAAACACCTAACCTTTAAGACGGTCTTTCTAATTTCAGCCACATCGG	NA	NA
15694872	TGCAGCAATCTAGGAACTCTGTTTAAGAACGATTGGCAGCGTG	NA	NA
15694881	TGCAGCAGCCATTGTAATTTGAATGTGCGCCGTCCGGATTACATAGCGCGCATG	NA	NA
15694946	TGCAGGCCTCCCAGGATCTGGAGCAGGCATACTAGCAAGAGGAATTTCTGCCGG	NA	NA

15698495	TGCAGGTATATCTCATTAAAAATGCCAGCCCATAATATTGATTGTATGCCTGTCAGTCAAATGAGTAAG	NA	NA
15701619	TGCAGGTGCACGAAGTCCTTTGGGGAGAGTCATTCATTAGTATTGAATGACTGGATTGACCTTCCACA	NA	NA
15704127	TGCAGCGTGTCAACCCCCTCTGGCGCGTCACCCTCACCCGTGCCAGTGGAGAGGACTGCTCCTCAACTG	NA	NA
15717728	TGCAGGGCTTGGGCTGCAATCCTGTTGTAAGAGAGCACTATGAACACAATCCTAAG	NA	NA

Chapter Four

Sex reversal explains some, but not all, climate mediated sex ratio variation within a viviparous reptile.

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Abstract

Sex determination directs development as male or female. Evolutionary transitions in sex determination have occurred frequently yet understanding how these transitions occur remains a major challenge in evolutionary biology. Here we explore sex reversal as a mechanism responsible for evolutionary transitions in sex determination using the only known example of a viviparous reptile with intraspecific variation in sex determination, *Carinascincus ocellatus*. Long-term field and experimental data shows that in a high elevation population, sex ratios do not deviate from parity regardless of developmental temperature, however, sex ratios in a low elevation population are male biased in cool temperatures and female biased in warm. We investigated the links between developmental temperature, sex reversal and the sex ratio. We found the occurrence of sex reversed (XX) males is associated with cool developmental temperatures and partially explains population-specific sex ratio response to temperature. We discuss the consequences of sex reversal in these populations and suggest avenues for further research.

Key words: Sex reversal; Sex determination; Sex ratio; *Niveoscincus*; GSD; TSD

Introduction

Sex determination occurs via a number of different mechanisms (Bachtrog et al. 2014). In vertebrates, the sex of an individual is determined either by gene(s) (genetic sex determination; GSD) or by the environment experienced during development (mostly temperature; TSD). In several systems, however, genes and the environment can interact to mediate sexual development (Quinn et al. 2007; Sarre et al. 2004; Shine et al. 2002; Valenzuela et al. 2003). When this occurs, temperature can override the genetic signal for sex determination resulting in a departure from the 1:1 sex ratios expected under GSD (Fisher 1930). Mechanistic models suggest that this occurs when the dosage of a gene that determines sex is temperature sensitive (Quinn et al. 2007; Quinn et al. 2011). Biased sex ratios then result when the homogametic sex (XX females or ZZ males), despite double dosage of a gene product, does not reach the required threshold for the development of sexual phenotype because of the temperature sensitivity of the gene product. Sex ratio biases are also possible when shifts occur in the threshold for sexual phenotype such that a single copy of a dosage dependent sex determining gene can now achieve this new threshold (Quinn et al. 2011). Irrespective of the direction of these effects, development is diverted down the alternate pathway (Quinn et al. 2007) and these processes result in individuals with a mismatch between sexual genotype (as determined by sex chromosomes) and sexual phenotype (as determined by the presence of primary and or secondary sexual characteristics), an outcome known as sex reversal (Holleley et al. 2016; Sarre et al. 2004).

Sex reversed individuals have been documented in a number of vertebrate species including fish (Nivelle et al. 2019; Shao et al. 2014), amphibians (Alho et al. 2010) and reptiles (Holleley et al. 2015; Quinn et al. 2007; Shine et al. 2002). Importantly, the presence of such individuals can have significant downstream ramifications for both the evolutionary and ecological trajectory of a population. For example, temperature-induced sex reversal of the

homogametic sex (e.g., ZZ genotype reversed to female phenotype) leads to mating between two homogametic individuals (e.g., a ZZ sex reversed female and a ZZ normal male). This results in the production of exclusively homogametic offspring. The sex of these offspring is determined by temperature because the sex-specific chromosome (the W chromosome in this example) is absent. Thermosensitivity is therefore a likely mechanism underlying evolutionary transitions in sex determination (Quinn et al. 2011; Sarre et al. 2011) because systems in which it occurs can transition from GSD with sex chromosomes to TSD with no sex chromosomes. This poses a risk to species with thermosensitivity in sex determination as climates warm, because maladaptive sex ratio biases are likely (Boyle et al. 2014; Sinervo et al. 2010) and loss of the sex determining chromosome can occur rapidly (Holleley et al. 2015).

Sex reversal of the heterogametic sex (ZW or XY) occurs in frogs (Rodrigues et al. 2018) and fish (Cui et al. 2018) and explains the rapid turnover in sex chromosomes observed across many vertebrate clades (Perrin 2009). Sex reversal of heterogametic individuals via temperature influences on sex determining gene products are theoretically possible in reptiles under the Quinn et al. (2011) dosage model, however, sex reversals of this type have not been reported in reptiles. Despite that we can understand and predict some of the consequences of sex reversal in wild populations, the frequency of evolutionary transitions in sex determination among reptiles, particularly lizards (Janzen and Phillips 2006; Pokorna and Kratochvil 2009) suggests sex reversals may occur to a larger extent than currently understood (Holleley et al. 2016). Long term field studies on closely related taxa displaying alternate forms of sex determination will allow greater understanding of the role of sex reversal in sex determination transitions and its ecological and evolutionary consequences to species.

The viviparous spotted snow skink, *Carinascincus ocellatus* (formerly *Niveoscincus*), provides an outstanding opportunity to explore the proximate and ultimate factors underpinning intraspecific lability in sex determination. Previous field-based, laboratory and theoretical work on this species have identified divergence in sex determining systems (Pen et al. 2010) which is rarely observed in amniotes, although has been observed in amphibians (Miura 2008) and fish (Kitano et al. 2009; Conover and Heins 1987). Populations at the lower and upper elevational extremes of the *C. ocellatus* range both possess sex-linked genetic sequence supporting XY heterogamety (Hill et al. 2018). Linkage between these sex-linked loci is greater in the high elevation population and sex chromosomes have more repeat and heterochromatin accumulation (Hill et al. 2018; Hill et al. 2021a). However long – term field and laboratory data show that while sex ratios at high elevation do not deviate from parity irrespective of seasonal temperature, indicative of GSD, sex ratios correlate with temperature at low altitude suggesting thermosensitivity in sex determination (Pen et al. 2010). Specifically, at low altitude, long term field studies show that sex ratios are female biased in warm seasons and male biased in cool seasons (Cunningham et al. 2017; Wapstra et al. 2009). An adaptive explanation for population divergence in sex ratio response to temperature is that the production of males or females is favoured at different low elevation temperatures because the concomitant variation in the length of the growing season has sex-specific fitness benefits. Specifically, warm seasons, and thus early birth, favour females at low elevations because birthdate strongly predicts the onset of maturity and thus reproductive output for females (Pen et al. 2010). The shorter reproductive season at high elevations means there are no benefits of early birth to either sex and sex ratios remain balanced. Despite our long-term field studies (Cunningham et al. 2017; Wapstra et al. 2004; Wapstra et al. 2009) informing a theoretical model (Pen et al. 2010) that explains this divergence, a complete picture of the mechanisms underpinning this transition remains elusive. Sex-specific

mortality has previously been ruled out as an unlikely mechanism behind sex ratio bias (Pen et al. 2010; Wapstra et al. 2004), which leaves sex reversal as a potential likely candidate (Holleley et al. 2016).

Here, we test whether the *C. ocellatus* sex ratio responses to temperature involves sex reversal of offspring. We compare the phenotypic and genetic sex of offspring from high and low elevations over a range of developmental temperatures. We tested for sex reversal using two experimental approaches. First we used an experimental protocol we have used previously to mimic basking opportunities across the extremes of the *C. ocellatus* range (Cadby et al. 2014; Gruber et al. 2018; Wapstra 2000; Wapstra et al. 2010), allowing developmental temperatures to be potentially regulated by basking behaviour. This protocol has previously reproduced sex ratios that are biased towards males under reduced basking and towards females under extended basking opportunities in low elevation *C. ocellatus* and at parity in high elevation *C. ocellatus* (Pen et al. 2010; Wapstra et al. 2004). Second, we complemented this with an approach in which we exposed females to controlled temperatures, restricting female opportunity to bask. By holding females at constant temperatures, we removed their ability to behaviourally manipulate their offspring's developmental environment thus mimicking experiments with oviparous species (e.g., *P. vitticeps*, Quinn et al. 2007; *B. duperreyi*, Shine et al. 2002). This allowed us to explore specific physiological responses to temperature with respect to sex determination by removing inter-individual variation in female basking frequency and body temperature (Cunningham et al. 2020). We predicted that sex reversal will occur in a manner consistent with long term sex ratio responses to temperature in *C. ocellatus* and that warmer temperatures would produce XY females and cooler temperatures would produce XX males in low elevation but not high elevation *C. ocellatus* where we expect genotypic sex to be concordant with phenotypic sex.

Materials and Methods

Study species and study sites

Carinascincus ocellatus is a small viviparous skink endemic to Tasmania. We studied populations representing the climatic extremes of this species' range from low elevation, warm coastal (42 34'S, 147 52' E; elevation 50 m) and high elevation, cold sub-alpine (41 51'S, 146 34'E; elevation 1200 m) regions. Long term data on these populations consistently documents their divergent sex determination systems: sex ratios correlate with ambient temperature in the low elevation, but not the high elevation population where they remain at parity (Cunningham et al. 2017; Wapstra et al. 2009). Reproduction follows a similar pattern across elevations and females reproduce annually (Wapstra et al. 1999). Pregnant females were collected shortly after ovulation (ovulation dates are 1st October and 15th October at low and high elevations, respectively; Wapstra et al. 1999). Females were allocated to either a 'thermoregulation' or a 'no thermoregulation' experiment which differed with respect to whether a radiant heat source was used, or temperature was regulated by an incubator (Table 1, see also below).

Table 1. Number of gravid high elevation and low elevation females of *Carinascincus ocellatus* assigned to experimental treatments.

Population	No Thermoregulation (incubator)			Thermoregulation (heat lamp)	
	High (33.0°C)	Med (29.5°C)	Low (26.0°C)	Long (10 hours)	Short (4 hours)
Low elevation	20	20	20	20	20
High elevation	20	20	20	20	20

In the 'no thermoregulation' experiment, females were held individually in terraria (150 X 200 X 100 mm^3), and placed in incubators and held under one of three experimental daytime

temperatures (low – 26 °C, medium – 29.5 °C, high – 33 °C; 8 am – 4 pm). These temperatures were chosen because they span the preferred body temperature of gravid *C. ocellatus* in the wild (Cadby et al. 2014). Temperature was lowered to 10 °C for the remaining 16 hours of the 24 hour period across all treatments to approximate ambient overnight temperatures. Females were held under LED strip lighting (14 hours light:10 hours dark) and UV lighting. To avoid positional effects, females were randomly shuffled within incubators three times a week and treatments were rotated through three incubators fortnightly. The 'thermoregulation' experiment was conducted as per Wapstra (2000). Briefly, females were held individually in terraria (200 X 300 X 100 mm^3), and provided with either 4 (short) or 10 (long) hours exposure to a radiant heat source per day which approximates the difference in basking opportunity experienced across altitudes in the wild (Wapstra et al. 1999). The ambient temperature fell to approximately 10 °C at night. Terraria were maintained under fluorescent tube lighting and UV lighting (14 hours light:10 hours dark) as for the 'no thermoregulation' treatment. Females were randomly re-positioned within the room three times a week. All females were supplied with water *ad libitum* and were offered mealworms and fruit supplemented with vitamins three times weekly.

Towards the end of gestation terraria were checked for offspring. Offspring were weighed at birth and their gestation length was recorded. Phenotypic sex was assessed via hemipene eversion at least twice: initially, two weeks after birth then two weeks after first sexing. Each sexing was performed by the same investigator (EW) and was blind to treatment and initial phenotypic sex. If initial and subsequent phenotypic sex differed, offspring sex was determined two weeks after the second sexing (2.3% of offspring: 'no thermoregulation' n = 6 low elevation, 0 high elevation; 'thermoregulation' n = 3 low elevation, 1 high elevation). Prior to release, tail tip samples were taken from offspring to genotype individuals for sex.

Genetic sex was assigned using a suite of SNP loci exhibiting sex linkage in either or both sex determining systems of *C. ocellatus* (n_{loci} = 45, $n_{offspring}$ = 428; Hill et al. 2018). Tail samples were sent to Diversity Arrays technology (https://www.diversityarrays.com; Kilian et al. 2012) for DNA extraction and targeted genotyping. Where a mismatch occurred between phenotypic and genetic sex, the individual was deemed to be sex reversed.

Statistical analysis

To confirm that offspring developed under different thermal regimes, we fit a linear model (lm) with log transformed gestation length as the response variable and treatment and population and their interaction as fixed factors for both the 'no thermoregulation' and 'thermoregulation' experiments. We analysed whether cohort phenotypic sex ratio deviated for any given treatment from parity using Pearson's chi-squared analyses on counts of male and female offspring. To test for differences between treatments in the extent of sex reversal, we fit a generalized linear mixed model (GLMM) with binomial error distribution using a type II Walds χ^2 test with offspring sex status (i.e., whether genotypic and phenotypic sex matched) as the dependent variable, and treatment, population and their interaction as fixed factors. We ran this for each experiment (thermoregulation and no thermoregulation) separately. We included maternal identity as a random effect, given mean clutch sizes of 1.8 and 3.1 at low and high elevation respectively (Atkins et al. 2007). Finally, because females across both populations exhibit the same mean reaction norm with respect to the thermal effects on gestation (Cunningham et al. 2020) we could combine the 'no thermoregulation' and 'thermoregulation' experiments to examine whether mean gestation length (as a proxy for the thermal environment) covaried more broadly with the proportion of sex reversed offspring from a particular experimental treatment. We achieved this by using logistic regression (glm) with a binomial distribution using a type II Walds χ^2 test with mean gestation length and population as fixed factors and offspring number per treatment size as a

weighting factor. Analyses were conducted using R (R Core Team 2017) using the 'lme4' (glmm, glm; Bates et al. 2015), 'stats' (lm; R Core Team 2017) and 'car' (type II Wald's test; Fox and Weisberg 2019) packages. Offspring whose phenotypic sex could not be assessed due to mortality were excluded from sex reversal analysis.

Results

There were significant differences in gestation length between individuals under the different thermal conditions and between individuals from the two populations. These effects were consistent across both experiments (thermoregulation and no thermoregulation). Specifically, individuals in the treatments with more restricted thermal opportunities had longer gestation lengths than those with prolonged thermal opportunities. Gestation length was also longer in individuals from the low elevation population compared to the high elevation population (Figure 1, Table 2). We found no significant interaction between these two main effects in either the 'no thermoregulation' or 'thermoregulation' experiment (Table 2).

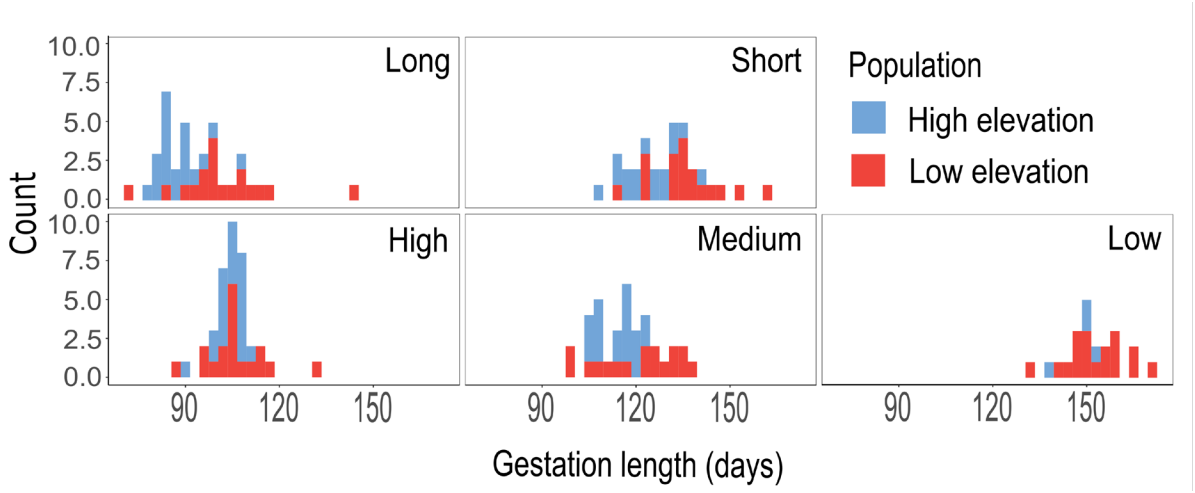

Figure 1. Histogram of gestation lengths for high elevation (blue) and low elevation (red) *Carinascincus ocellatus* females held in 'thermoregulation' (Long, Short) and 'no thermoregulation' (High, Medium, Low) experiments. Individual treatments within each experiment are indicated.

Table 2. Summary statistics from linear models testing the affect of treatment and population and their interaction on gestation length in high and low elevation populations of *Carinascincus ocellatus* with divergent sex determination.

No thermoregulation experiment		
Treatment	Population	Interaction
$F_{(2,1.65)}$=148.9 p <0.001	$F_{(1,0.02)}$=3.9 p=0.05	$F_{(2,0.005)}$=0.46 p=0.63
Thermoregulation experiment		
Treatment	Population	Interaction
$F_{(1,1.83)}$=181.5 p <0.001	$F_{(1,0.25)}$=25.1 p<0.001	$F_{(1,0.014)}$=1.4 p=0.23

We identified a substantial proportion of sex reversed *C. ocellatus* males (phenotypic males with an XX genotype) in both the 'no thermoregulation' and 'thermoregulation' experiments. Sex reversed individuals were identified in both populations (Table 3). No phenotypic females with an XY genotype were observed. Sex reversed individuals were more commonly observed in the cool treatment in both the 'no thermoregulation' and 'thermoregulation experiments (Table 3). In the 'no thermoregulation' experiment, offspring had a 21.5 and 5.9 times higher odds of being sex reversed relative to the high treatment (33.0°C) when under

the low (26°C) and medium (29.5°C) treatments respectively. In the 'thermoregulation' experiment, offspring had a 6.5 times higher odds of being sex reversed when under the short (4h) treatment relative to the long (10 h) treatment. These effects of the developmental environment on sex reversal were consistent across both populations (e.g., there was no interaction between treatment and population; Table 3).

Table 3. Number of offspring from gravid low elevation and high elevation *Carinascincus ocellatus* females held in 'no thermoregulation' or 'thermoregulation' experiments and number that were found to be sex reversed (XX males). Numbers in parentheses are the total number of offspring born (excludes mortalities). Summary statistics from type II Wald's test of GLMM testing the affect of treatment and population on sex reversal are included.

no thermoregulation experiment	Low elevation		High elevation	
	No. offspring	No. sex reversed	No. offspring	No. sex reversed
High (33.0°C)	48 (48)	1	60 (60)	0
Med (29.5°C)	41 (42)	2	57 (60)	3
Low (26.0°C)	31 (35)	6	11 (12)	1
Treatment $\chi^2_{(3)}$=8.5 p=0.04	Population $\chi^2_{(2)}$=0.90 p=0.64		Interaction $\chi^2_{(2)}$=0.61 p=0.74	
thermoregulation experiment	Low elevation		High elevation	
	No. offspring	No. sex reversed	No. offspring	No. sex reversed
Long (10 h)	37 (42)	2	56 (60)	1
Short (4 h)	37 (43)	8	45 (47)	7
Treatment $\chi^2_{(1)}$=7.3 p=0.007	Population $\chi^2_{(1)}$=1.1 p=0.30		Interaction $\chi^2_{(1)}$=0.26 p=0.61	

We then examined the links between gestation temperature and the proportion of sex reversals at the population level by regressing the proportion of sex reversals per experimental treatment and population on to that treatment/population mean gestation length (which acts as a proxy for developmental temperature). The proportion of sex reversed offspring was significantly related to mean gestation length ($\chi^2_{(1)}$=14.5, p<0.001); for every

increase in mean gestation length of one day, the odds of sex reversal increased by 5.0% (Figure 2). These effects of gestation length were consistent across both populations ($\chi^2_{(1)}$=0.29, p 0.59) with no overall difference in the extent of sex reversal between populations ($\chi^2_{(1)}$=0.06, p 0.80).

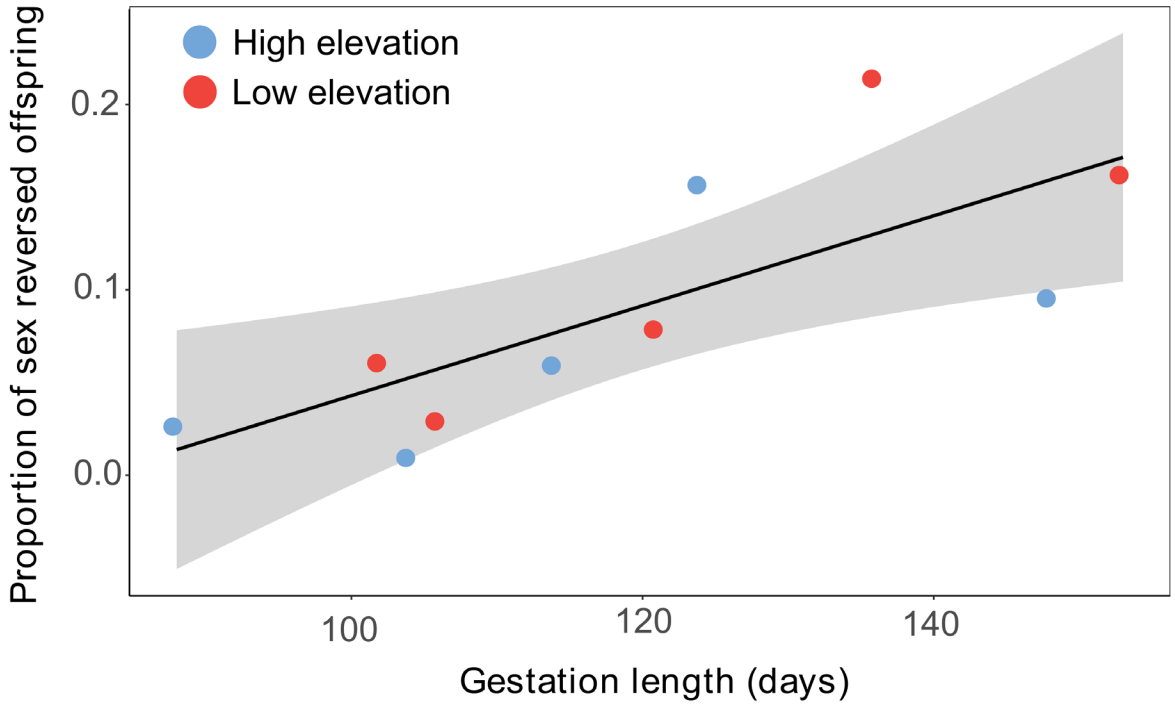

Figure 2. Relationship between gestation length (days) and the proportion of XX male (sex reversed) offspring in low elevation (red) and high elevation (blue) gravid *Carinascincus ocellatus* females held in thermal experiments.

Cohort sex ratios showed significant or borderline significant deviations from 50:50 in five treatments and ranged from 0.45 to 0.72 in the 'no thermoregulation' experiment and 0.36 to 0.59 in the 'thermoregulation' experiment (low elevation High: χ^2=0.75, p=0.39; Med: χ^2=0.38, p=0.54; Low: χ^2=3.12, p=0.08; high elevation High: χ^2=2.77, p=0.10; Med: χ^2=4.41, p=0.04; Low: χ^2=2.27, p=0.13; low elevation Long: χ^2=2.95p=0.09; Short: χ^2=0.95, p=0.33; high elevation Long: χ^2=0.86, p=0.35; Short: χ^2=1.72, p=0.19; Figure 3). This variation in treatment level sex ratios showed some congruence with the above patterns of sex reversal. Specifically, male biases were more often observed in cooler or shorter treatments

where sex reversal occurred at a higher frequency, and female biases observed in the warmer and longer treatments with fewer observed sex reversed males (Figure 3).

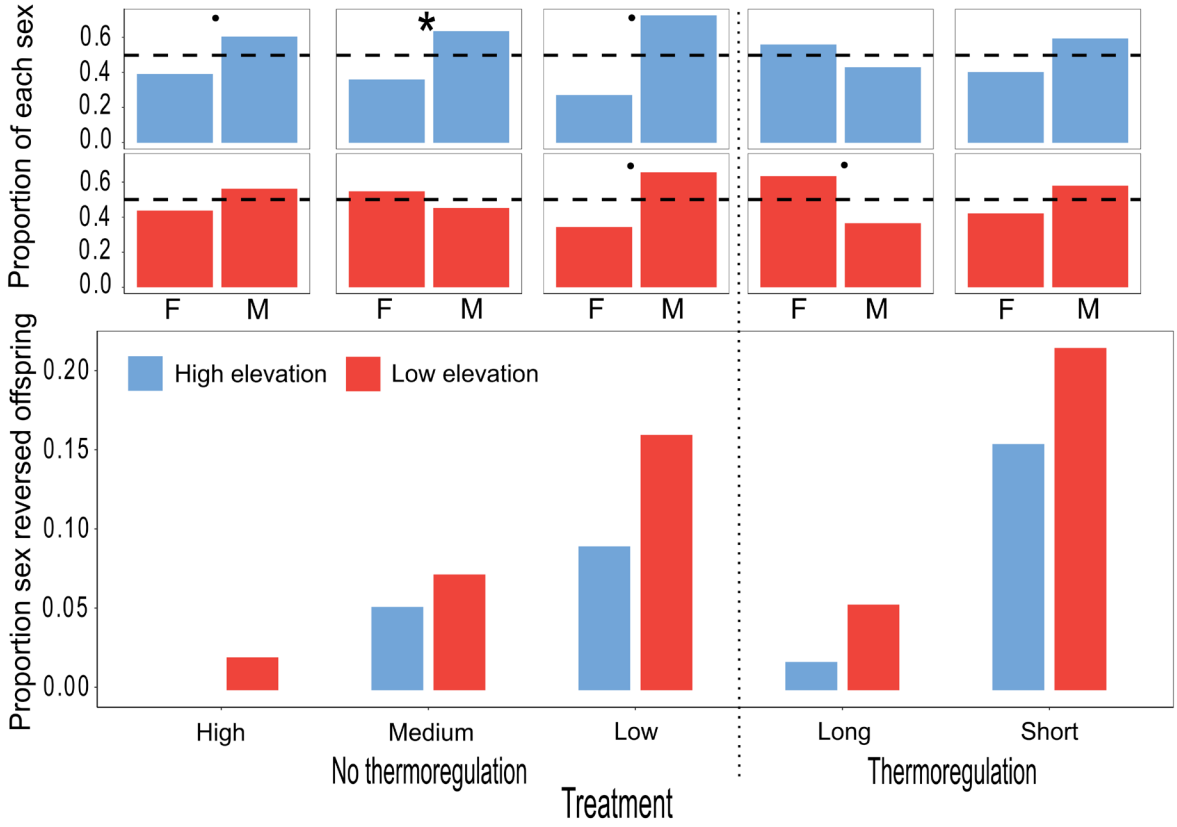

Figure 3. Proportion of sex reversed offspring (lower panel) and proportion of females and males (upper panel) born from gravid low elevation (red) and high elevation (blue) *Carinascincus ocellatus* females held in 'no thermoregulation' (High, Medium, Low) and 'thermoregulation' (Long, Short) thermal experiments. Black dashed line indicates sex ratio at 1:1. '*' indicates $p<0.05$, '.' Indicates $p<0.15$.

Discussion

Here we provide evidence that temperature-induced sex reversal occurs in *Carinascincus ocellatus*, the first report of this mechanism in a viviparous reptile. Sex reversal in *C. ocellatus* was unidirectional, with phenotypic males possessing the XX genotype but no evidence of XY females. While the proportion of sex reversed individuals was consistently higher in the low elevation population, this failed to reach significance and the proportion of sex reversals increased with decreasing temperature in both populations. We found qualitative evidence that variation in sex ratios correspond with the extent of sex reversal.

These results agree, in part, with our prediction of population-specific sex reversal in *C. ocellatus*. However, these effects do not appear to fully explain long term population-specific sex ratio responses to temperature, and we discuss the implications of this for understanding evolutionary transitions in sex determination.

The prevalence of sex reversal was significantly associated with cooler developmental temperatures and shorter basking opportunity for gravid females. This was consistent both when we examined differences in the extent of sex reversals between treatments and also broader scale variation in sex reversals as a function of gestation length (a proxy for developmental temperature; Cunningham et al. 2017; Pen et al. 2010; Wapstra et al. 2009). These results are consistent with data from our long-term field study which shows that there is an excess of males observed in cool seasons in low elevation *C. ocellatus* (Cunningham et al. 2017; Pen et al. 2010), suggesting a role for sex reversals as a mechanism responsible for male bias. These results fit with the gene dosage model of reptile sex determination proposed by Quinn et al. (2007). In this model, sexual phenotype is determined by the dosage of a sex determining gene as occurs in birds (Smith et al. 2009), rather than the presence or absence of a sex determining gene as occurs in therian mammals (Koopman et al. 1990). Evidence supporting this model of sex determination in other reptiles comes from both ZZ/ZW and XX/XY species (Quinn et al. 2007; Radder et al. 2008; Dissanayake et al. 2020). When an X-linked sex determining gene product in an XX/XY system is sensitive to temperature (e.g., through down regulation of gene transcription or denaturing of the resulting gene product), XX genotypes can fail to reach the threshold for female phenotype and are diverted down the male developmental pathway.

While our work provides evidence for sex reversal as the mechanism responsible for male biased sex ratios, our data also suggests that this mechanism operates in both populations and across a broader range of thermal regimes than we predicted. Indeed, we predicted sex

reversed individuals in frequencies consistent with observed long term sex ratio responses to temperature across altitudes in *C. ocellatus* populations (XX males in cool / short and XY females in warm / long treatments, respectively at low elevation, no sex reversal at high elevation; Cunningham et al. 2017; Pen et al. 2010). However, the observation of sex reversed males at both elevations and in warmer treatments suggests the mechanism responsible for long-term geographic divergence in sex ratio responses to climate is more complex than can be explained by a single process such as sex reversal. In addition, our results do not explain the female biased sex ratios observed in warmer seasons at low elevation. Specifically, the lack of XY reversals to female phenotype in warmer / longer treatments also suggests there is another factor contributing to biased sex ratios in *C. ocellatus* that was not immediately apparent from our manipulations of developmental temperatures and basking. Sex-specific mortality during development has been ruled out in this species (Wapstra et al. 2004). Given that sex reversal is operating in both populations, albeit to a lesser extent at high elevation, the mechanism responsible for female biases at low elevation may also be operating in both populations.

The observation of sex reversed males in both populations emphasises that the systems of sex determination in *C. ocellatus* are in the early stages of divergence and this chronology is supported by independent data (Hill et al. 2021b). Thermosensitivity of genetic sex determination is likely an ancestral state, that although maintained to present in both populations, has been modified in one relative to the other by selection for population-specific sex ratio responses to climate (Pen et al. 2010). However, sex reversal in both populations is evidence that the minor differences in sex chromosomes between the populations (Hill et al. 2018; Hill et al. 2021a) are not the only mechanism driving differences in sex ratios between the populations. The populations therefore also differ in other factors that influence sex ratios. For example, females from low elevation bask less and

achieve lower body temperatures than females from the high elevation when held under the same basking regimes (Cadby et al. 2014; Caldwell et al. 2017; Uller et al. 2011) therefore population differences in female basking behaviour might be expected to contribute to sex ratio response to temperature. Given we made efforts to control for population differences in maternal basking by holding females at constant temperatures, this suggests environmental temperature may translate to offspring developmental temperature differently in each population via female physiology. Further, clutches with sex reversed XX males also contained non-sex-reversed XX females, reflecting heritable variation in the threshold at which sex reversal occurs. Examining such within clutch/litter effects is experimentally possible with oviparous species but challenging with viviparous species.

Our selected thermal regimes may limit our ability to tease apart the intricacies of the interaction between maternal basking and physiology and offspring physiology and sex determining threshold in each population because they may represent limited thermal conditions compared to those that females experience in the wild despite that they encompassed measured gravid female preferred body temperatures (Cadby et al. 2014). A lack of female biased sex ratios in warm treatments and long basking opportunity supports this, notwithstanding that female biases have been achieved in past work (Wapstra et al. 2004) using the same long basking opportunity. However, attempts to replicate laboratory manipulations using free living animals frequently produce disparate results (Booksmythe et al. 2017; Gruber et al. 2018). The complexities of population-specific trends in sex ratio response to temperature may only become apparent with further experimentation.

The presence of sex reversals in our populations could have significant direct and indirect consequences for the ecological and evolutionary trajectory of these populations. For example, juvenile sex reversed *P. vitticeps* females exhibit a suite of phenotypic traits important to individual fitness (Li et al. 2016). Although some of these do not persist into

adulthood (Jones et al. 2020), sex reversed *P. vitticeps* females are fertile and produce more eggs per clutch than do normal ZW females (Holleley et al. 2015) thus influencing sex ratio variation in subsequent generations. Specifically, we found that sex reversals corresponded to some degree, but not completely, with variation in sex ratios which suggests that they have significant implications for the extent of sex ratio variation within and between years. However, sex determination during oviparous development is contained within the egg and there are no intra-clutch effects from circulating sex hormones. In contrast, the intrauterine environment experienced by offspring during viviparous development has a strong influence on fitness. Clutch sex ratios biased towards one sex may decrease the fitness of the minor sex because the circulating sex hormones can either masculinise females or feminise males depending on the sex ratio of the clutch (Uller 2003). This emphasises the need to understand the fitness consequences of sex reversal within the context of viviparity.

The presence of sex-reversed individuals has been suggested as a mechanism for transition from GSD to TSD. If XX male *C. ocellatus* suffer no fitness deficits and are fertile, successful mating between XX males and XX females will result in litters with all XX offspring; the sex of these offspring will be determined by temperature. If the temperature threshold at which sex reversal occurs is also evolving in this system (as it is in *P. vitticeps*; Holleley et al. 2015), divergent selection across altitudes on this sex reversal threshold could be driving the transition in sex determination by maintaining thermosensitive sex determination at low elevation, while favouring GSD at high elevation. If the frequency of XX males increases, there is the potential for a transition to TSD where all individuals are XX genotype and the Y chromosome is lost, analogous to the *P. vitticeps* system (Holleley et al. 2015; Quinn et al. 2011). However, future climates are predicted to become warmer, which in the *C. ocellatus* system whilst not necessarily eliminating XX sex reversal, will reduce its frequency in populations, making it unlikely that this system will undergo a climate

mediated transition to TSD via sex reversal. In addition, several factors including gene flow, heritable variation in the threshold for sex reversal and environmental fluctuations are likely to attenuate transitions to TSD (Schwanz et al. 2020). Further, current models based on long-term *C. ocellatus* sex ratio data suggest that sex ratios will become more female biased at low elevation under climate change, potentially promoting population growth and expansion (Cunningham et al. 2017).

Female biases in warm temperatures can be achieved without XY sex reversal, thus avoiding the costs of the YY genotypes that would result from XY females mating with XY males (Ezaz et al. 2006; Perrin 2009). For example, if the cohort genotypic sex ratio is XX biased, the phenotypic sex ratio can become biased towards males via sex reversal in cool seasons or towards females via warm developmental temperature in warm seasons. Our results support this hypothesis because although female biased sex ratios occur in warm seasons in low elevation *C. ocellatus*, all the female offspring in our study have the XX genotype, even in treatments with a slight female bias (e.g., long bask). Excess XX genotypes could occur due to several mechanisms. For example, sperm sex ratios biased towards the X chromosome would result in biased sex ratios at fertilisation, and sex reversed XX males mating in the population is also a plausible source of excess XX genotypes. Assessing XX male fitness and investigating the mechanism behind female biased sex ratios will increase our understanding of how sex reversal, sex ratios and climate interact and allow accurate predictions of the consequences of warming climate to populations. It will also further our understanding of the evolution of sex determination in XY systems and will provide insight into broader patterns of lability in sex determination across vertebrates.

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References

Alho JS, Matsuba C, Merila J. 2010. Sex reversal and primary sex ratios in the common frog (*Rana temporaria*). Mol. Ecol. 19: 1763-1773. doi: 10.1111/j.1365-294X.2010.04607.x

Atkins N, Swain R, Wapstra E, Jones SM. 2007. Late stage deferral of parturition in the viviparous lizard *Niveoscincus ocellatus* (Gray 1845): implications for offspring quality and survival. Biol. J. Linn. Soc. 90: 735-746. doi: 10.1111/j.1095-8312.2007.00770.x

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, Tree of Sex C. 2014. Sex determination: why so many ways of doing it? PLoS Biology 12: e1001899. doi: 10.1371/journal.pbio.1001899

Bates D, Machler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67: 1-48. doi: 10.18637/jss.v067.i01.

Booksmythe I, Mautz B, Davis J, Nakagawa S, Jennions MD. 2017. Facultative adjustment of the offspring sex ratio and male attractiveness: a systematic review and meta-analysis. Biol. Rev. 92: 108-134. doi: 10.1111/brv.12220

Boyle M, Hone J, Schwanz LE, Georges A. 2014. Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? Ecol. Evol. 4: 4522-4533. doi: 10.1002/ece3.1316

Cadby CD, Jones SM, Wapstra E. 2014. Geographical differences in maternal basking behaviour and offspring growth rate in a climatically widespread viviparous reptile. J. Exp. Biol. 217: 1175-1179. doi: 10.1242/jeb.089953

Caldwell AJ, While GM, Wapstra E. 2017. Plasticity of thermoregulatory behaviour in response to the thermal environment by widespread and alpine reptile species. Anim. Behav. 132: 217-227. doi: https://doi.org/10.1016/j.anbehav.2017.07.025

Conover DO, Heins SW 1987. Adaptive variation in environmental and genetic sex determination in a fish. Nature 326: 496-498. doi: 10.1038/326496a0

Cui Y, Wang W, Ma L, Jie J, Zhang Y, Wang H, Li H 2018. New locus reveals the genetic architecture of sex reversal in the Chinese tongue sole (*Cynoglossus semilaevis*). Heredity (Edinb) 121: 319-326. doi: 10.1038/s41437-018-0126-6

Cunningham GD, While GM, Olsson M, Ljungström G, Wapstra E. 2020. Degrees of change: between and within population variation in thermal reaction norms of phenology in a viviparous lizard. Ecology 101: e03136. doi: 10.1002/ecy.3136

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol. Lett. 13. doi: 10.1098/rsbl.2017.0218

Dissanayake DSB, Holleley CE, Deakin JE, Georges A 2021. High elevation increases the risk of Y chromosome loss in Alpine skink populations with sex reversal. Heredity. doi: 10.1038/s41437-021-00406-z

Ezaz T, Stiglec R, Veyrunes F, Graves JA. 2006. Relationships between vertebrate ZW and XY sex chromosome systems. Curr. Biol. 16: R736-743. doi: 10.1016/j.cub.2006.08.021

Fisher RA. 1930. The genetical theory of natural selection. New York: Dover Publications, Inc.

Fox J, Weisberg S. 2019. An R companion to applied regression. Sage, Thousand Oaks CA.

Gruber J, Cunningham GD, While GM, Wapstra E. 2018. Disentangling sex allocation in a viviparous reptile with temperature-dependent sex determination: a multifactorial approach. J. Evol. Biol. 31: 267-276. doi: 10.1111/jeb.13219

Hill PL, Burridge CP, Ezaz T, Wapstra E. 2018. Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. Genome Biol. Evol. 10: 1079-1087. doi: 10.1093/gbe/evy042

Hill P, Shams F, Burridge CP, Wapstra E, Ezaz T 2021a. Differences in homomorphic sex chromosomes are associated with population divergence in sex determination in *Carinascincus ocellatus* (Scincidae: Lygosominae). Cells 10: 291.

Hill PL, Wapstra E, Ezaz T, Burridge CP 2021b. Pleistocene divergence in the absence of gene flow among populations of a viviparous reptile with intraspecific variation in sex determination. Ecology and Evolution 00: 1-9. doi: DOI: 10.1002/ece3.7458

Holleley CE, O'Meally D, Sarre SD, Marshall Graves JA, Ezaz T, Matsubara K, Azad B, Zhang X, Georges A. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Holleley CE, Sarre SD, O'Meally D, Georges A. 2016. Sex reversal in reptiles: reproductive oddity or powerful driver of evolutionary change? Sex. Dev. 10: 279-287. doi: 10.1159/000450972

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J. Evol. Biol. 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Jones MEH, Pistevos JCA, Cooper N, Lappin AK, Georges A, Hutchinson MN, Holleley CE. 2020. Reproductive phenotype predicts adult bite-force performance in sex-reversed dragons (*Pogona vitticeps*). J. Exp. Zool. A Ecol. Integr. Physiol. Physiology 333: 252-263. doi: 10.1002/jez.2353

Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H, Caig V, Heller-Uszynska K, Jaccoud D, Hopper C, Aschenbrenner-Kilian M, Evers M, Peng K, Cayla C, Hok P, Uszynski G. 2012. Diversity Arrays Technology: a generic genome profiling technology on open platforms. In: Pompanon F, Bonin A, editors. Data Production and Analysis in Population Genomics: Methods and Protocols. Totowa, NJ: Humana Press. p. 67-89.

Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF, Absher DM, Grimwood J, Schmutz J, Myers RM, Kingsley DM, Peichel CL. 2009. A role for a neo-sex chromosome in stickleback speciation. Nature 461: 1079-1083. doi: 10.1038/nature08441

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348: 450-452. doi: 10.1038/348450a0

Li H, Holleley CE, Elphick M, Georges A, Shine R. 2016. The behavioural consequences of sex reversal in dragons. Proc. Royal Soc. 283. doi: 10.1098/rspb.2016.0217

Miura I. 2008. An evolutionary witness: the frog *Rana rugosa* underwent change of heterogametic sex from XY male to ZW female. Sex. Dev. 1: 323-331.

Nivelle R, Gennotte V, Kalala EJK, Ngoc NB, Muller M, Mélard C, Rougeot C. 2019. Temperature preference of Nile tilapia (*Oreochromis niloticus*) juveniles induces spontaneous sex reversal. PLOS One 14: e0212504. doi: 10.1371/journal.pone.0212504

Pen I, Uller T, Feldmeyer B, Harts A, While GM, Wapstra E. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Perrin N. 2009. Sex reversal: a fountain of youth for sex chromosomes? Evolution 63: 3043-3049. doi: 10.1111/j.1558-5646.2009.00837.x

Pokorna M, Kratochvil L. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles are sex chromosomes an evolutionary trap? Zool. J. Linn. Soc. 156: 168-183.

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JA. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, Sarre SD, Ezaz T, Graves JA, Georges A. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol. Lett. 7: 443-448. doi: 10.1098/rsbl.2010.1126

R Core Team. 2017. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

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. Biol Lett 4: 176-178. doi: 10.1098/rsbl.2007.0583

Rodrigues N, Studer T, Dufresnes C, Perrin N 2018. Sex-chromosome recombination in common frogs brings water to the fountain-of-youth. Molecular Biology and Evolution 35: 942-948. doi: 10.1093/molbev/msy008

Sarre SD, Ezaz T, Georges A. 2011. Transitions between sex-determining systems in reptiles and amphibians. Annu. Rev. Genomics Hum. Genet. 12: 391-406. doi: 10.1146/annurev-genom-082410-101518

Sarre SD, Georges A, Quinn A. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645. doi: 10.1002/bies.20050

Schwanz LE, Georges A, Holleley CE, Sarre SD. 2020. Climate change, sex reversal and lability of sex-determining systems. J. Evol. Biol. 33: 270-281. doi: 10.1111/jeb.13587

Shao C, Li Q, Chen S, Zhang P, Lian J, Hu Q, Sun B, Jin L, Liu S, Wang Z, Zhao H, Jin Z, Liang Z, Li Y, Zheng Q, Zhang Y, Wang J, Zhang G. 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Res. 24: 604-615. doi: 10.1101/gr.162172.113

Shine R, Elphick MJ, Donnellan S. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol. Lett. 5: 486-489. doi: 10.1046/j.1461-0248.2002.00351.x

Sinervo B, Mendez-de-la-Cruz F, Miles DB, Heulin B, Bastiaans E, Villagran-Santa Cruz M, Lara-Resendiz R, Martinez-Mendez N, Calderon-Espinosa ML, Meza-Lazaro RN, Gadsden H, Avila LJ, Morando M, De la Riva IJ, Victoriano Sepulveda P, Rocha CF, Ibarguengoytia N, Aguilar Puntriano C, Massot M, Lepetz V, Oksanen TA, Chapple DG, Bauer AM, Branch WR, Clobert J, Sites JW, Jr. 2010. Erosion of lizard diversity by climate change and altered thermal niches. Science 328: 894-899. doi: 10.1126/science.1184695

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461: 267-271. doi: 10.1038/nature08298

Uller T. 2003. Viviparity as a constraint on sex-ratio evolution. Evolution 57: 927-931. doi: 10.1554/0014-3820(2003)057[0927:Vaacos]2.0.Co;2

Uller T, While GM, Cadby CD, Harts A, O'Connor K, Pen I, Wapstra E. 2011. Altitudinal divergence in maternal thermoregulatory behaviour may be driven by differences in selection on offspring survival in a viviparous lizard. Evolution 65: 2313-2324. doi: 10.1111/j.1558-5646.2011.01303.x

Valenzuela N, Adams DC, Janzen FJ. 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am. Nat. 161: 676-683. doi: 10.1086/368292

Wapstra E. 2000. Maternal Basking Opportunity Affects Juvenile Phenotype in a Viviparous Lizard. Funct. Ecol. 14: 345-352. doi: 10.2307/2656411

Wapstra E, Olsson M, Shine R, Edwards A, Swain R, Joss JM. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. Biol. Lett. 271 Suppl 4: S230-232. doi: 10.1098/rsbl.2003.0152

Wapstra E, Swain R, Jones SM, O'Reilly J. 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata : Scincidae). Aust. J. Zool. 47: 539-550. doi: 10.1071/zo99038

Wapstra E, Uller T, Sinn DL, Olsson M, Mazurek K, Joss J, Shine R. 2009. Climate effects on offspring sex ratio in a viviparous lizard. J. Anim. Ecol. 78: 84-90. doi: 10.1111/j.1365-2656.2008.01470.x

Wapstra E, Uller T, While GM, Olsson M, Shine R. 2010. Giving offspring a head start in life: field and experimental evidence for selection on maternal basking behaviour in lizards. J. Evol. Biol. 23: 651-657. doi: 10.1111/j.1420-9101.2009.01924.x

Chapter Five

Pleistocene divergence in the absence of gene flow among populations of a viviparous reptile with intraspecific variation in sex determination.

All of the research contained within this chapter has been published as:

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Abstract

Polymorphisms can lead to speciation if there is differential mating success among conspecifics divergent for a trait. Polymorphism for sex determining system might be particularly expected to isolate gene pools, given strong selection for the production of viable males and females and the low success of heterogametic hybrids when sex chromosomes differ (Haldane's rule). Here we investigated whether populations exhibiting polymorphism for sex determination are genetically isolated, using the viviparous snow skink *Carinascincus ocellatus*. While a comparatively high elevation population has genotypic sex determination, in a lower elevation population there is an additional temperature component to sex determination. Based on 11,107 SNP markers, these populations appear genetically isolated. 'Isolation with Migration' analysis also suggests these populations diverged in the absence of gene flow, across a period encompassing multiple Pleistocene glaciations and likely greater geographic proximity of populations. However, further experiments are required to establish whether genetic isolation may be a cause or consequence of differences in sex determination. Given the influence of temperature on sex in one lineage, we also discuss the implications for the persistence of this polymorphism under climate change.

Keywords: Genetic sex determination; Temperature dependent sex determination; gene flow; Sex chromosome; *Niveoscincus*.

Introduction

Speciation occurs when lineages become reproductively isolated due to a trait polymorphism. If mating success is lower among individuals that differ for a given trait, lineages will diverge in the frequency of that trait and experience further reductions in gene flow, potentially initiating speciation. Trait polymorphisms that may particularly promote speciation include those related to mate choice (e.g., sexual dichromatism; Jenck et al. 2020; Portik et al. 2019), breeding phenology (Taylor and Friesen 2017), parity (Horreo et al. 2019), vocalisations (Campbell et al. 2019; Luo et al. 2017) and karyotype, particularly those involving chromosomes that determine sex (i.e., sex chromosomes; Bracewell et al. 2017; Faria and Navarro 2010; Kitano et al. 2009; O'Neill and O'Neill 2018; Zhang et al. 2015).

Sex determination, which directs gonadal differentiation in sexually reproducing organisms (Bachtrog et al. 2014; Hayes 1998), often has a strong chromosomal basis which is highly conserved within groups, reflecting strong selective constraint on the production of viable males and females. For example, the systems of genetic sex determination (GSD) are fixed in therian mammals and birds, represented by heterogametic XY male and ZW female chromosome systems, respectively (Graves 2006; Ohno 1967). However, sex determination is comparatively labile in reptiles (Alam et al. 2018; Janzen and Phillips 2006; Johnson Pokorna and Kratochvil 2016), where offspring sex is controlled either by genes (both male and female heterogametic systems), the environment (e.g., temperature dependant sex determination, TSD), or by a combination of genes and the environment (GSD with environmental effects, GSD + EE; Cornejo-Paramo et al. 2020; Ezaz et al. 2006; Holleley et al. 2015; Quinn et al. 2007; Radder et al. 2008; Sarre et al. 2004). Although transitions among these systems were initiated as an intraspecific polymorphism, it is unknown whether

they were accompanied by reproductive isolation. Within squamates, the family Scincidae shows evidence of conserved sex chromosomal regions between some lineages (Cornejo-Paramo et al. 2020; Dissanayake et al. 2020) in addition to temperature sensitivity in sex determination (Holleley et al. 2016). However, variation in the degree of sex chromosome differentiation, number of sex chromosomes (Ezaz et al. 2009) and system of heterogamety (Patawang et al. 2017) exists, and our understanding of the mechanisms of evolutionary transitions in sex determination and how they impact demographics remains poor.

Low mating success is expected among individuals when differences in sex determination reflect gross chromosomal differences (e.g., sex chromosome presence, composition, or system of heterogamety; Haldane 1922; Lima 2014; O'Neill and O'Neill 2018; Phillips and Edmands 2012). However, not all changes to the sex chromosomes result in incompatibilities. When genes and temperature interact to determine sex, a temperature override of the genetic sex determination signal can produce individuals whose sexual phenotype does not match their sexual genotype (known as sex reversal). This phenomenon is occurring in wild populations of the central bearded dragon, *Pogona vitticeps*, which has a thermosensitive ZW/ ZZ system of sex determination, resulting in the production of females (normally ZW) with a male genotype (ZZ) (Holleley et al. 2015). While the W chromosome has been lost in these sex reversed females, and the thermal threshold for sex reversal is evolving in this system (Holleley et al. 2015; Quinn et al. 2011), the Z chromosomes remain homologous and ZZ males can successfully breed with ZZ females under laboratory conditions (Holleley et al. 2015). For a transition in sex determining system to lead to postzygotic incompatibilities via hybrid inviability or sterility, the transition must involve changes to the sex chromosomes such that they show deleterious interactions on a hybrid background (Haldane 1922).

The viviparous Tasmanian spotted snow skink, *Carinascincus ocellatus* (formerly *Niveoscincus*), is an extraordinary example of a species exhibiting incipient divergence in sex

determination (Cunningham et al. 2017; Pen et al. 2010; Wapstra et al. 2004). This species is widely distributed across a broad altitudinal and climatic range in Tasmania, from sea level to 1200 m (Wapstra and Swain 2001; Wapstra et al. 1999). Long-term field data and laboratory experiments document variation in sex ratio with temperature at a comparatively 'warm coastal' population, but parity of sex ratios regardless of temperature at a 'cool sub-alpine' population (Pen et al. 2010; Wapstra et al. 2009). In addition, population-specific sex-linked DNA variation exists in both *C. ocellatus* sex determining systems and sex chromosomes in the two populations have minor structural differences (Hill, et al. 2021). Therefore, a high elevation population exhibits GSD (50:50 sex ratios facilitated by high elevation XY sex chromosomes in the absence of thermosensitivity), while a low elevation population has GSD+EE (biased sex ratios facilitated by low elevation XY sex chromosomes with thermosensitivity; Hill et al. 2018). In the GSD+EE population, warmer years result in a female biased sex ratio; cooler years result in a male bias (Cunningham et al. 2017; Pen et al. 2010). In *C. ocellatus*, the divergence in sex determination appears driven by climate-specific selection: early birth confers a fitness advantage to females at low elevation because birth date influences the onset of maturity and females have a higher lifetime reproductive fitness when born early (Pen et al. 2010). At high elevations the shorter reproductive season and longer period between birth and maturation preclude any advantage for either sex based on birth date (Pen et al. 2010). In addition, interannual weather fluctuations selects against GSD+EE at high altitudes to prevent extreme sex ratios (Pen et al. 2010). *C. ocellatus* populations would have experienced this climate-specific selection as they dispersed from refugia during the inter-glacial periods of the Pleistocene.

Although several studies have provided information regarding the genetic isolation of GSD and GSD+EE *C. ocellatus* populations, they each contain caveats (Cliff et al. 2015; Hill et al. 2018). Firstly, phylogeographic analysis of mitochondrial DNA (mtDNA) revealed a lack of

reciprocal monophyly between these populations and suggested that the ancestors of the GSD and GSD+EE lineages likely occupied shared lowland refugia during Pleistocene glaciations, including the Last Glacial Maximum, and were potentially interbreeding (Cliff et al. 2015). Furthermore, the species is presently more-or-less continuously distributed between the GSD and GSD+EE sites, with no obvious large-scale barriers to movement (Cliff et al. 2015), suggesting the possibility of contemporary gene flow. However, the lack of mtDNA genetic structuring among these populations may not refute contemporary genetic isolation of these sex determining systems, given the potential for mitochondrial incomplete lineage sorting to persist in large and recently diverged populations (Funk and Omland 2003). Secondly, a detailed genomic analysis identified loci with population-specific sex-linked variation (33 loci in the GSD and 42 loci in the GSD+EE populations; Hill et al. 2018), suggesting genetic isolation. Similarly, linkage disequilibrium amongst sex-linked SNPs common to both populations is greater in the GSD than GSD+EE population (Hill et al. 2018). This suggests disparate inhibition of sex chromosome recombination (and differentiation) among populations, despite some regions being conserved relative to other taxa (Cornejo-Paramo et al. 2020). However, crosses between individuals with different sex chromosomes could still maintain population-specific sex-linked loci, while homogenising autosomal variation depending on the strength of selection on hybrid incompatibilities (Presgraves 2018). Thirdly, while we have attempted to cross-breed these populations, and copulations occurred (suggesting no strong pre-mating isolation), there were no subsequent births, but breeding experiments involving within-population crosses also had low success. Therefore, we lack knowledge of whether the divergence of these sex determining systems may have impacted autosomal gene flow between their populations more generally (without precluding genetic isolation by other mechanisms).

Here we used 'Isolation with Migration' models (Hey and Nielsen 2004) and neutral, non-sex-linked single nucleotide polymorphisms (SNPs) to investigate whether autosomal gene flow has accompanied divergence of the GSD and GSD+EE populations of *C. ocellatus*. This approach is widely applicable to the exploration of whether gene flow between lineages has been disrupted by divergence in traits (Hey 2010; Hey et al. 2018; Hey and Nielsen 2004; Runemark et al. 2012). Furthermore, we used this approach to estimate the age of the divergence of GSD and GSD+EE lineages, to address whether gene flow occurred between them during their divergence. Reptiles' close link with the thermal environment makes them a compelling group for understanding the interactions between climate-mediated natural selection on sex determining systems and gene flow among lineages. We discuss our findings in the context of sex determination transitions against a background of Pleistocene climate fluctuations and infer the consequences to GSD and GSD+EE populations of *C. ocellatus* under climate change.

Methods

Study populations

We studied populations of *C. ocellatus* representing the climatic extremes of this species' range: a warmer low elevation population (42 34'S, 147 52' E; elevation 50 m) and a cooler high elevation population (41 51'S, 146 34'E; elevation 1200 m. Figure 1). These are the same populations that underpinned research on sex determination and sex-linked DNA sequences in this species (Cunningham et al. 2017; Hill et al. 2018; Wapstra et al. 2004; Wapstra et al. 2009). Mitochondrial genotypes for five and four individuals were included representing the GSD and GSD+EE populations, respectively, and likewise 42 and 44 individuals for nuclear SNPs.

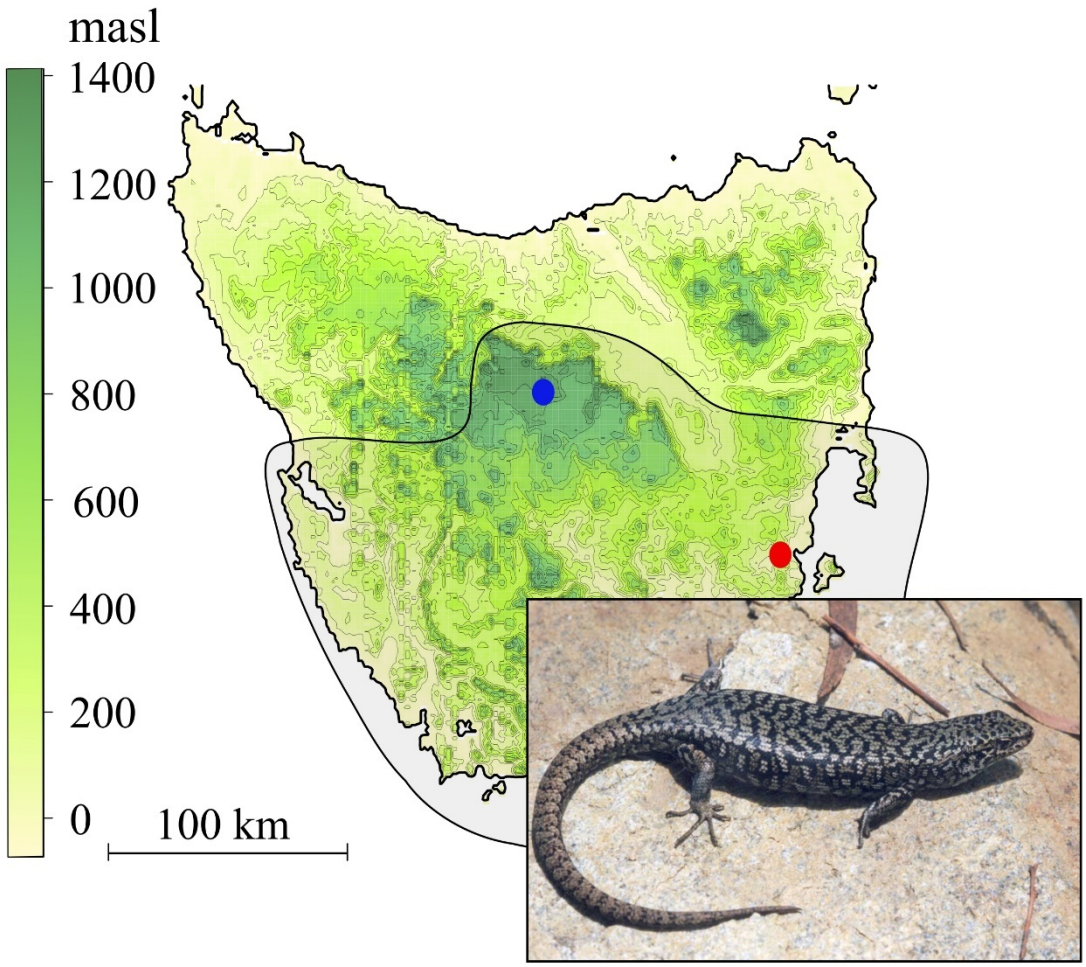

Figure 1. Locations of GSD (high elevation; blue circle) and GSD+EE (low elevation; red circle) populations of *Carinascincus ocellatus* with divergent sex determining systems. Altitudinal gradient is indicated and grey shaded region represents the mitochondrial clade to which these populations belong (Cliff et al. 2015). Inset: *C. ocellatus*.

Neutral autosomal SNP and mitochondrial markers

The concatenated mitochondrial sequences (NADH2 and NADH4) were obtained from Cliff et al. (2015). Neutral autosomal SNPs were derived from the dataset of Hill et al. (2018), obtained using a high-throughput double digest, restriction enzyme reduced representation sequencing approach (Kilian et al. 2012). All sex-linked SNPs from this dataset were removed for this analysis. Secondaries (additional SNPs on the same fragment) were removed from remaining loci using custom R script (R Development Core Team 2017); the SNP with the highest reproducibility and polymorphic information content from each locus was retained. SNP genotypes with an average reproducibility < 0.5, a call rate of < 0.9 and loci monomorphic within populations were also removed using the dartR package (Gruber and Georges 2019) in R. This left 11,107 SNPs with an average reproducibility of 0.99 and call rate of 0.98. These SNPs were used to calculate pairwise Fst, visualise the genetic similarity of the populations via a principle coordinates analysis in the dartR package (Gruber and Georges 2019) and to identify individuals of mixed origin using STRUCTURE v 2.3.4 (Pritchard et al. 2000). We used the admixture model as implemented in STRUCTURE with no prior information on geographic origin included. Runs were replicated five times and we assessed the likelihood values for K=1 – 5 using the Evanno method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). For each run we used a burnin of 10^5 iterations and a further run length of 10^6 iterations. SNPs putatively under selection (Fst in the 5th percentile) and those not in Hardy-Weinberg Equilibrium (HWE; $p < 0.05$) in either population were then filtered from the data using Genepop (Rousset 2008). From the remaining 9,453 loci, a set of 100 SNPs were chosen at random for coalescent analysis; linkage amongst these loci was ruled out ($R^2<0.5$) using the '*genetics*' package (Warnes et al. 2013) in R.

Isolation with Migration analysis

The level of gene flow accompanying divergence of GSD and GSD+EE populations, along with their duration of divergence, was assessed under the "Isolation with Migration" Bayesian framework of Hey and Nielsen (2004), employing IMa3 (Hey et al. 2018). Mitochondrial sequences and dart-tags containing the neutral nuclear SNPs were analysed concurrently to estimate lineage-splitting time and rates of gene flow between lineages in each direction (Figure 2). The HKY mutation model (Hasegawa et al. 1985) was employed for mtDNA sequence data, while the infinite sites model (Kimura 1969) was employed for nuclear SNPs (Hey and Nielsen 2004). Uniform priors were employed for divergence time and population size parameters, while exponential priors were employed for gene flow, given an expectation that low rates were likely (mean of prior distribution 6 $x10^{-06}$, approximating one individual per generation). Upper limits on uniform priors were initially set broadly, and then based on inspection of posterior distributions, were narrowed in a subsequent run to encompass the range of this posterior plus a margin of error; overly large priors reduce the precision of estimates given the use of a finite number of bins to represent the posterior distribution.

Isolation with Migration analysis was performed using Markov Chain Monte Carlo sampling with 112 chains distributed across 14 processors, and a geometric chain heating scheme with first and second heating parameters of 0.95 and 0.50, respectively. To reduce overall run-time, an initial analysis was run and traces inspected to ensure stationarity of the sampling distribution was achieved, and this was then used to seed four simultaneous analyses, each run for 24-hours following a 10 min burnin and using unique random number seeds, to ensure independence among runs. All runs were assessed for convergence using Tracer 1.7.1 (Rambaut et al. 2018) prior to combining the results. In total, 111,643 genealogies were retained for estimation of model parameters.

Information on mutation rate was employed to scale output into units of years (divergence time, gene flow). Mitochondrial mutation rates were employed in the analysis, against which mutation rates at the nuclear loci would be scaled. We followed the mean rate estimate of 1.52% divergence per million years based on calibrations from other squamates (Chapple et al. 2011) and used by Cliff et al. (2015). To account for potential variation in mutation rate (Ansari et al. 2019; Ho et al. 2005; Ho et al. 2007), we explored the consequences of using a faster rate of 2.3% divergence per million years, reported from Canary Islands skinks (Brown and Pestano 1998). Faster rates may be more applicable to reconstructing demographic history over recent (<100,000 yr) timescales (Burridge et al. 2008). Failure to entertain time-dependent rates of molecular change will lead to overestimation of divergence time and underestimation of gene flow (Burridge et al. 2008).

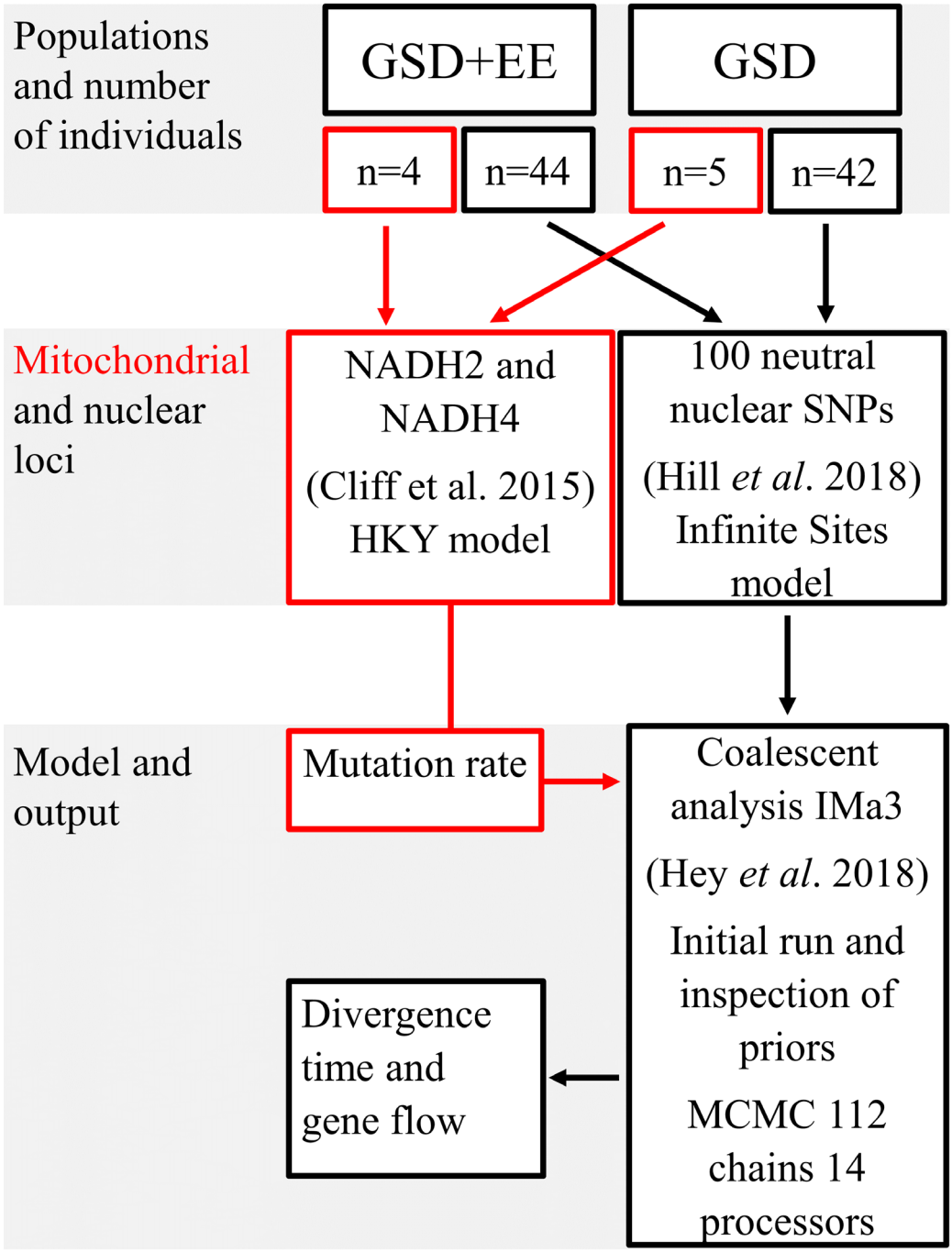

Figure 2. Flowchart for estimating divergence time and gene flow amongst GSD (high elevation) and GSD+EE (low elevation) populations of *Carinascincus ocellatus* using an Isolation with Migration (IMa3) framework. Red denotes steps specific to the mitochondrial locus.

Results

The divergence of GSD+EE (low elevation) and GSD (high elevation) populations of *C. ocellatus* occurred under negligible gene flow, and commenced between 0.61 and 0.92 Mya (highest posterior density 0.16-2.30 Mya under different mutation rates, Table 1; Figure 3). While analysis with a faster mutation rate (2.3% divergence/Myr) produced a more recent estimate of the divergence time (Table 1), divergence still occurred within the Pleistocene, and substantially pre-dated the Last Glacial Maximum. This result, and that of Cliff et al. (2015), indicates that GSD and GSD+EE lineages were likely sympatric, and definitely more proximate, at low elevation refugia through multiple Pleistocene glaciations, yet they still diverged under negligible gene flow.

Table 1. Posterior estimates of divergence (split) time, gene flow and effective population size (Ne) of GSD and GSD+EE populations of *Carinascincus ocellatus* based on mitochondrial mutation rates of 1.52% and 2.30% divergence per million years. Median values from posteriors are reported, along with 95% highest posterior densities (HPD) for population splitting time (values for migration posterior were sensitive to prior distribution, and hence are not reported). Note that migration rate (gene flow) posteriors are described "backwards in time".

Mutation rate (% per Myr)	Split time (Mya)	95% HPD interval	Gene flow (per gene per year)		Population size (Ne, million individuals)		
			GSD+EE to GSD	GSD to GSD+EE	GSD+EE	GSD	Ancestral
1.52	0.92	0.24 – 2.30	$3x10^{-8}$	$4x10^{-8}$	0.63	0.55	9.8
2.30	0.61	0.16 – 1.50	$3x10^{-8}$	$4x10^{-8}$	0.42	0.36	6.5

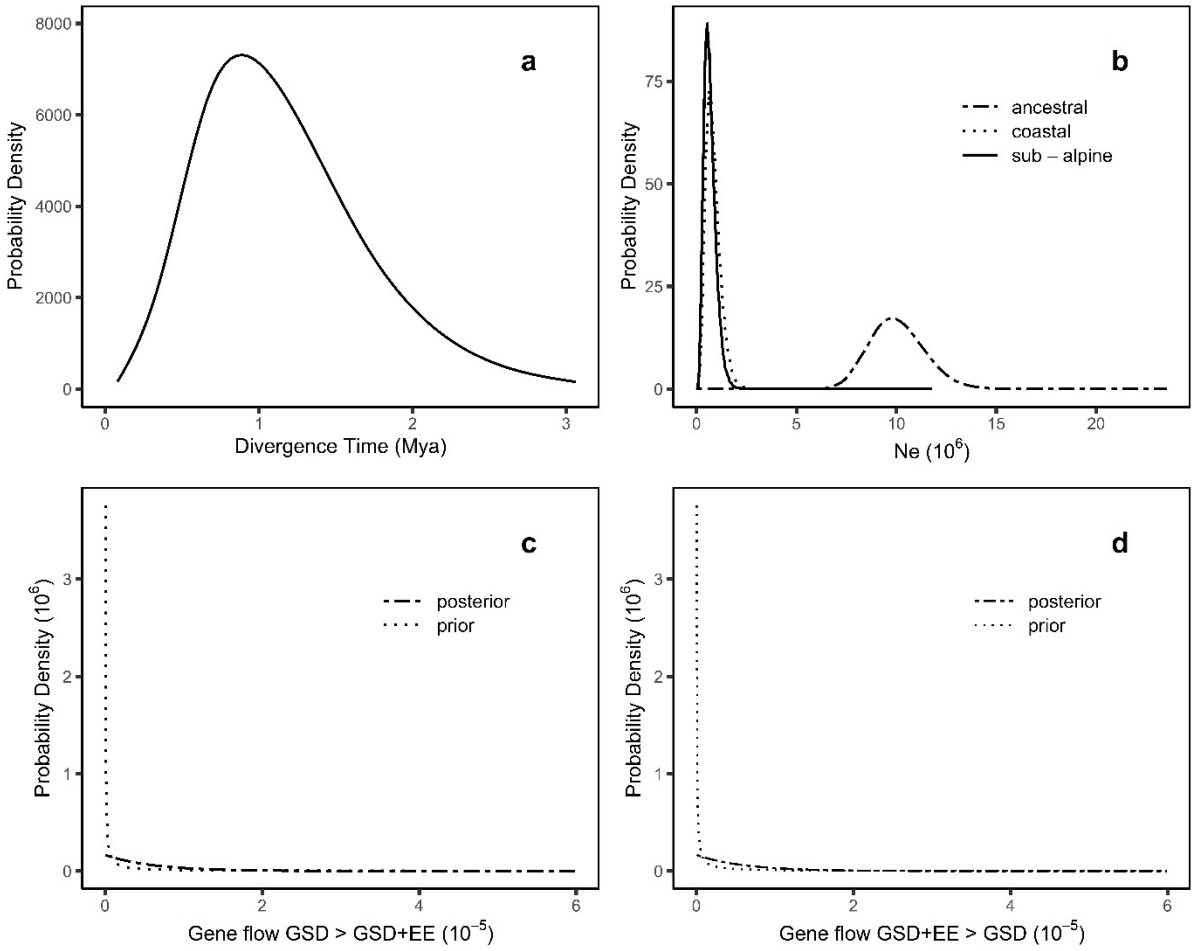

Figure 3. Estimated marginal posterior densities for parameters from the Isolation with Migration (IMa3) analysis of *Carinascincus ocellatus* populations with divergent sex determining systems. (a) divergence time of populations, (b) Effective population sizes (Ne) of ancestral, GSD and GSD+EE populations and gene flow from (c) GSD to GSD+EE and (d) GSD+EE to GSD populations.

Pairwise Fst between the populations is 0.24, consistent with negligible gene flow compared with both inter and intraspecific values reported for squamates (Dennison et al. 2012; Koc et al. 2017; Tucker et al. 2014). In the principle component analysis, the major axis of variation, PC1, explained 21.8% of the total variation in SNP genotypes and placed individuals into two distinct groups representing our populations, with PC2 explaining a further 1.7% (figure 4). These results were corroborated by STRUCTURE which assigned all individuals as pure GSD or pure GSD+EE in origin (K=2; figure 4).

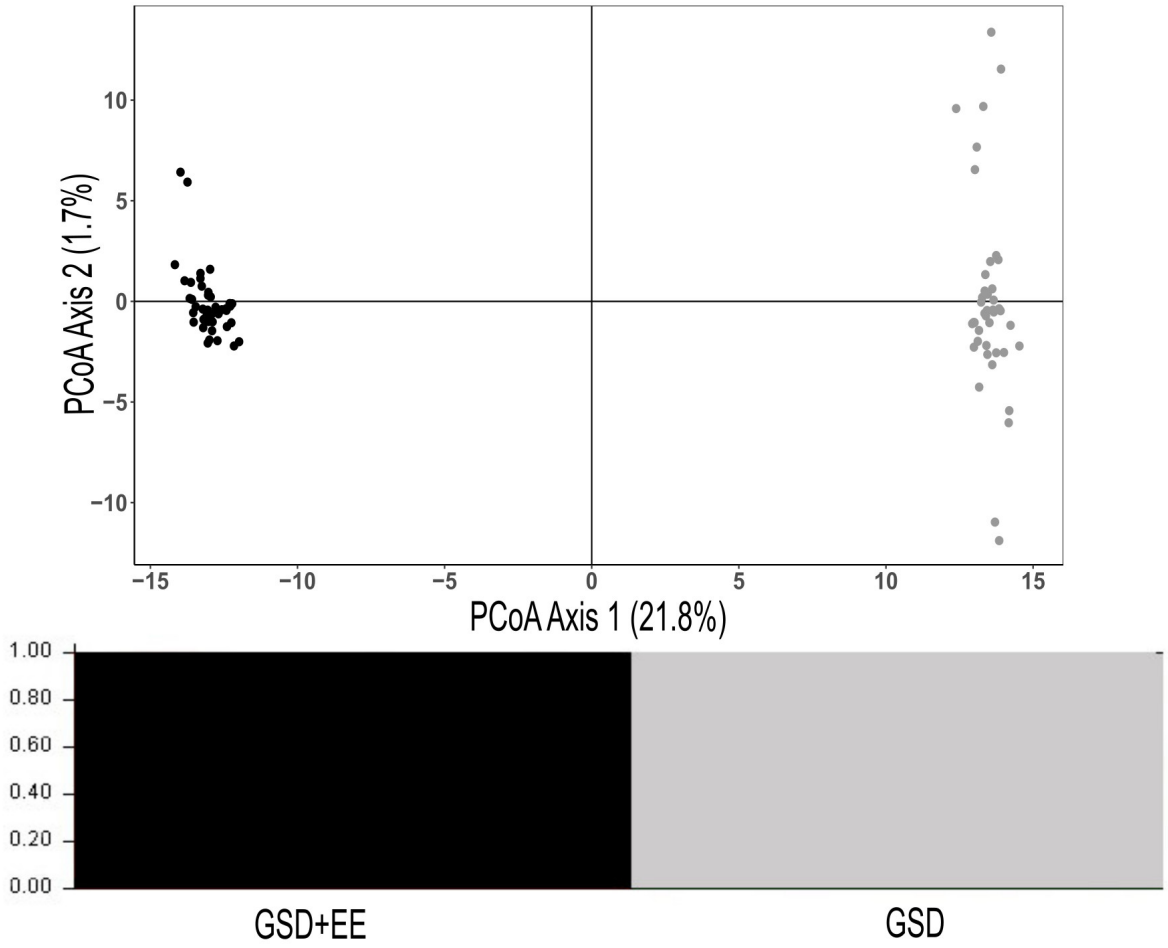

Figure 4. Principle Coordinates Analysis (upper panel) and STRUCTURE (lower panel) analysis conducted on 11,107 SNP genotypes of individuals collected from two populations of *Carinascincus ocellatus* with divergent sex determining systems. Populations are GSD (grey) and GSD+EE (black).

Discussion

The presence of contemporary genetic structure and an absence of gene flow during the Pleistocene divergence of GSD and GSD+EE populations of *C. ocellatus*, across recurrent periods of likely sympatry, raises the possibility that divergence in sex determining system promoted broader genetic isolation. When mutations occur on a sex chromosome that disrupts sex determination and leads to sex ratio skews (e.g., hybrid sterility in the heterogametic sex which leads to sex ratios in favour of the homogametic sex; Haldane's rule e.g., Olsson et al. 2004), compensatory mutations that return sex ratios to parity are favoured

(O'Neill and O'Neill 2018). These can occur on the alternative sex chromosome or the autosomes (Meiklejohn and Tao 2010). This process, driven by genetic conflict over the sex ratio, presents opportunities for further divergence of sex determination. If subsequent mating occurs between lineages diverging in sex determination, incompatibilities at the genomic or chromosomal level can result in post-zygotic isolation, inhibiting gene flow (Faria and Navarro 2010; Meiklejohn and Tao 2010; Zhang et al. 2015). The Pleistocene represents an important mediator of speciation in ectotherms (Avise et al. 1998). A mutation or a polymorphism for epigenetic regulation arising and segregating on sex chromosomes in the ancestral *C. ocellatus* population, once exposed to selection gradients across climate during Pleistocene glacial cycles, could rapidly inhibit gene flow between sex determining systems. Any of the population-specific sex-linked loci described for *C. ocellatus* (Hill et al. 2018) could be responsible for initiating the observed differences in sex determination, and potentially the isolation of their populations.

Although we have observed low gene flow during the divergence of sex determining systems in *C. ocellatus*, we cannot yet exclude the possibility that divergence in sex determination postdates the emergence of an alternative isolating trait. For instance, the impact of geographic distance on genetic isolation requires consideration; in essence, whether the genetic isolation we observe here exceeds that across a comparable geographic scale within a sex determining system. Furthermore, testing whether sex reversal contributes to observed population-specific sex determination in *C. ocellatus* is important, as sex reversed individuals can provide a conduit for gene flow between systems (Holleley et al. 2015). While morphologically distinct sex chromosomes are known to isolate lineages (Phillips and Edmands 2012), the degree of differentiation required for this to occur is unknown. In the case of *C. ocellatus*, sex chromosomes are similar between systems, with slightly more repeats and heterochromatin on Y chromosomes from the GSD population (Hill et al. 2021).

Likewise, the number of population-specific sex-linked markers (GSD n = 33, GSD+EE n = 42) is small relative to those still shared between populations (n = 201; Hill et al. 2018). High chromosomal similarity would also be expected if the difference in sex determining system is merely a polymorphism for a temperature threshold in a shared gene product (Quinn et al. 2011). On the other hand, close examination of the life history of this species has not revealed strong evidence for population-specific local adaptation in traits that may explain their genetic isolation (Cadby et al. 2014; Caldwell et al. 2017; Cliff et al. 2015; Wapstra and Swain 2001; Wapstra et al. 1999). For example, temperature reaction norms for gestation length and offspring development are remarkably similar in each population (Cunningham et al. 2020), however, further studies will reveal if local thermal adaptation has occurred since isolation resulting in population-specific thermosensitivity of sex determination. Regardless whether sex determination isolated populations or occurred subsequent to their isolation, our estimate of divergence time places a lower limit on the timeframe of their divergence.

With evidence for isolation of populations at the extremes of the species range, it is important to understand how different sex determining systems will impact population responses to increases and fluctuations in temperature over rapid timescales. As temperatures rise, sex ratios across the current *C. ocellatus* range will become increasingly female biased in populations with GSD+EE (Cunningham et al. 2017; Pen et al. 2010; Wapstra et al. 2009). Climate change is also shifting species' distributions (Bonebrake et al. 2018), with higher elevations becoming accessible to phenotypes that were historically excluded (Sinervo et al. 2010). Increased population growth due to an excess of females in GSD+EE populations (Wedekind 2002), will also promote range shifts (Boyle et al. 2014; Boyle et al. 2016). If the GSD and GSD+EE sex determining systems isolate these populations, this will inhibit the transmission of potentially beneficial autosomal alleles between populations on secondary contact, representing a potential impediment to their adaptation to changing environmental

conditions. Alternatively, as climates warm and temperature fluctuations become more extreme, and if dispersal is a limiting factor, a mismatch may occur between the climate experienced by populations and their ability to sustain fundamental metabolic processes, leading to local extinctions (Sinervo et al. 2010). Experiments designed to map the geographic distribution of sex determining systems in *C. ocellatus*, combined with modelling of future climate scenarios across its range, will confirm potential contact zones between alternative sex determining systems and regions of the current and future distribution where mismatches are most likely to occur between optimal sex determination and climate.

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Data Availability Statement

The data used in this study have been deposited to Dryad DOI https://doi.org/10.5061/dryad.h70rxwdgc

References

Alam S, Sarre S, Gleeson D, Georges A, Ezaz T. 2018. Did lizards follow unique pathways in sex chromosome evolution? Genes 9: 239 - 255. doi: doi:10.3390/genes9050239

Ansari MH, et al. 2019. Plio-Pleistocene diversification and biogeographic barriers in southern Australia reflected in the phylogeography of a widespread and common lizard species. Mol. Phylogenet. Evol. 133: 107-119. doi: 10.1016/j.ympev.2018.12.014

Avise JC, Walker D, Johns GC. 1998. Speciation durations and Pleistocene effects on vertebrate phylogeography. Proc. Biol. Sci. 265: 1707-1712. doi: 10.1098/rspb.1998.0492

Bachtrog D, et al. 2014. Sex determination: why so many ways of doing it? PLoS Biology 12: e1001899. doi: 10.1371/journal.pbio.1001899

Bonebrake TC, et al. 2018. Managing consequences of climate-driven species redistribution requires integration of ecology, conservation and social science. Biol. Rev. 93: 284-305. doi: 10.1111/brv.12344

Boyle M, Hone J, Schwanz LE, Georges A. 2014. Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? Ecol. Evol. 4: 4522-4533. doi: 10.1002/ece3.1316

Boyle M, Schwanz L, Hone J, Georges A. 2016. Dispersal and climate warming determine range shift in model reptile populations. Ecol. Model. 328: 34-43. doi: 10.1016/j.ecolmodel.2016.02.011

Bracewell RR, Bentz BJ, Sullivan BT, Good JM. 2017. Rapid neo-sex chromosome evolution and incipient speciation in a major forest pest. Nat. Commun. 8: 1593. doi: 10.1038/s41467-017-01761-4

Brown RP, Pestano J. 1998. Phylogeography of skinks (*Chalcides*) in the Canary Islands inferred from mitochondrial DNA sequences. Mol. Ecol. 7: 1183-1191. doi: 10.1046/j.1365-294x.1998.00442.x

Burridge CP, Craw D, Fletcher D, Waters JM. 2008. Geological dates and molecular rates: fish DNA sheds light on time dependency. Mol. Biol. Evol. 25: 624-633. doi: 10.1093/molbev/msm271

Cadby CD, Jones SM, Wapstra E 2014. Geographical differences in maternal basking behaviour and offspring growth rate in a climatically widespread viviparous reptile. Journal of Experimental Biology 217: 1175-1179. doi: 10.1242/jeb.089953

Caldwell AJ, While GM, Wapstra E 2017. Plasticity of thermoregulatory behaviour in response to the thermal environment by widespread and alpine reptile species. Animal Behaviour 132: 217-227. doi: 10.1016/j.anbehav.2017.07.025

Campbell P, et al. 2019. Vocal divergence is concordant with genomic evidence for strong reproductive isolation in grasshopper mice (*Onychomys*). Ecol. Evol. 9: 12886-12896. doi: 10.1002/ece3.5770

Chapple DG, Chapple SNJ, Thompson MB. 2011. Biogeographic barriers in south-eastern Australia drive phylogeographic divergence in the garden skink, *Lampropholis guichenoti*. J. Biogeogr. 38: 1761-1775.

Cliff HB, Wapstra E, Burridge CP. 2015. Persistence and dispersal in a Southern Hemisphere glaciated landscape: the phylogeography of the spotted snow skink (*Niveoscincus ocellatus*) in Tasmania. BMC Evol. Biol. 15: 121. doi: 10.1186/s12862-015-0397-y

Cornejo-Paramo P, et al. 2020. Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome Biol. Evol. 12: 924 - 930. doi: 10.1093/gbe/evaa104

Cunningham GD, While GM, Olsson M, Ljungström G, Wapstra E 2020. Degrees of change: between and within population variation in thermal reaction norms of phenology in a viviparous lizard. Ecology 101: e03136. doi: 10.1002/ecy.3136

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol. Lett. 13. doi: 10.1098/rsbl.2017.0218

Dennison S, Smith SM, Stow AJ. 2012. Long-distance geneflow and habitat specificity of the rock-dwelling coppertail skink, *Ctenotus taeniolatus*. Austral Ecol. 37: 258-267. doi: 10.1111/j.1442-9993.2011.02270.x

Dissanayake DSB, Holleley CE, Hill L, O'Meally D, Deakin J, Georges A. 2020. Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in-silico whole genome subtraction. BMC Genom. 21: 667.

Earl DA, vonHoldt BM 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361. doi: 10.1007/s12686-011-9548-7

Evanno G, Regnaut S, Goudet J 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611-2620. doi: 10.1111/j.1365-294X.2005.02553.x

Ezaz T, Sarre SD, O'Meally D, Graves JA, Georges A. 2009. Sex chromosome evolution in lizards: independent origins and rapid transitions. Cytogenet. Genome Res. 127: 249-260. doi: 10.1159/000300507

Ezaz T, Stiglec R, Veyrunes F, Graves JA. 2006. Relationships between vertebrate ZW and XY sex chromosome systems. Curr. Biol. 16: R736-743. doi: 10.1016/j.cub.2006.08.021

Faria R, Navarro A. 2010. Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends Ecol. Evol. 25: 660-669. doi: 10.1016/j.tree.2010.07.008

Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 34: 397-423. doi: 10.1146/annurev.ecolsys.34.011802.132421

Graves JA. 2006. Sex chromosome specialization and degeneration in mammals. Cell 124: 901-914. doi: 10.1016/j.cell.2006.02.024

Gregory Warnes with contributions from Gregor Gorjanc Friedrich Leisch and Michael Man. 2013. genetics: Population Genetics. R package version 1.3.8.1. https://CRAN.R-project.org/package=genetics.

Gruber B, Georges A. 2019. dartR: Importing and Analysing SNP and Silicodart Data Generated by Genome-Wide Restriction Fragment Analysis. Version 1.2.2.

Haldane JBS. 1922. Sex ratio and unisexual sterility in hybrid animals. J. Genet. 12: 101-109. doi: 10.1007/bf02983075

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22: 160-174. doi: 10.1007/BF02101694

Hayes TB. 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. J. Exp. Zool. 281: 373-399. doi: 10.1002/(SICI)1097-010X(19980801)281:5<373::AID-JEZ4>3.0.CO;2-L

Hey J. 2010. The divergence of chimpanzee species and subspecies as revealed in multipopulation isolation-with-migration analyses. Mol. Biol. Evol. 27: 921 - 933. doi: 10.1093/molbev/msp298

Hey J, et al. 2018. Phylogeny estimation by integration over isolation with migration models. Mol. Biol. Evol. 35: 2805 - 2818. doi: 10.1093/molbev/msy162

Hey J, Nielsen R. 2004. Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 167: 747 - 760. doi: 10.1534/genetics.103.024182

Hill PL, Burridge CP, Ezaz T, Wapstra E. 2018. Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. Genome Biol. Evol. 10: 1079-1087. doi: 10.1093/gbe/evy042

Hill P, Shams F, Burridge CP, Wapstra E, Ezaz T 2021. Differences in homomorphic sex chromosomes are associated with population divergence in sex determination in *Carinascincus ocellatus* (Scincidae: Lygosominae). Cells 10: 291.

Ho SYW, Phillips MJ, Cooper A, Drummond AJ. 2005. Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. Mol. Biol. Evol. 22: 1561-1568. doi: 10.1093/molbev/msi145

Ho SYW, Shapiro B, Phillips MJ, Cooper A, Drummond AJ. 2007. Evidence for time dependency of molecular rate estimates. Syst. Biol. 56: 515-522. doi: 10.1080/10635150701435401

Holleley CE, et al. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Holleley CE, Sarre SD, O'Meally D, Georges A. 2016. Sex reversal in reptiles: reproductive oddity or powerful driver of evolutionary change? Sex. Dev. 10: 279-287. doi: 10.1159/000450972

Horreo JL, Breedveld MC, Lindtke D, Heulin B, Surget-Groba Y, Fitze PS. 2019. Genetic introgression among differentiated clades is lower among clades exhibiting different parity modes. Heredity 123: 264-272. doi: 10.1038/s41437-019-0201-7

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J. Evol. Biol. 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Jenck CS, Lehto WR, Ketterman BT, Sloan LF, Sexton AN, Tinghitella RM. 2020. Phenotypic divergence among threespine stickleback that differ in nuptial coloration. Ecol. Evol. 10: 2900-2916. doi: 10.1002/ece3.6105

Johnson Pokorna M, Kratochvil L. 2016. What was the ancestral sex-determining mechanism in amniote vertebrates? Biol. Rev. 91: 1-12. doi: 10.1111/brv.12156

Kilian A, et al. 2012. Diversity Arrays Technology: a generic genome profiling technology on open platforms. In: Pompanon F, Bonin A, editors. Data Production and Analysis in Population Genomics: Methods and Protocols. Totowa, NJ: Humana Press. p. 67-89.

Kimura M. 1969. The number of heterozygous nucleotide sites maintained in a finite population due to steady flux of mutations. Genetics 61: 893-903.

Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF, Absher DM, Grimwood J, Schmutz J, Myers RM, Kingsley DM, Peichel CL. 2009. A role for a neo-sex chromosome in stickleback speciation. Nature 461: 1079-1083. doi: 10.1038/nature08441

Koc H, et al. 2017. Phylogenetic relationships of *D. rudis* (Bedriaga, 1886) and *D. bithynica* (Mehely, 1909) based on microsatellite and mitochondrial DNA in Turkey. Mitochondrial DNA A DNA Mapp. Seq. Anal. 28: 814-825. doi: 10.1080/24701394.2016.1197215

Lima TG. 2014. Higher levels of sex chromosome heteromorphism are associated with markedly stronger reproductive isolation. Nat. Commun. 5: 4743. doi: 10.1038/ncomms5743

Luo B, et al. 2017. Social call divergence in bats: a comparative analysis. Behav. Ecol. 28: 533-540. doi: 10.1093/beheco/arw184

Meiklejohn CD, Tao Y. 2010. Genetic conflict and sex chromosome evolution. Trends Ecol. Evol. 25: 215-223. doi: 10.1016/j.tree.2009.10.005

O'Neill MJ, O'Neill RJ. 2018. Sex chromosome repeats tip the balance towards speciation. Mol. Ecol. 27: 3783-3798. doi: 10.1111/mec.14577

Ohno S. 1967. Sex chromosomes and sex linked genes. Heidelberg, Germany: Springer - Verlag.

Olsson M, Ujvari B, Madsen T, Uller T, Wapstra E. 2004. Haldane rules: costs of outbreeding at production of daughters in sand lizards. Ecol. Lett. 7: 924 - 928. doi: 10.1111/j.1461-0248.2004.00652.x

Patawang I, Chuaynkern Y, Supanuam P, Maneechot N, Pinthong K, Tanomtong A. 2017. Cytogenetics of the skinks (Reptilia, Scincidae) from Thailand; IV: newly investigated karyotypic features of *Lygosoma quadrupes* and *Scincella melanosticta*. Caryologia 71: 29-34. doi: 10.1080/00087114.2017.1402249

Pen I, Uller T, Feldmeyer B, Harts A, While GM, Wapstra E. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Phillips BC, Edmands S. 2012. Does the speciation clock tick more slowly in the absence of heteromorphic sex chromosomes? Bioessays 34: 166-169. doi: 10.1002/bies.201100164

Portik DM, et al. 2019. Sexual dichromatism drives diversification within a major radiation of African amphibians. Syst. Biol. 68: 859-875. doi: 10.1093/sysbio/syz023

Presgraves DC. 2018. Evaluating genomic signatures of "the large X-effect" during complex speciation. Mol. Ecol. 27: 3822-3830. doi: 10.1111/mec.14777

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JA. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, Sarre SD, Ezaz T, Marshall Graves JA, Georges A. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol. Lett. 7: 443-448. doi: 10.1098/rsbl.2010.1126

R Development Core Team. 2017. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

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. Biol. Lett. 4: 176-178. doi: 10.1098/rsbl.2007.0583

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior summarization in bayesian phylogenetics using Tracer 1.7. Syst. Biol. 67: 901-904. doi: 10.1093/sysbio/syy032

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resources 8: 103 - 106. doi: 10.1111/j.1471-8286.2007.01931.x

Runemark A, Hey J, Hansson B, Svensson EI. 2012. Vicariance divergence and gene flow among islet populations of an endemic lizard. Mol. Ecol. 21: 117 - 129. doi: 10.1111/j.1365-294X.2011.05377.x

Sarre SD, Georges A, Quinn A. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645. doi: 10.1002/bies.20050

Sinervo B, et al. 2010. Erosion of lizard diversity by climate change and altered thermal niches. Science 328: 894-899. doi: 10.1126/science.1184695

Taylor RS, Friesen VL. 2017. The role of allochrony in speciation. Mol. Ecol. 26: 3330-3342. doi: 10.1111/mec.14126

Tucker DB, McBrayer LD, Harrison JS. 2014. Population structure of Florida scrub lizards (*Scleroporus woodi*) in an anthropogenically fragmented forest. Herpetologica 70: 266-278. doi: 10.1655/herpetologica-d-13-00041

Wapstra E, Olsson M, Shine R, Edwards A, Swain R, Joss JM. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. Biol. Lett. 271 Suppl 4: S230-232. doi: 10.1098/rsbl.2003.0152

Wapstra E, Swain R. 2001. Geographic and annual variation in life-history traits in a temperate zone Australian skink. J. Herpetol. 35: 194-203. doi: 10.2307/1566108

Wapstra E, Swain R, Jones SM, O'Reilly J. 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata : Scincidae). Aust. J. Zool. 47: 539-550. doi: 10.1071/zo99038

Wapstra E, et al. 2009. Climate effects on offspring sex ratio in a viviparous lizard. J. Anim. Ecol. 78: 84-90. doi: 10.1111/j.1365-2656.2008.01470.x

Wedekind C. 2002. Manipulating sex ratios for conservation: short-term risks and long-term benefits. Anim. Conserv. 5: 13-20. doi: 10.1017/s1367943002001026

Zhang L, Sun T, Woldesellassie F, Xiao H, Tao Y. 2015. Sex ratio meiotic drive as a plausible evolutionary mechanism for hybrid male sterility. PLoS Genet 11: e1005073. doi: 10.1371/journal.pgen.1005073

Chapter Six

General Discussion

The evolutionary lability of sex determination is perplexing. Development as male or female is near-ubiquitous amongst sexually reproducing organisms, yet the mechanisms that govern this process are diverse (Bachtrog et al. 2014). Sex determination systems also display a phylogenetically complex distribution, suggestive of multiple independent evolutionary transitions (Janzen and Phillips 2006; Pokorna and Kratochvil 2009). Classic theory (Bull 1981) and contemporary mechanistic models (Sander van Doorn and Kirkpatrick 2007; Perrin 2009; Pen et al. 2010; Sander van Doorn and Kirkpatrick 2010; Quinn et al. 2011; Schwanz et al. 2013; Blaser et al. 2014; Schwanz et al. 2020) explain why these transitions occur, however, understanding how they occur is difficult because of the evolutionary timescales involved (Bachtrog et al. 2014; Vicoso et al. 2013). The distribution of genetic and environmental sex determination and the variety in sex chromosome morphology and homology across plants and animals (Adkins-Regan and Reeve 2014; Charlesworth and Mank 2010; Gamble et al. 2015; Pennell et al. 2018; Quinn et al. 2011; Sarre et al. 2011), suggests it is unlikely that there is a universal mechanism responsible for transitions between systems. Our understanding of the general mechanisms responsible for transitions in sex determination is limited and remains a major challenge in evolutionary biology. However, characteristics of the genome, behaviour and physiology and aspects of population dynamics in sex determination transitions are important to investigate, because these mechanisms are often, but not ubiquitously, involved in transitions (Matsumoto et al. 2017; Pokorna and Kratochvil 2009; Sarre et al. 2011).

In this thesis, I combined next generation sequencing, traditional cytogenetic techniques, experimental manipulation of development, traditional population genetics tools and coalescent analysis to understand evolutionary transitions in sex determination using the Tasmanian spotted snow skink, *Carinascincus ocellatus* as a model. This species is an exceptional model for understanding the mechanisms that occur during a transition in sex determination because populations at the elevational range extremes have different systems. A high elevation population has genetic sex determination (GSD) and a low elevation population has thermosensitive GSD (GSD+EE, sensu Valenzuela et al. 2003). This difference is characterised by the population-specific response of offspring sex to temperature captured in both long-term field studies and in laboratory temperature manipulations during gestation (Cunningham et al. 2017; Pen et al. 2010; Wapstra et al. 2004; Wapstra et al. 2009). I show that the underlying architecture of this transition is very subtle at a molecular (Hill et al. 2018), chromosomal (Hill et al. 2021a) and physiological (**Chapter 4**) level. Whilst the divergence was initiated during Pleistocene glaciations, it is likely still ongoing (Hill et al. 2021b). In this general discussion, I summarise the main findings and integrate these with existing knowledge on sex determination across vertebrates to provide a mechanistic overview of sex determination transitions. I also discuss some questions that have emerged from this work and are important future directions for sex determination and sex chromosome research.

Minor changes to the genome underpin sex determination transitions

Sex ratios in high and low elevation populations of *C. ocellatus* show different responses to developmental temperature (Cunningham et al. 2017; Pen et al. 2010). Theoretical models based on sex allocation theory and life history theory predict that selection across the altitudinal gradient occupied by *C. ocellatus* favours emergence of sex determining genes and thus GSD at high elevation, and loss of sex determining genes, and the emergence of TSD at

low elevation (Pen et al. 2010). However, my research provides several lines of evidence that suggest the observed population divergence in sex determination is underpinned by subtle molecular, chromosomal and physiological mechanisms (**Chapter4**; Hill et al. 2018; Hill et al. 2021a). This is likely because of the short timeframe over which sex determination has diverged (Hill et al. 2021b).

Both populations of *C. ocellatus* have XY heterogamety (Hill et al. 2018; Hill et al. 2021a). This is evidenced by male-specific genetic sequence (Hill et al. 2018) and by homomorphic sex chromosomes with a male-specific region (Hill et al 2021a). The *C. ocellatus* Y chromosome is at least 79 million years old, based on sequence homology to the sex chromosomes of the water skink, *Eulamprus heatwolei* (Scincidae; Cornejo-Paramo et al. 2020). The sex chromosomes are the same chromosome pair (pair 7) as in two further scincids: *Bassiana duperreyi* and *Pseudemoia entrecasteauxii* (Donnellan 1985). Sex chromosome sequence homology among *C. ocellatus*, *B. duperreyi* and *P. entrecasteauxii* is yet to be explored and would be a worthwhile avenue for future research because it will allow reconstruction of the ancestral sex chromosomes in this lineage and broader taxa (Ezaz et al. 2017; Kostmann et al. 2020). The homology between *C. ocellatus* and *E. heatwolei* combined with the likely lack of homology between *C. ocellatus* and *Liopholis whitii* sex chromosomes is particularly relevant (Hill et al. 2021a) and reflects the broader, seemingly haphazard pattern of sex chromosome homology found between ZW birds, XY mammals and ZW, XY and GSD+EE reptiles (Ezaz et al. 2009a; Kawai et al. 2009; Matsubara et al. 2006).

The sex chromosomes in both populations of *C. ocellatus* retain shared sex-linked sequence (Hill et al. 2018). In addition, both *C. ocellatus* populations have retained a Y chromosome region that is likely an ancient region of recombination suppression (Hill et al. 2021a; Charlesworth et al. 2005; Ezaz and Deakin 2014; Matsubara et al. 2016; Ponnikas et al. 2018;

Reichwald et al. 2015). Genes are therefore important to sex determination in both populations, and each retains an ancestral molecular component to sex determination and a region of recombination suppression on the Y chromosome (Hill et al. 2018; Hill et al. 2021a). My results from Hill et al. (2018) and Hill et al. (2021a) suggest that the predicted emergence / loss of genes in *C. ocellatus* high and low elevation populations proposed by Pen et al. (2010), may be ongoing, and that only minor changes to the genome are required before population-specific sex ratio responses to temperature emerge. Alternatively, population divergence in sex determination has reached its evolutionary endpoint and fallen short of the predicted GSD / TSD dichotomy (Pen et al. 2010), because current population-specific systems are optimal. If minor genomic changes are all that is required for a transition in sex determination to occur, this can explain the high frequency of evolutionary transitions in sex determination (Capel 2017; Janzen and Phillips 2006; Pennell et al. 2018; Pokorna and Kratochvil 2009) and provides a mechanistic context for understanding the lability of sex determination in vertebrates.

The divergence in *C. ocellatus* has occurred recently and lineages are reproductively isolated despite potential sympatry during Pleistocene glaciations **(**Hill et al. 2021b; Cliff et al. 2015). While my results do not imply causation, the lack of gene flow between diverging populations may contribute to the rapid nature of this transition, and lineage isolation occurring at, or near, the time of divergence may constitute a common feature of older vertebrate transitions in sex determination. While the transition in sex determination in *C. ocellatus* is recent, population differences between sex-linked sequence and sex chromosomes, although minor, are apparent. One key difference concerns the continued accumulation of population-specific sex-linked sequence since high and low elevation populations diverged from their common ancestor (Hill et al. 2018). Selection across the altitudinal gradient occupied by *C. ocellatus* populations favours the sex-linkage of different

genomic regions. These regions are of interest to further research into the mechanisms underpinning sex determination, as they may be relevant to how sex determination functions in each population.

Another difference between the populations concerns the interaction between the X and Y chromosomes (Hill et al. 2018; Hill et al. 2021a). Linkage disequilibrium between sex-linked alleles is a direct measure of how often alleles on the sex chromosomes are inherited together and is therefore an indication of recombination suppression. Recombination suppression is a key evolutionary mechanism associated with morphological and genetic differentiation of sex chromosomes (Charlesworth et al. 2005; Rice 1987). I found both molecular and chromosomal evidence that recombination suppression between the X and Y chromosomes differs in the study populations. Specifically, recombination among sex-linked alleles is suppressed to a greater extent in the high elevation GSD population (Hill et al. 2018), and the Y chromosome has accumulated more heterochromatin and repeats (Hill et al. 2021a). This demonstrates the link between recombination suppression at a molecular level and the accumulation of heterochromatin and repeats on the Y chromosome. *C. ocellatus* high elevation X and Y chromosomes are more differentiated than their low elevation counterparts which is expected given sex ratios in the high elevation population imply GSD (Pen et al. 2010). My work reveals the link between population specific selection for different systems of sex determination (Pen et al. 2010) and sex chromosome evolution.

The presence of sex-linked sequence and sex chromosomes in both *C. ocellatus* populations raises the question of how temperature impacts the sex ratio in the low elevation population. The sex inherited at fertilisation can be overridden by temperature in reptiles, even species with heteromorphic sex chromosomes (Quinn et al. 2007; Shine et al. 2002). Offspring then develop as the phenotypic sex that is opposite to their genetic sex and are thus sex reversed.

In oviparous reptiles, such as *Pogona vitticeps* (Agamidae) and *Bassiana duperreyi* (Scincidae), sex reversal occurs in the homogametic sex (ZZ sex reversed female *P. vitticeps*, Holleley et al. 2015; XX sex reversed male *B. duperreyi*, Holleley et al. 2016). The fitness consequences of sex reversal have been demonstrated (Holleley et al. 2015) and have been used to model its evolutionary outcomes (Quinn et al. 2011). Mating of sex reversed individuals can lead to the loss of the sex-specific chromosome (Y or W) and the evolution of TSD (Holleley et al. 2015; Quinn et al. 2011). The demonstration of sex reversal in a viviparous system provides an opportunity to examine the generality of these models to a broader range of taxa.

Because sex ratios do not deviate from parity in the high elevation, GSD population of *C. ocellatus* (Cunningham et al. 2017; Pen et al. 2010), I predicted that I would not find sex reversal in this population. However, because sex ratios can be biased in either direction in the low elevation population, I predicted sex reversal of the homogametic sex (XX) to male in cool temperatures and of the heterogametic sex (XY) to female in warm temperatures. However, I found sex reversal of the homogametic sex (XX male) in both populations of *C. ocellatus* and no sex reversed (XY) females in either population. Sex reversal, along with conserved genetic sequence (Hill et al. 2018) and conserved Y chromosomal regions (Hill et al. 2021a), is therefore another aspect of sex determination that is shared between populations and is not reflected in the different sex ratio response to temperature observed in nature or in previous experiments (Pen et al. 2010; Wapstra et al. 2004; Wapstra et al. 2009). This suggests there is another mechanism contributing to the population-specific sex ratio

response to temperature observed in *C. ocellatus*. Such mechanisms may, for example, involve population differences in the epigenetic regulation of sex determination.

Sex reversal in *C. ocellatus* in my experiments is significantly correlated with temperature and restricted to the homogametic sex (XX, **Chapter 4**). My results therefore explain the mechanism behind observed male biases in the GSD+EE population of *C. ocellatus*. Cool experimental temperatures and reduced basking opportunity for females resulted in a higher proportion of sex reversed XX males, consistent with male biases in cool seasons at low elevation. However, the mechanism responsible for the female bias at warm temperatures in this population remains unknown. In addition, because sex reversal occurs in the GSD population, there must be an additional mechanism maintaining sex ratios at parity in this population.

The relationship between incubation temperature and sex reversal is not straightforward in reptiles with thermosensitive sex determination. For example, individual variation in thermal thresholds explains variation in sex reversal across the *P. vitticeps* distribution (Castelli et al. 2020). I also found evidence of variation in individual thermal thresholds for sex reversal in *C. ocellatus* (**Chapter 4**), which emphasizes the complexity of sex determination. Evidence of variation in thermal thresholds in *C. ocellatus* comes from two observations in **Chapter 4**. First, litters with XX sex reversed males also contained XX females, suggesting that litter mates had different thermal thresholds for sex reversal. Second, sex reversed XX males were found in the hotter / longer thermal treatments suggesting that the variation around thresholds for sex reversal is high. Evolving thermal thresholds for sex reversal are a likely component of the evolutionary transitions in sex determination and populations of *C. ocellatus* may be divergent for this trait. Thresholds for sex reversal are lower in offspring of sex reversed *P. vitticeps* females, resulting in ZZ offspring that reverse to female at lower temperatures than ZZ

offspring from ZW females (Holleley et al. 2015). Despite the challenges involved with examining such effects in viviparous species, assessing thresholds for sexual phenotype in *C. ocellatus* populations would be a valuable avenue for further research into how and why they vary and whether they contribute to evolutionary transitions in sex determination.

Sex determination in *Carinascincus ocellatus* can help infer the ancestral state in Squamata

At high elevation, selection for stable sex ratios is driving this *C. ocellatus* population towards GSD (Pen et al. 2010). While both populations possess sex-linked sequence, linkage between sex-linked loci is tighter (Hill et al. 2018) and sex chromosomes are more heterochromatic and repeat rich at high elevation (Hill et al. 2021a). However, while sex ratios in nature are always at parity in this population, in the laboratory sex reversal still occurs; at cool temperatures some high elevation XX genotypes reverse to male (**Chapter 4**). Therefore, both populations of *C. ocellatus* possess a genetic and thermal component to sex determination (Hill et al. 2018; Hill et al. 2021a; **Chapter 4**). Because transitions from environmental to genetic sex determination in squamates are higher than the reverse, GSD is likely more stable (Pennell et al. 2018) and the processes governing sex chromosome evolution are likely irreversible (Hill et al. 2021a). My results suggest sex determination in the low elevation population is thus closer to the sex determining system present in the most recent common ancestor (Hill et al. 2021a). Therefore, rather than strict GSD or TSD being ancestral in *C. ocellatus*, thermosensitive genetic sex determination may be ancestral.

The ancestral squamate sex determining system is suggested as TSD, however, the ancestral state of sex determination in Scincidae is equivocal and depends on whether putative TSD skinks are included (Gamble et al. 2015). Estimates of the ancestral state of sex determination in squamates as either TSD or GSD is inconsistent with the idea that sex determination exists along a continuum between TSD and GSD (Sarre et al. 2004). The likelihood that

thermosensitive sex determination is common in both XY and ZW squamates and across both oviparous and viviparous taxa (**Chapter 4**; Holleley et al. 2016) suggests that the ancestral state may exist on a continuum between TSD and GSD, rather than as either TSD or GSD.

Understanding broader patterns of sex determination transitions

Reptiles represent an ideal taxon for answering questions surrounding both sex determination transitions and sex chromosome evolution because sex determination and sex chromosomes are diverse in this group and transitions have occurred rapidly and frequently (Janzen and Phillips 2006; Pokorna and Kratochvil 2009). Sex determination and sex chromosomes among both distantly and closely related vertebrate lineages share evolutionary origins (Cornejo-Paramo et al. 2020; Dissanayake et al. 2020; Iannucci et al. 2019; Kawai et al. 2009; Rovatsos et al. 2014; Rovatsos et al. 2016), while others have evolved independently (Hill et al. 2021a; Ezaz et al. 2009a; Koubova et al. 2014; Matsubara et al. 2014) and understanding the complex phylogenetic patterns of homology remains challenging.

Research using *C. ocellatus* is important because it represents a recent transition in sex determination. Thermosensitive sex determination may be widespread in reptiles (Holleley et al. 2016), contributing to the diversity observed in sex determination in this group. Comparisons of sex determination and the sex chromosomes of *C. ocellatus* to those of congenerics and more distantly related species will facilitate informed conjecture into the ubiquity of mechanisms that allowed ancient transitions in sex determination and further illuminate the complex architecture of its evolution. Investigating the homology of sex chromosomes and the mechanisms that underpin sex determination in squamate lineages should be a continued focus of future research into the diversity of sex chromosomes and sex determination because it will help inform the earliest origins of sex chromosomes and sex determination.

Emerging Questions

How are female sex ratio biases achieved in *Carinascincus ocellatus* at low elevation without XY sex reversal?

Investigating the genetic sex ratios that underly the phenotypic sex ratios in high elevation GSD and low elevation GSD+EE populations of *C. ocellatus* may provide an explanation for population-specific sex ratio biases occurring in the wild. The gene dosage model (Quinn et al. 2007), which is often used to explain sex reversals assumes that a 50:50 genetic sex ratio at fertilisation underlies the interaction between genes and temperature. In thermosensitive XX/XY, the proportion of XX embryos at fertilization determines the number of females that can develop, but also the potential number of male sex reversals, depending on thermal conditions. If 50% of embryos are XX then 50% of progeny have the potential to sex reverse to male (e.g., *C. ocellatus* in cool temperatures). If all these embryos reverse, 100% phenotypic male litters can result (e.g., Wapstra et al. 2004). If no sex reversal occurs, litters are expected to comprise, on average, 50% females and 50% males. However, if the proportion of thermosensitive XX embryos at fertilization is 65%, for example, sex reversal of males can still occur at low temperatures (and still achieve 100% phenotypic male litters if all XX genotypes reverse), however, female biased sex ratios can occur at warmer temperatures (up to 65% female in this example), if all the XX genotypes achieve female phenotype. Note that a small number of male reversals (15%) brings the sex ratio to parity.

An excess of XX genotypes at fertilisation could occur several ways. First, if XX sex reversed males are viable and fertile and successfully mating with XX females in the wild, this would result in an excess of XX genotypes in the population. Clutches from XX males would be expected to contain only the XX genotype, analogous to ZZ offspring from ZZ sex

reversed female *P. vitticeps* (Holleley et al. 2015). Understanding XX male fitness is a logical next step for research into sex determination transitions because there are consequences to populations associated with sex reversal and biased sex ratios (**Chapter 4**; Boyle et al. 2014; Schwanz et al. 2020; Wedekind 2017). Sex ratios that become heavily biased towards either sex can have consequences for population growth and persistence if one sex becomes limiting (Boyle et al. 2014; Le Galliard et al. 2005; Valenzuela et al. 2019; Wedekind 2017), resulting in deterioration of genetic diversity and reduced adaptive potential, with consequences for species distributions (Mitchell and Janzen 2010).

A second possible source of an XX bias at fertilisation is X chromosome meiotic drive, where transmission of the X chromosome is favoured during spermatogenesis (Lindholm et al. 2016). This would result in X-biased sperm sex ratios (Helleu et al. 2019; Pokorna et al. 2014). This type of genomic conflict between the X and Y chromosome over transmission during meiosis can evolve rapidly and contribute to hybrid incompatibilities and species divergence (Meiklejohn and Tao 2010; O'Neill and O'Neill 2018). If the X chromosome is preferentially favoured over the Y during spermatogenesis in *C. ocellatus*, litters would comprise on average an excess of XX genotypes but also contain XY genotypes because males would still possess Y-bearing sperm. The population genotypic sex ratio of offspring would reflect the population sperm sex ratio.

Lastly, sperm sex ratio biases can occur subsequent to spermatogenesis via sperm competition. This has been documented in a wide range of vertebrates including mammals, birds and reptiles, and can occur in the female reproductive tract during sperm storage (Birkhead and Møller 1993; Friesen et al. 2020). Mating in *C. ocellatus* mostly takes place prior to winter hibernation and females store sperm until they ovulate after spring emergence in both populations (Jones et al. 1997; Wapstra et al. 1999). In another squamate, the painted dragon, *Ctenophorus pictus*, excess sons are produced from older, stored sperm (Olsson et al.

2007). *C. pictus* has ZW heterogamety (Ezaz et al. 2009b); male biases could therefore be a result of W chromosome-bearing sperm death during storage. If the equivalent mechanism is operating in *C. ocellatus* (increased death of Y-bearing sperm), this would result in an excess of X chromosome-bearing sperm and an excess of XX genotypes at fertilisation.

The female biased sex ratios consistently associated with warm seasons in the GSD+EE population of *C. ocellatus* are possible without XY reversals if sex ratios at fertilisation are XX biased. The capacity for sex reversal of the XX genotype to male at low temperature persists in the GSD population, albeit to a lesser extent than in the low elevation, GSD+EE population (**Chapter 4**). The lack of female bias in the GSD population may be a result of genetic sex ratios that are closer to 50:50. If sex reversal occurs in the wild at high elevation, it may be occurring at a low enough frequency that XX reversal to male phenotype may not appreciably bias the population phenotypic sex ratio towards males, and thus go undetected.

The prevalence of sex reversed adult males can be assessed by genotyping adult males from each population using loci I designed in Hill et al. (2018) or from experiments designed to test the survival and fitness of experimentally produced sex reversed males. Further, cohorts from each population across years can be genotyped to track genotypic sex ratios of litters using the panel of sex-linked markers developed in Hill et al. (2018) or a subset thereof. In addition, sperm sex ratios both at the time of mating and at various time points during storage in the female reproductive tract are easily investigated using the Y chromosome probe set developed in Hill et al. (2021a) or by testing the paternity of offspring from mating before and after hibernation as per Olsson et al. (2007), and represents a valuable extension of my work.

Why are the intrauterine effects of viviparity compatible with sex reversal?

TSD is favoured when there are sex-specific fitness benefits related to developmental temperature (Charnov and Bull 1977). However, viviparity is expected to constrain sex ratio evolution because of the intra-uterine effects on the fitness of the minority sex (Uller 2003). For example, in a female biased clutch, sex steroids can cross the fetal membrane of male siblings, resulting in their feminisation (Even et al. 1992; Uller and Olsson 2003). My results demonstrate that sex reversal occurs in a viviparous species. Further by manipulating female body temperature in experiments that either restricted or allowed females to thermoregulate, I showed that maternal basking does not buffer the prevalence of sex reversal (**Chapter 4**).

In *C. ocellatus*, development rate is correlated with basking opportunity in both populations (Cunningham et al. 2020), as are sex ratio biases in the GSD+EE population (Wapstra et al. 2004). However, because females bask differently at high and low elevation and this has implications for offspring fitness (Cadby et al. 2014; Caldwell et al. 2017; Uller et al. 2011), and because temperature-induced sex ratio biases at low elevation are driven by sex-specific fitness, it is surprising that population-specific maternal basking behaviour does not influence the sex ratio response via sex reversal (**Chapter 4**). The physiological mechanisms responsible for translating developmental temperature into offspring sex via temperature-induced sex reversal operate in both populations and are therefore ancestral. These mechanisms are yet to be resolved but may be similar to those that translate developmental temperature to offspring sex in oviparous taxa because sex reversal occurs both when females are free to bask and also when they are held at specific temperatures (**Chapter 4**).

My results challenge the idea that viviparity constrains sex ratio evolution (Uller 2003). Experiments using *C. ocellatus*, to assess the fitness of the minority sex in litters with biased sex ratios, will further refine our understanding of the mechanisms behind sex ratio biases in

viviparous taxa. Because the transition in sex determination is recent (Hill et al. 2021b), it is potentially ongoing and the mechanics of GSD and GSD+EE are retained in both populations to different extents. If fitness deficits of biased sex ratios to the minority sex are minor, or outweighed by the fitness benefits to the majority sex (e.g., *P. vitticeps*; Holleley et al. 2015), this may favour the evolution of thermosensitive sex determination irrespective of parity.

How do heteromorphic sex chromosomes evolve in taxa with thermosensitive sex determination?

Sex reversal and dosage of thermosensitive sex determining gene products has been implied in ZW reptiles (Quinn et al. 2007). My results provide strong evidence that genes and temperature also interact in a dosage-dependent manner in an XY system (Hill et al. 2018; Hill et al. 2021a; **Chapter 4**). Sex determination via dosage of thermosensitive sex determining genes in an XY species implies a crucial role for the X chromosome in sex determination. Male phenotype can be achieved without the Y chromosome, evidence that a gene or genes on the X chromosome have taken on a sex determining role via dosage in *C. ocellatus* and likely other XY skinks (e.g., *B. duperreyi*; Holleley et al. 2016; Shine et al. 2002). Dosage above a sex threshold (XX genotype) switches development onto the female pathway; dosage below this threshold (XY genotype or temperature-induced down regulation of the XX genotype) switches development onto the male pathway. In this case, the Y chromosome in *C. ocellatus* determines the male phenotype not because it carries sex determining genes, but because its presence in the zygote limits the dosage of an X chromosome gene product. No sex determining genes have been identified in squamates although candidates will likely emerge from the vast network of genes involved in sexual differentiation across vertebrates (Graves 2013; Pan et al. 2016; Uller and Helantera 2011). My results suggest that focus needs to shift in this regard and challenges the paradigm that

sex in vertebrates is governed by the presence, absence, or dosage of male-determining genes residing on male-determining chromosomes.

Dosage dependent sex determination raises questions surrounding the current model of sex chromosome evolution. This model proposes that sex chromosomes evolve from an autosomal pair when one member of the pair acquires a sex determining locus (Graves 1995; Waters et al. 2007). The sex-specific (Y or W) chromosome then begins to differentiate from its counterpart via recombination suppression and the accumulation of sexually antagonistic alleles to keep these alleles in the sex they benefit (Charlesworth 1991; Rice 1987). However, if sex in birds (Smith et al. 2009) and reptiles such as *P. vitticeps* (Quinn et al. 2007), and very likely *B. duperreyi* and *C. ocellatus* is determined via dosage of a gene on the X or the Z rather than the presence of a gene on the Y or the W, why and how did these species evolve differentiated — and in the case of *P. vitticeps*, *B. duperreyi* and most birds — heteromorphic sex chromosomes? This implies there are mechanisms involved in sex chromosome evolution in taxa that have dosage-dependent sex determination that result in the degeneration of the member of the sex chromosome pair that does not contain the sex determining gene.

Sex reversal of the homogametic sex changes the selective landscape of sex chromosomes (Charlesworth 2009; Ezaz and Deakin 2014; Johnson and Lachance 2012; Wilson Sayres 2018). For example, in *C. ocellatus*, due to sex reversal of the XX genotype (**Chapter 4**), the X chromosome is present in males to a greater extent than it would be if strict GSD were in place, meaning the X chromosome is sometimes exposed to male recombination rates and male-specific selection. This may have resulted in novel conditions that favour a departure from the classic model of sex chromosome evolution.

References

Adkins-Regan E, Reeve HK. 2014. Sexual dimorphism in body size and the origin of sex-determination systems. Am Nat183: 519-536. doi: 10.1086/675303

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, Tree of Sex Consortium. 2014. Sex determination: why so many ways of doing it? PLoS Biology 12: e1001899. doi: 10.1371/journal.pbio.1001899

Birkhead TR, Møller AP. 1993. Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. Biol. J. Linn. Soc. 50: 295-311. doi: 10.1111/j.1095-8312.1993.tb00933.x

Blaser O, Neuenschwander S, Perrin N 2014. Sex-Chromosome Turnovers: The Hot-Potato Model. 183: 140-146. doi: 10.1086/674026

Boyle M, Hone J, Schwanz LE, Georges A. 2014. Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? Ecol. Evol. 4: 4522-4533. doi: 10.1002/ece3.1316

Bull J. 1981. Evolution of environmental sex determination from genotypic sex determination. Heredity 47: 173-184.

Cadby CD, Jones SM, Wapstra E. 2014. Geographical differences in maternal basking behaviour and offspring growth rate in a climatically widespread viviparous reptile. J. Exp. Biol. 217: 1175-1179. doi: 10.1242/jeb.089953

Caldwell AJ, While GM, Wapstra E. 2017. Plasticity of thermoregulatory behaviour in response to the thermal environment by widespread and alpine reptile species. Anim. Behav. 132: 217-227. doi: 10.1016/j.anbehav.2017.07.025

Capel B. 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat. Rev. Genet. 18: 675-689. doi: 10.1038/nrg.2017.60

Castelli MA, Georges A, Cherryh C, Rosauer DF, Sarre SD, Contador-Kelsall I, Holleley CE. 2020. Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard. Divers. Distrib. 00. doi: 10.1111/ddi.13203

Charlesworth B. 1991. The evolution of sex chromosomes. Science 251: 1030-1033.

Charlesworth B. 2009. Fundamental concepts in genetics: effective population size and patterns of molecular evolution and variation. Nat. Rev. Genet. 10: 195-205. doi: 10.1038/nrg2526

Charlesworth D, Charlesworth B, Marais G. 2005. Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128. doi: 10.1038/sj.hdy.6800697

Charlesworth D, Mank JE. 2010. The birds and the bees and the flowers and the trees: lessons from genetic mapping of sex determination in plants and animals. Genetics 186: 9-31. doi: 10.1534/genetics.110.117697

Charnov EL, Bull J. 1977. When is sex environmentally determined? Nature 266: 828-830. doi: 10.1038/266828a0

Cliff HB, Wapstra E, Burridge CP. 2015. Persistence and dispersal in a Southern Hemisphere glaciated landscape: the phylogeography of the spotted snow skink (*Niveoscincus ocellatus*) in Tasmania. BMC Evol. Biol. 15: 121. doi: 10.1186/s12862-015-0397-y

Cornejo-Paramo P, Dissanayake DSB, Lira-Noriega A, Martinez-Pacheco ML, Acosta A, Ramirez-Suastegui C, Mendez-de-la-Cruz FR, Szekely T, Urrutia AO, Georges A, Cortez D. 2020. Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. Genome Biol. Evol. 12: 924 - 930. doi: 10.1093/gbe/evaa104

Cunningham GD, While GM, Olsson M, Ljungström G, Wapstra E. 2020. Degrees of change: between and within population variation in thermal reaction norms of phenology in a viviparous lizard. Ecology 101: e03136. doi: 10.1002/ecy.3136

Cunningham GD, While GM, Wapstra E. 2017. Climate and sex ratio variation in a viviparous lizard. Biol. Lett. 13. doi: 10.1098/rsbl.2017.0218

Dissanayake DSB, Holleley CE, Hill L, O'Meally D, Deakin J, Georges A. 2020. Identification of Y chromosome markers in the eastern three-lined skink (*Bassiana duperreyi*) using in-silico whole genome subtraction. BMC Genom. 21: 667.

Donnellan SC 1985. The evolution of sex chromosomes in scincid lizards. [PhD Thesis]: Macquarie University.

Even MD, Dhar MG, Saal FSV. 1992. Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. J Reprod Fertil. 96: 709-716.

Ezaz T, Deakin JE. 2014. Repetitive sequence and sex chromosome evolution in vertebrates. Adv. Evol. Biol. 2014: 9. doi: 10.1155/2014/104683

Ezaz T, Moritz B, Waters P, Graves JA, Georges A, Sarre SD. 2009a. The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. Chromosome Res. 17: 965-973. doi: 10.1007/s10577-009-9102-6

Ezaz T, Quinn AE, Sarre SD, O'Meally D, Georges A, Graves JA. 2009b. Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. Chromosome Res. 17: 91-98. doi: 10.1007/s10577-008-9019-5

Ezaz T, Srikulnath K, Graves JA. 2017. Origin of Amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? J. Hered. 108: 94-105. doi: 10.1093/jhered/esw053

Friesen C, Kahrl A, Olsson M. 2020. Sperm competition in squamate reptiles. Philos. Trans. R. Soc. Lond. B Biol. Sci. 375: 20200079. doi: 10.1098/rstb.2020.0079

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D. 2015. Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. Mol. Biol. Evol. 32: 1296-1309. doi: 10.1093/molbev/msv023

Graves JA. 2013. How to evolve new vertebrate sex determining genes. Dev. Dyn. 242: 354-359. doi: 10.1002/dvdy.23887

Graves JA. 1995. The origin and function of the mammalian Y chromosome and Y-borne genes - an evolving understanding. BioEssays 17: 311-320. doi: 10.1002/bies.950170407

Helleu Q, Courret C, Ogereau D, Burnham KL, Chaminade N, Chakir M, Aulard S, Montchamp-Moreau C. 2019. Sex ratio meiotic drive shapes the evolution of the Y chromosome in *Drosophila simulans*. Mol. Biol. Evol. 36: 2668-2681. doi: 10.1093/molbev/msz160

Hill PL, Burridge CP, Ezaz T, Wapstra E. 2018. Conservation of sex-linked markers among conspecific populations of a viviparous skink, *Niveoscincus ocellatus*, exhibiting genetic and temperature-dependent sex determination. Genome Biol. Evol. 10: 1079-1087. doi: 10.1093/gbe/evy042

Hill P, Shams F, Burridge CP, Wapstra E, Ezaz T 2021a. Differences in homomorphic sex chromosomes are associated with population divergence in sex determination in *Carinascincus ocellatus* (Scincidae: Lygosominae). Cells 10: 291.

Hill PL, Wapstra E, Ezaz T, Burridge CP 2021. Pleistocene divergence in the absence of gene flow among populations of a viviparous reptile with intraspecific variation in sex determination Ecology and Evolution 00: 1-9. doi: DOI: 10.1002/ece3.7458

Holleley CE, O'Meally D, Sarre SD, Graves JA, Ezaz T, Matsubara K, Azad B, Zhang X, Georges A. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. Nature 523: 79-82. doi: 10.1038/nature14574

Holleley CE, Sarre SD, O'Meally D, Georges A. 2016. Sex reversal in reptiles: reproductive oddity or powerful driver of evolutionary change? Sex. Dev. 10: 279-287. doi: 10.1159/000450972

Iannucci A, Altmanova M, Ciofi C, Ferguson-Smith M, Milan M, Pereira JC, Pether J, Rehak I, Rovatsos M, Stanyon R, Velensky P, Rab P, Kratochvil L, Pokorna MJ. 2019. Conserved sex chromosomes and karyotype evolution in monitor lizards (Varanidae). Heredity 123: 215-227. doi: 10.1038/s41437-018-0179-6

Janzen FJ, Phillips PC. 2006. Exploring the evolution of environmental sex determination, especially in reptiles. J. Evol. Biol. 19: 1775-1784. doi: 10.1111/j.1420-9101.2006.01138.x

Johnson NA, Lachance J. 2012. The genetics of sex chromosomes: evolution and implications for hybrid incompatibility. Ann. N.Y. Acad. Sci. 1256: E1-E22. doi: 10.1111/j.1749-6632.2012.06748.x

Jones SM, Wapstra E, Swain R. 1997. Asynchronous male and female gonadal cycles and plasma steroid concentrations in a viviparous lizard, *Niveoscincus ocellatus* (Scincidae), from Tasmania. Gen. Comp. Endocrinol. 108: 271-281. doi: 10.1006/gcen.1997.6971

Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S, Matsuda Y. 2009. The ZW sex chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 118: 43-51. doi: 10.1007/s00412-008-0176-2

Kostmann A, Kratochvil L, Rovatsos M. 2020. Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation. Preprint. doi: 10.1101/2020.08.29.273524

Koubova M, Johnson Pokorna M, Rovatsos M, Farkacova K, Altmanova M, Kratochvil L. 2014. Sex determination in Madagascar geckos of the genus *Paroedura* (Squamata: Gekkonidae): are differentiated sex chromosomes indeed so evolutionary stable? Chromosome Res. 22: 441-452. doi: 10.1007/s10577-014-9430-z

Le Galliard JF, Fitze PS, Ferriere R, Clobert J. 2005. Sex ratio bias, male aggression, and population collapse in lizards. Proc. Natl. Acad. Sci. U. S. A. 102: 18231-18236. doi: 10.1073/pnas.0505172102

Lindholm AK, Dyer KA, Firman RC, Fishman L, Forstmeier W, Holman L, Johannesson H, Knief U, Kokko H, Larracuente AM, Manser A, Montchamp-Moreau C, Petrosyan VG, Pomiankowski A, Presgraves DC, Safronova LD, Sutter A, Unckless RL, Verspoor RL, Wedell N, Wilkinson GS, Price TAR. 2016. The ecology and evolutionary dynamics of meiotic drive. Trends Ecol. Evol. 31: 315-326. doi: 10.1016/j.tree.2016.02.001

Matsubara K, Gamble T, Matsuda Y, Zarkower D, Sarre SD, Georges A, Graves JA, Ezaz T. 2014. Non-homologous sex chromosomes in two geckos (Gekkonidae: Gekkota) with female heterogamety. Cytogenet. Genome Res. 143: 251-258. doi: 10.1159/000366172

Matsubara K, O'Meally D, Azad B, Georges A, Sarre SD, Graves JA, Matsuda Y, Ezaz T. 2016. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. Chromosoma 125: 111-123. doi: 10.1007/s00412-015-0531-z

Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara C, Agata K, Matsuda Y. 2006. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. Proc. Natl. Acad. Sci. U. S. A. 103: 18190-18195. doi: 10.1073/pnas.0605274103

Matsumoto T, Yoshida K, Kitano J. 2017. Contribution of gene flow to the evolution of recombination suppression in sex chromosomes. J. Theor. Biol. 431: 25-31. doi: 10.1016/j.jtbi.2017.08.004

Meiklejohn CD, Tao Y. 2010. Genetic conflict and sex chromosome evolution. Trends Ecol. Evol. 25: 215-223. doi: 10.1016/j.tree.2009.10.005

Mitchell NJ, Janzen FJ. 2010. Temperature-dependent sex determination and contemporary climate change. Sex. Dev. 4: 129-140. doi: 10.1159/000282494

O'Neill MJ, O'Neill RJ. 2018. Sex chromosome repeats tip the balance towards speciation. Mol. Ecol. 27: 3783-3798. doi: 10.1111/mec.14577

Olsson M, Schwartz T, Uller T, Healey M. 2007. Sons are made from old stores: sperm storage effects on sex ratio in a lizard. Biol. Lett. 3: 491-493. doi: 10.1098/rsbl.2007.0196

Pan QW, Anderson J, Bertho S, Herpin A, Wilson C, Postlethwait JH, Schartl M, Guiguen Y. 2016. Vertebrate sex-determining genes play musical chairs. C. R. Biol. 339: 258-262. doi: 10.1016/j.crvi.2016.05.010

Pen I, Uller T, Feldmeyer B, Harts A, While GM, Wapstra E. 2010. Climate-driven population divergence in sex-determining systems. Nature 468: 436-438. doi: 10.1038/nature09512

Pennell MW, Mank JE, Peichel CL. 2018. Transitions in sex determination and sex chromosomes across vertebrate species. Mol. Ecol. 27: 3950-3963. doi: 10.1111/mec.14540

Perrin N 2009. Sex reversal: a fountain of youth for sex chromosomes? Evolution 63: 3043-3049. doi: 10.1111/j.1558-5646.2009.00837.x

Pokorna M, Altmanova M, Kratochvil L. 2014. Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. Chromosome Res. 22: 35-44. doi: 10.1007/s10577-014-9403-2

Pokorna M, Kratochvil L. 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? Zool. J. Linn. Soc. 156: 168-183.

Ponnikas S, Sigeman H, Abbott JK, Hansson B. 2018. Why do sex chromosomes stop recombining? Trends Genet. 34: 492-503. doi: 10.1016/j.tig.2018.04.001

Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JA. 2007. Temperature sex reversal implies sex gene dosage in a reptile. Science 316: 411. doi: 10.1126/science.1135925

Quinn AE, Sarre SD, Ezaz T, Graves JA, Georges A. 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. Biol. Lett. 7: 443-448. doi: 10.1098/rsbl.2010.1126

Reichwald K, Petzold A, Koch P, Downie BR, Hartmann N, Pietsch S, Baumgart M, Chalopin D, Felder M, Bens M, Sahm A, Szafranski K, Taudien S, Groth M, Arisi I, Weise A, Bhatt SS, Sharma V, Kraus JM, Schmid F, Priebe S, Liehr T, Görlach M, Than ME, Hiller M, Kestler HA, Volff J, Schartl M, Cellerino A, Englert C, Platzer M. 2015. Insights into sex chromosome evolution and aging from the genome of a short-lived fish. Cell 163: 1527-1538. doi: 10.1016/j.cell.2015.10.071

Rice WR. 1987. The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex chromosomes. Evolution 41: 911-914. doi: 10.2307/2408899

Rovatsos M, Altmanova M, Pokorna M, Kratochvil L. 2014. Conserved sex chromosomes across adaptively radiated *Anolis* lizards. Evolution 68: 2079-2085. doi: 10.1111/evo.12357

Rovatsos M, Vukic J, Kratochvil L. 2016. Mammalian X homolog acts as sex chromosome in lacertid lizards. Heredity 117: 8-13. doi: 10.1038/hdy.2016.18

Sander van Doorn GS, Kirkpatrick M 2007. Turnover of sex chromosomes induced by sexual conflict. Nature 449: 909-912. doi: 10.1038/nature06178

Sander van Doorn GS, Kirkpatrick M 2010. Transitions between male and female heterogamety caused by sex-antagonistic selection. Genetics 186: 629-645. doi: 10.1534/genetics.110.118596

Sarre SD, Ezaz T, Georges A. 2011. Transitions between sex-determining systems in reptiles and amphibians. Annu. Rev. Genomics Hum. Genet. 12: 391-406. doi: 10.1146/annurev-genom-082410-101518

Sarre SD, Georges A, Quinn A. 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26: 639-645. doi: 10.1002/bies.20050

Schwanz LE, Ezaz T, Gruber B, Georges A. 2013. Novel evolutionary pathways of sex-determining mechanisms. J. Evol. Biol. 26: 2544-2557. doi: 10.1111/jeb.12258

Schwanz LE, Georges A, Holleley CE, Sarre SD. 2020. Climate change, sex reversal and lability of sex-determining systems. J. Evol. Biol. 33: 270-281. doi: 10.1111/jeb.13587

Shine R, Elphick MJ, Donnellan S. 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. Ecol. Lett. 5: 486-489. doi: 10.1046/j.1461-0248.2002.00351.x

Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, Sinclair AH. 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461: 267-271. doi: 10.1038/nature08298

Uller T. 2003. Viviparity as a constraint on sex-ratio evolution. Evolution 57: 927-931. doi: 10.1554/0014-3820

Uller T, Helantera H. 2011. From the origin of sex-determining factors to the evolution of sex-determining systems. Q. Rev. Biol. 86: 163-180.

Uller T, Olsson M. 2003. Prenatal sex ratios influence sexual dimorphism in a reptile. J. Exp. Zool. A Comp. Exp. Bio. 295: 183-187.

Uller T, While GM, Cadby CD, Harts A, O'Connor K, Pen I, Wapstra E. 2011. Altitudinal divergence in maternal thermoregulatory behaviour may be driven by differences in selection on offspring survival in a viviparous lizard. Evolution 65: 2313-2324. doi: 10.1111/j.1558-5646.2011.01303.x

Valenzuela N, Adams DC, Janzen FJ. 2003. Pattern does not equal process: exactly when is sex environmentally determined? Am Nat 161: 676-683. doi: 10.1086/368292

Valenzuela N, Literman R, Neuwald JL, Mizoguchi B, Iverson JB, Riley JL, Litzgus JD. 2019. Extreme thermal fluctuations from climate change unexpectedly accelerate demographic collapse of vertebrates with temperature-dependent sex determination. Sci. Rep. 9. doi: 10.1038/s41598-019-40597-4

Vicoso B, Kaiser VB, Bachtrog D. 2013. Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. Proc. Natl. Acad. Sci. U. S. A. 110: 6453-6458. doi: 10.1073/pnas.1217027110

Wapstra E, Olsson M, Shine R, Edwards A, Swain R, Joss JM. 2004. Maternal basking behaviour determines offspring sex in a viviparous reptile. Biol. Lett. 271 Suppl 4: S230-232. doi: 10.1098/rsbl.2003.0152

Wapstra E, Swain R, Jones SM, O'Reilly J. 1999. Geographic and annual variation in reproductive cycles in the Tasmanian spotted snow skink, *Niveoscincus ocellatus* (Squamata : Scincidae). Aust. J. Zool. 47: 539-550. doi: 10.1071/zo99038

Wapstra E, Uller T, Sinn DL, Olsson M, Mazurek K, Joss J, Shine R. 2009. Climate effects on offspring sex ratio in a viviparous lizard. J. Anim. Ecol. 78: 84-90. doi: 10.1111/j.1365-2656.2008.01470.x

Waters PD, Wallis MC, Marshall Graves JA. 2007. Mammalian sex-Origin and evolution of the Y chromosome and SRY. Semin. Cell. Dev. Biol. 18: 389-400. doi: 10.1016/j.semcdb.2007.02.007

Wedekind C. 2017. Demographic and genetic consequences of disturbed sex determination. Phil. Trans. R. Soc. B 372. doi: 10.1098/rstb.2016.0326

Wilson Sayres MA. 2018. Genetic diversity on the sex chromosomes. Genome Biol. Evol. 10: 1064-1078. doi: 10.1093/gbe/evy039