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Temperature-Induced Sex Reversal in Reptiles: Prevalence, Discovery, and Evolutionary Implications

Sarah L. Whiteley[a, b] Meghan A. Castelli[a, b] Duminda S.B. Dissanayake[a, b]
Clare E. Holleley[b] Arthur Georges[a]

[a]Institute for Applied Ecology, University of Canberra, Canberra, ACT, Australia; [b]Australian National Wildlife Collection, CSIRO, Canberra, ACT, Australia

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Abstract

Sex reversal is the process by which an individual develops a phenotypic sex that is discordant with its chromosomal or genotypic sex. It occurs in many lineages of ectothermic vertebrates, such as fish, amphibians, and at least one agamid and one scincid reptile species. Sex reversal is usually triggered by an environmental cue that alters the genetically determined process of sexual differentiation, but it can also be caused by exposure to exogenous chemicals, hormones, or pollutants. Despite the occurrence of both temperature-dependent sex determination (TSD) and genetic sex determination (GSD) broadly among reptiles, only 2 species of squamates have thus far been demonstrated to possess sex reversal in nature (GSD with overriding thermal influence). The lack of species with unambiguously identified sex reversal is not necessarily a reflection of a low incidence of this trait among reptiles. Indeed, sex reversal may be relatively common in reptiles, but little is known of its prevalence, the mechanisms by which it occurs, or the consequences of sex reversal for species in the wild under a changing climate. In this review, we present a roadmap to the discovery of sex reversal in reptiles, outlining the various techniques that allow new occurrences of sex reversal to be identified, the molecular mechanisms that may be involved in sex reversal and how to identify them, and approaches for assessing the impacts of sex reversal in wild populations. We discuss the evolutionary implications of sex reversal and use the central bearded dragon (*Pogona vitticeps*) and the eastern three-lined skink (*Bassiana duperreyi*) as examples of how species with opposing patterns of sex reversal may be impacted differently by our rapidly changing climate. Ultimately, this review serves to highlight the importance of understanding sex reversal both in the laboratory and in wild populations and proposes practical solutions to foster future research.

The sex of an animal is one of its most fundamental traits as it shapes sex-specific morphology, physiology, and behaviour. In vertebrates, sex can be determined either genetically or by the environment. For species with genetic sex determination (GSD), the sexual phenotype is concordant with its chromosomal complement. For spe-

karger@karger.com
www.karger.com/sxd

Correspondence to:
Arthur Georges, georges@aerg.canberra.edu.au

Karger

cies with temperature-dependent sex determination (TSD), the sexual phenotype is determined by the environmental conditions that embryos experience in the absence of sex chromosomes [Valenzuela et al., 2003]. In some species with GSD, however, environmental factors (e.g., temperature) can override the genetic determinant and cause sex reversal during embryonic development, resulting in an individual with a sexual phenotype that is discordant with its genotype [Valenzuela et al., 2003; Holleley et al., 2016; Weber and Capel, 2018]. For species with male heterogamety (XX/XY), this usually results in reversal of the female genotype and generation of XX males, and for species with female heterogamety (ZZ/ZW), this results in reversal of the male genotype and generation of ZZ females. One fish and one amphibian species display reversal of the heterogametic sex (generation of ZW males in response to temperature), but this has not been observed in any amniote, presumably because of reduced fitness or viability arising from the production of YY or WW individuals [Dupoué et al., 2019; Wang et al., 2019; Hattori et al., 2020]. In reptiles, temperature is the only factor definitively demonstrated to influence sex, though some evidence exists for water restriction [Dupoué et al., 2019].

Sex reversal occurs commonly in fish [Wang et al., 2019; Hattori et al., 2020] and in some amphibians [Eggert, 2004; Flament, 2016; Nemesházi et al., 2020], but it has been confirmed in only 2 reptiles, the Australian central bearded dragon (*Pogona vitticeps*) and the eastern three-lined skink (*Bassiana duperreyi*) [Shine et al., 2002; Quinn et al., 2007; Holleley et al., 2015]. Sex reversal may also occur in the yellow-bellied water skink (*Eulamprus heatwolei*), the common collared lizard (*Crotaphytus collaris*), the multi-ocellated racerunner (*Eremias multiocellata*), the Japanese gecko (*Gekko japonicus*), the spotted snow skink (*Niveoscincus ocellatus*), and the Jacky dragon (*Amphibolurus muricatus*), though this has not yet been definitively confirmed [Tokunaga, 1985; Wang et al., 2015; Hill et al., 2018; Cornejo-Paramo et al., 2020; Wiggins et al., 2020; Whiteley et al., 2021a]. As a result, little is known about the mechanisms or consequences of sex reversal. The paucity of reptile species with confirmed sex reversal systems is not necessarily a reflection of a low incidence of this trait. Identifying and confirming genotypic sex in reptiles can be challenging, requiring considerable time and resources to develop reliable assays. Homomorphic sex chromosomes are common in reptiles, necessitating the use of advanced cytological techniques [Ezaz et al., 2005] or sequencing technologies to identify sex-specific sequences [Sankovic et al., 2006; Chen et al., 2012; Traut et al., 2013; Palmer et al., 2019; Cornejo-Paramo et al., 2020; Dissanayake et al., 2020]. There is also limited understanding as to the individual- and population-level consequences of sex reversal in the wild, particularly under changing climatic regimes [Bókony et al., 2017; Schwanz et al., 2020]. The occurrence of sex reversal in a species has considerable implications for conservation management, particularly given the climatic perturbations caused by global warming.

In this review, we outline the various techniques that allow new examples of sex reversal to be identified, candidate molecular mechanisms for sex reversal, and approaches for assessing the impacts of sex reversal in wild populations, with particular attention to the difficulties of confirming both genotypic and phenotypic sex in reptiles. We discuss the evolutionary implications of sex reversal using *P. vitticeps* and *B. duperreyi* as case studies to assess how different species with sex reversal may be impacted by a rapidly changing climate.

Molecular Mechanisms Driving Sex Reversal

How sex reversal occurs at a molecular level is currently not known, though it may involve temperature sensing through calcium and reactive oxygen species signalling in *P. vitticeps* [Weber and Capel, 2018; Castelli et al., 2020; Whiteley et al., 2021b]. While these ancient and ubiquitous environmental sensing mechanisms are very promising candidates for the transduction of an environmental signal to a sex determining signal, experimental demonstration is lacking [Castelli et al., 2020].

Calcium and redox signalling pathways are conserved between phylogenetically disparate species, and thus further insight about sex reversal mechanisms may be gleaned from well-studied TSD models such as the red-eared slider turtle, (*Trachemys scripta*). In this species, calcium signalling at high temperatures was causally demonstrated to be required for female development [Weber et al., 2020]. In *T. scripta, P. vitticeps*, and the American alligator (*Alligator mississippiensis*), the epigenetic modifier KDM6B and potentially its splicing variants play an important role in the temperature-driven regulation of sex determination [Deveson et al., 2017]. In *T. scripta,* KDM6B initiates the male developmental cascade via demethylation of the promoter region of the male pathway-initiating gene *DMRT1* [Ge et al., 2017, 2018]. Despite little being currently understood about sex reversal, it is clear that there are similarities between *P. vitticeps* and distantly related alligator and turtle species with TSD. Ongoing research effort is required to better understand what genes and pathways are conserved between these evolutionarily disparate lineages.

The reptile phylogeny is marked by numerous transitions between TSD and GSD systems even between closely related lineages [Pokorna and Kratochvil, 2016]. This, coupled with the broad phylogenetic distribution of developmental processes associated with thermolabile sex (asynchronous gonadal and genital development), lay the groundwork for future investigations of the occurrence of sex reversal [Whiteley, 2018, 2021a]. Ultimately, sex reversal must occur by repression of the sex signals originating from the sex chromosomes and amplification of signals for the development of the opposite sex driven by incubation temperature. The epigenetic mechanisms by which this occurs remain unknown and is a compelling area of ongoing research.

Detection of Sex Reversal

Sex-reversed individuals can be either generated in the laboratory or found in the wild. However, many reptiles possess poorly differentiated sex microchromosomes, and many more have homomorphic sex macrochromosomes, so instances of sex reversal do not easily come to attention. This can complicate both the detection of sex reversal and the identification of the mechanisms driving it, because both genetic and environmental factors may contribute to sex determination even in the absence of discernible sex chromosomes [Girondot et al., 1994].

The first indications of sex reversal in a species may be gleaned from incubation experiments where eggs are incubated across a range of temperatures and the offspring sex ratios are obtained. Many TSD species exhibit sex ratio patterns where females are produced at high and low temperatures, and balanced sex ratios are produced at intermediate temperatures [Nagahama et al., 2020]. Modelling has led to the hypothesis that species with this pattern may possess an underlying ZZ/ZW system [Quinn et al., 2011]. Indeed, there is evidence to suggest this may be the case for the Jacky dragon, *A. muricatus* [Whiteley et al., 2021a].

Cytogenetic techniques present opportunities to detect new examples of sex reversal by identification of sex chromosomes and a mismatch between the chromosome complement and sexual phenotype. Early research involved using approaches like AFLPs, CGH, and BAC-mapping [Traut et al., 2001; Lee et al., 2011; Ezaz et al., 2013; Deakin et al., 2019]. Now, a variety of sequencing-based approaches are possible, particularly since the advent of next-generation sequencing, which allows for techniques like assaying the sex-biased expression of candidate genes with RNA-seq [Ayers et al., 2013] or mapping read depth in whole genome sequencing to identify regions on sex chromosomes [Chen et al., 2012; Bidon et al., 2015]. Reduced representation sequencing approaches such as RAD-seq, ddRAD-seq, and DArT-seq [Kilian et al., 2012; Gamble and Zarkower, 2014; Gamble, 2016; Hill et al., 2018; Nemesházi et al., 2020] can also be useful. However, these techniques target only a subset of the genome and may fail to identify markers in species with limited genetic differentiation between the sexes [Lowry et al., 2017]. It is also becoming increasingly important to incorporate both cytogenetic and sequencing-based approaches to obtain the most complete picture of sex chromosomes in a species [Deakin et al., 2019]. For example, in *B. duperreyi,* heteromorphic sex chromosomes were first detected using cytogenetic approaches [Shine et al., 2002] and then sex reversal was first demonstrated using PCR targeting a sequence unique to the Y chromosome [Radder et al., 2008], which was later refined using similar methods [Quinn et al., 2009]. New markers for this species have also been identified using an in silico whole genome subtraction approach [Dissanayake et al., 2020].

Laboratory experimentation is necessary to quantitatively characterise temperature-sex relationships, typically through examining sexual outcomes resulting from controlled crosses and incubation experiments. However, it is equally important to demonstrate the occurrence of sex reversal in wild populations and whether the trigger of sex reversal established in the laboratory also affects natural populations. Evidence is growing in some fish species that multiple cues may trigger sex reversal, but these cues may not always be biologically relevant in natural populations [Eggert, 2004; García-Cruz et al., 2020; Sakae et al., 2020]. Some studies in TSD reptiles show evidence of interactions between different environmental variables as well as maternal effects like egg size and yolk hormones [Radder et al., 2009; Ballen et al., 2016; Dupoue et al., 2019]. So while laboratory-based experiments will always be essential, appropriate field studies will be required to avoid misinterpretation of what could be a laboratory artefact caused by conditions that do not occur in the wild.

The identification of species with sex reversal can be difficult, and a lack of clear direction for how to definitively demonstrate sex reversal in a species has likely contributed to a lack of identification. We present a workflow to provide guidance for the process of identifying sex re-

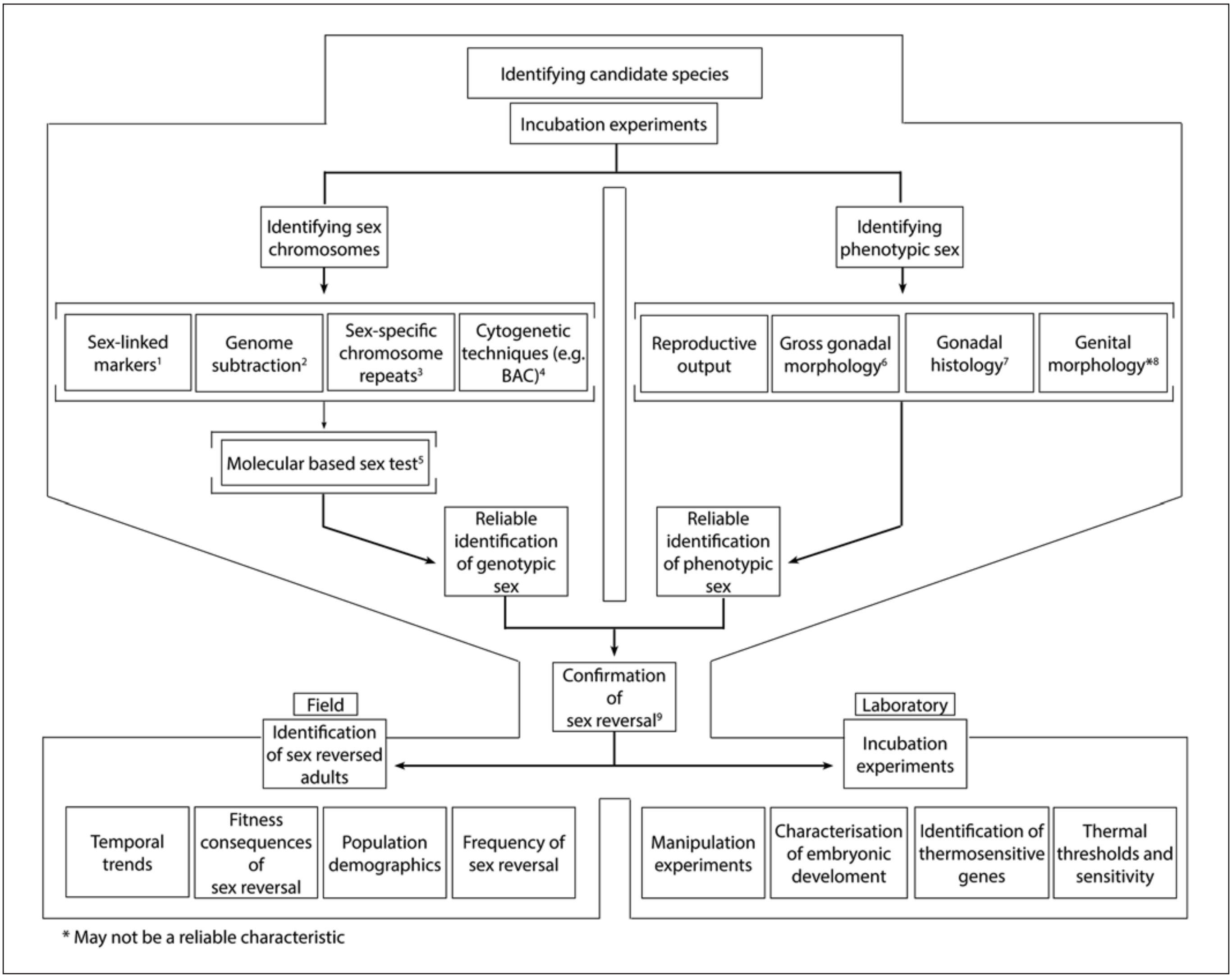

Fig. 1. Workflow proposing strategies for identifying new examples of sex reversal. Reliable confirmation of both genotypic and phenotypic sex is ultimately required to identify sex reversal in a new species, which is defined as an individual being discordant between its genotypic and phenotypic sex. Potential research applications in both field and laboratory studies are also suggested following the identification of sex reversal. See references for further details on how these approaches and techniques have been used. 1, Trenkel et al. [2020]; 2, Dissanayake et al. [2020]; 3, Rovatsos et al. [2015]; 4, Deakin et al. [2019]; 5, Hill et al. [2018]; 6, 7, Antonio-Rubio et al. [2015]; 8, Harlow [1996]; 9, Quinn et al. [2007].

versal and propose numerous avenues for research in both field and laboratory settings (Fig. 1). Ultimately, reliable identification of both genotypic and phenotypic sex is required to definitively demonstrate the occurrence of sex reversal in a species (Fig. 1).

Examples of Reptile Sex Reversal

P. vitticeps has a ZZ/ZW GSD system, but high incubation temperatures (>32°C) result in reversal of males (ZZ genotype) to phenotypic females in the laboratory and in the wild [Ezaz et al., 2005; Quinn et al., 2007; Holleley et al., 2015]. *B. duperreyi* has an XX/XY system of GSD in which low incubation temperatures (<20°C) result in reversal of the female XX genotype to phenotypic male [Radder et al., 2008]. The directionality of sex reversal in these 2 cases may not be coincidental for it avoids the production of WW and YY individuals and the associated fitness consequences in both cases. The contrasting GSD systems and sex reversal conditions of these species make them ideal to compare the effects of sex reversal in wild

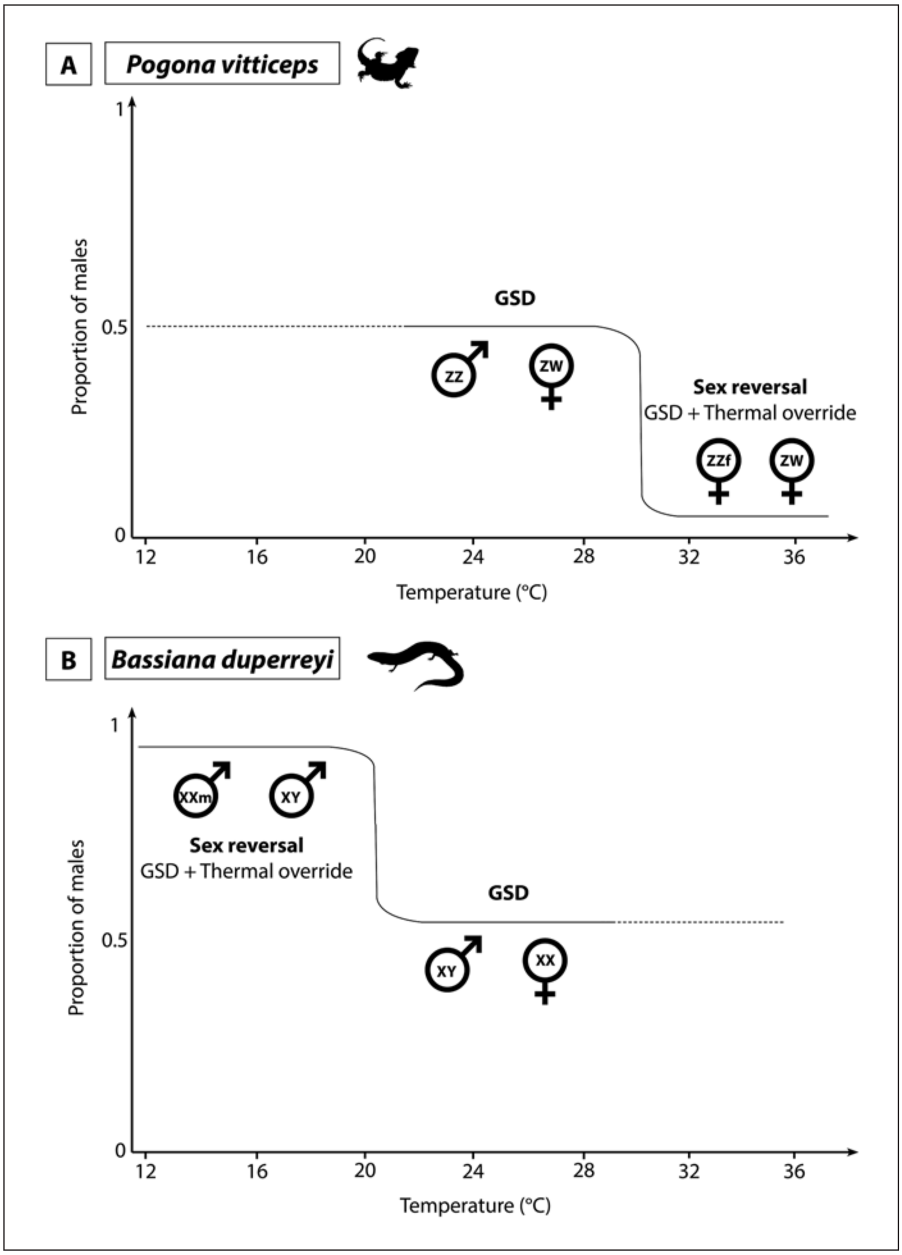

Fig. 2. Schematic representation of sex reversal characteristics in *P. vitticeps* and *B. duperreyi*. In *P. vitticeps* (**A**) sex reversal occurs when an individual with a male genotype (ZZ) is incubated at temperatures above 32°C, causing it to develop as a phenotypic female. In *B. duperreyi* (**B**) an individual with a female phenotype (XX) incubated at low temperatures will reverse its sex and develop as a phenotypic male.

populations (Fig. 2). We focus our attention on these 2 species as the only definitive and well-studied cases of reptile sex reversal in both the laboratory and the wild.

In both *P. vitticeps* and *B. duperreyi,* sex-reversed individuals occur in the wild, albeit at a lower proportion than either of their concordant counterparts. In *P. vitticeps*, sex-reversed ZZ females are fertile, with greater fecundity than concordant ZW females [Holleley et al., 2015], and there are other attributes with implications for their fitness, like increased levels of activity and boldness [Li et al., 2016; Jones et al., 2020]. The offspring of sex-reversed females have a greater propensity to reverse, which may influence sex ratios across populations. There is conflicting evidence for this in wild populations. The occurrence of sex reversal was reported to increase in a wild population between 2003 and 2011 [Holleley et al., 2015], but a second long-term, range-wide study did not find evidence of an increase in the rate of sex reversal over a 38-year period [Castelli et al., 2020]. Specifically, sex reversal occurred in 12 of the 38 years, at rates ranging from

6 to 27% of phenotypically female individuals [Castelli et al., 2020]. In *B. duperreyi*, 28% sex-reversed XX males arose in nests [Holleley et al., 2016] and survived to adulthood [Dissanayake et al., 2021], but whether the sex-reversed males are fertile remains to be determined. Unlike *P. vitticeps*, gravid females and viable eggs are not evidence of fertility of sex-reversed individuals, because the father of the clutch is typically unknown. Controlled mating and incubation experiments will be required to confirm that sex-reversed XX males are in fact capable of producing viable sperm and fertilizing eggs. Alternatively, parentage studies of exhaustively sampled wild populations may be able to detect sex-reversed fathers of clutches, but this approach is complicated in the case of *B. duperreyi* by communal nesting. Behaviour and survivorship of sex-reversed *B. duperreyi* has not yet been studied, so the fitness consequences of reversal are unclear. Ultimately, if these phenotypic effects result in altered survivability or reproductive success then sex reversal can affect population demographic processes and transitions from GSD to TSD.

Population Processes and Transitions between Sex Determining Systems

The mechanisms of sex determination are rapidly evolving in many vertebrate species [Bachtrog et al., 2014]. However, it is not understood whether this rapid evolution of sex determination mechanisms in reptiles is enough to accommodate current climatic change or indeed if species are exhibiting sex reversal under pressure from changing environmental conditions. Modelling shows that as the frequency of sex reversal increases in a population, a likely response is a reduction in frequency and possible losses of the W or Y chromosome under Fisher's frequency-dependent selection [Fisher, 1930; Charnov and Bull, 1977]. Alternatively, an evolutionary response that alters the temperature at which sex reversal occurs could happen; however, effective heritability in temperature thresholds is low so the former scenario is more likely [McGaugh and Janzen, 2011]. As a result, evolution of thermal thresholds is slow, whereas decline in frequency and ultimately loss of the Y or W chromosome is driven by the much stronger frequency-dependent selection [Fisher, 1930]. Such loss of the W chromosome is predicted to happen in *P. vitticeps* under a warming climate in small, isolated populations [Schwanz et al., 2020]. We also predict the complete loss of the Y chromosome from the wild population of *B. duperreyi* at higher elevational sites should the climate ever cool [Dissanayake et al., 2021]. Indeed, Fisher's frequency-dependent selection alone is sufficient to drive transitions between GSD and TSD under changing climate scenarios [Holleley et al., 2015]. However, conventional selection under a range of proposed scenarios [see Schwanz and Georges, this issue] may still be required to maintain TSD once it has been achieved.

Latitude and altitude, as landscape correlates of average temperature conditions, are useful for generating expectations about the frequency and spatial distribution of sex reversal. Indeed, cooler alpine areas have the highest rates of sex reversal in *B. duperreyi*, which decrease with decreasing elevation and associated increases in mean air temperature [Dissanayake et al., 2021]. The field observations agree well with controlled laboratory experiments incubating eggs at different temperatures [Shine et al., 2002; Radder et al., 2008]. Increasing global temperatures are likely to alleviate the demographic impact of sex ratio skew in *B. duperreyi* because temperatures consistently higher than 23°C result in a genetic influence only, producing a 50:50 sex ratio. However, even short term decreases in temperature (<20°C) during the natural incubation period, likely to persist during global warming, will be sufficient to cause sex reversal in high elevation populations [Dissanayake et al., 2021]. So far, we know little about the sex reversal frequency in natural nests compared to the adults in higher elevational sites where nest temperatures are below 20°C. Our proposed models for *B. duperreyi* show that the frequency of the XY genotype is predicted to decline with decreasing incubation temperature as the system maintains a 1:1 sex ratio equilibrium. In fact, under current climate regimes within the species range, some with averages below 18°C, we expect the complete loss of the Y chromosome at some elevational sites [Dissanayake et al., 2021].

The case of *P. vitticeps* appears more complex under warming climatic conditions. The overproduction of females by the reversal of the ZZ genotype to a female phenotype will deliver a disadvantage to the ZW females under Fisher's frequency-dependent selection. This will potentially drive the frequency of the ZW genotype down as the system comes to a local equilibrium. We expect to see (i) an increase in the frequency of ZZ reversal and (ii) a decrease in the frequency of the ZW genotype with increasing latitude across the widespread range of *P. vitticeps*. However, widespread sex reversal and overabundance of females will result in selection for the rarer sex [see Schwanz and Georges, this issue], which under warming conditions would be ZZ males, resulting in evolution of the sex reversal threshold. Although the frequency of sex reversal is spatially clustered in *P. vitticeps*, no trend in the frequency of sex reversal was observed with latitude, and sex reversal was absent in the hottest parts of the species range [Castelli et al., 2021]. Thus, local adaptation in the propensity to reverse sex may provide a better explanation for the distribution of reversal in this species [Castelli et al., 2021]. Individuals that do not sex reverse at sex-reversing temperatures (possessing a higher individual threshold for sex reversal) have a reproductive advantage, and their offspring may inherit this higher sex reversal threshold [Schwanz et al., 2020]. In this way, a transition to a TSD system and loss of the W chromosome may not occur under climate change if local adaptation in the pivotal temperature for sex reversal occurs fast enough to avoid the action of frequency-dependent selection in bringing the sex ratio to equilibrium. The fact that *P. vitticeps* has accommodated different climatic regimes across the landscape through local adaptation in the propensity to sex reverse over evolutionary time does not necessarily translate to an adequate capacity to respond in the same way under rapid climate change. Climate change may threaten numerous species with environmentally sensitive sex determination, largely by skewing population sex ratios [Mitchell and Janzen, 2010; Refsnider and Janzen, 2016; Bókony et al., 2017; Booth et al., 2020; Breitenbach et al., 2020; Geffroy and Wedekind, 2020]. The complex ways that climate change may interact with sex reversal in different species remain to be investigated fully.

Conclusion

As little is currently understood about sex reversal in reptiles, this understudied area warrants far greater attention. As long as sex reversal is confirmed only in 2 species, it will remain unclear as to whether sex reversal is widespread throughout the reptile phylogeny or if it occurs only rarely. We suggest there is likely an unappreciated diversity in sex reversal cues and mechanisms in reptiles, which may impact wild populations, particularly in the face of a rapidly changing climate. Future research will greatly benefit from marrying field and lab-based research to better understand all aspects of sex reversal. There is much to be gained from identifying sex reversal in additional species and establishing new sex reversal model systems. This will inform on the commonalities and differences in molecular mechanisms underlying sex reversal and its fitness consequences. Ultimately by understanding sex reversal, we can comprehend the complex ways in which the environment can interact with sex to drive the evolution of sex determination systems in reptiles and vertebrates more broadly.

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Conflict of Interest Statement

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Author Contributions

S.L.W. led the writing of this review, to which all other authors contributed substantially.

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