Sex and stress: Is stress both a mediator and a consequence of sex reversal in the central bearded dragon (*Pogona vitticeps*)?

by

Meghan A. Castelli

BSc (Hons)

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy (PhD)
July 2021

Institute for Applied Ecology
University of Canberra, Australia

Abstract

Among vertebrates, sex determining systems are diverse and range on a continuum from entirely genetic sex determination (GSD) to purely environmental sex determination (ESD). The central bearded dragon (*Pogona vitticeps*) possesses a heterogametic (ZZ male/ZW female) system of genetic sex determination, but high egg incubation temperatures induce sex reversal, in which ZZ genotypic males develop as female. The biological mechanism by which temperature is translated into a sexual outcome is not fully understood in reptiles but is proposed to involve the vertebrate stress axis, a highly conserved environmental sensory mechanism which generates physiological responses to stress through glucocorticoid hormone production. Here I demonstrate using developmental transcriptomes and chemical manipulation experiments that the stress axis is unlikely to mediate sex reversal, and instead find evidence for the involvement of oxidative stress responses or circadian rhythm regulation in high temperature sex reversal. The relevance of these molecular studies is contextualised by a range-wide study of sex reversal and population genetic structure which demonstrates the lack of a clear relationship between climate and sex reversal in the wild. I have proposed that the threshold temperature for sex reversal (and thus the underlying genetic network which determines the threshold) varies across the landscape, having evolved in response to higher average incubation temperatures in warmer regions of the species range. Together, these studies demonstrate that not only is temperature sex reversal in reptiles a molecular process likely driven by sensory mechanisms other than the stress axis, but it also has complex evolutionary dynamics in the wild.

Authorship of Thesis

Except where clearly acknowledged in footnotes, quotations and the bibliography, I certify that I am the sole author of the thesis submitted today entitled:

Sex and stress: Is stress both a mediator and a consequence of sex reversal in the central bearded dragon (*Pogona vitticeps*)?

I further certify that to the best of my knowledge the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis.

The material in the thesis has not been the basis of an award of any other degree or diploma except where due reference is made in the text of the thesis.

The thesis complies with University requirements for a thesis as set out in the *Examination of Higher Degree by Research Theses Policy:* https://www.canberra.edu.au/research/current-research-students/hdr-policy-and-procedures

Candidate's Signature — 05/02/2021 Date

Primary Supervisor's Signature — 05/02/2021 Date

Publications

The following chapters have been peer reviewed and published in scientific journals.

Chapter 2: Cellular calcium and redox regulation: The mediator of vertebrate environmental sex determination?

Castelli, M. A., Whiteley, S. L., Georges, A., & Holleley, C. E. (2020). Cellular calcium and redox regulation: the mediator of vertebrate environmental sex determination? Biological Reviews, 95(3), 680–695. https://doi.org/10.1111/brv.12582

Chapter 4: Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*)

Castelli, M., Georges, A., & Holleley, C. E. (2021). Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (Pogona vitticeps). Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, Early view. https://doi.org/10.1002/jez.2441

Chapter 6: Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard

Castelli, M. A., Georges, A., Cherryh, C., Rosauer, D. F., Sarre, S. D., Contador-Kelsall, I., & Holleley, C. E. (2020). Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard. Diversity and Distributions, Early view. https://doi.org/10.1111/ddi.13203

Declaration of Co-Authored Publications

Declaration for Thesis Chapter 2: Cellular calcium and redox regulation: The mediator of vertebrate environmental sex determination?

DECLARATION BY CANDIDATE

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
Synthesis of idea, literature review and writing	50%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Sarah L. Whitelely	Co-first author. Equal party to synthesis and writing.	Yes
Arthur Georges	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No
Clare E. Holleley	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No

_____ 20/01/2021

Candidate's Signature Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Location(s):	University of Canberra, Institute for Applied Ecology

Signatures	Date
Arthur Georges.	20/1/21
	20/1/21
	20/1/21

Declaration for Thesis Chapter 4: Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*)

DECLARATION BY CANDIDATE

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
Experimental design, data collection, analysis and writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Arthur Georges	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No
Clare E. Holleley	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No

20/01/2021

Candidate's Signature Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

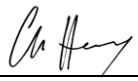
(10) there are no other authors of the publication according to these criteria;

(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Location(s):	University of Canberra, Institute for Applied Ecology

Signatures	Date
Arthur Georges.	20/1/21
	20/1/21

Declaration for Thesis Chapter 5: Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard

DECLARATION BY CANDIDATE

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contributions (%)
Data collection, analysis, and led manuscript writing	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Contributor is also a UC student (Yes/No)
Caitlin Cherryh	Code development and data analysis in collaboration with the candidate	No
Arthur Georges	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No
Clare E. Holleley	Contribution did not exceed that normally expected of a supervisor, covering all aspects of the work.	No
Stephen Sarre	Contributed to data analysis with the candidate and writing	No
Isabella Contador-Kelsall	Contributed to data collection	No
Dan Rosauer	Contribution did not exceed that normally expected of an advisor, covering all aspects of the work.	No

_____ 20/01/2021

Candidate's Signature Date

DECLARATION BY CO-AUTHORS

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

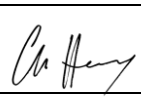
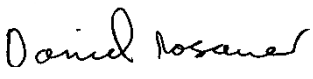
(16) there are no other authors of the publication according to these criteria;

(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Location(s):	University of Canberra, Institute for Applied Ecology

Signatures	Date
Arthur Georges.	20/1/21
	20/1/21
	20/1/21
Caitlin Cherryh	20/1/21
	20/1/21

Signatures	Date
	28/1/2021

Acknowledgements

The personal and professional support from my friends, family, and supervisors made this thesis possible.

Firstly, I would like to thank Arthur Georges and Clare Holleley for their patience and guidance. Without their scientific talent and ability to see the big picture this body of work would not have developed as it has. The mentorship you both provided will travel with me for the rest of my life and I hope one day to get to a point where I am returning the kindness you've shown me.

I'd like to thank my mum, dad and sister for their never-ending support. Sharon, Alex and Giovanna, completing a PhD so far from home was difficult, but it never felt like I was too far away from you all. There wouldn't be much to show if you hadn't been there every time I needed to talk or brainstorm out loud, this is as much your work as it is mine.

My comrades Sarah Whiteley and Sarah Bates were great sources of emotional support, scientific advice, and caffeine. The days were made easier knowing that we would have a coffee break, and I am so lucky to have gotten to know two remarkable scientists just at the beginning of their careers.

For their animal husbandry, assistance in experimental design and general willingness to have a chat, I'd like to thank Jacqui Richardson and Wendy Ruscoe. Their hard work maintained the breeding colony on which most of my work was based.

I'd like to thank Jennifer Marshall Graves, Craig Smith, Ira Deveson, Margarita Medina, Anthony Davidson, Hardip Patel, Sarah Whiteley, Susan Wagner, Duminda Dissanayake, and Kristoffer Wild for their feedback on my writing in various manuscripts. Thank you to Matt Young, Sarah Whiteley, and Duminda Dissanayake for teaching me lab protocols.

For their assistance with museum collection access and tissue loans, I would like to thank Marc Jones, Mark Hutchinson, Carolyn Kovach and Alejandro Velasco Castrillon of the South Australian Museum, Katie Date, Joanna Sumner and Jane Melville of Museum Victoria, Gavin Dally and Dane Trembath of the Museum and Art Gallery of the Northern Territory, Andrew Amey and Jessica Worthington-Wilmer of the Queensland Museum and Jodi Rowley and Stephen Mahony of the Australian Museum.

I would also like to thank Kristine French, Kimberley Maute, Adam Bernich and volunteers associated with the University of Wollongong for assisting in sample collection

from the field. For their assistance with developing analysis, I thank Ian Marang (University of Sydney), Mike Kearney (University of Melbourne), Kerensa McElroy, Andrew Young (Commonwealth Scientific and Industrial Research Organisation) and Craig Moritz (Australian National University).

I would like to thank everyone at the Australian National Wildlife Collection, and in particular Leo Joseph and Margaret Cawsey.

Thank you to the people at the University of Canberra who provided the infrastructure and support to allow this PhD to proceed, in particular Llara Weaver and Barbara Harriss.

Though I don't know your names, thank you to the many anonymous reviewers who provided essential and practical feedback on my manuscripts. Your feedback was always appreciated.

Lastly, I'd like to thank all of the animals that formed the basis of my research, their use has I hope provided an insight into evolution and development that is novel and meaningful to the wider community.

Table of Contents

List of Figures

List of Tables

List of Appendix Materials

List of Abbreviations

Abbreviation	Definition
CORT	Corticosterone: glucocorticoid hormone involved in energy mobilisation and regulation of the stress response.
ESD	Environmental sex determination: sex determination is influenced by environmental cues.
FPT	Female-producing temperature: temperature at which the majority of embryos in an ESD species develop as female.
GR	Glucocorticoid receptor: Protein which binds to and mediates the physiological actions of glucocorticoids.
GRE	Glucocorticoid responsive element: Element of the genome which glucocorticoid receptors can bind to and effect changes in gene expression.
GSD	Genetic sex determination: sex determination is directed by genetic factors.
HPA	Hypothalamic-pituitary-adrenal axis: brain-gonad axis responsible for regulating glucocorticoid hormone production in birds, mammals and reptiles.
HPI	Hypothalamic-pituitary-interrenal axis: brain gonad axis responsible for regulating glucocorticoid hormone production in fish and amphibians.
HPG	Hypothalamic-pituitary-gonadal axis: responsible for regulating gonadotrophic hormone production and plays a role in sexual maturation.
HSP	Heat shock protein: Family of proteins which have roles in cytoprotection, transcript and protein stabilization in response to temperature changes.
MPT	Male-producing temperature: the temperature at which the majority of embryos in an ESD species develop as male.
ROS	Reactive oxygen species: Highly reactive secondary signalling molecules produced as a by-product of metabolism

SCN	Suprachiasmatic nucleus: A region of the hypothalamus responsible for regulation of the peripheral circadian clocks.
SNP	Single nucleotide polymorphism: a diagnostic difference in a single DNA nucleotide.
TPH	Temporary pseudo-hermaphroditism: the retention of hemipenes by gonadally female reptiles. Typically resolves before hatching but can persist post-hatching.
TSD	Temperature sex determination: a subset of ESD, in which sex determination is influenced by temperature.
TSP	Temperature-sensitive period: the period during incubation in which developing embryos are sexually labile in response to temperature.

Chapter 1 Introduction

1.1 Sex determining systems: From cue to outcome

The outcome of sex determination, typically a male or female phenotype, is the end point of a cascade of developmental changes which can be directed by genetic or environmental factors. Though the sexual phenotypes themselves (males with testes or females with ovaries) are highly conserved among animals, there is great diversity in the genetic and environmental factors which have taken on master or influential roles in the determination of sexual fate (Grossen et al., 2011; Cutting et al., 2013; Marshall Graves, 2013; Herpin & Schartl, 2015). In species with genetic sex determination (GSD), sex is determined by genes on the sex chromosomes. In species with environmental sex determination (ESD), sex is determined not by genetics but by external environmental cues. These two general sex determination modes are not exclusive, sex reversal can occur in which an underlying GSD system is overridden by environmental influence; nor are they fixed on an evolutionary timescale, as transitions from one to the other have occurred frequently (Sarre et al., 2011; Bachtrog et al., 2014; Capel 2017). Mammals and birds are two phylogenetically distinct homeothermic clades in which GSD has independently evolved, but with contrasting systems of male and female heterogamety (Marshall Graves & Shetty, 2001). Mammals possess a male heterogametic system (XX/XY) in which sex determination is controlled by a dominant factor on the Y chromosome (Koopman et al., 1990, 1991; Marshall Graves, 2013), though this is not universally the case (Soullier et al., 1998; Grützner et al., 2004). In contrast, birds display a female heterogametic system (ZZ/ZW) in which sex is most likely determined by the differential dosage of a gene located on one or both of the sex chromosomes (Smith et al., 2009; Major & Smith, 2016).

The remaining vertebrate clades display greater within-group diversity in sex determination mode. Among reptiles and teleost fish, male (XX/XY) and female (ZZ/ZW) heterogamety, as well as ESD have arisen multiple times (Pokorná & Kratochvíl, 2009; Van Doorn, 2014). Teleost fish display perhaps the greatest diversity in sex determination modes, possessing male and female heterogametic GSD, ESD and several modes of sequential hermaphroditism (Todd et al., 2016; Baroiller & D'Cotta, 2016). Among the reptilians, tuatara and crocodiles display only ESD as do the majority of turtle species, although male and female heterogamety also occurs in turtles (Ferguson & Joanen, 1982, 1983; Cree et al.,

1995; Pieau et al., 1999; Kawai et al., 2007; Badenhorst et al., 2013). Squamate reptile lineages display both male and female heterogametic systems in addition to ESD (Viets et al., 1993; Harlow & Shine, 1999; Gamble et al., 2015, 2017), and are thought to have undergone several independent transitions between ESD and GSD (Sarre et al., 2011; Holleley et al., 2016), although some lineages possess highly stable GSD systems (Cornejo-Páramo et al., 2020; Kostmann et al. 2021). Among reptiles, the only definitively demonstrated form of ESD is temperature sex determination (TSD), in which it is temperature during a specific period of embryonic development that determines sex (Singh et al., 2020). Sex determining systems and their cues are diverse, but tend to enlist a conserved suite of genes, which can be regulated or recruited in different ways (Cutting et al., 2013).

Plasticity in sex determination may allow a closer match between sex and the environment, where there are sex-specific fitness differences and environmental conditions vary on a scale which may affect individual fitness. First proposed in 1977, the Charnov-Bull hypothesis predicts that the evolution of ESD will be favoured when there are sex-specific fitness differences and environmental conditions are patchy, providing some "patches" with optimal developmental conditions for males, and some for females (Charnov & Bull, 1977). These testable parameters have been expanded to focus on the effects of incubation temperature on growth rate and size at sexual maturity (Warner, Uller, et al., 2009; Schwanz et al., 2016), with experimental evidence bearing out these ideas (Warner et al., 2008). TSD has evolved repeatedly among ectotherms, and so for each species with TSD, the fitness benefits of sexual plasticity must outweigh the effects of possible sex ratio skews. It is predicted that over time, selection for the rarer sex should maintain sex ratio parity (Darwin, 1871; Düsing, 1884; Fisher, 1930; Edwards, 2000; Warner, 2011; Schwanz et al., 2020).

Rapid anthropogenic climate change may push the cost-benefit dynamic of TSD towards a greater cost, with change potentially occurring at a rate greater than compensatory evolutionary processes can occur. Studies in both fish and marine turtles have already identified sex ratio skews in warming water and rookery conditions (Jensen et al., 2018; Honeycutt et al., 2019), and even in the absence of changes in mean conditions, greater fluctuations in nest temperature may generate sex ratio skews in turtles (Valenzuela et al., 2019). Without oppositional evolutionary processes (such as evolution of the threshold temperature or compensatory maternal nesting behaviours), predictions for the survival of TSD species into the future are dire, with some modelling studies positing extinction due to extreme sex ratio skews (Mitchell et al., 2010; Mitchell & Janzen, 2010). Pivotal temperatures (the temperature at which sex ratios are 1:1) and the transitional range of temperature (range

of temperatures between completely female and completely male production) vary across latitude in turtles with TSD (Ewert et al., 1994, 2005; Carter et al., 2019), strongly suggesting that evolution in the threshold temperature to maintain sex ratio parity has already occurred. Indeed, threshold temperature heritability has been demonstrated in TSD turtles (Bull et al., 1982; Morjan, 2003; McGaugh et al., 2011) but the rapidity with which thresholds may evolve in response to increasing average temperatures depends on both the magnitude of sex ratio skews and the capacity for maternal nesting choices to buffer (Bull et al., 1982; Doody et al. 2006; Pezaro et al., 2017) . Determining the capacity for thermal thresholds to evolve would provide knowledge on the molecular pathways involved in sex reversal and TSD. I will directly address this question in my thesis, examining patterns of sex reversal in the wild to infer the rapidity with which evolutionary turnover in sex determining systems can occur.

The differential regulation of androgens and estrogens has long been known as a critical effector of sex differentiation in vertebrates, and reptiles in particular (Lance, 2009; Angelopoulou et al., 2012). Typically, estrogen application results in ovarian development in a broad range of taxa (Bull et al., 1988; Crews et al., 1989; Radder et al., 2008; Ehl et al., 2017). However, the addition of estrogens does not always lead to feminization, and some sex steroid manipulations lead to counter-intuitive results, highlighting the variety of ways in which sex steroids are regulated and utilised to determine sex within reptiles. The application of an aromatase inhibitor, to prevent estrogen production, produces gonadal males in most studies, including those on squamate lizards and turtles (Crews & Bergeron, 1994; Wibbels & Crews, 1994; Wennstrom & Crews, 1995; Shine et al., 2007; Warner & Shine, 2008). In the central bearded dragon (*Pogona vitticeps*), an agamid with temperature sex reversal, inhibiting aromatase production in genetic females does not result in testis development but does cause the retention of male hemipenes into adulthood (Ehl et al., 2017). In the leopard gecko (*Eublepharis macularis*), a species with TSD, estrogen application causes male development at female-producing temperatures (FPT) (Janes et al., 2007). In the TSD snapping turtle (*Chelydra serpentina*), testosterone application at male-producing temperature (MPT) caused feminization, and the additional application of fadrozole abrogated this effect, once again causing male development (Schroeder & Rhen, 2019). Furthermore, the addition of non-aromatizable androgen at MPT caused male development (Schroeder & Rhen, 2019).

Not only are endogenously produced sex steroids critical in sexual development, their deposition into and uptake from the yolk has the potential to alter the outcome of sexual development, representing another opportunity for maternal contribution aside from nesting behaviours. Levels of sex steroid hormones in the yolk are known to correlate with sex ratios

in some TSD species (Bowden et al., 2000; Lovern & Wade, 2003), and in others egg size is associated with sex ratio (Shine et al., 2002; Radder et al., 2009), although this is not universal (Warner & Shine, 2005). Sex steroids in the yolk and incubation temperature may additively influence sex determination, as in the pond slider turtle (*Trachemys scripta*) it was proposed that eggs laid later in the breeding season and containing more estradiol required lower exposure to FPT in order to feminize (Carter, Sadd, et al., 2018). It is clear that the regulation of androgens and estrogens is critical in reptile sexual differentiation, although the strength and directionality of their effects varies greatly (Wibbels et al., 1994; Crews, 1996), and interactions between environmentally-cued endogenous steroid production and yolk steroid utilisation add another layer of complexity, even within a single species let alone between species with contrasting sex determination systems (Elf, 2003). The production of sex steroids is regulated by upstream hormonal and epigenetic processes, with the epigenetic regulation of aromatase production implicated in several species with TSD (Navarro-Martín et al., 2011; Parrott et al., 2014; Matsumoto et al., 2016). These upstream factors are becoming better elucidated, with the focus now on temperature-responsive epigenetic modifiers and the recruitment of conserved stress response pathways in gonad development (Deveson et al., 2017; Ge et al., 2017; Ge et al., 2018; Weber et al., 2020).

Epigenetic processes which respond rapidly to the environment on a molecular level imprint environmental information on the genome and have long been understood to be involved in the differential regulation of genes involved in sex determination. Both DNA and histone methylation have been implicated in sexual differentiation in ESD species (Piferrer, 2013). DNA methylation is associated with incubation temperature in reptiles both during development and in adulthood (Paredes et al., 2016; Venegas et al., 2016; Dong et al., 2019). Histone methylation, particularly around certain promoter or repressor sequences of key genes, is associated with ESD (Matsumoto et al., 2016). Differential splicing of the mRNA of critical epigenetic remodelling genes is proposed to have a controlling role in temperature sex determination in reptiles (Standora & Spotila, 1985; Harry et al., 1992).

The differential expression and alternative splicing of Jumonji and AT-rich interacting domain 2 (*jarid2*) and lysine demethylase 6B (*kdm6b*) in temperature sex-reversed females of the central bearded dragon (*Pogona vitticeps*), red-eared slider turtle (*Trachemys scripta elegans*), and American alligator (*Alligator mississippiensis*) have emerged as rapidly temperature-responsive epigenetic controllers of sex which have a role in reshaping the epigenome of the developing gonad (Deveson et al., 2017; Ge et al., 2018; Bock et al., 2020). These histone demethylases have roles in gene repression and epigenetic remodelling,

particularly during embryonic development (Peng et al., 2009; Sanulli et al., 2015; Holoch & Margueron, 2017). While clearly involved in the regulation of TSD, the proteins JARID2 and KDM6B are not known to be inherently thermosensitive, and their expression and alternative splicing are likely controlled by factors more proximal to the environmental sex-determining cue. Cold-inducible RNA binding protein (CIRBP) is a cold shock and stress response protein that is known to be inducible by a range of environmental stressors (De Leeuw et al., 2007; Zhong & Huang, 2017), and is also associated with sex in the TSD snapping turtle (*Chelydra serpentina*) (Chojnowski & Braun, 2012; Schroeder et al., 2016; Radhakrishnan et al., 2017). Further layers of temperature-specific control have recently come to light, with temperature-specific isoforms of CIRBP being generated by the inherently temperature-sensitive splicing activity of CDC-like kinases (Haltenhof et al., 2020). Thus, epigenetic and post-transcriptional processes that control environmental sex determination are starting to become clear, what remains unknown is the proximal environmental sensory mechanism that initiates these regulatory processes. This question is central to my thesis and I will assess several candidate mechanisms for the biological sensory mechanism responsible for environmental sex determination.

The vertebrate stress axis is a conserved biological response system that integrates and responds to the environment through the production of glucocorticoids, and mediates ESD in some fish (Goikoetxea et al., 2017). In response to stressful environmental stimuli, the hypothalamus secretes corticotropin releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary (Figure 1-1). This hormone enters the bloodstream and stimulates the adrenal glands to produce glucocorticoid hormones, which have roles in energy mobilisation, physiological response to stresses, and a range of social and reproductive behaviours, with plasma corticosterone levels in wild reptiles generally increasing within 10 minutes of stressful stimuli (Moore & Jessop, 2003; Wada, 2008; Sinervo & Miles, 2011). The stress axis is mediated by negative feedback mechanisms at several levels, including the production of corticotropin releasing hormone binding protein (CRHBP) to bind and inactivate CRH (Kalin, 2018), and the production of 11β-hydroxysteroid dehydrogenase (11β-HSD), which catalyses the conversion of active to inactive glucocorticoids (Stewart, 2003). The role of the stress axis in mediating environmental interactions, and in particular the opportunity for cross-talk between the stress axis and sex steroid production, have led to its recruitment as the biological sensory mechanism of ESD in many fish species.

In fish, the production of cortisol (the main glucocorticoid hormone produced by fish) occurs in response to a variety of environmental conditions, including temperature, social stress and even background colour (Goikoetxea et al., 2017). All gonochoristic fish with ESD display a pattern of masculinization in response to environmental stress, mostly commonly this is with increasing water temperature (Ospina-Álvarez & Piferrer, 2008). Studies which investigate natural corticosterone production, artificially elevate corticosterone or manipulate stress axis gene expression in gonochoristic fish with TSD have confirmed that corticosterone elevation causes complete gonadal masculinization (Hattori et al., 2009; Yamaguchi et al., 2010; Hayashi et al., 2010; Mankiewicz et al., 2013; Adolfi et al., 2019; Castañeda Cortés et al., 2019; Miller et al., 2019; Castañeda-Cortés et al., 2020; García-Cruz et al., 2020; Hara et al., 2020). Experimental evidence is emerging that the stress axis also mediates adult sex change in hermaphroditic fish (Chen et al., 2020). Two main molecular pathways are supported which explain the masculinizing effect of cortisol in fish, the first being aromatase suppression *via* the action of upstream glucocorticoid responsive elements (GRE) (Kitano et al., 1999; Yamaguchi et al., 2010; Todd et al., 2016). The second pathway involves the upregulation of 11β-HSD, which converts cortisol to its inactive form (cortisone), but which also catalyses the conversion of testosterone to 11-ketotestosterone (11-KT), a more potent androgen (Fernandino et al., 2013; Todd et al., 2016).

The role of the stress axis in reptile TSD is posited to involve similar mechanisms, i.e. stressful incubation conditions result in an increase in corticosterone production and sex ratio skews. However, the experimental evidence does not bear this out. Corticosterone or dexamethasone (corticosterone agonist) application in reptiles, turtles and squamate lizards has mostly failed to result in sex ratio changes (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015), although one study reports possible feminization or male-specific mortality in response to corticosterone in the agamid *Amphibolurus muricatus* (Warner, Radder, et al., 2009). Corticosterone levels in two species of crocodilian are also not elevated at high temperature (FPT), either through development (Marcó et al., 2015) or during the temperature sensitive period of development (Medler & Lance, 1998). Though the evidence is currently equivocal for the role of stress hormones in sex determination in reptiles, the well-established role for the stress axis in mediating fish ESD and a relative lack of study on the topic in reptiles means the question is far from answered. While evidence exists for both brain-driven (stress axis) and gonad-autonomous (temperature-specific splicing) sex determination in reptiles, it may be that this is species-specific.

Aside from the vertebrate stress axis, several independent and seemingly disparate hypotheses for the mechanism by which ESD occurs have been proposed in the decades since ESD was first characterised (Charnier, 1966). All of these hypotheses identify a crucial role for growth and metabolism in sex determination. Growth-dependent sex determination has been proposed in several taxa, in which it is growth rate, either in and of itself (Kraak & De Looze, 1992), or as a function of temperature (Haig, 1991; Smith & Joss, 1994), food availability (Johnson et al., 2017), or social factors (Hattori, 1991) that determines sex. Even in mammals, metabolic hypotheses have been proposed to explain the masculinizing effects of the eutherian master sex-determining SRY gene (Mittwoch, 1989, 2004). Growth rate is directly related to metabolism and reactive oxygen species (ROS) production (Sun et al., 2015), and each of the environmental factors discussed here can influence ROS production *via* their effects on growth rate and metabolism (Clarke & Fraser, 2004; Halliwell & Gutteridge, 2015). Feedback mechanisms which sense and respond to ROS produced at high temperatures, in particular the antioxidant response, are critical in regulating the levels of potentially damaging ROS in the cell, and themselves can act as signals of oxidative state (Kobayashi et al. 2009; Cyr & Domann, 2011; Morgan & Liu, 2011). Similarly, intracellular calcium (Ca^{2+}) levels are tightly regulated and known to be sensitive to a variety of environmental cues (Hilton et al., 2015). Through their reciprocal regulation, both ROS and calcium levels form a cellular environmental sensory system which is capable of signalling changes in epigenetic regulation and gene expression (Chapter 2; Castelli, Whiteley, et al., 2020). The involvement of calcium signalling in sex determination has been recently borne out through calcium imaging experiments in *T. scripta* (Weber et al., 2020), but the involvement of metabolic rate and ROS production in sex determination has not been experimentally demonstrated. The common line of thought in these works could be explained if cellular calcium and redox regulation were the inherently environmentally sensitive cellular sensor of stress which has been co-opted for use in sex determination.

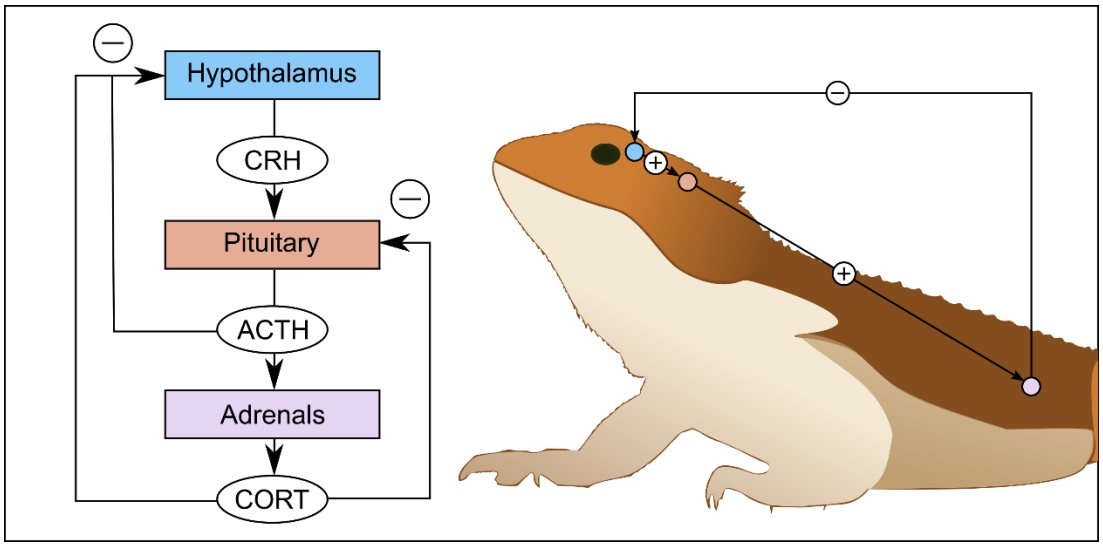


Figure 1-1. The hypothalamus-pituitary-adrenal (HPA) axis, showing the hormonal pathway of corticoid production and negative feedback control in this system. In response to a stressful event, the hypothalamus produces corticotropin releasing hormone (CRH), which stimulates the production of adrenocorticotropic hormone (ACTH) by the pituitary, which in turn stimulates corticosteroid (CORT) production by the adrenal glands. Expression of corticosteroids inhibits ACTH secretion by the pituitary, and CRH production by the hypothalamus, returning the system to homeostasis after a stressful event (Kay, 1998).

The central bearded dragon (*Pogona vitticeps*) is an ideal model species in which to investigate the involvement of the HPA axis in sex determination. This widespread Australian agamid displays a ZZ/ZW system of genetic sex determination (Ezaz et al., 2005), but also exhibits thermosensitive sex reversal, in which ZZ genetic males develop as phenotypic females at high incubation temperature (Quinn et al., 2007; Holleley et al., 2015). The master and effector genetic sex determination genes in this species are not yet known, and although it is probably a dosage-based rather than dominance-based system (Quinn et al., 2007). The ZZ/ZW system of *P. vitticeps* is not homologous with the sex chromosomes of birds, geckos or snakes (Ezaz et al., 2009). While the mechanisms of genetic sex determination are still being elucidated, the epigenetic effectors of temperature sex reversal are becoming better characterised. The genes *kdm6b* and *jarid2* are upregulated and uniquely spliced sex-reversed females, in all of the tissues investigated (Deveson et al., 2017). While these epigenetic effectors are likely to be involved in gonad differentiation, the biological sensory mechanism which signals for their upregulation and alternative splicing is not known.

Behavioural and gene expression evidence suggests that stress axis upregulation or dysregulation (loss of negative feedback) is a consequence of sex reversal in this species, and so may be involved in the mediation of sexual fate. Sex-reversed females display upregulated

gene expression of proopiomelanocortin (*pomc)*, a precursor protein to adrenocorticotropic hormone (ACTH), which is released into the bloodstream and stimulates the adrenal glands to produce glucocorticoid hormones (Cawley et al., 2016). Additionally, sex-reversed females display downregulation of genes with immune functions (Deveson et al., 2017), and given that immune responses can be altered by chronic or acute stress (Mclaren et al., 2003; Padgett & Glaser, 2003; Tort, 2011), this further indicates that stress may be chronically elevated in sex-reversed *P. vitticeps*. Behavioural differences between the sexes are also suggestive of dysregulation of the stress response in sex-reversed females, as they display higher levels of activity and boldness than either concordant males or concordant females (Li et al., 2016). These behavioural characteristics may indicate either a heightened or lowered stress response in sex-reversed females, as the link between glucocorticoid production and environmental stressors is species-specific (Atwell et al., 2012; Clary et al., 2014). The foundational knowledge of the sex determining system of this species, their molecular signatures of sex reversal, and behavioural consequences of sex reversal make the central bearded dragon an ideal model species in which to study the biological sensory mechanisms of sex determination.

1.2 Thesis aims

I aim to examine the biological sensory mechanism responsible for the translation of an environmental cue into a sexual outcome, using the central bearded dragon (*P. vitticeps*) as a model species.

My specific objectives are to:

1. Synthesise a literature review which assesses the potential for cellular calcium and redox regulation to be the ultimate regulator of sex reversal and ESD among vertebrates, and how this relates to hormonal stress in fish ESD systems.
2. Explore the potential for the brain to drive temperature sex determination in *P. vitticeps* using embryonic transcriptome data and looking for signatures of brain-gonad communication in the stress axis, hypothalamic-pituitary-gonadal axis, and other novel forms of brain-gonad communication.
3. Determine if the application of stress hormones *in ovo* can induce sex reversal in *P. vitticeps*, and if the stress axis is basally upregulated in adult sex-reversed females.

4. Determine if the application of antioxidants *in ovo* can induce sex reversal in *P. vitticeps*, to elucidate the role of reactive oxygen species production and the antioxidant response in temperature sex reversal.
5. Examine the evolutionary potential for *P.* vitticeps to adapt to a warming climate by establishing a spatial and temporal profile of sex reversal across the range of *P. vitticeps* in the wild, and determine whether genetic or climatic factors can explain the distribution of sex reversal.

Chapter 2 Cellular calcium and redox regulation: The mediator of vertebrate environmental sex determination?

This chapter has been published in **Biological Reviews**.

Castelli, M. A., Whiteley, S. L., Georges, A., & Holleley, C. E. (2020). Cellular calcium and redox regulation: the mediator of vertebrate environmental sex determination? Biological Reviews, 95(3), 680–695. https://doi.org/10.1111/brv.12582

Apart from formatting, this chapter has not been altered in any way. The references have been consolidated at the end of the thesis.

2.1 Abstract

Many reptiles and some fish determine offspring sex by environmental cues such as incubation temperature. The mechanism by which environmental signals are captured and transduced into specific sexual phenotypes has remained unexplained for over 50 years. Indeed, environmental sex determination (ESD) has been viewed as an intractable problem because sex determination is influenced by myriad genes that may be subject to environmental influence. Recent demonstrations of ancient, conserved epigenetic processes in the regulatory response to environmental cues suggest that the mechanisms of ESD have a previously unsuspected level of commonality, but the proximal sensor of temperature that ultimately gives rise to one sexual phenotype or the other remains unidentified. Here, we propose that in ESD species, environmental cues are sensed by the cell through highly conserved ancestral elements of regulation of calcium and redox (CaRe) status, then transduced to activate ubiquitous signal transduction pathways, or influence epigenetic processes, ultimately to drive the differential expression of sex genes. The early evolutionary origins of CaRe regulation, and its essential role in eukaryotic cell function, gives CaRe a propensity to be independently recruited for diverse roles as a ‘cellular sensor’ of environmental conditions. Our synthesis provides the first cohesive mechanistic model

connecting environmental signals and sex determination pathways in vertebrates, providing direction and a framework for developing targeted experimentation.

Key words: oxidative stress, reactive oxygen species, calcium signalling, temperature dependent sex determination, epigenetics.

2.2 Introduction

The mechanisms by which sex is determined and the processes by which sexual phenotypes subsequently differentiate (sexual differentiation) have been a focus of enquiry for many centuries (Mittwoch, 2000, 2013). The structures of the testes and ovaries are highly conserved across vertebrates (Morrish & Sinclair, 2002; Schroeder et al., 2016), so it is not surprising that the genes and regulatory processes governing gonad formation and differentiation share a high degree of commonality (Sarre et al., 2004; Cutting et al., 2013; Capel, 2017). Despite the conservation of gonadal morphology, sex in vertebrates is influenced by a wide variety of mechanisms, broadly divided into genetic sex determination (GSD) and environmental sex determination (ESD), as well as mixed systems in which genes and environment interact to determine sex (Bachtrog et al., 2014). ESD systems occur in species from 15% of vertebrate orders. They use several different environmental cues including light regime, social stress, pH and temperature (Bachtrog et al., 2014).

Decades of research on model and non-model organisms have documented the extraordinary variety of sex-determining environmental signals, and characterised different downstream elements of sex differentiation pathways in ESD systems. However, recent work implicating ancient, conserved epigenetic mechanisms in the regulatory response to environmental cues suggests that the mechanisms of ESD have a previously unsuspected level of commonality (Rhen & Schroeder, 2010; Deveson et al., 2017; Ge et al., 2018). This poses the fundamental question: what is the mechanism by which such a wide variety of environmental cues are transduced to determine sex by a common molecular sensor?

The conservation of epigenetic elements in ESD suggests the action of a biochemical sensor common to all ESD species. Such a sensor must be (*i*) inherently environmentally sensitive, (*ii*) capable of interacting with components of known sex differentiation pathways, and (*iii*) conserved in function yet plastic enough to be recruited to capture and transduce different environmental signals for different phenotypic outcomes.

Here, we propose a general model in which sex determination is mediated by cellular calcium (Ca^{2+}) and redox (reactive oxygen species; ROS) status, which are subject to

environmental influence. Elements of this hypothesis have been discussed in six recent papers that explicitly posited the involvement of either ROS production or Ca^{2+} flux in directing the outcomes of ESD (Yatsu et al., 2015, 2016; Czerwinski et al., 2016; Corona-Herrera et al., 2018; Lin et al., 2018; Hayasaka et al., 2019). We suggest that these two interrelated signalling systems (Richter & Kass, 1991) work together to initiate sex determination.

Here, we refer to calcium and redox status collectively as CaRe status, and propose a model for its biological action in ESD. We review evidence that CaRe status (and its subsequent effects on CaRe-sensitive regulatory pathways) is an environmentally sensitive mediator of complex biochemical cascades, and therefore a promising candidate for the capture and transduction of environmental signals into a sexual outcome. We propose that these CaRe-sensitive regulatory pathways have been co-opted independently and repeatedly to determine sex in different vertebrate lineages, acting as the crucial missing link between sex and the environment.

2.3 Calcium and redox regulation in the cell

2.3.1 Roles of ROS and Ca^{2+}

ROS and Ca^{2+} constitute some of the most important signalling molecules in the cell, and are both involved in a staggering variety of essential cellular processes (Gordeeva et al., 2003; Camello-Almaraz et al., 2006; Görlach et al., 2015). The subtle ways in which these interactions can be modulated allows cellular responses to be fine-tuned according to the cellular context (Yan et al., 2006; Metcalfe & Alonso-Alvarez, 2010).

ROS are highly reactive by-products of cellular respiration, and can cause cellular damage when production exceeds that of the cell's antioxidant capacities (Martindale & Holbrook, 2002; Temple et al., 2005). ROS are produced mainly in the electron transport chain in the mitochondria, but can be generated elsewhere in the cell. They are typically rapidly dismuted through a series of antioxidant reactions (Camello-Almaraz et al., 2006; Yan et al., 2006; Hamanaka & Chandel, 2010). If ROS production outweighs the antioxidant capacity of the cell, the redox environment can be altered to an oxidizing state (Treidel et al., 2016). However, at physiologically moderate levels (eustress), ROS possess vital cellular signalling roles in growth, homeostasis, reproduction, and programmed apoptosis (Covarrubias et al., 2008; Dowling & Simmons, 2009; Sies et al., 2017). When acting in their capacity as signalling molecules, ROS can influence protein conformation and function

through the oxidative modification of accessible cysteine residues and reversible changes to disulphide bonds (Hammond et al., 2001; Covarrubias et al., 2008; Morgan & Liu, 2011; Cremers & Jakob, 2013). Even subtle subcellular alterations in redox state can drive differential gene expression (Sen & Packer, 1996; Antelmann & Helmann, 2010) through physiological or epigenetic mechanisms (Cyr & Domann, 2011; Timme-Laragy et al., 2018), and ultimately influence cell and tissue-specific environmental responses.

In close concert with redox signals, Ca^{2+} flux co-regulates many cellular signalling and environmental sensing functions (West et al., 2001; Contreras et al., 2010; Görlach et al., 2015; Plattner & Verkhratsky, 2015), and displays considerable evolutionary flexibility in recruitment to these different functions (Hilton et al., 2015). Ca^{2+} concentrations inside the cell are tightly controlled by numerous calcium pumps and channels on the plasma membrane (Ermak & Davies, 2002), and are mediated by Ca^{2+} release from internal stores in the mitochondria and endoplasmic and sarcoplasmic reticula (Røttingen & Iversen, 2000; Berridge et al., 2003; Brostrom & Brostrom, 2003). Ca^{2+}-mediated signalling is crucial for orchestrating cell signalling cascades, which are highly sensitive to and modulated by the amplitude, duration, and subcellular localisation of Ca^{2+} (Røttingen & Iversen, 2000; Dupont & Sneyd, 2017). Such finely tuned signal transduction cascades, which primarily involve protein phosphorylation or dephosphorylation, allow Ca^{2+} to control a wide variety of highly specific responses to environmental variables (Brostrom & Brostrom, 2003; Sharma et al., 2014).

2.3.2 Environmental sensitivity of Ca^{2+} and ROS

We propose that CaRe status is the most promising candidate for encoding extrinsic environmental signals in the cell, and provide a framework in which CaRe status determines sex in environmentally sensitive species. On a biochemical level, ROS and Ca^{2+} levels in the cell are affected by many environmental factors, such as temperature (Ahn & Thiele, 2003), ultraviolet (UV) light (Schieven et al., 1993; Gniadecki et al., 2000), and hypoxia (Chandel et al., 2000). CaRe status can therefore indicate the presence and magnitude of an environmental signal and initiate a cellular response.

Ca^{2+} signalling has been implicated in temperature-dependent sex determination (TSD) through the temperature-sensitive regulation of transient receptor potential (TRP) cation channel expression in two TSD alligator species (American alligator, *Alligator mississippiensis* and Chinese alligator, *Alligator sinensis* (Yatsu et al., 2015; Lin et al., 2018)

and a freshwater turtle *Mauremys reevesii* (Ye et al., 2019). These plasma membrane channels control the flow of Ca^{2+} ions into the cell, and are thermosensitive at least in mammals (Hilton et al., 2015), although TRP channel function is unknown for other vertebrates (Hilton et al., 2015; Yatsu et al., 2015). Within the TRP family, *TRPV4* exhibits temperature-specific differential expression in *A. mississippiensis* (Yatsu et al., 2015), and three other TRP family genes (*TRPV2*, *TRPC6*, and *TRPM6*) displayed temperature- and sex-biased expression in *A. sinensis* (Lin et al., 2018). It was suggested that these channels act as the initial temperature sensor mechanism in alligators that regulates the expression of downstream sexual development genes through Ca^{2+} signalling (Lin et al., 2018). The application of TRPV4 antagonist drugs in *A. mississippiensis* partially interfered with male development, producing testes-like gonads with incomplete Mullerian ducts (Yatsu et al., 2015). This suggests that TRPV4 operates alongside other, as yet unidentified, thermosensitive mechanisms acting in concert with Ca^{2+}, such as those involving ROS. In the turtle *M. reevesii*, the application of a TRPV1 and TRPM8 inhibitor altered sex ratios under certain incubation conditions, and although the authors accredited this to inhibited thermoregulatory behaviour rather than altered sex gene expression, the result could be due to inference with Ca^{2+} signalling (Ye et al., 2019).

TRP channels also respond to different wavelengths of visible light (Y. Wang et al., 2016), and other research has proposed the effect of light on intracellular calcium concentrations to be mediated by ROS production (Lavi et al., 2003). Additionally, the oxidation of cysteine residues can sensitize and activate TRPA1 (Materazzi et al., 2012) and TRPV1 (Kozai et al., 2013; Ogawa, Kurokawa, & Mori, 2016), further substantiating the link between the two messenger systems in response to various stimuli. TRP channels are also sensitive to and can be modulated by steroid hormones, particularly in sperm cells (Kumar et al., 2015).

ROS production is directly influenced by the environment, primarily through the metabolism-enhancing effects of temperature (Clarke & Fraser, 2004; Halliwell & Gutteridge, 2015), although pH (Maurer et al., 2005; Wang et al., 2009), UV light (de Jager et al., 2017) and photoperiod-influenced circadian rhythms (Hirayama et al., 2007) can also alter oxidative state. Developmental rate in some reptiles accelerates with temperature, as does mitochondrial respiration (Sun et al., 2015), so it is feasible that that ROS could accumulate more quickly at a higher temperature, activating responses to oxidative stress. Further, antioxidant capacity in embryos varies in response to incubation temperature in a TSD turtle (red-eared slider, *Trachemys scripta elegans*), indicating that metabolic rate and ROS accumulation vary with

temperature (Treidel et al., 2016). Additionally, yolk deposition of antioxidants is greater in birds with shorter developmental periods (Deeming & Pike, 2013), suggesting that even in a homeothermic taxon, faster development results in greater oxidative stress. In some fish species, water temperature affects redox status and oxidative damage, although the effects have not been investigated in the context of sex determination (Birnie-Gauvin et al., 2017).

Environmental cues do not necessarily need to be abiotic, as many species of fish display forms of socially cued sex change, commonly through the reorganisation of dominance hierarchies (Todd et al., 2016). Oxidative stress has been shown to correlate with social status in species of fish (Border et al., 2019) and primates (Beaulieu et al., 2014), probably through the increased behavioural costs of defending and maintaining dominance. Signals of differential calcium regulation and responses to oxidative stress were both observed in dominant male bluehead wrasse (*Thalassoma bifasciatum*), further indicating differential regulation of these messenger systems during sex change (Todd et al., 2019).

Combined with evidence on the environmental sensitivity of calcium channels, these studies show that a wide range of environmental conditions, including temperature, during development can alter both redox state and calcium flux. This raises the possibility that CaRe status could have a role as a cellular sensor for a broad range of environmental cues responsible in developmental programming and variation in different species.

2.4 Connections between CaRe status and sex determination

2.4.1 Signal transduction pathways

As discussed above, CaRe status is clearly a strong candidate for the capture of environmental signals by the cell. We propose here that the signal captured by CaRe status is then transduced *via* ubiquitous signalling pathways that influence epigenetic processes to govern sex differentiation.

The interactions between CaRe status and cellular organisation and function are complex, and so can interact with a variety of pathways involved in sex determination. Here we discuss CaRe-sensitive candidates likely to transduce an environmental signal; the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), heat shock response and antioxidant response pathways, and explore the potential interactions between CaRe status and another candidate pathway for ESD, the vertebrate stress axis (Table 2-1)

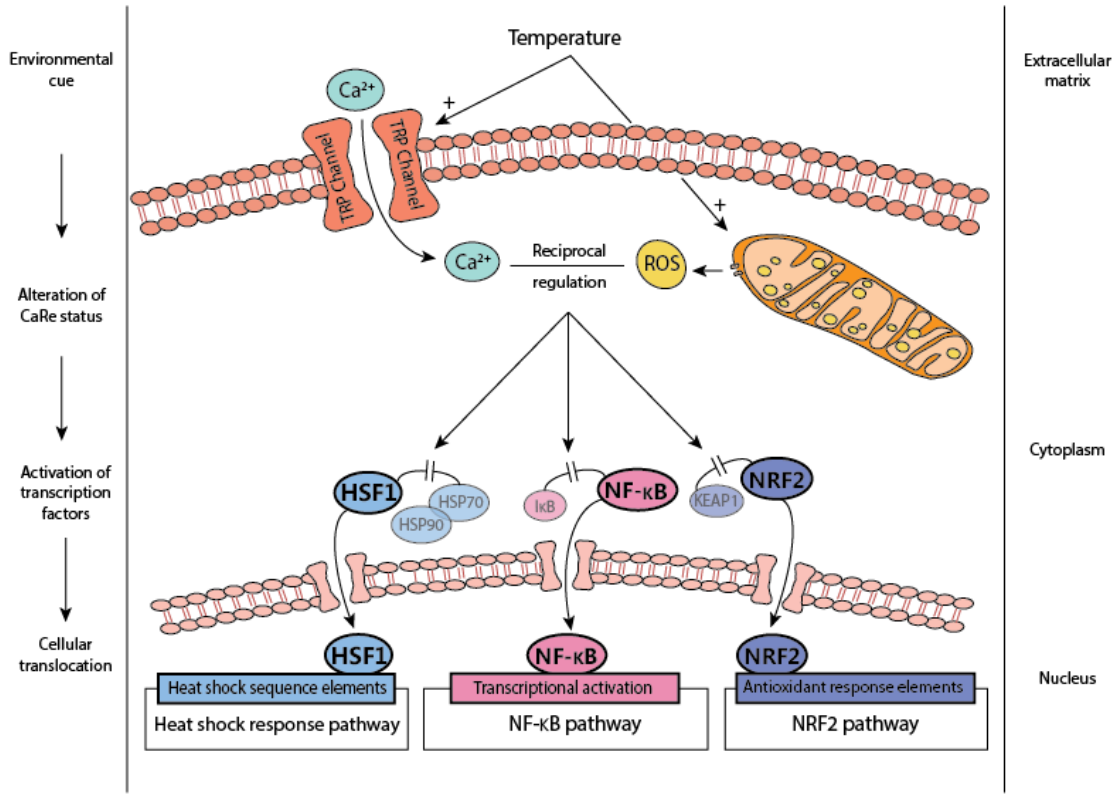


Figure 2-1. A subset of environmental response pathways hypothesised to be involved in environmental sex determination, activated by external signals integrated into the cell as calcium and redox (CaRe) status. This simplified model outlines how an environmental cue, in this case temperature, can alter CaRe status by causing an influx of Ca^{2+} ions through innately thermosensitive transient receptor potential (TRP) channels, and an increase in reactive oxygen species (ROS) production by mitochondria through increased metabolic rate. With their reciprocal co-regulation, both Ca^{2+} and ROS can act in concert to activate transcription factors [heat shock factor 1 (HSF1); nuclear factor erythroid-related factor 2 (NRF2)] and pathways [nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)], which then translocate from the cytoplasm to the nucleus to alter the transcription of target genes involved in sex determination. HSP, heat shock protein; KEAP1, Kelch-like ECH-associated protein 1.

2.4.1.1 The NF-κB pathway

The NF-κB pathway is involved in a wide variety of cellular processes and can be activated by Ca^{2+} influx, ROS, and ROS-induced glutathione production (Røttingen & Iversen, 2000; Hammond et al., 2001; Antonsson et al., 2003; Morgan & Liu, 2011) (Figure 2-1).

The NF-κB pathway has well-established associations with numerous sex determination genes in mammalian development. However, its role has been less well studied in ESD taxa (Delfino & Walker, 1998; Josso & di Clemente, 2003; Hong et al., 2003) (Table 2-1). Analysis of the transcriptome during development in two TSD species (the alligator *A. sinensis* and painted turtle, *Chrysemys picta*) showed that differential expression of various genes in the NF-κB pathway is associated with temperature at key developmental stages, but this has not been backed up by functional studies (Radhakrishnan et al., 2017; Lin et al., 2018).

A single study directly demonstrated a role for NF-κB in vertebrate sex determination using the zebrafish (*Danio rerio*) (Pradhan et al., 2012). While the genetics of sex determination in laboratory strains of *D. rerio* lacking a W chromosome (Wilson et al., 2014) are not yet well understood, it appears to have a polygenic basis that is sensitive to environmental factors such as temperature and hypoxia (Ribas et al., 2017; Santos et al., 2017). *Danio rerio* is unusual in that a juvenile ovary initially forms, and either continues to mature as an ovary, or transitions into testes through the promotion of selective apoptosis (Uchida et al., 2004; W. Chen et al., 2017). Manipulating the induction or inhibition of the NF-κB pathway prior to gonadal commitment led to a female or male bias, respectively, demonstrating its role in suppressing the apoptotic pathways that trigger the transition to testis development (Pradhan et al., 2012). Sex cell-specific apoptosis is a well-established mechanism in sex determination in *D. rerio* (Uchida et al., 2002), as well as in other teleosts (Y. He et al., 2009; Yamamoto et al., 2014; Sarida et al., 2019) and other model organisms such as *Drosophila melanogaster* (DeFalco et al., 2003) and *Caenorhabditis elegans* (Gumienny et al., 1999; Kuwabara & Perry, 2001; Peden et al., 2007). Manipulating the NF-κB pathway thus presents opportunities for exploring the link between CaRe regulation and ESD (Figure 2-2).

Table 2-1 Calcium and redox (CaRe)-sensitive elements, their functions relating to epigenetic modulation, cellular localisation and their roles in environmental sex determination (ESD) or temperature sex determination (TSD).

Candidate element	Cellular functions and known roles in environmental sex determination		References
Nuclear to cytoplasmic translocation			
CIRBP Cold-inducible RNA-binding protein	**Functions**	• Translocation to cytoplasm induced by numerous environmental stressors including temperature and oxidative state • Typically associates with cytoplasmic stress granules where it acts as a mRNA chaperone	(De Leeuw et al., 2007; Rhen & Schroeder, 2010; Schroeder et al., 2016; Radhakrishnan et al., 2017; Zhong & Huang, 2017)
	ESD roles	• Candidate gene for TSD in *Chelydra serpentina* • Thermosensitive expression in *Chrysemys picta* and *Apalone spinifera*	
hnRNPs Heterogeneous ribonucleoprotein particle family	**Functions**	• Involved in numerous cellular processes including splicing regulation, pre-mRNA processing, nuclear export of mRNA, chromatin remodelling • Interacted with *p38 MAPK* stress induced signalling pathway, and the EED subunit of the PRC2 complex	(Harry et al., 1990, 1992; Huelga et al., 2012; H. J. Kim et al., 2017)
	ESD roles	• Thermosensitive expression in *Caretta caretta* • Posited as candidates for the regulation of TSD	
Cytoplasmic to nuclear translocation			
NRF2 Nuclear factor (erythroid-derived 2)-like 2	**Functions**	• Regulates expression of antioxidant genes under oxidative stress through transactivation of antioxidant response elements	(Covarrubias et al., 2008; Loboda et al., 2016)
HSF1 Heat shock factor 1	**Functions**	• Transcriptional regulator of all heat shock proteins • Redox and temperature regulated • Induced by *p38 MAPK* phosphorylation	(Harry et al., 1990; Kohno et al., 2010; Tedeschi et al., 2015; Bentley et al., 2017; Lin et al., 2018; Furukawa et al., 2019)
	ESD roles	• Role of heat shock response established for majority of TSD species • Involved in female sexual development in *Oryzias latipes*	
HSPs Heat shock protein family	**Functions**	• Molecular chaperone for steroids and hormones, participates in cell signalling • Roles in maintaining protein stability, folding, and transmembrane transport	(Harry et al., 1990; Brostrom & Brostrom, 2003; He et al., 2009; Kohno et al., 2010; Tedeschi et al., 2016, 2015; Casas et al., 2016; Czerwinski et al., 2016; Bentley et al., 2017; Lin et al., 2018; Tao et al., 2018; Wang et al., 2019)
	ESD roles	• Thermosensitive expression in *Alligator mississippiensis* and *Alligator sinensis* • Markers of thermal stress, and thermosensitive expression in *Caretta caretta* • Downregulation of *HSP10*-associated apoptosis during sex reversal in *Monopterus albus* • Various HSPs associated with social sex change in *Amphiprion bicinctus* • *HSP90* upregulated in *Oreochromis niloticus* undergoing temperature-induced sex reversal	
Protein kinases Family includes mitogen-activated, cAMP-dependent, calcium/calmodulin-dependent	**Functions**	• Multitude of cellular roles centring on ability to catalyse protein phosphorylation, so playing an integral role in numerous signal transduction cascades	(Radhakrishnan et al., 2017; Lin et al., 2018; Tsakogiannis et al., 2018)
	ESD roles	• Temperature-dependent expression in *Alligator sinensis* and *Chrysemys picta* • Male-biased expression in *Pagellus erythrinus* and *Pagrus pagrus*	
JAK-STAT pathway Janus kinase/signal transducers and activators of transcription	**Functions**	• Redox-regulated signalling cascade for stress response	(Simon et al., 1998; Radhakrishnan et al., 2017; Todd et al., 2019)
	ESD roles	• Components of pathway show thermosensitive expression in *Chrysemys picta* • Progressive upregulation during sex change in *Thalassoma bifasciatum*	
NF-κB pathway Nuclear factor kappa light-chain-enhancer of activated B cells	**Functions**	• Redox-regulated signalling cascade for environmental stress response • Activation has anti-apoptotic effects	(Pradhan et al., 2012; Ravi et al., 2014; Radhakrishnan et al., 2017; Lin et al., 2018)
	ESD roles	• Components of pathway show thermosensitive expression in *Chrysemys picta* and *Alligator sinensis* • Crucial for sexual differentiation in *Danio rerio*	

		• Male-biased expression in *Lates calcarifer*	
No subcellular translocation known/not applicable			
JARID2 & JMJD3 Jumonji and AT-rich interaction domain-containing 2 (*JARID2*) and lysine demethylase 6B (*JMJD3/KDM6B*)	**Functions**	• Members of the Jumonji chromatin remodelling gene family • *JARID2* mediates Polycomb repressive complex (PRC2) deposition of silencing H3K27me3 marks • *JMJD3* catalyses demethylation of H3K27me3	(Díaz & Piferrer, 2015; Akashi et al., 2016; Deveson et al., 2017; Radhakrishnan et al., 2017; Ge et al., 2018; Todd et al., 2019)
	ESD roles	• Retained intron associated with sex reversal in *Pogona vitticeps*, *Alligator mississippiensis* and *Trachemys scripta elegans* • TSD in *Trachemys scripta elegans*, *Chrysemys picta*, and *Apalone spinifera* • Thermal adaptation in *Anolis* lizards (*A. allogus, A. homolechis, A. sagrei*) • Associated transition to masculine phenotype during sex change in *Thalassoma bifasciatum* • Upregulated in response to temperature in *Dicentrarchus labrax*	
AP1 Transcription factor, activator protein-1	**Functions**	• Acts as a point of integration of many signalling pathways involved in responses to environmental signals (e.g. MAPKs, NF-κB, HSPs) • Redox controlled switch determines ability to bind DNA	(Yin et al., 2017)
TRPs Transient receptor potential cation channels	**Functions**	• Innately thermosensitive channels that allow the passive transfer of Ca^{2+} across the plasma membrane	(Yatsu et al., 2015; Liu et al., 2015; Lin et al., 2018; Todd et al., 2019)
	ESD roles	• Known thermosensitivity, temperature-dependent expression in *Alligator sinensis* and *Alligator mississippiensis* • Calcium signalling enrichment during sex change in *Thalassoma bifasciatum*	
TET enzymes Ten-eleven translocation methylcytosine dioxygenases	**Functions**	• Redox-dependent DNA methylation	(Todd et al., 2019)
	ESD roles	• Expression strongly associated with sex change in *Thalassoma bifasciatum*	
DNMTs DNA methyltransferases	**Functions**	• Sensitive to redox state and calcium concentration • Action influenced by the redox microenvironment of chromatin	(van der Wijst et al., 2015; Tsakogiannis et al., 2018; Todd et al., 2019)
	ESD roles	• Associated with sex change in *Thalassoma bifasciatum* • Sex-biased expression in *Pagellus erythrinus* and *Pagrus pagrus*	

Abbreviations: MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; PRC2, polycomb repressive complex 2

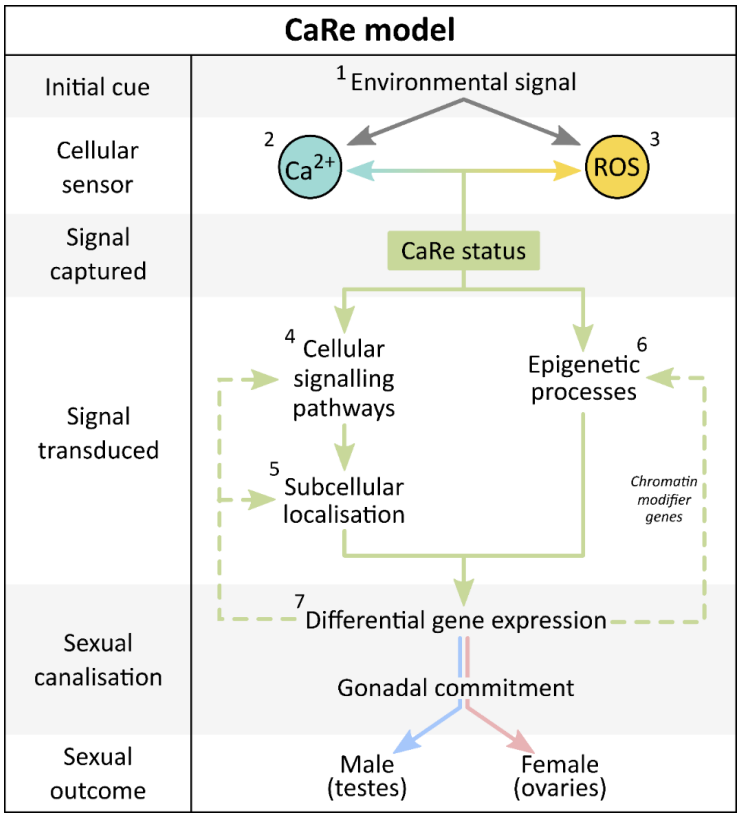


Figure 2-2. Generalised model for the influence of environment on sexual fate in vertebrates, identifying target stages for manipulation techniques that facilitate rigorous testing of the model. Solid lines indicate the top-down influence from environmental cue to sexual outcome, while dashed lines indicate areas where there is potential for feedback loops to occur. Incubation/rearing conditions during the environmentally sensitive period can be expanded to include not just the environmental stimulus the species is known to respond to, but other calcium and redox (CaRe)-altering stimuli, such as ultraviolet (UV) light, green light, or pH (1). Ca^{2+} flux can be manipulated either through the addition of calcium (typically accompanied by the calcium transporter ionomycin) or through altering the function of transient receptor potential (TRP) channels, either through RNA interference or the administration of TRP channel agonist and antagonist drugs (2). Reactive oxygen species (ROS) production can be manipulated by the direct addition of oxidants (e.g. H_2O_2) or antioxidants, or by application of ROS-inducing drugs (e.g. doxorubicin) (3). A range of approaches could be taken to interfere with cellular signal transduction pathways, which would vary depending on the pathway of interest (4). Subcellular localisation is similarly pathway specific, but for example small peptides can be used to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation (Gupta et al., 2010) (5). The role of epigenetic regulators can be investigated using agents for histone demethylation (e.g. 5-azacytidine), or through agents that inhibit the epigenetic regulatory machinery, for example polycomb repressive complex 2 (PRC2) inhibitors (Danishuddin et al., 2019) (6). Genes suspected to be involved in the determination of sexual fate can be downregulated through gene knock-down, RNA interference, or the addition of downstream products including hormones or hormone disruptors (e.g. oestrogen, testosterone, corticosterone, or fadrozole) (7).

2.4.1.2 Heat shock proteins and the heat shock response

Several authors have proposed a role in TSD for heat shock proteins (HSPs) (Harry et al., 1990; Kohno et al., 2010; Bentley et al., 2017) (Table 2-1). These proteins are chaperones and regulators of transcription factor binding, functions which are essential for maintaining cell function at extreme incubation temperatures (Haslbeck & Vierling, 2015; Ikwegbue et al., 2018).

Heat shock causes Ca^{2+} concentration to rise according to time and temperature, and concurrently increases levels of the oxidising agent hydrogen peroxide (Soncin et al., 2000; Ahn & Thiele, 2003). This change in CaRe status can activate heat shock factor 1 (HSF1), which in turn regulates expression of heat shock protein genes (notably *HSP70*), whose actions are required for protection against heat-induced cell damage (Soncin et al., 2000; Ahn & Thiele, 2003; Tedeschi et al., 2015, 2016) (Figure 2-1). Incubation temperature affects the expression of many HSPs in reptiles (Table 2-1), however, no consistent patterns have emerged even between closely related species, suggesting that HSPs exhibit considerable evolutionary flexibility (Harry et al., 1990; Kohno et al., 2010; Haslbeck & Vierling, 2015; Czerwinski et al., 2016; Bentley et al., 2017). Inconsistent patterns of expression of HSPs across species, and their role as molecular chaperones across a wide range of temperatures, might explain the variety of ESD responses to temperature across species (Hilton et al., 2015; Tedeschi et al., 2016).

Particularly interesting is that environmental triggers of HSPs extend beyond temperature. Some members of the HSP family show differential expression during socially induced sex change in the two-banded anemonefish (*Amphiprion bicinctus*) (Casas et al., 2016), and HSP10 is associated with female to male sex reversal (the trigger of sex reversal is not yet known) in the rice field eel (*Monopterus albus*), where it plays a role in inhibiting apoptosis in male germ cells (He et al., 2009). Given HSPs demonstrated roles in sex determination across ESD taxa, and responsiveness to diverse environmental stimuli, they are promising candidates for further study (Figure 2-2).

2.4.1.3 Oxidative stress and the antioxidant response

Cellular responses to oxidative stress commonly involve induction of the cell's inbuilt antioxidant defence system (Kobayashi et al., 2009). The response is generally initiated by nuclear factor erythroid-related factor 2 (NRF2), whose action is critical for the oxidative stress response and cytoprotection (Brigelius-Flohé & Flohé, 2011; Loboda et al., 2016).

Ordinarily NRF2 persists in the cytoplasm at low levels bound in an inactive state with KEAP1 (Kelch-like ECH-associated protein 1). However, in a state of oxidative stress the bond with KEAP1 is broken, allowing NRF2 to translocate to the nucleus where it binds to antioxidant responsive elements. This initiates expression of genes such as thioredoxins, peroxiredoxins, and glutaredoxins that are critical to launching an antioxidant response to oxidative stress (Nguyen et al., 2009) (Figure 2-1).

These antioxidants quench ROS and cross-talk with proteins involved in the NF-κB pathway (Morgan & Liu, 2011). Glutathione is particularly crucial in the oxidative stress response, as the ratio of its oxidised and reduced states (GSH:GSSG ratio) is responsible for sensing the redox status of the cell (Storey, 1996; Hammond et al., 2001; Robert et al., 2007; Cyr & Domann, 2011). Glutathione directly modifies chromatin structure *via* histone glutathionylation, increasing the binding of transcription factors and upregulating gene expression (Olaso et al., 2013). This has been demonstrated in mammals, in which glutathione enhances decondensation of the paternal genome in a newly fertilised egg (Reyes et al., 1989; Sutovsky & Schatten, 2005; Sánchez-Vázquez et al., 2007).

Broadly, the response of antioxidant genes to environmental changes may be able to affect chromatin structure, essentially 'priming' key regions for binding by transcription factors, such as components of the NF-κB pathway (Hammond et al., 2001), and the polycomb repressive complex PRC2, which is likely to be involved in reptile sex reversal (Deveson et al., 2017; Georges & Holleley, 2018). The antioxidant response can therefore induce changes in gene expression and protein function which may contribute to the broader processes taking place during sex determination and differentiation in environmentally sensitive species (Table 2-1).

2.4.1.4 Synergism between hormonal and oxidative stress

The hypothalamic–pituitary–adrenal (HPA) axis in reptiles, birds and mammals or inter-renal (HPI) axis in fish and amphibians has a role in sex determination in a range of taxa [see reviews in (Goikoetxea et al., 2017; Geffroy & Douhard, 2019)]. Among gonochoristic (single-sex) fish, cortisol-mediated sex determination in response to temperature is well supported by experimental application of cortisol (Hattori et al., 2009; Hayashi et al., 2010; Castañeda Cortés et al., 2019; Miller et al., 2019). Cortisol has not yet been experimentally demonstrated to be a mediator of sex change in sequentially hermaphroditic teleost fish, but transcriptomic evidence suggests cortisol upregulation, supporting a role for the HPI axis in the repression of aromatase and the regulation of downstream epigenetic effectors of gene

regulation (Fernandino et al., 2013; Solomon-Lane et al., 2013; Goikoetxea et al., 2017; Todd et al., 2019).

Even in these fish species in which the stress axis has been co-opted as the environmental sensory mechanism, CaRe pathways may play a synergistic role in initiating, maintaining or mediating sex determination or sex change. Hormonal stress results in oxidative stress *via* an increase in metabolic rate (Spiers et al., 2015), and Ca^{2+} has a very strong association with sexual reproduction in fish (Persson et al., 1998; J. D. Johnson & Chang, 2002; Norberg et al., 2004). For example, a social cue such as the removal of a dominant male induces HPI activation and glucocorticoid production in the dominant female of some species (Goikoetxea et al., 2017). Elevated hormonal stress then results in aromatase repression and elevated androgen production through glucocorticoid receptor (GR) nuclear localisation and glucocorticoid receptor element (GRE) occupation in key genomic regions (Adolfi et al., 2019; Todd et al., 2019). Concurrently, hormonal stress leads to oxidative stress through elevated metabolism and energy production (Spiers et al., 2015), and alteration in CaRe status through one or more of the mechanisms described herein. There is extensive cross-talk between the hormonal stress axis and CaRe-sensitive pathways, creating opportunities for the two to synergise. CaRe-sensitive HSPs chaperone GRs, and GRs further interact extensively with the NF-κB pathway in a stimulus-, time-, and cell-specific manner to control responses to stimuli (Bekhbat et al., 2017). Whether CaRe pathways play a causative or synergistic role with stress hormones in species that have co-opted the HPI axis for sex determination (as many teleost fish clearly have) is not yet known, but there is evidence to suggest that these interactions exist.

Among crocodilians, turtles, and squamates there is little, and contradictory, evidence for the involvement of stress hormones in ESD. Temperature sex-reversed adult bearded dragons (*Pogona vitticeps*) display greatly upregulated pro-opiomelanocortin (*POMC*) gene expression in the brain, suggesting stress axis upregulation (Deveson et al., 2017). However, in other reptiles, manipulating incubation temperature and yolk corticosteroids during the embryonic period of sex determination has not demonstrated a causal link between temperature and glucocorticoid production (Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015; Marcó et al., 2015). Additionally, gonads of TSD reptiles cultured in isolation from the brain were still found to respond to temperature, suggesting that the effect of temperature on the HPA axis is not the temperature-sensitive mechanism in reptiles (Moreno-Mendoza et al., 2001; Shoemaker-Daly et al., 2010; Mork et al., 2014). Thus, there is substantial evidence that the stress axis plays a role in ESD in teleost fish, but evidence for

stress axis activation as a cause or consequence of sex reversal among reptiles remains equivocal. It is therefore unlikely that the stress axis is central to the temperature-sensitive mechanism in all vertebrates, but a common role for CaRe mechanisms is plausible in both teleost fish and reptiles with ESD.

2.4.2 Subcellular localisation

A commonality among many of the candidate pathways and proteins discussed herein is that their mode of action requires cellular translocation in response to changes in CaRe status (Nelson et al., 2004; Awad et al., 2013) (Figure 2-1, Table 2-1). A change in localisation of transcription factors is necessarily upstream of any changes in nuclear organisation and gene expression. For example, in mammals the testis-inducing transcription factor (SOX9) must be translocated from the cytoplasm to the nucleus for normal testes development to occur. Otherwise, the developing gonads retain ovary-like characteristics even when expression levels of *SOX9* are maintained (Y. Chen et al., 2017). This process in mammals is regulated by the CaRe-sensitive catabolite activator protein cyclic AMP (cAMP) and protein kinase A phosphorylation (Malki, Berta, et al., 2005; Malki, Nef, et al., 2005), and by Ca^{2+}-calmodulin nuclear entry pathways (Hanover et al., 2009). It is plausible that a similar process, linked more directly to environmental conditions, occurs in vertebrates with ESD. While numerous candidates whose function relies on changes in cellular localisation have been associated with ESD, functional studies in this context are currently lacking, so future experimentation would benefit from considering these processes (Figure 2-2).

2.4.3 Alternative splicing and epigenetic remodelling

As well as the signal transduction pathways discussed above, there are other mechanisms that can also modulate gene expression in response to environmentally driven changes in CaRe status (Table 2-1). While these are as yet poorly understood, evidence is building that post-transcriptional processes including alternative splicing and epigenetic remodelling are involved in ESD.

In the 1990s, differential splicing was proposed to control TSD after differential expression of heterogeneous ribonucleoprotein particles (hnRNPs) was discovered in two TSD turtles (diamondback terrapin, *Malaclemys terrapin* and loggerhead turtle, *Caretta caretta*) (Harry et al., 1990, 1992; Jeyasuria & Place, 1998) (Table 2-1). Splicing factors in the hnRNP family were suggested to regulate expression of key genes in a temperature-

dependent manner at crucial stages in development, although the mechanism by which thermosensitivity is conferred on hnRNPs was (and remains) unidentified (Harry et al., 1992; Matthew Michael et al., 1995; Van Oordt et al., 2000; Huelga et al., 2012).

Subsequently, sex-specific associations with a single nucleotide polymorphism, embryonic expression profiles, and protein localisation in the TSD snapping turtle (*Chelydra serpentina*) suggested that *CIRBP* (cold-inducible RNA-binding protein; *CIRP, A18 hNRNP*) was critical for determining sex (Schroeder et al., 2016). This gene has thermosensitive expression in the pond slider turtle (*Trachemys scripta*) (Chojnowski & Braun, 2012) and Chinese alligator (*A. sinensis*) (Lin et al., 2018), so this gene may be involved in TSD more broadly. CaRe status may be involved in the regulation of *CIRBP*, as it can be activated by a variety of environmental stressors that cause changes in CaRe, including osmotic shock, hypoxia, heat, and oxidative stress (Zhong & Huang, 2017). *CIRBP* may also be involved in mediating CaRe-regulated feedback loops, as upon activation it can function as an RNA chaperone or post-transcriptional regulator of many CaRe-sensitive genes (Peng et al., 2006; De Leeuw et al., 2007; Y. Zhang et al., 2016; Zhong & Huang, 2017).

Recent work supports the early evidence for a role of alternative splicing of key chromatin remodelling genes in TSD in reptiles. A sex-associated retained intron event in two members of the Jumonji gene family *JARID2* and *JMJD3* (also called *KDM6B*) occurs in three thermally sensitive reptile species (*Pogona vitticeps*, *Alligator mississippiensis*, and *Trachemys scripta*; Deveson et al., 2017). In *P. vitticeps*, intron retention (IR) occurs only in sex-reversed females produced at high incubation temperatures. There is variation among these species in the pattern of sex-associated IR, perhaps arising from different ancestral genetic sex determination systems (Deveson et al., 2017). In a fish that undergoes socially cued sex change, the bluehead wrasse *Thalassoma bifasciatum*, *JARID2* and other cofactors within the PRC2 (*EZH2*, *SUZ12*, *EED*, *RNF2*) are transiently downregulated during female to male transition (Todd et al., 2019). Both *JARID2* and *JMJD3* also exhibit thermosensitive expression in the brains of sex-reversed (neomale) Nile tilapia (*Oreochromis niloticus*) (Zhao et al., 2019). The PRC2 complex is also involved in orchestrating the commitment of sexual fate in GSD species, primarily through chromatin remodelling on the sex chromosomes (Garcia-Moreno et al., 2018).

JARID2 and JMJD3 regulate the tri-methylation of histone H3, lysine 27 (H3K27), and are involved in orchestrating embryonic development and sexual differentiation (Sanulli et al., 2015; Holoch & Margueron, 2017) (Figure 2-3). Knockdown of *JMJD3* in a TSD turtle (*T. scripta elegans*) at male-producing temperatures triggers female development in 80% of

embryos that survive (Ge et al., 2018). JMJD3 mediates transcription of the male-determining gene *DMRT1* (Ge et al., 2017) by demethylating the repressive H3K27me3 near its promoter (Ge et al., 2018). Downregulation of *JMJD3* by upstream mechanisms responding to high temperature results in persistent tri-methylation of H3K27, which suppresses *DMRT1* and promotes the female developmental pathway (Figure 2-3). Upregulation of *JMJD3* in response to lower temperature results in de-methylation of H3K27me3 near the *DMRT1* promoter, activating *DMRT1* expression and promoting the male developmental pathway (Figure 2-3). In alligators, switching embryos from a low female-producing temperature to a high male-producing temperature results in downregulation of *JARID2* and *JMJD3*, further demonstrating the commonality of these chromatin remodelling pathways in reptiles (Yatsu et al., 2016). The interplay between thermo-responsive intron retention and activity of *JMJD3* (Deveson et al., 2017; Ge et al., 2018) is not well understood (Georges & Holleley, 2018). However, these recent findings have dramatically shifted the focus of inquiry from direct thermosensitivity of candidate sex-determining genes to higher-order thermosensitive epigenetic processes that differentially downregulate or upregulate influential sex genes (Georges & Holleley, 2018).

CaRe status may be directly linked to the epigenetic processes discussed above. ROS release from mitochondria (Ying et al., 2018) and hydrogen peroxide exposure (Niu et al., 2015) can alter histone methylation, and the oxidative status of a *JMJD3*-regulating transcription factor (STAT6) directly alters *JMJD3* (He et al., 2016). JARID2 and the associated epigenetic remodelling complex PRC2, and JMJD3, exhibit a wide range of responses to oxidative and other cellular stressors, triggered by environmental signals such as heat shock (Marasca et al., 2018). The actions of hnRNPs also change depending on their oxidation status. For example, the activity of hnRNPk (a chaperone and inhibitor of HSF1 binding to heat shock elements) alters depending on the oxidation status of a single redox-sensitive cysteine residue, affecting the activation of heat shock response genes (H. J. Kim et al., 2017). Alternatively, epigenetic processes may be mediated by the CaRe-responsive signalling pathways detailed above. The NF-κB pathway is known to control some histone methylation marks, perhaps *via* the transcriptional regulation of *KDM2B*, another lysine demethylase (Nakshatri et al., 2015), and HSF1 has been demonstrated to open chromatin structure to assist the recruitment of other transcription factors (Inouye et al., 2007). These examples point to a promising area of future research, directed at the CaRe-sensitive epigenetic processes driving ESD.

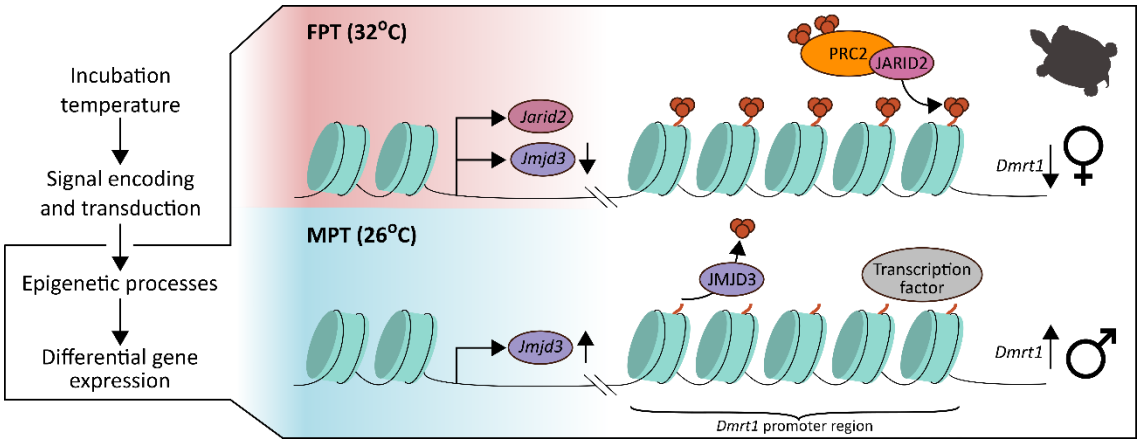


Figure 2-3. A schematic diagram showing the action of Jumonji family genes in altering the expression of a key sex gene in the red-eared slider turtle (Trachemys scripta elegans) based on the work of Ge et al., (2017, 2018). At female-producing temperatures (FPT), the chromatin modifier JMJD3, a histone demethylase, is downregulated, presumably under the influence of calcium and redox (CaRe)-mediated upstream signal transduction pathways. This allows the polycomb repressive complex 2 (PRC2) complex to deposit heritable methylation marks on histone 3 lysine 27 (H3K27me3), in part due to the action of JARID2. The methylation marks deposited in the DMRT1 promoter give permanence to the trimethylation and repression through cell division, ultimately leading to ovary development. At male-producing temperatures (MPT), JMJD3 is upregulated, likely under the influence of upstream CaRe-mediated signal transduction pathways. JMJD3 removes the H3K27me3 marks deposited by the PRC2 complex on the DMRT1 promoter, which then opens this region for transcription by as yet unidentified transcription factors, so altering the developmental trajectory toward a male fate. [After Georges & Holleley (2018)]. Image credit (turtle silhouette) Roberto Díaz Sibaja under PhyloPic Creative Commons attribution unported license 3.0.

2.5 Evolutionary significance of CaRe regulation

Tightly controlled regulation of intracellular levels of Ca^{2+} and ROS is essential for life, and has been since the emergence of the earliest eukaryotes (Maynard Case et al., 2007). The regulatory mechanisms by which Ca^{2+} and ROS are sensed, and the genetic pathways involved in responding to these signalling molecules, are therefore highly conserved (Aguirre et al., 2005). The evolution of sexual reproduction itself has been proposed as an adaptive response to mitigate the subcellular damage caused by increased production of ROS in an oxygen-rich environment (Nedelcu & Michod, 2003). An alternative view is that ROS production by bacterial endosymbionts may have driven the evolution of sexual reproduction as a mechanism to allow for DNA repair through recombination (Hörandl & Speijer, 2018).

In a facultatively sexual multicellular alga (*Volvox carteri*), temperature-induced ROS production triggered sexual reproduction (Nedelcu et al., 2004), and treatment with antioxidants completely inhibited temperature-induced sexual reproduction (Nedelcu & Michod, 2003). There is a fundamental association between ROS and the regulation of sexual reproduction in all three eukaryotic domains (Gapper & Dolan, 2006). ROS are known to control sexual/asexual reproductive modes in fungi (Lara-Ortíz, Riveros-Rosas & Aguirre, 2003), affect germination and gametogenesis in plants (Chailakhyan & Khrianin, 1987; Traverso et al., 2013), and influence reproductive phenotypes in multicellular animals (Shibata et al., 2003).

Canalisation of the downstream regulatory pathways of gonad development, indicated by the relative commonality of gonadal structure, releases upstream elements of the regulation from selection. Provided functional ovaries or testes result, diversity in the upstream regulatory processes will be tolerated by selection (Georges et al., 2010; Capel, 2017). The resultant evolutionary flexibility might account for the phylogenetic variability of ESD systems, which has been difficult to explain (Sarre et al., 2004; Bachtrog et al., 2014; Pennell et al., 2018). In particular, the independent re-emergence of TSD from GSD can be seen as a gain of sensitivity to the environment without the disruption of underlying CaRe mechanisms, which are essential for life (Pokorná & Kratochvíl, 2009; Georges et al., 2010; Janes et al., 2010). Sensitivity to CaRe status can therefore be rapidly regained if there is selective pressure to do so. This may require only small-scale biochemical changes, allowing rapid responses in shorter evolutionary time scales compared with larger scale genetic or physiological changes.

2.6 Applying the CaRe model in theory and practice

2.6.1 Summary of the model

We have provided a simplified and generalised framework that proposes a critical role for CaRe regulation in environmentally sensitive sex determination systems. The CaRe model we present posits that an environmental influence, for example temperature, acts as a cue to stimulate a regulatory cascade that ultimately delivers a sexual outcome (testes or ovaries) (Figure 2-2). Such temperature cues act upon thermosensitive ion channels to regulate Ca^{2+} flux, interacting with ROS production driven by metabolic rate, resulting in a CaRe status that captures the environmental signal. CaRe status is decoded and transmitted to the nucleus *via*

signal transduction pathways, such as the NF-κB and heat shock response pathways, potentially moderated by antioxidant activity (Figure 2-1). Each of these signal transduction pathways is likely to involve changes in subcellar localisation of key transcription factors such as HSF1, which can influence expression of genes responsible for developmental outcomes (Kim et al., 2009) (Figure 2-1). CaRe status can also be transmitted *via* epigenetic or post-translational modifications, so that a diverse array of CaRe-sensitive cellular pathways can ultimately drive differential gene expression and direct sexual outcomes.

2.6.2 Testing hypotheses derived from the model

While our model is necessarily speculative, it forms a basis for the generation of testable hypotheses and the re-examination of existing data. Models such as this have proven immensely successful in setting priorities and giving direction to research on the genes and gene products responsible for sexual differentiation (Morrish & Sinclair, 2002; Smith & Sinclair, 2004).

Functional analysis will be critical for determining the role of CaRe in ESD systems and elucidating the species-specific pathways involved. Our model identifies target stages at different levels of the pathway for manipulation techniques, which can be applied to a wide range of study species (Figure 2-2). Manipulation of such ubiquitous signal transduction pathways is likely to present practical barriers (e.g. lethality), so we suggest that functional manipulation should exploit the wide variety of targeted inhibitor drugs and enhancers in both *in vitro* and *in vivo* experiments. We might borrow approaches from the biomedical and cancer research fields, in which these regulatory pathways are becoming well characterised and techniques for their manipulation are becoming more accessible. Gene editing techniques such as the clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) system (Cong & Zhang, 2014), combined with drug manipulation and transcriptomic approaches, will increase understanding of the role of these ubiquitous signal transduction pathways in both model and non-model species with ESD.

Understanding the mechanisms by which environmental signals are transduced to determine sex will have broader implications beyond the evolution of ESD systems. Practical applications could include manipulation of sex ratios in aquaculture systems, which frequently rear ESD species. Precise control of sex ratios in farmed species could increase efficiency of food production for a growing human population (Budd et al., 2015). More broadly, a better understanding of ESD is increasingly important for assessing the biological

impacts of climate change on environmentally sensitive species (Parmesan & Yohe, 2003; Umina et al., 2005; Botkin et al., 2007; Sinervo, 2010; IPCC, 2013). Already populations of ESD species are experiencing skewed sex ratios caused by rising global temperatures (Mitchell & Janzen, 2010; Refsnider & Janzen, 2016; Bókony et al., 2017; Hays et al., 2017; Honeycutt et al., 2019). By understanding how an environmental signal is transduced to a sexual outcome, novel conservation management strategies could be devised to avoid or mitigate these impacts of climate change.

2.7 Conclusions

(1) A universal cellular sensor in ESD systems must be (*i*) inherently environmentally sensitive, (*ii*) capable of interacting with components of known sex determination pathways, and (*iii*) highly conserved in function yet plastic enough to be recruited for the transduction of different environmental signals for different phenotypic outcomes.
(2) CaRe status meets these requirements for a cellular sensor, and associated CaRe-sensitive pathways are promising candidates for the transduction of the environmental cue to orchestrate sex determination and differentiation in ESD species. Several lines of evidence support our model that CaRe-sensitive pathways have been independently and repeatedly co-opted as the mechanism by which an environmental signal is transduced to a sexual outcome in ESD species.
(3) The CaRe model is so far the only unifying model that has been proposed for ESD in vertebrates. Continued investigation of the role of CaRe regulation in ESD through explicit testing of CaRe mechanisms proposed in this review will not only advance understanding of evolutionary developmental biology and genetics, but may also at last identify the cellular sensing mechanism of ESD.
(4) We posit that what has been viewed as an intractable problem of identifying the environmentally sensitive element(s) among myriad possible candidates with putative influences on sexual differentiation, instead involves the more tractable challenge of identifying highly conserved ancestral elements of cellular machinery under the influence of equally highly conserved signalling pathways.
(5) We present this model as a basis for future experimentation that goes beyond simply examining gene expression. Our model incorporates signal reception, capture of the signal by the cell, receipt of the signal by established cellular signal transduction pathways, and the

transduction of signals to the epigenome to direct gene expression leading to discrete sexual outcomes.

Chapter 3 Transcriptional architecture of the brain-gonad axis is consistent with gonad-autonomous sex determination in a reptile with temperature sex reversal

3.1 Abstract

In a broad array of fish and reptiles, sex is determined by environmental cues during development, this is known as environmental sex determination (ESD). In fish, the vertebrate stress axis is well-known to mediate ESD, but the biological mediator of ESD in reptiles is not yet known. Here we present matched brain and gonad transcriptomes from early bipotential *P. vitticeps* embryos incubated at sex-reversing and non-sex reversing temperatures, examining the possibility for the involvement of previously proposed brain-gonad axes (stress axis, estrogen communication) in temperature sex reversal. We find no evidence for the involvement of the stress axis or brain estrogen production in signalling temperature sex reversal, instead finding evidence in both the brain and gonad for the differential regulation of the circadian rhythm at different temperatures. We propose that this may represent a co-option of elements of the circadian rhythm in sex determination, namely the regulatory genes *clk4* and *cirbp*, genes both known to be involved in sex determination. Other candidate sex-determining genes, including epigenetic regulators (*kdm6b* and *jarid2*) are globally upregulated in both the brain and gonad in response to temperature, and likely have organ-specific roles in sex reversal.

3.2 Introduction

Environmental sex determination (ESD) occurs broadly in fish and reptiles in response to a range of environmental cues, but whether the biological sensory mechanism responsible for converting the environmental cue into a sexual outcome is conserved across vertebrates is not known. The broad occurrence of socially-cued sex change and corticosterone-mediated sex determination in gonochoristic and hermaphroditic fish strongly suggests that ESD is brain-

driven, through the action of the stress axis (Todd et al., 2016; Baroiller & D'Cotta, 2016; Goikoetxea et al., 2017). The biological mediator of sex in reptiles in reptiles is not yet definitively known, though it is hypothesized that similar to fish, developing reptiles experience stress axis activation and elevated glucocorticoid production in the egg, resulting in aromatase production and estrogen-driven ovary development (J. Chen et al., 2020). Despite the well-established occurrence of stress axis-driven ESD in fish, corticosterone experiments (both observational and manipulation) in turtle, alligator, and lizards are inconclusive (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015; Marcó et al., 2015; Chapter 4; Castelli et al., 2021).

Whether ESD in reptiles is driven by the environmental responsiveness of the stress axis is still equivocal, and there is conflicting evidence from organ culture studies as to whether sex determination is brain-driven. In the red-eared slider turtle (*Trachemys scripta elegans*), gonads differentiate according to temperature when cultured in isolation from the body (Shoemaker-Daly et al., 2010; Mork et al., 2014). In contrast, the cultured brain of the olive ridley sea turtle (*Lepidochelys olivacea*) responds to temperature whereas the isolated gonad does not differentiate according to temperature (Merchant-Larios & Villalpando, 1990; Salame-Mendez et al., 1998). Further, the gonads of the American alligator (*Alligator mississippiensis*) fail to differentiate according to temperature in isolated organ culture (Lance & Bogart, 1994). Thus, there is evidence both for and against the role of the brain in determining sex in ESD reptiles, but the brain-gonad axis which may be responsible for translating the environmental cue into a sexual outcome is not yet known.

The vertebrate stress axis is the most prominent hypothesis for brain-driven sex determination in reptiles, but there is evidence for two alternative hypotheses: brain aromatase production and direct neural communication. The brain estrogen hypothesis is predicated on the production of aromatase and estrogens becoming elevated in the brain, followed by conferral of this steroidogenic activity to the gonads. The isolated gonads of *L. olivacea* are unresponsive to temperature, while the isolated diencephalon is temperature-responsive, producing more estrogen than the isolated gonads and showing higher efficiency for transforming testosterone to estrogen (Merchant-Larios & Villalpando, 1990; Salame-Mendez et al., 1998). Other studies in turtle also indicate that aromatase activity in the brain is sexually dimorphic earlier than, or in the absence of, a sexually dimorphic pattern of aromatase activity in the gonads (Jeyasuria & Place, 1998; Willingham et al., 2000). However, this suggests a role for locally produced brain estrogen in sex determination, where it could be involved principally in sexual differentiation of the brain. Neural control over sex

has also been proposed as the biological system mediating ESD, through the release of neurotransmitters in gonadal nerves (Merchant-Larios et al., 1989; Lance, 1997; Gutiérrez-Ospina et al., 1999; Gerendai et al., 2005).

The central bearded dragon (*Pogona vitticeps*) displays a ZZ/ZW system of genetic sex determination with thermal override, with incubation temperatures above 32^{o}C generating sex-reversed ZZ females (Ezaz et al., 2005; Quinn et al., 2007; Holleley et al., 2015). Despite the ambiguous role of the stress axis in reptile ESD, adult *P. vitticeps* display marked transcriptomic differences between the sex reversed (ZZ female, ZZf) and concordant (ZZ male, ZZm; ZW female, ZWf) sexes in the regulation of the stress axis. Adult sex-reversed females display marked upregulation of the stress hormone signalling precursor pro-opiomelanocortin (*pomc*) in the brain, and downregulation of corticotropin-releasing hormone binding protein (*crhbp*) in the liver (Deveson et al., 2017). This indicates that the stress axis is differentially regulated as a consequence of sex reversal, and given the role of the stress axis in mediating sex determination in fish, is highly suggestive that glucocorticoid production might be involved in sex determination.

Here, we assess whether brain-driven communication systems; namely, the stress axis, brain estrogen production, and neural communication, may be involved in the initiation of sex reversal in response to temperature. Using a matched brain and gonad transcriptome dataset from developing *P. vitticeps* embryos prior to gonadal commitment, we search for enrichment of *a priori* defined brain-driven communication pathways and for the enrichment of novel brain communication pathways. If the brain senses environmental temperature and directs gonadal fate in this sex reversal system, we predict that in high temperature incubated embryos there should be (a) enrichment of stress axis genes in both the brain and gonad, (b) enrichment of estrogen-producing enzymes in the brain, or (c) evidence of neuronal activity in the gonads. We also conduct a global assessment of differentially expressed genes to determine whether novel brain-gonad axes show potential for involvement in temperature sex reversal.

3.3 Methods

3.3.1 Specimen and sample collection

Using the brains and gonads from embryos incubated at non-sex-reversing (28°C), and sex-reversing (36°C) temperatures, the brain and gonad-specific gene expression responses to high temperature incubation were investigated. Specimen incubation and sample collection occurred as detailed by Whiteley et al. (2021). In short, eggs were obtained from breeding groups of heterozygous (ZW concordant) females and homozygous (ZZ sex-reversed) female bearded dragons, with three females paired with one male to control for paternal effects. Viable eggs (visible ring of vasculature) were incubated within two hours of lay at 28°C (±1°C) in a damp vermiculite substrate (4 parts water to 5 parts vermiculite by weight). Clutches from heterozygous (ZW) mothers were incubated at 28°C until sampling. Clutches from homozygous (ZZ) mothers were incubated for 28°C for 10 d before half of the eggs were selected randomly and switched to 36°C incubation until sampling.

Embryos were sampled at stage 6 of development (*sensu* Whiteley et al., 2021), corresponding to 16 days post oviposition (dpo) at 28°C and 13 dpo at 36°C. Embryos were euthanised *via* intracranial injection of sodium pentobarbitone (60 mg/mL in isotonic saline). Under a dissection microscope, gonads were removed and snap frozen in liquid nitrogen, as were whole heads. When incubated at a constant temperature, *P. vitticeps* gonads are morphologically bipotential at stage 6 and are not typically fully morphologically differentiated as either testes or ovaries until stage 12 (Whiteley et al., 2017, 2018).

Embryos from heterozygous (ZW mothers) were genotyped to determine their genotypic sex. Blood samples from the vasculature inside the eggshells were collected on FTA Elute Micro cards at the time of embryo sampling (Whiteley et al., 2017). To determine genotypic sex, DNA was extracted using manufacturer protocols and a PCR test developed for use in *P. vitticeps* was conducted to amplify size-polymorphic fragments of the Z and W chromosome (Quinn et al., 2010; Holleley et al., 2015).Four experimental groups exist in this study design: ZZ offspring of ZW mothers incubated at 28°C (genetic males), ZW offspring of ZW mothers incubated at 28°C (genetic females), ZZ offspring of ZZ mothers incubated at 28°C (genetic males), and ZZ offspring of ZZ mothers incubated at 36°C (sex-reversing females).

3.3.2 RNA extraction and sequencing

Brain RNA extraction was conducted using the PureLink Mini RNA Extraction Kit (Thermo Fisher, 12183018A). Whole heads were kept on dry ice until dissection and were left at room temperature for 1-2 min to allow the surface tissue of the head to thaw slightly, while still keeping the brain frozen. The surrounding tissue, including skin, eyes and skeletal matter, were then dissected away and the brain (except the olfactory bulbs, which are delicate and difficult to dissect out of frozen matter) was removed and placed into chilled kit lysis buffer with 1% 2-mercaptoethanol (2mL total volume). RNA extraction followed the kit specifications for extraction of animal tissue (100-200mg) with on-column DNase treatment as described in the kit protocol. RNA was quantified and 260/280 ratios determined using a NanoDrop One (ThermoFisher) spectrophotometer. RNA of sufficiently high quality and quantity was prepared for paired-end sequencing at the Australian Genome Research Facility (AGRF) and sequencing was run on two lanes of the Illumina Nova-Seq 6000 platform.

Matched gonads underwent RNA extraction and sequencing as detailed by (Whiteley et al., 2021). In short, RNA from single gonads was extracted using the Qiagen RNeasy Micro Kit (No. 74004) following kit specifications. Samples were sequenced at the Kinghorn Centre for Clinical Genomics (Garvan Institute of Medical Science) on the Illumina HiSeq 2500 platform.

3.3.3 Gene expression profiling

Reads were trimmed using *trimgalore* set to default parameters The alignment program *STAR 2.5.3* and RNA quantification program *RSEM 1.3.0* were used to align reads to the *Pogona vitticeps* reference genome (GCF_900067755.1_pvi1.1_genomic.fna), using an index created with the *P. vitticeps* gene-finding format file (GCF_900067755.1_pvi1.1_genomic.gff) (Georges et al., 2015).

3.3.4 Differential expression analysis

Differential expression analysis was conducted in RStudio vers. 3.6.1 *EdgeR* (vers. 3.30.3). The brain and gonad datasets were processed separately in *EdgeR* and the differential expression outputs compared on a gene-by-gene basis. Genes with fewer than 10 counts across at least three samples were considered lowly expressed, and were filtered out from further analysis. Library size normalization was applied and differential expression analysis

conducted between each treatment and genotype, using the Benjamini-Hochberg (BH) method to adjust significance for false discovery rate (FDR).

Differential expression analysis was only conducted between different treatments and genotypes of the same organ (i.e. brain or gonad). Brain and gonad datasets were not explicitly compared because organ-specific differences in expression would obscure temperature- and genotype-responsive gene expression. Identification of genes shared between brain and gonad (i.e. upregulated in both brain and gonad for a particulate genotype or temperature), or uniquely regulated in the brain or gonad, was conducted by classifying the output of differential expression analysis as either "significant" or "non-significant" using a false discovery rate (FDR) cut-off of 0.05 and the log-fold change as either "positive" or "negative" in the brain and gonad datasets separately. These outputs were combined on a gene-by-gene basis to generate gene expression patterns for each gene (e.g. upregulated in 36°C-incubated ZZ brain, not differentially regulated in the gonad). Gene ontology and KEGG pathway analysis was conducted using the online platform shinyGo vers. 0.61 (http://bioinformatics.sdstate.edu/go/) with "human" as reference, a p-value cut-off of 0.05 and 30 significant terms.

The two male groups (ZZ, incubated at 28°C) from different maternal genotypes (ZZ sex-reversed females or ZW concordant females) were combined for analysis, as there were no genes differentially expressed in the gonad dataset and only one gene differentially expressed in the brain dataset. We therefore make comparisons of one 28°C ZZ male group (heterozygous and homozygous mothers, 28ZZ, n = 9), one 28°C ZW female group (heterozygous mothers, 28ZW, n = 6) and one 36°C ZZ female group (homozygous mothers, 36ZZ, n = 4).

3.3.5 K-means clustering

K-means clustering analysis was conducted to determine clusters of genes which display similar expression across the three experimental groups. Analysis was conducted according to Whiteley et al. (2021). In short, normalised counts per million (CPM) values from both the brain and gonad datasets were extracted using the *EdgeR* package and the "kmeans" function in the R package *stats* (vers. 4.0.2) was used to conduct cluster estimation.

3.4 Results and discussion

3.4.1 Genetic sex determination is not brain-driven

Between the ZZ and ZW embryos incubated at 28°C, there was greater differential expression in the gonad than the brain, with 62 genes upregulated in the 28ZZ gonad and 47 upregulated in the 28ZW gonad. Zero genes were differentially expressed between the 28ZW and 28ZZ brains (Figure 3-1, Figure 3-2). Aromatase (*loc110077216*), the estrogen-producing enzyme responsible for ovary differentiation and maintenance in reptiles, was upregulated in the 28ZW gonad (Lance, 2009). Furthermore, the ovarian maintenance gene forkhead box L2 (*foxl2*) was also upregulated in the 28ZW gonad (Pisarska et al., 2011). Thus, we can confidently say that female gonad differentiation is already proceeding at stage 6 of development in ZW females, under the influence of a genetic sex determination factor. The genes upregulated in the 28ZZ gonad include doublesex and mab-3 related transcription factor 1 (*dmrt1*), hydroxysteroid 17-beta dehydrogenase 3 (*hsd17b3*) and WNT inhibitory factor 1 (*wif1*). *Dmrt1* is a known male development gene in reptiles (Janes et al., 2014; Ge et al., 2017), and is located autosomally in *P. vitticeps* (Ezaz et al., 2009) and *hsd17b3* is a testosterone-generating steroid, mutations in which are associated with testosterone deficiency and disorders of sex development in humans (Engeli et al., 2017). *Wif1* possesses androgen-mediated roles in development (Keil et al., 2012).

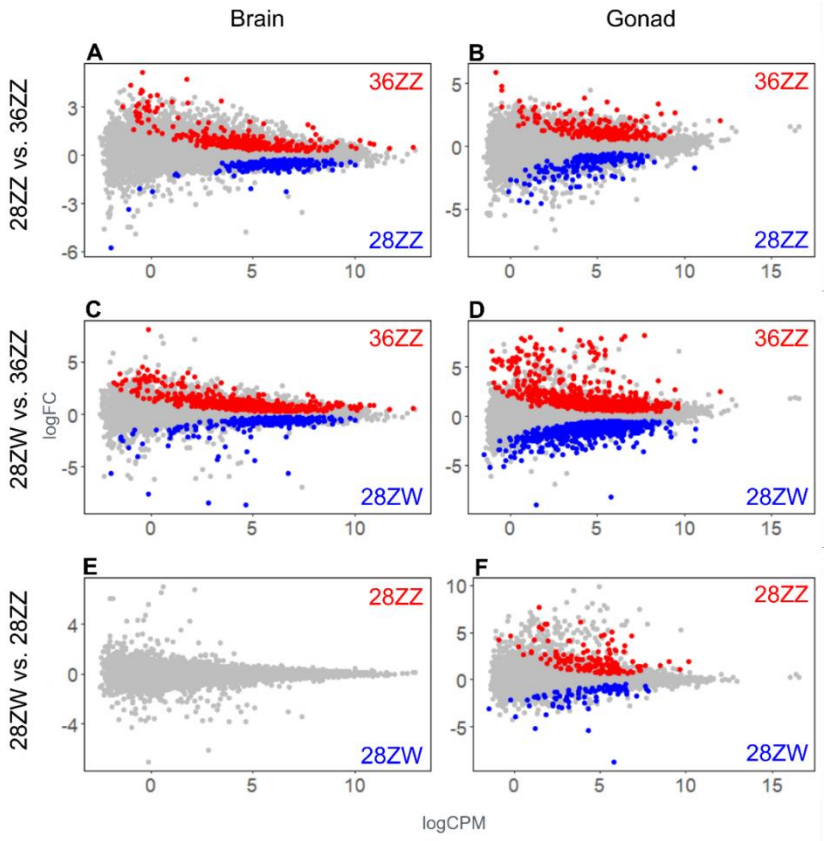


Figure 3-1. MA plots of brain and gonad transcriptomic data comparing the log counts per million (logCPM) and log fold change (logFC) of genes in each between-group comparison. Differentially expressed genes (FDR < 0.05) are coloured either red or blue, and genes which are not differentially expressed are grey. High temperature incubation (36ZZ) results in comparable levels of differential expression in both the brain and gonad when compared to both the 28ZZ (A, B) and 28ZW (C, D) groups. In comparison, the genetic sexes (28ZW vs. 28ZZ) display fewer differences when incubated at the same temperature, with no genes differentially expressed in the brain (E) and some genes differentially expressed in the gonad (F).

KEGG analysis of the genes which are uniquely upregulated in the 28ZZ gonad returned enrichment for the terms “steroid biosynthesis” and “thyroid hormone synthesis”, further demonstrating the initiation of steroidogenic activity in the gonad. Thus, gonadal sex differentiation has already commenced in stage 6 embryos which are developing according to their chromosomal sex, with estrogenic activity in the 28ZW gonad and androgenic activity in the 28ZZ gonad in the absence of any discernible differences in brain transcriptional architecture. Genes uniquely upregulated in the 28ZW gonad did not display enrichment for gonad development or steroid biosynthesis terms.

	Brain	Gonad	Brain & gonad
36°C ZZ vs 28°C ZZ	293	206	28 *clk4 cirbp kdm6b jarid2*
	200	168 *wif1*	13
36°C ZZ vs 28°C ZW	500	563 *dmrt1*	98 *clk4 cirbp kdm6b jarid2*
	208	709 *aromatase foxl2*	62
28°C ZW vs 28°C ZZ	0	84 *aromatase foxl2*	0
	0	143 *dmrt1 wif1 amh*	0

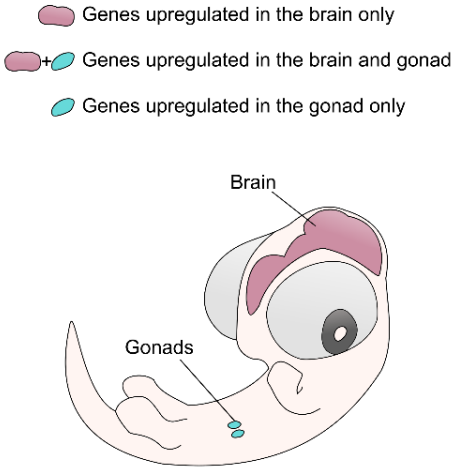


Figure 3-2. The number of genes differentially expressed between each experimental group (28ZW, 28ZZ and 36ZZ) and in each expression category (brain, gonad or brain and gonad). Between ZZ and ZW embryos incubated at 28°C, no genes are differentially expressed between the brain groups. The genes differentially expressed between the gonad groups indicate that ovary and testis differentiation has already begun at this stage of development in the absence of differential gene regulation in the brain. The candidate sex determining genes and epigenetic modifiers known to be involved in sex reversal (*clk4*, *cirbp*, *jarid2* and *kdm6b*) are upregulated in both the brain and the gonad in the high temperature incubated ZZ embryos.

3.4.2 Temperature sex reversal

3.4.2.1 Aromatase expression and sex steroid production

The estrogen-generating enzyme aromatase (*loc110077216*) is not differentially expressed in the brain in any group comparison and has uniformly absent expression in the brain. 28ZW gonads display upregulation of aromatase in comparison to all other gonad groups, even 36ZZ gonads. Estrogen receptor 1 (*esr1*), estrogen related receptor alpha (*esrra*) and estrogen related receptor gamma (*esrrg*) are not differentially expressed in any comparison in either the brain or gonad, although the estrogen receptor 2 (*esr2*) gene is upregulated in the 28ZW gonad compared to the 36ZZ gonad. The androgen and estrogen inactivating enzyme hydroxysteroid 17-beta dehydrogenase 4 (*hsd17b4*) (Rasiah et al., 2009) is upregulated in the 36ZZ brain in comparison to 28ZW brains, which may indicate some *de novo* sex steroid biosynthesis in the 36ZZ brain, though in the absence of aromatase production or activity we cannot conclude this definitively.

Given that at high temperature incubation (36ZZ) there is negligible aromatase expression in the brain, and no upregulation of aromatase in the gonad, brain aromatase mRNA production or protein activity is not likely to be the factor initiating temperature sex reversal. In comparison, estrogen is upregulated in the 28ZW gonad, indicating its importance in ovary formation in the genetic sex determination pathway. This is in general agreement with studies in both alligator (Gabriel et al., 2001; Milnes et al., 2002) and newt (Kuntz et al., 2004), which found aromatase expression to be sexually dimorphic expressed in gonads but not in the brain at different incubation sex-determining temperatures.

There is greater evidence supporting a role for brain aromatase in turtle ESD. Aromatase activity levels were higher in the brain of embryos at FPT during the TSP in *Trachemys scripta elegans*, with no differences in aromatase activity detected between gonads incubated at FPT or MPT (Willingham et al., 2000). In cultured *Lepidochelys olivacea* diencephalons, testosterone and estrogen content was responsive to incubation temperature and the authors propose a model in which aromatase expression in the brain directly or indirectly controls morphological differentiation of the gonad via neural factors (Salame-Mendez et al., 1998). Differential regulation of aromatase expression in the brain compared to the gonad was found in the diamondback terrapin (*Malaclemys terrapin*) (Jeyasuria & Place, 1998), and brain-gonad estrogen communication occurs in *M. terrapin* and the common snapping turtle (*Chelydra serpentina*) (Place et al., 2001). However, this may only be a sign of early brain sexual differentiation, rather than gonadal sex determination, so the role of brain aromatase in sex determination more broadly in ESD species is unclear. We can conclude that for *P. vitticeps*, the brain does not act to direct gonad fate *via* aromatase synthesis or signalling.

3.4.2.2 Stress axis genes and glucocorticoid production

We investigated a suite of stress axis genes for differential expression in the brain and gonad to determine if the stress axis was transcriptionally activated or repressed at high incubation temperature. The genes pro-opiomelanocortin (*pomc*), corticotropin releasing hormone binding protein (*crhbp*), corticotropin releasing hormone receptor 2 (*crhr2*) and glucocorticoid receptor (*nr3c1*) were not differentially expressed between the 36ZZ group and either the 28ZW or 28ZZ groups. Corticotropin releasing hormone (*crh*) was upregulated in the 28ZW gonad compared to both 28ZZ gonad and 36ZZ gonad, indicating a role for *crh* in the formation of the ZW ovary, although the absence of a difference in expression between the brains indicates that the high expression of *crh* in the gonad is not due to stress axis

activation and there may instead be local and glucocorticoid-independent roles for *crh* in ovary development (Whiteley et al., 2021). The expression of *pomc* is stimulated in response to the production of glucocorticoids (GCs), and acts as part of the negative feedback loop to reduce GC production, and was upregulated in the brains of adult sex-reversed females (Deveson et al., 2017), but a similar upregulation in response to incubation temperature was not observed in embryos here.

Serum/glucocorticoid regulated kinase 1 (*sgk1*) was upregulated in 28ZZ and 28ZW brain compared to the 36ZZ brain. The transcription of this gene is stimulated by glucocorticoids, indicating that glucocorticoid production may be lower in embryos incubated at high temperature, although a broad range of stressors such as oxidative stress and calcium levels can stimulate the production of *sgk1* (Schoenebeck et al., 2005). In summary, there is very little transcriptional evidence that the stress axis is acting differentially in response to temperature in stage 6 *P. vitticeps* embryos, corroborating the current experimental literature on this topic (Chapter 4; Castelli et al., 2021; Iungman et al., 2015; Marcó et al., 2015; Medler & Lance, 1998b; Uller et al., 2009; Warner, Radder, et al., 2009; Wibbels & Crews, 1992).

3.4.2.3 Neural development/activation in the gonad

Neurotransmission in the gonad may be heightened at high temperature incubation, though evidence for this is not strong. The enzyme acetylcholinesterase (*ache*), responsible for the termination of neurotransmission by acetylcholine, is upregulated in the 36ZZ gonad compared to the 28ZW gonad, which may indicate greater neuronal signal transduction in the gonad at higher incubation temperatures (Soreq & Seidman, 2001), and supports findings in the TSD turtle *L. olivacea* which detect acetylcholinesterase in the gonad during the temperature-sensitive period (Gutiérrez-Ospina et al., 1999). However, *ache* expression does not differ between the 28ZZ and 36ZZ gonads. There are no other indications of greater neuron growth or activity in the gonads at high incubation temperature. Thus, we cannot conclusively exclude neuronal signalling as the inducer of sex determination but we do not identify unequivocal evidence for this hypothesis in this dataset.

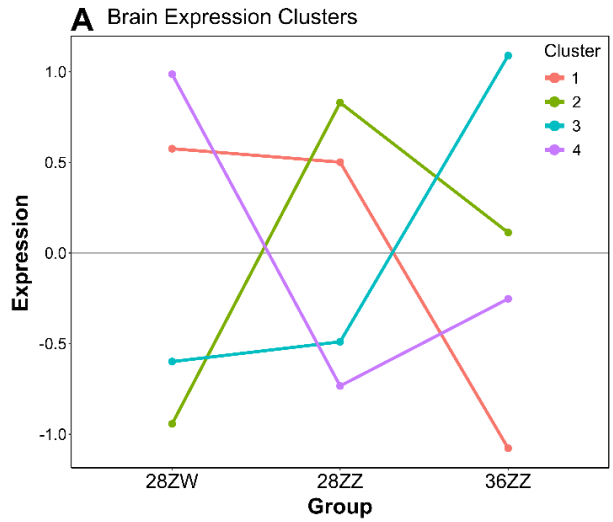
3.4.2.4 Epigenetic regulators and post-transcriptional modifiers

Epigenetic modifier genes are well known to be involved in reptile sex determination. Upregulated globally (in both the brain and gonad) at 36°C, Jumonji and AT-rich interaction domain containing 2 (*jarid2*) is a transcriptional repressor with known involvement in TSD in *P. vitticeps*. Similarly, lysine demethylase 6B (*kdm6b*) was upregulated in both the brain and

gonad at 36°C. Cold-inducible RNA binding protein (*cirbp*) is also upregulated in response to high temperature, and the global upregulation of these three genes is indicative of their critical roles in response to heat shock. Eukaryotic initiation elongation factor 4A2 (*eif4a2*) was also upregulated in both the 36ZZ brain and gonad, an mRNA regulatory protein previously identified to be associated with TSD in *A. mississippiensis* (Yatsu et al., 2016; Wilczynska et al., 2019). Given the global nature of their expression, their upregulation in the gonad is more likely to be involved in the initiation of sex reversal as opposed to their upregulation in the brain.

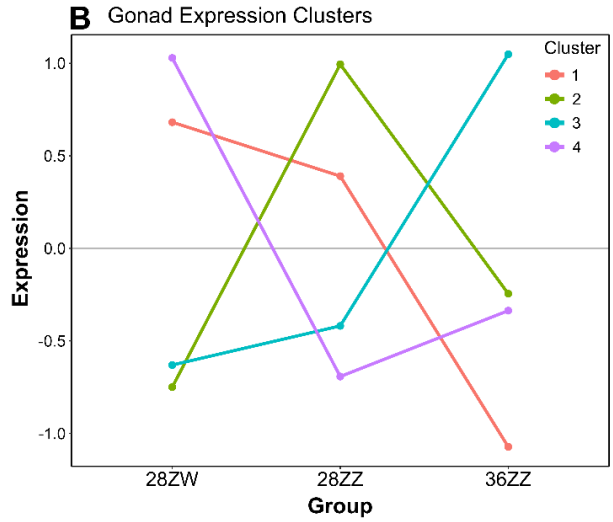
K-means clustering supports a global role for RNA regulation in the embryonic response to temperature (Figure 3-3). Genes in cluster 3 (associated with higher expression in the 36ZZ group) that were shared in both the brain and gonad k-means analysis were enriched for the KEGG terms “ribosome”, “spliceosome”, “RNA transport” and “protein processing in endoplasmic reticulum”, among others. The enrichment of these terms indicates not only greater levels of RNA and protein production (“ribosome”, “protein processing in endoplasmic reticulum”, and “RNA transport”), but also a change in the way that RNA transcripts are spliced (“spliceosome”) to perform alternative functions. The majority of this response may be due to the energetic demands of increased growth rate at high temperature (Reid et al, 2009), as evidenced by the enrichment of the terms “metabolic pathways” and “oxidative phosphorylation” in cluster 3 (Figure 3-3).

A novel marker of histone deposition has been found to be upregulated in the brain, and may represent a mechanism by which brain-specific tissue remodelling in response to high temperature is made stable. Histone H1X is upregulated within the 36ZZ brain. As the most recently identified member of the histone H1 linker histone family, the functions of histone H1X are not fully understood, however it has been shown that H1X is more abundant at included exons as opposed to excluded exons, as well as being more abundant in retained introns (Mayor et al., 2015). Interestingly, the significant and broadly demonstrated retention of specific *Kdm6b* introns in reptiles with TSD (Deveson et al., 2017; Weber et al., 2020) has been proposed to mediate sex determination in these species. Histone H1X may therefore be the epigenetic marker by which those key introns are marked, a mechanism by which epigenetic alteration persists throughout development and into adulthood. Why this process of histone H1X-mediated intron retention would occur only in the brain is not clear, but may indicate that the brain undergoes a relatively greater amount of intron retention associated with high temperature incubation than the gonad.



C Shared brain & gonad cluster 3 (up in 36ZZ)

Functional Category	Enrichment (FDR)
Ribosome	<0.001
Spliceosome	<0.001
RNA transport	0.001
Non-alcoholic fatty liver disease	0.001
Huntington disease	0.001
Metabolic pathways	0.001
Alzheimer disease	0.001
Protein processing in endoplasmic reticulum	0.005
Parkinson disease	0.009
Proteoglycans in cancer	0.009
Oxidative phosphorylation	0.014
Retrograde cannabinoid signaling	0.014
ECM-receptor interaction	0.037



D Shared brain & gonad cluster 1 (down in 36ZZ)

Functional Category	Enrichment (FDR)
Insulin signaling pathway	<0.001
Autophagy	<0.001
AMPK signaling pathway	0.002
Hedgehog signaling pathway	0.002
Circadian rhythm	0.005
Renal cell carcinoma	0.005
Oocyte meiosis	0.005
Rap1 signaling pathway	0.005
ErbB signaling pathway	0.009
Ubiquitin mediated proteolysis	0.010
mRNA surveillance pathway	0.015
Ras signaling pathway	0.021
Endocytosis	0.021
Insulin resistance	0.027
Sphingolipid signaling pathway	0.027
Vasopressin-regulated water reabsorption	0.027
Central carbon metabolism in cancer	0.041

Figure 3-3. K-means clustering analysis of brain (A) and gonad (B) transcriptome datasets of 28ZW, 28ZZ and 36ZZ embryos at k = 4. Cluster 3 genes are associated positively with the 36ZZ group (C) and cluster 1 genes are associated negatively with the 36ZZ group (D). KEGG analysis of the genes shared between the brain and gonad in cluster 3 identified enrichment for the terms “spliceosome”, “RNA transport” and “oxidative phosphorylation” among others (C). KEGG analysis of the genes shared between the brain and gonad in cluster 1 identified enrichment for the terms “circadian rhythm” and “ubiquitin-mediated proteolysis” among others (D).

3.4.2.5 Circadian rhythm: A novel temperature response pathway

Genes related to the circadian rhythm are broadly differentially regulated in both the brain and gonad in response to high temperature incubation. This is likely to carry into adulthood, as sex-reversed *P. vitticeps* adults also display significant downregulation of several key circadian regulation genes in the brain (Deveson et al., 2017). Connections have been made

between the temperature-sensing CDC-like kinases, circadian regulation and sex determination (Haltenhof et al., 2020). Here, CLK4 is upregulated in both the brain and the gonad of 36ZZ embryos, and likely participates in both regulation of circadian rhythm and alternative splicing in the TSD pathway. The peripheral circadian clocks within the body are all calibrated by the suprachiasmatic nucleus (SCN) within the hypothalamus, and the circadian system is one of the most conserved biological pathways for the incorporation of and entrainment to environmental conditions (Tosini et al., 2001), making it a good candidate for functional involvement in TSD.

The gene *cul4a* is involved in circadian regulation through the cyclical ubiquitination and degradation of the core clock protein CRY1, and is downregulated in 36ZZ brain compared to both the 28ZZ and 28ZW brain. A cryptochrome 1-like protein (*loc110088812*) is upregulated in the 36ZZ brain and gonad compared to the 28ZW brain and gonad, possibly *via* release from CUL4A degradation. The core circadian clock gene *per3* is globally downregulated in both the brain and gonad, suggesting a total perturbation of the circadian rhythm in developing 36ZZ embryos. Suppressed *per3* gene expression at 36°C is consistent with findings from the ruin lizard *Podarcis sicula* (Magnone et al., 2005), in which the rhythmicity of *per2* gene expression was abolished and expression was high in hypothermic compared to euthermic conditions. The lower expression of *per3* at 36°C could reflect either increased degradation of the transcript at higher temperature or activation of *period* gene suppressing factors.

Across fish and reptiles, perturbation of circadian rhythm genes is common either during or after the thermosensitive period. The clock-interacting pacemaker (*cipc*) gene is upregulated after 3 hour heat shock in *Caretta caretta* (Bentley et al., 2017). A suite of circadian genes are perturbed by hormone-induced sex reversal in the flounder (Zou et al., 2020). The circadian regulator *nr1d1* was upregulated in high temperature treated (early sex-reversing) tilapia (Zhao et al., 2019). Among both reptiles and fish, perturbations in circadian gene expression seem to be common. The circadian rhythm being capable of incorporating information from the environment in the brain and transferring this to other cells in the body, has not previously been considered to have a determining role in sex determination, we consider this to be unlikely. The circadian rhythm gene expression is known to be under control of *cirbp*, although typically it's thought that attenuation of *cirbp* lowers circadian gene expression (Hoekstra et al., 2019), and in this case we see high levels of *cirbp* and low expression of several circadian rhythm genes.

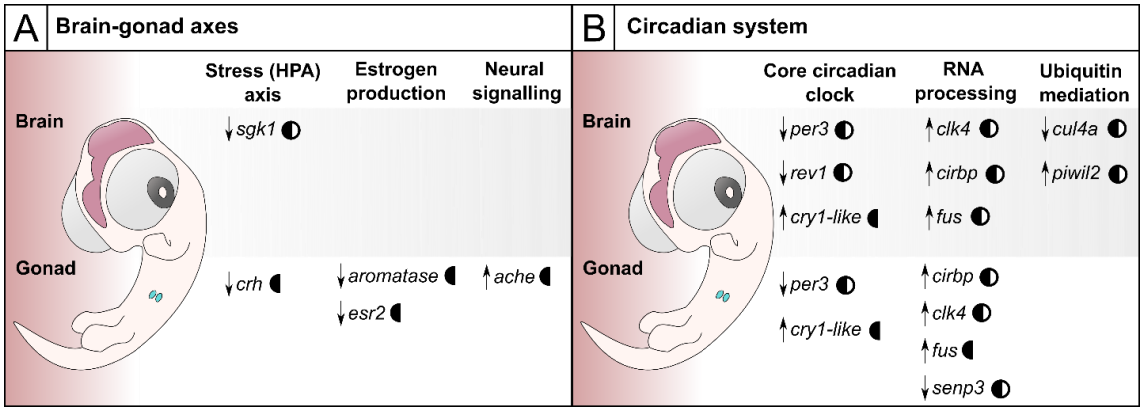


Figure 3-4. The expression patterns of genes within 36ZZ embryos relating (A) previously proposed hypotheses for brain-driven sex determination and (B) the circadian system, a novel candidate for brain-driven sex determination. The directionality of gene expression is displayed by an arrow to the left of the gene, and whether the gene is upregulated or downregulated in relation to either the 28ZW (◖) or 28ZZ (◗) groups, or both (◐).

3.5 Conclusion

Here we show that in the sex-reversing agamid species *P. vitticeps*, there is extensive sex-specific and temperature-sensitive gene expression in both the brain and the gonad, demonstrating that both organs are environmentally responsive, even in an early stage of development. To assess the role of the brain in mediating temperature sex reversal during the temperature sensitive period, we focused our analyses on known brain-gonad axes. Under genetic influence at a developmentally neutral temperature (28°C), gonad differentiation (as determined by the expression of canonical ovary- and testis-formation genes) proceeds in the absence of differential gene expression in the brain. We conclude that neither stress axis activation nor brain aromatase production are involved in the initiation of sex reversal, owing to the relative lack of differential expression of core genes in these axes. Both hypotheses have been prominent candidates for the mediation of TSD in reptiles, though the evidence for both is conflicting and based primarily on non-squamate reptiles (Merchant-Larios & Villalpando, 1990; Lance & Bogart, 1994; Salame-Mendez et al., 1998; Shoemaker-Daly et al., 2010; Mork et al., 2014; Chapter 4; Castelli et al., 2021). We do identify extensive differential regulation of circadian rhythm genes in both the brain and the gonad, representing a novel hypothesis for brain-driven mediation of sex in ESD species. If the circadian rhythm does play a role in initiating or effecting sex reversal, this may explain the occurrence of light-induced sex determination in many species of fish (Corona-Herrera et al., 2018; Hayasaka et al., 2019; García et al., 2020).

Chapter 4 Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*)

This chapter has been published in the **Journal of Experimental Zoology Part A: Ecological and Integrative Zoology**.

Castelli, M., Georges, A., & Holleley, C. E. (2021). Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*). Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, Early view. https://doi.org/10.1002/jez.2441

Apart from formatting, this chapter has not been altered in any way. The references have been consolidated at the end of the thesis.

4.1 Abstract

Environmental sex determination (ESD) is common among ectothermic vertebrates. The stress axis and production of stress hormones (corticosteroids) regulates ESD in fish, but evidence of a similar influence in reptiles is sparse and conflicting. The central bearded dragon (*Pogona vitticeps*) has a system of sex determination involving the interplay between sex chromosomes (ZZ/ZW female heterogamety) and the thermal environment. High egg incubation temperatures induce sex reversal of the ZZ genotype, feminising chromosomally male individuals. Here we show that corticosterone elevation is not associated with sex reversal in the central bearded dragon, either during embryonic development or adulthood. We also demonstrate experimentally that sex determination is not affected by corticosterone injection into the yolk. This strongly suggests that stress axis upregulation by high temperature during incubation does not cause sex reversal in *P. vitticeps*. Our work is in general agreement with other research in reptiles, which suggests that the stress axis does not mediate sex in reptiles with ESD. Alternative biological systems may be responsible for capturing environmental conditions during reptile development, such as cellular calcium and redox regulation or the action of temperature-sensitive splicing factors.

4.2 Introduction

In many lineages of fish, amphibians and reptiles, environmental conditions experienced during early development influence sex (Bachtrog et al., 2014), a developmental process known as environmental sex determination (ESD). Among vertebrates, modes of sex determination are most diverse in fish, including conventional genetic sex determination (GSD), pure ESD, GSD with environmental influence, or sex change post-maturity (Todd et al., 2016; Baroiller & D'Cotta, 2016). A wide range of environmental cues determine sex in fish, including temperature, pH, salinity, social status or light regime (Baroiller & D'Cotta, 2016). The predominant environmental cue in reptile ESD is temperature (temperature dependent sex determination; TSD), although moisture and egg size can also affect sex (Radder et al., 2009; Wyneken & Lolavar, 2015; Dupoué et al., 2019). Temperature has the capacity to direct sexual outcomes in the absence of sex chromosomes, in combination with an underlying genetic predisposition or even in the presence of differentiated sex chromosomes (Sarre et al., 2004). The epigenetic effectors of ESD are becoming well characterised in reptiles (Deveson et al., 2017; Ge et al., 2018; Weber et al., 2020), but the biological sensory mechanism which receives and translates the environmental signal into a biological cue is not yet known (Georges & Holleley, 2018).

The diversity among vertebrates in the environmental cues that are influential in sex determination (Bachtrog et al., 2014) and the poor concordance between modes of sex determination and phylogeny, particularly in reptiles (Sarre et al., 2004), suggests that ESD has evolved repeatedly and independently many times, with frequent transitions in sex determining modes. Whether the biological translation mechanism of ESD is common to all species, or whether different mechanisms have been recruited during each emergence of ESD is not yet known. In fish, the vertebrate stress axis (hypothalamic-pituitary-interrenal/HPI axis) plays a fundamental role in ESD in both gonochoristic (Hattori et al., 2009, 2020; Mankiewicz et al., 2013; Adolfi et al., 2019; Castañeda Cortés et al., 2019) and sequentially hermaphroditic fish (Solomon-Lane et al., 2013; Goikoetxea et al., 2017; Chen et al., 2020). Typically, environmental influence leads to masculinization in fish (Ospina-Álvarez & Piferrer, 2008), and the molecular pathway by which cortisol initiates androgen production is well understood (Goikoetxea et al., 2017). The production of cortisol can generate an increase in the enzyme HSD11B2/3. This enzyme inactivates cortisol as part of the negative feedback system of the stress axis, but also catalyses the conversion of testosterone to 11-

ketotestosterone, a potent androgen (Chen et al., 2020). The molecular pathway of cortisol involvement in sex determination in fish is thus well-characterised.

A causative role for the stress axis and glucocorticoids in reptile ESD not been conclusively demonstrated, though it must be noted that far less experimental research has been conducted in reptiles. Only five explicitly test this hypothesis. There was no effect of corticosterone application on sex (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015) and no association between embryonic corticosterone production and male or female-producing temperatures (Marcó et al., 2015). One study did conclude that corticosterone may have a feminizing effect in a TSD agamid lizard (*Amphibolurus muricatus*), though sex-specific mortality in response to corticosterone application could not be excluded (Warner, Radder, et al., 2009). Here we apply comparable methods to a third reptile species with high temperature sex reversal and investigate the role of corticosterone in influencing sexual outcome.

The central bearded dragon (*Pogona vitticeps*) is an agamid with both a ZZ/ZW system of GSD (Ezaz et al., 2005) and thermosensitivity (Holleley et al., 2015; Deveson et al., 2017). Adult ZZ sex-reversed females display transcriptomic signatures of an upregulated stress response, including upregulation of the stress hormone signalling precursor proopiomelanocortin (*POMC*) and downregulation of the negative feedback regulator corticotropin releasing hormone binding protein (*CRHBP*) (Deveson et al., 2017). Behavioural repertoires of juvenile sex-reversed females differ from those of either concordant sex, also indicative of sustained stress axis upregulation (Li et al., 2016). This evidence suggests that stress axis activation and glucocorticoid production are key effectors of the sex-reversed phenotype in adulthood, and so may also be involved in the initiation of sex reversal in the embryo. We predicted that (a) corticosterone and other indicators of prolonged hormonal stress will be naturally elevated in adult sex reversed females, and (b) experimental injection of corticosterone at low temperatures will initiate sex reversal. Contrary to our predictions, we did not find evidence that the stress axis mediates or is a long-term consequence of sex reversal in *P. vitticeps*. We did observe that acute exposure to high incubation temperature elevates embryonic corticosterone levels, but not until after sex determination has occurred.

4.3 Materials and Methods

4.3.1 Colony details

Captive bred dragons from a breeding colony at the University of Canberra were used in this study. All dragons were caged communally in terrariums with sand substrate, water available *ad libitum*, and on equal feeding regimens. All adults were of known chromosomal sex (using the sex test of Holleley et al., 2015), and were either concordant males (ZZm), concordant females (ZWf) or sex-reversed females (ZZf).

4.3.2 Animal breeding and egg allocation

Breeding groups consisting of one male and 2-4 females were set up to generate eggs. Both sex-reversed females (ZZf) and concordant females (ZWf) were used in this study. Gravid females were allowed to lay naturally, and eggs were recovered within 24 hr of oviposition. Fertile eggs which had visible vascular systems were weighed and placed in boxes with divisions filled with moist vermiculite (5 vermiculite: 4 water) and immediately incubated at a constant 28°C or 36°C until egg treatment. Eggs from each clutch were allocated systematically to temperature and treatment blind to the genotypic sex of the embryo.

4.3.3 Egg incubation and injection

Eggs were injected with corticosterone (Sigma C2505) in sesame oil vehicle (Sigma S3547) or sesame oil alone three days after lay. Uninjected controls were not altered. Firstly, the egg was candled to confirm the position of the embryo at the top of the egg. Injections were performed using disposable BD Insulin Syringes (Product #324910), which have 5 µL divisions and a 6 mm 31-gauge needle. The needle was inserted to its full length at a position approximately halfway down the lateral (long) side of the egg, to position the needle in the approximate centre of the egg, depositing the vehicle or corticosterone solutions into the yolk. Once the injection was complete, a small amount of cyanoacrylate superglue (Selleys® QuickFix Gel SupaGlue^{TM}) was placed over the injection site, to prevent infection and desiccation. Eggs were then returned to their incubator and incubated at a constant 28°C or 36°C until either embryonic sampling or hatching. Doses of corticosterone were either 10 µg CORT total in 5 µL sesame oil or 25 µg CORT total in 5 µL sesame oil. We estimated the endogenous levels of yolk corticosterone in *P. vitticeps* based on those of two Australian agamids, *Amphibolurus muricatus* and *Ctenophorus pictus*. Using *A. muricatus*, we assumed

an average corticosterone concentration of 7.8 pg mg^{-1} of yolk mass (Warner et al., 2007). Using *C. pictus* we assumed an average corticosterone concentration of 446.16 pg g^{-1} of total egg mass (Uller et al., 2009; Hansson & Olsson, 2018). The average total egg mass of *P. vitticeps* is approximately 4.2g (unpublished data), and so making the conservative assumption that the egg is 100% yolk at the time of lay then the total amount of yolk corticosterone per egg in *P. vitticeps* is likely to range from 0.0019 - 0.0328 µg, significantly lower than even the low dose (10 µg) applied in these experiments.

4.3.4 Experiment 1: Tissue corticosterone levels during development

Eggs from 12 males and 12 females were used in this experiment. For each clutch, within 24 hr of oviposition half of the eggs were transferred to constant temperature incubators set at 28°C and the other half at 36°C. A total of 150 eggs were used in this experiment, split into the control (no injection; n = 24 at 28°C, n = 31 at 36°C), vehicle control (5 µL sesame oil; n = 24 at 28°C, n = 27 at 36°C) or high CORT (25 µg CORT in 5 µL sesame oil; n = 29 at 28°C only) treatments. Both control and vehicle injections were conducted at both 28°C and 36°C, and CORT injections were only conducted in eggs at 28°C.

Embryos from both incubation temperatures and all treatments and controls were sampled at embryonic development stages 7 and 12. Stage 7 embryos still possess bipotential gonads when incubated at constant temperature, and we thus consider sex to still be labile and responsive to temperature at this point (Whiteley et al., 2017, 2018). Stage 7 embryos were sampled at 20 d post lay (dpl) for embryos at 28°C and 10 dpl for embryos at 36°C. Stage 12 embryos incubated at constant temperature possess fully differentiated gonads (ovaries or testes) and we thus consider sex to be fixed at this point during development (Whiteley et al., 2017, 2018). Stage 12 embryos were sampled at 35 dpl at 28°C and 20 dpl at 36°C (see 4.3.6 Hormonal and metabolite sampling).

4.3.5 Experiment 2: The effect of corticosterone manipulation on sex

A total of 168 eggs were used in this experiment. Eggs within each clutch were systematically allocated to control, vehicle control, low CORT and high CORT treatments at a ratio of 1:1:3:3. Eggs were allocated to control (no injection; n = 27), vehicle control (5 µL sesame; n = 18), low CORT (10 µg CORT in 5 µL sesame oil; n = 61), or high CORT (25 µg CORT in

5 µL sesame oil; n = 62). The high CORT treatment is approximately equivalent (by egg weight) to the dose applied to lizard eggs in (Warner et al., 2009).

In this experiment all eggs were brought to hatching. Hatchlings were removed from the incubator one day after hatching and placed into plastic tubs with newspaper substrate and cardboard hides. Hatchlings were provided with water *ad libitum* and were kept alive for 3 d before euthanasia and sampling. They were not fed during this time to prevent antagonistic competitive interactions between hatchlings. Before euthanasia via intraperitoneal injection of sodium pentobarbital, blood samples were taken to conduct plasma corticosterone measurements (see Hormonal and metabolite sampling).

4.3.6 Hormonal and metabolite sampling

Blood samples from adult dragons were taken between 10 am and 4 pm, allowing the dragons to behaviourally thermoregulate and reach an appropriate body temperature for blood sampling without the need for prior handling and positioning by researchers. Blood samples were taken from 20 sex-reversed females, 20 concordant females and 20 concordant males for corticosterone analysis. For DNA damage analysis, plasma samples of 14 sex-reversed females, 18 concordant females and 12 concordant males were used. It was assumed that as the dragons were bred in captivity and have become habituated to the presence of humans, the presence of the researcher in the terrarium room was likely not a major stressor, and so sampling time was considered to begin when the animal was first handled. Effort was made to keep handling time to a minimum.

Blood was drawn from the caudal vein, syringed into heparinised microcapillary tubes (Thomas Scientific 1202K16), sealed with capillary wax (Thomas Scientific 1202K13) and placed on ice until centrifugation in a microhematocrit centrifuge for 15 min. All samples were fractionated and frozen at -20°C within 8 hr of collection. If the tail had been punctured three times, the caudal vein had not been found and the handling time reached 7 min, sampling was abandoned and the animal returned to its cage.

In experiment 2, 113 hatchlings were successfully blood sampled for comparisons of basal corticosterone levels between treatments. Before euthanasia, the tip of the tail was cut and blood from the tail drawn into a heparinised microcapillary tube (Thomas Scientific 1202K16). The tube was sealed with microcapillary wax (Thomas Scientific 1202K13) and spun down in a microhematocrit centrifuge for 15 min. Plasma was separated from the red blood cell pellet and frozen at -20°C for later analysis of corticosterone.

Sampled embryos were euthanised via intracranial injections of sodium pentobarbital before being snap frozen in liquid nitrogen. Steroid fractions were extracted from subsamples of the embryo (see Corticosterone and DNA damage measurements).

4.3.7 Corticosterone and DNA damage measurements

Corticosterone and DNA damage levels were assessed using the Arbor Assays® DetectX Corticosterone EIA kit (K014) and DetectX DNA Damage EIA kit (K059) according to the manufacturer's instructions applied to blood plasma. Plasma samples were diluted to an appropriate level to ensure that measurements fell within the standard curve generated during each plate run. Mostly, dilutions were performed to a factor of 12, but ranged from a 5 to 25-fold dilution factor, depending on the amount of plasma available and whether previous runs determined the sample to have exceedingly low or high amounts of corticosterone. A subset of samples were included on every plate to assess inter-assay variability. The endpoint absorbance values at 420 nm absorbance were read on a Bio-Rad Benchmark Plus Microplate Spectrophotometer and the raw values exported. Corticosterone and DNA Damage levels were calculated from the raw data using the MyAssays (www.myassays.com) calculator tools designed specifically for these two assays.

Steroid hormone extractions for tissue were conducted according to the Arbor Assays® protocol for tissue steroid extractions. In short, 5-15 mg of trunk tissue was pulverised using a handheld mortar and pestle in 1.5 mL acetonitrile (HPLC grade, 100%). The sample was centrifuged at 10,160xg for 10 min at 4°C and the supernatant was transferred to a clean tube. 3 mL hexane (HPLC grade, 100%) was added to each sample and the tube was vortexed for 5 min, to solubilise the tissue-derived lipids in the hexane layer. The hexane was poured off and the acetonitrile transferred to a clean container before evaporation of the acetonitrile using a Christ SpeedVac system. One sample in each extraction batch was spiked with a known quantity of corticosterone to evaluate steroid recovery.

Dried hormone samples were kept frozen at -80°C for no more than three days until use in the Arbor Assays® DetectX Corticosterone Enzyme Immunoassay kit (K014). The extracted hormone fraction was dissolved in 10 µL of 100% ethanol, then with 40 µL of the diluted assay buffer. The sample was vortexed well and incubated at room temperature for 5 min three times, to ensure reconstitution of the hormone. The samples were again diluted with 350 µL diluted assay buffer and immediately run in duplicate in the assay. The manufacturer's protocol for the corticosterone EIA kit indicates that there is the potential for

cross-reactivity mainly with 1-dehydrocorticosterone and desoxycorticosterone, metabolites of corticosterone with no glucocorticoid activity.

4.3.8 Genotypic and phenotypic sexing

Clutches from both ZWf mothers and ZZf mothers were used in these experiments, so hatchling *P. vitticeps* were phenotypically sexed using the hemipene transillumination (HTI) method while alive, and subsequently after euthanasia via manual eversion of the hemipenes. Sampled embryos were not phenotypically sexed, as all embryos possess hemipenes which are only regressed in females before hatching (Whiteley et al., 2017).

To confirm genotypic sex in both hatchlings and embryos, a blood sample was placed on an FTA Elute or Classic card and DNA was extracted using the Whatman Elute protocol or Qiagen DNeasy kit. A PCR sex test was conducted to determine if the individual was ZZ or ZW following the method of Holleley et al., (2015) and primers characterised by Quinn et al., (2010).

A subset of gonads from each treatment and genotype (total of 15 individuals) from experiment 1 were used to confirm gonadal identity *via* histology. Five hatchlings of each genotype (ZZ and ZW) in each treatment were used (aside from control ZW, in which three hatchlings were used) to confirm that gonadal morphology matched with the presence or absence of hemipenes. The adrenal-kidney-gonadal complex was removed and fixed in 10% neutral buffered formalin for 24 hr then transferred to 70% ethanol. Histology was performed using haematoxylin and eosin (H&E) staining as described in Whiteley et al., (2018).

4.3.9 Statistical analysis

All statistical analyses were conducted in RStudio (R version 4.0.2). Data were analysed for normality, homogeneity of variance by analysis of residuals. Outliers were identified and data analysis performed both with and without outliers included to determine their impact on the result. Where data were not normally distributed (tissue corticosterone measurements from experiment 1), a log10 transformation was applied and the data analysed using a factorial ANOVA which accounted for specimen genotype (ZZ/ZW) and treatment.

4.4 Results

4.4.1 Experiment 1: Tissue corticosterone throughout development

The purpose of this experiment was to examine, by way of a factorial design, the impact of treatments involving combinations of corticosterone injections, stage and temperature (treatment) on embryonic tissue corticosterone (response), after accounting for differences between the sexes (Figure 4-1). Assumptions of homoscedasticity and normality were accommodated by a log 10 transformation. There was no significant interaction between treatment and sex as the main factors ($F = 0.883$; $df = 5,79$; $p = 0.497$), so it was appropriate to consider the significance of the main effects. There was no significant difference between the two sexes ($F=0.232$; $df = 1,79$; $p = 0.631$). There was a significant effect of the treatment on embryonic corticosterone levels ($F = 4.805$; $df = 5,79$; $p < 0.001$). Neither corticosterone treatment nor incubation temperature affected embryo mortality in this experiment ($\chi^2 = 4.147$, $df = 4$, $p = 0.387$).

The data were re-analysed as a single-factor ANOVA (on treatment) followed by Tukey multiple comparisons. Control and vehicle injection treatments did not differ significantly and were pooled. The 36°C control stage 12 embryos had significantly higher corticosterone levels than either of the 28°C control stage 7 embryos (Tukey HSD = 0.388, $df = 85$, $p < 0.001$). and the 28°C CORT stage 7 embryos (Tukey HSD = 0.302, $df = 85$, $p = 0.009$) (Figure 4-1). There were no other significant differences between groups (Figure 4-1).

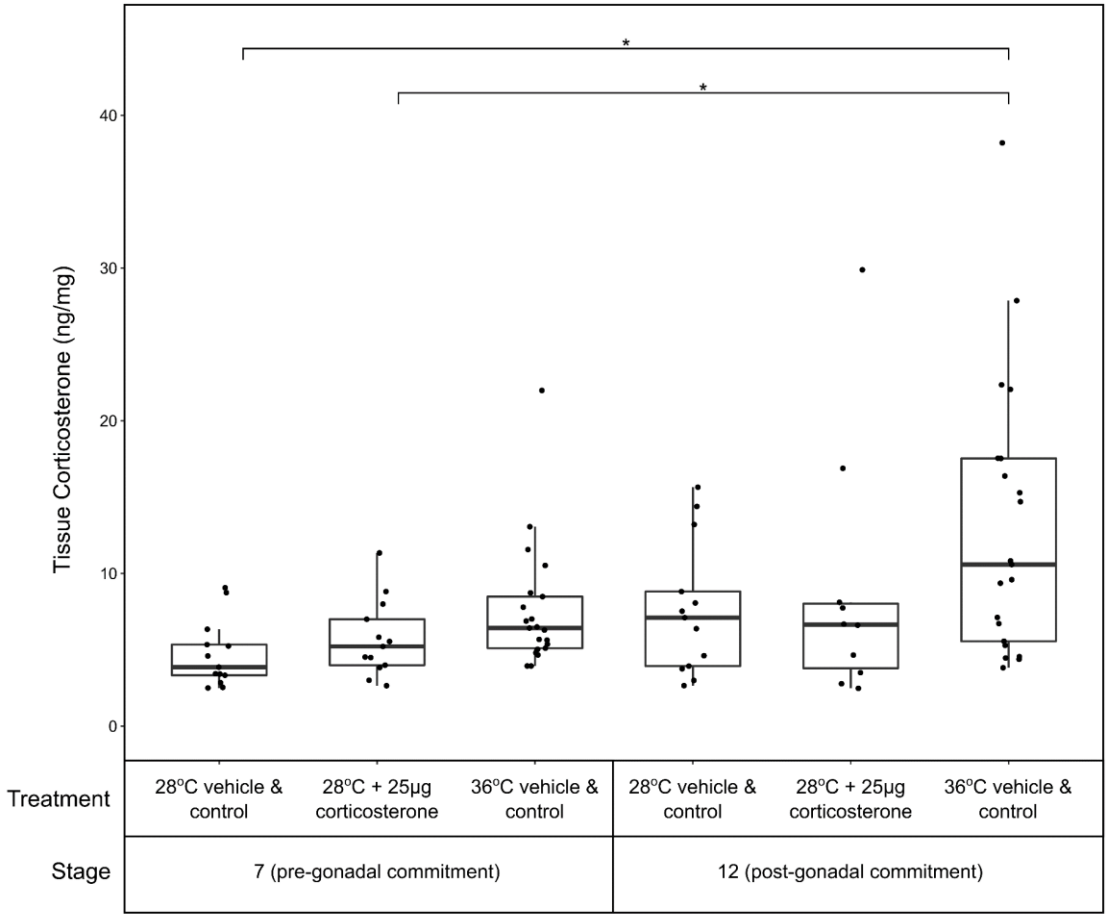


Figure 4-1. Embryonic tissue corticosterone levels are elevated in response to high temperature (36°C) incubation after gonadal commitment. Analysis was conducted on log10 transformed values. A factorial ANOVA with Tukey's pairwise comparisons determined that 36°C incubated stage 12 embryos had higher tissue corticosterone levels than both 28oC incubated control (Tukey HSD = 0.388, df = 85, $p < 0.001$). and 28°C corticosterone-treated (Tukey HSD = 0.302, df = 85, $p = 0.009$) embryos. This indicates that the embryonic stress axis can be responsive to incubation temperature, but only after the thermosensitive period, strongly suggesting that the vertebrate stress axis does not initiate sex reversal in Pogona vitticeps. Each treatment group contains both ZZ and ZW embryos.

4.4.2 Experiment 2: Effects of corticosterone on sex

The purpose of this experiment was to examine, by way of a factorial design, the impact of corticosterone injection concentration and genotype (treatments) on hatchling (3 d post-hatch) plasma corticosterone (response). Of the hatchlings which were produced (n = 158), sufficient plasma was recovered to allow corticosterone measurements from 21 control hatchlings, 8 vehicle injected hatchlings, 43 low CORT hatchlings and 40 high CORT hatchlings. Corticosterone treatment did not affect mortality in this experiment ($\chi^2 = 1.244$; df = 3; p = 0.743). There was a significant treatment and sex interaction (F = 5.196; df = 2, 104; p = 0.007). Opposite to what we would predict, the ZW control group displayed significantly higher corticosterone levels than the group experimentally treated with 25µg corticosterone injection in ZW individuals (Tukey HSD = 46.63; df = 106; p = 0.026) (Figure 4-2). There were no other significant differences between treatment classes or genotype (Figure 4-2).

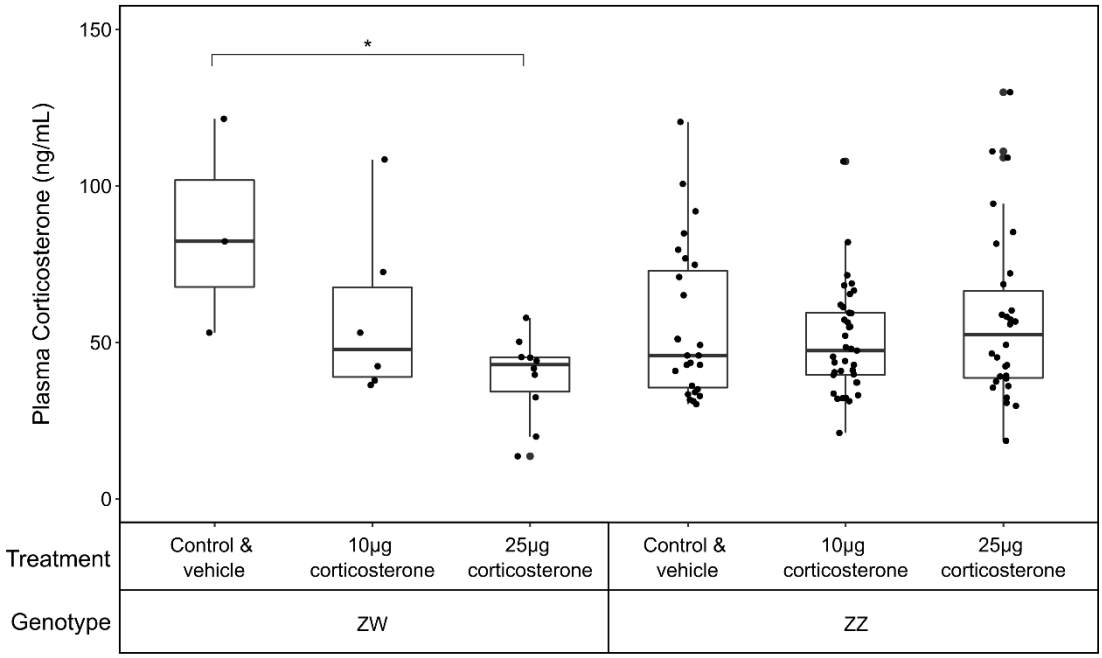


Figure 4-2. Hatchling basal plasma corticosterone (CORT) levels are not elevated by corticosterone yolk injections early in development. Genotypic female (ZW) and genotypic male (ZZ) eggs were treated with two doses of corticosterone (10 µg or 25 µg) during development. No hatchlings were sex-reversed as a result and all hatchlings had a physical sex concordant with their genotypic sex. Vehicle-treated (sesame oil injections) and uninjected control hatchlings were pooled for analysis. Control ZWf hatchlings had significantly higher basal plasma corticosterone levels than 25 µg CORT-treated ZWf hatchlings (Tukey HSD = 46.63, df = 107, p = 0.026), but there were no other significant differences between treatment groups, demonstrating that CORT injections early in development do not affect basal CORT levels.

No sex-reversed individuals were identified in either the low CORT or high CORT treatment groups (Figure 4-3). All ZZ individuals were phenotypically male and all ZW individuals were phenotypically female. Histological examination of gonads confirmed typical ovary structure in ZW females and typical testes structure in ZZ males, regardless of treatment group.

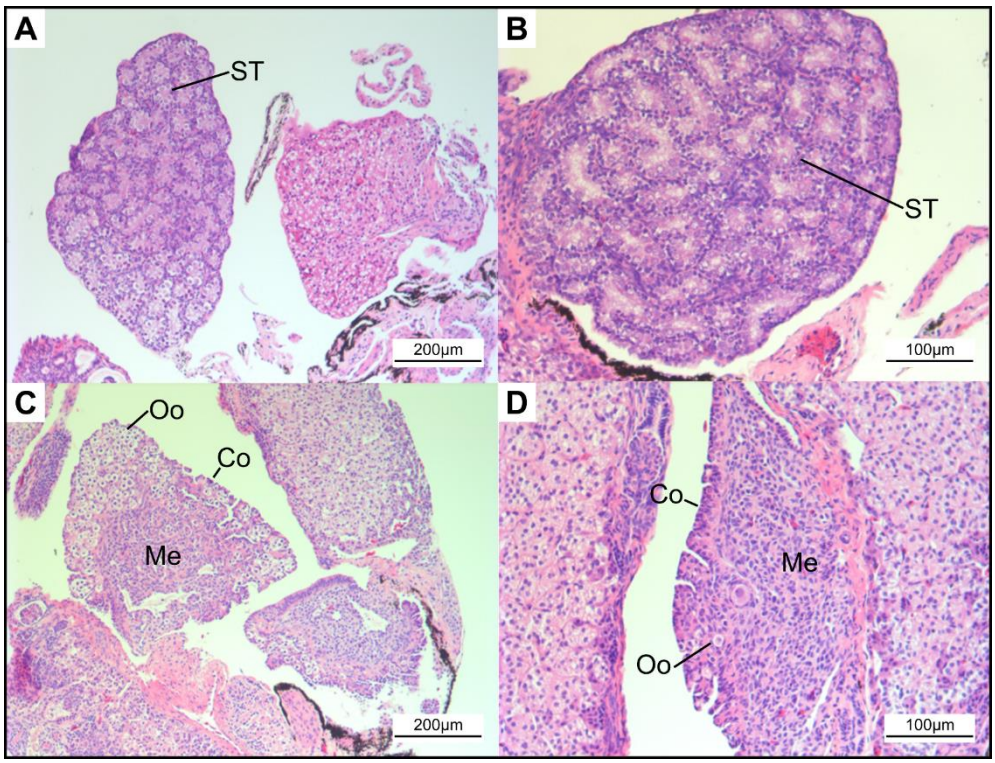


Figure 4-3. Gonadal phenotype matches genetic sex in corticosterone-treated *P. vitticeps* hatchlings incubated at a non sex-reversing temperature. Both control ZZ hatchlings (A) and ZZ hatchlings treated with corticosterone early in development (B) have normal testes with seminiferous tubules (ST). Both control ZW hatchlings (C) and ZW hatchlings treated with corticosterone early in development (D) have normal ovaries, with a clear medulla (Me) and cortex (Co) and developing oogonia (Oo).

4.4.3 Corticosterone measurements in adults

The purpose of this experiment was to examine, by way of a factorial ANOVA, the differences in plasma corticosterone levels and DNA damage (an indicator of chronic stress) in adult specimens of the three sex classes, ZZm (male), ZZf (reversed to female) and ZWf (female). Mean basal levels of plasma corticosterone between the three sex classes did not differ significantly ($F=2.737$; $df = 2,57$; $p = 0.073$) (Figure 4-4). Exclusion of the two outliers did not alter the outcome ($F=2.849$; $df = 2,55$; $p=0.067$). There were no significant differences

between levels of oxidised guanine in adult ZW females, ZZf sex reversed females and ZZm males (F=0.987; df = 2,41; p = 0.381) (Figure 4-4). Exclusion of the two outliers did not alter the outcome (F=0.819; df = 2,39; p=0.448).

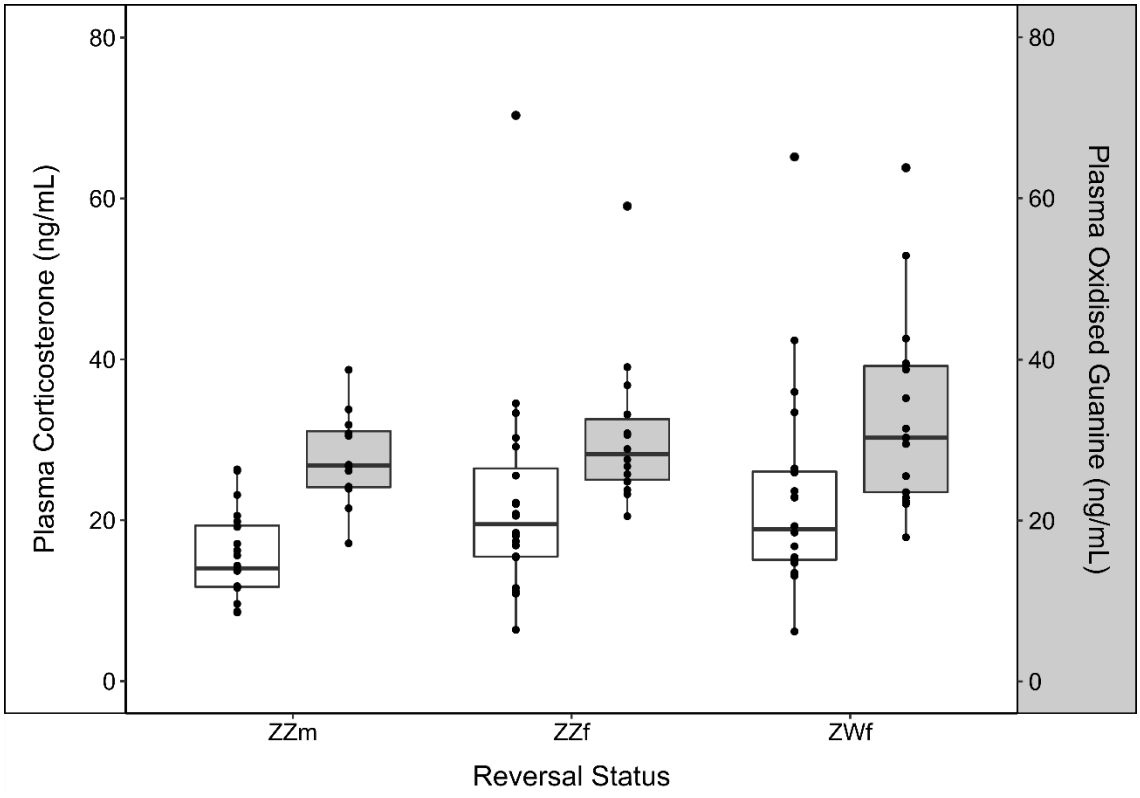


Figure 4-4. Adult basal plasma corticosterone (white) and oxidised guanine (grey) levels of concordant female (ZWf), sex-reversed female (ZZf) and concordant male (ZZm) bearded dragons (*Pogona vitticeps*). A one-way ANOVA detected no significant difference between mean corticosterone or oxidised guanine levels in any of the sexes, indicating that there is no difference in basal hormonal stress levels or DNA damage levels (as an indicator of long-term chronic stress) between the sexes.

4.5 Discussion

We set out to determine whether the vertebrate stress axis and corticosterone production are either a cause or consequence of sex reversal in *P. vitticeps*. We found no difference in the basal corticosterone levels of sex-reversed adult dragons or embryos at high temperature during a thermolabile period of development. Exposure to high incubation temperature does induce a temporary acute stress response to temperature *in ovo* during late embryonic development (stage 12, after gonadal commitment). However, this corticosterone elevation

does not occur during a period of development where sexual differentiation is labile, and it does not persist post-hatching or in adults. Our results are consistent with previous work in other reptile species that do not support a role for the vertebrate stress axis in reptile ESD (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015; Marcó et al., 2015).

We did not observe either acute (corticosterone) or chronic (DNA damage) stress axis upregulation in adult sex reversed females (Figure 4-4). Nor did we observe upregulation of corticosterone at the sexually labile period of development (stage 7) (Figure 4-1). Here we can confidently conclude that sex reversed females are not inherently more stressed than concordant males and females. This result is contrary to our predictions based on a previous RNA-seq study of sex reversal. This discrepancy could be for several reasons. The previously observed upregulation *POMC* (a pre-protein which signals for stress hormone production) in sex-reversed females could have been biased by random acute upregulation of the stress response during manual handling and/or low sample sizes (Deveson et al., 2017). However, similar *POMC* upregulation occurs in temperature treated fish with a sex reversal system (Yao et al., 2021), corroborating the Deveson et al., (2017) result. We assumed that the observed *POMC* upregulation in sex-reversed females (Deveson et al., 2017) is indicative of a stress response because one of its derived proteins (ACTH) is released into the circulatory system and signals glucocorticoid secretion from the adrenals. However, POMC has a range of other non-stress related biological functions. For example, the POMC pre-protein can be cleaved for roles in melanin production and steroidogenesis (Denver, 2009; D'Agostino & Diano, 2010; Cawley et al., 2016; Narayan, 2017). Thus, it is likely that *POMC* upregulation is a genuine consequence of sex reversal, but activation of the stress response via corticosterone is not.

High incubation temperature did result in elevated corticosterone levels, but only later in development after gonadal commitment. Specifically, stage 12 embryos incubated at 36°C have significantly higher levels of corticosterone than stage 7 embryos incubated at 28°C. They were not significantly higher, however, than stage 12 28°C incubated embryos. The timing of this increase in corticosterone in response to temperature is important because it occurs after the bipotential gonad has transitioned to a committed gonad (Whiteley et al., 2018). At constant incubation temperature, by developmental stage 12 the embryo has exited the thermosensitive window where sex can be influenced by environmental stimuli. While nothing is known about the ontogeny of the stress axis in reptile embryos, in chickens the adrenals are capable of low-level autonomous corticosteroid production throughout

development. Corticosteroid production does not come under cerebral control until two thirds of the way through development in the egg (Jenkins & Porter, 2004). Our result indicates a similar ontogeny of the stress axis in the beaded dragon. Proximal exposure to high temperature in the egg activates the stress axis late in development when the cerebral components of the stress axis (hypothalamus and pituitary) are integrated fully with the adrenal.

Direct injection of corticosterone (10µg and 25µg doses) did not induce sex reversal in the absence of high temperature exposure (Figure 4-3). Utilising sex reversal to detect the effect of our treatment on sex determination is more powerful and more definitive than previous work which relied upon detecting differences in resulting sex ratios (Warner, Radder, et al., 2009; Iungman et al., 2015; Marcó et al., 2015). Thus, here we are confident in the absence of an effect of our treatment on sex determination.

Experimental approaches that rely upon injecting compounds into the yolk of an egg make the assumption that this is an effective mode of delivery for the embryo. It must be considered that yolk hormone manipulation may not have been taken up by the embryo here and in other reptile experiments (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015), and the lack of a detectable increase in tissue corticosterone concentrations in treated embryos in this experiment (Figure 4-1) may suggest this. However, strong evidence from this literature supports the successful delivery of corticosterone to the embryo and the lack of an effect of corticosterone on sex. Using identical delivery methods, Wibbels & Crews, (1992) demonstrated that a number of estrogenic compounds generated females at male-producing temperatures, but that neither corticosterone nor metyrapone (a corticosterone suppressing drug) affected sex. Despite the absence of an effect of corticosterone/dexamethasone treatment on sex, Iungman et al., (2015), Warner et al., (2009) and Uller et al., (2009) all detected phenotypic differences in hatchlings as a result of treatment.

Given the demonstrated phenotypic effects of corticosterone treatment in similar contexts, the lack of increase in tissue corticosterone could be explained by what we know of yolk hormone movement. The metabolism of corticosterone by the embryo is rapid (Carter, Bowden, et al., 2018), and so is conversion of the steroid from its lipid-soluble to water-soluble forms *via* reversible sulfonylation (Moore & Johnston, 2008; Paitz & Bowden, 2008). Our tissue corticosterone extractions and measurements targeted only lipophilic free corticosterone, and it is possible that this method obscured differences in levels of corticosterone metabolites. However, increasing corticosterone dosage resulted in decreasing

plasma corticosterone concentrations in ZW hatchlings (Figure 4-2). This suggests that corticosterone was successfully delivered to the embryo and that gross administration of high concentrations may result in a negative feedback loop, suppressing endogenous corticosterone production. A similar manipulation study involving the application of estrogen to alligator eggs raised the possibility of such a mechanism having a suppressive effect on the steroid of interest (Milnes et al., 2002). Further experimentation and method improvement would remove this ambiguity.

We have demonstrated that corticosterone production is not elevated at high temperatures during the temperature sensitive period of sex determination, and that stress axis dysregulation is not an inherent consequence of sex reversal. This agrees with most other studies in reptiles in which no association has been found between corticosterone and sex (Wibbels & Crews, 1992; Uller et al., 2009; Warner, Radder, et al., 2009; Iungman et al., 2015; Marcó et al., 2015). The theoretical basis of stress-induced sex reversal in fish, which has been borne out in many experimental papers, relies on the androgen-inducing effect of corticosterone production (Straková et al., 2020). In our model species high temperatures induce feminization, and so the paradigm for stress-induced sex reversal in fish cannot be applied directly to *P. vitticeps*. We suggest that the mediator of sex in ESD reptiles is unlikely to be corticosterone but reptilian environmental sex determination may involve cellular, rather than hormonal stress. For example, the reciprocal regulation of calcium levels and reactive oxygen species have the potential to encode and transduce environmental stimuli and influence sexual outcomes (Chapter 2; Castelli, Whiteley, et al., 2020). Recent experimental evidence supports this hypothesis, with calcium levels found to regulate the epigenetic regulators of sex determination in a TSD turtle (Weber et al., 2020). Due to the apparent lack of a responsive stress axis during the temperature sensitive period in *P. vitticeps*, a biochemical and gonad-autonomous sensor may be a better candidate for the biological transducer of environmental cues in ESD reptiles.

Chapter 5 Experimental support for a role for oxidative stress in reptile sex reversal

5.1 Abstract

Environmental conditions during early development determine the sex of many reptile and fish species, and some amphibian species. Known as environmental sex determination (ESD), the environmental conditions that act as sex-determining cues vary widely. Highly conserved and environmentally sensitive processes such as the reciprocal regulation of calcium and redox (CaRe) signalling are a promising candidate for the biological translation mechanism in ESD species. The central bearded dragon (*P. vitticeps*) has a ZZ/ZW system of genetic sex determination in which chromosomally male (ZZ) individuals can sex reverse their sexual phenotype under the influence of high incubation temperature. Here we applied antioxidants (ascorbic acid, superoxide dismutase, and glutathione) to the embryo early in development at low (male-generating) incubation temperatures and assessed the sex of the resultant hatchlings *via* gonadal histology. We demonstrate that antioxidant injection can cause partial or complete feminization of the gonads of chromosomally male individuals, but stress that our conclusions are limited by high mortality in treatment groups (41-45%) compared to control and vehicle groups (5-11%), and by the low effect size of antioxidant treatment (one individual fully sex-reversed, one displaying partial gonadal feminization). Our results are suggestive that increased ROS production at high temperature incubation may comprise part of the environmental sensing mechanisms of ESD, but further experimental work must be conducted to demonstrate this.

5.2 Introduction

Environmental sex determination (ESD) is present in many fish and reptile, and some amphibian species, having emerged independently many times during the evolution of these clades (Bachtrog et al., 2014; Capel 2017). The environmental signals that direct the outcome of gonad development vary among ESD species (Saillant et al., 2003; Bachtrog et al., 2014). Among reptiles with ESD, the only unequivocally demonstrated determinant of sex is incubation temperature (Rhen & Schroeder, 2010), although evidence for the effects of

moisture (Wyneken & Lolavar, 2015; Dupoué et al., 2019) and egg size (Shine et al., 2002; Radder et al., 2009) is emerging. Some species display sex reversal; they possess genetic sex determination (GSD) with well characterized sex chromosomes, but these can be overridden by incubation temperature (Quinn et al., 2007; Radder et al., 2008; Holleley et al., 2015).

The upstream biological sensory systems which translate environmental sex-determining signals into a sexual outcome are still not well known, but the downstream hormonal effectors of sex (differential regulation of androgens and estrogens) are well-characterised (Barske & Capel, 2008). Upstream of hormonal regulation, the molecular pathways controlling this differential regulation involve alternately spliced epigenetic regulators, the action of which appears to be conserved across at least three reptile orders (Deveson et al., 2017; Ge et al., 2018; Weber et al., 2020). Structurally thermosensitive proteins such as CLK4 (Haltenhof et al., 2020) are direct temperature-sensing proteins capable of interacting with the epigenetic modifiers of sex (e.g. *KDM6B* and *JARID2*), and are good candidates for the upstream regulators of these temperature-specific splicing patterns (Deveson et al., 2017; Ge et al., 2018; Weber et al., 2020).

Based on experimental and transcriptomic evidence suggesting roles for calcium signalling and ROS production separately in ESD (Yatsu et al., 2015, 2016; Czerwinski et al., 2016; Corona-Herrera et al., 2018; Lin et al., 2018; Hayasaka et al., 2019), calcium and redox (CaRe) status in the cell has been proposed to regulate the action of ubiquitous signalling pathways and epigenetic modifiers of sex (Chapter 2; Castelli, Whiteley, et al., 2020). In short, the temperature-sensitive influx of calcium ions and the metabolism-driven production of reactive oxygen species (ROS) are critical cellular messengers involved in the capture of environmental information and could therefore be involved in influencing sexual phenotype. Indeed, intracellular calcium levels participate in sex determination in the red-eared slider turtle (*Trachemys scripta elegans*) *via* the phosphorylation of a cellular signalling protein which goes on to influence the expression of the epigenetic modifier *KDM6B* (Weber et al., 2020).

ROS are by-products of metabolism that in moderate amounts are critical cellular messengers (Forman et al., 2003; Torres & Forman, 2003), but which in excess can damage cellular structures through irreversible oxidation (Hörandl & Speijer, 2018). Metabolic rate is closely tied to temperature (Clarke & Fraser, 2004; Halliwell & Gutteridge, 2015), and varies in embryonic turtles with incubation temperature (Sun et al., 2015). The antioxidant response pathway is a conserved response to cellular stresses and moderates the activation of conserved signalling pathways such as the JAK-STAT pathway, that in turn mediates sex in *T. scripta*

(Martindale & Holbrook, 2002; Weber et al., 2020). Evidence for the role of responses to oxidative stress in ESD species has been identified through gene expression studies in both alligator and fish with ESD (Yatsu et al., 2016; Corona-Herrera et al., 2018), yet experimental evidence for this relatively new hypothesis has not yet been published.

We sought to determine whether cellular responses to oxidative stress form part of the sex differentiation pathway in reptiles with thermolabile sex using the central bearded dragon (*Pogona vitticeps*). This Australian agamid has female heterogamety (ZZ/ZW) with reversal of the ZZ genotype to a female phenotype at high temperatures (above 32°C) (Quinn et al., 2007; Holleley et al., 2015). We found that application of both enzymatic and non-enzymatic antioxidants can cause gonadal dysregulation and even complete gonadal feminization in *P. vitticeps*, lending support to the hypothesis that ancient and conserved responses to environmental stress have a role in the sex differentiation pathway.

5.3 Materials and Methods

All procedures were approved by the University of Canberra Animal Ethics Committee (AEC 17-14) and reciprocally approved by the CSIRO Wildlife and Large Animal Ethics Committee. Captive bred dragons from a breeding colony at the University of Canberra were used in this study. Adults were of known chromosomal sex (using the PCR sex test of Holleley et al., 2015), and were either concordant males (ZZm), concordant females (ZWf) or sex-reversed females (ZZf). Breeding groups consisted of one male and 2-4 females.

Gravid females laid naturally and eggs were recovered within 24 hr of laying. Eggs were placed in compartmentalised boxes filled with moist vermiculite (5 parts vermiculite to 4 parts water by weight) and incubated at a constant 28°C until experimental treatment within 24 hours of lay. Eggs from each clutch were allocated systematically to temperature and treatment blind to the genotypic sex of the embryo. 32 eggs were used from four different ZW mothers, and 90 eggs were used from three different ZZ mothers.

5.3.1 Egg incubation and injection

Solutions for injection were made fresh as each clutch was laid. Two treatments were used in this experiment. The cocktail solution contained 3.8 mg ascorbic acid (Sigma-Aldrich, Australia: PHR1008), 3.8 mg reduced L-glutathione (Sigma-Aldrich, Australia: G4251) and 0.1 mg superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, Australia: S7571). The single compound solution contained only 3.8 mg reduced glutathione. Superoxide

dismutase was dissolved in sterile 0.9% saline and frozen at -20˚C in 1.25 mg dosage aliquots until use. Each dose was delivered in 20 µL total injection volume, dissolved in sterile 0.9% saline. Superoxide dismutase was chosen as an enzymatic antioxidant for its superoxide-quenching capability, glutathione for its role as a broadly acting antioxidant and signaller of cell redox state, and ascorbic acid for its functions as a broad antioxidant (Storey, 1996; Surai et al., 1996; Hammond et al., 2001; Robert et al., 2007; Cyr & Domann, 2011).

Injections were conducted using disposable syringes with 5 µL divisions and a 6 mm 31-gauge needle (BD, United States of America: Product #324910). The egg was candled to confirm the position of the embryo at the top of the egg, within a ring of visible vasculature. The needle was inserted into the egg within the vasculature ring, and very close to the embryo, for injection of vehicle or treatment solutions. Once injection was complete, the injection site was sealed with cyanoacrylate superglue (Selleys® QuickFix Gel SupaGlue™). Eggs were then returned to the incubator at 28°C until hatching. In total, 122 eggs containing a mix of both ZZ and ZW embryos were used in this experiment, with four experimental groups: un-injected controls (n = 21), saline vehicle injections (n = 19), antioxidant cocktail injections (n = 44) and reduced glutathione injections (n = 38).

5.3.2 Identification of genotypic and phenotypic sex

Hatchling phenotypic sex was determined by hemipenal transillumination (Brown, 2009). After euthanasia, manual eversion of the hemipenes was conducted to confirm this. Genotypic sex of hatchlings from ZW mothers was confirmed using blood samples from the hatchlings stored on FTA Elute cards (GE Healthcare, United States of America: WB120410). DNA was extracted following manufacturer's instructions, and a PCR sex test confirmed whether the individual possessed a ZZ or ZW sex chromosome complement (Holleley *et al.*, 2015).

Histological examination of the gonads for a subset of the ZZ hatchlings from each treatment (n = 59 across all treatments/controls) confirmed that gonadal sex matched the determinations made earlier using transillumination and hemipenal extrusion. The adrenal-kidney-gonadal complex was fixed in 10% neutral buffered formalin for 24 hours, then transferred to 70% ethanol. The tissues were sectioned and stained with haematoxylin and eosin as described in Whiteley *et al.* (2018).

5.3.3 Statistical analysis

Pearson's chi-square tests were used to compare mortality and sex reversal rates in R (vers. 4.0.2) and RStudio (vers. 1.3.2093).

5.4 Results

There were significant differences in mortality rates between the cocktail (41% mortality) and glutathione (45% mortality) treatment groups compared to the un-injected control (5% mortality) and saline injection (11% mortality) groups ($\chi^2 = 15.812$, df = 3, $p = 0.001$). All ZZ hatchlings which had histology conducted in the control (n = 13) and saline (n = 11) treatment groups possessed normal testes, with seminiferous tubules and no indication of sex reversal.

Of the antioxidant-treated ZZ hatchlings that had their gonadal sex confirmed histologically, one hatchling in the cocktail treatment group (n = 20) was fully sex-reversed, possessing two typical ovaries and highly reduced hemiclitores, typical of the latest stages of hemipenal regression in females (Whiteley et al., 2017) (Figure 5-1). Sex reversal has not been observed to occur at 28°C (Holleley et al., 2015), and so we attribute this sex reversal event to the effect of the treatment, however all other hatchlings in this treatment possessed testes and fully developed hemipenes (as would be expected of ZZ individuals incubated at 28°C).

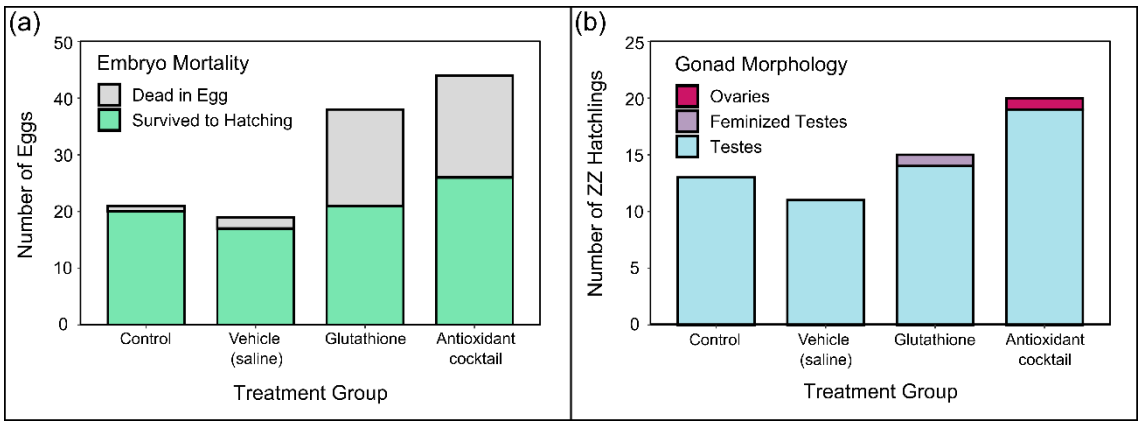


Figure 5-1. Antioxidant application early in development can result in partial or complete feminization of the gonads of genetically male bearded dragons (*Pogona vitticeps*) incubated at a non-sex reversing temperature (28°C). (*a*) The application of an antioxidant cocktail (glutathione, superoxide dismutase and ascorbic acid) or glutathione alone caused significantly higher mortality than was observed in either control or saline-injected eggs. (*b*) Of the surviving hatchlings which were genotypically male (ZZ), the injection of glutathione caused the development of one individual with feminized testes. One individual in the cocktail group was fully sex-reversed, possessing two typical ovaries.

One hatchling in the glutathione treatment group (n = 15) possessed fully developed hemipenes and feminized testes (Figure 5-2). Both testes displayed an atypical, polarized appearance that has not been observed in previous histological examinations of *P. vitticeps* (Whiteley et al., 2017, 2018). Half of each gonad comprised seminiferous tubules, while the other half displayed an unusual structure that possessed ovary-like characteristics including a homogeneous inner medulla and a thickened outer cortex (Figure 5-2). This is a novel gonadal phenotype and distinct from embryonic ovotestis phenotypes commonly observed in embryonic sex-reversing *P. vitticeps*. We refer to this novel gonadal phenotype as a "feminized testis". All other hatchlings in this treatment possessed normally developed testes and fully developed hemipenes.

Likely due to the low effect size of the treatments, there is no significant difference between the rates of gonadal reversal (full or partial) between all control and treatment groups ($\chi^2 = 0.031$, df = 3, $p = 0.999$). Both individuals which displayed gonadal reversal (full or partial) were offspring of ZZ sex-reversed mothers.

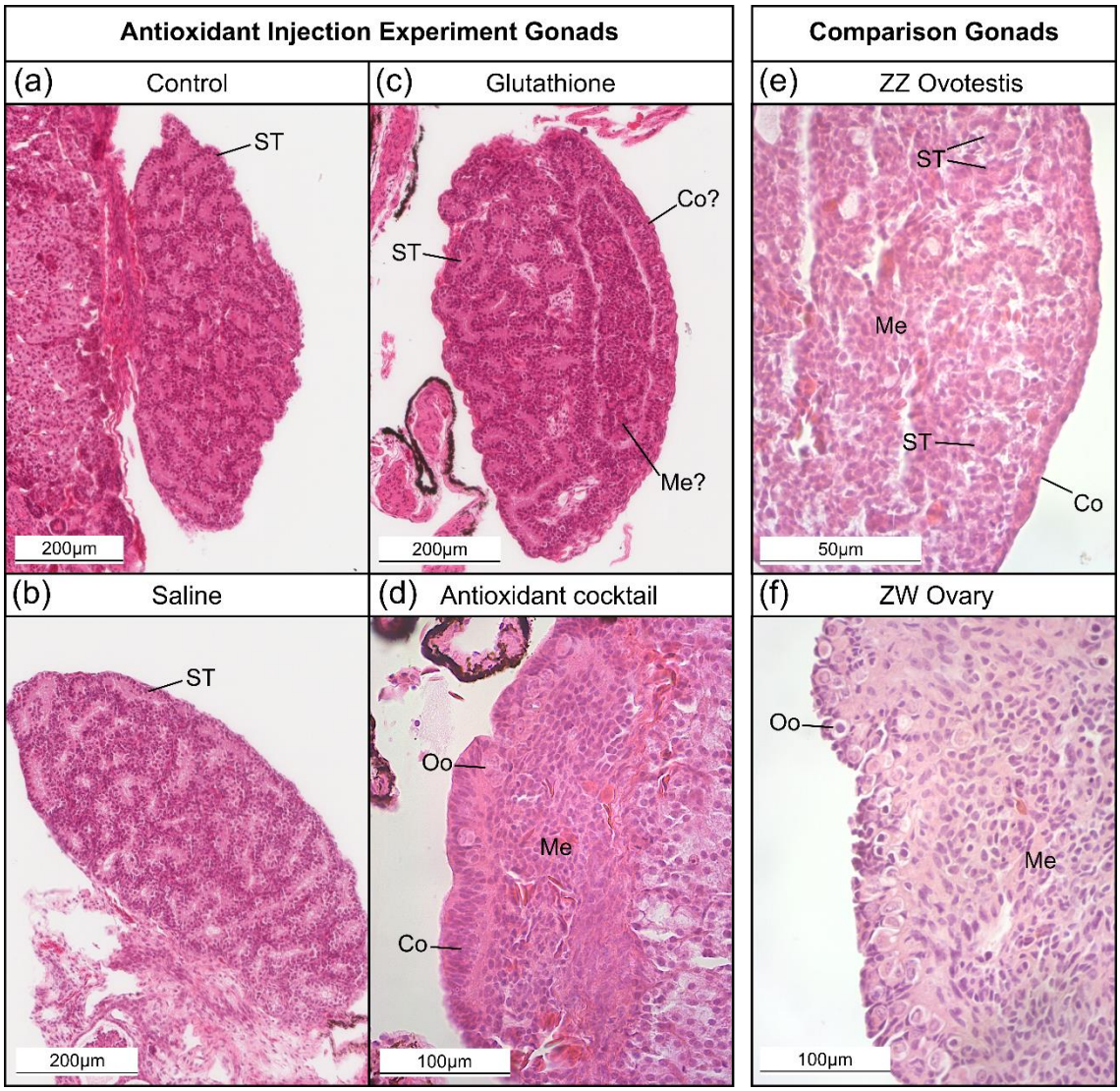


Figure 5-2. Gonadal histology of *Pogona vitticeps* treated with antioxidants. All individuals in both (a) control (n = 13) and (b) vehicle (n = 11) groups developed testes, characterized by bundles of seminiferous tubules (ST). One individual in the (*c*) glutathione treatment group (n = 15) possessed feminized testes, in which half of the gonad was composed of ST and the other half possessed a structure reminiscent of an ovary, with a homogeneous inner medulla (Me?) and thickened outer cortex (Co?). One individual in the (d) antioxidant cocktail treatment (n = 20) was fully sex-reversed, possessing typical ovaries with an inner medulla (Me), thickened outer cortex (Co) and developing oogonia (Oo). For reference, (e) is an ovotestes of an embryonic ZZ individual incubated at 36°C and sampled at stage 12 of development. The gonad displays both a thickening outer cortex and remnant ST. A typical hatchling ZW ovary (f) displays a homogeneous inner medulla and numerous Oo in the outer cortex.

5.5 Discussion

Here we demonstrate that the artificial elevation of antioxidants *in ovo* can generate gonadal feminization and sex reversal, with the important caveat that effect size is low and mortality

rate as a result of treatment is high (41-45%). Observed in two individuals in this study, this feminization (one partial gonadal feminization and one complete sex reversal) mimics that observed at high temperature incubation, indicating that cellular responses to reactive oxygen species (ROS) production may be involved in sex reversal in this species. The instances of sex reversal and testis feminization detected here demonstrate a low effect size of these treatments, but nonetheless demonstrate that antioxidants can interfere with sex. The molecular pathway by which these treatments can interfere with testis development and promote ovarian development was not determined, but there are several possibilities.

The activity of proteins is dependent on their structural conformation, and antioxidants in their reduced and oxidised forms are capable of modulating protein function through their interactions with redox-sensitive cysteine thiols (Covarrubias et al., 2008). For example, both reduced and oxidised glutathione are crucial signalling molecules in the NF-κB cellular signalling pathway through their reactions with specific protein thiols (Morgan & Liu, 2011). Redox sensing is also a feature of transient receptor protein (TRP) channels, which are known to influence sex through their control over intracellular calcium levels (Yatsu et al., 2015; Weber et al., 2020). The oxidation of cysteine thiols within TRP proteins by ROS and their subsequent reduction by certain antioxidants has the potential to alter the sensitivity of the channel to temperature, revealing potential synergism between the calcium and redox pathways (Ogawa, Kurokawa, & Mori, 2016; Ogawa, Kurokawa, Fujiwara, et al., 2016).

Antioxidants can directly participate in epigenetic remodelling. Ascorbic acid is synthesised in the yolk membrane in embryonic chicken (Surai, 1999) and the liver of adult reptiles (Menon & Rozman, 2007) and can regulate epigenetic remodelling through its effects on lysine demethylase activity (KDM family genes) (Wang et al., 2009; Wang et al., 2011). The addition of vitamin C may have stimulated KDM6B activity, mimicking the upregulation of *KDM6B* observed in adult sex-reversed *P. vitticeps* (Deveson et al., 2017) and initiating epigenetic remodelling to result in the sex-reversed phenotype (Figure 5-2). Superoxide dismutase is also known to mediate the expression of *JMJD3* through the production of hydrogen peroxide (H_2O_2) and its subsequent interactions with a redox-sensitive cysteine thiol in STAT6, a transcription factor responsible for *JMJD3* expression (C. He et al., 2016). Glutathione may have facilitated epigenetic remodelling in both the cocktail and glutathione treatments through the glutathionylation of histone H3, which is known to promote an open and accessible chromatin structure (Olaso et al., 2013; Kietzmann et al., 2017).

It must be noted that the low effect size of gonad feminization in response to both the glutathione and cocktail treatments limits the scope of our conclusions. However, all control

and saline-treated ZZ hatchlings were male, and sex reversal has not been observed at 28°C in this species (Holleley et al., 2015). However, individual variation in the threshold for sex reversal occurs (Holleley et al., 2015) and this threshold likely has the capacity to evolve in a population specific manner (Chapter 6; Castelli, Georges, et al., 2020), so interindividual variation in sex reversal threshold may explain the low effect size. Alternatively, it may be that ROS and the antioxidant response are involved in sex reversal, but only have minimal contribution to determining sex, requiring concomitant activation of other pathways. The significant levels of mortality (40-44%) associated with antioxidant treatments demonstrate their efficacy, indicating their interference with cell proliferation and apoptotic pathways (Covarrubias et al., 2008), and it may be that the dosage window in which these antioxidants both have an effect on sex and don't cause embryo death is small. Thus, sex reversal may have been initiated in embryos which suffered mortality as a result of treatment. It is interesting to note that the single antioxidant treatment induced partial feminization, whereas the cocktail treatment induced ovary development, suggesting that a broad-spectrum antioxidant treatment may have been more successful in exceeding the threshold for sex reversal.

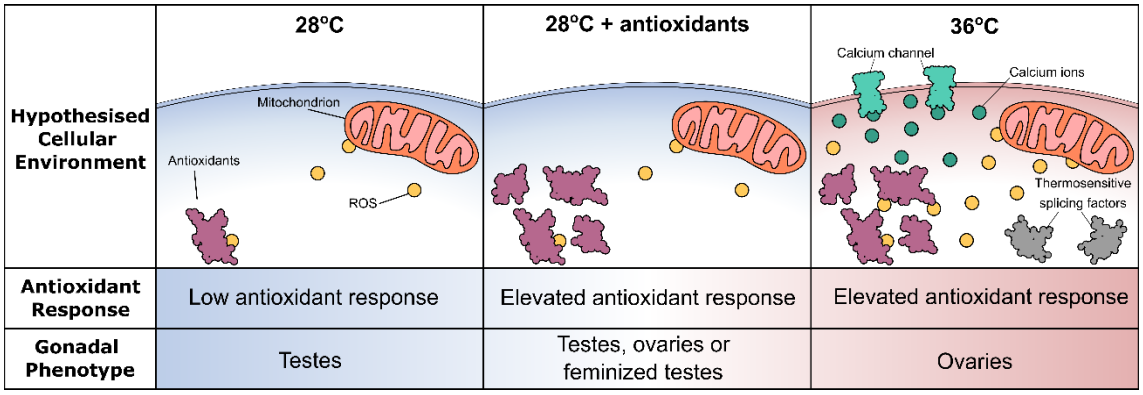


Figure 5-3. The hypothesised involvement of oxidative stress and the antioxidant response in sex reversal of genetically male (ZZ) *Pogona vitticeps* embryos. At 28ºC, we propose that growth rates and metabolic rate are low and the antioxidant response to reactive oxygen species (ROS) production is accordingly low. When embryos incubated at 28ºC are supplemented with antioxidants, the artificially elevated antioxidant response is sufficient to generate partial or complete sex reversal in some individuals. At 36ºC, a number of concomitant responses to heat stress occur, including the antioxidant response to ROS production, calcium ion influx through transient receptor potential (TRP) channels (Yatsu et al., 2015, 2016; Weber et al., 2020) and the conformational changes of thermosensitive proteins such as CLK1 (Haltenhof et al., 2020). The simultaneous occurrence of these multiple heat response pathways is sufficient to generate sex reversal in a majority of ZZ individuals (Quinn et al., 2007; Holleley et al., 2015).

As the epigenetic mechanisms of sex determination are becoming characterised, evidence is emerging that the initial sensor and translator of environmental conditions may be biochemical in nature (Chapter 2; Castelli, Whiteley, et al., 2020). This study provides compelling evidence in favour of such a paradigm but does not provide a complete picture as to the mechanisms by which application of these antioxidants was able to interfere with sex in some individuals. Further experiments focusing on the effects of these and other antioxidants such as catalase and hydrogen peroxidase may shed light on these mechanisms, perhaps in an organ culture system to avoid the significant mortality associated with *in ovo* application (Salame-Mendez et al., 1998). Furthermore, the antioxidant response pathway may only be one aspect of the sex-determining signalling initiated by ROS production at high temperatures (Figure 5-3). If a single environmental stimulus generates a multitude of response pathways that all contribute to determining the threshold at which an individual becomes male or female, we suggest from these results that the antioxidant response may be one of these response pathways.

Chapter 6 Evolving thermal thresholds explain the distribution of sex reversal in an Australian dragon lizard

This chapter has been published in **Diversity and Distributions**.

Castelli, M. A., Georges, A., Cherryh, C., Rosauer, D. F., Sarre, S. D., Contador-Kelsall, I., & Holleley, C. E. (2020). Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard. Diversity and Distributions, Early view. https://doi.org/10.1111/ddi.13203

Apart from formatting, this chapter has not been altered in any way. The references have been consolidated at the end of the thesis.

6.1 Abstract

Aim

Species with temperature-dependent sex determination (TSD) are particularly vulnerable to climate change because a resultant skew in population sex ratio can have severe demographic consequences and increase vulnerability to local extinction. The Australian central bearded dragon (*Pogona vitticeps*) has a thermosensitive ZZ male/ZW female system of genetic sex determination (GSD). High incubation temperatures cause reversal of the ZZ genotype to a viable female phenotype. Nest temperatures in the wild are predicted to vary on a scale likely to produce heterogeneity in the occurrence of sex reversal, and so we predict that sex reversal will correlate positively with inferred incubation conditions.

Location

Mainland Australia

Methods

Wild-caught specimens of *P. vitticeps* vouchered in museum collections and collected during targeted field trips were genotypically and phenotypically sexed to determine the distribution of sex reversal across the species range. To determine whether environmental conditions or genetic structure can explain this distribution, we infer the incubation conditions experienced

by each individual and apply a multi-model inference approach to determine which conditions associate with sex reversal. Further, we conduct reduced representation sequencing on a subset of specimens to characterise the population structure of this broadly distributed species.

Results

Here we show that sex reversal in this widespread Australian dragon lizard is spatially restricted to the eastern part of the species range. Neither climatic variables during the inferred incubation period nor geographic population genetic structure explain this disjunct distribution of sex reversal. The main source of genetic variation arose from isolation by distance across the species range.

Main Conclusions

We propose that local genetic adaptation in the temperature threshold for sex reversal can counteract the sex-reversing influence of high incubation temperatures in *P. vitticeps*. Our study demonstrates that complex evolutionary processes need to be incorporated into modelling biological responses to future climate scenarios.

Key words

Climate change, environmental sex determination, sex reversal, threshold evolution

6.2 Introduction

In most vertebrates, the outcome of sexual differentiation is binary, with individuals developing phenotypically as male or female following distinct developmental trajectories. Although the outcomes of sexual differentiation are highly conserved among vertebrates, great diversity exists in the genetic and environmental factors that have acquired master or influential roles in the determination of sexual fate (Bachtrog et al., 2014). These signals can be genetic or environmental, but rather than a dichotomy, a continuum occurs from genetic sex determination (GSD) to environmental sex determination (ESD) in that the critical influence can be genetic, environmental or an interaction between the two (Sarre et al., 2004).

Transitions between genetic and environmental sex determination systems have occurred frequently among vertebrates on an evolutionary timescale (Sarre et al., 2011; Warner, 2011; Van Doorn, 2014; Bachtrog et al., 2014), and underlying these transitions is evolution in the threshold temperature for sex reversal (Quinn et al., 2011). The intrinsic and extrinsic factors which are capable of driving evolution towards one or the other extreme of the continuum have been widely modelled computationally and demonstrated experimentally

(Sarre et al., 2004). Temperature sex determination (TSD) is the most common mode of ESD and is observed widely among fish, reptiles and amphibians (Bachtrog et al., 2014). Mechanistic models under different climatic scenarios have identified the importance of factors other than temperature alone in generating primary sex ratios, including maternal behaviour, nesting phenology, fecundity, and threshold temperature (Mitchell et al., 2010; Boyle et al., 2014; Schwanz et al., 2020). Species with TSD are particularly vulnerable to climate change because skews in the population sex ratio in response to extreme conditions can have severe demographic consequences, increasing vulnerability to local extinction (Mitchell & Janzen, 2010). Significantly skewed sex ratios caused by climate warming already occurs in contemporary populations of TSD reptiles (Jensen et al., 2018) and fish (Honeycutt et al., 2019). The tipping-point at which skewed sex ratios lead to demographic collapse is not yet known.

Species with GSD are typically assumed to be resilient to sex ratio skew (Bókony et al., 2019). The occurrence of sex reversal (temperature overriding an underlying GSD system) challenges this view. Sex reversal occurs in wild populations of two Australian reptiles: the central bearded dragon (*Pogona vitticeps*; Holleley et al., 2015), the eastern three-lined skink (*Bassiana duperreyi*; Holleley et al., 2016), and is suspected in six more species of lizard and turtle (Holleley et al., 2016; Wiggins et al., 2020). Sex reversal is also a frequent phenomenon in fish (Baroiller & D'Cotta, 2016). Sex reversal provides a mechanism by which increasingly extreme conditions leads to the loss of the heterogametic chromosome (the Y or the W) and a transition from GSD to TSD (Holleley et al., 2015; Schwanz et al., 2020). Persistence of the population then requires one of two things; behavioural modifications that alter the nest environment, or evolution in the threshold for sex reversal temperature (Düsing, 1884; Fisher, 1930; Edwards, 2000). Though maternal behaviours can buffer against the effects of climate, this is not universally true and these behaviours are not always heritable (Ewert et al., 2005; Doody et al., 2006; Warner & Shine, 2008; Refsnider et al., 2013; Refsnider & Janzen, 2016). Additionally, evolutionary responses take time and require existing heritable variation upon which to act (Schwanz et al., 2020). The time lag implicit in these evolutionary forces can leave thermosensitive species (including those with GSD and sex reversal) extremely vulnerable to local extinction under rapid or extreme climate change via demographic skews.

The central bearded dragon (*Pogona vitticeps*) displays a sex determination system characterized by a ZZ/ZW GSD system of female heterogamety (Ezaz et al., 2005) and sex reversal of the ZZ male genotype to phenotypically female under the influence of high temperature (Quinn et al., 2007; Holleley et al., 2015). Sex reversal occurs rarely at 31°C, but

rises in frequency with temperature until 36°C where almost 100% of ZZ genotype animals are feminized (Holleley et al., 2015). This Australian agamid is distributed across a range of climatic types spanning mesic to xeric climatic regions across central, southeastern and northeastern Australia (Cogger, 2018; Rej & Joyner, 2018), providing a unique opportunity to study the dynamics of genetic and environmental influences on sex determination. Some populations of *P. vitticeps* may be in the early stages of transition from GSD to TSD via loss of the W chromosome (Holleley et al., 2015).

Here, we examine the distribution of sex reversal across the geographic range of *P. vitticeps*, using historical museum specimens and contemporary wild caught individuals. Nest temperatures are predicted to vary across this range (Figure 6-1). We predict that sex reversal frequency in the wild will be positively correlated with inferred incubation temperatures if there is a common and static threshold for temperature sex reversal. Conversely, if there is local adaptation in the threshold for sex reversal, we predict that the frequency of sex reversal will be independent of incubation temperature across the species range.

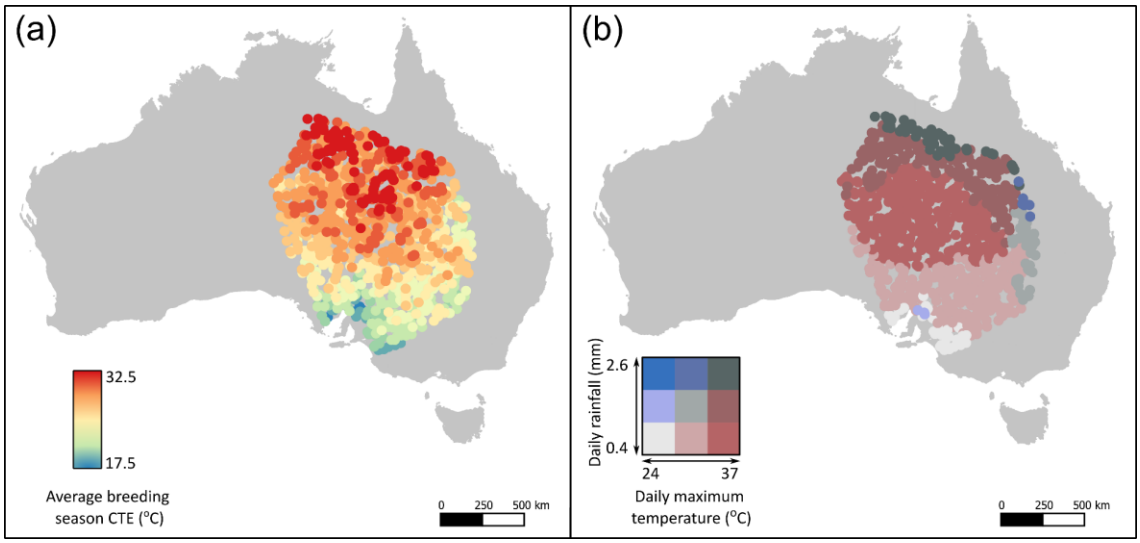


Figure 6-1. Inferred nest incubation conditions across the range of *Pogona vitticeps* within the average breeding season (September-February inclusive) from 1975-2014. (A) The average constant temperature equivalent (CTE) at 15 cm nest depth. (B) Ambient temperature and rainfall conditions obtained for the same time period. We predict that sex reversal will occur most often in areas where incubation conditions are highest, and therefore expect to see a latitudinal gradient in the occurrence of sex reversal, with more cases of sex reversal occurring in the north of the species range.

6.3 Methods

6.3.1 Specimen collection and phenotypic sex identification

A total of 534 wild-caught dragons, collected from field trips (n = 337 dragons) and supplemented by vouchered museum specimens (n = 197) collected over a period of 38 years from 1980 to 2018, were sampled in this study (Supplementary File 1). Tissues from dragons collected during field trips were taken as either tail or toe clips, frozen blood or dried blood on DNA-preserving card storage systems (Whatman™ FTA™ Elute Cards, Macherey-Nagel Nucleocards, or PerkinElmer 226 Five Spot cards). Liver samples from museum specimens were sampled by museum staff at the time of collection, and either stored in ethanol at room temperature or frozen (-80°C). Specimens that were wild-caught and released in the field were phenotypically sexed by hemipenal eversion. In this approach, the hemipenes of adult males are reliably everted by running the thumb up the ventro-lateral surface of the base of the tail, while gently bending the tail to expose the cloacal aperture. Sex identification was confirmed secondarily by examining the animal for gravidity, observing nesting behaviour, and examining male secondary sexual characteristics, such as larger head size and dimorphism in femoral pores. The sex of museum specimen and road-killed animals was identified by dissection of the abdomen and inspection the gonads. The few juvenile or hatchling animals captured or located during the study were only included if gonadal sex could be determined by dissection.

6.3.2 Molecular sex identification

Genotypic sex (ZZ/ZW) was determined by extracting DNA from tissue and conducting a PCR sex test (Quinn et al., 2010; Holleley et al., 2015). Three DNA extraction methods were performed as appropriate for each tissue type: 1. Qiagen's Gentra® Puregene® DNA purification kit for tail and toe clips, frozen liver, frozen blood, 2. Macherey-Nagel Nucleospin® Tissue kit for blood on frozen PerkinElmer 226 cards and Macherey-Nagel Nucleocards; 3. Whatman Elute quick extraction protocol for blood on Whatman™ FTA™ Elute Cards. Manufacturer's instructions were followed in all cases. Purified DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Australia) and standardised to a concentration of 25 ng/µL prior to PCR. DNA extraction and PCR setup was automated using the epMotion 5075 platform (Eppendorf, Germany), and included both

positive and negative controls following Whiteley et al., (2017). PCR products were visualised on a 1.5% agarose gel. ZZ genotypic individuals displayed one band at 524 bp (both Z fragments) and ZW genotypic individuals displayed two bands, one at 524 bp (Z fragment) and the other at 357bp (W fragment). Internal ZZ and ZW control samples were run on every gel. Individuals with a ZZ genotype and a female phenotype were classified as sex-reversed. Chi-square tests were used to determine if there were sex biases in this dataset between males and females across the entire period of the study. Additionally, chi square tests were conducted to determine if the proportion of concordant females and sex-reversed females varied across the years in which at least 15 phenotypic females were collected.

6.3.3 Prediction generation: Constant temperature equivalent

The rate of embryonic development of *P. vitticeps* is dependent on temperatures experienced by the egg within the nest, with embryos developing faster at hotter temperatures (Whiteley et al., 2017). For embryos developing at higher temperatures, this means that the period during which sex can be determined by temperature is shorter. Constant temperature equivalent (CTE) calculation allows the fluctuations in temperature experienced by a typical buried egg to be converted to a constant temperature which results in the same amount of embryonic development. First, the daily soil temperature was calculated over a single laying season (September to December in year y and January to February in year y + 1) using NicheMapR (Kearney & Porter, 2017; Kearney, 2020) and user-supplied weather input data (SILO climate gridded dataset). Secondly, the daily CTE was calculated using methods described in (Georges, 1989) and (Georges et al., 1994). To generate range-wide long-term trends in CTE, the procedure was repeated over forty consecutive breeding seasons from 1975-2014, and for 1000 locations randomly selected from within the range of *P. vitticeps*.

The microclimate model was driven by the Scientific Information for Land Owners (SILO) climate gridded dataset (Jeffrey et al., 2001). For all the 1000 randomly selected points we extracted minimum and maximum temperature, relative humidity at time of minimum and maximum temperature, rainfall, vapour pressure, and solar radiation (used to estimate cloud cover, as outlined in NicheMapR documentation). In addition, we used a daily gridded mean wind speed dataset at both the minimum and maximum wind temperature (McVicar et al., 2008; McVicar, 2011). Elevation was extracted from the GEODATA 9 second Digital Elevation Model (DEM-9S) Version 3 (Hutchinson et al., 2008). For each

location, soil properties were extracted from the Soil and Landscape Grid of Australia (Viscarra Rossel et al., 2014a, 2014b, 2015; Grundy et al., 2015). The proportion of clay, silt and sand at each location was used to determine the soil parameters based on Table 9.1 from Campbell & Norman (1998), included in NicheMapR (Kearney, 2020). We used a custom R program (R Core Team, 2020) including the packages *sp* (Pebesma & Bivand, 2005; Bivand et al., 2013), *Rfast* (Papadakis et al., 2020), *zoo* (Zeileis & Grothendieck, 2005), *timezone* (Rundel, 2013) and *raster* (Hijmans, 2020) to collate climate variables into NicheMapR, run the microclimate model and extract the hourly soil temperature output for ten soil depths between 0 and 200cm. We assumed a nest depth 15cm (Pianka, 2005), and that nests were unshaded.

To calculate the daily CTE, we used the method described in Georges (1989) and Georges et al. (1994). The model assumes that daily soil temperature varies on a cycle about a stationary mean (equation 1), and as such provides a daily estimate of nest temperature corrected for fluctuations. Given the hourly soil temperatures for one day, T is the temperature at time t in degrees Celsius, M is the mean soil temperature, and R is the maximum deviation from M. T_0 is the minimum temperature at which embryonic development is possible. In *P. vitticeps*, T_0 = 16.2°C (unpublished data). The CTE (T') occurs at time t'. Equation 2 is solved iteratively for t' until $t'_{old} - t'_{new} < 0.0001$. If this did not occur within 10,000 iterations, we used the value of t' that occurred when the difference between iterations was smallest. The value for t' is then substituted into equation 1 and solved for T'.

$$(1) \quad T = R\cos(t) + M, \qquad 0 \leq R < M \text{ (Georges, 1989)}$$

$$(2) \quad t' = \frac{\pi}{2} - \frac{R}{M - T_0}\sin(t') \text{ (Georges, 1989)}$$

If soil temperature dropped below T_0, development ceased for part of that day. In that case, t_0 was calculated using equation 3 to estimate the time at which soil temperature drops to T_0 and development ceases. Then, t' was calculated iteratively using equation 4 until $t'_{old} - t'_{new} < 0.0001$. If this did not occur within 10,000 iterations, we used the value of t' that occurred when the difference between iterations was smallest. Finally, the CTE was calculated by substituting t' into equation 1. For further details on the derivation of these equations, please refer to (Georges, 1989).

$$(3) \quad t_0 = \cos^{-1}\left[\frac{T_0 - M}{R}\right], \qquad 0 < t_0 \leq \pi \text{ (Georges, 1989)}$$

$$(4) \quad t' = \frac{t_0}{2} + \frac{R}{2(M - T_0)}\sin(t_0) - \frac{R}{M - T_0}\sin(t') \text{ (Georges, 1989)}$$

For some days there was little variation in soil temperature and $M = T_0$, or $M = T_0$ and $R = 0$. On these days, the CTE could not be calculated using the method described in Georges (1989) and Georges et al. (1994). When this occurred, we set CTE = $T' = M$. We calculated the daily CTE for each day in September to February. The mean CTE for a breeding season is the mean of all daily CTEs from September to February (n = 181 days) for that random location.

The high degree of correlation (Pearson's Correlation; R = 0.890, t = 61.739, df = 998, p <0.001) between the average maximum temperature for each location from 1975-2014 and the corresponding CTE estimates (derived in part from maximum temperature) led us to use only the extracted SILO data for estimation of inferred incubation conditions for each collected specimen rather than the more derived CTE values.

6.3.4 Geographic correlates of sex reversal

Multinomial logistic regression (R packages: *nnet* and *tidy*) was used to determine whether sex-reversed individuals displayed different geographical distributions when compared to the concordant sexes (where genetic sex matches phenotypic sex). A multinomial logistic regression was constructed using latitude, longitude and elevation as the predictor variables, with the sex of each specimen as the response variable, either concordant male (ZZm), concordant female (ZWf) or sex-reversed female (ZZf). The three categorical outcomes were then compared on a pairwise basis, to determine if there was an association between latitude or longitude and sex.

Cluster analysis software SaTScan v9.6 (SaTScanv.9.6., 2018) was used to identify if sex reversal was clustered in the landscape. A Bernoulli probability model and moving window analysis was applied to identify significant clustering in the spatial distribution of sex-reversed compared to all concordant individuals. The maximum spatial cluster size was set at 50% of the size of the population, a generally accepted standard (Kim & Jung, 2017), with high abundance clusters defined as containing at least ten instances of sex reversal.

6.3.5 Environmental correlates of sex reversal: Inferred incubation conditions

Inferred incubation conditions (Table 6-1) were determined for each individual using its estimated age, location of capture and parameters of the reproductive cycle of *P. vitticeps*. Temperature variables (maximum temperature and diel range) were selected for their direct

relationship to sex reversal (Holleley et al., 2015) and rainfall for its effects on temperature. The deviation of these conditions during the inferred incubation period from the long term average for each site was also included in analysis, to account for the possibility that it may not be absolute temperature which causes sex reversal, but rather deviation from long term conditions, to which populations in different areas may have become adapted. Age was estimated qualitatively, and by using snout-vent length (SVL). Adult individuals were assumed to be between 3-5 years old, juveniles between 1-2 years old and hatchlings less than 6 months old. The breeding season (and therefore time during which incubation conditions could reasonably be experienced by each individual) was determined to be from September-February inclusive based on patterns of gravidity in dissected wild female specimens and patterns of egg laying in a breeding colony (Supplementary Figure 3). For adults, inferred incubation conditions during the third, fourth and fifth breeding season prior to collection were averaged. For juveniles, inferred incubation conditions during the first and second breeding season prior to collection were averaged. Hatchlings were excluded from analysis.

Table 6-1. Environmental variables included as predictors of sex in *Pogona vitticeps*. These variables were all included in a single binomial logistic regression model. The response variable was a binary outcome, either being a sex-reversed female (ZZf), or a concordant male (ZZm).

Variable	Description
Average daily maximum temperature during incubation	Average maximum temperature (°C) for days during the inferred incubation period.
Average daily rainfall during incubation	Average daily rainfall (mm) for days during the inferred incubation period.
Average diel temperature range during incubation	Average diel temperature range (°C) for days during the inferred incubation period.
Deviation from long term average daily maximum	Average deviation of the *daily maximum temperature* from the long term mean of daily maximums during the breeding season for that site from 1910 until 5 years before specimen collection.
Deviation from long term average rainfall	Average deviation of the *daily rainfall* from the long term mean of daily rainfall during the breeding season for that site from 1910 until 5 years before specimen collection.
Deviation from long term average diel temperature	Average deviation of the *diel temperature range* from the long term mean of diel range during the breeding season for that site from 1910 until 5 years before specimen collection.

Environmental data during the inferred incubation period of each individual were extracted from the Scientific Information for Land Owners (SILO) database of Australian climate data, in gridded format (~25 km^2). We needed to estimate the home range size to ensure that we could reasonably assume that each specimen was incubated within the grid in which it was collected. There are no published data on the home range and post-hatching dispersal of *P. vitticeps*, but the data available in other Australian terrestrial reptiles indicates that home ranges are likely to be less than 25km^2 (Shine & Lambeck, 1989; Piza-Roca et al., 2018). Thus, we have extracted and analyzed inferred incubation conditions from only the SILO grid which contains the collection location of the specimen, assuming that this grid also contained the location in which the individual developed in the egg. All data analyses were conducted in R v.4.0.2 (R Core Team, 2020), and significance was set at $p = 0.05$ for all tests.

To identify environmental variables associated with the occurrence of sex reversal, we used multiple model inference analysis [R package MuMIn v.1.42.14; (Barton, 2019)]. A binomial logistic regression model containing all of the model predictors (Table 6-1) was generated, and the MuMIn package then used to generate models with all possible combinations of the predictor variables. Akaike Information Criteria (AICc, corrected for small sample sizes) values assigned to each model were then used to determine the model/s which best explained variation in the response variable. Both minimum and maximum air temperature were considered for inclusion in analysis, but owing to the high degree of correlation displayed between these two variables and the more direct link between high temperature and sex reversal, we decided to include only maximum daily temperature in analysis. The inferred incubation conditions of each ZZ individual (excluding hatchlings) were entered into a single binomial logistic regression model and MuMIn used to perform model averaging and to determine which variables best explain the binomial regression model.

6.3.6 Population structure

To determine whether population structure across the species geographic range may explain patterns in sex reversal, reduced representation sequencing was conducted on a sample of 218 individuals (13 ZZf, 73 ZWf and 132 ZZm; Supplementary Figure 4). Each individual was genotyped by reduced representation Illumina short-read sequencing using commercial provider Diversity Arrays Technology (DArT Pty Ltd, Canberra, ACT, Australia). Briefly, SNP genotyping was performed using a combination of complexity reduction by using two

restriction enzymes, implicit fragment size selection and next-generation sequencing (Kilian et al., 2012). A pair of restriction enzymes PstI (recognition sequence 5'-CTGCA|G-3') and SphI (5'-GCATG|C-3') was used for complexity reduction by double digestion. Sequences were processed using proprietary DArT analytical pipelines (Kilian et al., 2012) to yield SNP markers polymorphic within the set of samples. Calling quality was assured by high average read depth per locus (medium coverage, 10X). In addition, approximately one-third of the samples were processed twice from DNA to allelic calls as technical replicates. Scoring consistency (repeatability) was used as the main selection criteria for high-quality and low error-rate markers. Refer to (Georges et al., 2018) for further detail. Additional filtering of loci and individuals and preliminary analysis was undertaken using the R package *dartR* (v.1.1.11; Gruber et al., 2017). Loci with a call rate of less than 95%, repeatability of less than 99%, monomorphic loci and secondary SNPs were removed prior to analysis. Individuals with a call rate of less than 75% (n = 2) were also removed from analysis. Structure across the landscape was visualised using principal coordinates analysis (PCoA) in the *dartR* package and isolation by distance was assessed using Mantel's test in the R package *adegenet* (Jombart, 2008).

6.4 Results

6.4.1 Geographic distribution of sex reversal

Among the 534 wild-caught individuals sampled across the species range, 294 (55%) were ZZ males, and 240 (45%) were phenotypic females. Of those females, 28 (12%) possessed a ZZ genotype and were therefore sex-reversed. The bias towards males in our data was significant ($\chi^2 = 5.46$, df = 1, $p = 0.019$) possibly due to capture-bias and the higher visibility of males which display prominently. Sex-reversed females were collected in 12 non-consecutive years of the 38 years sampled. The earliest case of sex reversal was in South Australia in 1983 and the most recent in New South Wales in 2018 (Figure 6-2). There was no range-wide temporal trend in sex reversal. The proportion of females which were sex reversed varied from 6-27% but without significant difference between years ($\chi^2 = 6.031$, df = 5, $p = 0.303$) among the six years in which at least 15 phenotypic females were collected.

Sex reversal was detected across a substantial proportion of the range of *P. vitticeps* but was not randomly distributed (Figure 6-2).Spatial cluster analysis using SaTScan identified a single large cluster in the south-eastern part of the sampling area where sex-

reversal occurred at a rate higher than would be expected if sex-reversed individuals were distributed randomly throughout the study range. The radius of the cluster was 410.83 km, containing 23 out of the 28 cases of sex reversal (Expected cases: 12.9, Log-Likelihood Ratio: 8.182, $p = 0.039$). We found that the latitudinal, longitudinal and elevational distributions of concordant males (ZZm) and concordant females (ZWf) did not differ (Table 6-2). Sex-reversed individuals (ZZf) were found not to differ in terms of latitudinal or elevational distribution compared to both ZZm and ZWF, but were significantly different in their longitudinal distributions, being largely absent from lower longitudes (the western part of the species range; Table 6-2, Figure 6-2).

In a previous report detailing the extent of wild sex reversal (Holleley et al., 2015), the area examined spanned a minimum convex polygon (MCP) of ~260,000 km² (12.2% of the known species range), and sex-reversed females were found to occupy an MCP of ~26,000 km² (9.9% of the sampled range). Here we report a substantial increase in the sampling area, with an MCP of ~1.7 million km² (79.5% of the species range) sampled, of which sex-reversed females were found in a ~400,000 km² MCP representing approximately 24.2% of the sampled range.

Table 6-2. Multinomial logistic regression comparing the latitudinal, longitudinal and elevational distributions of sex-reversed females (ZZf) to both concordant males (ZZm) and concordant females (ZW) of *Pogona vitticeps*. The longitudinal distribution of sex-reversed females is significantly different to both concordant sexes.

Comparison to ZZf	Term	Estimate	Standard Error	Statistic	*p* value
ZZm	Latitude	0.009	0.075	0.113	0.910
ZZm	Longitude	-0.095	0.017	-5.530	<0.001
ZZm	Elevation	0.002	0.003	0.736	0.462
ZWf	Latitude	0.063	0.076	0.831	0.406
ZWf	Longitude	-0.087	0.017	-5.000	<0.001
ZWf	Elevation	0.003	0.003	1.120	0.264

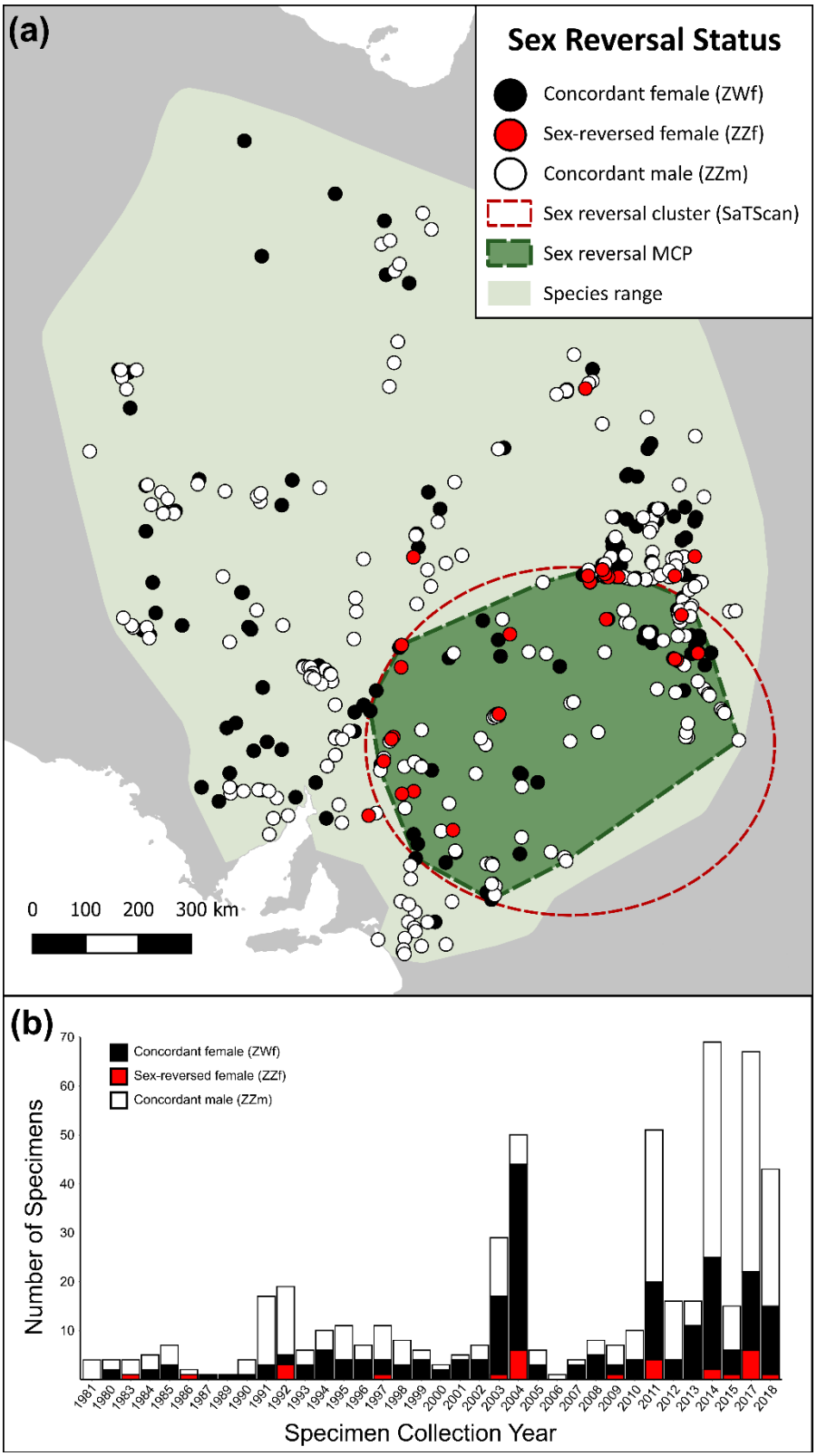


Figure 6-2. (a) The distribution of sex reversal across the range of *Pogona vitticeps*. Cluster analysis identified a single cluster in which sex reversal occurred at a higher frequency than expected under the assumption of a uniform spatial distribution. (Expected cases: 12.9, Log-Likelihood Ratio: 8.182, p = 0.039). (b) Temporal trends in sex reversal are difficult to determine owing to uneven sampling effort, but the earliest sex reversal was detected in 1983.

6.4.2 Environmental correlates of sex reversal

Overall, the binomial logistic regression comparing ZZ males and ZZ females using all six inferred incubation condition predictor variables had exceptionally poor predictive ability (R^2 = 0.032) (Table 6-3). An unbiased model inference approach using MuMIn extracted nine "best" models to explain the binomial logistic regression. These models differed by less than two AICc values from the best model with the lowest AICc value and are therefore indistinguishable in terms of their explanatory power (Burnham & Anderson, 2002). Five of these were single-variable models, three contained two variables and one was the null model. That the null model was also included indicates that no combination of inferred incubation conditions was able to explain the binomial regression model of the frequency of sex-reversal (ratio of ZZ females to ZZ males) more parsimoniously than the null model. This suggests that of the inferred incubation conditions included in the model, none are capable of adequately explaining the occurrence of sex reversal.

Table 6-3. Results of multi-model inference analysis comparing all possible combinations of the six environmental variables used in binomial regression to predict ZZ sex. The response variable is a binary outcome (sex-reversed female or normal male). Only models which were within two AICc values of the lowest AICc are reported. Log likelihood (LogLik) values, Akaike information criteria (AICc) values, change in AIC (△AICc) from the most likely model and model weight are provided.

Model	LogLik	AICc	△AICc	Weight
Null	-94.67	191.36	0.00	0.08
Average daily rainfall during incubation	-94.18	192.4	1.04	0.04
Average diel temperature range during incubation	-94.19	192.41	1.05	0.04
Average daily rainfall deviation from long term	-94.24	192.51	1.15	0.04
Average diel temperature range during incubation + Deviation from long term average daily maximum	-93.27	192.61	1.25	0.04
Deviation from long term average daily maximum + Average daily rainfall during incubation	-93.43	192.94	1.58	0.03
Deviation from long term average daily diel temperature	-94.51	193.07	1.71	0.03
Average diel temperature range during incubation + Deviation from long term average daily maximum	-93.50	193.07	1.71	0.03
Average daily maximum temperature during incubation	-94.60	193.24	1.88	0.03

6.4.3 Population structure

Pre-filtering, this dataset contained 197,890 SNP loci for 218 individuals with a missing data rate of 34.29%. After filtering and quality control, the dataset was reduced to 11,128 SNPs for 216 individuals with a missing data rate of 2.1%.

We were unable to detect any broadscale geographic population structure. In a PCoA analysis, the first and second axes explain only 2.4% and 1.3% of variation respectively (Figure 6-3), indicating that despite the large sampling area, there is limited population differentiation. A relatedness matrix constructed using *dartR* identified a higher degree of relatedness in all specimens collected within a geographically isolated area south of the Murray River in South Australia. In the PCoA analysis, these individuals do cluster and appear to be genetically divergent from the rest of the sampled specimens, but low variation explained by the PCoA indicates that this divergence is minor. There was significant evidence of weak isolation by distance (Wright, 1943) across the entire range of the species (Mantel's test; r = 0.571, p = 0.001, Figure 6-3) suggesting a stepping-stone model of dispersal for this species (Kimura, 1953).

6.5 Discussion

Here, we present the most detailed *in situ* account of naturally occurring sex reversal in a terrestrial vertebrate. Over a 38-year period encompassing a study area of 1.7 million km² (almost 80% of the species range) we show that sex reversal in *P. vitticeps* occurred across a quarter of the species range but was not directly explained by inferred incubation conditions. Sex-reversed ZZ females comprise 12% of all phenotypic females collected, demonstrating that the sex-reversed phenotype is a substantial demographic in this species.

Counter to our predictions based on the established relationship between high temperature and sex reversal in a laboratory setting (Quinn et al., 2007; Holleley et al., 2015), we did not observe an association between environmental temperatures and sex in the wild. There is a noticeable lack of sex reversal in the northern and north-western part of the species range where nest CTE and ambient temperatures are the hottest (Figure 6-1). Instead, we observed a significant cluster of sex reversal in the south-eastern portion of the species range, where inferred incubation conditions are relatively cooler than in the north, and where long term CTE's do not approach the 32°C sex reversal threshold. It must be noted that while we cannot conclude definitively that sex reversal is absent from the north of the species range,

owing to lower sample size in this area, our clustering methods do account statistically for sampling heterogeneity, and still identified a significant cluster. The occurrence of sex reversal in wild populations is clearly more complex than the simple relationship with temperature observed under controlled laboratory conditions, even after correcting for diel variation in temperature in natural nests (Georges, 1989; Georges et al., 1994), and could be explained by differences in the thermal threshold for sex reversal.

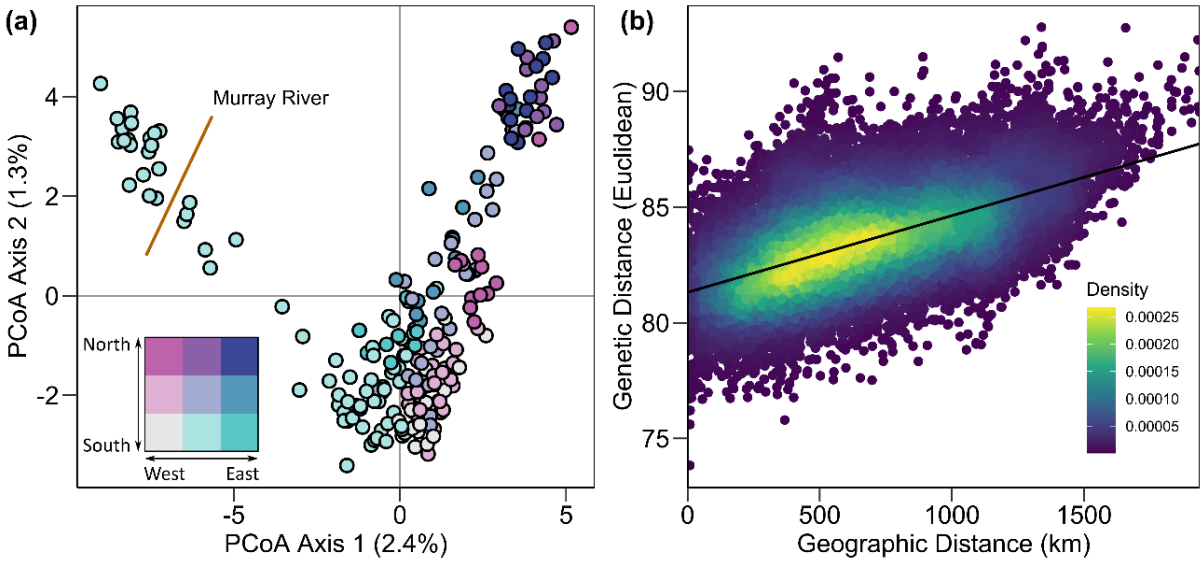


Figure 6-3. Genetic structure in the central bearded dragon (*Pogona vitticeps*). The structure displays a pattern of isolation by distance, but no strong genetic differentiation between populations throughout the species range. Slight genetic differentiation is apparent among specimens collected south of the Murray River, a geographical barrier to gene flow. (a) Principal coordinates analysis indicates that there is little genetic differentiation between individuals from across the species range. Axes 1 and 2 explain only 2.4% and 1.3% of variation, respectively, and this combined with the lack of clear delineation of groups of individuals, indicates that gene flow is occurring between groups of individuals across the species range, resulting in a geographical gradient of genetic differentiation. (b) Significant and relatively strong isolation by distance was detected (Mantel's test: r = 0.571, p = 0.001), indicating that across the species range, *P. vitticeps* genetic similarity decreases with increasing distance. PCoA and isolation by distance analysis were generated from a filtered SNP dataset of 11,128 binary SNPs and 216 individuals selected from across the species range.

We argue that the geographically disjunct distribution of sex reversal is most likely explained by local genetic adaptation to environmental conditions and evolution in the thermal threshold for reversal. Such a mechanism is involved in transitions between GSD and TSD (Quinn et al., 2011), and our population genetic data demonstrate that isolation by distance is the primary pattern of genetic differentiation in this species, providing a pattern of

underlying genetic variation. In contrast to other reptiles with very large species distributions, *P. vitticeps* has no obvious barriers to dispersal and no significant population structure (Melville et al., 2001, 2017; Sovic et al., 2016; Atkins et al., 2019). The Murray River in South Australia was the only weak and porous barrier to dispersal that we detected. We propose that the thermal threshold for sex reversal varies in a gradient pattern across the landscape, matching the pattern of isolation by distance. We predict that threshold temperatures will be higher in the northern part of the species range, where incubation temperatures are the hottest.

For evolution of the thermal threshold to occur, heritable variation in the propensity to reverse must exist. Preliminary observations suggest that this is the case in *P. vitticeps.* Offspring of sex-reversed *P. vitticeps* have a lower threshold for sex reversal than the offspring of ZW females (Holleley et al., 2015). Additionally, individual females can produce offspring that are completely resistant to sex reversal at all temperatures (Holleley et al., 2015). The exact molecular mechanisms of high temperature sex reversal are not known, but a genetic component to the threshold for reversal could be controlled by a single gene of major influence [e.g. CIRBP in the snapping turtle, *Chelydra serpentina* (Schroeder et al., 2016)] or have a polygenic basis [e.g. European sea bass, *Dichentrarchus labrax* (Faggion et al., 2019)]. There is also likely to be an epigenetic component to the sex reversal threshold. Epigenetic mechanisms already implicated in sex reversal include differential expression and temperature-specific splicing of chromatin modifying genes JARID2 and JMJD3/KDM6B (Deveson et al., 2017; Ge et al., 2018).

Compensatory maternal choice in nesting phenology, depth and location cannot fully explain the distribution of sex reversal in *P. vitticeps*. While we acknowledge that uncertainty in nest depth, location and specimen age may contribute to low power in this analysis, another study in a TSD reptile found significant environmental associations at this scale with a similar sampling and analytical approach (Ewert et al., 2005). Additionally, studies in thermally sensitive reptiles are mixed in terms of whether maternal choices are capable of buffering the effects of climate (Ewert et al., 2005; Doody et al., 2006; Warner & Shine, 2008; Refsnider et al., 2013; Refsnider & Janzen, 2016). We consider it very unlikely that behavioural repertoires to avoid sex reversal would be 100% effective for *P. vitticeps* in the hottest parts of the species range and yet less effective in the relatively cooler regions in which sex reversal was detected (Figure 6-2). Given the underlying genetic variation in this species (Figure 6-3) and role of threshold evolution in mediating transitions between GSD and TSD (Quinn et al., 2011), we consider it likely that the sex reversal threshold varies across the species range,

buffering against the sex-reversing conditions of northern Australian nesting habitats (Figure 6-1).

Understanding the capacity of the threshold for sex reversal to evolve is essential for predicting demographic outcomes under altered climate scenarios, including the likelihood of transitions in sex determining modes. Contemporary climate change could accelerate the transition to TSD through greater production of ZZ females and local/widespread loss of the W chromosome. Conversely, conferral of a reproductive advantage to high temperature males could decelerate the transition to TSD (Schwanz et al., 2020). Here, a poor relationship between temperature and the incidence of sex reversal suggests that local genetic adaptation to extreme conditions has already occurred as a sex ratio-balancing mechanism. Populations that resist sex reversal then become a source of ZW immigrants, and even small rates of ZW immigration (1% p.a.) significantly decelerate the transition to TSD (Schwanz et al., 2020). Thus, local thermal adaptation and an influx of ZW immigration could allow *P. vitticeps* to remain stably as a mixed sex determination system in the long term. Importantly, the existence of multiple evolutionary processes working in opposition means that the outcome of transitions between sex determining modes cannot be predicted based on climatic data alone (Schwanz et al., 2020).

The capacity of thermal thresholds to evolve is a critical component of the biological response to climate change, particularly in ectothermic animals. We demonstrate here that threshold evolution may have already occurred across the geographic range of a species with temperature sex reversal. Understanding this component of resilience to changing thermal regimes is essential to future conservation efforts.

Chapter 7 Synopsis

7.1 Research Summary

In this thesis, I identified and addressed key questions relating to the mechanisms and effects of sex reversal, both at the individual level and on an evolutionary scale. Using the central bearded dragon (*P. vitticeps*) as a model species, and utilising transcriptomic, genomic, and climatic data, I have demonstrated that temperature sex reversal is complex at both the molecular and landscape level.

I provide experimental and transcriptomic evidence that the vertebrate stress axis is not a cause or a consequence of sex reversal (Chapter 3; Chapter 4; Castelli et al., 2021). Tissue corticosterone levels in embryos during the temperature sensitive period did not vary with temperature, and yolk corticosterone injection did not result in sex reversal (Chapter 4; Castelli et al., 2021). Brain and gonad transcriptomic data from the temperature sensitive period supports this conclusion, with no evidence for stress axis activation in embryos prior to gonadal commitment in response to high temperature incubation (Chapter 3). I conclude that the stress axis does not mediate ESD, in agreement with all studies in reptiles except for one, which was potentially confounded by high levels of sex-specific mortality (Warner, Radder, et al., 2009).

Using brain and gonad transcriptomic data, I provide broad support for a gonad-autonomous model of sex determination (Chapter 3; Chapter 5; Castelli et al., 2021), but do identify a new candidate for brain-driven sex determination in the circadian system (Chapter 3). Neither the stress axis nor brain estrogen production are associated with sex-reversing temperatures, but core circadian clock genes and peripheral regulators of ubiquitination and RNA processing are differentially regulated in embryos incubated at high sex-reversing temperatures (Chapter 3). The involvement of the circadian rhythm in sex determination appears to occur in both fish and reptiles with ESD (Bentley et al., 2017; Zhao et al., 2019; Zou et al., 2020) and represents a promising brain-driven communication system which may have been co-opted to determine sexual phenotype in species with ESD.

I provide early evidence to suggest that the antioxidant response to high temperature incubation and reactive oxygen species (ROS) production is involved in sex reversal (Chapter 5). This supports the role of CaRe regulation in mediating sexual fate, a gonad-autonomous hypothesis for environmental sex determination presented in Chapter 2 (Castelli, Whiteley, et

al., 2020). Although I cannot conclude definitively that oxidative stress pathways are involved in sex reversal, owing to the high mortality and low effect size associated with *in ovo* antioxidant treatment, the generation of a fully sex-reversed individual through antioxidant application is promising. The evidence I present here points towards a sex determination system that is gonad-autonomous and does not support a role for sexually dimorphic gene expression in the brain as a primary sex determining mechanism in *P. vitticeps*.

The chronic upregulation of the stress axis as a consequence of sex reversal, while predicted from earlier adult transcriptomic data (Deveson et al., 2017), was not supported by my data which examined stress axis activity during the temperature sensitive period (TSP). Additionally, neither basal levels of plasma corticosterone (as a measure of acute stress) or oxidised guanine (as a measure of chronic stress and DNA damage) differed between concordant males, concordant females or sex-reversed females. This indicates that hormonal stress is not a consequence of temperature sex reversal in *P. vitticeps*.

I provide landscape-scale genetic and climatic evidence to suggest that oppositional evolutionary forces have acted to alter the threshold temperature for sex reversal in wild populations (Chapter 6; Castelli, Georges, et al., 2020). By creating a spatial profile of wild-caught *P. vitticeps* specimens from field trips and museum collections, I generated a comprehensive geographic record of sex reversal. I used reduced representation sequencing on a subset of these specimens and estimated egg incubation temperature to assess the potential for threshold variation across the species range. Based on the detection of a spatial cluster of sex-reversed individuals but the absence of an association between sex reversal and climatic conditions in the wild, I proposed that there are different threshold temperatures for sex reversal across the range of *P. vitticeps*. This may explain why no sex-reversed individuals were observed in the hottest part of the species range (Chapter 6, Castelli, Georges, et al., 2020).

7.2 Future research directions

The question of whether vertebrate environmental sex determination is brain-driven or gonad-autonomous in reptiles is still unanswered, but the scope of candidates for brain-driven axes has been considerably narrowed by this thesis. I have generated compelling evidence that the vertebrate stress axis is not involved in mediating temperature sex reversal in *P. vitticeps*, instead finding evidence for the role of oxidative stress responses or the circadian rhythm in temperature sex reversal. The roles of these systems in mediating temperature sex reversal

could be explored further using a range of gene editing, chemical application, and organ culture approaches. Furthermore, the genetic and epigenetic underpinnings of thermal sensitivity and the ability for these thermal thresholds to evolve could be explored by taking advantage of what I have proposed is a natural gradient in threshold temperature across the species range.

The lack of an effect of corticosterone treatment during development in *P. vitticeps* may indicate that the mediator of ESD differs among vertebrate species (Chapter 4; Castelli, Georges, and Holleley 2021). Fish gonads contain germ cells, providing them with plasticity not present in other vertebrates into adulthood (Hattori et al., 2020) and amniotic and anamniotic vertebrates differ in the organisation of their steroidogenic tissues (Denver, 2009). Further work on amphibians (as semi-terrestrial anamniotes) would be illuminating as to whether the stress axis mediates sex determination beyond fish. So far, no corticosterone application experiments have been published to explore the role of the stress axis in amphibian ESD. Both GSD and ESD occur in amphibians, and depending on the species different response patterns to temperature have been reported, with high temperature causing masculinization or feminization (Flament, 2016; Phuge, 2017; Lambert et al., 2018). Amphibian temperature sex reversal has even been confirmed, with high temperatures resulting in the generation of ZW males in the newt *Pleurodeles waltl* (Chardard & Dournon, 1999). In *P. waltl*, brain aromatase is not involved in sex determination (Kuntz et al., 2004), but the role of the stress axis has not yet been investigated, leaving the question of whether ESD is brain-driven in amphibians still unanswered. Stress hormones and intermediary signalling molecules in the stress axis are known to mediate life history transitions in frogs (Denver, 1997, 1999), and so the co-option of the stress axis for determination of sexual phenotype in amphibians is also plausible. To address this question, corticosterone application experiments could be conducted across a thermal rearing gradient (Chapter 4; Castelli et al., 2021), with corticosterone treatments applied through diet, as is typical for corticosterone application experiments in fish larvae (Yamaguchi et al., 2010).

I provided initial experimental evidence in support of a role for oxidative stress and the antioxidant response in temperature sex reversal (Chapter 5). Further experimental work testing this hypothesis in favour of the stress axis should be pursued. Ideally, this should occur in an organ culture system, in which the bipotential gonads are isolated and cultured across a thermal gradient. The value in organ culture experiments is two-fold; firstly, if a bipotential gonad cultured in isolation differentiates according to incubation temperature, it can be concluded that sex reversal in *P. vitticeps* is gonad-autonomous and does not involve

extragonadal signals. Secondly, it allows the direct and repeated application of chemical treatments, which may avoid the significant level of mortality observed in my *in ovo* antioxidant experiments (Chapter 5). This, combined with the low effect size of the antioxidant treatments, limits my ability to conclude that antioxidant responses have a definitive role in reptile sex reversal.

The application of antioxidants in isolation and in combination could be repeated in organ culture, but the question of whether redox regulation is involved in sex determination could be answered more directly by the application of ROS themselves. The oxidants hydrogen peroxide (H_2O_2) and potassium superoxide (KO_2) are frequently applied to cells in culture to induce a state of oxidative stress, as they readily cross the cell membrane and are biologically relevant oxidising compounds (Gille & Joenje, 1992). Oxidative stress could also be induced by certain heavy metals or pesticides, but this approach is not preferred because these compounds many non-target genotoxic effects and may therefore interfere with a number of pathways other than the oxidative stress pathway (Koch & Hill, 2017). The possibility of brain communication with the gonad (either through the hypophyseal-pituitary axes or direct gonad innervation) has still not been ruled out definitively, and so an organ culture system could also be used to answer this question by culturing brains and gonads separately to determine if there is an organ-specific response to temperature. Furthermore, the embryonic adrenals could be cultured and treated with a variety of chemical factors, as the adrenals have been demonstrated to produce corticosterone in a temperature-dependent manner in late-stage lizard embryos (Girling & Jones, 2006). The development of brain culture protocols could be beneficial not only to sex determination research but do brain development research more broadly. *P. vitticeps* is an emerging model species in brain structure and evolution research, and the development of brain organ culture could allow in-depth experimentation and hypothesis testing in this field (Tosches et al., 2018; Norimoto et al., 2020; Schede et al., 2020).

The potential for crossover with classical hormone manipulation experiments also comes to mind, to explore the hierarchy of mechanisms which ultimately lead to differential sex gene expression and sex reversal. The application of a range of hormones, hormonal precursors and antagonists has been critical in determining the roles of estrogen and testosterone in gonad development (Crews et al., 1989; Wibbels & Crews, 1992; Lance & Bogart, 1992). Utilising an organ culture system for improved survivability, a number of combinations of treatments could be applied to determine the point in the sex determining cascade at which sex hormones and the candidate sensory mechanisms I propose in this thesis

(e.g. CaRe status, circadian rhythm) are influential. For example, cultured *P. vitticeps* ZZ gonads incubated at 28°C (non-sex-reversing temperature) could be treated separately and with combinations of estrogen, fadrozole (an estrogen inhibitor), oxidants (potassium superoxide, hydrogen peroxide), and calcium ions. For example, the application of oxidants and calcium ions together may cause sex reversal, but the addition of fadrozole to a calcium ion and oxidant treatment may prevent sex reversal, if it is assumed that increasing calcium flux and oxidant concentration will signal for the downstream upregulation of estrogen. Experimental manipulations such as these could be further substantiated by recently developed imaging techniques that detect ROS (Habibalahi et al., 2020) or calcium ions (Perry et al., 2015; Weber et al., 2020), either in cell or organ culture.

Ultimately, the best evidence to support the CaRe model for ESD (Chapter 2; Castelli, Whiteley, et al., 2020) would be to produce sex-reversed (or non-sex reversed) animals by interfering with calcium and redox regulation and raising the resulting individuals to reproductive maturity. While *ex ovo* techniques do exist (Dohle et al., 2009; Dorrell et al., 2012; Nomura et al., 2015), and would allow for repeated exposure of the embryo to the chemical of interest (as organ culture allows), preliminary research from our research team suggests that survival of the embryo is limited and development is delayed in an *ex ovo* system. Alternative techniques to chemical experimentation include CRISPR-Cas9 gene editing technology, recently demonstrated to be successful in a reptile (Rasys et al., 2019). In this study, the Cas9 solution was injected into the developing ovarian follicle while still inside the mother. I have demonstrated here that injection of saline solution on top of the embryo on day of lay (stage 1; Whiteley et al., 2017) does not result in elevated mortality from un-injected controls (Chapter 5), meaning that the physical delivery of CRISPR gene editing compounds is feasible with day of lay embryos. Using CRISPR, the candidate calcium channels and ROS-generating metabolic enzymes could be edited to abrogate their function. However, there will be challenges associated with targeting such ubiquitous signalling pathways which have many roles in cell function, and survivability of the embryo may suffer if complete gene knockout is conducted.

A complementary approach to chemical application or gene editing to target particular pathways in the CaRe framework, incubation experiments could be conducted which expose the developing *P. vitticeps* embryo to stressors other than temperature. Moisture content of the substrate in which the egg is incubated and water availability to gravid mothers have been found to affect sex in both oviparous turtles and in viviparous GSD reptiles (Wyneken & Lolavar, 2015; Dupoué et al., 2019). In addition, light regime is known to influence the sex of

fish with ESD (Corona-Herrera et al., 2018; García et al., 2020), and light exposure during reptile development can affect hatchling phenotypes (Y. P. Zhang et al., 2016). Rather than conducting incubation experiments across a temperature gradient, a gradient of light intensity or moisture could be applied to determine if alternative environmental stressors can cause sex reversal, and if they do, whether the same molecular pathways are activated as in response to temperature (e.g. *jarid2*, *kdm6b* and *cirbp* upregulation and intron retention).

Incubation across a light exposure gradient would be a particularly interesting experiment, as I demonstrated the involvement of the differential regulation of the circadian axis in response to high temperature incubation in this thesis (Chapter 3). Naturally laid *P. vitticeps* egg clutches are buried in substrate throughout the duration of their incubation, and all incubation experiments conducted in this thesis were in dark temperature-controlled incubators. If the circadian rhythm has indeed been co-opted as a mediator of sex determination in *P. vitticeps*, I would predict that chronic light exposure (the canonical environmental input for entrainment of the circadian rhythm) should induce sex reversal. Indeed, light exposure during *in ovo* development has been demonstrated to affect some morphological characteristics in reptiles (Zhang et al., 2016), and may even act synergistically with temperature, as temperature and light often co-vary seasonally.

Similar to CaRe regulation (Chapter 2; Castelli, Whiteley, et al., 2020), the circadian rhythm is an ancient and conserved environmental sensory system. Involving cyclical epigenetic regulation and the ubiquitination and degradation of proteins, rhythms in physiology and behaviour are entrained to the environment *via* the circadian rhythm using light and temperature inputs (Tosini et al., 2001; Yadlapalli et al., 2018). Already, two circadian proteins have been identified as central to the temperature response in reptiles with ESD: CLK4 (Haltenhof et al., 2020) and CIRBP (Schroeder et al., 2016). In this thesis, I demonstrate that not only are these circadian genes (*clk4* and *cirbp*) differentially regulated prior to gonadal commitment, but a suite of other core clock genes are differentially regulated at high temperature, in both the brain and the gonad (Chapter 3). Whether these circadian genes may have been recruited independently from the circadian system and are acting autonomously to determine sex in the gonad, or whether the core circadian clock in the brain (suprachiasmatic nucleus; SCN) is responsible for generating the sex-reversing signal in *P. vitticeps* is an avenue of research worth pursuing.

I demonstrated the global upregulation in both the brain and gonad of epigenetic modifiers well-known to be involved in reptile TSD (Chapter 2). For example, I confirmed that at high incubation temperature *kdm6b* and *jarid2* are upregulated in both the brain and the

gonad prior to sex determination, as they are in adult sex-reversed females (Deveson et al., 2017). The organ-specific functions of these proteins in response to temperature, and whether these functions in organs other than the gonad contribute to the development of secondary sex characteristics is not known. It may be that their sex-reversing function in the gonad (and subsequent hormone production) is the main driver of secondary sex characteristics, whereas their function in other organs is primarily protective, maintaining baseline cellular function and organization in the presence of a physiological stressor. The complete reversal of *P. vitticeps* in response to estrogen treatment during development (Ehl et al., 2017) allows this idea to be tested, as the secondary behavioural and phenotypic characteristics of sex (head size, body size, presence of femoral pores, displaying and copulatory behaviours) should be identical between temperature- and estrogen-reversed females if all secondary sex characteristics are due solely to hormone production in the gonads. Together, this represents a significant contribution to the sex determination literature and raises a number of future directions for research.

The physiological effects of *pomc* upregulation in adult sex-reversed females are still unknown (Deveson et al., 2017). I demonstrated here that basal levels of corticosterone and oxidized guanine levels (an indicator of chronic stress) are not upregulated in adult sex-reversed females (Chapter 4; Castelli et al., 2021). However, there may be differences in the responsiveness of the stress axis when presented with acute stressors, such as capture and handling. To build on this finding, behavioural experiments could be conducted to determine whether corticosteroids are elevated in sex-reversed females in response to an acute stressor. A close relative, the eastern bearded dragon (*Pogona barbata*) did not show any sex differences in corticosterone response between phenotypic males or females, either at capture or several hours after capture (Cree et al., 2000), but we do not know whether *P. barbata* displays sex reversal like *P. vitticeps*, a distinction which may have obscured differences in stress hormone levels. Alternatively, the *pomc* transcript requires cleaving by various pro-protein convertases which generates ACTH (which then goes on to signal glucocorticoid production). The alternative cleavage of *pomc* post-transcriptionally generates proteins with different functions, including various melanocyte-stimulating hormones, lipotropins and endorphins (D'Agostino & Diano, 2010; Cawley et al., 2016), any of which could have roles in mediating the behaviour of sex-reversed females.

I postulated that across the species range of *P. vitticeps*, there exists a difference in the threshold temperature for sex reversal (Chapter 6; Castelli, Georges, et al., 2020). To confirm this, controlled temperature incubation experiments should be conducted using populations

outside of the area in which sex reversal is currently known to occur. By comparing the reaction norms of sex over the same temperatures between different populations, differences in the threshold for sex reversal could be definitively identified, and the heritability of sex reversal thresholds in this species quantified. I predicted that in the north and west of the species range, thresholds for sex reversal will be higher than that in the eastern populations (Chapter 6; Castelli, Georges, et al., 2020), from which wild animals were taken and bred in the lab to determine the threshold temperature of 32^{o}C (Quinn et al., 2007; Holleley et al., 2015). Demonstrating the heritability of the threshold temperature could then be accomplished by breeding between populations with defined temperature reaction norms.

Whether the underlying genetic variation responsible for differences in threshold temperature is polygenic (as in the sea bass *Dicentrarchus labrax*; Piferrer, 2013) or perhaps controlled by a single locus (as in the snapping turtle *Chelydra serpentina;* Schroeder et al., 2016), is not known. Compounding the complexity of identifying sources of underlying variation in sex reversal threshold, it is possible that heritable epigenetics may also contribute to the threshold (Bošković & Rando, 2018; Lind & Spagopoulou, 2018). Taking a genomic approach, the quantitative trait loci (QTLs), single nucleotide polymorphisms (SNPs), patterns of histone modification and DNA methylation which differ between the two populations could be explored. Using whole-genome, chromatin immunoprecipitation (ChIP-seq) and methylation-sensitive sequencing technologies, the genetic loci which underpin the temperature threshold (either through DNA sequence variation or epigenetic regulation) could be elucidated. This could then be combined with further developmental transcriptome work, to determine which of the genes which vary between populations are differentially expressed, spliced or otherwise regulated in the developing gonad of embryos from each of these populations. Furthermore, the maternal influences of nest site choice and nesting phenology on offspring sex ratios must be investigated further in this species (Chapter 6; Castelli, Georges, et al., 2020). Taking a combined approach of controlled breeding experiments, genomics, epigenomics and transcriptomics, and taking advantage of a natural existing variation in threshold temperature, would allow the targeted identification of elements of the genome which are involved in temperature sex reversal in this species and in reptiles more generally.

7.3 Thesis conclusion

In the study of sex determination, we are faced with a range of possibilities at a range of scales for the factors controlling sex determination, many of which present as binary options. Is the sex determination system genetic or environmental? Is environmental sex determination brain-driven or gonad-autonomous? Is there a single biological system which senses environmental cues and transmits this information, or do a number of concomitant systems cumulatively determine gonadal phenotype? Much like the paradigm of sex determination itself, these mechanisms may exist not in a binary state but as a continuum, varying across taxa depending on the sex determining system of that species and the environmental stimuli most relevant to sex-specific fitness in that species.

Appendix

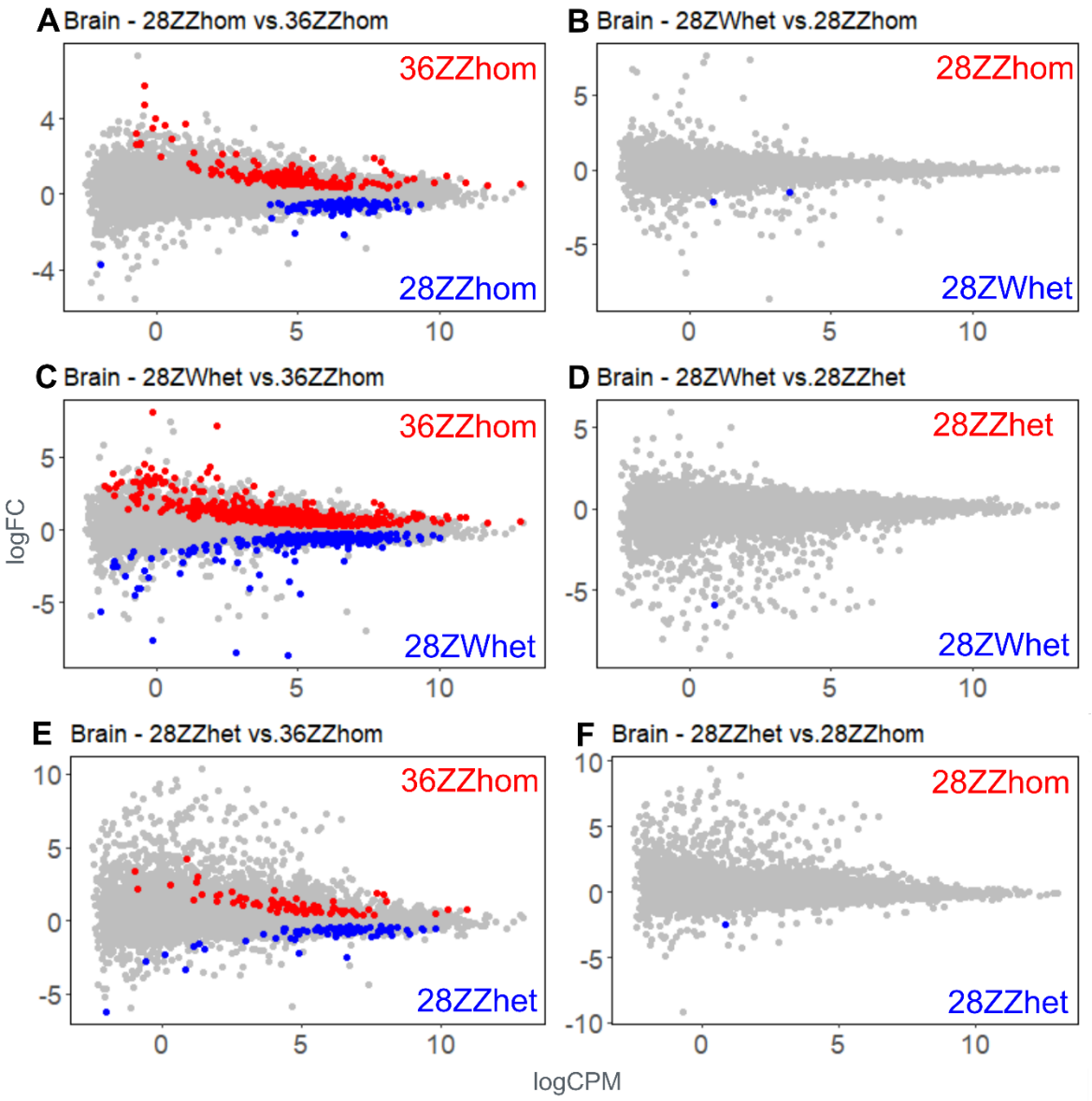
Supplementary Table 1. Mortality associated with corticosterone treatments in experiments 1 and 2 of Chapter 4 of this thesis.

Incubation experiment 1: Tissue corticosterone throughout development

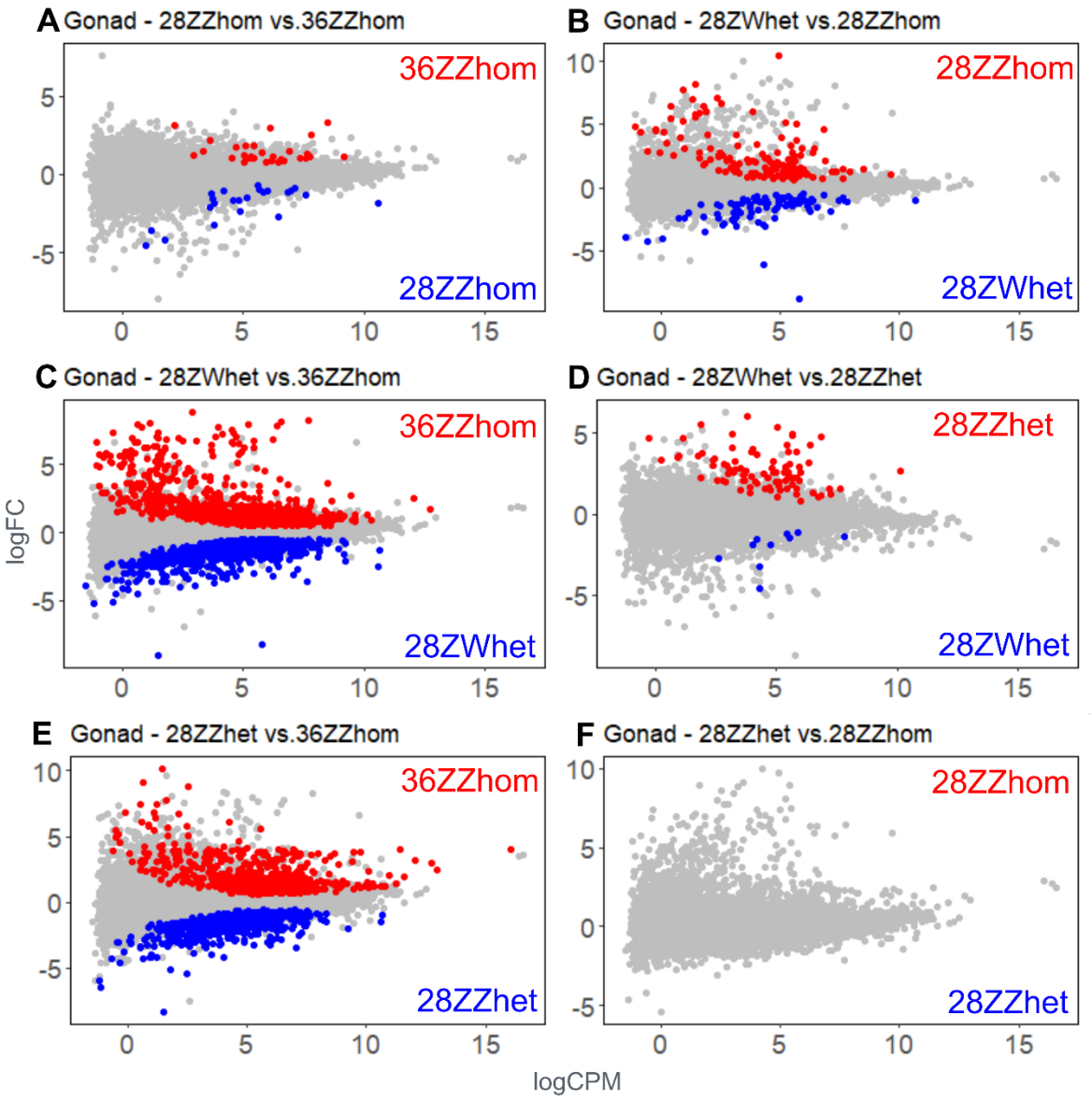
Groups (Stages 7 and 12 for each group combined)	Total Eggs Allocated	Number of Embryonic Deaths	% Embryonic Deaths
28°C-Control	27	4	14.8%
28°C-Vehicle	26	3	11.5%
28°C-Corticosterone 25µg	35	7	20%
36°C-Control	33	2	6.1%
36°C-Vehicle	29	2	6.9%

Incubation experiment 2: Effects of corticosterone on sex

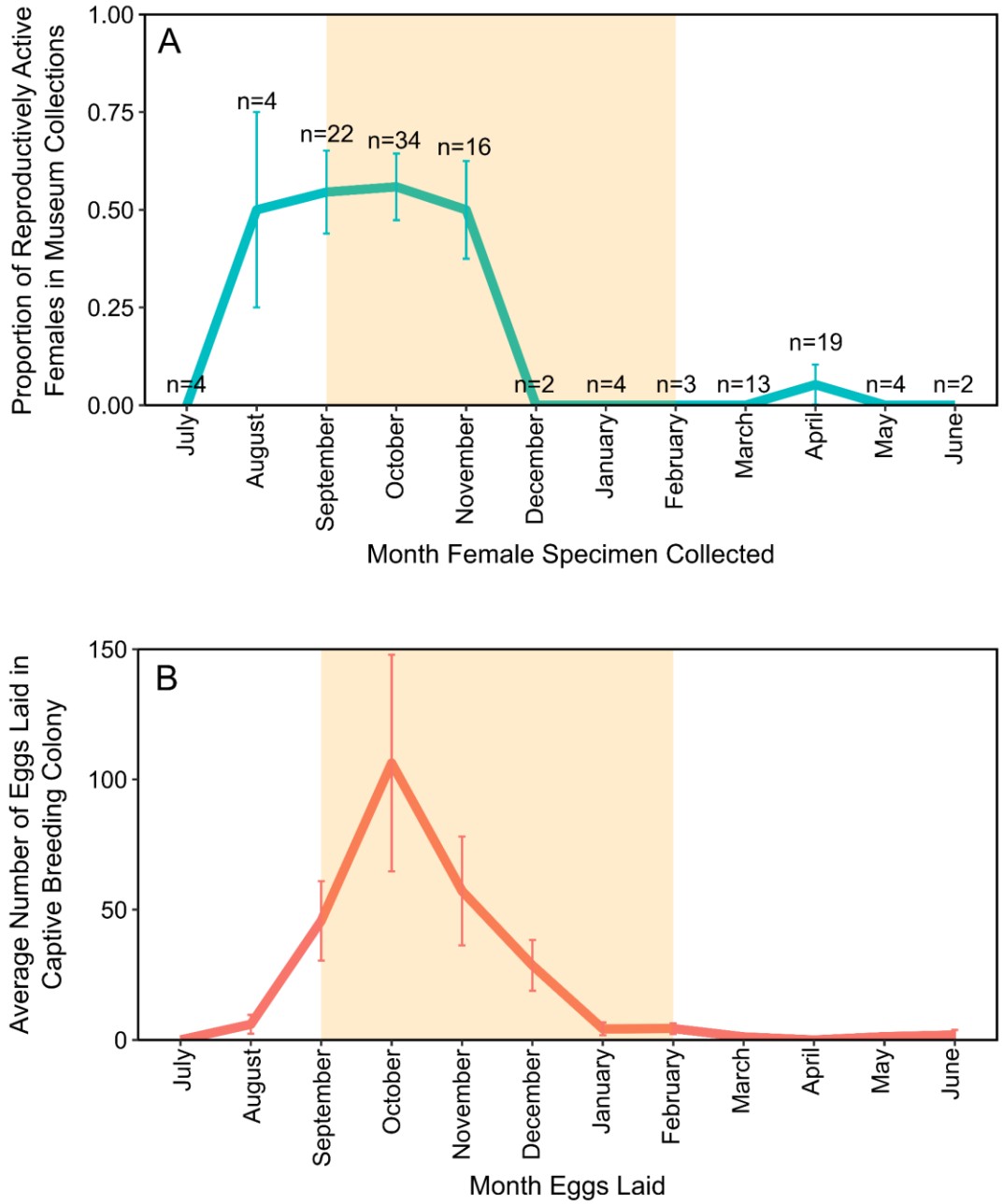
Groups	Total Eggs Allocated	Number of Embryonic Deaths	% Embryonic Deaths
Control	27	1	3.7%
Vehicle	18	2	11.1%
Corticosterone 10µg	61	3	4.9%
Corticosterone 25µg	62	4	6.5%



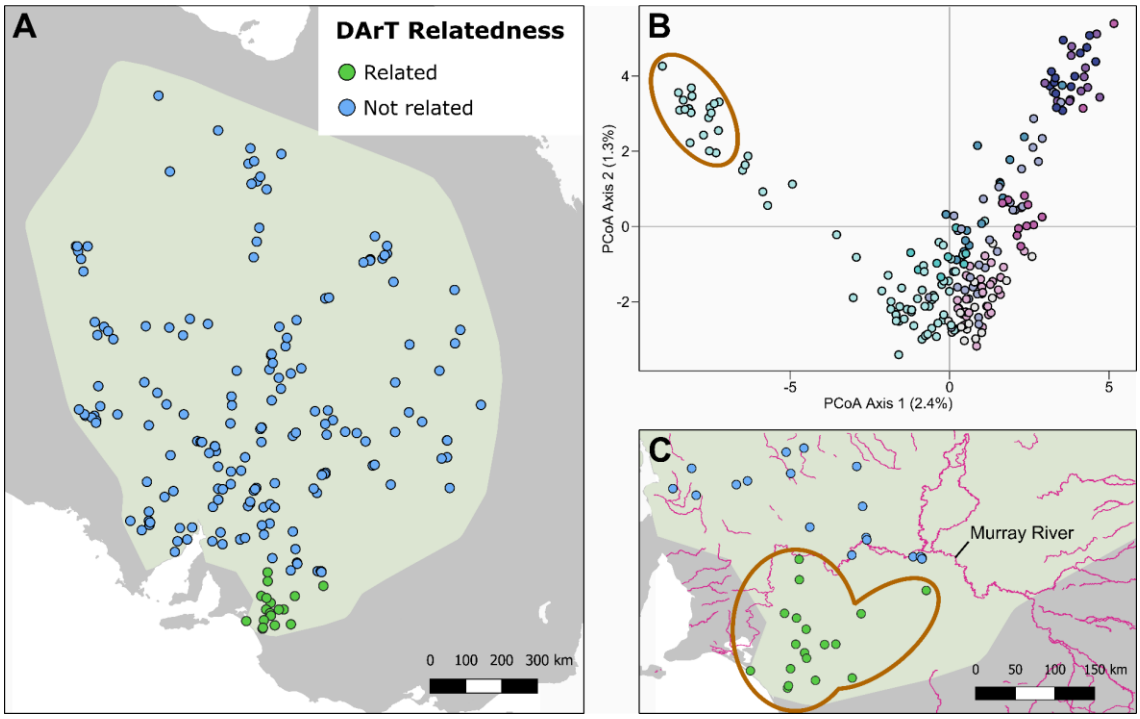
Supplementary Figure 1. MA plots of brain transcriptomic data comparing the log counts per million (logCPM) and log fold change (logFC) of genes in each between-group comparison in Chapter 3 of this thesis.The genes which are differentially expressed (FDR < 0.05) in each comparison are coloured either red or blue. Crucially, in the comparison between males from mothers of different genotypes (F), there is only one gene differentially expressed between the two groups. The brains of ZZ embryos incubated at 28°C are highly similar in this dataset, and so further analysis will be conducted on combined 28ZZ groups



Supplementary Figure 2. MA plots of gonad transcriptomic data comparing the log counts per million (logCPM) and log fold change (logFC) of genes in each between-group comparison in Chapter 3 of this thesis.The genes which are differentially expressed (FDR < 0.05) in each comparison are coloured either red or blue. Crucially, in the comparison between males from mothers of different genotypes (F), no genes are differentially expressed between the two groups. The bipotential gonads of ZZ embryos incubated at 28°C are indistinguishable in this dataset, and so further analysis will be conducted on combined 28ZZ groups.



Supplementary Figure 3. Timing of reproductive activity in colony-bred and wild-caught *Pogona vitticeps* defines the egg incubation period (orange box) used in Chapter 6 of this thesis. (A) The proportion of reproductively active females in museum collections (± SE) was determined by dissection and was defined as the specimen possessing either enlarged ovarian follicles or fully developed eggs. (B) The average number of eggs (± SE) laid in a captive colony per month at the University of Canberra was averaged over 11 non-consecutive breeding seasons. We defined the egg incubation period to be from the beginning of September to the end of February, when the majority of eggs will have been laid and exposed to environmental conditions in the nest.



Supplementary Figure 4. (A) The collection locations of wild-caught *Pogona vitticeps* which were successfully sequenced and analysed (n = 216) for population structure using reduced representation sequencing in Chapter 6 of this thesis. The gl.grm function in the analysis package dartR identified a group of individuals collected south of the Murray River which were all more closely related to each other than any of the other specimens. (B) PCoA analysis identified little genetic variation in the dataset, with the first and second axes explaining only 2.4% and 1.3% of variation respectively. Despite this, the group of related individuals clearly cluster differently to the majority of other specimens. (C) Detailed map of the area in which the related individuals were identified, with major tributaries displayed in pink. All of the related specimens were collected south of the Murray River, a major river which could provide a considerable geographical barrier to dispersal in this terrestrial reptile.

References

Adolfi, M. C., Fischer, P., Herpin, A., Regensburger, M., Kikuchi, M., Tanaka, M., & Schartl, M. (2019). Increase of cortisol levels after temperature stress activates *dmrt1a* causing female-to-male sex reversal and reduced germ cell number in medaka. *Molecular Reproduction and Development*, *86*(10), 1405–1417. https://doi.org/10.1002/mrd.23177

Aguirre, J., Ríos-Momberg, M., Hewitt, D., & Hansberg, W. (2005). Reactive oxygen species and development in microbial eukaryotes. *Trends in Microbiology*, *13*(3), 111–118. https://doi.org/10.1016/j.tim.2005.01.007

Ahn, S. G., & Thiele, D. J. (2003). Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. *Genes and Development*, *17*(4), 516–528. https://doi.org/10.1101/gad.1044503

Akashi, H. D., Cádiz Díaz, A., Shigenobu, S., Makino, T., & Kawata, M. (2016). Differentially expressed genes associated with adaptation to different thermal environments in three sympatric Cuban *Anolis* lizards. *Molecular Ecology*, *25*(10), 2273–2285. https://doi.org/10.1111/mec.13625

Angelopoulou, R., Lavranos, G., & Manolakou, P. (2012). Sex determination strategies in 2012: Towards a common regulatory model? *Reproductive Biology and Endocrinology*, *10*, 13. https://doi.org/10.1186/1477-7827-10-13

Antelmann, H., & Helmann, J. D. (2010). Thiol-based redox switches and gene regulation. *Antioxidants and Redox Signaling*, *14*(6), 1049–1063. https://doi.org/10.1089/ars.2010.3400

Antonsson, A., Hughes, K., Edin, S., & Grundstrom, T. (2003). Regulation of c-Rel nuclear localization by binding of Ca^{2+}/calmodulin. *Molecular and Cellular Biology*, *23*(4), 1418–1427. https://doi.org/10.1128/mcb.23.4.1418-1427.2003

Atkins, Z. S., Amor, M. D., Clemann, N., Chapple, D. G., While, G. M., Gardner, M. G., Haines, M. L., Harrisson, K. A., Schroder, M., & Robert, K. A. (2019). Allopatric divergence drives the genetic structuring of an endangered alpine endemic lizard with a sky-island distribution. *Animal Conservation*, *23*(1), 104–118.

https://doi.org/10.1111/acv.12519

Atwell, J. W., Cardoso, Gonçalo, C. C., Whittaker, D. J., Campbell-Nelson, S., Robertson, K. W., & Ketterson, E. D. (2012). Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. *Behavioral Ecology, 23*(5), 960-969. https://doi.org/10.1093/beheco/ars059

Awad, E. M., Khan, S. Y., Sokolikova, B., Brunner, P. M., Olcaydu, D., Wojta, J., Breuss, J. M., & Uhrin, P. (2013). Cold induces reactive oxygen species production and activation of the NF-kappa B response in endothelial cells and inflammation *in vivo*. *Journal of Thrombosis and Haemostasis*, *11*(9), 1716–1726. https://doi.org/10.1111/jth.12357

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T.-L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., Vamosi, J. C., & The Tree of Sex Consortium. (2014). Sex determination: Why so many ways of doing it? *PLoS Biology*, *12*(7), e1001899. https://doi.org/10.1371/journal.pbio.1001899

Badenhorst, D., Stanyon, R., Engstrom, T., & Valenzuela, N. (2013). A ZZ/ZW microchromosome system in the spiny softshell turtle, *Apalone spinifera*, reveals an intriguing sex chromosome conservation in Trionychidae. *Chromosome Research 21*, 137-147. https://doi.org/10.1007/s10577-013-9343-2

Baroiller, J.-F., & D'Cotta, H. (2016). The reversible sex of gonochoristic fish: Insights and consequences. *Sexual Development*, *10*(5–6), 242–266. https://doi.org/10.1159/000452362

Barske, L. A., & Capel, B. (2008). Blurring the edges in vertebrate sex determination. *Current Opinion in Genetics and Development*, *18*(6), 499–505. https://doi.org/10.1016/j.gde.2008.11.004

Barton, K. (2019). *MuMIn: Multi-model inference. R package 1.43.15*. https://cran.r-project.org/package=MuMIn

Beaulieu, M., Mboumba, S., Willaume, E., Kappeler, P. M., & Charpentier, M. J. E. (2014). The oxidative cost of unstable social dominance. *Journal of Experimental Biology*, *217*(15), 2629–2632. https://doi.org/10.1242/jeb.104851

Bekhbat, M., Rowson, S. A., & Neigh, G. N. (2017). Checks and balances: The glucocorticoid receptor and NFκB in good times and bad. *Frontiers in Neuroendocrinology*, *46*, 15–31. https://doi.org/10.1016/j.yfrne.2017.05.001

Bentley, B. P., Haas, B. J., Tedeschi, J. N., & Berry, O. (2017). Loggerhead sea turtle embryos (*Caretta caretta*) regulate expression of stress-response and developmental genes when exposed to a biologically realistic heat stress. *Molecular Ecology*, *26*(11), 2978–2992. https://doi.org/10.1111/ijlh.12426

Berridge, M. J., Bootman, M. D., & Roderick, H. L. (2003). Calcium signalling: Dynamics, homeostasis and remodelling. *Nature Reviews Molecular Cell Biology*, *4*(7), 517–529. https://doi.org/10.1038/nrm1155

Birnie-Gauvin, K., Costantini, D., Cooke, S. J., & Willmore, W. G. (2017). A comparative and evolutionary approach to oxidative stress in fish: A review. *Fish and Fisheries*, *18*(5), 928–942. https://doi.org/10.1111/faf.12215

Bivand, R. S., Pebesma, E. J., & Gomez-Rubio, V. (2013). *Applied Spatial Data Analysis with R* (2nd ed.). Springer-Verlag New York.

Bock, S. L., Hale, M. D., Leri, F. M., Wilkinson, P. M., Rainwater, T. R., & Parrott, B. B. (2020). Post-transcriptional mechanisms respond rapidly to ecologically relevant thermal fluctuations during temperature-dependent sex determination. *Integrative Organismal Biology*, *2*(1), obaa033. https://doi.org/https://doi.org/10.1093/iob/obaa033

Bókony, V., Kövér, S., Nemesházi, E., Liker, A., & Székely, T. (2017). Climate-driven shifts in adult sex ratios via sex reversals: The type of sex determination matters. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1729), 20160325. https://doi.org/10.1098/rstb.2016.0325

Bókony, V., Milne, G., Pipoly, I., Székely, T., & Liker, A. (2019). Sex ratios and bimaturism differ between temperature-dependent and genetic sex-determination systems in reptiles. *BMC Evolutionary Biology*, *19*, 57. https://doi.org/10.1186/s12862-019-1386-3

Border, S. E., Deoliveira, G. M., Janeski, H. M., Piefke, T. J., Brown, T. J., & Dijkstra, P. D. (2019). Social rank, color morph, and social network metrics predict oxidative stress in a cichlid fish. *Behavioral Ecology*, *30*(2), 490–499. https://doi.org/10.1093/beheco/ary189

Bošković, A., & Rando, O. J. (2018). Transgenerational epigenetic inheritance. *Annual Review of Genetics*, *52*(August), 21–41. https://doi.org/10.1146/annurev-genet-120417-031404

Botkin, D. B., Saxe, H., Araújo, M. B., Betts, R., Bradshaw, R. H. W., Cedhagen, T., Chesson, P., Dawson, T. P., Etterson, J. R., Faith, D. P., Ferrier, S., Guisan, A., Hansen, A. S., Hilbert, D. W., Loehle, C., Margules, C., New, M., Sobel, M. J., & Stockwell, D. R. B. (2007). Forecasting the effects of global warming on biodiversity. *BioScience*, *57*(3), 227–236. https://doi.org/10.1641/b570306

Bowden, R. M., Ewert, M. A., & Nelson, C. E. (2000). Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proceedings of the Royal Society B: Biological Sciences*, *267*(1454), 1745–1749. https://doi.org/10.1098/rspb.2000.1205

Boyle, M., Hone, J., Schwanz, L. E., & Georges, A. (2014). Under what conditions do climate-driven sex ratios enhance versus diminish population persistence? *Ecology and Evolution*, *4*(23), 4522–4533. https://doi.org/10.1002/ece3.1316

Brigelius-Flohé, R., & Flohé, L. (2011). Basic principles and emerging concepts in the redox control of transcription factors. *Antioxidants and Redox Signaling*, *15*(8), 2335–2381. https://doi.org/10.1089/ars.2010.3534

Brostrom, M. A., & Brostrom, C. O. (2003). Calcium dynamics and endoplasmic reticular function in the regulation of protein synthesis: Implications for cell growth and adaptability. *Cell Calcium*, *34*(4–5), 345–363. https://doi.org/10.1016/S0143-4160(03)00127-1

Brown, D. (2009). Hemipenal transillumination as a sexing technique in varanids. *Biawak*, *3*(1), 26–29.

Budd, A., Banh, Q., Domingos, J., & Jerry, D. (2015). Sex control in fish: Approaches, challenges and opportunities for aquaculture. *Journal of Marine Science and Engineering*, *3*(2), 329–355. https://doi.org/10.3390/jmse3020329

Bull, J. J., Gutzke, W. H. N., & Crews, D. (1988). Sex reversal by estradiol in three reptilian orders. *General and Comparative Endocrinology*, *70*(3), 425–428. https://doi.org/10.1016/0016-6480(88)90117-7

Bull, J. J., Vogt, C., & Bulmer, M. G. (1982). Heritability of sex ratio in turtles with environmental sex determination. *Evolution*, *36*(2), 333–341. https://doi.org/10.2307/2408052

Camello-Almaraz, C., Gomez-Pinilla, P. J., Pozo, M. J., & Camello, P. J. (2006). Mitochondrial reactive oxygen species and Ca^{2+} signaling. *American Journal of Physiology - Cell Physiology*, *291*(5), C1082–C1088. https://doi.org/10.1152/ajpcell.00217.2006

Campbell, G., & Norman, J. (1998). *An Introduction to Environmental Biophysics*. Springer-Verlag.

Capel, B. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nature Reviews Genetics*, *18*(11), 675–689. https://doi.org/10.1038/nrg.2017.60

Carter, A. L., Bodensteiner, B. L., Iverson, J. B., Milne-Zelman, C. L., Mitchell, T. S., Refsnider, J. M., Warner, D. A., & Janzen, F. J. (2019). Breadth of the thermal response captures individual and geographic variation in temperature-dependent sex determination. *Functional Ecology*, *33*(10), 1928–1939. https://doi.org/10.1111/1365-2435.13410

Carter, A. W., Bowden, R. M., & Paitz, R. T. (2018). Evidence of embryonic regulation of maternally derived yolk corticosterone. *Journal of Experimental Biology*, *221*(22), jeb182600. https://doi.org/10.1242/jeb.182600

Carter, A. W., Sadd, B. M., Tuberville, T. D., Paitz, R. T., & Bowden, R. M. (2018). Short heatwaves during fluctuating incubation regimes produce females under temperature-dependent sex determination with implications for sex ratios in nature. *Scientific Reports*, *8*, 3. https://doi.org/10.1038/s41598-017-17708-0

Casas, L., Saborido-Rey, F., Ryu, T., Michell, C., Ravasi, T., & Irigoien, X. (2016). Sex change in clownfish: Molecular insights from transcriptome analysis. *Scientific Reports*, *6*, 35461. https://doi.org/10.1038/srep35461

Castañeda-Cortés, D. C., Zhang, J., Boan, A. F., Langlois, V. S., & Fernandino, J. I. (2020). High temperature stress response is not sexually dimorphic at the whole-body level and is dependent on androgens to induce sex reversal. *General and Comparative*

Endocrinology, *299*, 113605. https://doi.org/10.1016/j.ygcen.2020.113605

Castañeda Cortés, D. C., Padilla, L. F. A., Langlois, V. S., Somoza, G. M., & Fernandino, J. I. (2019). The central nervous system acts as a transducer of stress-induced masculinization through corticotropin-releasing hormone B. *Development*, *146*(8), dev172866. https://doi.org/10.1242/dev.172866

Castelli, M. A., Georges, A., Cherryh, C., Rosauer, D. F., Sarre, S. D., Contador-Kelsall, I., & Holleley, C. E. (2020). Evolving thermal thresholds explain the distribution of temperature sex reversal in an Australian dragon lizard. *Diversity and Distributions*, *Early view*. https://doi.org/10.1111/ddi.13203

Castelli, M. A., Georges, A., & Holleley, C. E. (2021). Corticosterone does not have a role in temperature sex reversal in the central bearded dragon (*Pogona vitticeps*). *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *Early view*. https://doi.org/10.1002/jez.2441

Castelli, M. A., Whiteley, S. L., Georges, A., & Holleley, C. E. (2020). Cellular calcium and redox regulation: the mediator of vertebrate environmental sex determination? *Biological Reviews*, *95*(3), 680–695. https://doi.org/10.1111/brv.12582

Cawley, N. X., Li, Z., & Loh, Y. P. (2016). Biosynthesis, trafficking, and secretion of pro-opiomelanocortin-derived peptides. *Journal of Molecular Endocrinology*, *56*(4), T77–T97. https://doi.org/10.1530/JME-15-0323

Chailakhyan, M. K., & Khrianin, V. N. (1987). *Sexuality in Plants and its Hormonal Regulation* (K. V. Thimann (ed.); 1st ed.). Springer-Verlag New York.

Chandel, N. S., Trzyna, W. C., McClintock, D. S., & Schumacker, P. T. (2000). Role of oxidants in NF-κB activation and TNF-α gene transcription induced by hypoxia and endotoxin. *The Journal of Immunology*, *165*(2), 1013–1021. https://doi.org/10.4049/jimmunol.165.2.1013

Chardard, D., & Dournon, C. (1999). Sex reversal by aromatase inhibitor treatment in the newt *Pleurodeles waltl*. *Journal of Experimental Zoology*, *283*(1), 43–50. https://doi.org/10.1002/(SICI)1097-010X(19990101)283:1<43::AID-JEZ6>3.0.CO;2-G

Charnier, M. (1966). Action de la température sur la sex-ratio chez l'embryon d'*Agama agama* (Agamidae, Lacertilien). *Comptes Rendus Des Séances de La Societé de Biologie de l'Ouest Africain*, *160*, 620–622.

Charnov, E. L., & Bull, J. J. (1977). When is sex environmentally determined? *Nature*, *266*(828–830). https://doi.org/10.1016/0021-9797(80)90501-9

Chen, J., Peng, C., Yu, Z., Xiao, L., Yu, Q., Li, S., Zhang, H., Lin, H., & Zhang, Y. (2020). The administration of cortisol induces female-to-male sex change in the protogynous Orange-Spotted Grouper, *Epinephelus coioides*. *Frontiers in Endocrinology*, *11*, 12. https://doi.org/10.3389/fendo.2020.00012

Chen, W., Liu, L., & Ge, W. (2017). Expression analysis of growth differentiation factor 9 (Gdf9/*gdf9*), anti-müllerian hormone (Amh/*amh*) and aromatase (Cyp19a1a/*cyp19a1a*) during gonadal differentiation of the zebrafish, *Danio rerio*. *Biology of Reproduction*, *96*(2), 401–413. https://doi.org/10.1095/biolreprod.116.144964

Chen, Y., Yu, H., Pask, A. J., Shaw, G., & Renfree, M. B. (2017). Prostaglandin D2 regulates SOX9 nuclear translocation during gonadal sex determination in tammar wallaby, *Macropus eugenii*. *Sexual Development*, *11*(3), 143–150. https://doi.org/10.1159/000473782

Chojnowski, J. L., & Braun, E. L. (2012). An unbiased approach to identify genes involved in development in a turtle with temperature-dependent sex determination. *BMC Genomics*, *13*, 308. https://doi.org/10.1186/1471-2164-13-308

Clarke, A., & Fraser, K. P. P. (2004). Why does metabolism scale with temperature? *Functional Ecology*, *18*(2), 243–251. https://doi.org/10.1111/j.0269-8463.2004.00841.x

Clary, D., Skyner, L. J., Ryan, C. P., Gardiner, L. E., Anderson, W. G., & Hare, J. F. (2014). Shyness-boldness, buit not exploration, predicts glucocorticoid stress response in Richardson's Ground Squirrels (*Urocitellus richardsonii*). *Ethology*, *120*(11), 1101-1109. https://doi.org/10.1111/eth.12283

Cogger, H. G. (2018). *Reptiles and amphibians of Australia* (7th ed.). CSIRO Publishing.

Cong, L., & Zhang, F. (2014). Genome engineering using CRISPR-Cas9 system. In S. Pruett-

Miller (Ed.), *Chromosomal Mutagenesis Methods in Molecular Biology (Methods and Protocols), vol 1239* (pp. 197–217). Humana Press. https://doi.org/https://doi.org/10.1007/978-1-4939-1862-1_10

Contreras, L., Drago, I., Zampese, E., & Pozzan, T. (2010). Mitochondria: The calcium connection. *Biochimica et Biophysica Acta - Bioenergetics*, *1797*(6–7), 607–618. https://doi.org/10.1016/j.bbabio.2010.05.005

Cornejo-Páramo, P., Dissanayake, D. S. B., Lira-Noriega, A., Martínez-Pacheco, M. L., Acosta, A., Ramírez-Suástegui, C., Méndez-de-la-Cruz, F., Székely, T., Urrutia, A. O., Georges, A., & Cortez, D. (2020). Viviparous reptile regarded to have temperature-dependent sex determination has old XY chromosomes. *Genome Biology and Evolution 12*(6), 924-930. https://doi.org/10.1093/gbe/evaa104

Corona-Herrera, G. A., Arranz, S. E., Martínez-Palacios, C. A., Navarrete-Ramírez, P., Toledo-Cuevas, E. M., Valdez-Alarcón, J. J., Martínez-Chávez, C. C., Corona-Herrera, G. A., Arranz, S. E., Martínez-Palacios, C. A., Navarrete-Ramírez, P., Toledo-Cuevas, E. M., Valdez-Alarcón, J. J., & Martínez-Chávez, C. C. (2018). Experimental evidence of masculinization by continuous illumination in a temperature sex determination teleost (Atherinopsidae) model: Is oxidative stress involved? *Journal of Fish Biology*, *93*(2), 229–237. https://doi.org/10.1111/jfb.13651

Covarrubias, L., Hernández-García, D., Schnabel, D., Salas-Vidal, E., & Castro-Obregón, S. (2008). Function of reactive oxygen species during animal development: Passive or active? *Developmental Biology*, *320*(1), 1–11. https://doi.org/https://doi.org/10.1016/j.ydbio.2008.04.041

Cree, A., Amey, A. P., & Whittier, J. M. (2000). Lack of consistent hormonal responses to capture during the breeding season of the bearded dragon, *Pogona barbata*. *Comparative Biochemistry and Physiology Part A - Molecular and Integrative Physiology*, *126*(2), 275–285. https://doi.org/10.1016/S1095-6433(00)00210-5

Cree, A., Thompson, M. B., & Daugherty, C. H. (1995). Tuatara sex determination. *Nature, 375*, 543. https://doi.org/10.1038/375543a0

Cremers, C. M., & Jakob, U. (2013). Oxidant sensing by reversible disulfide bond formation. *Journal of Biological Chemistry*, *288*(37), 26489–26496.

https://doi.org/10.1074/jbc.R113.462929

Crews, D. (1996). Temperature-dependent sex determination: The interplay of steroid hormones and temperature. *Zoological Science*, *13*(1), 1–13. https://doi.org/10.2108/zsj.13.1

Crews, D., & Bergeron, J. M. (1994). Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *Journal of Endocrinology*, *143*(2), 279–289. https://doi.org/10.1677/joe.0.1430279

Crews, D., Wibbels, T., & Gutzke, W. H. N. (1989). Action of sex steroid hormones on temperature-induced sex determination in the snapping turtle (*Chelydra serpentina*). *General and Comparative Endocrinology*, *76*(1), 159–166. https://doi.org/10.1016/0016-6480(89)90042-7

Cutting, A., Chue, J., & Smith, C. A. (2013). Just how conserved is vertebrate sex determination? *Developmental Dynamics*, *242*(4), 380–387. https://doi.org/10.1002/dvdy.23944

Cyr, A. R., & Domann, F. E. (2011). The redox basis of epigenetic modifications: From mechanisms to functional consequences. *Antioxidants and Redox Signaling*, *15*(2), 551–589. https://doi.org/10.1089/ars.2010.3492

Czerwinski, M., Natarajan, A., Barske, L., Looger, L. L., & Capel, B. (2016). A timecourse analysis of systemic and gonadal effects of temperature on sexual development of the red-eared slider turtle *Trachemys scripta elegans*. *Developmental Biology*, *420*(1), 166–177. https://doi.org/10.1016/j.ydbio.2016.09.018

D'Agostino, G., & Diano, S. (2010). alpha-Melanocyte stimulating hormone: Production and degradation. *Journal of Molecular Medicine*, *88*(12), 1195–1201. https://doi.org/10.1007/s00109-010-0651-0.alpha-Melanocyte

Danishuddin, M., Subbarao, N., Faheem, M., & Khan, S. N. (2019). Polycomb repressive complex 2 inhibitors: Emerging epigenetic modulators. *Drug Discovery Today*, *24*(1), 179–188. https://doi.org/10.1016/j.drudis.2018.07.002

Darwin, C. (1871). *The Descent of Man, and Selection in Relation to Sex*. London: John Murray.

de Jager, T. L., Cockrell, A. E., & Du Plessis, S. S. (2017). Ultraviolet light induced generation of reactive oxygen species. *Advances in Experimental Medicine and Biology*, *996*, 15–23. https://doi.org/10.1007/978-3-319-56017-5_2

De Leeuw, F., Zhang, T., Wauquier, C., Huez, G., Kruys, V., & Gueydan, C. (2007). The cold-inducible RNA-binding protein migrates from the nucleus to cytoplasmic stress granules by a methylation-dependent mechanism and acts as a translational repressor. *Experimental Cell Research*, *313*(20), 4130–4144. https://doi.org/10.1016/j.yexcr.2007.09.017

Deeming, D. C., & Pike, T. W. (2013). Embryonic growth and antioxidant provision in avian eggs. *Biology Letters*, *9*(6), 20130757.

DeFalco, T. J., Verney, G., Jenkins, A. B., McCaffery, J. M., Russell, S., & Van Doren, M. (2003). Sex-specific apoptosis regulates sexual dimorphism in the *Drosophila* embryonic gonad. *Developmental Cell*, *5*(2), 205–216. https://doi.org/10.1016/S1534-5807(03)00204-1

Delfino, F., & Walker, W. H. (1998). Stage-specific nuclear expression of NF-κB in mammalian testis. *Molecular Endocrinology*, *12*(11), 1696–1707. https://doi.org/10.1210/me.12.11.1696

Denver, R. J. (1997). Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. *Hormones and Behavior*, *31*(2), 169–179. https://doi.org/10.1006/hbeh.1997.1383

Denver, R. J. (1999). Evolution of the corticotropin-releasing hormone signaling system and its role in stress-induced phenotypic plasticity. *Annals of the New York Academy of Sciences*, *897*(1), 46–53. https://doi.org/10.1111/j.1749-6632.1999.tb07877.x

Denver, R. J. (2009). Structural and functional evolution of vertebrate neuroendocrine stress systems. *Annals of the New York Academy of Sciences*, *1163*(1), 1–16. https://doi.org/10.1111/j.1749-6632.2009.04433.x

Deveson, I. W., Holleley, C. E., Blackburn, J., Marshall Graves, J. A., Mattick, J. S., Waters, P. D., & Georges, A. (2017). Differential intron retention in *Jumonji* chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Science Advances*, *3*(6), e1700731. https://doi.org/10.1126/sciadv.1700731

Díaz, N., & Piferrer, F. (2015). Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. *BMC Genomics*, *16*(1), 679. https://doi.org/10.1186/s12864-015-1862-0

Dohle, D. S., Pasa, S. D., Gustmann, S., Laub, M., Wissler, J. H., Jennissen, H. P., & Dünker, N. (2009). Chick *ex ovo* culture and *ex ovo* CAM assay: How it really works. *Journal of Visualized Experiments : JoVE*, *33*. https://doi.org/10.3791/1620

Dong, J., Xiong, L., Ding, H., Jiang, H., Zan, J., & Nie, L. (2019). Characterization of deoxyribonucleic methylation and transcript abundance of sex-related genes during temperature-dependent sex determination in *Mauremys reevesii*. *Biology of Reproduction*, *102*(1), 27–37. https://doi.org/10.1093/biolre/ioz147

Doody, J. S., Guarino, E., Georges, A., Corey, B., Murray, G., & Ewert, M. (2006). Nest site choice compensates for climate effects on sex ratios in a lizard with environmental sex determination. *Evolutionary Ecology*, *20*(4), 307–330. https://doi.org/10.1007/s10682-006-0003-2

Dorrell, M. I., Marcacci, M., Bravo, S., Kurz, T., Tremblay, J., & Rusing, J. C. (2012). Ex ovo model for directly visualizing chick embryo development. *The American Biology Teacher*, *74*(9), 628–634. https://doi.org/10.1525/abt.2012.74.9.6

Dowling, D. K., & Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1663), 1737–1745. https://doi.org/10.1098/rspb.2008.1791

Dupont, G., & Sneyd, J. (2017). Recent developments in models of calcium signalling. *Current Opinion in Systems Biology*, *3*, 15–22. https://doi.org/10.1016/J.COISB.2017.03.002

Dupoué, A., Lourdais, O., Meylan, S., Brischoux, F., Angelier, F., Rozen-Rechels, D.,

Marcangeli, Y., Decencière, B., Agostini, S., & Le Galliard, J. F. (2019). Some like it dry: Water restriction overrides heterogametic sex determination in two reptiles. *Ecology and Evolution*, *9*(11), 6524–6533. https://doi.org/10.1002/ece3.5229

Düsing, C. (1884). Die Regulierung des Geschlechtsverhältnisses: Bei der Vermehrung der Menschen, Tiere und Pflanzen. *Jenaische Zeitschrift Für Naturwissenschaft*, *17*(593–940).

Edwards, A. W. (2000). Carl Düsing (1884) on the regulation of the sex-ratio. *Theoretical Population Biology*, *58*, 255–257. https://doi.org/10.1006/tpbi.2000.1482.

Ehl, J., Vukić, J., & Kratochvíl, L. (2017). Hormonal and thermal induction of sex reversal in the bearded dragon (*Pogona vitticeps*, Agamidae). *Zoologischer Anzeiger*, *271*, 1–5. https://doi.org/10.1016/j.jcz.2017.11.002

Elf, P. K. (2003). Yolk steroid hormones and sex determination in reptiles with TSD. *General and Comparative Endocrinology*, *132*(3), 349–355. https://doi.org/10.1016/S0016-6480(03)00098-4

Engeli, R. T., Tsachaki, M., Hassan, H. A., Sager, C. P., Essawi, M. L., Gad, Y. Z., Kamel, A. K., Mazen, I., & Odermatt, A. (2017). Biochemical analysis of four missense mutations in the *HSD17B3* gene associated with 46,XY disorders of sex development in Egyptian patients. *Journal of Sexual Medicine, 14*(9), 1165–1174. https://doi.org/10.1016/j.jsxm.2017.07.006

Ermak, G., & Davies, K. J. A. (2002). Calcium and oxidative stress: From cell signaling to cell death. *Molecular Immunology*, *38*(10), 713–721. https://doi.org/10.1016/S0161-5890(01)00108-0

Ewert, M. A., Jackson, D. R., & Nelson, C. E. (1994). Patterns of temperature-dependent sex determination in turtles. *Journal of Experimental Zoology*, *270*(1), 3–15. https://doi.org/10.1002/jez.1402700103

Ewert, M. A., Lang, J. W., & Nelson, C. E. (2005). Geographic variation in the pattern of temperature-dependent sex determination in the American snapping turtle (*Chelydra serpentina*). *Journal of Zoology*, *265*(1), 81–95. https://doi.org/10.1017/S0952836904006120

Ezaz, T., Moritz, B., Waters, P., Marshall Graves, J. A., Georges, A., & Sarre, S. D. (2009). The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. *Chromosome Research*, *17*(8), 965–973. https://doi.org/10.1007/s10577-009-9102-6

Ezaz, T., Quinn, A. E., Miura, I., Sarre, S. D., Georges, A., & Marshall Graves, J. A. (2005). The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Research*, *13*(8), 763–776. https://doi.org/10.1007/s10577-005-1010-9

Faggion, S., Vandeputte, M., Chatain, B., Gagnaire, P. A., & Allal, F. (2019). Population-specific variations of the genetic architecture of sex determination in wild European sea bass *Dicentrarchus labrax* L. *Heredity*, *122*(5), 612–621. https://doi.org/10.1038/s41437-018-0157-z

Ferguson, M. W. J., & Joanen, T. (1982). Temperature of egg incubation determines sex in *Alligator mississippiensis*. *Nature*, *296*(5860), 850–853. https://doi.org/10.1038/296850a0

Ferguson, M. W. J., & Joanen, T. (1983). Temperature-dependent sex determination in *Alligator mississippiensis*. *Journal of Zoology*, *200*(2), 143–177. https://doi.org/10.1111/j.1469-7998.1983.tb05781.x

Fernandino, J. I., Hattori, R. S., Moreno Acosta, O. D., Strüssmann, C. A., & Somoza, G. M. (2013). Environmental stress-induced testis differentiation: Androgen as a by-product of cortisol inactivation. *General and Comparative Endocrinology*, *192*, 36–44. https://doi.org/10.1016/j.ygcen.2013.05.024

Fisher, R. A. (1930). *The Genetical Theory of Natural Selection*. Clarendon Press.

Flament, S. (2016). Sex reversal in amphibians. *Sexual Development*, *10*(5–6), 267–278. https://doi.org/10.1159/000448797

Forman, H. J., Fukuto, J., & Torres, M. (Eds.). (2003). *Signal transduction by reactive oxygen and nitrogen species*. Kluwer Academic Publishers.

Furukawa, F., Hamasaki, S., Hara, S., Uchimura, T., Shiraishi, E., Osafune, N., Takagi, H., Yazawa, T., Kamei, Y., & Kitano, T. (2019). Heat shock factor 1 protects germ cell

proliferation during early ovarian differentiation in medaka. *Scientific Reports*, *9*(1), 6927. https://doi.org/10.1038/s41598-019-43472-4

Gabriel, W. N., Blumberg, B., Sutton, S., Place, A. R., & Lance, V. A. (2001). Alligator aromatase cDNA sequence and its expression in embryos at male and female incubation temperatures. *Journal of Experimental Zoology*, *290*(5), 439–448. https://doi.org/10.1002/jez.1087

Gamble, T., Castoe, T. A., Nielsen, S. V, Banks, J. L., Card, D. C., Schield, D. R., Schuett, G. W., & Booth, W. (2017). The discovery of XY sex chromosomes in a boa and python. *Current Biology*, *27*, 2148–2153. https://doi.org/10.1016/j.cub.2017.06.010

Gamble, T., Coryell, J., Ezaz, T., Lynch, J., Scantlebury, D. P., & Zarkower, D. (2015). Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems. *Molecular Biology and Evolution*, *32*(5), 1296–1309. https://doi.org/10.1093/molbev/msv023

Gapper, C., & Dolan, L. (2006). Control of plant development by reactive oxygen species. *Plant Physiology*, *141*, 341–345. https://doi.org/10.1104/pp.106.079079

García-Cruz, E. L., Yamamoto, Y., Hattori, R. S., de Vasconcelos, L. M., Yokota, M., & Strüssmann, C. A. (2020). Crowding stress during the period of sex determination causes masculinization in pejerrey *Odontesthes bonariensis*, a fish with temperature-dependent sex determination. *Comparative Biochemistry and Physiology Part A - Molecular and Integrative Physiology*, *245*, 110701. https://doi.org/10.1016/j.cbpa.2020.110701

Garcia-Moreno, S. A., Plebanek, M. P., & Capel, B. (2018). Epigenetic regulation of male fate commitment from an initially bipotential system. *Molecular and Cellular Endocrinology*, *468*, 19–30. https://doi.org/10.1016/j.mce.2018.01.009

García, L. N., Marín, A. F., & Chapman, F. A. (2020). Effects of different color artificial lights on growth, survival, and sex ratio on an experimental population of freshwater ornamental emperor tetra fish *Nematobrycon palmeri*. *AACL Bioflux*, *13*(2), 1048–1054.

Ge, C., Ye, J., Weber, C., Sun, W., Zhang, H., Zhou, Y., Cai, C., Qian, G., & Capel, B. (2018). The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. *Science*, *360*(6389), 645–648.

https://doi.org/10.1126/science.aap8328

Ge, C., Ye, J., Zhang, H., Zhang, Y., Sun, W., Sang, Y., Capel, B., & Qian, G. (2017). *Dmrt1* induces the male pathway in a turtle species with temperature-dependent sex determination. *Development*, *144*(12), 2222–2233. https://doi.org/10.1242/dev.152033

Geffroy, B., & Douhard, M. (2019). The adaptive sex in stressful environments. *Trends in Ecology & Evolution*, *34*(7), 628–640. https://doi.org/10.1016/j.tree.2019.02.012

Georges, A. (1989). Female turtles from hot nests: Is it duration of incubation or proportion of development at high temperatures that matters? *Oecologia*, *81*(3), 323–328. https://doi.org/10.1007/BF00377078

Georges, A., Ezaz, T., Quinn, A. E., & Sarre, S. D. (2010). Are reptiles predisposed to temperature-dependent sex determination? *Sexual Development*, *4*, 7–15. https://doi.org/10.1159/000279441

Georges, A., Gruber, B., Pauly, G. B., White, D., Adams, M., Young, M. J., Kilian, A., Zhang, X., Shaffer, B., & Unmack, P. J. (2018). Genomewide SNP markers breathe new life into phylogeography and species delimitation for the problematic short-necked turtles (Chelidae: *Emydura*) of eastern Australia. *Molecular Ecology*, *27*(24), 5195–5123. https://doi.org/https://doi.org/10.1111/mec.14925

Georges, A., & Holleley, C. E. (2018). How does temperature determine sex? *Science*, *360*(6389), 601–602. https://doi.org/10.1126/science.aat5993

Georges, A., Li, Q., Lian, J., O'Meally, D., Deakin, J., Wang, Z., Zhang, P., Fujita, M., Patel, H. R., Holleley, C. E., Zhou, Y., Zhang, X., Matsubara, K., Waters, P., Graves, J. A. M. M., Sarre, S. D., Zhang, G., O'Meally, D., Deakin, J., … Zhang, G. (2015). High-coverage sequencing and annotated assembly of the genome of the Australian dragon lizard *Pogona vitticeps*. *GigaScience*, *4*(1), 45. https://doi.org/10.1186/s13742-015-0085-2

Georges, A., Limpus, C., & Stoutjesdijk, R. (1994). Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. *Journal of Experimental Zoology*, *270*(5), 432–444. https://doi.org/10.1002/jez.1402700504

Gerendai, I., Banczerowski, P., & Halász, B. (2005). Functional significance of the innervation of the gonads. *Endocrine*, *28*(3), 309–318. https://doi.org/10.1385/ENDO:28:3:309

Gille, J. J. P., & Joenje, H. (1992). Cell culture models for oxidative stress: Superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mutation Research*, *275*(3–6), 405–414. https://doi.org/10.1016/0921-8734(92)90043-O

Girling, J. E., & Jones, S. M. (2006). In vitro steroid production by adrenals and kidney-gonads from embryonic southern snow skinks (*Niveoscincus microlepidopotus*): Implications for the control of the timing of parturition? *General and Comparative Endocrinology, 145*(2), 169-176. https://doi.org/10.1016/j.ygcen.2005.08.012

Gniadecki, R., Thorn, T., Vicanova, J., Petersen, A., & Wulf, H. C. (2000). Role of mitochondria in ultraviolet-induced oxidative stress. *Journal of Cellular Biochemistry*, *80*(2), 216–222. https://doi.org/10.1002/1097-4644(20010201)80:2<216::AID-JCB100>3.0.CO;2-H

Goikoetxea, A., Todd, E. V, & Gemmell, N. J. (2017). Stress and sex: Soes cortisol mediate sex change in fish? *Reproduction*, *154*(6), 149–160. https://doi.org/10.1530/REP-17-0408

Gordeeva, A. V., Zvyagilskaya, R. A., & Labas, Y. A. (2003). Cross-talk between reactive oxygen species and calcium in living cells. *Biochemistry*, *68*(10), 1077–1080. https://doi.org/10.1023/A:1026398310003

Görlach, A., Bertram, K., Hudecova, S., & Krizanova, O. (2015). Calcium and ROS: A mutual interplay. *Redox Biology*, *6*, 260–271. https://doi.org/10.1016/j.redox.2015.08.010

Grossen, C., Neuenschwander, S., & Perrin, N. (2011). Temperature-dependent turnovers in sex-determination mechanisms: A quantitative model. *Evolution*, *65*(1), 64–78. https://doi.org/10.1111/j.1558-5646.2010.01098.x

Gruber, B., Unmack, P. J., Berry, O., & Georges, A. (2017). dartR: An R package to facilitate analysis of SNP data generated from reduced representation genome sequencing. *Molecular Ecology Resources*, *18*(3), 691–699. https://doi.org/10.1111/1755-0998.12745

Grundy, M. J., Viscarra Rossel, R. A., Searle, R. D., Wilson, P. L., Chen, C., & Gregory, L. J. (2015). Soil and landscape grid of Australia. *Soil Research*, *53*(8), 835–844. https://doi.org/10.1071/SR15191

Grützner, F., Rens, W., Tsend-Ayush, E., El-Mogharbel, N., O'Brien, P. C. M., Jones, R. C., Ferguson-Smith, M. A., & Marshall Graves, J. A. (2004). In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature*, *432*(7019), 913–917. https://doi.org/10.1038/nature03021

Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R., & Hengartner, M. O. (1999). Genetic control of programmed cell death in the nematode *Caenorhabditis elegans* hermaphrodite germline. *Development*, *126*(5), 1011–1022.

Gupta, S. C., Sundaram, C., Reuter, S., & Aggarwal, B. B. (2010). Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, *1799*(10–12), 775–787. https://doi.org/10.1016/j.bbagrm.2010.05.004

Gutiérrez-Ospina, G., Jiménez-Trejo, F. J., Favila, R., Moreno-Mendoza, N. A., Granados Rojas, L., Barrios, F. A., Díaz-Cintra, S., & Merchant-Larios, H. (1999). Acetylcholinesterase-positive innervation is present at undifferentiated stages of the sea turtle *Lepidochelis olivacea* embryo gonads: Implications for temperature-dependent sex determination. *Journal of Comparative Neurology*, *410*(1), 90–98. https://doi.org/10.1002/(SICI)1096-9861(19990719)410:1<90::AID-CNE8>3.0.CO;2-7

Habibalahi, A., Moghari, M. D., Campbell, J. M., Anwer, A. G., Mahbub, S. B., Gosnell, M., Saad, S., Pollock, C., & Goldys, E. M. (2020). Non-invasive real-time imaging of reactive oxygen species (ROS) using auto-fluorescence multispectral imaging technique: A novel tool for redox biology. *Redox Biology*, *34*, 101561. https://doi.org/10.1016/j.redox.2020.101561

Haig, D. (1991). Developmental asynchrony and environmental sex determination in alligators. *Journal of Theoretical Biology*, *150*(3), 373–383. https://doi.org/https://doi.org/10.1016/S0022-5193(05)80435-7

Halliwell, B., & Gutteridge, J. M. C. (2015). *Free Radicals in Biology and Medicine*. Oxford University Press.

Haltenhof, T., Kotte, A., De Bortoli, F., Schiefer, S., Meinke, S., Emmerichs, A. K., Petermann, K. K., Timmermann, B., Imhof, P., Franz, A., Loll, B., Wahl, M. C., Preußner, M., & Heyd, F. (2020). A conserved kinase-based body-temperature sensor globally controls alternative splicing and gene expression. *Molecular Cell*, *78*(1), 57–69. https://doi.org/10.1016/j.molcel.2020.01.028

Hamanaka, R. B., & Chandel, N. S. (2010). Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends in Biochemical Sciences*, *35*(9), 505–513. https://doi.org/10.1016/j.tibs.2010.04.002

Hammond, C. L., Lee, T. K., & Ballatori, N. (2001). Novel roles for glutathione in gene expression, cell death, and membrane transport of organic solutes. *Journal of Hepatology*, *34*(6), 946–954. https://doi.org/10.1016/S0168-8278(01)00037-X

Hanover, J. A., Love, D. C., & Prinz, W. A. (2009). Calmodulin-driven nuclear entry: Trigger for sex determination and terminal differentiation. *Journal of Biological Chemistry*, *284*(19), 12593–12597. https://doi.org/10.1074/jbc.R800076200

Hansson, A., & Olsson, M. (2018). The influence of incubation temperature on phenotype of Australian Painted Dragons (*Ctenophorus pictus*). *Herpetologica*, *74*(2), 146–151. https://doi.org/10.1655/HERPETOLOGICA-D-17-00052.1

Hara, S., Sawamura, R., & Kitano, T. (2020). Cortisol induces masculinization of XX medaka through gonadal soma-derived growth factor (GSDF) and anti-Müllerian hormone receptor type 2 (AMHR2). *Fisheries Science*, *87*, 85–91. https://doi.org/10.1007/s12562-020-01479-1

Harlow, P. S., & Shine, R. (1999). Temperature-dependent sex determination in the frillneck lizard, *Chlamydosaurus kingii* (Agamidae). *Herpetologica*, *55*(2), 205–212. http://www.jstor.org/stable/3893081

Harry, J. L., Briscoe, D. A., & Williams, K. L. (1992). Putting the heat on sex determination. *Genetica*, *87*(1), 1–6. https://doi.org/10.1007/BF00128767

Harry, J. L., Williams, K. L., & Briscoe, D. A. (1990). Sex determination in loggerhead turtles: Differential expression of two hnRNP proteins. *Development*, *109*(2), 305–312.

Haslbeck, M., & Vierling, E. (2015). A first line of stress defense: Small heat shock proteins and their function in protein homeostasis. *Journal of Molecular Biology*, *427*(7), 1537–1548. https://doi.org/10.1016/j.jmb.2015.02.002

Hattori, A. (1991). Socially controlled growth and size-dependent sex change in the anemonefish *Amphiprion frenatus* in Okinawa, Japan. *Japanese Journal of Ichthyology*, *38*(2), 165–177.

Hattori, R. S., Castañeda-Cortés, D. C., Arias Padilla, L. F., Strobl-Mazzulla, P. H., & Fernandino, J. I. (2020). Activation of stress response axis as a key process in environment-induced sex plasticity in fish. *Cellular and Molecular Life Sciences*, *77*(21), 4223–4236. https://doi.org/10.1007/s00018-020-03532-9

Hattori, R. S., Fernandino, J. I., Kishii, A., Kimura, H., Kinno, T., Oura, M., Somoza, G. M., Yokota, M., Strüssmann, C. A., & Watanabe, S. (2009). Cortisol-induced masculinization: Does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? *PLoS ONE*, *4*(8), e6548. https://doi.org/10.1371/journal.pone.0006548

Hayasaka, O., Takeuchi, Y., Shiozaki, K., Anraku, K., & Kotani, T. (2019). Green light irradiation during sex differentiation induces female-to-male sex reversal in the medaka *Oryzias latipes*. *Scientific Reports*, *9*(1), 2383. https://doi.org/10.1038/s41598-019-38908-w

Hayashi, Y., Kobira, H., Yamaguchi, T., Shiraishi, E., Yazawa, T., Hirai, T., Kamei, Y., & Kitano, T. (2010). High temperature causes masculinization of genetically female medaka by elevation of cortisol. *Molecular Reproduction and Development*, *77*(8), 679–686. https://doi.org/10.1002/mrd.21203

Hays, G. C., Mazaris, A. D., Schofield, G., & Laloë, J. O. (2017). Population viability at extreme sex-ratio skews produced by temperature-dependent sex determination. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1848), 20162576. https://doi.org/10.1098/rspb.2016.2576

He, C., Larson-Casey, J. L., Gu, L., Ryan, A. J., Murthy, S., & Carter, A. B. (2016). Cu,Zn-superoxide dismutase-mediated redox regulation of Jumonji domain containing 3 modulates macrophage polarization and pulmonary fibrosis. *American Journal of*

Respiratory Cell and Molecular Biology, *55*(1), 58–71. https://doi.org/10.1165/rcmb.2015-0183OC

He, Y., Shang, X., Sun, J., Zhang, L., Zhao, W., Tian, Y., Cheng, H., & Zhou, R. (2009). Gonadal apoptosis during sex reversal of the rice field eel: Implications for an evolutionarily conserved role of the molecular chaperone heat shock protein 10. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *314*(4), 257–266. https://doi.org/10.1002/jez.b.21333

Herpin, A., & Schartl, M. (2015). Plasticity of gene-regulatory networks controlling sex determination: Of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Reports*, *16*(10), 1260–1274. https://doi.org/10.15252/embr.201540667

Hijmans, R. J. (2020). *Raster: Geographic data analysis and modeling* (R package version 3.0-12). R package version 3.0-12.

Hilton, J. K., Rath, P., Helsell, C. V. M., Beckstein, O., & Van Horn, W. D. (2015). Understanding thermosensitive transient receptor potential channels as versatile polymodal cellular sensors. *Biochemistry*, *54*(15), 2401–2413. https://doi.org/10.1021/acs.biochem.5b00071

Hirayama, J., Cho, S., & Sassone-Corsi, P. (2007). Circadian control by the reduction/oxidation pathway: Catalase represses light-dependent clock gene expression in the zebrafish. *Proceedings of the National Academy of Sciences*, *104*(40), 15747–15752. https://doi.org/10.1073/pnas.0705614104

Hoekstra, M. M. B., Emmenegger, Y., Hubbard, J., & Franken, P. (2019). Cold-inducible RNA-binding protein (CIRBP) adjusts clock-gene expression and REM-sleep recovery following sleep deprivation. *ELife*, *8*, 1–31. https://doi.org/10.7554/eLife.43400

Holleley, C. E., O'Meally, D., Sarre, S. D., Marshall Graves, J. A., 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*(7558), 79–82. https://doi.org/10.1038/nature14574

Holleley, C. E., Sarre, S. D., O'Meally, D., & Georges, A. (2016). Sex reversal in reptiles: Reproductive oddity or powerful driver of evolutionary change? *Sexual Development*,

10(5–6), 279–287. https://doi.org/10.1159/000450972

Holoch, D., & Margueron, R. (2017). Mechanisms regulating PRC2 recruitment and enzymatic activity. *Trends in Biochemical Sciences*, *42*(7), 531–542. https://doi.org/https://doi.org/10.1016/j.tibs.2017.04.003

Honeycutt, J. L., Deck, C. A., Miller, S. C., Severance, M. E., Atkins, E. B., Luckenbach, J. A., Buckel, J. A., Daniels, H. V., Rice, J. A., Borski, R. J., & Godwin, J. (2019). Warmer waters masculinize wild populations of a fish with temperature-dependent sex determination. *Scientific Reports*, *9*(1), 6527. https://doi.org/10.1038/s41598-019-42944-x

Hong, C. Y., Park, J. H., Seo, K. H., Kim, J.-M., Im, S. Y., Lee, J. W., Choi, H.-S., & Lee, K. (2003). Expression of MIS in the testis is downregulated by tumor necrosis factor alpha through the negative regulation of SF-1 transactivation by NF-κB. *Molecular and Cellular Biology*, *23*(17), 6000–6012. https://doi.org/10.1128/mcb.23.17.6000-6012.2003

Hörandl, E., & Speijer, D. (2018). How oxygen gave rise to eukaryotic sex. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1872), 20172706. https://doi.org/10.1098/rspb.2017.2706

Huelga, S. C., Vu, A. Q., Arnold, J. D., Liang, T. D., Liu, P. P., Yan, B. Y., Donohue, J. P., Shiue, L., Hoon, S., Brenner, S., Ares, M., & Yeo, G. W. (2012). Integrative genome-wide analysis reveals cooperative regulation of alternative splicing by hnRNP proteins. *Cell Reports*, *1*(2), 167–178. https://doi.org/10.1016/j.celrep.2012.02.001

Hutchinson, M. F., Stein, J. L. A., Stein, J. L. A., Anderson, H., & Tickle, P. K. (2008). *GEODATA 9 Second DEM and D8 Digital Elevation Model Version 3 and Flow Direction Grid*. Geoscience Australia. http://pid.geoscience.gov.au/dataset/ga/66006

Ikwegbue, P., Masamba, P., Oyinloye, B., & Kappo, A. (2018). Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. *Pharmaceuticals*, *11*(1), 2. https://doi.org/10.3390/ph11010002

Inouye, S., Fujimoto, M., Nakamura, T., Takaki, E., Hayashida, N., Hai, T., & Nakai, A. (2007). Heat shock transcription factor 1 opens chromatin structure of interleukin-6

promoter to facilitate binding of an activator or a repressor. *Journal of Biological Chemistry*, *282*(45), 33210–33217. https://doi.org/10.1074/jbc.M704471200

IPCC. (2013). Climate Change 2013: The Physical Science Basis. Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. In *Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA*. https://doi.org/10.1029/2001JD001516

Iungman, J. L., Somoza, G. M., & Piña, C. I. (2015). Are stress-related hormones involved in the temperature-dependent sex determination of the broad-snouted caiman? *South American Journal of Herpetology*, *10*(1), 41–49. https://doi.org/10.2994/SAJH-D-14-00027.1

Janes, D. E., Bermudez, D., Guillette, L. J. J., & Wayne, M. L. (2007). Estrogens induced male production at a female-producing temperature in a reptile (Leopard Gecko, *Eublepharis macularis*) with temperature-dependent sex determination. *Journal of Herpetology*, *41*(1), 9–15. https://doi.org/10.1670/0022-1511(2007)41[9:EIMPAA]2.0.CO;2

Janes, D. E., Organ, C. L., & Edwards, S. V. (2010). Variability in sex-determining mechanisms influences genome complexity in reptilia. *Cytogenetic and Genome Research*, *127*(2–4), 242–248. https://doi.org/10.1159/000293283

Janes, D. E., Organ, C. L., Stiglec, R., O'Meally, D., Sarre, S. D., Georges, A., Graves, J. A. M., Valenzuela, N., Literman, R. A., Rutherford, K., Gemmell, N., Iverson, J. B., Tamplin, J. W., Edwards, S. V, & Ezaz, T. (2014). Molecular evolution of *Dmrt1* accompanies change of sex-determining mechanisms in reptilia. *Biology Letters*, *10*(12), 20140809. https://doi.org/10.1098/rsbl.2014.0809

Jeffrey, S. J., Carter, J. O., Moodie, K. B., & Beswick, A. R. (2001). Using spatial interpolation to construct a comprehensive archive of Australian climate data. *Environmental Modelling and Software*, *16*(4), 309–330. https://doi.org/10.1016/S1364-8152(01)00008-1

Jenkins, S. A., & Porter, T. E. (2004). Ontogeny of the hypothalamo-pituitary-adrenocortical axis in the chicken embryo: A review. *Domestic Animal Endocrinology*, *26*(4), 267–275. https://doi.org/10.1016/j.domaniend.2004.01.001

Jensen, M. P., Allen, C. D., Eguchi, T., Bell, I. P., LaCasella, E. L., Hilton, W. A., Hof, C. A. M., & Dutton, P. H. (2018). Environmental warming and feminization of one of the largest sea turtle populations in the world. *Current Biology*, *28*(1), 154–159. https://doi.org/10.1016/j.cub.2017.11.057

Jeyasuria, P., & Place, A. R. (1998). Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: A role for P450 aromatase (CYP19). *Journal of Experimental Zoology*, *281*(5), 428–449. https://doi.org/10.1002/(SICI)1097-010X(19980801)281:5<428::AID-JEZ8>3.0.CO;2-Q

Johnson, J. D., & Chang, J. P. (2002). Agonist-specific and sexual stage-dependent inhibition of gonadotropin-releasing hormone-stimulated gonadotropin and growth hormone release by ryanodine: Relationship to sexual stage-dependent caffeine-sensitive hormone release. *Journal of Neuroendocrinology*, *14*(2), 144–155. https://doi.org/10.1046/j.0007-1331.2001.00756.x

Johnson, N. S., Swink, W. D., & Brenden, T. O. (2017). Field study suggests that sex determination in sea lamprey is directly influenced by larval growth rate. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1851), 20170262. https://doi.org/10.1098/rspb.2017.0262

Jombart, T. (2008). *adegenet*: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Josso, N., & di Clemente, N. (2003). Transduction pathway of anti-Müllerian hormone, a sex-specific member of the TGF-β family. *Trends in Endocrinology & Metabolism*, *14*(2), 91–97. https://doi.org/10.1016/S1043-2760(03)00005-5

Kalin, N. H. (2018). Corticotropin-releasing hormone binding protein: Stress, psychopathology, and antidepressant treatment response. *American Journal of Psychiatry*, *175*(3), 204–206. https://doi.org/10.1176/appi.ajp.2018.18010059

Kawai, A., Nishida-Umehara, C., Ishijima, J., Tsuda, Y., Ota, H., & Matsuda, Y. (2007). Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenetics and Genome Research*, *117*(1-4), 92-102. https://doi.org/10.1159/000103169

Kay, I. (1998). *Introduction to animal physiology*. BIOS Scientific Publishers Ltd.

Kearney, M. R. (2020). *NicheMapR: Implementation of Niche Mapper software for biophysical modelling* (R Package Version 2.0.0). R Package Version 2.0.0.

Kearney, M. R., & Porter, W. P. (2017). NicheMapR - An R package for biophysical modelling: The microclimate model. *Ecography*, *40*(5), 664–674.

Keil, K. P., Mehta, V., Branam, A. M., Abler, L. L., Buresh-Stiemke, R. A., Joshi, P. S., Schmitz, C. T., Marker, P. C., & Vezina, C. M. (2012). Wnt inhibitory factor 1 (*Wif1*) is regulated by androgens and enhances androgen-dependent prostate development. *Endocrinology*, *153*(12), 6091–6103. https://doi.org/10.1210/en.2012-1564

Kietzmann, T., Petry, A., Shvetsova, A., Gerhold, J. M., & Görlach, A. (2017). The epigenetic landscape related to reactive oxygen species formation in the cardiovascular system. *British Journal of Pharmacology*, *174*(12), 1533–1554. https://doi.org/10.1111/bph.13792

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. *Methods in Molecular Biology*, *888*, 67–89. https://doi.org/10.1007/978-1-61779-870-2_5

Kim, D. H., Doyle, M. R., Sung, S., & Amasino, R. M. (2009). Vernalization: Winter and the timing of flowering in plants. *Annual Review of Cell and Developmental Biology*, *25*, 277–299. https://doi.org/10.1146/annurev.cellbio.042308.113411

Kim, H. J., Lee, J. J., Cho, J. H., Jeong, J., Park, A. Y., Kang, W., & Lee, K. J. (2017). Heterogeneous nuclear ribonucleoprotein K inhibits heat shock-induced transcriptional activity of heat shock factor 1. *Journal of Biological Chemistry*, *292*(31), 12801–12812. https://doi.org/10.1074/jbc.M117.774992

Kim, S., & Jung, I. (2017). Optimizing the maximum reported cluster size in the spatial scan statistic for ordinal data. *PLoS ONE*, *12*(7), e0182234. https://doi.org/10.1371/journal.pone.0182234

Kimura, M. (1953). “Stepping stone” model of population. *Annual Report of the National Institute of Genetics Japan*, *3*, 62–63.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., & Abe, S. I. (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). *Journal of Molecular Endocrinology*, *23*(2), 167–176. https://doi.org/10.1677/jme.0.0230167

Kobayashi, M., Li, L., Iwamoto, N., Nakajima-Takagi, Y., Kaneko, H., Nakayama, Y., Eguchi, M., Wada, Y., Kumagai, Y., & Yamamoto, M. (2009). The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Molecular and Cellular Biology*, *29*(2), 493–502. https://doi.org/10.1128/mcb.01080-08

Koch, R. E., & Hill, G. E. (2017). An assessment of techniques to manipulate oxidative stress in animals. *Functional Ecology*, *31*(1), 9–21. https://doi.org/10.1111/1365-2435.12664

Kohno, S., Katsu, Y., Urushitani, H., Ohta, Y., Iguchi, T., & Guillette, L. J. (2010). Potential contributions of heat shock proteins to temperature-dependent sex determination in the American alligator. *Sexual Development*, *4*(1–2), 73–87. https://doi.org/10.1159/000260374

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature*, *351*(6322), 117–121. https://doi.org/10.1038/351117a0

Koopman, P., Münsterberg, A., Capel, B., Vivian, N., & Lovell-Badge, R. (1990). Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature*, *348*(6300), 450–452. https://doi.org/10.1038/348450a0

Kostmann, A., Kratochvíl, L., & Rovatsos, M. (2021). Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation. *Proceedings of the Royal Society B: Biological Sciences 288*(1943), 20202139. 10.1098/rspb.2020.2139

Kozai, D., Ogawa, N., & Mori, Y. (2013). Redox regulation of transient receptor potential channels. *Antioxidants & Redox Signaling*, *21*(6), 971–986.

https://doi.org/10.1089/ars.2013.5616

Kraak, S. B. M., & De Looze, E. M. A. (1992). A new hypothesis on the evolution of sex determination in vertebrates; Big females ZW, big males XY. *Netherlands Journal of Zoology*, *43*(3–4), 260–273. https://doi.org/10.1163/156854293X00034

Kumar, A., Kumari, S., Majhi, R. K., Swain, N., Yadav, M., & Goswami, C. (2015). Regulation of TRP channels by steroids: Implications in physiology and diseases. *General and Comparative Endocrinology*, *220*, 23–32. https://doi.org/10.1016/j.ygcen.2014.10.004

Kuntz, S., Chesnel, A., Flament, S., & Chardard, D. (2004). Cerebral and gonadal aromatase expressions are differently affected during sex differentiation of *Pleurodeles waltl*. *Journal of Molecular Endocrinology*, *33*(3), 717–727. https://doi.org/10.1677/jme.1.01588

Kuwabara, P. E., & Perry, M. D. (2001). It ain't over till it's ova: Germline sex determination in *C. elegans*. *BioEssays*, *23*(7), 596–604. https://doi.org/10.1002/bies.1085

Lambert, M. R., Smylie, M. S., Roman, A. J., Freidenburg, L. K., & Skelly, D. K. (2018). Sexual and somatic development of wood frog tadpoles along a thermal gradient. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *329*(2), 72–79. https://doi.org/10.1002/jez.2172

Lance, V. A. (1997). Sex determination in reptiles: An update. *American Zoologist*, *37*(6), 504–513. http://www.jstor.org/stable/3884126

Lance, V. A. (2009). Is regulation of aromatase expression in reptiles the key to understanding temperature-dependent sex determination? *Journal of Experimental Zoology Part A - Ecological and Integrative Physiology*, *311A*(5), 314–322. https://doi.org/10.1002/jez.465

Lance, V. A., & Bogart, M. H. (1992). Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *General and Comparative Endocrinology*, *86*(1), 59–71. https://doi.org/10.1016/0016-6480(92)90126-5

Lance, V. A., & Bogart, M. H. (1994). Studies on sex determination in the American alligator

Alligator mississippiensis. *Journal of Experimental Zoology*, *270*(1), 79–85. https://doi.org/https://doi.org/10.1002/jez.1402700109

Lavi, R., Shainberg, A., Friedmann, H., Shneyvays, V., Rickover, O., Eichler, M., Kaplan, D., & Lubart, R. (2003). Low energy visible light induces reactive oxygen species generation and stimulates an increase of intracellular calcium concentration in cardiac cells. *Journal of Biological Chemistry*, *278*(42), 40917–40922. https://doi.org/10.1074/jbc.M303034200

Li, H., Holleley, C. E., Elphick, M., Georges, A., & Shine, R. (2016). The behavioural consequences of sex reversal in dragons. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1832), 20160217. https://doi.org/10.1098/rspb.2016.0217

Lin, J. Q., Zhou, Q., Yang, H. Q., Fang, L. M., Tang, K. Y., Sun, L., Wan, Q. H., & Fang, S. G. (2018). Molecular mechanism of temperature-dependent sex determination and differentiation in Chinese alligator revealed by developmental transcriptome profiling. *Science Bulletin*, *63*(4), 209–212. https://doi.org/10.1016/j.scib.2018.01.004

Lind, M. I., & Spagopoulou, F. (2018). Evolutionary consequences of epigenetic inheritance. *Heredity*, *121*(3), 205–209. https://doi.org/10.1038/s41437-018-0113-y

Liu, H., Lamm, M. S., Rutherford, K., Black, M. A., Godwin, J. R., & Gemmell, N. J. (2015). Large-scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biology of Sex Differences*, *6*(1), 26. https://doi.org/10.1186/s13293-015-0044-8

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., & Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: An evolutionarily conserved mechanism. *Cellular and Molecular Life Sciences*, *73*(17), 3221–3247. https://doi.org/10.1007/s00018-016-2223-0

Lovern, M. B., & Wade, J. (2003). Yolk testosterone varies with sex in eggs of the lizard, *Anolis carolinensis*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *295A*(2), 206–210. https://doi.org/10.1002/jez.a.10225

Magnone, M. C., Jacobmeier, B., Bertolucci, C., Foà, A., & Albrecht, U. (2005). Circadian expression of the clock gene *Per2* is altered in the ruin lizard (*Podarcis sicula*) when

temperature changes. *Molecular Brain Research*, *133*(2), 281–285. https://doi.org/10.1016/j.molbrainres.2004.10.014

Major, A. T., & Smith, C. A. (2016). Sex reversal in birds. *Sexual Development*, *10*(5–6), 288–300. https://doi.org/10.1159/000448365

Malki, S., Berta, P., Poulat, F., & Boizet-Bonhoure, B. (2005). Cytoplasmic retention of the sex-determining factor SOX9 via the microtubule network. *Experimental Cell Research*, *309*(2), 468–475. https://doi.org/10.1016/j.yexcr.2005.07.005

Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Méjean, C., Berta, P., Poulat, F., & Boizet-Bonhoure, B. (2005). Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *EMBO Journal*, *24*(10), 1798–1809. https://doi.org/10.1038/sj.emboj.7600660

Mankiewicz, J. L., Godwin, J., Holler, B. L., Turner, P. M., Murashige, R., Shamey, R., Daniels, H. V., & Borski, R. J. (2013). Masculinizing effect of background color and cortisol in a flatfish with environmental sex-determination. *Integrative and Comparative Biology*, *53*(4), 755–765. https://doi.org/10.1093/icb/ict093

Marasca, F., Bodega, B., & Orlando, V. (2018). How polycomb-mediated cell memory deals with a changing environment: Variations in PcG complexes and proteins assortment convey plasticity to epigenetic regulation as a response to environment. *BioEssays*, *40*(4), e1700137. https://doi.org/10.1002/bies.201700137

Marcó, M. V. P., Piña, C. I., Somoza, G. M., Jahn, G. A., Pietrobon, E. O., & Iungman, J. L. (2015). Corticosterone plasma levels of embryo and hatchling broad-snouted caimans (*Caiman latirostris*) incubated at different temperatures. *South American Journal of Herpetology*, *10*(1), 50–57. https://doi.org/10.2994/sajh-d-14-00026.1

Marshall Graves, J. A. (2013). How to evolve new vertebrate sex determining genes. *Developmental Dynamics*, *242*(4), 354–359. https://doi.org/10.1002/dvdy.23887

Marshall Graves, J. A., & Shetty, S. (2001). Sex from W to Z: Evolution of vertebrate sex chromosomes and sex determining genes. *Journal of Experimental Zoology*, *290*(5), 449–462. https://doi.org/10.1002/jez.1088

Martindale, J. L., & Holbrook, N. J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. *Journal of Cellular Physiology*, *192*(1), 1–15. https://doi.org/10.1002/jcp.10119

Materazzi, S., Fusi, C., Benemei, S., Pedretti, P., Patacchini, R., Nilius, B., Prenen, J., Creminon, C., Geppetti, P., & Nassini, R. (2012). TRPA1 and TRPV4 mediate paclitaxel-induced peripheral neuropathy in mice via a glutathione-sensitive mechanism. *Pflugers Archiv European Journal of Physiology*, *463*(4), 561–569. https://doi.org/10.1007/s00424-011-1071-x

Matsumoto, Y., Hannigan, B., & Crews, D. (2016). Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (*cyp19a1*) gene in species with temperature-dependent sex determination. *PLoS One*, *11*(11), e0167362. https://doi.org/10.1371/journal.pone.0167362

Matthew Michael, W., Choi, M., & Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: A signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, *83*(3), 415–422. https://doi.org/10.1016/0092-8674(95)90119-1

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M., & Slonczewski, J. L. (2005). pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. *Journal of Bacteriology*, *187*(1), 304–319. https://doi.org/10.1128/JB.187.1.304-319.2005

Maynard Case, R., Eisner, D., Gurney, A., Jones, O., Muallem, S., & Verkhratsky, A. (2007). Evolution of calcium homeostasis: From birth of the first cell to an omnipresent signalling system. *Cell Calcium*, *42*(4–5), 345–350. https://doi.org/10.1016/j.ceca.2007.05.001

Mayor, R., Izquierdo-Bouldstridge, A., Millán-Ariño, L., Bustillos, A., Sampaio, C., Luque, N., & Jordan, A. (2015). Genome distribution of replication-independent histone H1 variants shows H1.0 associated with nucleolar domains and H1X associated with RNA polymerase II-enriched regions. *Journal of Biological Chemistry*, *290*(12), 7474–7491. https://doi.org/10.1074/jbc.M114.617324

McGaugh, S. E., Bowden, R. M., Kuo, C. H., & Janzen, F. J. (2011). Field-measured heritability of the threshold for sex determination in a turtle with temperature-dependent

sex determination. *Evolutionary Ecology Research*, *13*(1), 75–90.

Mclaren, G. W., Macdonald, D. W., Georgiou, C., Mathews, F., Newman, C., & Mian, R. (2003). Leukocyte coping capacity: A novel technique for measuring the stress response in vertebrates. *Experimental Physiology*, *88*(4), 541–546. https://doi.org/10.1113/eph8802571

McVicar, T. R. (2011). *Near-surface wind speed v10*. CSIRO. https://doi.org/10.25919/5c5106acbcb02

McVicar, T. R., Van Niel, T. G., Li, L. T., Roderick, M. L., Rayner, D. P., Ricciardulli, L., & Donohue, R. J. (2008). Wind speed climatology and trends for Australia, 1975-2006: Capturing the stilling phenomenon and comparison with near-surface reanalysis output. *Geophysical Research Letters*, *35*(20), L20403. https://doi.org/10.1029/2008GL035627

Medler, K. F., & Lance, V. A. (1998). Sex differences in plasma corticosterone levels in alligator (*Alligator mississippiensis*) embryos. *Journal of Experimental Zoology*, *280*(3), 238–244. https://doi.org/10.1002/(SICI)1097-010X(19980215)280:3<238::AID-JEZ5>3.0.CO;2-O

Melville, J., Haines, M. L., Boysen, K., Hodkinson, L., Kilian, A., Date, K. L. S., Potvin, D. A., & Parris, K. M. (2017). Identifying hybridization and admixture using SNPs: Application of the DArTseq platform in phylogeographic research on vertebrates. *Royal Society Open Science*, *4*(7), 161061. https://doi.org/10.1098/rsos.161061

Melville, J., Schulte, J. A., & Larson, A. (2001). A molecular phylogenetic study of ecological diversification in the Australian lizard genus *Ctenophorus*. *Journal of Experimental Zoology*, *291*(4), 339–353. https://doi.org/10.1002/jez.1133

Menon, J., & Rozman, R. (2007). Oxidative stress, tissue remodeling and regression during amphibian metamorphosis. *Comparative Biochemistry and Physiology Part C - Toxicology & Pharmacology*, *145*(4), 625–631. https://doi.org/10.1016/j.cbpc.2007.02.011

Merchant-Larios, H., & Villalpando, I. (1990). Effect of temperature on gonadal sex differentiation in the sea turtle *Lepidochelys olivacea*: An organ culture study. *Journal of Experimental Zoology*, *254*(3), 327–331.

Merchant-Larios, H., Fierro, I. V., & Urruiza, B. C. (1989). Gonadal morphogenesis under controlled temperature in the sea turtle *Lepidochelys olivacea*. *Herpetological Monographs*, *3*, 43–61. https://doi.org/https://doi.org/10.2307/1466985

Metcalfe, N. B., & Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: The role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology*, *24*(5), 984–996. https://doi.org/10.1111/j.1365-2435.2010.01750.x

Miller, K. A., Kenter, L. W., Breton, T. S., & Berlinsky, D. L. (2019). The effects of stress, cortisol administration and cortisol inhibition on black sea bass (Centropristis striata) sex differentiation. *Comparative Biochemistry and Physiology Part A - Molecular and Integrative Physiology*, *227*, 154–160. https://doi.org/10.1016/j.cbpa.2018.10.009

Milnes, M. R., Roberts, R. N., & Guillette, L. J. (2002). Effects of incubation temperature and estrogen exposure on aromatase activity in the brain and gonads of embryonic alligators. *Environmental Health Perspectives*, *110*(SUPPL. 3), 393–396. https://doi.org/10.1289/ehp.02110s3393

Mitchell, N. J., Allendorf, F. W., Keall, S. N., Daugherty, C. H., & Nelson, N. J. (2010). Demographic effects of temperature-dependent sex determination: Will tuatara survive global warming? *Global Change Biology*, *16*(1), 60–72. https://doi.org/10.1111/j.1365-2486.2009.01964.x

Mitchell, N. J., & Janzen, F. J. (2010). Temperature-dependent sex determination and contemporary climate change. *Sexual Development*, *4*(1–2), 129–140. https://doi.org/10.1159/000282494

Mittwoch, U. (1989). Sex differentiation in mammals and tempo of growth: Probabilities vs. switches. *Journal of Theoretical Biology*, *137*(4), 445–455. https://doi.org/10.1016/S0022-5193(89)80039-6

Mittwoch, U. (2000). Three thousand years of questioning sex determination. *Cytogenetic and Genome Research*, *91*(1–4), 186–191. https://doi.org/10.1159/000056842

Mittwoch, U. (2004). The elusive action of sex-determining genes: Mitochondria to the rescue? *Journal of Theoretical Biology*, *228*(3), 359–365. https://doi.org/10.1016/j.jtbi.2004.02.001

Mittwoch, U. (2013). Sex determination. *EMBO Reports*, *14*(7), 588–592. https://doi.org/10.1038/embor.2013.84

Moore, I. T., & Jessop, T. S. (2003). Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Hormones and Behavior*, *43*(1), 39–47. https://doi.org/10.1016/S0018-506X(02)00038-7

Moore, M. C., & Johnston, G. I. H. (2008). Toward a dynamic model of deposition and utilization of yolk steroids. *Integrative and Comparative Biology*, *48*(3), 411–418. https://doi.org/10.1093/icb/icn079

Moreno-Mendoza, N., Harley, V. R., & Merchant-Larios, H. (2001). Temperature regulates SOX9 expression in cultured gonads of *Lepidochelys olivacea*, a species with temperature sex determination. *Developmental Biology*, *229*(2), 319–326. https://doi.org/10.1006/dbio.2000.9952

Morgan, M. J., & Liu, Z. (2011). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research*, *21*(1), 103–115. https://doi.org/10.1038/cr.2010.178

Morjan, C. L. (2003). How rapidly can maternal behavior affecting primary sex ratio evolve in a reptile with environmental sex determination? *American Naturalist*, *162*(2), 205–219. https://doi.org/10.1086/376583

Mork, L., Czerwinski, M., & Capel, B. (2014). Predetermination of sexual fate in a turtle with temperature-dependent sex determination. *Developmental Biology*, *386*(1), 264–271. https://doi.org/10.1016/j.ydbio.2013.11.026

Morrish, B. C., & Sinclair, A. H. (2002). Vertebrate sex determination: Many means to an end. *Reproduction*, *124*(4), 447–457. https://doi.org/10.1530/rep.0.1240447

Nakshatri, H., Appaiah, H. N., Anjanappa, M., Gilley, D., Tanaka, H., Badve, S., Crooks, P. A., Mathews, W., Sweeney, C., & Bhat-Nakshatri, P. (2015). Nf-kB-dependent and -independent epigenetic modulation using the novel anti-cancer agent DMAPT. *Cell Death and Disease*, *6*, e1608. https://doi.org/10.1038/cddis.2014.569

Narayan, E. J. (2017). Evaluation of physiological stress in Australian wildlife: Embracing pioneering and current knowledge as a guide to future research directions. *General and*

Comparative Endocrinology, *244*, 30–39. https://doi.org/10.1016/j.ygcen.2015.12.008

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., & Piferrer, F. (2011). DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European Sea Bass. *PLoS Genetics*, *7*(12), e1002447. https://doi.org/10.1371/journal.pgen.1002447

Nedelcu, A. M., Marcu, O., & Michod, R. E. (2004). Sex as a response to oxidative stress: A twofold increase in cellular reactive oxygen species activates sex genes. *Proceedings of the Royal Society B: Biological Sciences*, *271*(1548), 1591–1596. https://doi.org/10.1098/rspb.2004.2747

Nedelcu, A. M., & Michod, R. E. (2003). Sex as a response to oxidative stress: The effect of antioxidants on sexual induction in a facultatively sexual lineage. *Proceedings of the Royal Society B: Biological Sciences*, *270*, S136–S139. https://doi.org/10.1098/rsbl.2003.0062

Nelson, D. E., Ihekwaba, A. E. C., Elliott, M., Johnson, J. R., Gibney, C. A., Foreman, B. E., Nelson, C., See, V., Horton, C. A., Spiller, D. G., Edwards, S. W., McDowell, H. P., Unitt, J. F., Sullivan, E., Grimley, R., Benson, N., Broomhead, D., Kell, D. B., & White, M. R. H. (2004). Oscillations in NF-κB signaling control the dynamics of gene expression. *Science*, *306*(5696), 704–708. https://doi.org/10.1126/science.1099962

Nguyen, T., Nioi, P., & Pickett, C. B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *Journal of Biological Chemistry*, *284*(20), 13291–13295. https://doi.org/10.1074/jbc.R900010200

Niu, Y., Desmarais, T. L., Tong, Z., Yao, Y., Costa, M., Hospital, B. C., Medical, C., Hospital, B. C., & Gastroenterol, C. O. (2015). Oxidative stress alters global histone modification and DNA methylation. *Free Radical Biology and Medicine*, *82*, 22–28. https://doi.org/10.1016/j.freeradbiomed.2015.01.028.Oxidative

Nomura, T., Yamashita, W., Gotoh, H., & Ono, K. (2015). Genetic manipulation of reptilian embryos: Toward an understanding of cortical development and evolution. *Frontiers in Neuroscience*, *9*. https://doi.org/10.3389/fnins.2015.00045

Norberg, B., Brown, C. L., Halldorsson, O., Stensland, K., & Björnsson, B. T. (2004).

Photoperiod regulates the timing of sexual maturation, spawning, sex steroid and thyroid hormone profiles in the Atlantic cod (*Gadus morhua*). *Aquaculture*, *229*(1–4), 451–467. https://doi.org/10.1016/S0044-8486(03)00393-4

Norimoto, H., Fenk, L. A., Li, H. H., Tosches, M. A., Gallego-Flores, T., Hain, D., Reiter, S., Kobayashi, R., Macias, A., Arends, A., Klinkmann, M., & Laurent, G. (2020). A claustrum in reptiles and its role in slow-wave sleep. *Nature*, *578*(7795), 413–418. https://doi.org/10.1038/s41586-020-1993-6

Ogawa, N., Kurokawa, T., Fujiwara, K., Polat, O. K., Badr, H., Takahashi, N., & Mori, Y. (2016). Functional and structural divergence in human TRPV1 channel subunits by oxidative cysteine modification. *Journal of Biological Chemistry*, *291*(8), 4197–4210. https://doi.org/10.1074/jbc.M115.700278

Ogawa, N., Kurokawa, T., & Mori, Y. (2016). Sensing of redox status by TRP channels. *Cell Calcium*, *60*(2), 115–122. https://doi.org/10.1016/j.ceca.2016.02.009

Olaso, G., Capdevila, M., Gimeno, A., Pérez-Quilis, C., Hake, S. B., Bönisch, C., Viña, J., García-Giménez, J. L., Dasí, F., Palacios, Ò., Wiedemann, S. M., Pallardó, F. V., & Markovic, J. (2013). Histone H3 glutathionylation in proliferating mammalian cells destabilizes nucleosomal structure. *Antioxidants and Redox Signaling*, *19*(12), 1305–1320. https://doi.org/10.1089/ars.2012.5021

Ospina-Álvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLoS ONE*, *3*(7), e2837. https://doi.org/10.1371/journal.pone.0002837

Padgett, D. A., & Glaser, R. (2003). How stress influences the immune response. *Trends in Immunology*, *24*(8), 444–448. https://doi.org/10.1016/S1471-4906(03)00173-X

Paitz, R. T., & Bowden, R. M. (2008). A proposed role of the sulfotransferase/sulfatase pathway in modulating yolk steroid effects. *Integrative and Comparative Biology*, *48*(3), 419–427. https://doi.org/10.1093/icb/icn034

Papadakis, M., Tsagris, M., Dimitriadis, M., Fafalios, S., Tsamardinos, I., Fasiolo, M., Borboudakis, G., Burkhardt, J., Zou, C., Lakiotaki, K., & Chatzipantsiou, C. (2020). *Rfast: A collection of efficient and extremely fast R functions* (R Package Version 1.9.9).

Paredes, U., Radersma, R., Cannell, N., While, G. M., & Uller, T. (2016). Low incubation temperature induces DNA hypomethylation in lizard brains. *Journal of Experimental Zoology Part A - Ecological and Integrative Physiology*, *325*(6), 390–395. https://doi.org/10.1002/jez.2024

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, *421*, 37–42. https://doi.org/10.1038/nature01286

Parrott, B. B., Kohno, S., Cloy-McCoy, J. A., & Guillette, L. J. Jr. (2014). Differential incubation temperatures result in dimorphic DNA methylation patterning of the *SOX9* and aromatase promoters in gonads of alligator (*Alligator mississippiensis*) embryos. *Biology of Reproduction*, *90*(1), 1-11. https://doi.org/10.1095/biolreprod.113.111468

Pebesma, E. J., & Bivand, R. S. (2005). Classes and methods for spatial data in R. *R News*, *5*(2).

Peden, E., Kimberly, E., Gengyo-Ando, K., Mitani, S., & Xue, D. (2007). Control of sex-specific apoptosis in *C. elegans* by the BarH homeodomain protein CEH-30 and the transcriptional repressor UNC-37/Groucho. *Genes and Development*, *21*(23), 3195–3207. https://doi.org/10.1101/gad.1607807

Peng, J. C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., & Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. *Cell*, *139*(7), 1290–1302. https://doi.org/10.1016/j.cell.2009.12.002

Peng, Y., Yang, P. H., Tanner, J. A., Huang, J. D., Li, M., Lee, H. F., Xu, R. H., Kung, H. F., & Lin, M. C. M. (2006). Cold-inducible RNA binding protein is required for the expression of adhesion molecules and embryonic cell movement in *Xenopus laevis*. *Biochemical and Biophysical Research Communications*, *344*(1), 416–424. https://doi.org/10.1016/j.bbrc.2006.03.086

Pennell, M. W., Mank, J. E., & Peichel, C. L. (2018). Transitions in sex determination and sex chromosomes across vertebrate species. *Molecular Ecology*, *27*(19), 3950–3963. https://doi.org/10.1111/mec.14540

Perry, J. L., Ramachandran, N. K., Utama, B., & Hyser, J. M. (2015). Use of genetically-

encoded calcium indicators for live cell calcium imaging and localization in virus-infected cells. *Methods*, *90*, 28–38. https://doi.org/10.1016/j.ymeth.2015.09.004.Use

Persson, P., Sundell, K., Björnsson, B. T., & Lundqvist, H. (1998). Calcium metabolism and osmoregulation during sexual maturation of river running Atlantic salmon. *Journal of Fish Biology*, *52*(2), 334–349. https://doi.org/10.1111/j.1095-8649.1998.tb00801.x

Pezaro, N., Doody, J. S., & Thompson, M. B. (2017). The ecology and evolution of temperature-dependent reaction norms for sex determination in reptiles: A mechanistic conceptual model. *Biological Reviews*, *92*(3), 1348–1364. https://doi.org/10.1111/brv.12285

Phuge, S. K. (2017). High temperatures influence sexual development differentially in male and female tadpoles of the Indian skipper frog, *Euphlyctis cyanophlyctis*. *Journal of Biosciences*, *42*(3), 449–457. https://doi.org/10.1007/s12038-017-9689-2

Pianka, E. R. (2005). Zur Okologie und Naturgeschichte der Zwergbartagame (*Pogona minor*) in der Great Victoria Desert (Australien). *Draco*, 63–66. https://doi.org/10.1017/CBO9781107415324.004

Pieau, C., Dorizzi, M., & Richard-Mercier, N. (1999). Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cellular and Molecular Life Sciences*, *55*(6–7), 887–900. https://doi.org/10.1007/s000180050342

Piferrer, F. (2013). Epigenetics of sex determination and gonadogenesis. *Developmental Dynamics*, *242*(4), 360–370. https://doi.org/10.1002/dvdy.23924

Pisarska, M. D., Barlow, G., & Kuo, F.-T. (2011). Minireview: Roles of the forkhead transcription factor FOXL2 in granulosa cell biology and pathology. *Endocrinology*, *152*(4), 1199–1208. https://doi.org/10.1210/en.2010-1041

Piza-Roca, C., Strickland, K., Schoeman, D., & Frere, C. H. (2018). Eastern water dragons modify their social tactics with respect to the location within their home range. *Animal Behaviour*, *144*, 27–36. https://doi.org/10.1016/j.anbehav.2018.08.001

Place, A. R., Lang, J., Gavasso, S., & Jeyasuria, P. (2001). Expression of $P450_{arom}$ in *Malaclemys terrapin* and *Chelydra serpentina*: A tale of two sites. *Journal of*

Experimental Zoology, *290*(7), 673–690. https://doi.org/10.1002/jez.1118

Plattner, H., & Verkhratsky, A. (2015). The ancient roots of calcium signalling evolutionary tree. *Cell Calcium*, *57*(3), 123–132. https://doi.org/10.1016/j.ceca.2014.12.004

Pokorná, M., & Kratochvíl, L. (2009). Phylogeny of sex-determining mechanisms in squamate reptiles: Are sex chromosomes an evolutionary trap? *Zoological Journal of the Linnean Society*, *156*(1), 168–183. https://doi.org/10.1111/j.1096-3642.2008.00481.x

Pradhan, A., Khalaf, H., Ochsner, S. A., Sreenivasan, R., Koskinen, J., Karlsson, M., Karlsson, J., McKenna, N. J., Orbán, L., & Olsson, P. E. (2012). Activation of NF-κB protein prevents the transition from juvenile ovary to testis and promotes ovarian development in zebrafish. *Journal of Biological Chemistry*, *287*(45), 37926–37938. https://doi.org/10.1074/jbc.M112.386284

Quinn, A. E., Ezaz, T., Sarre, S. D., Marshall Graves, J. A., & Georges, A. (2010). Extension, single-locus conversion and physical mapping of sex chromosome sequences identify the Z microchromosome and pseudo-autosomal region in a dragon lizard, *Pogona vitticeps*. *Heredity*, *104*(4), 410–417. https://doi.org/10.1038/hdy.2009.133

Quinn, A. E., Georges, A., Sarre, S. D., Guarino, F., Ezaz, T., & Marshall Graves, J. A. (2007). Temperature sex reversal implies sex gene dosage in a reptile. *Science*, *316*(5823), 411–411. https://doi.org/10.1126/science.1135925

Quinn, A. E., Sarre, S. D., Ezaz, T., Marshall Graves, J. A., & Georges, A. (2011). Evolutionary transitions between mechanisms of sex determination in vertebrates. *Biology Letters*, *7*(3), 443–448. https://doi.org/10.1098/rsbl.2010.1126

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Radder, R. S., Pike, D. A., Quinn, A. E., & Shine, R. (2009). Offspring sex in a lizard depends on egg size. *Current Biology*, *19*(13), 1102–1105. https://doi.org/10.1016/j.cub.2009.05.027

Radder, R. S., Quinn, A. E., Georges, A., Sarre, S. D., & Shine, R. (2008). Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard.

Biology Letters, *4*(2), 176–178. https://doi.org/10.1098/rsbl.2007.0583

Radhakrishnan, S., Literman, R., Neuwald, J., Severin, A., & Valenzuela, N. (2017). Transcriptomic responses to environmental temperature by turtles with temperature-dependent and genotypic sex determination assessed by RNAseq inform the genetic architecture of embryonic gonadal development. *PLoS ONE*, *12*(3), e0172044. https://doi.org/10.1371/journal.pone.0172044

Rasiah, K. K., Gardiner-garden, M., Padilla, E. J. D., Möller, G., Kench, J. G., Alles, M. C., Eggleton, S. A., Stricker, P. D., Adamski, J., Sutherland, R. L., Henshall, S. M., & Hayes, V. M. (2009). *HSD17B4* overexpression, an independent biomarker of poor patient outcome in prostate cancer. *301*(1–2), 89–96. https://doi.org/10.1016/j.mce.2008.11.021

Rasys, A. M., Park, S., Ball, R. E., Alcala, A. J., Lauderdale, J. D., & Menke, D. B. (2019). CRISPR-Cas9 gene editing in lizards through microinjection of unfertilized oocytes. *Cell Reports*, *28*(9), 2288-2292.e3. https://doi.org/10.1016/j.celrep.2019.07.089

Ravi, P., Jiang, J., Liew, W. C., & Orbán, L. (2014). Small-scale transcriptomics reveals differences among gonadal stages in Asifan seabass (*Lates calcarifer*). *Reproductive Biology and Endocrinology*, *12*, 5. https://doi.org/10.1186/1477-7827-12-5

Refsnider, J. M., Bodensteiner, B. L., Reneker, J. L., & Janzen, F. J. (2013). Nest depth may not compensate for sex ratio skews caused by climate change in turtles. *Animal Conservation*, *16*(5), 481–490. https://doi.org/10.1111/acv.12034

Refsnider, J. M., & Janzen, F. J. (2016). Temperature-dependent sex determination under rapid anthropogenic environmental change: Evolution at a turtle's pace? *Journal of Heredity*, *107*(1), 61–70. https://doi.org/10.1093/jhered/esv053

Reid, K. A., Margaritoulis, D., & Speakman, J. R., (2009) Incubation temperature and energy expenditure during development in loggerhead sea turtle embryos. *Journal of Experimental Marine Biology and Ecology, 378*(1-2), 62-68. https://doi.org/10.1016/j.jembe.2009.07.030

Rej, J. E., & Joyner, T. A. (2018). Niche modeling for the genus *Pogona* (Squamata: Agamidae) in Australia: Predicting past (late Quaternary) and future (2070) areas of

suitable habitat. *PeerJ*, *6*, e6128. https://doi.org/10.7717/peerj.6128

Reyes, R., Rosado, A., Hernández, O., & Delgado, N. M. (1989). Heparin and glutathione: Physiological decondensing agents of human sperm nuclei. *Gamete Research*, *23*(1), 39–47. https://doi.org/10.1002/mrd.1120230105

Rhen, T., & Schroeder, A. (2010). Molecular mechanisms of sex determination in reptiles. *Sexual Development*, *4*(1–2), 16–28. https://doi.org/10.1159/000282495

Ribas, L., Liew, W. C., Díaz, N., Sreenivasan, R., Orbán, L., & Piferrer, F. (2017). Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proceedings of the National Academy of Sciences*, *114*(6), E941–E950. https://doi.org/10.1073/pnas.1609411114

Richter, C., & Kass, G. E. N. (1991). Oxidative stress in mitochondria: Its relationship to cellular Ca^{2+} homeostasis, cell death, proliferation and differentiation. *Chemico-Biological Interactions*, *77*(1), 1–23. https://doi.org/10.1016/0009-2797(91)90002-O

Robert, K. A., Brunet-Rossinni, A., & Bronikowski, A. M. (2007). Testing the "free radical theory of aging" hypothesis: Physiological differences in long-lived and short-lived colubrid snakes. *Aging Cell*, *6*(3), 395–404. https://doi.org/10.1111/j.1474-9726.2007.00287.x

Røttingen, J. A., & Iversen, J. G. (2000). Ruled by waves? Intracellular and intercellular calcium signalling. *Acta Physiologica Scandinavica*, *169*(3), 203–219. https://doi.org/10.1046/j.1365-201x.2000.00732.x

Rundel, C. (2013). *Timezone: Utility function for determining timezone based on location* (R Package Version 0.1). R Package Version 0.1.

Saillant, E., Fostier, A., Haffray, P., Menu, B., & Chatain, B. (2003). Saline preferendum for the European sea bass, *Dicentrarchus labrax*, larvae and juveniles: Effect of salinity on early development and sex determination. *Journal of Experimental Marine Biology and Ecology*, *287*(1), 103–117. https://doi.org/10.1016/S0022-0981(02)00502-6

Salame-Mendez, A., Herrera-Munoz, J., Moreno-Mendoza, N., & Merchant-Larios, H. (1998). Response of diencephalon but not the gonad to female-promoting temperature

with elevated estradiol levels in the sea turtle *Lepidochelys olivacea*. *Journal of Experimental Zoology*, *280*(4), 304–313. https://doi.org/10.1002/(SICI)1097-010X(19980301)280:4<304::AID-JEZ4>3.0.CO;2-S

Sánchez-Vázquez, M. L., Rosado, A., Merchant-larios, H., Ramírez, G., Reyes, R., & Delgado, N. M. (2007). DNA unpacking in guinea pig sperm chromatin by heparin and reduced glutathione. *Archives of Andrology*, *40*(1), 15–28. https://doi.org/10.3109/01485019808987924

Santos, D., Luzio, A., & Coimbra, A. M. (2017). Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. *Aquatic Toxicology*, *191*, 141–163. https://doi.org/10.1016/j.aquatox.2017.08.005

Sanulli, S., Justin, N., Teissandier, A., Ancelin, K., Portoso, M., Caron, M., Michaud, A., Lombard, B., da Rocha, S. T., Offer, J., Loew, D., Servant, N., Wassef, M., Burlina, F., Gamblin, S. J., Heard, E., & Margueron, R. (2015). Jarid2 methylation via the PRC2 complex regulates H3K27me3 deposition during cell differentiation. *Molecular Cell*, *57*(5), 769–783. https://doi.org/10.1016/j.molcel.2014.12.020

Sarida, M., Hattori, R. S., Zhang, Y., Yamamoto, Y., & Strüssmann, C. A. (2019). Spatiotemporal correlations between *amh* and *cyp19a1a* transcript expression and apoptosis during gonadal sex differentiation of pejerrey, *Odontesthes bonariensis*. *Sexual Development*, *13*(2), 99–108. https://doi.org/10.1159/000498997

Sarre, S. D., Ezaz, T., & Georges, A. (2011). Transitions between sex-determining systems in reptiles and amphibians. *Annual Review of Genomics and Human Genetics*, *12*(1), 391–406. https://doi.org/10.1146/annurev-genom-082410-101518

Sarre, S. D., Georges, A., & Quinn, A. (2004). The ends of a continuum: Genetic and temperature-dependent sex determination in reptiles. *BioEssays*, *26*(6), 639–645. https://doi.org/10.1002/bies.20050

Schede, H. H., Schneider, C. G., Stergiadou, J., Borm, L. E., Ranjak, A., Yamawaki, T. M., David, F. P. A., Lonnerberg, P., Laurent, G., Tosches, M. A., Codeluppi, S., & La Manno, G. (2020). Spatial tissue profiling by imaging-free molecular tomography. *BioRxiv*, 2020.08.04.235655. http://biorxiv.org/content/early/2020/08/04/2020.08.04.235655.abstract

Schieven, G. L., Kirihara, J. M., Gilliland, L. K., Uckun, F. M., & Ledbetter, J. A. (1993). Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signaling in lymphocytes. *Molecular Biology of the Cell*, *4*(5), 523–530. https://doi.org/10.1091/mbc.4.5.523

Schoenebeck, B., Bader, V., Xin, R. Z., Schmitz, B., Lübbert, H., & Stichel, C. C. (2005). Sgk1, a cell survival response in neurodegenerative diseases. *Molecular and Cellular Neuroscience*, *30*(2), 249–264. https://doi.org/10.1016/j.mcn.2005.07.017

Schroeder, A. L., Metzger, K. J., Miller, A., & Rhen, T. (2016). A novel candidate gene for temperature-dependent sex determination in the common snapping turtle. *Genetics*, *203*(1), 557–571. https://doi.org/10.1534/genetics.115.182840

Schroeder, A. L., & Rhen, T. (2019). Role for androgens in determination of ovarian fate in the common snapping turtle, *Chelydra serpentina*. *General and Comparative Endocrinology*, *281*, 7–16. https://doi.org/10.1016/j.ygcen.2019.05.003

Schwanz, L. E., Cordero, G. A., Charnov, E. L., & Janzen, F. J. (2016). Sex-specific survival to maturity and the evolution of environmental sex determination. *Evolution*, *70*(2), 329–341. https://doi.org/10.1111/evo.12856

Schwanz, L. E., Georges, A., Holleley, C. E., & Sarre, S. D. (2020). Climate change, sex reversal and lability of sex-determining systems. *Journal of Evolutionary Biology*, *33*(3), 270–281. https://doi.org/10.1111/jeb.13587

Sen, C., & Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB*, *10*(7), 709–720. https://doi.org/https://doi.org/10.1096/fasebj.10.7.8635688

Sharma, A., Nguyen, H., Geng, C., Hinman, M. N., Luo, G., & Lou, H. (2014). Calcium-mediated histone modifications regulate alternative splicing in cardiomyocytes. *Proceedings of the National Academy of Sciences*, *111*(46), E4920–E4928. https://doi.org/10.1073/pnas.1408964111

Shibata, Y., Branicky, R., Oviedo Landaverde, I., & Hekimi, S. (2003). Redox regulation of germline and vulval development in *Caenorhabditis elegans*. *Science*, *302*(5651), 1779–1782. https://doi.org/10.1126/science.1087167

Shine, R., Elphick, M. J., & Donnellan, S. (2002). Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. *Ecology Letters*, *5*(4), 486–489. https://doi.org/10.1046/j.1461-0248.2002.00351.x

Shine, R., & Lambeck, R. (1989). Ecology of frillneck lizards, *Chlamydosaurus kingii* (Agamidae), in tropical Australia. *Wildlife Research*, *16*(5), 491–500. https://doi.org/10.1071/WR9890491

Shine, R., Warner, D. A., & Radder, R. (2007). Windows of embryonic sexual lability in two lizard species with environmental sex determination. *Ecology*, *88*(7), 1781–1788. https://doi.org/10.1890/06-2024.1

Shoemaker-Daly, C. M., Jackson, K., Yatsu, R., Matsumoto, Y., & Crews, D. (2010). Genetic network underlying temperature-dependent sex determination is endogenously regulated by temperature in isolated cultured *Trachemys scripta* gonads. *Developmental Dynamics*, *239*(4), 1061–1075. https://doi.org/10.1002/dvdy.22266

Sies, H., Berndt, C., & Jones, D. P. (2017). Oxidative stress. *Annual Review of Biochemistry*, *86*, 715–748. https://doi.org/10.1146/annurev-biochem-061516-045037

Simon, A. R., Rai, U., Fanburg, B. L., & Cochran, B. H. (1998). Activation of the JAK-STAT pathway by reactive oxygen species. *American Journal of Physiology - Cell Physiology*, *275*(6), 1640–1652. https://doi.org/10.1152/ajpcell.1998.275.6.c1640

Sinervo, B. (2010). Erosion of lizard diversity by climate change and altered thermal niches. *Science*, *328*(5980), 894–899. https://doi.org/10.1126/science.1184695

Sinervo, B., & Miles, D. B. (2011). Hormones and behavior of reptiles. In *Hormones and Reproduction of Vertebrates* (Vol. 3). Elsevier. https://doi.org/10.1016/B978-0-12-374930-7.10008-1

Singh, S. K., Debojyoti, D., & Rhen, T. (2020) Embryonic temperature programs phenotype in reptiles. *Frontiers in Physiology, 11*, 35. https://doi.org/10.3389/fphys.2020.00035

Smith, C. A., & Joss, J. M. (1994). Sertoli cell differentiation and gonadogenesis in *Alligator mississippiensis*. *Journal of Experimental Zoology*, *270*(1), 57–70. https://doi.org/https://doi.org/10.1002/jez.1402700107

Smith, C. A., Roeszler, K. N., Ohnesorg, T., Cummins, D. M., Farlie, P. G., Doran, T. J., & Sinclair, A. H. (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature*, *461*(7261), 267–271. https://doi.org/10.1038/nature08298

Smith, C. A., & Sinclair, A. H. (2004). Sex determination: Insights from the chicken. *BioEssays*, *26*(2), 120–132. https://doi.org/10.1002/bies.10400

Solomon-Lane, T. K., Crespi, E. J., & Grober, M. S. (2013). Stress and serial adult metamorphosis: Multiple roles for the stress axis in socially regulated sex change. *Frontiers in Neuroscience*, *7*, 210. https://doi.org/10.3389/fnins.2013.00210

Soncin, F., Asea, A., Zhang, X., Stevenson, M. A., & Calderwood, S. K. (2000). Role of calcium activated kinases and phosphatases in heat shock factor-1 activation. *International Journal of Molecular Medicine*, *6*(6), 705–710. https://doi.org/10.3892/ijmm.6.6.705

Soreq, H., & Seidman, S. (2001). Acetylcholinesterase - new roles for an old actor. *Nature Reviews Neuroscience*, *2*, 294–302.

Soullier, S., Hanni, C., Catzeflis, F., Berta, P., & Laudet, V. (1998). Male sex determination in the spiny rat *Tokudaia osimensis* (Rodentia: Muridae) is not *Sry* dependent. *Mammalian Genome*, *9*(7), 590–592. https://doi.org/10.1007/s003359900823

Sovic, M. G., Fries, A. C., & Gibbs, H. L. (2016). Origin of a cryptic lineage in a threatened reptile through isolation and historical hybridization. *Heredity*, *117*(5), 358–366. https://doi.org/10.1038/hdy.2016.56

Spiers, J. G., Chen, H. J. C., Sernia, C., & Lavidis, N. A. (2015). Activation of the hypothalamic-pituitary-adrenal stress axis induces cellular oxidative stress. *Frontiers in Neuroscience*, *8*, 456. https://doi.org/10.3389/fnins.2014.00456

Standora, E. A., & Spotila, J. R. (1985). Temperature dependent sex determination in sea turtles. *Copeia*, *1985*(3), 711–722. https://doi.org/https://doi.org/10.2307/1444765

Stewart, P. M. (2003). Tissue-specific Cushing's syndrome, 11β-hydroxysteroid dehydrogenases and the redefinition of corticosteroid hormone action. *European Journal*

of Endocrinology, *149*(3), 163–168. https://doi.org/10.1530/eje.0.1490163

Storey, K. B. (1996). Oxidative stress: Animal adaptations in nature. *Brazilian Journal of Medical and Biological Research*, *29*(12), 1715–1733.

Straková, B., Rovatsos, M., Kubička, L., & Kratochvíl, L. (2020). Evolution of sex determination in amniotes: Did stress and sequential hermaphroditism produce environmental determination? *BioEssays*, *42*(10), 2000050. https://doi.org/10.1002/bies.202000050

Sun, B. J., Li, T., Gao, J., Ma, L., & Du, W. G. (2015). High incubation temperatures enhance mitochondrial energy metabolism in reptile embryos. *Scientific Reports*, *5*, 8861. https://doi.org/10.1038/srep08861

Surai, P. F., Noble, R. C., & Speake, B. K. (1996). Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embyro. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism 1304*(1), 1-10. https://doi.org/10.1016/S0005-2760(96)00099-9

Surai, P. F. (1999). Tissue-specific changes in the activities of antioxidant enzymes during the development of the chicken embryo. *British Poultry Science*, *40*(3), 397–405. https://doi.org/10.1080/00071669987511

Sutovsky, P., & Schatten, G. (2005). Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biology of Reproduction*, *56*(6), 1503–1512. https://doi.org/10.1095/biolreprod56.6.1503

Tao, W., Chen, J., Tan, D., Yang, J., Sun, L., Wei, J., Conte, M. A., Kocher, T. D., & Wang, D. (2018). Transcriptome display during tilapia sex determination and differentiation as revealed by RNA-Seq analysis. *BMC Genomics*, *19*(1), 363. https://doi.org/10.1186/s12864-018-4756-0

Tedeschi, J. N., Kennington, W. J., Berry, O., Whiting, S., Meekan, M., & Mitchell, N. J. (2015). Increased expression of *Hsp70* and *Hsp90* mRNA as biomarkers of thermal stress in loggerhead turtle embryos (*Caretta caretta*). *Journal of Thermal Biology*, *47*, 42–50. https://doi.org/10.1016/j.jtherbio.2014.11.006

Tedeschi, J. N., Kennington, W. J., Tomkins, J. L., Berry, O., Whiting, S., Meekan, M. G., & Mitchell, N. J. (2016). Heritable variation in heat shock gene expression: A potential mechanism for adaptation to thermal stress in embryos of sea turtles. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20152320. https://doi.org/10.1098/rspb.2015.2320

Temple, M. D., Perrone, G. G., & Dawes, I. W. (2005). Complex cellular responses to reactive oxygen species. *Trends in Cell Biology*, *15*(6), 319–326. https://doi.org/10.1016/j.tcb.2005.04.003

Timme-Laragy, A. R., Hahn, M. E., Hansen, J. M., Rastogi, A., & Roy, M. A. (2018). Redox stress and signaling during vertebrate embryonic development: Regulation and responses. *Seminars in Cell & Developmental Biology*, *80*, 17–28. https://doi.org/10.1016/j.semcdb.2017.09.019

Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish. *Sexual Development*, *10*(5–6), 223–241. https://doi.org/10.1159/000449297

Todd, E. V., Ortega-Recalde, O., Liu, H., Lamm, M. S., Rutherford, K. M., Cross, H., Black, M. A., Kardailsky, O., Graves, J. A. M., Hore, T. A., Godwin, J. R., & Gemmell, N. J. (2019). Stress, novel sex genes and epigenetic reprogramming orchestrate socially controlled sex change. *Science Advances*, *5*(7), eaaw7006. https://doi.org/10.1126/sciadv.aaw7006

Torres, M., & Forman, H. J. (2003). Redox signaling and the MAP kinase pathways. *BioFactors*, *17*(1–4), 287–296. https://doi.org/10.1002/biof.5520170128

Tort, L. (2011). Stress and immune modulation in fish. *Developmental & Comparative Immunology*, *35*(12), 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002

Tosches, M. A., Yamawaki, T. M., Naumann, R. K., Jacobi, A. A., Tushev, G., & Laurent, G. (2018). Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomes in reptiles. *Science*, *360*(6391), 881-888. https://doi.org/10.1126/science.aar4237

Tosini, G., Bertolucci, C., & Foà, A. (2001). The circadian system of reptiles: A

multioscillatory and multiphotoreceptive system. *Physiology and Behavior*, *72*(4), 461–471. https://doi.org/10.1016/S0031-9384(00)00423-6

Traverso, J. A., Pulido, A., Rodríguez-García, M. I., & Alché, J. D. (2013). Thiol-based redox regulation in sexual plant reproduction: New insights and perspectives. *Frontiers in Plant Science*, *4*, 465. https://doi.org/10.3389/fpls.2013.00465

Treidel, L. A., Carter, A. W., & Bowden, R. M. (2016). Temperature experienced during incubation affects antioxidant capacity but not oxidative damage in hatchling red-eared slider turtles (*Trachemys scripta elegans*). *The Journal of Experimental Biology*, *219*, 561–570. https://doi.org/10.1242/jeb.128843

Tsakogiannis, A., Manousaki, T., Lagnel, J., Sterioti, A., Pavlidis, M., Papandroulakis, N., Mylonas, C. C., & Tsigenopoulos, C. S. (2018). The transcriptomic signature of different sexes in two protogynous hermaphrodites: Insights into the molecular network underlying sex phenotype in fish. *Scientific Reports*, *8*(1), 3564. https://doi.org/10.1038/s41598-018-21992-9

Uchida, D., Yamashita, M., Kitano, T., & Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *Journal of Experimental Biology*, *205*(6), 711–718.

Uchida, D., Yamashita, M., Kitano, T., & Iguchi, T. (2004). An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. *Comparative Biochemistry and Physiology Part A - Molecular and Integrative Physiology*, *137*(1), 11–20. https://doi.org/10.1016/S1095-6433(03)00178-8

Uller, T., Hollander, J., Astheimer, L., & Olsson, M. (2009). Sex-specific developmental plasticity in response to yolk corticosterone in an oviparous lizard. *Journal of Experimental Biology*, *212*(8), 1087–1091. https://doi.org/10.1242/jeb.024257

Umina, P. A., Weeks, A. R., Kearney, M. R., McKechnie, S. W., & Hoffmann, A. A. (2005). Evolution: A rapid shift in a classic clinal pattern in *Drosophila* reflecting climate change. *Science*, *308*(5722), 691–693. https://doi.org/10.1126/science.1109523

Valenzuela, N., Literman, R., Neuwald, J. L., Mizoguchi, B., Iverson, J. B., Riley, J. L., &

Litzgus, J. D. (2019). Extreme thermal fluctuations from climate change unexpectedly accelerate demographic collapse of vertebrates with temperature-dependent sex determination. *Scientific Reports*, *9*(1), 4254. https://doi.org/10.1038/s41598-019-40597-4

van der Wijst, M. G. P., Venkiteswaran, M., Chen, H., Xu, G. L., Plösch, T., & Rots, M. G. (2015). Local chromatin microenvironment determines DNMT activity: From DNA methyltransferase to DNA demethylase or DNA dehydroxymethylase. *Epigenetics*, *10*(8), 671–676. https://doi.org/10.1080/15592294.2015.1062204

Van Doorn, G. S. (2014). Evolutionary transitions between sex-determining mechanisms: A review of theory. *Sexual Development*, *8*(1–3), 7–19. https://doi.org/10.1159/000357023

Van Oordt, W. V. D. H., Diaz-Meco, M. T., Lozano, J., Krainer, A. R., Moscat, J., & Caceres, J. F. (2000). The MKK3/6-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *Journal of Cell Biology*, *149*(2), 307–316. https://doi.org/10.1083/jcb.149.2.307

Venegas, D., Marmolejo-Valencia, A., Valdes-Quezada, C., Govenzensky, T., Recillas-Targa, F., & Merchant-Larios, H. (2016). Dimorphic DNA methylation during temperature-dependent sex determination in the sea turtle *Lepidochelys olivacea*. *General and Comparative Endocrinology*, *236*, 35–41. https://doi.org/10.1016/j.ygcen.2016.06.026

Viets, B. E., Tousignant, A., Ewert, M. A., Nelson, C. E., & Crews, D. (1993). Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. *Journal of Experimental Zoology*, *265*(6), 679–683. https://doi.org/10.1002/jez.1402650610

Viscarra Rossel, R. A., Chen, C., Grundy, M. J., Searle, R., Clifford, D., & Campbell, P. H. (2015). The Australian three-dimensional soil grid: Australia's contribution to the GlobalSoilMap project. *Soil Research*, *53*(8), 845–864. https://doi.org/10.1071/SR14366

Viscarra Rossel, R. A., Chen, C., Grundy, M., Searle, R., Clifford, D., Odgers, N., Holmes, K., Griffin, T., Liddicoat, C., & Kidd, D. (2014a). *Soil and landscape grid national soil attribute maps - bulk density - whole earth (3" resolution) - release 1. v5.* https://doi.org/10.4225/08/546EE212B0048

Viscarra Rossel, R. A., Chen, C., Grundy, M., Searle, R., Clifford, D., Odgers, N., Holmes,

K., Griffin, T., Liddicoat, C., & Kidd, D. (2014b). *Soil and Landscape Grid National Soil Attribute Maps - Silt (3" resolution) - Release 1. v5*. https://doi.org/10.4225/08/546F29646877E

Wada, H. (2008). Glucocorticoids: Mediators of vertebrate ontogenetic transitions. *General and Comparative Endocrinology*, *156*(3), 441–453. https://doi.org/10.1016/j.ygcen.2008.02.004

Wang, J., Liu, Y., Jiang, S., Li, W., Gui, L., Zhou, T., Zhai, W., Lin, Z., Lu, J., & Chen, L. (2019). Transcriptomic and epigenomic alterations of Nile tilapia gonads sexually reversed by high temperature. *Aquaculture*, *508*, 167–177. https://doi.org/10.1016/j.aquaculture.2019.04.073

Wang, J., Hevi, S., Kurash, J. K., Lei, H., Gay, F., Bajko, J., Su, H., Sun, W., Chang, H., Xu, G., Gaudet, F., Li, E., & Chen, T. (2009). The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nature Genetics*, *41*(1), 125–129. https://doi.org/10.1038/ng.268

Wang, T., Chen, K., Zeng, X., Yang, J., Wu, Y., Shi, X., Qin, B., Zeng, L., Esteban, M. A., Pan, G., & Pei, D. (2011). The histone demethylases Jhdm1a/1b enhance somatic cell reprogramming in a vitamin-C-dependent manner. *Cell Stem Cell*, *9*(6), 575–587. https://doi.org/10.1016/j.stem.2011.10.005

Wang, W. N., Zhou, J., Wang, P., Tian, T. T., Zheng, Y., Liu, Y., Mai, W. jun, & Wang, A. L. (2009). Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. *Comparative Biochemistry and Physiology Part C - Toxicology and Pharmacology*, *150*(4), 428–435. https://doi.org/10.1016/j.cbpc.2009.06.010

Wang, Y., Huang, Y. Y., Wang, Y., Lyu, P., & Hamblin, M. R. (2016). Photobiomodulation (blue and green light) encourages osteoblastic-differentiation of human adipose-derived stem cells: Role of intracellular calcium and light-gated ion channels. *Scientific Reports*, *6*, 33719. https://doi.org/10.1038/srep33719

Warner, D. A. (2011). Sex Determination in Reptiles. In *Hormones and Reproduction of Vertebrates* (Vol. 3). Elsevier. https://doi.org/10.1016/B978-0-12-374930-7.10001-9

Warner, D. A., Lovern, M. B., & Shine, R. (2007). Maternal nutrition affects reproductive output and sex allocation in a lizard with environmental sex determination. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1611), 883–890. https://doi.org/10.1098/rspb.2006.0105

Warner, D. A., Lovern, M. B., & Shine, R. (2008). Maternal influences on offspring phenotypes and sex ratios in a multi-clutching lizard with environmental sex determination. *Biological Journal of the Linnean Society*, *95*(2), 256–266. https://doi.org/10.1111/j.1095-8312.2008.01058.x

Warner, D. A., Radder, R. S., & Shine, R. (2009). Corticosterone exposure during embryonic development affects offspring growth and sex ratios in opposing directions in two lizard species with environmental sex determination. *Physiological and Biochemical Zoology*, *82*(4), 363–371. https://doi.org/10.1086/588491

Warner, D. A., & Shine, R. (2005). The adaptive significance of temperature-dependent sex determination: Experimental tests with a short-lived lizard. *Evolution*, *59*(10), 2209–2221. https://doi.org/10.1111/j.0014-3820.2005.tb00929.x

Warner, D. A., & Shine, R. (2008). The adaptive significance of temperature-dependent sex determination in a reptile. *Nature*, *451*(7178), 566–568. https://doi.org/10.1038/nature06519

Warner, D. A., Uller, T., & Shine, R. (2009). Fitness effects of the timing of hatching may drive the evolution of temperature-dependent sex determination in short-lived lizards. *Evolutionary Ecology*, *23*, 281–294. https://doi.org/10.1007/s10682-007-9222-4

Weber, C., Zhou, Y., Lee, J. G., Looger, L. L., Qian, G., Ge, C., & Capel, B. (2020). Temperature-dependent sex determination is mediated by pSTAT3 repression of *Kdm6b*. *Science*, *368*(6488), 303–306. https://doi.org/10.1126/science.aaz4165

Wennstrom, K. L., & Crews, D. (1995). Making males from females: The effects of aromatase inhibitors on a parthenogenetic species of whiptail lizard. *General and Comparative Endocrinology*, *99*(3), 316–322. https://doi.org/10.1006/gcen.1995.1115

West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., Takasu, M. A., Tao, X., & Greenberg, M. E. (2001). Calcium regulation of neuronal

gene expression. *Proceedings of the National Academy of Sciences*, *98*(20), 11024–11031. https://doi.org/10.1073/pnas.191352298

Whiteley, S. L., Holleley, C. E., Blackburn, J., Deveson, I. W., Wagner, S. W., Marshall Graves, J. A., & Georges, A. (2021). Two transcriptionally distinct pathways drive female development in a reptile with temperature induced sex reversal. *PLoS Genetics*, *In review*.

Whiteley, S. L., Holleley, C. E. C. E., Ruscoe, W. A. W. A., Castelli, M., Whitehead, D. L. D. L., Lei, J., Georges, A., & Weisbecker, V. (2017). Sex determination mode does not affect body or genital development of the central bearded dragon (*Pogona vitticeps*). *EvoDevo*, *8*, 25. https://doi.org/10.1186/s13227-017-0087-5

Whiteley, S. L., Weisbecker, V., Georges, A., Gauthier, A. R. G., Whitehead, D. L., & Holleley, C. E. (2018). Developmental asynchrony and antagonism of sex determination pathways in a lizard with temperature-induced sex reversal. *Scientific Reports*, *8*, 14892 |. https://doi.org/10.1038/s41598-018-33170-y

Wibbels, T., Bull, J. J., & Crews, D. (1994). Temperature-dependent sex determination: A mechanistic approach. *Journal of Experimental Zoology*, *270*(1), 71–78. https://doi.org/10.1002/jez.1402700108

Wibbels, T., & Crews, D. (1992). Specificity of steroid hormone-induced sex determination in a turtle. *Journal of Endocrinology*, *133*(1), 121–129. https://doi.org/10.1677/joe.0.1330121

Wibbels, T., & Crews, D. (1994). Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with temperature-dependent sex determination. *Journal of Endocrinology*, *141*(2), 295–299. https://doi.org/10.1677/joe.0.1410295

Wiggins, J. M., Santoyo-Brito, E., Scales, J. B., & Fox, S. F. (2020). Gene dose indicates presence of sex chromosomes in collared lizards (*Crotaphytus collaris*), a species with temperature-influenced sex determination. *Herpetologica*, *76*(1), 27–30. https://doi.org/https://doi.org/10.1655/Herpetologica-D-19-00036

Wilczynska, A., Gillen, S. L., Schmidt, T., Meijer, H. A., Jukes-Jones, R., Langlais, C.,

Kopra, K., Lu, W. T., Godfrey, J. D., Hawley, B. R., Hodge, K., Zanivan, S., Cain, K., Le Quesne, J., & Bushell, M. (2019). EIF4A2 drives repression of translation at initiation by Ccr4-Not through purine-rich motifs in the 5′UTR. *Genome Biology*, *20*(1), 1–21. https://doi.org/10.1186/s13059-019-1857-2

Willingham, E., Baldwin, R., Skipper, J. K., & Crews, D. (2000). Aromatase activity during embryogenesis in the brain and adrenal-kidney-gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. *General and Comparative Endocrinology*, *119*(2), 202–207. https://doi.org/10.1006/gcen.2000.7505

Wilson, C. A., High, S. K., McCluskey, B. M., Amores, A., Yan, Y. L., Titus, T. A., Anderson, J. L., Batzel, P., Carvan, M. J., Schartl, M., & Postlethwait, J. H. (2014). Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains. *Genetics*, *198*(3), 1291–1308. https://doi.org/10.1534/genetics.114.169284

Wright, S. (1943). Isolation by distance. *Genetics*, *28*(2), 114–138.

Wyneken, J., & Lolavar, A. (2015). Loggerhead sea turtle environmental sex determination: Implications of moisture and temperature for climate change based predictions for species survival. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *324*(3), 295–314. https://doi.org/10.1002/jez.b.22620

Yadlapalli, S., Jiang, C., Bahle, A., Reddy, P., Meyhofer, E., & Shafer, O. T. (2018). Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature*, *555*(7694), 98–102. https://doi.org/10.1038/nature25740

Yamaguchi, T., Yoshinaga, N., Yazawa, T., Gen, K., & Kitano, T. (2010). Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinology*, *151*(8), 3900–3908. https://doi.org/10.1210/en.2010-0228

Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S., & Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLoS ONE*, *9*(7), e102574. https://doi.org/10.1371/journal.pone.0102574

Yan, Y., Wei, C., Zhang, W., Cheng, H., & Liu, J. (2006). Cross-talk between calcium and reactive oxygen species signaling. *Acta Pharmacologica Sinica*, *27*(7), 821–826.

https://doi.org/10.1111/j.1745-7254.2006.00390.x

Yao, Z. L., Chen, H. J., Zhao, Y., Cao, Z. J., Wang, H., & Ji, X. S. (2021). A time course transcriptome analysis of brains from sex-undifferentiated Nile tilapia discloses genes associated with high-temperature-induced masculinization. *Aquaculture*, *530*, 735762. https://doi.org/10.1016/j.aquaculture.2020.735762

Yatsu, R., Miyagawa, S., Kohno, S., Parrott, B. B., Yamaguchi, K., Ogino, Y., Miyakawa, H., Lowers, R. H., Shigenobu, S., Guillette, L. J., & Iguchi, T. (2016). RNA-seq analysis of the gonadal transcriptome during *Alligator mississippiensis* temperature-dependent sex determination and differentiation. *BMC Genomics*, *17*(1), 77. https://doi.org/10.1186/s12864-016-2396-9

Yatsu, R., Miyagawa, S., Kohno, S., Saito, S., Lowers, R. H., Ogino, Y., Fukuta, N., Katsu, Y., Yasuhiko, O., Tominaga, M., Jr, L. J. G., & Iguchi, T. (2015). TRPV4 associates environmental temperature and sex determination in the American alligator. *Scientific Reports*, *5*, 18581. https://doi.org/10.1038/srep18581

Ye, Y. Z., Ma, L., Sun, B. J., Li, T., Wang, Y., Shine, R., & Du, W. G. (2019). The embryos of turtles can influence their own sexual destinies. *Current Biology*, *29*(16), 2597–2603. https://doi.org/10.1016/j.cub.2019.06.038

Yin, Z., Machius, M., Nestler, E. J., & Rudenko, G. (2017). Activator Protein-1: Redox switch controlling structure and DNA-binding. *Nucleic Acids Research*, *45*(19), 11425–11436. https://doi.org/10.1093/nar/gkx795

Ying, Z., Xiang, G., Zheng, L., Tang, H., Duan, L., Lin, X., Zhao, Q., Chen, K., Wu, Y., Xing, G., Lv, Y., Li, L., Yang, L., Bao, F., Long, Q., Zhou, Y., He, X., Wang, Y., Gao, M., … Liu, X. (2018). Short-term mitochondrial permeability transition pore opening modulates histone lysine methylation at the early phase of somatic cell reprogramming. *Cell Metabolism*, *28*(6), 935–945. https://doi.org/10.1016/j.cmet.2018.08.001

Zeileis, A., & Grothendieck, G. (2005). Zoo: S3 infrastructure for regular and irregular time series. *Journal of Statistical Software*, *14*(6). https://doi.org/10.18637/jss.v014.i06

Zhang, Y. P., Li, S. R., Ping, J., Li, S. W., Zhou, H. Bin, Sun, B. J., & Du, W. G. (2016). The effects of light exposure during incubation on embryonic development and hatchling

traits in lizards. *Scientific Reports*, *6*, 38527. https://doi.org/10.1038/srep38527

Zhang, Y., Wu, Y., Mao, P., Li, F., Han, X., Zhang, Y., Jiang, S., Chen, Y., Huang, J., Liu, D., Zhao, Y., Ma, W., & Songyang, Z. (2016). Cold-inducible RNA-binding protein CIRP/hnRNP A18 regulates telomerase activity in a temperature-dependent manner. *Nucleic Acids Research*, *44*(2), 761–775. https://doi.org/10.1093/nar/gkv1465

Zhao, Y., Mei, Y., Chen, H. J., Zhang, L. T., Wang, H., & Ji, X. S. (2019). Profiling expression changes of genes associated with temperature and sex during high temperature-induced masculinization in the Nile tilapia brain. *Physiological Genomics*, *51*(5), 159–168. https://doi.org/10.1152/physiolgenomics.00117.2018

Zhong, P., & Huang, H. (2017). Recent progress in the research of cold-inducible RNA-binding protein. *Future Science OA*, *3*(4), FSO246. https://doi.org/10.4155/fsoa-2017-0077

Zou, Y., Wu, Z., Fan, Z., Liang, D., Wang, L., Song, Z., & You, F. (2020). Analyses of mRNA-seq and miRNA-seq of the brain reveal the sex differences of gene expression and regulation before and during gonadal differentiation in 17β-estradiol or 17α-methyltestosterone-induced olive flounder (*Paralichthys olivaceus*). *Molecular Reproduction and Development*, *87*(1), 78–90. https://doi.org/10.1002/mrd.23303