

# The maternal influence on fitness correlates in a lizard with sex reversal



by

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A thesis submitted to the University of Canberra  
to fulfil the requirements for the degree of  
Doctor of Philosophy

Centre for Conservation Ecology and Genomics  
Faculty of Science and Technology  
University of Canberra  
Ngunnawal Country  
Australia  
2023

*FOR WILLIAM R. D. SOUTH*

# Abstract

Sex in vertebrates is typically understood to follow two primary modes of sex determination: genotypic sex determination and environmental sex determination. In genotypic sex determination (GSD), the genes that trigger sex differentiation are located on sex-specific chromosomes and are typically either male heterogametic (XX female and XY male) or female heterogametic (ZZ male and ZW female). Organisms with environmental sex determination (ESD) have no sex chromosomes, and the presence of an environmental cue (e.g., temperature) triggers sexual differentiation. Despite this conceptual dichotomy in sex determination, several lineages of vertebrates with GSD have shown a temperature override of sexual differentiation pathways producing sex-reversed individuals. These sex-reversed individuals have a mismatched sexual genotype and phenotype (e.g., ZZ female). Within reptiles, several species have exhibited sex reversal under laboratory and natural conditions, and it has been proposed that the propensity for sex reversal may partly explain the multiple evolutionary transitions between GSD and ESD (in the form of temperature-dependent sex determination (TSD)) seen in this taxon. Identifying how or if sex reversal influences fitness related traits of a species is critical to understanding how modes of sex determination evolve or persist in a population. Here, I use a combination of laboratory and field-based studies to understand the consequences of sex reversal in the Central Bearded Dragon lizard (*Pogona vitticeps*) by 1) quantifying reproductive output and the propensity to sex reverse between captive concordant and sex-reversed females; 2) quantifying morphology, growth, survival, and locomotor performance of the offspring of both concordant and sex-reversed females; 3) quantifying the nesting behaviours of free-ranging *P. vitticeps*, and 4) using predictive models to understand the relative risk of sex reversal.

Using a captive colony of *P. vitticeps*, I measured the reproductive rate of concordant (ZW) and sex-reversed (ZZ) females across two reproductive seasons and analysed historical data. I found that sex-reversed females produce fewer eggs per reproductive season than concordant females. This is contrary to previous published findings. I show that one hyper-fecund, sex-reversed female drove the results from the previous study. Additionally, I note that the pivotal temperature for offspring of concordant mothers is lower than previously suggested and not significantly different than sex-reversed mothers. I also found that sex-reversed females

produce larger eggs suggesting that there may be a trade-off between reproductive rate and egg size. These results suggest that sex reversal does not provide a reproductive advantage.

Then, I used the offspring produced from the previous study to quantify fitness-related phenotypes. I found that offspring of sex-reversed mothers are larger with better body condition at hatching than those of concordant mothers, but this difference dissipates quickly after hatching. Maternal sex genotype did not influence growth, survival, performance, or critical thermal limits. However, developmental temperatures did influence locomotor performance as well as the critical thermal minimums of offspring. The larger size at hatching afforded to offspring of sex-reversed mothers may provide an advantage allowing survival to adulthood increasing the persistence of sex reversal in a population.

I next used free-ranging, concordant female *P. vitticeps* to quantify nesting behaviours and nest site microclimate variables. I provide the first documentation of the nesting ecology in this species. Although sample sizes were relatively low, I found that nesting concordant females chose open canopy locations but varied the depth of their nests across the reproductive season, which alters the temperatures experienced by the developing embryos. I show that late season nests have a higher risk of sex reversal than early nests.

So far, sex reversal has only been documented in approximately 24 percent of the range of *P. vitticeps*, while ambient temperatures suggest that sex reversal should extend well beyond. To address my final aim, I deployed temperature loggers and collected microhabitat data from open and shaded potential nest sites at eight locations to determine the relative risk of sex reversal across the species' range. I used these data to test the accuracy of and then inform the microclimate model NicheMapR (Shiny app interface) to predict the risk of sex reversal at these point locations. I found that areas where no sex reversal has been recorded may have refugia that allow females to mitigate the risk of sex reversal. Furthermore, I show that open canopy areas where sex reversal has been documented may be at the greatest risk if females continue to choose open canopy nest sites.

Overall, my research suggests a change in the perception of sex reversal in *P. vitticeps* in the context of evolutionary transitions. Sex reversal does not convey a reproductive advantage, nor does it provide much of an advantage past hatching. Although free-ranging females choose nest sites that may induce sex reversal late in the reproductive season, they may be able to mitigate the risk of sex reversal by altering their behaviours or shifting their reproductive phenology. In full, the phenotypes associated with sex reversal in *P. vitticeps*

alone are unlikely to provide the momentum to advance this species towards a transition in mode of sex determination.

# Acknowledgements

First, I would like to acknowledge and pay respect to Aboriginal peoples past and present as the traditional custodians of the lands where I conducted my research specifically the Ngunnawal and Ngambri peoples whose lands the University of Canberra's Bruce campus is built upon. I also acknowledge the Wathi Wathi, Mutthi Mutthi, Nari Nari, Wilyakali, Kunja, Wangkangurru, Lower Southern Arrernte, Arrernte, Adnyamathanha, and Yandruwandha peoples who are the traditional custodians of the lands where my fieldwork took place.

The process of this thesis has been an all-consuming storm over the past five years. However, it has provided me the opportunities to chase my dream, see the world, make life-long friends, and given me the opportunity to meet my amazing partner. While this body of work has nearly destroyed me, I truly would not trade the experience for anything. I am incredibly proud of myself for seeing this to the end after the many ups, downs, lockdowns, and shutdowns.

I would like to thank William South, who entered my PhD journey a little late but has supported me every step of the way. He did not hesitate to join me on a field trip to Cunnamulla to monitor a gravid lizards after only dating for a couple of months. On another trip to Fowler's Gap, he proved to be the most frustrating volunteer I have ever had, but I would ask him out in the field again. He endured me attempting to work from home in lockdown twice without too many complaints, so I cannot complain too much about his fieldwork skills. He is the kindest and most patient partner a person could ask for. I am so grateful for our little family, and I could not have completed this work without his support.

I am incredibly grateful for my supervisory committee. My primary supervisor, Stephen Sarre, has been incredibly supportive throughout my longer than normal PhD. He has always had an open door and open ear to hear my complaints and worries. He has told me so many times that I am "incredibly resilient" that I now genuinely believe that. He has boosted my confidence as a researcher and writer without ever being critical or harsh, although sometimes I could have used it. My secondary supervisor Lisa Schwanz has provided me with so much help with statistics and experimental design while providing words of encouragement every step of the way. She also has brought me into her lab group, which has been a supportive group of thermal ecologists after I have been surrounded by geneticists who have no idea what my research means. Those songs of the day have sometimes really made my day. I am also incredibly grateful that we could discuss The Wheel of Time. It was in incredible

journey, and it is nice to be able to discuss things outside of research. My advisor Janine Deakin has been so supportive since joining my committee. While her expertise lies outside of the scope of my research, her people management skills and empathy are unmatched.

I cannot thank Katie Cohen enough for being such an amazing mentor, colleague, and friend. Her blind faith in my abilities is astounding, but I have never met a more supportive person. She has given me hope for the future of education in academia and has been an incredible role model. Her passion for teaching biology is contagious, and I am fully infected. There are few people you meet in life who refuel your passions and motivate you to be a better person, and Katie has done that for me.

It truly takes a village to complete a PhD. My fellow PhD students have given me so much support likely from the shared trauma of trying to complete a PhD during the COVID era. I cannot thank E. Stringer, M. Young, J. Fraser, M. Jeromson, K. Abicair, D. Doudin, K. Hemming, S. Deering, K. Soennichsen, A. Cormican, J. Thomson, M. Medina and all the other students and staff in CCEG and CAWS who have been there to support me. My fellow students have become my dearest friends and made me feel at home when home is 15000kms away.

I could not have done any of my fieldwork without the help of all the volunteers, M. Wild J. Soroka, H. Warrick, K. Joyner, R. Sarre, F. Knappe, S. Hinderaker, N. Walbridge, R. Johns, and D. Rathdod. I must thank A. Georges for providing me the opportunity to join Team Pogona. The Wildlife Genetics Lab group and Team Pogona made me realise that genetics truly is not that scary. I thank M. Castelli, S. Whiteley, J. Bartlett, and L. Weaver for the help in the lab, and A. Trujillo-Gonzalez for being a beautiful human providing endless positivity and joy. Jacqui Richardson gave me so much support with animal care after I produced hundreds hatchlings to be put into her care. Clare Holleley gave me support and advice for Chapter 2 and taught me that “data doesn’t step on toes.” I am also incredibly grateful to have worked on Australian Wildlife Conservancy property and for John and Melissa Barton for their genuine hospitality, field assistance, and general life lessons in the bush to an oblivious American. Jane Ebner and the SciTech admin team made fieldwork so easy and listened to me whinge when I needed to vent. Barbara Harriss was the first person to show me around the faculty and has been such a supportive person. She has put the students first and fought for us all with a smile and caring attitude. These people have all made a lasting impact on me and my thesis.

I must acknowledge the impact that Beatrice Dewenter made on my life during my PhD. It is hard to believe that she is gone from this world, and it is truly not the same without her

here. I will always have fond memories of lamenting the woes of PhD life with munching on strong liquorice. I could not have completed my thermal limits work without her help support and use of her equipment. We should be celebrating completing our doctorates together.

I honestly could not have gotten my work done without the support of Kris Wild. Nothing can bring two Americans closer together like a passion for lizards and isolation in a shearer's quarters in the middle of the Outback for six months. Kris is an amazing researcher, mentor, and friend. We have cheered each other on throughout our PhDs, and he has taught me so much. Every time I had doubts about my research, he gets so excited about my results that it makes me excited about my research again. I am glad to have had him and Mallory be a little slice of home for me here in Australia.

Finally, I of course must thank my family back in Alabama. My mom and dad have always supported me regardless of the task and moving across the world to chase lizards was no exception. I cannot thank them enough for the years of support and encouragement. My mom has truly been my other rock. I also must thank Maddie, Aunt Tammy, and Nana Graham for always being supportive also. Also, my Nana Tula, who has always been my cheerleader. I would not be half the person I am today without her love, support, and encouragement.

My research was conducted under University of Canberra Animal Ethics Project 2081. Funding was provided by a Commonwealth Research Training Program Scholarship, a Discovery Grant from the Australian Research Council (DP170101147) awarded to Arthur Georges (lead), Clare E. Holleley, Janine Deakin, Tariq Ezaz, Stephen Sarre, Lisa Schwanz, Paul Waters, and Jennifer Marshall Graves, ACT Herpetological Society, Ecological Society of Australia's Holsworth Wildlife Research Endowment, and a small grant from UC Faculty of Science and Technology.



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# List of abbreviations

Abbreviation	Definition
$CT_{\min}$	Critical thermal minimum: lower extreme temperature at which an animal loses righting response
$CT_{\max}$	Critical thermal maximum: upper extreme temperature at which an animal loses righting response
DOH	Day of hatching
ESD	Environmental sex determination: sex determination is influenced by environmental factors
GSD	Genotypic sex determination: sex determination if determined by genetic factors
HLL	Hind limb length
<i>rmsd</i>	Root-mean-square deviation: Measure to compare the differences between observed and predicted values
SVL	Snout-vent length: measure of body length
TL	Tail length
$T_{\text{pant}}$	Panting threshold: proxy for $CT_{\max}$ : Temperature at which an animal begins to pant showing heat stress
$T_{\text{piv}}$	Pivotal temperature: Temperature at which sex ratios are 1:1
TSD	Temperature-dependant sex determination: Temperature determines the sex of an individual
XXf	Concordant female, male heterogamety
XYm	Concordant male, male heterogamety
XXm	Sex-reversed male, male heterogamety
ZZm	Concordant male, female heterogamety
ZWf	Concordant female, female heterogamety
ZZf	Sex-reversed female, female heterogamety

# Chapter 1. General Introduction



Wild female *Pogona vitticeps* found east of Alice Springs, Northern Territory, Australia



## 1.1 Vertebrate sex determining systems

An organism's phenotypic sex can be one of the most influential traits as it provides a basis for physiology, morphology, and reproduction and extends across ontogeny into maturity (Bachtrog et al. 2014). Traits associated with phenotypic sex can ultimately influence total fitness of the individual. The evolution of gonochorism (male and female individuals) in sexual reproducing species has provided a means to increase genetic diversity in their progeny and reduce exposure to deleterious alleles that accrue in organisms with asexual modes of reproduction (Kratochvíl et al. 2021; Otto 2009). The evolution of anisogamy (differing sized male and female gametes) through disruptive selection (Bulmer and Parker 2002; da Silva 2018; Parker et al. 1972) has provided a further pathway into differentiating the sexes and means to which each parent can differentially invest into their offspring (Kodric-Brown and Brown 1987).

For many vertebrates, sex is determined at the time of fertilisation by the presence (or sometimes absence) of specific sex chromosomes, which contain genes that trigger male or female gonadal developmental pathways (Bachtrog et al. 2014; Pokorná and Kratochvíl 2016). This genotypic sex determination (GSD) typically occurs in two forms: male heterogamety (XX female and XY male) and female heterogamety (ZZ male and ZW female); although, there are variations in these forms (Pennell et al. 2018). The X-Y system is ubiquitous in mammals, where the sex determining gene (*SRY*) is generally located on the Y chromosome (Kashimada and Koopman 2010). In birds, which all share a Z-W system, a dosage response of genes located on the Z chromosome will induce sexual development (Ioannidis et al. 2021). While we know that specific chromosomes are associated with a given sex in many species, the genes that determine sex are not fully understood outside of these two groups (Bachtrog et al. 2014). Other clades of vertebrates with GSD can have either of these systems with even some closely related lineages exhibiting different modes (Ezaz et al. 2009; Holleley et al. 2016; Sarre et al. 2004). The persistence of this mode of determination is often attributed to its ability to produce a 1:1 offspring sex ratio and independent assortment of chromosomes in meiotic division (Schwanz and Georges 2021). Persistence of a system whereby master genes determine sex may seem like an inflexible mechanism; however, there is evidence for rapid evolution between male and female heterogamety when new genes arise that benefit one sex over the other (Bachtrog et al. 2014; van Doorn and Kirkpatrick 2010). While the benefits of GSD are fairly evident, some clades have evolved away from having

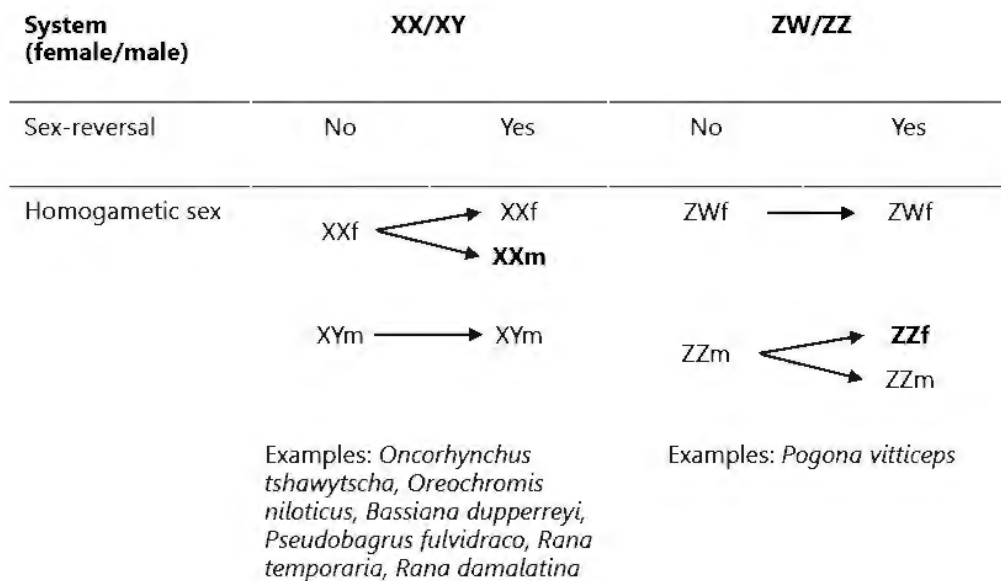
GSD or have evolved no sex chromosomes.

Many fish, amphibians, and reptiles do not have sex chromosomes. Rather, their sex is determined by stimuli in their environment that trigger sexual differentiation. Pathways of this mode, known as environmental sex determination (ESD; Valenzuela & Lance, 2004), can occur at various points in ontogeny (Bachtrog et al. 2014) and be induced by various environmental stimuli (Valenzuela et al. 2003). Teleost fish, for example, display a wide variety of ESD forms including postzygotic behavioural cues from conspecifics that can lead to the masculinisation or feminisation of an individual at later life stages or abiotic factors such as temperature or pH experienced during development that alter the sex ratios (reviewed in Devlin & Nagahama, 2002).

Temperature-dependent sex determination (TSD) is a common form of ESD in vertebrates (Devlin and Nagahama 2002; Valenzuela and Lance 2004). Under this mode, the temperature experienced during a thermosensitive period (usually the first third of embryonic development) determines the sex of the individual (Valenzuela et al. 2003). TSD has attracted significant research since its discovery by Charnier (1966) in the lizard *Agama agama*, and it has been shown to be common throughout most reptilian clades where it seems to have evolved multiple times (Deeming 2004; Sarre et al. 2004; Schwanz and Georges 2021). The evolutionary significance of TSD is commonly seen as a response to sex-differential fitness between developmental environments (Charnov and Bull 1977, 1989; Schwanz and Georges 2021) and has sparked extensive studies into the biological drivers of TSD (Valenzuela & Lance, 2004; and reviewed in Schwanz & Georges, 2021). Recently, more attention has been paid to the influence climate change may have on species with TSD and the risk of skewed sex ratios as the global climate continues to rise (Carter and Janzen 2021; Edmands 2021; Grayson et al. 2014; Hays et al. 2017; Mitchell et al. 2008, 2010; Valenzuela et al. 2019).

## 1.2 Evolutionary Transitions between modes of sex determination

Both GSD and ESD have evolved across multiple lineages with both modes found in closely related taxa (Bachtrog et al. 2014). While these two developmental pathways may seem independent, they are not generally considered dichotomous; instead, they more closely resemble a continuum or spectrum with GSD and ESD at the ends (Holleley et al. 2016; Sarre



**Figure 1.1.** Types of sex reversal in X-Y and Z-W systems. Phenotypic sexes are denoted with “f” for female or “m” for male. Sex-reversed genotype-phenotype combinations are bolded. Figure is modified from Piferrer and Anastasiadi 2021.

et al. 2004, 2011; Whiteley, Castelli, et al. 2021). Those species that fall within the continuum can experience sex reversal under specific environmental conditions exhibiting a mismatch between phenotypic sex and genotypic sex. For example, a GSD species with female heterogamety (ZZ/ZW sex chromosomes) may experience environmental conditions (e.g., high temperatures) during development that override the genotypic control of the sex differentiating signalling pathways to produce a ZZ female (Fig. 1.1; Piferrer & Anastasiadi, 2021). The evolution of these pathways shows that genotype and environment can interact, leading to the development or loss of sex chromosomes (Grossen et al. 2011; Perrin 2009). In vertebrates, this discordance between genotype and phenotype has been shown in fish, amphibians, and reptiles and can arise from many environmental factors such as exogenous hormones, pH, and temperature (Bachtrog et al. 2014; Hattori et al. 2020; Nemesházi et al. 2021; Whiteley, Castelli, et al. 2021). The physiological pathways of these overrides are not clear in species with sex reversal, but it has been posited that cellular calcium and redox regulation may convey the environmental signal in vertebrates with ESD or environmental sex reversal (Castelli et al. 2020).

Species with sex reversal may sit at any place on the continuum with the possibility of evolving toward ESD or GSD (Holleley et al. 2016; Sarre et al. 2004, 2011). These

evolutionary transitions could occur in only a few generations under appropriate conditions (Schwanz et al. 2020). The transition is so rapid because sex-reversed ZZ females can only produce ZZ descendants (as they only mate with ZZ males) whose development into male or female is determined by developmental temperature. Thus, a ZZ female's lineage effectively displays ESD. This process shows that the W chromosome can be lost from a population in a single generation if the environmental conditions cause a sufficient level of sex reversal. Moreover, if sex reversal provides a reproductive advantage, selection may act in its favour and push populations or the species as a whole towards ESD (Holleley et al. 2015; Sarre et al. 2004; Schwanz et al. 2020). The loss of the W chromosome could be beneficial to the species if the W had accrued a series of deleterious alleles (Blaser et al. 2013; Grossen et al. 2011; Perrin 2009; Schwanz et al. 2013). As selection on this theoretical species continues, new sex-related traits may arise on a specific chromosome, which could give rise to new sex chromosomes (Grossen et al. 2011). Understanding factors that drive these transitions will elucidate the evolutionary trajectories of species and potentially provide insight into conservation or management of species under changing climatic conditions as temperature is the most common environmental cue (Holleley et al. 2016; Sarre et al. 2011).

### 1.3 Phenotypic influence of temperature-induced sex reversal in vertebrates

Much of the research into sex reversal revolves around the cellular mechanisms that induce the process or the effect that sex reversal may have on sex ratios (Bókony et al. 2021; Edmands 2021). These studies are invaluable in understanding how sex reversal occurs and provide implications as to how it may shift a species' mode of sex determination in future generations. However, the phenotypic consequences of this phenomenon outside the effects of endocrine disrupting compounds (e.g., exogenous hormones) have only been described in a few species (Senior et al. 2012). Moreover, the impacts of sex reversal on phenotypes (i.e., growth, performance, survival, behaviours) that could influence the prevalence or persistence of sex reversal have only rarely been explored (Bókony et al. 2021; Dussenne et al. 2022; Jones et al. 2020; Lambert et al. 2019; Li et al. 2016; Nemesházi et al. 2020; Wild et al. 2022). Arguably, these traits are equally as important as they can inform the ecological significance of sex reversal and can be used to improve predictive evolutionary models (Bókony et al. 2021). Below, I briefly describe some of the species that have shown

temperature-induced sex reversal in wild populations and the trait differences that have been described.

In fishes, sex reversal has been recorded in wild populations of medaka (*Oryzias latipes*, Adrianichthyidae), Nile tilapia (*Oreochromis niloticus*, Cichlidae), the pejerrey (*Odontesthes bonariensis*, Atherinopsidae), and the Patagonian pejerrey (*Odontesthes hatcheri*, Atherinopsidae) (Baroiller et al. 1995; Hattori et al. 2007; Matsuda et al. 2002; Nanda et al. 2002; Strüssmann et al. 1997). These species exhibit male heterogamety (X-Y) with XX males occurring in wild populations where high temperatures induce the masculinisation (Baroiller and D’Cotta 2016). While *O. latipes* has been extensively studied in the context of its genetics, the influence of sex reversal on phenotypic traits in this group has not been reported outside of XX male fertility (Hattori et al. 2007). Sex-reversed-male *O. niloticus* are fertile (Sissao et al. 2019), behave more aggressively than XY males, and seem equally as attractive to females as XY males (Dussenne et al. 2022). XX males in both *O. bonariensis* and *O. hatcheri* are also fertile (del Fresno et al. 2023; Strüssmann et al. 2021), yet further phenotypic variation has yet to be explored.

Sex reversal in amphibians has been shown in a few wild populations of anurans (Alho et al. 2010; Lambert et al. 2019; Mikó et al. 2021). However, only agile frogs (*Rana dalmantina*, Ranidae) have been shown to have temperature-induced reversal (Mikó et al. 2021). *Rana dalmantina* has X-Y GSD, and short periods of high temperatures experienced during larval development produce XX males (Mikó et al. 2021; Nemesházi et al. 2020). Sex-reversed juvenile males have reduced body mass, development, fat reserves, and survival compared to XY males and XX females in this species (Mikó et al. 2021), which suggests that temperature sex reversal creates males that are less-fit (Bókonyi et al. 2021). While not yet empirically tested, sex reversal in wild populations of both *Rana temporaria* (Ranidae, X-Y GSD) and *Rana clamitans* (Ranidae, X-Y GSD) may also involve temperature (Alho et al. 2010; Lambert et al. 2019).

Non-avian reptiles have shown much promise in understanding the consequences of temperature-induced sex reversal and its implications in evolutionary transitions in modes of sex determination (Holleley et al. 2016; Sarre et al. 2011; Whiteley, Castelli, et al. 2021). Temperature-induced sex reversal have been documented across several lineages of reptiles with new documented cases increasing as genetic sexing techniques arise (Cornejo-Páramo et al. 2020; Hansson et al. 2023; Hill et al. 2022; Li et al. 2022; Wang et al. 2015; Whiteley,

Castelli, et al. 2021; Wiggins et al. 2020). However, studies of the resulting phenotypic consequences of sex reversal have been limited to two species of Australian lizards - the Eastern three-lined skink (*Bassiana duperreyi*, Scincidae) and the central bearded dragon (*Pogona vitticeps*: Agamidae). The Eastern three-lined skink is an alpine oviparous skink that has X-Y GSD where sex-reversed XX males are produced when eggs of are incubated at cool temperatures (Dissanayake et al. 2021; Quinn et al. 2009; Radder et al. 2008). Although the influence of developmental temperatures on phenotypes have been extensively studied in *B. duperreyi* (Amiel and Shine 2012; Elphick and Shine 1998; Flatt et al. 2001; Shine et al. 1997, 2016; Shine and Elphick 2001; Telemeco et al. 2009), only one study has explored the influence of sex reversal, where they found that juvenile XX males have similar metabolic rates and growth to XY males (Wild et al. 2023). More is known about sex reversal in the central bearded dragon, which has a Z-W system with a high temperature induction of ZZ females (Holleley et al. 2015). Thus far, several phenotypes of sex-reversed *P. vitticeps* have been described. As *P. vitticeps* is the focal species for the present thesis, I describe the current knowledge regarding sex reversal in the species in more detail below (Ch. 1.5).

## 1.4 Reptiles as models for developmental plasticity and evolution of sex determining systems

The environment experienced by a developing embryo can alter its phenotype (developmental plasticity) and, in so doing, potentially increase its fitness in its postnatal environment (Pigliucci 2005; West-Eberhard 2003). As ectotherms, non-avian reptiles are reliant on their environmental temperatures from embryo through adulthood to regulate their physiological processes. This makes them particularly sensitive to environmental perturbations (Deeming 2004; Sinervo et al. 2010). The influence of temperature on plastic traits has been studied extensively across many reptilian taxa (Noble et al. 2018; Refsnider et al. 2019; While et al. 2018). Furthermore, these plastic traits induced by temperature may provide beneficial phenotypes and may be adaptive and favoured by natural selection (Ghalambor et al. 2007). If temperature-induced sex-reversal produces individuals with plastic traits that have a fitness advantage, they may have the ability to shift their population's mode of sex determination in a relatively short amount of time (Nemesházi et al. 2020; Sarre et al. 2004, 2011; Schwanz et al. 2020). Alternatively, if these traits are maladaptive, the population could be pushed toward extinction or towards skewed sex ratios under climate warming conditions (Boyle, Hone, et

al. 2014; Edmands 2021). As such, understanding these traits in thermolabile systems can help us understand the how sex determining systems evolve and persist.

Oviparous reptiles typically exhibit little or no parental care. After oviposition, the developing embryos have little capacity to move during development and are therefore subject to the environmental conditions that were chosen by their mother potentially experiencing wide variations in temperature, moisture, and other environmental conditions across only small-scale differences in microhabitats (Du et al. 2011; Telemeco et al. 2013; Ye et al. 2019). Although this suggests that the mothers are abandoning their offspring, extensive studies across taxa have shown that maternal nest site selection can have major impacts on the phenotype, developmental rate, survival, and sex ratios of the offspring that are non-random (Deeming 2004; Pruett et al. 2019; Refsnider 2016; Refsnider et al. 2019; Refsnider and Janzen 2010; Shine 2005; Warner and Shine 2008a; While et al. 2018). Thus, the parental influence is exerted through nest site selection.

Nest temperature is a major factor in nest site selection. This characteristic can affect all phenotypes of the offspring (Noble et al. 2018). For TSD species (and presumably mixed determining systems), the maternally chosen nest will have several variables (i.e., moisture, canopy cover, nest depth, and egg position) that can affect the nest temperature and in turn the sex ratio of a given clutch (Janzen 1994; Mitchell et al. 2013; Refsnider and Janzen 2010; Schwanz and Janzen 2008; Warner and Shine 2007). In nature, TSD mothers may have a wide variety of suitable nesting habitats; therefore, the location and aspects of the nest site selected could directly influence offspring fitness and, consequently, the mother's own lifetime reproductive fitness (Charnov and Bull 1977; Warner and Shine 2008a, 2008b). Since primary sex ratios could also be subject to frequency-dependent selection (Ayala and Campbell 1974), maternal nesting behaviours reflect complex selective forces. For nest selection to be adaptive, females should be choosing a nest site that maximises the fitness of their offspring and minimises maternal mortality (Warner and Shine 2009). These nesting behaviours are crucial to understanding how females may mitigate against sex reversal or how sex-reversed females may avoid skewed sex ratios in their nests.

The temperatures afforded by the microhabitat variables chosen by the nesting females may induce plastic phenotypes that can persist into adulthood (Noble et al. 2018; Packard et al. 1985; Reedy et al. 2013). For example, incubation temperatures can influence development, body size, sprint speed, and behaviour of individuals (Andrews 2018; Noble et

al. 2018; Pearson and Warner 2016, 2018; While et al. 2018). These offspring traits are frequently used as fitness correlates or fitness-related phenotypes as they may increase the chance of survival to reproduction (Noble et al. 2018). Such traits would be particularly useful in understanding how sex reversal may sway a population towards an alternate mode of sex determination.

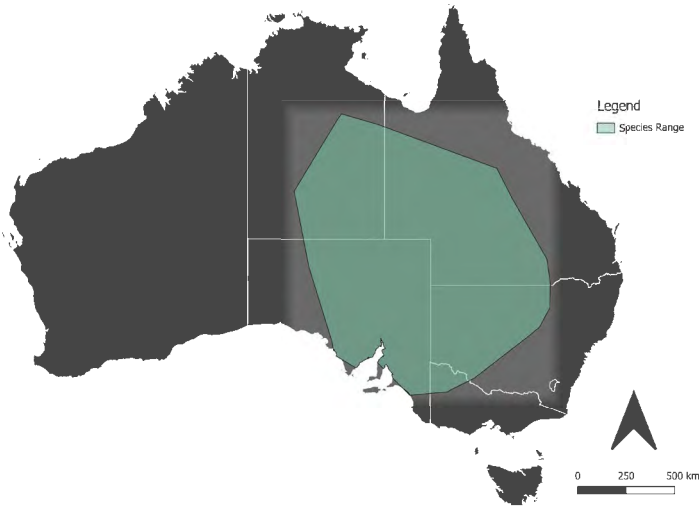
Differing reproductive output would greatly contribute to transitions in modes of sex determination. For example, if sex-reversed individuals have a fecundity advantage, GSD can be lost with only 35-40% sex reversal under climate warming scenarios (Schwanz et al. 2020). Furthermore, the diet, behaviours during gestation, external environmental conditions, and active choices made by expecting mothers have all been shown to be important drivers of adaptive phenotypic variation in their offspring (Marshall and Uller 2007; Moore et al. 2019; Mousseau and Fox 1998). The mothers may also provide anticipatory effects by choosing environments that may enhance their offspring's survival in heterogeneous environments (Uller et al. 2013).

Overall, reptiles provide an excellent opportunity for examining developmental plasticity and the influence of temperature on phenotypes. Of the reptiles, only lizards have been shown to have sex reversal and all those have been documented in wild populations as well as the laboratory (Hansson et al. 2023; Hill et al. 2018, 2022; Whiteley, Castelli, et al. 2021; Wiggins et al. 2020). Such instances provide the opportunity to test the influence of developmental temperatures on fitness related phenotypes in natural populations and laboratory conditions.

## 1.5 Study Species *Pogona vitticeps*

In this thesis, I utilise the Central Bearded Dragon lizard (*Pogona vitticeps*, Agamidae) as a model to study the influence of sex reversal on fitness-related phenotypes. *Pogona vitticeps* is a widely distributed, medium-sized lizard with a range that spans most of the central arid zone





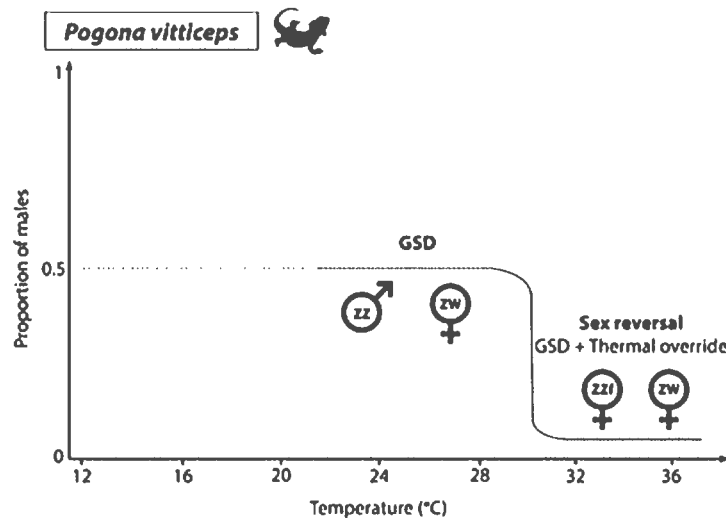
**Figure 1.2.** Species range of *P. vitticeps*.

of Australia (Cogger, 2018; Fig. 1.2). These lizards are typically female heterogametic GSD (ZW female, ZZ male; Ezaz et al. 2005). They also exhibit temperature-induced sex reversal in both the laboratory and in free-ranging populations (Castelli, Georges, Cherryh, et al. 2021; Holleley et al. 2015; Quinn et al. 2007; Wild et al. 2022).

Thermolabile sex determination in *P. vitticeps*, is induced by warmer developmental temperatures (>33°C; Fig. 1.3) which produces chromosomally male (ZZ genotype) yet phenotypically female individuals that are viable (Holleley et al. 2015; Quinn et al. 2007). This species has been shown to readily reproduce in captivity and is common in the pet trade.

However, little is known about their ecology (see Wild et al., 2022), and physiological studies have primarily been documented in veterinarian journals (Crouch et al. 2021; Raiti 2012; Schmidt-Ukaj et al. 2017). Thus, the species is an ideal study system into which to explore how sex reversal can influence fitness differences in adults and

their offspring. Furthermore, they can be used to study critical behavioural traits that can



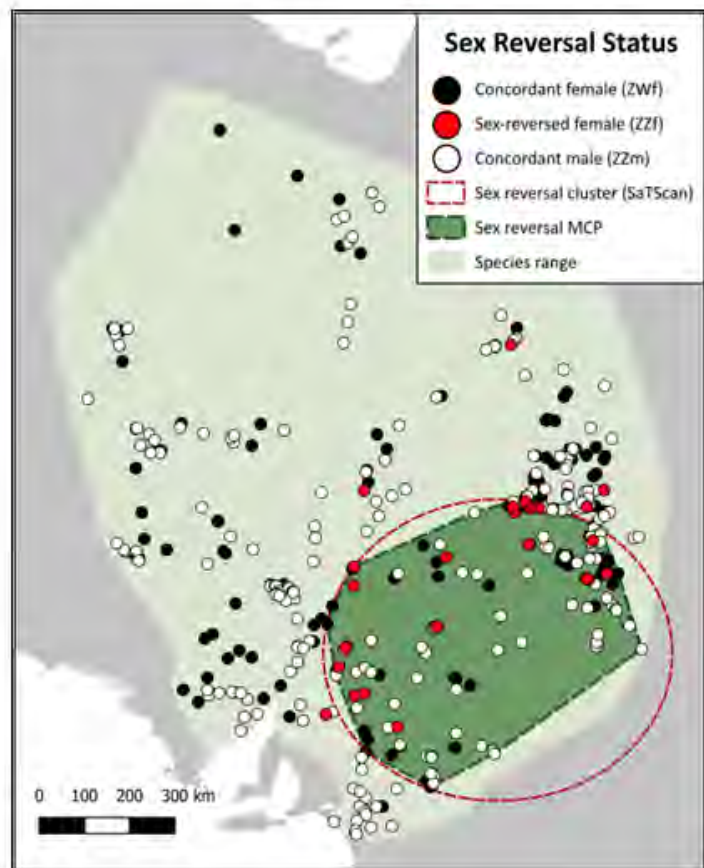
**Figure 1.3.** Thermal reaction norm of sex determination in *P. vitticeps*. This shows that the proportion of males decreases as incubation temperatures exceed 30°C. Modified from Whiteley et al. (2021).

influence evolutionary transitions in mode of sex determination (as suggested in Wild et al., 2022).

While sex reversal in this species occurs in the wild (Fig. 1.4; Castelli et al., 2021; Wild et al., 2022), it does not appear to have reached the default proportion of individuals (>50%) required to induce an evolutionary transition to TSD (Wild et al. 2022). However, previous lab-based studies suggest that sex reversal conveys a strong reproductive advantage (sex-reversed females produced almost twice as many eggs as concordant females) and a lower threshold temperature for sex reversal in offspring (Holleley et al. 2015). Li et al. (2016) also showed that

captive subadult and adult sex-reversed females are significantly bolder than either concordant females or males and have similar activity levels as males. These reproductive and behavioural phenotypes provide key advantages that could aid in transitions to TSD (Schwanz et al. 2020). However, other studies have shown no difference in head morphology or bite force (Jones et al. 2020), laboratory metabolic rate in juveniles (Wild et al. 2023), or behaviours in free-ranging individuals (Wild et al. 2022). Although beneficial traits have been observed in sex-reversed animals, it is unclear whether the sex reversal status of parents can influence their offspring's fitness-related phenotypes.

The work done thus far with *P. vitticeps* has given some understanding to how the presence of sex reversal may contribute to a rapid transition from GSD to TSD (Holleley et al. 2015; Schwanz et al. 2020). However, most of our knowledge regarding sex-reversal has been conducted on adults, leaving a critical knowledge gap in our understanding of the role of this



**Figure 1.4.** The range of *P. vitticeps* including the distribution of sex reversal or “sex reversal cluster.” Dots represent the genotyped samples from sampling events and museum specimens extending from 1980-2018. Figure modified from Castelli et al. (2021).

phenomenon across ontogeny. Early advantages provided by sex reversal could improve the chances sex-reversed offspring surviving to a reproductive age. If these traits are quantified first in a laboratory setting, we can interpolate how they may be influencing sex reversal in free-ranging populations.

While incubation temperature is the determining factor of sex reversal, the nesting ecology of *P. vitticeps* has not previously been explored. Nest site selection will play a fundamental role in determining nest temperatures, which could sway the sex ratios of a nest if they exceed the pivotal temperature. Previous models have looked to explain the distribution of sex reversal in wild populations, but they used nesting data from *Pogona minor* to determine the nest depth in their models (Castelli, Georges, Cherryh, et al. 2021). While this species is closely related and share some overlap in distribution, *P. minor* are smaller in body size than *P. vitticeps* (Cogger 2018), which could influence the nesting ecology. To best understand the relative risk of sex reversal in *P. vitticeps*, their nesting ecology must be explored. The microclimate data collected from nest sites can be used to inform predictive models to investigate how nest site selection influences transitions in modes of sex determination.

## 1.6 Thesis aims and structure

The overarching aim of this thesis is to provide a better understanding of the influence of maternal sex genotype (ZWf or ZZf) on fitness-related traits and if they may influence evolutionary transitions in modes of sex determination in *P. vitticeps*. I utilise captive and free-ranging *P. vitticeps* to address the following objectives:

1. Determine if there are reproductive advantages between sex-reversed and concordant mothers.
2. Determine if an offspring's maternal genotype and incubation temperature can induce beneficial fitness-related phenotypes.
3. Quantify the nesting ecology of free-ranging females.
4. Characterise the spatial and temporal soil temperatures across the range of *P. vitticeps* to determine the capacity for nest site selection to influence sex ratios.

To address these aims, I present three data chapters (Chapters 2-4) and a general discussion (Chapter 5) as outlined below. I have written each data chapter as individual units prepared for publication in scientific journals. The target journal is indicated and followed by an

author's contribution statement prior to the abstract of each chapter. I have written each data chapter in accordance with the general guidelines for the journal to be targeted. As each chapter revolves around the central theme of my thesis, there is some overlap between chapters particularly in each introduction. Supplemental materials for each chapter directly follow the discussion of each chapter as they would in publication. The reference list for this thesis encompasses all five chapters.

I address my first aim in Chapter 2 where I revisit the differences in fecundity seen between concordant and sex-reversed mothers as described in Holleley et al. (2015). I collected reproductive data from the captive colony of *P. vitticeps* across two seasons. I showed that concordant females have higher fecundity than sex-reversed females, which is opposite to the trends previously found (Holleley et al. 2015). I further investigate maternal age, inbreeding coefficient, and generation in captivity as potential causes for the contrasting conclusions reached between the two studies. This chapter shows that sex-reversal does not enhance reproductive output and may even inhibit it.

In Chapter 3, I explore the influence of maternal sex genotype, incubation temperature, and individual sex genotype on morphology, growth, and performance traits in the resultant offspring from the eggs generated from Chapter 2. I found that sex-reversed mothers produced larger offspring with better body condition at hatching than concordant mothers, but this size difference quickly dissipates. Maternal sex genotype did not influence locomotor performance or thermal limits, but warm incubation temperatures did increase performance traits under warmer conditions. My results show that sex-reversed mothers may provide their offspring with a head start; however, they are unlikely to provide enough of an advantage to push populations toward a transition in their mode of sex determination.

The nesting ecology of *P. vitticeps* has not previously been described. These behaviours can influence nest temperatures and may increase proportion of sex reversal in a population or mitigate the risk altogether. In Chapter 4, I combine field derived nesting data, observed microclimate data, and predicted microclimate data to address my final aim. The current records of sex reversal suggest that it is confined to the more central areas of the species' range in the "sex reversal cluster." However, ambient temperatures in the northern and north-western regions suggest that reversal should be present. I provided the first report on the nesting behaviours of free-ranging concordant *P. vitticeps* within the sex reversal cluster and show that they choose nest sites with more open canopies throughout their reproductive season. I also quantified potential nest microclimate variables from eight geographic locations

in shaded and open areas across the range of *P. vitticeps* to better understand the soil temperatures available to nesting females and predicted the risk of sex reversal at these point locations using the NicheMapR Shiny app microclimate models informed by my observed microclimate data. Overall, I found that shaded sites in locations in the north-western portion of the species' range and early nesting by females may mitigate the risk of sex reversal and explain the disparity of sex reversal in this region.

Finally, I provide a synopsis of key findings in Chapter 5 in relation to my original aims for this thesis. I then provide future research directions that could be pursued to better understand how sex reversal may be influencing other behavioural traits and outline other physiological differences between sex-reversed and concordant *P. vitticeps* that may further influence evolutionary transitions in their mode of sex determination.

## Chapter 2. Temperature-induced sex reversal does not increase reproductive output in the Central Bearded Dragon (*Pogona vitticeps*)



Female *P. vitticeps* with daisies

**Author Contributions:** Sampling design, data collection, analysis, and figure were performed by PRP. Historic data and insight into previous studies were provided by Clare E. Holleley. The first draft of manuscript was written by PRP. Comments from SDS, LES, CEH, and JED contributed to the final version of the manuscript.

## 2.1 Abstract

In vertebrates, sex is typically determined by an individual's genes (genotypic sex determination) or by their environment (environmental sex determination) including the temperature of incubation (temperature-dependent sex determination). However, some species have a mixed determination system where environmental factors can override the genetic signal for sex determination yielding sex-reversed individuals. These systems are excellent models for understanding traits that drive transitions in modes of sex determination. The central bearded dragon lizard (*Pogona vitticeps*) is one such species. This agamid typically has GSD with female heterogamety (ZZ/ZW), but sex can be reversed at high temperatures such that ZZ individuals, which are typically male, develop as females. Previous studies have shown that sex-reversed female *P. vitticeps* have higher fecundity and more aggressive behaviour than their concordant counterparts of a similar age. If correct, this characteristic could increase the likelihood of a transition to ESD in wild populations as the global climate increases. Here, I examined the reproductive traits of sex-reversed and concordant female *P. vitticeps* that vary in age, levels of domestication, and inbreeding within a captive colony to assess the proposition that maternal sex genotype affects reproductive and other traits. Contrary to previous studies, sex-reversed females produce fewer eggs and smaller clutches on average per reproductive year than concordant females regardless of the effects of captive breeding. These data suggest that rather than being advantageous, sex reversal in *P. vitticeps* may even be disadvantageous. These trends remained when combined with earlier published data suggesting that one sex-reversed female disproportionately influenced the previous findings. My results suggest that reproductive traits of sex-reversed *P. vitticeps* alone do not provide a reproductive advantage and are therefore unlikely to promote a transition in mode of sex determination.

## 2.2 Introduction

Sex is determined in a variety of ways across all animals, but there are two predominant sex-determining modes that are most widespread: genotypic sex determination (GSD) and environmental sex determination (ESD) (Bachtrog et al. 2014). Despite this dichotomous view, some populations exhibit a mixed sex-determining mode containing sex-reversed individuals whose phenotypic sex does not match their genotypic sex, a state that may facilitate a rapid transition from GSD systems to ESD (Holleley et al. 2015). Furthermore, evolutionary transitions in sex-determining modes are predicted to be sensitive to the relative fitness of sex-reversed individuals (Bókony et al. 2021; Nemesházi et al. 2021). Specifically, a fitness advantage of sex-reversed individuals over their concordant counterparts would sway a population toward an ESD mode of sex determination (Schwanz et al. 2020; Schwanz and Georges 2021; Wild et al. 2022). However, the influence sex reversal has on a fitness related traits remains virtually unknown, which inhibits the ability to predict how easily these evolutionary transitions can occur.

Given the sensitivity of evolutionary transitions to the relative fitness of sex reversed individuals, this is a crucial comparison to examine in order to gain an understanding of how sex reversal persists or drives transitions to ESD (Grossen et al. 2011; Holleley et al. 2015, 2016; Schwanz et al. 2020; Nemesházi et al. 2021; Nemesházi and Bókony 2022). There are many potential disadvantages of reversal, including novel chromosomal combinations yielding potentially inviable offspring (YY or WW) (Blaser et al. 2013) or chromosomally-driven behaviour and physiology in sex-reversed individuals being maladaptive compared with phenotypes expressed in the concordant sex (Holleley et al. 2016; Nemesházi and Bókony 2022). In agile frogs, sex reversal reduces growth, development, and survival (Mikó et al. 2021). A relative disadvantage in the fitness of sex-reversed animals would impede an evolutionary transition to ESD. In contrast, fitness advantages that could accelerate evolutionary transitions occur in bearded dragons (Holleley et al., 2015; Li et al., 2016).

Transitions between sex determining modes are important to understand as they may drive populations toward loss of sex chromosomes and heightened sensitivity to changing environments (Whiteley, Castelli, et al. 2021). Increased mating of homogametic individuals can drive the heterogametic sex out of the population, leading to the loss of an entire chromosome (Schwanz et al. 2020). Furthermore, transitions to ESD could increase



population vulnerability to global warming if sex ratios become increasingly biased increasing the risk of demographic collapse.

Across animal taxa, ectothermic vertebrates seem to be more susceptible to transitions in sex determining modes (Quinn et al. 2011; Sarre et al. 2011), perhaps because the direct physiological dependence of embryos on the environment enables sex reversal (Angilletta 2009). Moreover, oviparity increases vulnerability as eggs and embryos are exposed to the environment during key developmental stages (Deeming 2004) making them more likely to experience extreme temperatures and increasing the risk of sex reversal. For example, female tadpoles of agile frogs (*Rana dalmatina*) undergo masculinisation when exposed to short heat waves, yielding phenotypic males with a female (XX) genotype (Mikó et al. 2020). Similarly, warm developmental temperatures induce the somatic expression of the sex determining gene DMRT1 yielding XX male medaka rice fish (*Oryzias latipes*) (Nanda et al. 2002). Sex reversal has been documented in many fish and some frogs (Hattori et al. 2007; Nemeshazi and Bokony 2022) but is seemingly rare in reptiles (Whiteley, Castelli, et al. 2021). However, this apparent rarity may be understating its prevalence since many reptile lineages have both ESD and GSD (Gamble 2010; Gamble and Zarkower 2012) and the existence of sex reversal can be difficult to discern. As global temperatures increase, developing embryos of species affected by temperature are at risk of increased levels of reversal in populations unless there are behavioural or genetic mitigation tactics (Castelli, Georges, Cherryh, et al. 2021). Such increases could lead to changes in sex determining mode from GSD to TSD and the potential for increased vulnerability to global warming.

The central bearded dragon (*Pogona vitticeps*) is a widespread, oviparous Australian Agamid lizard that exhibits temperature-induced sex reversal in both the laboratory and the wild (Quinn et al. 2007; Holleley et al. 2015). Typically, this species has GSD with female heterogamety with ZZ males (ZZm) and ZW females (ZWf) (Ezaz et al., 2005). However, as developmental temperatures increase from 32°C, higher proportions of ZZ embryos are reversed from phenotypic male (ZZm) to sex-reversed phenotypic female (ZZf) (Quinn et al., 2007; Holleley et al., 2015). Previous studies have shown that ZZf individuals exhibit increased fecundity and boldness (Holleley et al. 2015; Li et al. 2016). Specifically, sex-reversed females of a similar age produce almost twice as many eggs as those produced by concordant females, and the eggs of sex-reversed mothers are more sensitive to incubation temperatures and have a lower pivotal (50:50 sex ratio) temperature (Holleley et al. 2015).

These traits could favour reproduction in sex-reversed females and enhance the spread of sex-reversed females leading to a decreased prevalence of the W chromosomes within local populations (Holleley et al. 2015; Schwanz et al. 2020) although recent field data suggest that sex reversal could be an evolutionarily stable state in this species (Wild et al. 2022).

Here, I use a combination of breeding experiments and analyses of historical breeding data to examine the influence of sex reversal on reproductive traits in *P. vitticeps*. My aim was to determine whether the reproductive trends between the two maternal sex genotypes seen in the formative work on *P. vitticeps* sex reversal (Holleley et al. 2015) remain true in a larger and more heterogeneous sample of females. Additionally, I tested for the effects of captive breeding (varied age, provenance, domestication, and inbreeding) on the same reproductive traits to determine if they could cause biases in measured reproductive output. This combination provides a robust dataset that allowed me to quantify differences in reproductive traits of concordant and sex-reversed *P. vitticeps*.

## 2.3 Methodology

### 2.3.1 *Breeding design and egg collection*

Eggs from both concordant (ZWf) and sex-reversed (ZZf) *P. vitticeps* were collected from the University of Canberra breeding colony across two reproductive seasons (October 2019-February 2020, October 2020-February 2021). Breeding groups were assigned after brumation and comprised of one concordant male (ZZm) and two or three females of the same genotype (ZZf or ZWf). Breeding groups were kept together throughout to ensure the production of fertile eggs, and each breeding group was kept the same across both reproductive seasons. Housing conditions and diet were consistent among all breeding groups.

Throughout the experiment breeding enclosures were provided with a 10:14, light:dark cycle with full spectrum UV bulbs and heat lamps. Breeding groups were fed live invertebrates dusted with calcium powder 3 days per week ad libitum and a mixture of shredded vegetables 3 days per week ad libitum. Females were hand fed approximately 10 grams of meat mince mixed with Wombaroo Reptile Supplement (Wombaroo Food Products, Glen Osmond, South Australia) once per week during the breeding season.

Females were palpated gently each week to check for developing eggs. When the eggs reach approximately 2cm, cages were monitored daily for nesting behaviours. Females were allowed to nest naturally in their cages in a sand substrate. Eggs were removed from the cage within 3 hours, checked for viability via presence of vascularisation, numbered with a non-toxic marker, weighed, and randomly assigned to one of two constant incubation regimes (28°C or 34°C) if viable. A random subset of viable eggs from the second reproductive season was destructively sampled for additional experiments and were not incubated.

Once assigned to a temperature, eggs were placed into plastic boxes containing moist vermiculite (~ -200 kPa; 5 vermiculite:4 water) then placed into a partially sealed plastic bag to maintain moisture levels throughout incubation. Incubation boxes were placed into programmable incubators set to the assigned temperature regime ( $\pm 1^\circ\text{C}$ ; see below). Eggs were checked weekly for health and development, then daily as they approached 6 weeks in the 34°C treatment and 10 weeks in the 28°C. Hatchlings were removed from the boxes, assigned a unique identification number, measured, sexed, then transported to the animal care facility. After 10 weeks, blood samples were taken using Whatman FTA Elute cards and stored at room temperature for genotyping.

### 2.3.2 *Determining offspring sex*

Phenotypic sex of the hatchlings was determined by hemipenile transillumination (Brown 2009). Genotypic sex (ZZ or ZW) was determined by PCR detection of the W chromosome using DNA extracted from blood or tissue following protocols described in Wild et al. (2022). Sex reversal was determined by a discordance between genotypic sex and phenotypic sex (Holleley et al. 2015).

### 2.3.3 *Historical data*

Historical data from the captive breeding colony were analysed to determine whether factors associated with captive breeding (maternal age, generation in captivity, inbreeding) influenced reproductive capabilities between maternal genotypes. Specifically, I determined the age of each captive bred female used and constructed a pedigree for all individuals used. This analysis provided inbreeding coefficients and the level of domestication for each lizard. Additionally, reproductive records from the breeding seasons of 2011-2012 to 2018-2019

were analysed to identify historic trends in reproduction and enable comparisons with the findings of the current study.

### 2.3.4 *Statistical analyses*

First, I ran simple models for the effect of maternal sex genotype on the probability of reproduction, fecundity (total viable eggs produced in a season), clutching rate, average eggs per clutch (total eggs per year divided by total clutches per year), egg mass, and egg survival. Reproductive year was included in each model. Then, I ran more complex models to evaluate the effects of maternal genotype combined with colony-related factors (maternal age, generation in captivity, and inbreeding coefficient) on the fixed terms and reproductive year was included in each model. Maternal age and generation in captivity were pooled into “classes” to allow comparisons between all groups. Maternal age classes were defined as “very young” (1-2 years), “young” (3-4 years), “old” (5-6 years), and “very old” (>6 years), and generation class was defined as “class 1” (0-2 generations from wild caught) or “class 2” (3-4 generations). Inbreeding coefficients were calculated from pedigree-based kinship matrices. Tests for interactions between maternal genotype and captive effects revealed no significant interactions in any model and were therefore excluded from further analyses. Maternal identity was included as a random effect in all mixed effect models. All statistical analyses were run in R v. 4.2.2 (R Core Team 2022).

Generalised linear mixed models (GLMMs) were used to quantify the influence of fixed effects on a female’s probability of reproducing (binomial error and logit link) with maternal ID as a random effect. GLMMs with Poisson distributions were used to determine the influence of fixed effects on fecundity and clutch rate. Egg survival was also fitted to a binomial GLMM (nested random effect of clutch with maternal ID) and included additional fixed effects of incubation temperature and egg mass. Wald  $\chi^2$  tests were used to analyse differences between fixed effects of all GLMMs. Linear mixed-effect models (LMMs) were used to analyse average number of eggs per clutch and individual egg mass (nested random effect of clutch with maternal ID) using Satterthwaite approximations to obtain degrees of freedom. Type III ANOVAs were used to test for differences between fixed effects for all LMMs. When ANOVAs or  $\chi^2$  tests were statistically significant, I used post-hoc tests for pairwise comparisons.

Rates of sex reversal were calculated by obtaining the proportion of ZZ individuals that reversed 34°C from both concordant and sex-reversed females. A two-sample test for equality of proportions was used to determine  $\chi^2$  and significance between the two maternal sex genotypes.

## 2.4 Results

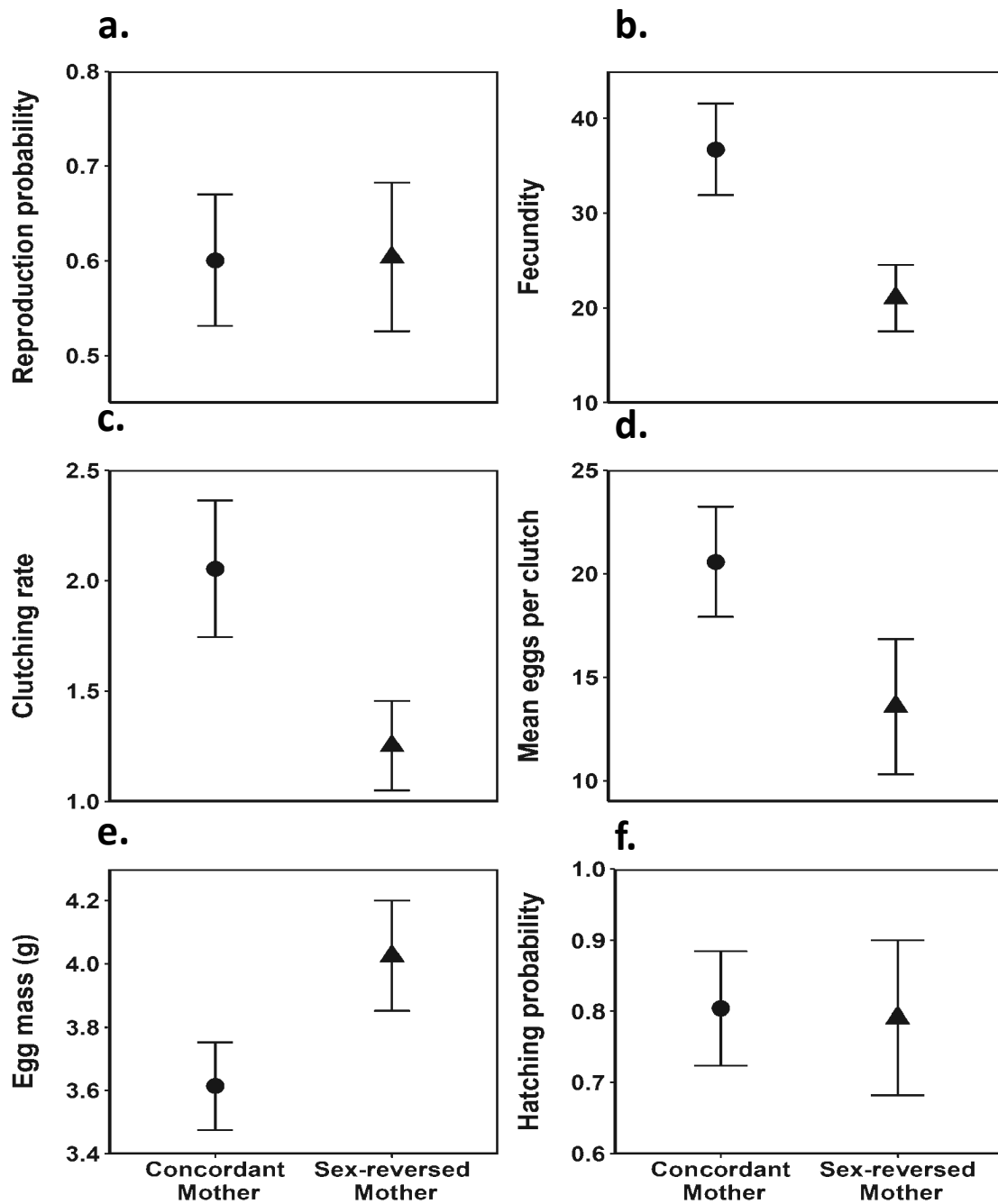
### 2.4.1 Influence of maternal genotype

A total of 981 eggs were produced in 44 clutches from 25 mothers (concordant=16, sex-reversed=11) across two reproductive seasons with 745 of those eggs viable and incubated. Twenty-eight females (ZWf=15, ZZf= 13) in the experiment did not reproduce. Maternal sex genotype did not influence the probability of reproduction (Fig 2.1a, Table 2.1).

**Table 2.1.** Differences in reproductive traits between concordant (ZWf) and sex-reversed (ZZf) mothers shown as non-standardised effect sizes derived from GLMMs or LMMs with 95% confidence intervals (CI).

Trait	Sex-reversed vs concordant mothers	34°C vs. 28°C Incubation	Egg Mass
Reproduction Probability	0.0137 (-0.838, 0.866)	-	-
Fecundity	<b>-0.666</b> <b>(-1.1, -0.233)</b>	-	-
Clutch Rate	-0.312 (-0.926, 0.302)	-	-
Average Eggs per Clutch	<b>-6.98</b> <b>(-11, -2.98)</b>	-	-
Egg Mass (g)	0.412 (-0.0264, 0.85)	-	-
Hatching Probability	-0.398 (-0.82, 0.0235)	-0.398 (-0.82, 0.0235)	<b>1.33</b> <b>(0.603, 2.05)</b>

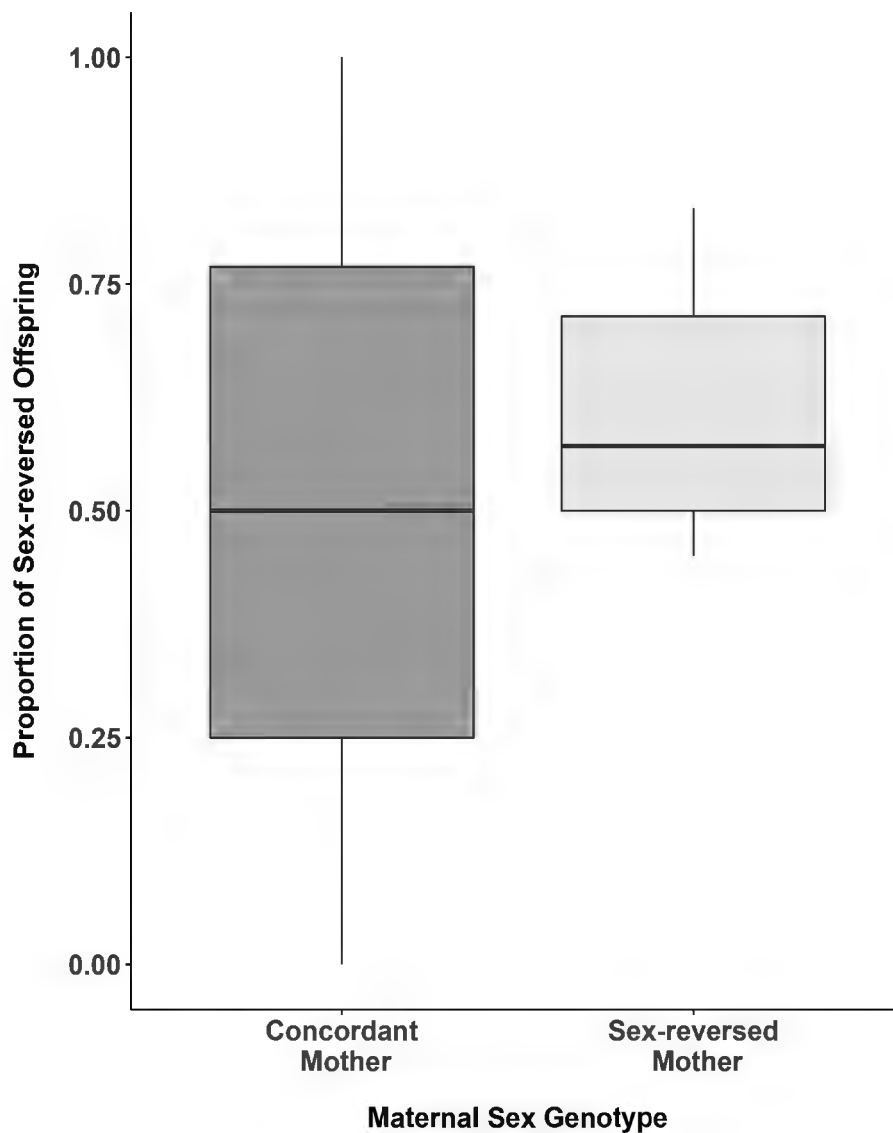
Significant differences (i.e., CI excluding 0) are marked with bold text.



### Maternal Sex Genotype

**Figure 2.1.** Estimated marginal means (least squared means) of maternal sex genotype on reproductive traits across two reproductive seasons of *Pogona vitticeps*. **a.** Probability of reproducing, **b.** fecundity (rate of reproduction), **c.** rate of clutches, **d.** average number of eggs per clutch, **e.** average egg mass, and **f.** probability of hatching. Statistical results are reported in tables 1. Error bars represent  $1 \pm \text{S.E.}$

Overall, concordant mothers produced 1.95 (se  $\pm$  0.43,  $p = .003$ , Table 2.1, Fig 2.1b) times more eggs, more eggs per clutch (6.98 se  $\pm$  2.04;  $p = .002$ , Table 2.1, Fig 2.1d), than sex-reversed mothers across a breeding season. There was no differences in clutching rate (Table 2.1, Fig 2.1c). However, sex-reversed mothers on average had 0.41g (se  $\pm$  0.32) heavier eggs than concordant mothers ( $p = .078$ ; Table 2.1).



**Figure 2.2.** Proportion of sex reversal of ZZ embryos produced from concordant (ZWf) and sex-reversed (ZZf) female *P. vitticeps* at 34°C incubation regime.

Overall, 74.36% of incubated eggs hatched. Larger eggs had a 3.74% higher chance of survival than smaller eggs, and eggs in cooler incubation temperatures had a 1.49% higher chance of hatching than eggs in warmer temperatures (back transformed estimate from Table

1). Neither maternal genotype nor year had a significant effect on hatching probability (Table 1).

Of the 176 ZZ offspring produced, 103 reversed to female at 34°C. Although the reversal rate of offspring produced by sex-reversed mothers was higher (61.6%; n=56) than those produced by concordant mothers (51.7%; n=47), the difference was not significant (Fig. 2.2,  $\chi^2 = 1.24$ ,  $p = .265$ ).

#### 2.4.2 *Influence of maternal sex genotype and captive breeding*

Analysis of colony-related factors for the females in this study (maternal age, generation from wild caught, and inbreeding) excluded all wild caught females (n= 4 ZW females) and their eggs (n= 151). No significant interactions were found between maternal sex genotype and any colony-related factors. Furthermore, no colony-related factors (number of generations in captivity, maternal age class, inbreeding coefficient) had a significant influence on any reproductive trait (Table S2.1). All models maintained a similar trend to the simple models except for average eggs per clutch where maternal sex genotype no longer had a significant effect (Tables S2.1).

#### 2.4.3 *Historical reproductive data*

Across the 5 years of historic reproductive data, concordant mothers produced 1.14 (SE± 0.05, back transformed estimate from Table 2.2) times more eggs than sex-reversed mothers across the breeding season ( $p = .005$ ; Table 2, Fig. 2.3a).

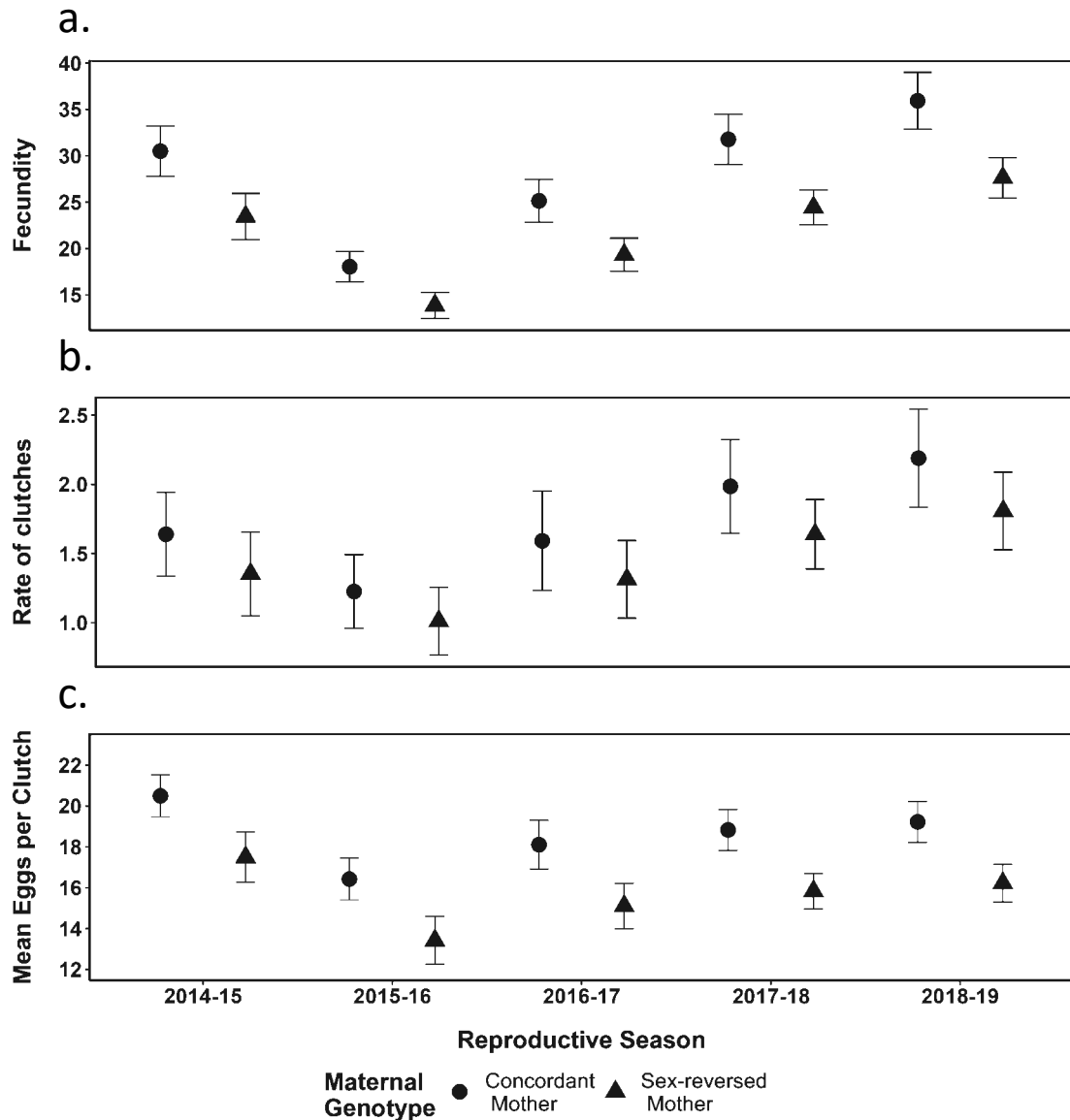


**Table 2.2.** Differences in reproductive traits between concordant (ZWf) and sex-reversed (ZZf) mothers across five historic reproductive seasons shown as non-standardised effect sizes derived from LMMs with 95% confidence intervals (CI).

Trait	Sex-reversed vs concordant mothers	2014-15 vs 2015-16	2014-15 vs 2016-17	2014-15 vs 2017-18	2014-15 vs 2018-19
Fecundity	<b>-0.262</b> <b>(-0.445,</b> <b>-0.080)</b>	-0.525 (-0.716, -0.334)	-0.193 (-0.406, 0.0198)	0.041 (-0.159, 0.241)	0.164 (-0.040, 0.368)
Clutch rate	-0.192 (-0.502, 0.119)	-0.291 (-0.824, 0.260)	-0.0296 (-0.582, 0.523)	0.192 (-0.279, 0.662)	0.289 (-0.173, 0.751)
Average eggs per clutch	<b>-3.00</b> <b>(-4.86,</b> <b>-1.13)</b>	<b>-4.07</b> <b>(-6.69,</b> <b>-1.44)</b>	-2.39 (-5.22, 0.448)	-1.67 (-4.23, 0.894)	-1.27 (-3.85, 1.31)

Significant differences (i.e., CI excluding 0) are marked with bold text.

Clutching rate did not differ (Table 2.2, Fig. 2.3b). However, concordant mothers laid 3 (SE $\pm$  0.95) more eggs per clutch than sex-reversed ( $p = .002$ , Table 2.2, Fig. 2.3c). After the analysis of the historic data showed conflicting results, I revisited the data from Holleley et al., (2015) and found that one sex-reversed female in their study produced 105 eggs across 5 clutches in a single reproductive season. I ran a LMM on the data presented in Holleley *et al.*, 2015 and excluded this hyper-productive female and found no difference between in fecundity per reproductive season (Figure S2.1,  $p = .572$ ).



**Figure 2.3.** Estimated marginal means (least squared means) of maternal sex genotype on reproductive traits across five years of reproductive seasons (2014-2019). **a.** fecundity, **b.** clutching rate, **c.** average number of eggs per clutch. Error bars represent  $1 \pm \text{S.E.}$

## 2.5 Discussion

I report on the reproductive capabilities of sex-reversed and concordant *P. vitticeps* and show that sex reversal does not convey higher fecundity on females. Indeed, I found that concordant females produced significantly more eggs per season, as well as eggs per clutch, than sex-reversed females; a result that contradicts previous findings arising from an earlier study of the same colony of dragons (Holleley et al. 2015). I also show that my results are not an

artifact of inbreeding, varied age class, or domestication as concordant females produced more eggs in each case and the analysis of five additional years of historical data show findings like those emerging from my experiments (Fig. 2.3). In summary, I failed to confirm a reproductive advantage for sex-reversed females over concordant females, as previously posited, but do identify one particularly fecund sex-reversed female as the critical source of the original finding of high fecundity in this sex genotype. My larger sample sizes remove the skewed effect created by that one female.

Furthermore, I show that while embryos of sex-reversed mothers have a higher reversal rate, they do not necessarily have a higher propensity for sex reversal. Rather, ZZ embryos from concordant mothers have a lower pivotal temperature (Fig. 2.2) than previously suggested, which is similar to that of embryos from sex-reversed mothers. These findings again are reflective of my larger sample size where the previous study included only 7 ZZ offspring (concordant male and sex-reversed female) from concordant mothers and 20 ZZ offspring (concordant male and sex-reversed female) from sex-reversed mothers (Holleley et al. 2015). Overall, my data suggest that reversal to female from male does not provide a reproductive advantage in this species and imply that sex reversal is not likely to promote transitions from GSD to TSD in the wild.

Often, fecundity in laboratory animals can be confounded by artifacts of captive breeding such as artificial selection of highly productive breeders (Williams and Hoffman 2009) or by inbreeding depression in colonies with limited mate choice (Foerster et al. 2003). Furthermore, maternal age and body size are often closely correlated with female fecundity (Kroeger et al. 2020) such that age influences offspring number, size, or health and the number of eggs an individual produces (McGinley 1989). The higher rates of reproduction, typically exhibited by smaller, short-lived animals, can also convey a fitness advantage by increasing the chance of survival to reproductive age for an individual's offspring (McGinley 1989). Multiple reproduction events in a single season can further this advantage (Foerster et al. 2003; Williams and Hoffman 2009). I found no interaction between maternal genotype and other factors related to captive breeding in either my experimental or breeding record analyses suggesting that maternal sex genotype, not artifacts of captive breeding, is likely to be the primary driver of fecundity in this species.

In populations that experience mixed modes of sex determination, fecundity could be a key driver of the persistence or dominance of a single mode of sex determination in a population

(Schwanz et al. 2020). Predictive models for wild populations of *P. vitticeps* show that higher fecundity among sex-reversed females under climate warming scenarios could lead to the loss of the W chromosome with only 35-40% sex reversal of ZZ embryos (Schwanz et al. 2020). This would result in a transition from a GSD to TSD mode. Conversely, if sex reversal were to confer equal or lower fecundity, the ZW genotype will remain in a population even when there is 100% reversal of the ZZ genotype. The result would be a mixed population of sex-reversed (ZZ) and concordant (ZW) females (Schwanz et al. 2020). The lower fecundity observed among sex-reversed females could help explain the low rates of sex reversal and stable sex reversal seen in regions of *P. vitticeps*'s native range (Castelli, Georges, Cherryh, et al. 2021; Wild et al. 2022). Without a reproductive advantage, sex-reversed females may produce fewer viable offspring than concordant females. As a result, there would be fewer sex-reversed offspring surviving to reproductive age, thereby enhancing the relative abundance of the ZW genotype.

While measures of fecundity may be lower in sex-reversed females, their eggs tended to be heavier than concordant females and greater egg mass is linked to an increase in hatching probability in both sex genotypes. While there was no difference in hatching success between sex-reversed and concordant mothers, the greater mass of eggs produced by sex-reversed mothers may suggest that sex-reversed mothers are investing more resources in their offspring, which could provide an offspring fitness advantage in the post-hatching environment (Brown and Shine 2009). Further laboratory and field studies are required to determine if maternal sex genotype can confer such a fitness advantage to their offspring and to explore the influence sex reversal might have on early ontological stages of offspring that might influence survival and reproduction.

As global temperatures rise, there is a need to understand how species with sex reversal interact with their environment. Nest site selection in particular could have a profound impact on nest temperature ( Refsnider 2016; Nelson et al. 2018) and hence the likelihood of sex reversal. As I have shown here, the pivotal temperature at which 50 percent of ZZ embryos are reversed is actually lower for concordant females, which could indicate more of reversal within clutches as global temperatures rise. Many studies have been conducted on the potential effects climate change may have on TSD reptile species and how mothers may mitigate the risk of skewed sex ratios, but so far, none have investigated species with sex reversal. Additionally, anecdotal data of constant 36°C incubation regime in the laboratory is

near lethal for these embryos. Therefore, the nesting ecology of species like *P. vitticeps* must be explored to understand when and where females are nesting and if nest site selection may be driving rates of reversal without killing the embryos. If females are unable to mitigate skewed sex ratios, then higher rates of sex-reversed individuals will occur.

Although a transition in sex determining mode does not seem imminent, my results reveal other consequences of sex reversal for population stability. Higher rates of sex reversal in wild populations under warming climate conditions means fewer eggs are likely to be produced each reproductive season. While not immediately concerning for population stability, this lower reproductive rate of sex-reversed female could lead to lower numbers within populations over time if there is a transition to TSD. However, as previous models have shown, the ZW genotype will persist provided that there is immigration of ZW females into a population (Schwanz et al. 2020). My results can aid in future predictive models for this species to understand how populations may decline or persist under climate change.

## 2.6 Supplementary Material

### 2.6.1 *Supplementary tables*

**Table S2.1.** Differences in reproductive traits and hatching probability between concordant (ZWf) and sex-reversed (ZZf) mothers across generation in captivity (Gen.), maternal age class, inbreeding coefficient as well as incubation temperature and egg mass for hatching probability shown as non-standardised effect sizes derived from GLMMs or LMMs with 95% confidence intervals (CI).

Trait	Sex-reversed vs. Concordant Mothers	Gen. Class 1 vs. Gen. Class 2 *	Old vs. Very Young mothers †	Old vs. Young mothers †	Old vs. Very Old mothers †	Not Inbred vs. Inbred §	34C vs. 28C Incubation	Egg Mass
Probability of Reproduction	0.0469 (-0.98, 1.07)	1.11 (-0.273, 2.5)	-0.244 (-1.84, 1.35)	0.312 (-1.69, 1.62)	-0.359 (-2.31, 1.59)	0.0323 (-0.649, 0.354)	-1.22 (-3.55, 1.11)	-0.378 (-3.17, 2.42)
Fecundity	<b>-0.683</b> <b>(-1.13, -0.231)</b>	0.566 (-0.0958, 1.23)	-0.489 (-1.23, 0.256)	-0.22 (-1.06, 0.622)	0.399 (-0.404, 1.2)	-0.514 (-1.12, 0.0953)	-	-
Clutch Rate	-0.461 (-1.02, 0.0985)	0.0594 (-0.772, 0.891)	0.199 (-0.772, 1.17)	0.312 (-0.771, 1.39)	0.736 (-0.975, 0.6)	-0.188 (-0.649, 0.354)	-	-
Average eggs per clutch	-4.93 (-9.79, -0.0748)	4.48 (-2.29, 11.3)	-7.52 (-12.3, 4.58)	-5.52 (-15.2, 0.138)	-3.88 (-14.3, 3.2)	-4.57 (-10.9, 1.78)	-	-
Egg Mass	0.221 (-0.274, 0.716)	0.0136 (-0.699, 0.727)	0.425 (-0.727, 1.02)	0.195 (-0.388, 1.24)	0.144 (-0.699, 0.727)	-0.988 (-0.727, 1.12)	-	-
Hatching Probability	-0.499 (-3.02, 2.02)	-1.23 (-4.9, 2.43)	-2.34 (-6.88, 2.21)	-0.88 (-5.36, 3.6)	-0.266 (-5.18, 4.65)	1.21 (-2.39, 4.81)	-0.432 (-0.95, 0.0854)	<b>1.31</b> <b>(0.449, 2.16)</b>

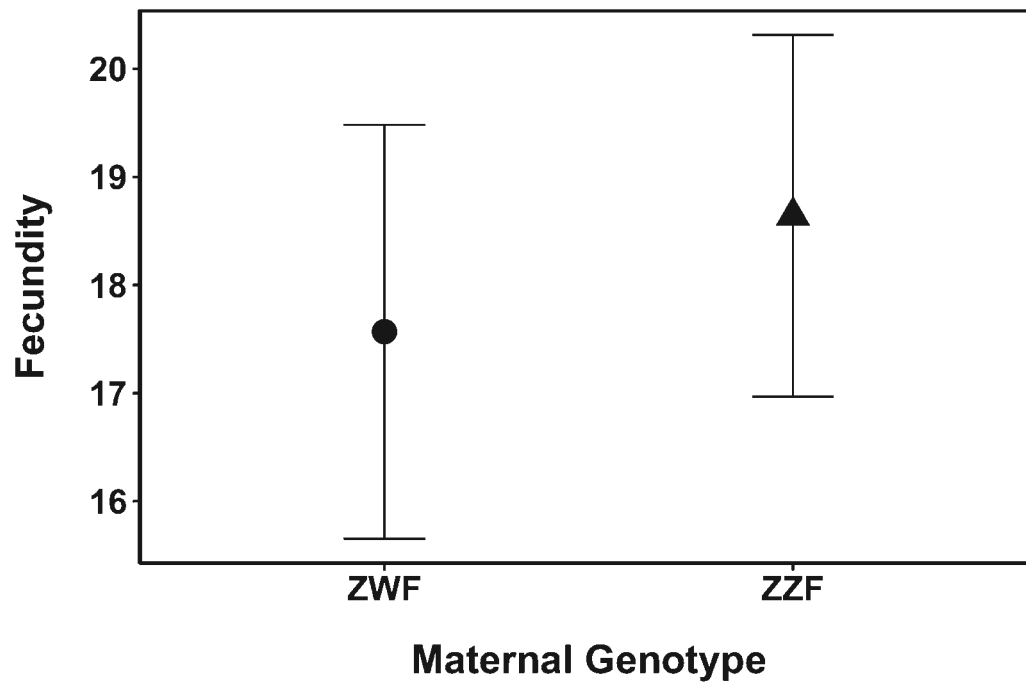
Significant differences (i.e., CI excluding 0) are marked with bold text.

\* Generation class: Class 1 = 0-2 generations in captivity, Class 2 = 3-5 generations

† Maternal age class: Very young = 1-2 years, Young = 3-4 years, Old = 5-6 years, Very old = 6+ years

§ Inbreeding coefficient: Not Inbred- inbreeding coefficient = 0, Inbred- inbreeding coefficient = 0.031

2.6.2 *Supplementary Figures*



**Figure S2.1.** Estimated marginal means of total fecundity of ZWf and ZZf from 2011-12 and 2013-14 reproductive season reported in Holleley et al (2015) excluding the hyper productive female. Error bars represent  $1 \pm \text{S.E.}$





## Chapter 3. Maternal sex reversal provides early benefits for offspring fitness-related phenotypes



*Pogona vitticeps* emerging from its egg.

**Author Contributions:** Sampling design, data collection, analysis, and figures were performed by PRP. Comments from SDS, LES, and JED contributed to the final version of the manuscript.

### 3.1 Abstract

Sex is generally determined by genetics or the environment in vertebrates but, in some, sex determining genes interact with developmental temperatures to produce sex-reversed individuals. This phenomenon provides an opportunity to observe how fitness-related traits could influence transitions between modes of sex determination. The Central Bearded Dragon (*Pogona vitticeps*) typically has genotypic sex determination. However, sex-reversed females are produced when incubated at hot temperatures in the laboratory and occur at low to moderate frequency in some wild populations. The presence of sex reversal in this species could lead to an evolutionary transition between modes of sex determination but this is likely to require a positive relationship between sex reversal and fitness – a relationship that has not yet been explored. Here, I quantified the effects of maternal sex reversal on morphology, growth, locomotor performance, and critical thermal limits of captive bred juvenile *P. vitticeps* to evaluate how these fitness-related traits may influence the persistence or spread of sex reversal. I show that sex-reversed mothers produce larger offspring with better body condition at hatching. Although this size difference dissipates quickly, it does not affect offspring locomotor performance or critical limits. The maternal effects are complicated by the influence of developmental temperatures on locomotor performance as well as the critical thermal minimums of offspring. The larger size at hatching exhibited by the offspring of sex-reversed mothers may allow sex-reversed offspring to better survive to adulthood and enhance transitions from GSD to TSD modes of sex determination.

## 3.2 Introduction

Sex in vertebrates is generally determined by genes located on sex-specific chromosomes (genetic sex determination, GSD) (Bachtrog et al. 2014). However, many species lack sex chromosomes and, in such cases, sex is determined by their environment (Valenzuela and Lance 2004). Environmental sex determination is seen across many taxa where abiotic (e.g., temperature) or external biotic (e.g., behavioural cues) factors determine the sex of an individual (Bachtrog et al. 2014). Temperature-dependent sex determination (TSD) is a common form of environmental sex determination where the temperature experienced during embryonic development determines the sex of the individual (Valenzuela and Lance 2004). Closely related taxa can have differing modes of sex determination, which suggests evolutionary transitions in modes of sex determination could be easily triggered in some lineages (Sarre et al. 2004; Quinn et al. 2011). It is not entirely clear why these transitions occur, but for some species, thermal extremes or other environmental cues may offer a pathway to transition.

Several species of lizards exhibit a combination of GSD and TSD modes, which may offer insight into how shifts in sex determining modes evolve (Quinn et al. 2007; Van Dyke et al. 2021; Whiteley, Castelli, et al. 2021). For these species, the thermal conditions experienced by a developing embryo can override genetic sex determination producing 'sex-reversed' individuals (Quinn et al. 2007; Holleley et al. 2015; Whiteley, Castelli, et al. 2021). When sex-reversed homogametic individuals (XX males or ZZ females) mate with homogametic conspecifics (XX females or ZZ males), the heterogametic sex chromosome (Y or W), becomes rarer and can eventually be lost from the population, which, in turn, will cause the loss of GSD and a transition to TSD (Holleley et al. 2016). A mechanistic basis for this sort of transition has been proposed (Quinn et al. 2011), and such transitions may be accelerated if there are fitness advantages for sex-reversed individuals or if there is a benefit to having TSD (Sarre et al. 2004; Schwanz et al. 2013, 2020; Whiteley, Castelli, et al. 2021). Were such an advantage to exist, sex reversal would increase the potential for GSD species to transition to a system of TSD. The intensity of this advantage could influence the time required for a transition to occur (Schwanz et al. 2020). Thus, species with thermal sex reversal provide the opportunity to investigate how sex determination evolves and to determine whether some populations are pre-disposed to transition between sex determination modes (Whiteley, Castelli, et al. 2021). With global temperatures rising, transitions in sex determination may

become more frequent in ectothermic species that are dependent on their environmental temperatures for embryonic development (Janzen 1994; Holleley et al. 2016; Schwanz et al. 2020).

While developmental temperatures can determine the sex of the individual under TSD or sex reversal, they also strongly influence other phenotypes (e.g., morphology, performance, behaviour, thermal limits, etc. as reviewed by Refsnider et al. 2019). Many temperature-induced plastic phenotypes have fitness implications for individuals (Noble et al. 2018). For example, body morphometrics (e.g., snout-vent length and body condition) predict locomotor performance and overall survival (Losos 2009; Pearson and Warner 2016, 2018; While et al. 2018). While temperature can influence many phenotypes, the adaptive significance of plastic phenotypes is not always clear (West-Eberhard 2003). One suggestion is that environmentally-induced phenotypes can prime developing embryos for their post-hatching environment (environmental matching hypothesis) (Mousseau and Fox 1998). Empirical studies of this relationship are critical because temperature-induced phenotypes could provide a fitness advantage to offspring of sex-reversed parents or to offspring that are themselves sex-reversed, yet examination of these patterns is lacking.

The Central Bearded Dragon (*Pogona vitticeps*, Agamidae) is a widespread lizard native to the central arid zone of Australia that has GSD, with thermal sex reversal in some wild populations (Holleley et al. 2015). These lizards are typically female heterogametic (ZW female, ZZ male; Ezaz et al. 2005), but they also have temperature-induced sex reversal where warmer developmental temperatures produces genetically male, but phenotypically female, individuals (ZZ females) (Quinn et al. 2007; Holleley et al. 2015). While sex reversal in this species occurs in the wild (Castelli, Georges, Cherryh, et al. 2021; Wild et al. 2022), it has not reached the default proportion of individuals (>50%) required to induce an evolutionary transition to TSD (Wild et al. 2022). However, previous lab-based studies suggest that sex reversal conveys beneficial phenotypes in subadult and adult captive *P. vitticeps* (Holleley et al. 2015; Li et al. 2016), which could induce transitions to TSD under a lower rate of sex reversal (Schwanz et al. 2020). In contrast, I have recently shown (Chapter 2 of this thesis) that sex reversal does not actually increase female reproductive output as previously thought. Moreover, while beneficial traits have been seen in sex-reversed animals, it is unclear whether the sex reversal status of parents can influence their offspring's fitness-related phenotypes .

Here, I test the hypothesis that maternal sex genotype (concordant or sex-reversed) of *P. vitticeps* can increase fitness-related phenotypes of their offspring and also revisit the hypothesis that sex-reversal provides a potential fitness benefit by investigating the traits at early life stages. First, I incubated eggs from concordant and sex-reversed captive *P. vitticeps* under two incubation regimes (28°C and 34°C) to produce offspring of all possible sex genotype-phenotype combinations (Fig. 3.1) and measured key morphological phenotypes at hatching, 5-7 weeks, and 10-12 weeks post hatching. Then, I assessed locomotor performance across five temperatures to examine performance advantages. Finally, I determined the critical thermal minimum ( $CT_{min}$ ) and panting threshold ( $T_{pant}$ , proxy for critical thermal maximum) for a subset of offspring. I show that maternal sex genotype can indeed influence some measures of fitness related phenotypes but that incubation temperature also influences these traits. This series of experiments provides a robust insight into how offspring are influenced by their mother's sex genotype, their own developmental temperature, and their own sex genotype to draw conclusions as to how these may influence transitions in modes of sex determination for this species.

### 3.3 Methodology

#### 3.3.1 *Animal breeding and egg incubations*

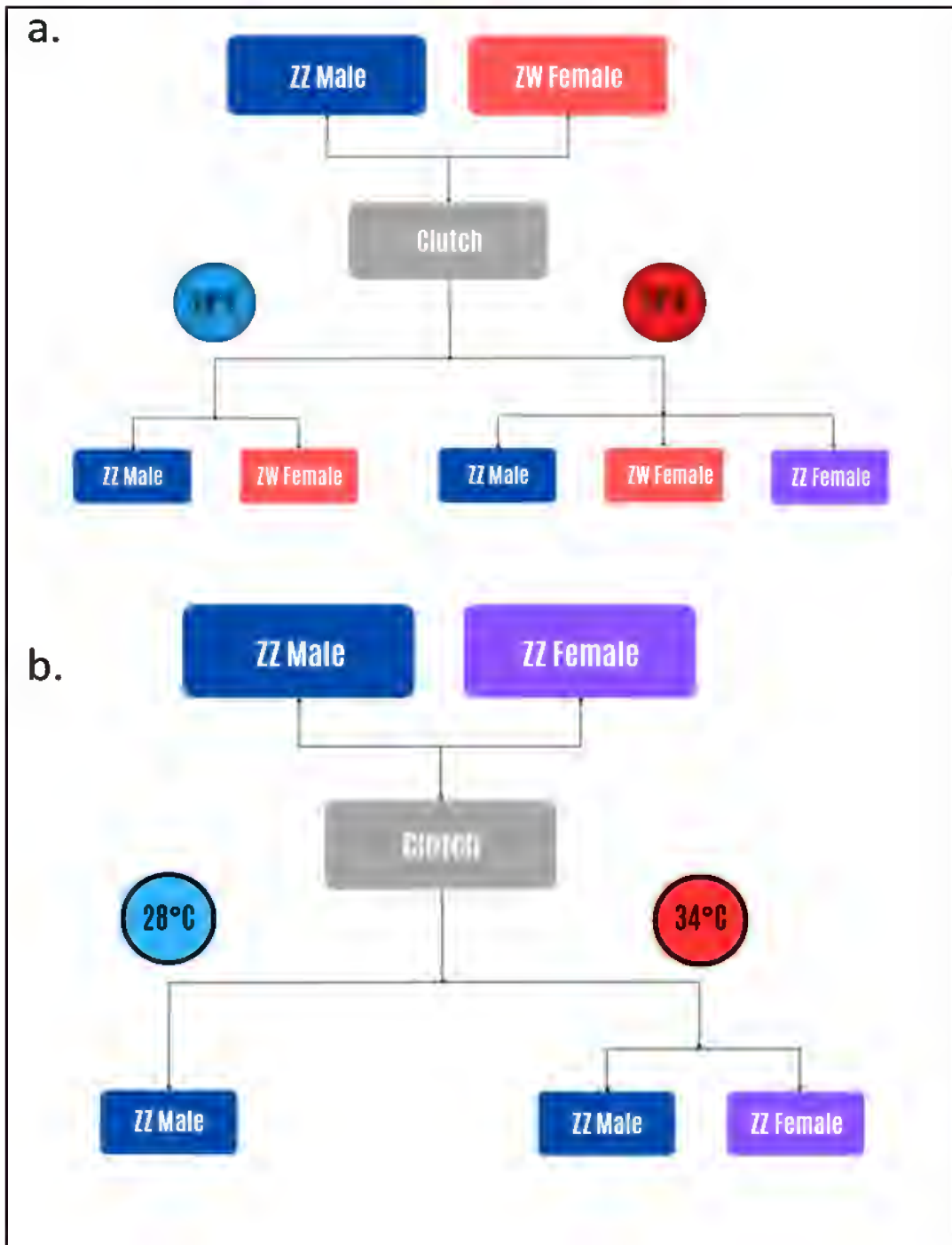
Eggs from ZW and ZZ female *P. vitticeps* were collected for incubation from the University of Canberra breeding colony during the 2019-2020 and 2020-2021 breeding seasons (cohorts one & two respectively). Breeding groups were composed of two to three females of the same genotype and one male per cage (see Fig. 3.1 for breeding design and outcomes). To decrease the likelihood of transgenerational effects, males paired with ZW females had ZW mothers, and males paired with ZZ females had ZZ mothers.

Females were introduced to males in August (2019) post-brumation and prior to the onset of breeding seasons. Breeding groups were maintained across both breeding seasons and were provided with a 10:14, light:dark cycle with full spectrum UV bulbs and heat lamps. They were fed live invertebrates dusted with calcium powder 3 days per week ad libitum and a mixture of shredded vegetables 3 days per week ad libitum. Females were hand fed approximately 10 grams of meat mince mixed with Wombaroo Reptile Supplement

(Wombaroo Food Products, Glen Osmond, South Australia) once per week during the breeding season to ensure females maintained a healthy weight during reproduction.

Females were palpated gently each week to check for developing eggs. When developing eggs reach approximately 2cm, enclosures were monitored daily for nesting behaviours, and females were allowed to nest naturally in their cages in a sand substrate. Eggs were removed from the enclosures within 3 hours of laying, checked for viability via presence of vascularisation, numbered with a non-toxic marker, weighed, and randomly assigned to one of two constant incubation regimes (28°C or 34°C) if assessed as viable.

These two temperature regimes were chosen as they can produce every possible genotype-phenotype from both genotypes of mothers (Fig. 3.1). Once assigned to a temperature, viable eggs were placed into a clear plastic “incubation box” capable of holding 18 eggs in individual spaces and containing moist vermiculite (~-200 kPa; 120% water to vermiculite by mass). Each cell of the container had a vent hole in the lid to aid in gas exchange. The container was placed into a sealed plastic bag with one corner cut to retain moisture levels throughout incubation. Incubation boxes were placed into programmable incubators set to the assigned temperatures ( $\pm 1^\circ\text{C}$ ). Incubators contained a sand heat sink and an open water container to minimise temperature fluctuations and water loss. Egg boxes were checked weekly for egg health throughout the early stages of incubation. Daily checks began as eggs approached 6 weeks in the 34°C treatment and 10 weeks in the 28°C. Dates were noted if eggs died or became mouldy.



**Figure 3.1.** Breeding design and outcome for *Pogona vitticeps* incubated at 28°C or 34°C. **a.** Concordant male (ZZ Male) crossed with concordant female (ZW Female). **b.** Concordant male (ZZ Male) crossed with sex-reversed female (ZZ Female).



### 3.3.2 *Hatchling morphology and husbandry*

At day of hatch (DOH), each hatchling was assigned a unique identification number that was written on its dorsum. Morphometrics data (snout-vent length [SVL], tail length [TL], hind-limb length [HLL], and mass) and phenotypic sex were recorded. Phenotypic sex was determined by hemipenile transillumination (Brown 2009). Hatchlings were housed with clutch mates from the same incubation temperature in open-topped, plastic tubs with a sand substrate and no more than six individuals per tub. Each tub contained cardboard tubes and paper egg carton pieces were provided for hides. Full-spectrum UV bulbs and heat bulbs were placed alternating between tubs (heat from one side, UV from the other). Hatchlings were fed live, gut-loaded crickets twice per day *ad libitum* and twice per week the crickets were dusted with calcium powder. Beginning at 6-7 weeks finely grated vegetables were introduced in addition to crickets. Hatchlings were provided with shallow water dishes that were replenished daily and misted twice per day with water. Individual identification numbers were regularly checked and reapplied as hatchlings moulted.

### 3.3.3 *Locomotor performance*

Morphometrics and phenotypic sex were re-measured and assessed for all hatchlings between 5 and 7 weeks after hatching. Locomotor performance in the form of sprint speed was then assessed across five ecologically relevant temperatures (20, 25, 30, 35, and 40°C). Hatchlings were held in plastic cups and acclimated to the assigned temperature for at least one hour before their sprint trials. A subset of individuals in each trial were checked for skin temperature with an infrared thermometer (Doucette et al. 2022). Sprint speed was measured on a one-metre-long wooden racetrack equipped with infrared sensors (Trackmate Racing™, British Columbia, Canada) placed at the “start” (0cm) and every 25 cm along the track, which relayed the data to Trackmate Racing software when the infrared beams were broken. A starting area was provided at the beginning of the track to allow placement of the lizard without tripping the sensors. A covered hide was placed at the end of the track giving the lizards shelter to run towards.

Sprint trials consisted of five sprints at each of the five temperatures with five-minute rest periods between sprints. No more than two temperature trials were performed in a given day and only when temperature differences across the day did not exceed 10°C. The lizards were chased by tapping the tip of their tail with a paintbrush to the end of the track then placed

back into the incubator for their rest period to maintain the desired temperature. Number of stops per sprint and whether the individual ran or not were recorded as measures of performance behaviour.

### 3.3.4 *Critical thermal limits*

After the completion of sprint performance, a subset of individuals from the second reproductive season were assessed for thermal limits. As individuals varied in age, they were weighed and measured before being subjected to the  $CT_{\min}$  and  $T_{\text{pant}}$  trials to control for morphometric differences in the statistical analyses. The critical thermal limits ( $CT_{\min}$  and panting threshold  $T_{\text{pant}}$ ) for hatchlings were determined using modified method from Senior et al. (2019) and von May et al. (2019). Lizards were placed in partially sealed, transparent plastic containers (chambers) that were submerged in a water bath that was heated or cooled. Temperature of the water bath was controlled by a programable water circulator (Unisat Heater Circulator - 2000W, Thermoline Scientific, Australia), and two small aquarium pumps were used to maintain an even temperature around the submerged chambers. Three cooling coils (TIC-400 Immersion Cooler, Thermoline Scientific, Australia) were used for the  $CT_{\min}$  trials to achieve a  $0.3^{\circ}\text{C}$  per minute cooling rate. Thermometers were placed in the water bath and into the chambers.  $CT_{\min}$  trials were performed first followed by  $T_{\text{pant}}$  two days later to minimise physiological stress.

A K-type thermocouple connected to thermal data logger (Lascar EL-USB-2, Hong Kong) programmed to take the temperature every second was inserted into the cloaca and taped in place using paper masking tape to measure internal body temperature. The animal was then placed into the chamber ensuring that the water levels in the bath were above the height of the lizard. Lizards were acclimated for 30 minutes at  $30^{\circ}\text{C}$  then cooled ( $CT_{\min}$ ) or heated ( $T_{\text{pant}}$ ) at a rate of  $0.3^{\circ}\text{C}$  per minute. Time that the acclimation period began was recorded.

Loss of righting response (LRR) was used to determine  $CT_{\min}$  (as specified in Taylor et al. 2021). I used silicone coated forceps to flip the lizards onto their backs once the chamber temperatures reached  $10^{\circ}\text{C}$  and every 1.5 minutes ( $\sim 1/2^{\circ}\text{C}$  change).  $CT_{\min}$  was determined when lizards could not right themselves. Once  $CT_{\min}$  was reached, the lizards were immediately removed from the chamber and placed on a heating pad to recover. The time of removal, chamber temperature, and water temperature were recorded.

$T_{\text{pant}}$  was determined by visually monitoring each lizard for signs of mouth gaping once the chamber temperatures reached 39°C with  $T_{\text{pant}}$  recorded when the lizard's mouth gaped and began to exhibit gular movements (panting). At that point, the lizard was removed from the chamber and placed into a container at room temperature to recover.

### 3.3.5 *Genotyping for offspring sex*

Blood was drawn from the caudal vein of the lizards after 10 weeks post hatching and dispensed onto FTA Elute card to determine the genetic sex of the individual (Castelli et al. 2021b). I used a PCR-based molecular sex test described in Holleley et al. 2015 using the H2 and F primers. ZW individual show two bands where ZZ show only one (Figure S1). Individuals that showed discordance between genotype and phenotypic sex were classified as sex-reversed.

### 3.3.6 *Statistical analyses*

All statistical analyses were run in R v. 4.2.2 (R Core Team 2022). Comparisons between maternal sex genotype, incubation temperature, and offspring sex genotype are inherently rank deficient as sex-reversed (ZZf) mothers cannot produce concordant female (ZWf) offspring (Fig. 3.1). Therefore, I analysed concordant offspring of both maternal sex genotypes together (Fig. S3.2), and I analysed all warm-incubated offspring data together (Fig. S3.3). For concordant offspring, I compared the influence of maternal sex genotype, incubation temperature, individual sex genotype, and interactions on the response variables. For warm-incubated offspring, I compared the influence of maternal sex genotype, individual sex genotype, and their interaction on the response variables. Nonsignificant interactions were excluded from the model. Cohort year was used in as a covariate in all analyses except thermal limits as they were only assessed in the second cohort. If significance ( $p < .05$  or CI excludes 0) was detected, post-hoc analyses were performed to determine individual differences.

Linear mixed-effect models (LMMs) were used to analyse all hatchling morphometric data, growth data, locomotor performance, and thermal limits. All models of DOH morphometrics included egg mass as a covariate. DOH-mass was used as a covariate for DOH-SVL, TL, and HLL. Body condition was determined by taking the residuals of a linear regression between SVL and mass (determined for DOH, five-weeks, and ten-weeks). DOH-

SVL was used as a covariate for DOH-mass. Nested random effect of clutch number within parents (1|parents/clutch) was used in all DOH models. Five-week morphometric analysis included DOH-mass as a covariate for SVL and DOH-SVL as a covariate for TL, HLL, and mass. Ten-week morphometric analysis included five-week mass as a covariate for SVL and five-week SVL as a covariate for TL, HLL, and mass.

Sprint performance across the five temperatures and critical thermal limits were also analysed with LMMs. Sprint data were not normally distributed and were log-transformed before analysis. Trial temperatures were treated as categorical variables. Nested random effect of trial, individual, clutch number, and parents (1|parents/clutch/individual/trial) were included for performance models.

$CT_{min}$  and  $T_{pant}$  were determined as the mean temperature of the final 60 seconds of these trials as the data loggers did not have digital interfaces to allow for the precise second of the lizards were removed from the chambers. LMMs were used to analyse both  $CT_{min}$  and  $T_{pant}$  with mass before trial used as a covariate. Individual age (1|age) and nested clutch within parents (1|parents/clutch) were used as random effects for these models.

Generalised linear mixed-effect models (GLMMs; family= binomial, logit-link function) were used to analyse survival to five and ten weeks, the probability of sprinting, and the probability of completing the full 100cm sprint. GLMMs (family = Poisson) were used to analyse the number of stops made during each of the five temperature sprint trials. Nested random effect of clutch number within parents (1|parents/clutch) was used in all survival GLMMs. DOH-SVL was used as a covariate in GLMMs of five-week survival and five-week SVL was used in ten-week survival GLMMs. Nested random effect of trial, individual, clutch number, and parents (1|parents/clutch/individual/trial) were included for GLMMs of total stops.

## 3.4 Results

### 3.4.1 *Development*

I incubated 745 eggs produced across 44 clutches by 25 mothers (16 concordant and 11 sex-reversed). Maternal sex genotype did not influence incubation duration (Table S3.1), but incubation duration at the warm temperature of 34°C was shorter relative to incubation at

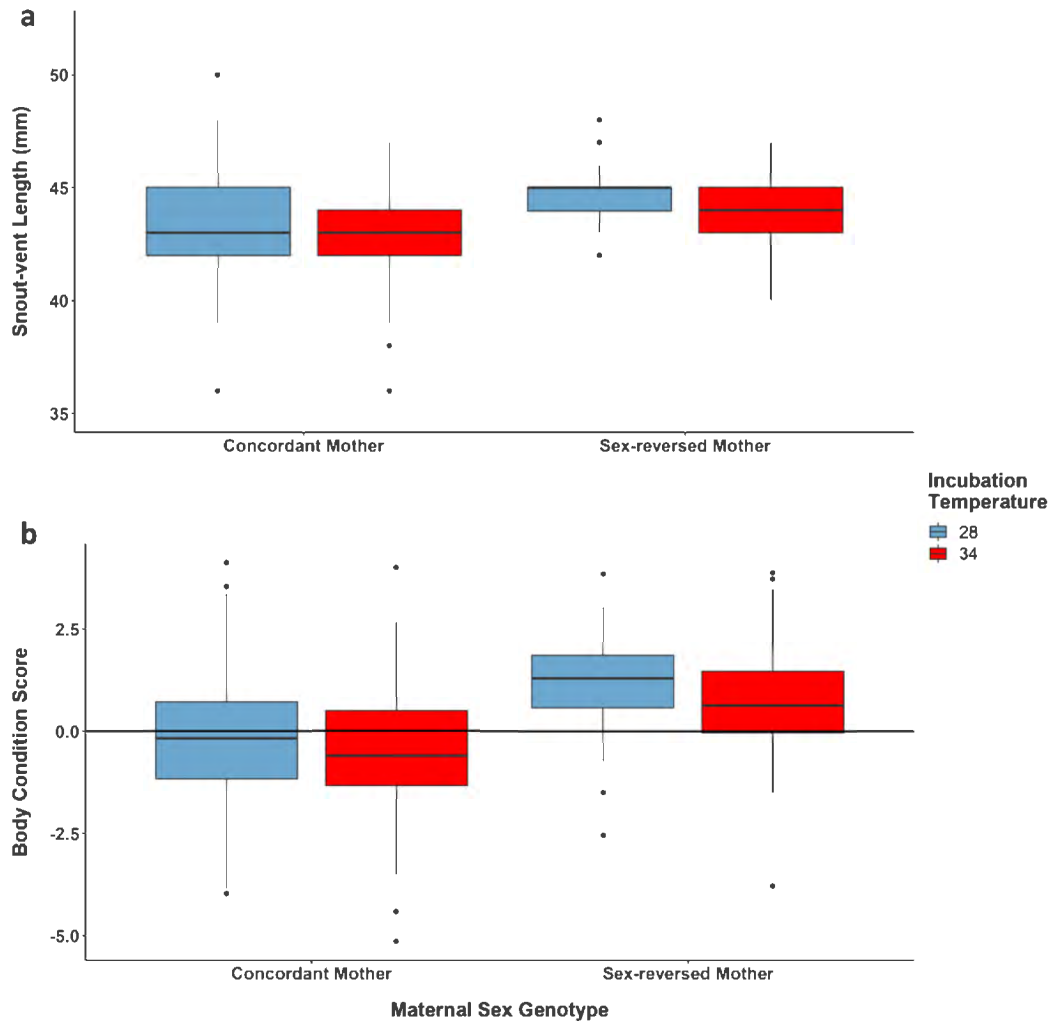
28°C. Egg survival across all incubated eggs was 74.36% as reported in Chapter 2 of this thesis (Chapter 2, Fig 2.2f). Of these, 34 individuals for which complete records could not be obtained were excluded from analysis. Sex ratios and reversal rate are reported in Chapter 2 of this thesis.

### 3.4.2 *Offspring morphology and growth*

Sex-reversed mothers had larger offspring at hatching with better body condition (heavy for their size) than concordant mothers for all models (Figure 3.2, Tables S3.1, S3.2). Incubation at 34°C produced smaller offspring than 28°C (Table S3.1). Offspring sex genotype did not influence any morphological traits at hatching (Table S3.1, S3.2).

Concordant offspring of sex-reversed mothers had lower survival to five weeks than concordant offspring of concordant mothers (Table S3.1). However, male offspring had higher survival than concordant females overall (Table S3.1). No measurements of growth were influenced by the fixed terms for any models (Table S3.1 & S3.2).

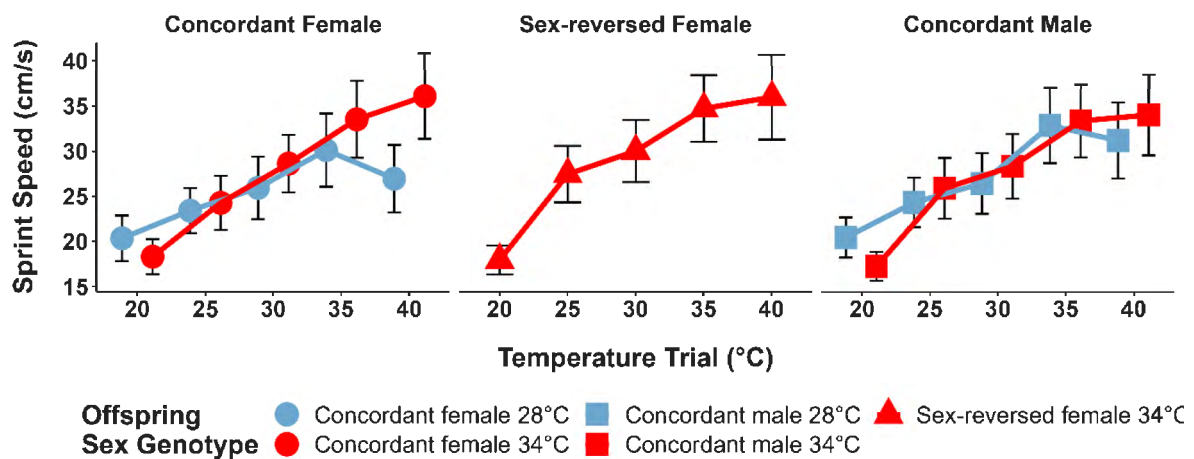
Survival to 10-12 weeks was not significantly influenced by any fixed terms. Sex-reversed female offspring had a higher SVL growth rate than concordant female offspring (Table S3.2). Cool-incubated concordant offspring had higher snout-vent length and hind-limb length growth rates as well as better body condition than warm-incubated concordant offspring (Table S3.1).



**Figure 3.2.** Average snout-vent length (a) and body condition (b) at hatching of *P. vitticeps* across maternal sex genotype and incubation temperature.

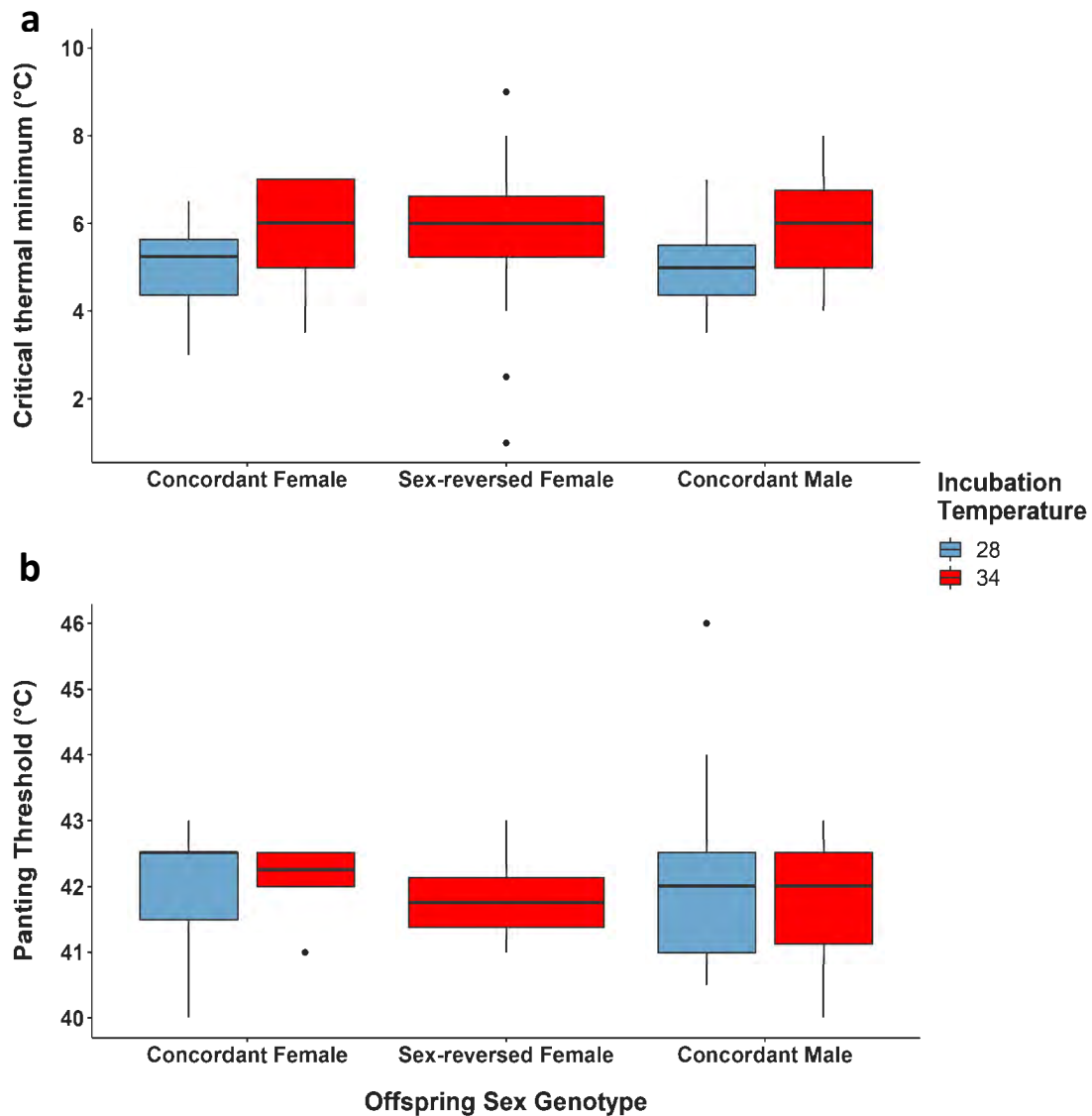
### 3.4.3 Locomotor performance and critical thermal limits

Locomotor performance was assessed across 274 concordant offspring (from both 28°C and 34°C incubation; Fig. S3.2) and 191 warm-incubated offspring (34°C incubation only; Fig. S3.3). Maternal sex genotype had no influence on the probability of completing the first sprint across the five different temperatures for any incubation regime (Tables S3.3, S3.4). For both types of mothers, males had a higher probability of completing the first 25cm sprint than concordant females (Tables S3.3, S3.4). No fixed effects influenced the probability of offspring completing the 100cm sprint (Tables S3.3, S3.4). Average number of stops for individuals was not influenced by any fixed terms.



**Figure 3.3.** Average sprint speed of juvenile *P. vitticeps* for first 25cm split across five different temperature regimes from raw data. Error bars represent +/- 1 standard error.

As not all individuals completed the full 100cm race, I used the first 25cm sprint to determine differences between the fixed effects in both models. Concordant (ZZ male) offspring of sex-reversed mothers ran faster than concordant offspring (ZZ male and ZW female) of concordant mothers (Table S3.3). However, warm incubated offspring of concordant mothers (all sex genotypes) ran faster than those of sex-reversed mothers (ZZ males and females; Table S3.4). There was also a significant interaction between offspring sex genotype and incubation temperature for concordant offspring (Table S3.3), which shows that cool-incubated male offspring perform better than cool-incubated concordant female offspring but warm-incubated concordant female offspring perform better than warm-incubated male offspring (Fig 3.3).



**Figure 3.4.** Box plots of the thermal limits for juvenile *P. vitticeps* between incubation regimes and across the three offspring sex genotypes (concordant females, sex-reversed females, and concordant males). Lines within the boxes represent the mean. **a.** Critical thermal minimum. **b.** Panting threshold (proxy for critical thermal maximum).

Maternal sex genotype did not influence CTmin but cool-incubated offspring had a lower CTmin than those that were warm-incubated (Fig. 3.4a, Table S3.5, S3.6) indicating a key role for incubation temperature in determining this trait. Panting threshold was not influenced by any fixed effects (Fig. 3.4b, Table S3.5, S3.6).



## 3.5 Discussion

Here, I show that maternal sex genotype can influence fitness-related phenotypes of their progeny therefore providing the potential to influence the fitness of sex-reversed individuals. I also show that incubation temperature itself influences these traits as previously seen across various taxa without sex reversal (Deeming 2004; Noble et al. 2018; While et al. 2018; Refsnider et al. 2019) and may be contributing to perceived fitness advantages. The offspring of sex-reversed mothers are larger and have better body condition than those of concordant mothers at hatching. However, these traits dissipate quickly with age and are no longer apparent after five weeks. Incubation temperature seems to be the primary influence of variation in performance traits and  $CT_{min}$  rather than maternal sex genotype or individual genotype. My results suggest that traits passed from sex-reversed mothers may provide their offspring with a head start and that warm developmental temperatures, which can induce sex reversal, also convey beneficial phenotypes. However, the combination of these two variables may not provide enough of an advantage to advance populations towards an evolutionary transition in their mode of sex determination if concordant females are present in a population.

I found that maternal investment in offspring was different between sex-reversed and concordant mothers. Rather than producing more eggs per reproductive season, as found by Holleley et al. 2015, sex-reversed mothers produced larger eggs (See Chapter 2 of this thesis) and larger offspring at hatching than concordant mothers. Larger offspring at hatching can be associated with higher survival (Lovern and Jenssen 2001). Additionally, it is possible that the larger eggs of sex-reversed mothers contain higher quality yolks with differing nutrient levels than concordant mothers affecting the phenotypes of their offspring (Van Dyke and Griffith 2018). This head start provided by sex-reversed mothers could increase offspring survival to maturity (Warner and Lovern 2014) and increase the proportion of sex-reversed females in a population over time.

The influence of incubation temperature on performance traits has been heavily tested across squamate taxa though results vary between species (Noble et al. 2018). My study shows that warmer developmental temperatures increase sprint performance overall and raise  $CT_{min}$ . While warmer developmental temperature seem to provide a more positive influence, I provide evidence supporting the environmental matching hypothesis (Monaghan 2008)

between the two temperature treatments. Offspring from the cooler incubation regime performed better at cooler temperatures than at hotter temperatures and had significantly lower  $CT_{min}$  (Fig. 4.3a). However, I see the opposite trends for the warm incubated offspring. As the warm developmental temperature required for sex reversal in this species is inherently tied to warm ambient temperatures, the temperatures needed to produce sex-reversed females may also make them faster sprinters in their post-hatching environment providing them some further advantage over any cooler-incubated concordant counterparts.

Although I see the potential for advantages of warm developmental temperatures in laboratory settings, female *P. vitticeps* have a breeding season from September to February where early nest temperatures are typically below the reversal threshold while the potential for reversal is seen in late season nests (December-February; see Chapter 4 for details of nest temperatures). This would mean that under current climate conditions only concordant offspring will be produced in the cooler months in the early reproductive season. The matching seen between warm incubation and warm locomotor performance could provide late hatching sex-reversed offspring with some advantage when competing with early hatching, cool-incubated offspring that have had time to acclimate to their shared post-developmental environment. Though Pearson and Warner (2018) found that warm incubation temperatures produce faster hatchling *Anolis sagrei* lizards, they found that early hatching dramatically increased survival over late hatching in a natural setting. Their results could be applied here to predict that the evolutionary stability of sex reversal that is seen in some natural populations (Wild et al. 2022) could be, at least in part, due to sex-reversed offspring being produced only under late season conditions.

As global temperatures rise, the proportion of sex reversal across the range of *P. vitticeps* is also likely to increase (Holleley et al. 2015; Schwanz et al. 2020). The warmer nest temperatures alone could shift this system from GSD to TSD (Schwanz et al. 2020). However, modest levels of immigration will maintain a mixed GSD system in simulated populations with warm nest temperatures, so transitions to TSD would require strongly advantageous traits in sex reversed individual compared with concordant individuals (Schwanz et al. 2020). Combining my results with the behavioural phenotypes (boldness and higher activity) associated with laboratory raised sex-reversed females (Li et al. 2016) could provide the means for sex-reversed individuals to successfully disperse through a population just as bolder, faster invasive species disperse into novel environments (Bensky and Bell

2022). However, by showing that a warmer developmental environment provides the same beneficial phenotypes to concordant female offspring, my data suggest that warmer nest temperatures could also increase the migration of ZW females and thus act to maintain the W chromosome despite shifts in climate.

Overall, I tested the proposition that maternal sex reversal and incubation temperature provide offspring with fitness-enhancing phenotypes that could increase the prevalence of sex reversal in a population. I show that maternal sex reversal does convey benefits at hatching that dissipate within five weeks, while incubation temperature provides offspring with longer lasting locomotor benefit that follows the environmental matching hypothesis. While these benefits may provide some advantages to sex-reversed offspring, warm-incubated concordant female offspring have the same locomotor capabilities as sex-reversed female offspring and would experience the same post hatching environment. As concordant mothers produce more eggs per clutch (Chapter 2), any warm incubated concordant offspring would likely provide heavy competition for the sex-reversed offspring in the post-developmental environment. Furthermore, the cool-incubated, concordant offspring (of both concordant and sex-reversed mothers), which hatch early in the season, are likely to dominate the same environment and provide a selective pressure against late produced offspring. This seasonal balance coupled with the higher fecundity of concordant females may explain how sex-reversed females are able to persist stably in current populations without shifting the mode of sex determination and how the W chromosome may be preserved in future populations.

## 3.6 Supplementary Materials

### 3.6.1 *Supplementary Tables*

**Table S3.1.** Differences in offspring morphological traits between concordant (ZWF) and sex-reversed (ZZF) mothers, incubation regime, their interaction, and male and concordant female offspring shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs concordant mothers	34°C vs. 28°C incubation	Maternal sex genotype by incubation interaction	Male vs. concordant female offspring
Hatching Measure				
Incubation Duration	0.196 (-1.52, 1.91)	<b>-29.5</b> <b>(-30, -29.1)</b>	-0.898 (-1.81, 0.00892)	0.624 (-0.0812, 1.33)
SVL	<b>0.97</b> <b>(0.441, 1.5)</b>	<b>-0.278</b> <b>(-0.544, -0.0125)</b>	-0.283 (-0.93, 0.36)	0.164 (-0.13, 0.457)
TL	<b>2.45</b> <b>(0.182, 4.72)</b>	<b>2.31</b> <b>(1.61, 3)</b>	-1.42 (-2.93, 0.093)	0.162 (-0.517, 0.842)
HLL	-0.15 (-0.607, 0.307)	0.184 (-0.0245, 0.393)	-0.185 (-0.636, 0.267)	0.074 (-0.131, 0.279)
Mass	-0.0736 (-0.24, 0.0933)	0.0165 (-0.0378, 0.0708)	-0.0399 (-0.157, 0.0777)	<b>0.0566</b> <b>(0.00337, 0.11)</b>
Body Condition	<b>1.12</b> <b>(0.531, 1.71)</b>	-0.225 (-0.526, 0.0767)	-0.241 (-0.893, 0.411)	0.127 (-0.168, 0.421)
5-week				
Survival	<b>-2.51</b> <b>(-4.69, -0.334)</b>	0.979 (-0.28, 2.24)	1.57 (-1.2, 4.33)	<b>1.34</b> <b>(0.0481, 2.63)</b>
SVL Growth	-0.0315 (-0.0638, 0.000727)	0.00187 (-0.0156, 0.0194)	0.00746 (-0.0318, 0.0468)	-0.00771 (-0.024, 0.00862)
TL Growth	-0.0289 (-0.0853, 0.0276)	0.0149 (-0.0137, 0.0435)	-0.000102 (-0.0629, 0.0627)	-0.0097 (-0.0372, 0.0179)
HLL Growth	-0.0106 (-0.0274, 0.00616)	-0.0119 (-0.0208, -0.00308)	0.00833 (-0.0113, 0.028)	-0.00298 (-0.0112, 0.00521)
Mass Growth	0.00396 (-0.00411, 0.012)	-0.00103 (-0.0052, 0.00314)	-0.00608 (-0.0155, 0.00333)	-0.00181 (-0.00572, 0.0021)
Body Condition	-0.227 (-0.707, 0.252)	-0.473 (-1.27, 0.325)	0.449 (-0.647, 1.55)	0.198 (-0.266, 0.662)
10-week				
Survival	-1.84 (-3.69, 0.0027)	0.367 (-0.598, 1.33)	1.47 (-0.787, 3.72)	0.217 (-0.72, 1.16)
SVL Growth	-0.00196 (-0.0303, 0.0263)	<b>-0.0189</b> <b>(-0.0324, -0.00529)</b>	0.02 (-0.0096, 0.0495)	-4.08e-05 (-0.0132, 0.0132)
TL Growth	<b>0.0525</b> <b>(0.0159, 0.0892)</b>	0.0194 (-0.0022, 0.041)	-0.0497 (-0.0965, -0.00288)	-0.0146 (-0.0361, 0.00689)
HLL Growth	-0.0018 (-0.0107, 0.00715)	<b>-0.00947</b> <b>(-0.0159, -0.00306)</b>	0.000715 (-0.0123, 0.0137)	-0.00531 (-0.0119, 0.00125)
Mass Growth	0.00135 (-0.00649, 0.0092)	0.000646 (-0.00333, 0.00463)	-0.000409 (-0.00901, 0.00819)	-0.0018 (-0.00563, 0.00202)
Body Condition	-0.88 (-2.41, 0.652)	<b>-1.29</b> <b>(-2, -0.569)</b>	1.24 (-0.322, 2.8)	-0.102 (-0.802, 0.598)

Significant differences (i.e., CI excluding 0) are marked with bold text.

**Table S3.2.** Differences in offspring morphological traits incubated at 34°C between concordant (ZWf) and sex-reversed (ZZf) mothers, sex-reversed (ZZ) and concordant (ZW) female offspring, and male and concordant female offspring shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. concordant mothers	Sex-reversed female vs. concordant female offspring	Male vs. concordant female offspring
Hatching Measure			
SVL	<b>1.08</b> ( <b>0.424, 1.73</b> )	-0.0666 (-0.458, 0.325)	0.0936 (-0.329, 0.516)
TL	1.81 (-0.511, 4.13)	-0.856 (-1.7, -0.0141)	-0.222 (-1.13, 0.688)
HLL	-0.131 (-0.638, 0.375)	0.0106 (-0.245, 0.266)	0.0595 (-0.216, 0.335)
Mass	-0.103 (-0.252, 0.0465)	0.00577 (-0.0648, 0.0763)	0.0303 (-0.0459, 0.106)
Body Condition	<b>1.08</b> ( <b>0.42, 1.75</b> )	-0.067 (-0.459, 0.325)	0.0761 (-0.346, 0.498)
5-week Measure			
Survival	-1.23 (-3.32, 0.865)	0.195 (-1.28, 1.67)	2.12 (-0.272, 4.51)
SVL Growth	-0.0211 (-0.0556, 0.0134)	-0.0195 (-0.0439, 0.00496)	-0.00598 (-0.0311, 0.0191)
TL Growth	-0.043 (-0.0932, 0.00717)	0.00241 (-0.0311, 0.0359)	-0.0217 (-0.0568, 0.0134)
HLL Growth	-0.00213 (-0.0203, 0.016)	0.000476 (-0.0107, 0.0117)	0.00212 (-0.00941, 0.0137)
Mass Growth	0.00396 (-0.00411, 0.012)	-0.00103 (-0.0052, 0.00314)	-0.00181 (-0.00572, 0.0021)
Body Condition	0.146 (-0.582, 0.875)	-0.321 (-0.887, 0.245)	0.175 (-0.404, 0.753)
10-week Measure			
Survival	-0.377 (-2.42, 1.67)	-0.879 (-2.22, 0.458)	-0.185 (-1.65, 1.28)
SVL Growth	0.00156 (-0.0257, 0.0289)	<b>0.0254</b> ( <b>0.00656, 0.0443</b> )	0.00599 (-0.0145, 0.0265)
TL Growth	-0.000675 (-0.0407, 0.0393)	-0.00323 (-0.0253, 0.0189)	0.00156 (-0.0229, 0.026)
HLL Growth	0.00403 (-0.00478, 0.0128)	-0.000569 (-0.00749, 0.00635)	-0.00529 (-0.0128, 0.00226)
Mass Growth	0.00195 (-0.00401, 0.00791)	-0.00122 (-0.00572, 0.00329)	-0.00123 (-0.00612, 0.00366)
Body Condition	-0.136 (-1.54, 1.27)	0.612 (-0.314, 1.54)	0.0606 (-0.952, 1.07)

Significant differences (i.e., CI excluding 0) are marked with bold text.

**Table S3.3.** Differences in offspring morphological traits incubated at 34°C between concordant (ZWf) and sex-reversed (ZZf) mothers, sex-reversed (ZZ) and concordant (ZW) female offspring, and male and concordant female offspring shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. Concordant mothers	Sex-reversed female vs. concordant female offspring	Male vs. concordant female offspring
<b>Hatching Measure</b>			
Snout-vent Length	<b>1.08</b> <b>(0.424, 1.73)</b>	-0.0666 (-0.458, 0.325)	0.0936 (-0.329, 0.516)
Tail Length	1.81 (-0.511, 4.13)	-0.856 (-1.7, -0.0141)	-0.222 (-1.13, 0.688)
Hind-limb length	-0.131 (-0.638, 0.375)	0.0106 (-0.245, 0.266)	0.0595 (-0.216, 0.335)
Mass	-0.103 (-0.252, 0.0465)	0.00577 (-0.0648, 0.0763)	0.0303 (-0.0459, 0.106)
Body condition	<b>1.08</b> <b>(0.42, 1.75)</b>	-0.067 (-0.459, 0.325)	0.0761 (-0.346, 0.498)
<b>5-week Measure</b>			
Survival	-1.23 (-3.32, 0.865)	0.195 (-1.28, 1.67)	2.12 (-0.272, 4.51)
Snout-vent Length growth	-0.0211 (-0.0556, 0.0134)	-0.0195 (-0.0439, 0.00496)	-0.00598 (-0.0311, 0.0191)
Tail growth	-0.043 (-0.0932, 0.00717)	0.00241 (-0.0311, 0.0359)	-0.0217 (-0.0568, 0.0134)
Hind-limb growth	-0.00213 (-0.0203, 0.016)	0.000476 (-0.0107, 0.0117)	0.00212 (-0.00941, 0.0137)
Mass growth	0.00396 (-0.00411, 0.012)	-0.00103 (-0.0052, 0.00314)	-0.00181 (-0.00572, 0.0021)
Body condition	0.146 (-0.582, 0.875)	-0.321 (-0.887, 0.245)	0.175 (-0.404, 0.753)
<b>10-week Measure</b>			
Survival	-0.377 (-2.42, 1.67)	-0.879 (-2.22, 0.458)	-0.185 (-1.65, 1.28)
Snout-vent Length growth	0.00156 (-0.0257, 0.0289)	<b>0.0254</b> <b>(0.00656, 0.0443)</b>	0.00599 (-0.0145, 0.0265)
Tail growth	-0.000675 (-0.0407, 0.0393)	-0.00323 (-0.0253, 0.0189)	0.00156 (-0.0229, 0.026)
Hind-limb growth	0.00403 (-0.00478, 0.0128)	-0.000569 (-0.00749, 0.00635)	-0.00529 (-0.0128, 0.00226)
Mass growth	0.00195 (-0.00401, 0.00791)	-0.00122 (-0.00572, 0.00329)	-0.00123 (-0.00612, 0.00366)
Body condition	-0.136 (-1.54, 1.27)	0.612 (-0.314, 1.54)	0.0606 (-0.952, 1.07)

Significant differences (i.e., CI excluding 0) are marked with bold text.

**Table S3.4.** Differences in offspring performance traits between concordant (ZWF) and sex-reversed (ZZF) mothers, incubation regime, male and concordant female offspring, and the five temperatures performance was assessed shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. concordant mothers	34°C vs. 28°C incubation regime	Male vs. concordant female offspring	Maternal sex genotype by incubation temperature interaction	Incubation Temperature : Offspring Sex Genotype Interaction	20C Race vs. 25C Race	20C Race vs. 30C Race	20C Race vs. 35C Race	20C Race vs. 40C Race
25cm Running Probability	0.152 (-0.623, 0.927)	-0.07 (-0.435, 0.295)	<b>0.375</b> ( <b>0.02, 0.731</b> )	-	-	-0.14 (-0.601, 0.321)	-0.12 (-0.589, 0.35)	-0.17 (-0.629, 0.287)	-0.24 (-0.705, 0.228)
100 cm Running Probability	0.218 (-0.266, 0.702)	-0.18 (-0.406, 0.0426)	-0.00427 (-0.229, 0.221)	-	-	-0.11 (-0.358, 0.146)	<b>0.29</b> ( <b>0.0155, 0.563</b> )	0.14 (-0.126, 0.399)	0.13 (-0.143, 0.395)
Maximum Burst Sprint	6.18 (-6.580, 18.90)	5.83 (-0.802, 12.50)	4.31 (-2.26, 10.90)	5.95 (-8.790, 20.700)	<b>-10.90</b> ( <b>-21.100, -0.711</b> )	<b>16.30</b> ( <b>12.90, 19.80</b> )	<b>26.10</b> ( <b>22.600, 29.600</b> )	<b>36.70</b> ( <b>33.200, 40.100</b> )	<b>37.30</b> ( <b>33.800, 40.800</b> )
25 cm Sprint	0.214 (-0.0428, 0.471)	<b>0.147</b> ( <b>0.00942, 0.284</b> )	<b>0.133</b> ( <b>0.000804, 0.2660</b> )	-	<b>-0.213</b> ( <b>-0.403, -0.023</b> )	<b>0.233</b> ( <b>0.195, 0.272</b> )	<b>0.333</b> ( <b>0.295, 0.372</b> )	<b>0.464</b> ( <b>0.426, 0.503</b> )	<b>0.418</b> ( <b>0.379, 0.457</b> )
100 cm Sprint	0.118 (-0.092, 0.33)	0.00333 (-0.095, 0.1)	0.0172 (-0.085, 0.12)	-	<b>-0.202</b> ( <b>-0.392, -0.0109</b> )	<b>0.317</b> ( <b>0.28, 0.35</b> )	<b>0.471</b> ( <b>0.44, 0.5</b> )	<b>0.612</b> ( <b>0.58, 0.65</b> )	<b>0.586</b> ( <b>0.55, 0.62</b> )
Stops per trial	-0.0787 (-0.183, 0.0256)	-0.06 (-0.302, 0.182)	0.0461 (-0.0614, 0.154)	-	-	<b>-0.074</b> ( <b>-0.111, -0.0363</b> )	<b>-0.18</b> ( <b>-0.218, -0.141</b> )	-0.27 (-0.312, -0.233)	-0.25 (-0.29, -0.21)

Significant differences (i.e., CI excluding 0) are marked with bold text.



**Table S3.5.** Differences in offspring morphological traits incubated at 34°C between concordant (ZWf) and sex-reversed (ZZf) mothers, sex-reversed (ZZ) and concordant (ZW) female offspring, concordant male and concordant female offspring, and the five temperatures performance was assessed shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. concordant mothers	Sex-reversed female vs. concordant female offspring	Concordant male vs. concordant female offspring	20C Race vs. 25C Race	20C Race vs. 30C Race	20C Race vs. 35C Race	20C Race vs. 40C Race
25cm Running Probability	0.0713 (-0.788, 0.93)	0.411 (-0.152, 0.973)	<b>0.592</b> ( <b>0.0196</b> , <b>1.16</b> )	0.21 (-0.394, 0.814)	-0.0712 (-0.66, 0.518)	-0.134 (-0.703, 0.435)	-0.0747 (-0.666, 0.516)
100 cm Running Probability	0.348 (-0.138, 0.834)	0.214 (-0.124, 0.553)	0.0619 (-0.26, 0.384)	0.11 (-0.202, 0.422)	<b>0.352</b> ( <b>0.0181</b> , <b>0.686</b> )	<b>0.362</b> ( <b>0.034</b> , <b>0.689</b> )	0.0808 (-0.235, 0.397)
Maximum Burst Sprint	-2.34 (-11.80, 7.10)	5.74 (-1.690, 13.20)	-0.0913 (-7.620, 7.43)	<b>21.4</b> ( <b>17.20</b> , <b>25.60</b> )	<b>30.0</b> ( <b>25.80</b> , <b>34.30</b> )	<b>42.30</b> ( <b>38.0</b> , <b>46.50</b> )	<b>40.50</b> ( <b>36.30</b> , <b>44.80</b> )
25 cm Sprint	-0.0249 (-0.243, 0.192)	0.0275 (-0.119, 0.174)	0.00289 (-0.144, 0.15)	<b>0.274</b> ( <b>0.283</b> , <b>0.373</b> )	<b>0.418</b> ( <b>0.371</b> , <b>0.463</b> )	<b>0.526</b> ( <b>0.528</b> , <b>0.618</b> )	<b>0.552</b> ( <b>0.528</b> , <b>0.618</b> )
100 cm Sprint	-0.0964 (-0.278, 0.0851)	0.114 (-0.0257, 0.254)	0.00747 (-0.136, 0.151)	<b>0.399</b> ( <b>0.360</b> , <b>0.438</b> )	<b>0.536</b> ( <b>0.497</b> , <b>0.576</b> )	<b>0.724</b> ( <b>0.685</b> , <b>0.764</b> )	<b>0.709</b> ( <b>0.669</b> , <b>0.749</b> )
Stops per trial	0.209 (-0.0236, 0.441)	-0.0755 (-0.233, 0.0823)	0.0105 (-0.146, 0.167)	<b>-0.0845</b> ( <b>-0.129</b> , <b>-0.0401</b> )	<b>-0.17</b> ( <b>-0.216</b> , <b>-0.123</b> )	<b>-0.328</b> ( <b>-0.376</b> , <b>-0.281</b> )	<b>-0.314</b> ( <b>-0.362</b> , <b>-0.267</b> )

Significant differences (i.e., CI excluding 0) are marked with bold text.

**Table S3.6.** Differences in offspring critical thermal minimum and panting threshold between concordant (ZWF) and sex-reversed (ZZF) mothers, incubation regime, and concordant male and concordant female offspring was assessed shown as non-standardised effect sizes with 95% confidence intervals (CI).

Trait	Sex-reversed vs. concordant mothers	34°C vs. 28°C incubation regime	Concordant male vs. concordant female offspring
Average CT <sub>min</sub>	0.0587 (-0.823, 0.940)	<b>1.32 (0.531, 2.100)</b>	0.329 (-0.468, 1.130)
Minimum CT <sub>min</sub>	0.0816 (-0.827, 0.991)	<b>0.936 (0.167, 1.710)</b>	0.291 (-0.474, 1.06)
Average T <sub>pant</sub>	-0.673 (-1.77, 0.427)	-0.0292 (-0.900, 0.841)	0.755 (-0.161, 1.67)
Maximum T <sub>pant</sub>	<b>-1.10 (-1.90, -0.320)</b>	0.127 (-0.600, 0.850)	0.620 (-0.160, 1.40)

Significant differences (i.e., CI excluding 0) are marked with bold text.

**Table S3.7.** Differences in offspring critical thermal minimum and panting threshold incubated at 34°C between concordant (ZWf) and sex-reversed (ZZf) mothers, sex-reversed (ZZ) and concordant (ZW) female offspring, and concordant male and concordant female offspring shown as non-standardised effect sizes with 95% confidence intervals (CI).

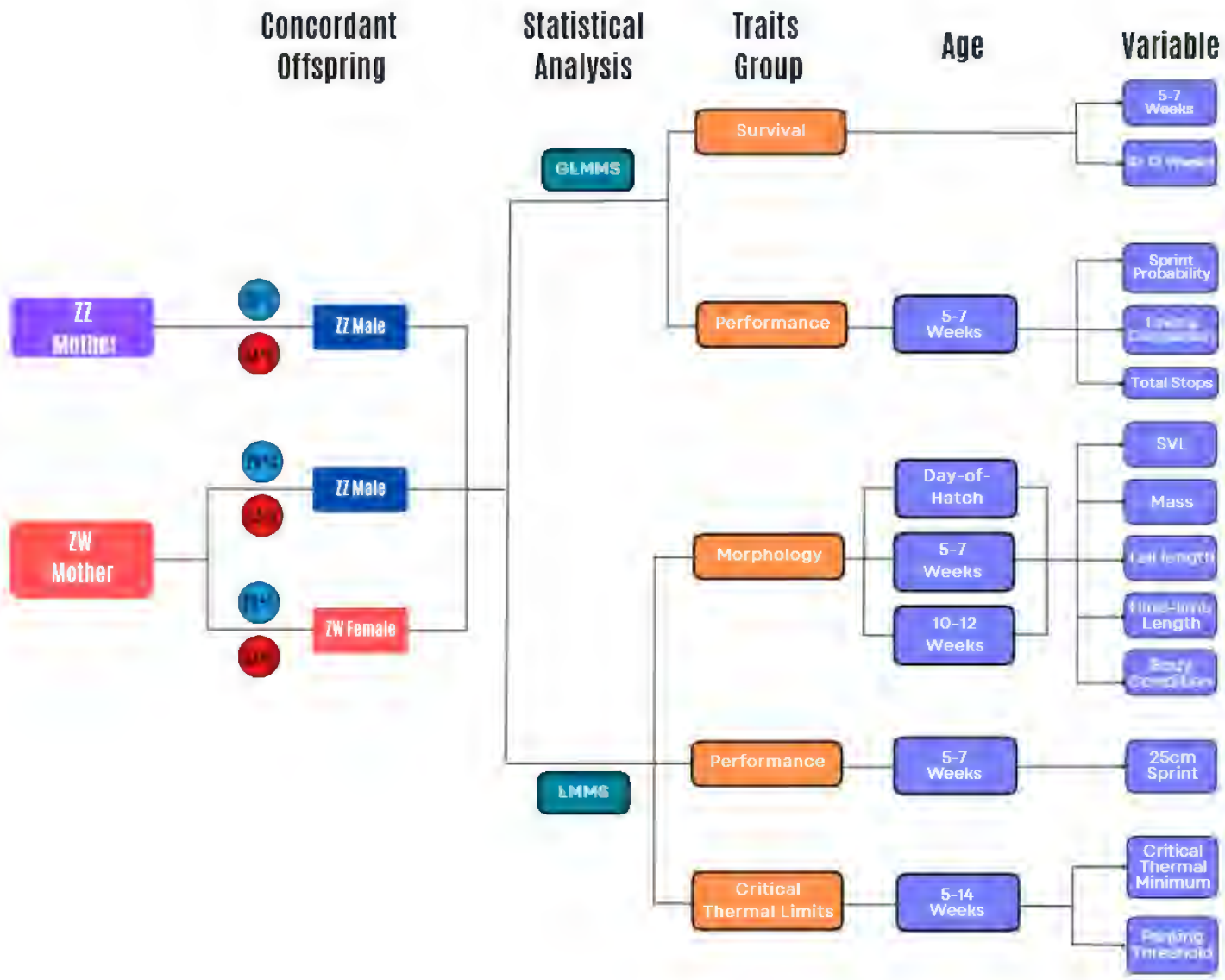
Trait	Sex-reversed vs. concordant mothers	Sex-reversed female vs. concordant female offspring	Concordant male vs. concordant female offspring
Average CT <sub>min</sub>	-0.539 (-1.79, 0.709)	0.456 (-0.514, 1.43)	<b>1.34 (0.194, 2.48)</b>
Minimum CT <sub>min</sub>	0.369 (-0.992, 1.73)	<b>-0.138 (1.28, 1.01)</b>	0.518 (-0.890, 1.93)
Average T <sub>pant</sub>	0.285 (-0.834, 1.40)	0.686 (-0.294, 1.66)	0.411 (-0.0245, 0.0626)
Maximum T <sub>pant</sub>	-0.172 (-1.30, 0.959)	0.244 (-0.424, 0.913)	0.567 (-0.209, 1.34)

Significant differences (i.e., CI excluding 0) are marked with bold text.

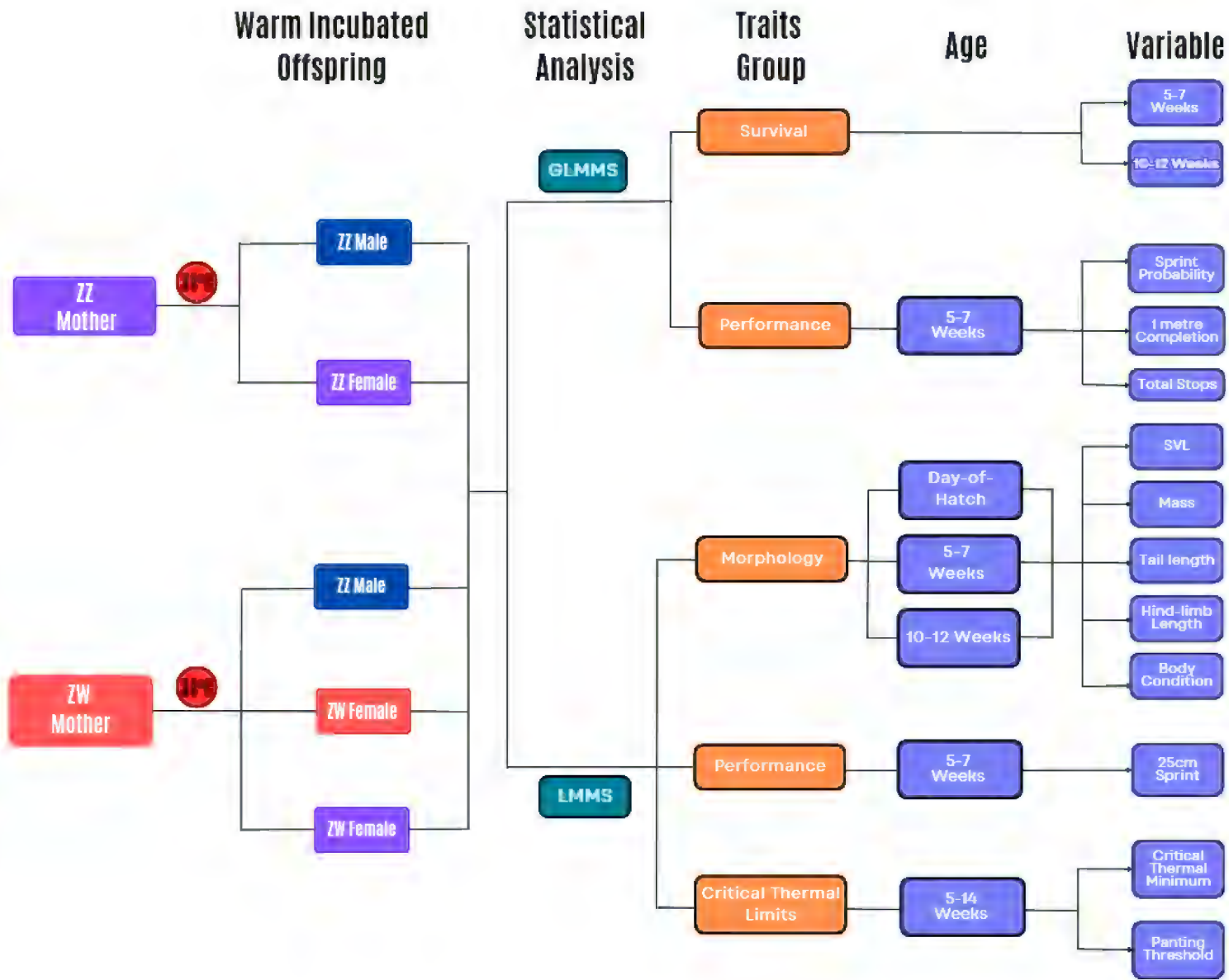
3.6.2 *Supplementary Figures*



**Figure S3.1.** Gel image of H2F PCR sex test for determining genotypic sex of *Pogona vitticeps*. One band (A) indicates ZZ individuals and two bands (B) indicate ZW individuals.



**Figure S3.2.** Flow diagram of how traits of concordant offspring were analysed.



**Figure S3. 3.** Flow diagram of how traits of warm-incubated offspring were analysed.



# Chapter 4. Predicting risk of temperature-driven sex reversal through microhabitat modelling



Panoramic view of Witjira National Park, South Australia



**Author Contributions:** Sampling design, data collection, analysis, and figures were performed by PRP. Comments from SDS, LES, JED, and Kristoffer H. Wild contributed to the final version of the manuscript.

## 4.1 Abstract

Nest site selection by oviparous reptiles can affect many environmental characteristics experienced by incubating eggs and can alter phenotypes, including sex, in ways that persist into adulthood. Such selection of nest sites could become critical as the warmer soil temperatures caused by global heating may skew sex ratios in thermally sensitive species or increase nest mortality. Maternal nesting behaviour may therefore be essential to mitigating the risks posed by a heating climate through the choice of cooler nest sites or shifts in the timing of reproduction. The central bearded dragon lizard (*Pogona vitticeps*) has thermally labile sex determination, with ZZ male/ZW female genetic sex determination (GSD) and a high temperature override that produces sex-reversed (ZZ) females. This phenomenon has been observed in natural populations, yet little is known of their nesting behaviours and if these behaviours may mitigate or perpetuate sex reversal in free-ranging populations. I quantified microclimate variables from five nest sites chosen by free-ranging females and potential nest sites across eight geographic locations within the species' range. I used these data to inform microclimate models using NicheMapR Shiny app web interface to predict the risk of sex reversal at these points. My observed nesting data shows that females preferentially chose relatively open habitats that provide warmer incubation temperatures across the reproductive season, which may explain the presence of sex reversal in some populations. I show that the NicheMapR models frequently under-predicted soil temperatures likely due to our relatively minimal parameter entries, and I offer a general correction that improves the predictions and can be utilised by future studies without relying on intense microhabitat sampling. Adjusted predictions and observed soil temperature data show that canopy cover and early nesting in some of the hottest regions of the species' range may offer refuge to buffer sex reversal. This study informs on the relative risk of reversal at point locations and highlights the necessity of nesting behaviour studies across the range of this species.

## 4.2 Introduction

The microhabitat selected by nesting oviparous reptiles can affect critical nest characteristics like temperature and moisture (Packard et al. 1985; Refsnider 2016; Noble et al. 2018). The thermal environment experienced by a developing reptilian embryo is of particular interest as it can affect the rate of development and alter phenotypic traits (i.e., body size, sprint speed, thermal preference, behaviour, sex) which may persist into adulthood (Deeming 2004; Warner and Shine 2005; Angilletta 2009; Pearson and Warner 2016, 2018; Andrews 2018; Noble et al. 2018; While et al. 2018). In particular, maternally-selected thermal environments can directly influence the sex of offspring for many reptiles with thermolabile sex determination (Sarre et al. 2004). Such reptiles may have a wide choice of nesting habitats available, and their behaviours (phenology, test nests, depth of nest, canopy cover, etc.) and ultimate choice of nesting site may, therefore, directly influence the sex of their offspring and their lifetime reproductive fitness (Warner and Shine 2008a; Telemeco et al. 2009; Warner and Shine 2009; Refsnider and Janzen 2010; Le Henanff et al. 2013). Since primary sex ratios are also subject to frequency-dependent selection (Ayala and Campbell 1974; Schwanz and Georges 2021), complex selection may operate on maternal nesting behaviours.

Identifying nesting behaviours and the temperature profiles of nests associated with those behaviours is central to understanding how species may respond to the impact of global warming on nest temperatures (Janzen 1994; Doody et al. 2006; Mitchell et al. 2008). A failure by females to adjust nesting behaviour in response to climatic warming could lead to higher nest temperatures and skewed population sex ratios (Boyle, Schwanz, et al. 2014), or in extreme cases, increased egg mortality, leading to increased risk of population decline (Boyle, Hone, et al. 2014; Mitchell et al. 2010). While nesting behaviours have been well documented in birds, those of non-avian reptiles are mostly limited to turtles and relatively understudied in free-ranging squamates (Refsnider 2016). Nesting is typically a cryptic activity presumably to deter predation of the female or of her nests (Refsnider 2016; Spencer 2002). Therefore, locating nests of solitary cryptic species can prove logistically challenging (Doody et al. 2020). Likewise, the thermal landscape available to a reptile seeking nest sites is difficult to quantify at even modest scales (Refsnider and Janzen 2010), making the broadscale identification of nesting sites using environmental data problematic. As a result, long-term, range-wide studies of the nesting behaviours of reptiles are rare (Carter et al. 2019)

and, to our knowledge, non-existent in squamates.

Predictive modelling of thermal environments provides one potential approach to mapping the thermal landscape available to a nesting reptile (Castelli, Georges, Cherryh, et al. 2021; Mitchell et al. 2010; Carter et al. 2018, 2019). These models typically use ambient air temperature or calculated solar irradiation to predict key environmental parameters, such as soil temperature, across time and space. However, they can present challenges in incorporating microhabitat variations such as shade, aspect, and soil moisture at sufficiently fine scales to provide information relevant to the organism of interest. The statistical package NicheMapR (Kearney and Porter 2016) provides one such approach, and has been shown to provide strong predictive microclimate models (Mitchell et al. 2008; Kearney et al. 2009; Kearney and Porter 2016; Klinges et al. 2022; Castelli, Georges, Cherryh, et al. 2021; Briscoe et al. 2022). Microclimate models in NicheMapR use first principles from biophysical ecology to estimate environmental variables (Gates 1980; Porter and Gates 1969; Kearney et al. 2009). With these models, users have the flexibility to incorporate specific parameters (i.e., canopy cover, substrate thermal conductivity, soil moisture, etc.) to generate predictions at a 5-km resolution from gridded thermal databases (e.g., Australian Gridded Climate Data; Jones et al. 2009) and hindcast soil temperatures at a given location. Additionally, there are options for forecasting future temperatures under varied climate warming scenarios (Klinges et al. 2022). While these models are incredibly useful, they require extensive knowledge of biophysical ecology principles and programming to produce the desired outcomes (Briscoe et al. 2023; Meyer et al. 2023). Recently, an R Shiny app web interface of NicheMapR's models has been published providing a powerful, easy-to-use tool to reach a broader range of researchers and ecological management. By empirically verifying the microclimate predictions, it becomes possible to develop a broad-scale understanding of the thermal nesting environments available to reptiles and to reveal the associated risks or advantages of nest site selection.

I examine the utility of microhabitat modelling to understand nest site selection and heat-induced sex reversal in the central bearded dragon lizard (*Pogona vitticeps*). *P. vitticeps* is a ground nesting, oviparous Agamid lizard that has a distribution across the arid and semi-arid zones of central Australia where some populations may be imperilled by warming nest temperatures (Schwanz et al. 2020). This species has been the subject of considerable laboratory experimentation regarding its reproduction and sex determination (Whiteley, Castelli, et al. 2021). However, little is known about their ecology or nesting behaviours

(Wild et al. 2022). This species typically exhibits genetic sex determination (GSD) with female heterogamety (ZZ males, ZW females; Ezaz et al. 2005), but also has temperature-induced sex reversal in laboratory and free-ranging populations (Quinn et al. 2007; Holleley et al. 2015). Sex reversal in this species occurs at warmer developmental temperatures (temperatures  $>33$  °C with pivotal temperatures [50% reversal] at  $\sim 33.5$ °C) where the temperature overrides the sex chromosomes producing viable female individuals with a male (ZZ) sex chromosome arrangement (Quinn et al. 2007). High levels of sex reversal in wild populations could cause a transition from a genetic mode of sex determination to one involving only temperature-dependence (Schwanz et al. 2020). Thus, if increased ambient temperatures caused by climate change increases the proportion of sex-reversed individuals, there would be large consequences for the evolution of the species (Schwanz et al. 2020). The distribution of sex reversal is seemingly spatially restricted to approximately 24.2% of the range of *P. vitticeps* (referred to henceforth as the “sex reversal cluster”; Castelli et al. 2021). Yet, there are no documented cases in the north-western range, which experiences temperatures that could induce sex reversal and sampling (Castelli, Georges, Cherryh, et al. 2021). Although recent studies have shown that current levels of reversal within the sex reversal cluster may be evolutionarily stable (Wild et al. 2022), modest increases in nest temperatures ( $\sim 2$ °C) could shift stable populations towards an evolutionary switch in their mode of sex determination (Schwanz et al. 2020). Additionally, if greater than 50 percent of ZZ embryos are reversed leading to population-wide loss of the W chromosome, sex ratios will be female biased until the threshold for thermal sensitivity increases or a new genetic sex determining factor arises (Schwanz et al. 2020).

Here, I use field-microclimate data to inform microclimate models to predict the risk of sex reversal at point locations within the range of *P. vitticeps*. This will provide foundational data that can be incorporated into future studies without extensive field sampling. First, I used radio-telemetry to follow gravid females and identify their nests, establish the nest depth, and determine the temperature profile of those nests. Then, I measured soil temperatures at various depths and canopy cover during the reproductive season and across the species' range. I used the NicheMapR Soil Microclimate Hindcaster for Australia web-based shiny app to predict the temperatures at a range of depths that spans the measured nest depths and empirically derive a correction factor that improves the accuracy of the predictions. I used the corrected model to predict how nesting phenology and behaviours may influence the rate of sex reversal in this species. This combination of empirically

collected and modelled microclimate data provides a framework for determining how sex reversal may persist or progress as global temperatures rise.

## 4.3 Methods

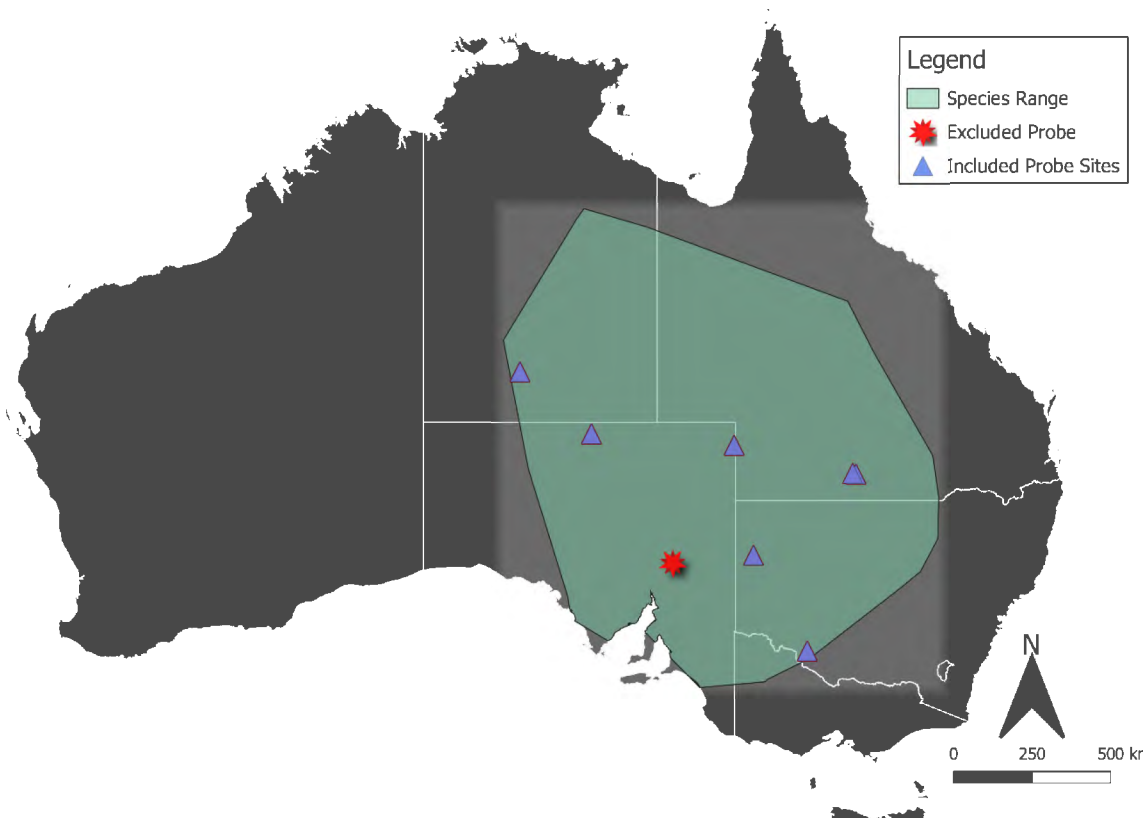
### 4.3.1 *Field Soil Temperatures*

Nest characteristics were collected from five nests of free-ranging concordant (ZW) *P. vitticeps* females. Gravid females were radio-tracked at Bowra Wildlife Sanctuary approximately 17 km west of Cunnamulla, Queensland, Australia until they were observed nesting (see Wild et al. 2022 for radio-telemetry and genotyping methodology). For each nest, ThermoChron iButton<sup>®</sup> data loggers (model DS1921G; accuracy/resolution  $\pm 1^\circ\text{C}/0.5^\circ\text{C}$ ; Maxim Integrated) were placed at the top, middle, and bottom of the nest to characterise the temperatures experienced by the clutch. Slope, aspect, and canopy cover were measured from three paired random locations within six metres of each nest to further characterise the nest site. An iButton was randomly placed at one of the three locations at the same depth as the bottom of the nest.

To broaden our understanding of the available microclimates of each nest across the geographic range of *P. vitticeps*, I recorded the soil temperatures at multiple depths at eight locations spanning a large portion of their range (Fig 4.1). Records extended over six months (October 2019 – March 2020), encompassing the majority of the reproductive season of *P. vitticeps* (Greer, 1989). I used iButtons attached to wooden dowels at depths of 0, 10, 25, and 55 cm (henceforth referred to as range-probes) and set to record temperatures every two hours. Each location had a “shaded” probe and an “open” probe to provide insight to the influence of canopy cover on the soil temperatures. For some locations, soil characteristics combined with extreme drought in previous years rendered the soil impenetrable beyond 25 cm. Probes at these locations were shortened to accommodate the environment. For each probe, slope and aspect were determined and a hemispherical photo taken with a digital camera with 180° fish-eye lens to determine canopy openness. With these data, I can assess temperatures of potential nesting areas across time, depth, and varied canopy to understand the potential risk of sex reversal.

To test the variability of microclimates at a finer scale, I deployed five circular arrays of single-depth probes in October 2020 at Fowler’s Gap Arid Zone Research Station (UNSW)

located 112 kilometres north of Broken Hill, NSW, Australia. Each array consisted of 17 probes (henceforth array-probes) where iButtons were attached to PVC pipe, buried 15 cm deep, and recorded temperatures every hour. The array-probes were arranged with one in the centre and one at each cardinal direction 10 and 25 metres from the centre. Arrays were placed across a variety of habitat types with varied levels of canopy cover. Slope and aspect



**Figure 4.1.** Species distribution of *Pogona vitticeps* and the location of temperature range-probe sites denoted. Excluded range-probe denoted was removed from analysis because the range-probes were found on the surface upon retrieval and exposure time could not be determined.

were recorded, and a hemispherical photo taken for each array-probe and were retrieved in December 2020 (61 days).

For all probes, canopy cover (percent canopy openness) was determined by hemispherical photos and analysed using Gap Light Analyzer software (Simon Fraser University, Cary Institute of Ecosystem Studies, 1999; Doody et al. 2006). Prior to analysis, photos were edited using Adobe Photoshop Lightroom (version 6.0) to remove any light anomalies that could affect the analysis of canopy openness. Minimum shade was calculated from the percent

canopy openness and categorised as open (0-49% cover) or shaded (50-100% cover).

At four range-probe locations, open canopy range-probes were unable to be recovered owing to device failure caused by the extreme environments in which they were deployed. Additionally, range-probes placed in Ikara-Flinders National Park were found on the surface upon retrieval and the duration of exposure could not be determined and was therefore also excluded from further analysis. When percent canopy cover was calculated, the “shade” range-probe for Innamincka Regional Reserve had only 4.46 percent canopy cover and was therefore treated as open canopy.

Empirical data were analysed using R v. 4.2.2 (R Core Team 2022). Linear mixed models were used to compare empirical temperatures of nests, range-probes, and array-probes between season, canopy cover, and depth. For nest models, nest identification nested in female identification was added as a random effect. For both types of probe models, location or array number was included as a random effect. To assess the risk of sex reversal from empirical data, I determined the total number of two-week windows where the temperatures exceeded the pivotal temperature ( $>33^{\circ}\text{C}$ ) of ZZ sex reversal for at least eight hours per day, which should encompass the thermosensitive period of the embryos (Whiteley et al. 2017). I used generalised linear mixed-models (family = Poisson) with locality or nest identification as random effects to determine differences between the influence of depth and canopy on the frequency of reversal windows. Tukey’s HSD post-hoc analyses were performed to determine contrasts between groups when significant.

#### 4.3.2 *Predicting Soil Temperatures*

I used the Soil Microclimate Hindcaster for Australia (R Shiny app interface of NicheMapR’s *micro\_aust* function) to predict the soil temperatures for each probe in the corresponding year of the observed temperatures. This model predicts soil temperatures using the Australian Gridded Climate Data (5 km resolution) from Jones et al. (2009) and the near-surface wind speed from McVicar et al. (2008). The latitude, longitude, year, slope, aspect, and percent shade were entered for each location where empirical data were collected. Models were run with a clear sky and fine-scale topography (30 m resolution at 100 pixels). Soil properties were set to the program’s defaults.

The shiny app interface predicted temperatures at 0, 2.5, 5, 10, 15, 20, 30, 50, 100, and 200cm depths every hour for a two-year period for each location. As some observed depths

were not able to be selected for predictions through the app, I utilised the nearest depths to make comparisons (i.e., observed 25cm was compared to predicted 30cm). Predicted temperature data were compared to observed (iButton) temperature data from each probe or nest. I determined correlation coefficients (*r*-values) and root-mean-squared deviations (*rmsd*) between the observed soil temperatures and the predicted soil temperatures for all probes and nests.

### 4.3.3 *Correction Equation and Adjusted Predictions*

I generated a general correction for the predicted soil temperatures produced by the *micro\_aust* microclimate model using a random subset of 75% of the predicted and observed range- and array-probe data. I examined linear models of predicted temperatures vs. observed temperatures for all probes (excluding nest data,  $n=91$ ). The intercepts and slopes of the linear models were averaged across all sites to create a linear equation to apply to the predicted temperatures and derive adjusted temperatures. I applied the correction equation to the remaining 25% of the data to test the extent to which the equation improved the *rmsd* of the data.

Given the robustness of the adjustment (see Results), I wanted to know how well the predicted soil temperatures estimated risk of sex reversal and to extend those estimations to depths and time of year for which soil or nest temperature observations were not available. To do this, I applied our correction formula to the predicted temperatures for the five nests, associated random sites, and the 16 range-probe sites (including those which were lost or excluded) to generate adjusted predictions. As with our observed data, I determined the total number of two-week windows where the temperatures exceeded the pivotal temperature using the adjusted temperatures predicted at 10, 15, 20, 30, and 50 cm depths, which were within the range of observed depths in natural nests. I used generalised linear mixed-models (GLMM; family = Poisson) with locality or nest identification as random effects to determine the influence of depth, canopy, month, and year on the frequency of reversal windows. For nest GLMMs, I also included “type” to determine if female-chosen nest sites differed from random sites. Post-hoc analyses were performed to determine differences between groups when significant.



## 4.4 Results

### 4.4.1 Empirical Soil Temperatures

Nests and random site temperatures had varied microclimate measurements as each was laid in different times of the reproductive season with different microhabitat variables (Table S4.1). Early season nests temperatures (September-November) were on average 2.94°C cooler than late season (December-February,  $p < .0001$ , Table 4.1). Early season nest temperatures rarely exceeded pivotal temperatures  $T_{piv}$  (~33.5°C), but late season nest temperatures frequently experienced long periods of 8 or more hours above  $T_{piv}$  (Fig. S4.1).

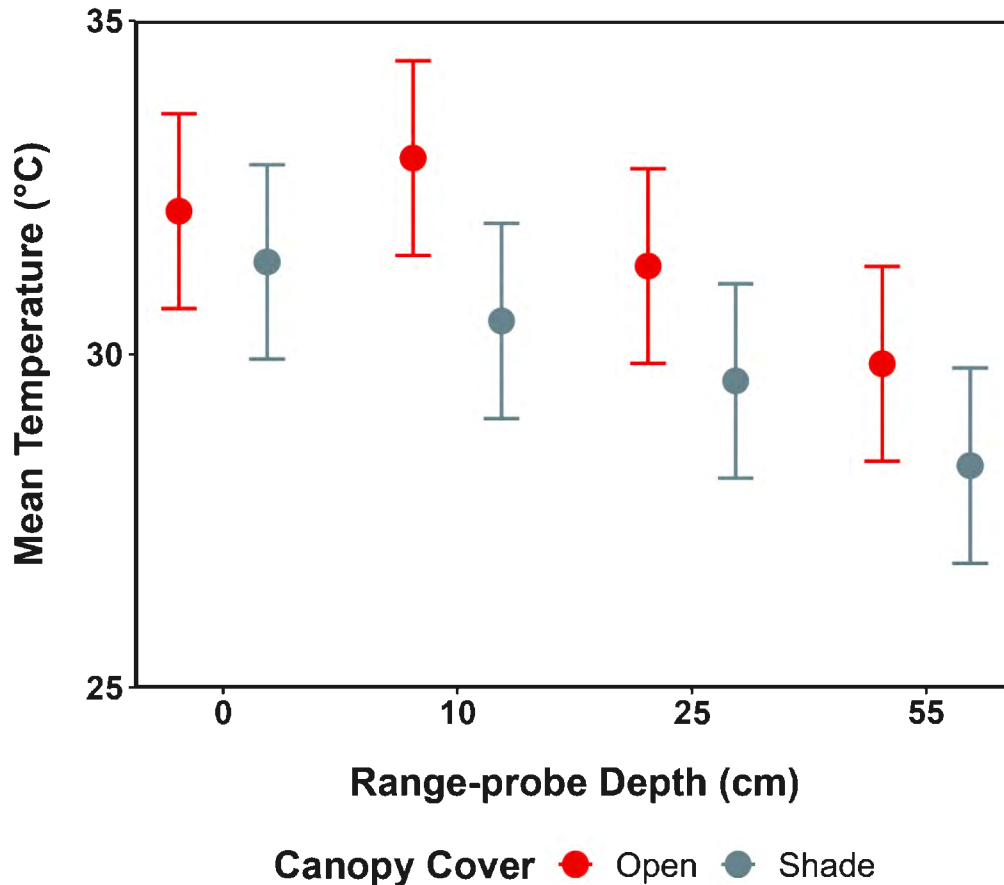
**Table 4.1.** Table of estimates from linear-mixed effect model for influence of parameters on observed nest temperature. Bolded p-values denote significance.

	Estimate	SE	DF	T	P
Intercept	-4.72	6.95	5.84	-0.679	0.523
Late Season	2.59	0.118	23700	21.90	<b>&lt; .0001</b>
Minimum Shade	1.01	0.0612	14400	16.50	<b>&lt; .0001</b>
Depth	-0.0843	0.0225	23700	-3.74	<b>&lt; .001</b>
Random	-1.97	0.175	16300	-11.20	<b>&lt; .0001</b>
Minimum Shade-Depth Interaction	0.00322	0.000808	23700	3.98	<b>&lt; .0001</b>

Six months of soil temperatures spanning the breeding season of *P. vitticeps* (October 2019 - March 2020) from 10 of the 16 deployed range-probes (7 locations) were used for analysis. Observed temperatures across all locations and all depths ranged from 2.5 to 70.5°C. Average temperatures of open canopy probes were 1.61°C warmer than shaded (Fig. 4.2, and average temperatures significantly decreased with depth in both open and shaded probes (excepting 0cm to 10cm; Fig. 4.2, Table S4.1).

Three months (mid-October to mid-December 2020) of soil temperature data during the breeding season were collected from the five arrays at Fowler's Gap, NSW. Four of the 85

deployed array-probes failed to record data. Across the 81 array-probes, temperatures ranged from 12.5 to 45.5°C. The average minimum shade available across all array-probes was 11.99% .



**Figure 4.2.** Estimated marginal mean temperatures from linear mixed-effects model of the 10 collected range-probes across four depths and two canopy covers.

#### 4.4.2 Accuracy of Predicted Temperatures

Microclimate models provided poor predictions of soil temperature for the 10 range-probes (7 locations, 3-4 depths each;  $r$ -values = -0.0912 - 0.696; root mean square deviation ( $rmsd$ ) = 4.21 - 17.20; Table S4.3). Surface temperatures had the poorest predictive ability, with the lowest correlation coefficients (all  $r$ -values < 0.407). Conversely, the microclimate models predicted temperatures within the arrays at Fowler’s Gap relatively well compared to the range probes with all  $r$ -values > 0.80 (Table S4.4) and  $rmsd$  between 1.14 and 11.51 (Table

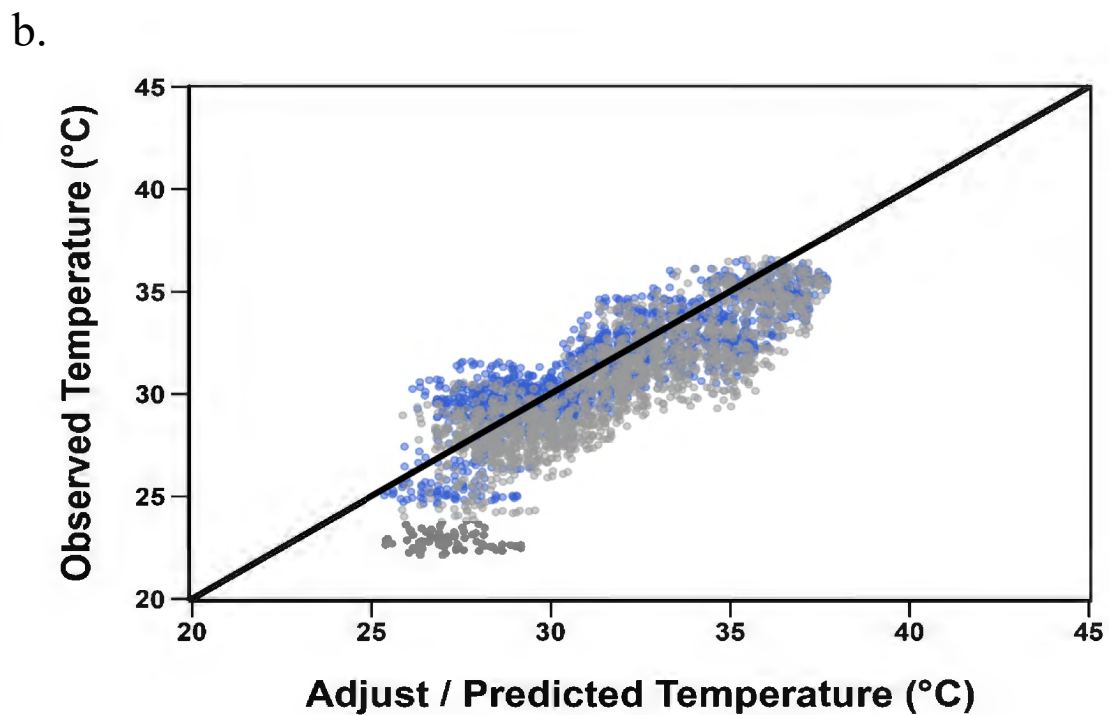
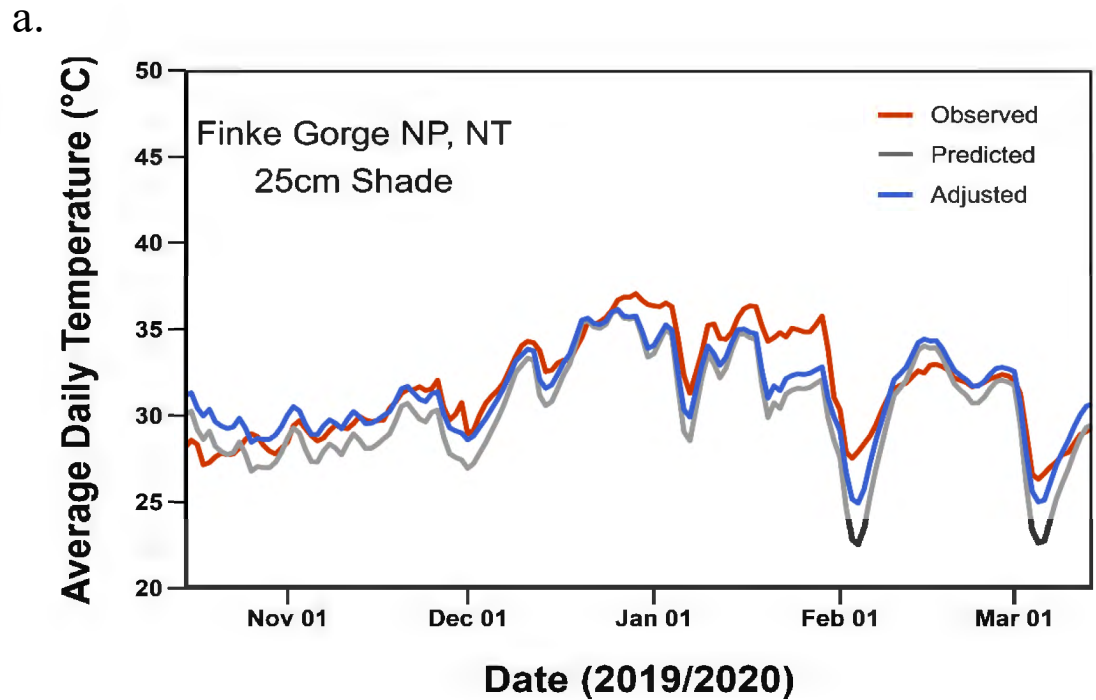
S4.5) with 61.8% of predictions within 2°C of observed temperatures.

#### 4.4.3 *Adjusted predictions*

The average intercept and slope were 6.40 and 0.823 respectively for the 75% of the range-probe and nest data used to generate the correction equation. These data were used to create the following correction equation where  $x$  is the predicted temperature from NicheMapR, and  $y$  is the resulting adjusted temperature.

$$y = 6.402224 + 0.823445x$$

When the correction equation was applied to the remaining 25% of the data, the fit was considerably improved (adjusted: *rmsd*: 1.04 – 14.53, mean = 3.02; pre-adjustment: *rmsd*: 1.22 to 15.93, mean = 3.35). Linear models of observed vs. predicted were compared to those of observed vs. adjusted to determine the differences in the slopes as an estimate of robustness of the correction. The average slope from linear models of the observed and adjusted temperatures was extremely close to 1 (slope = 0.998) and considerably better than that found between the observed and predicted temperatures. I applied the correction to each range-probe site and array-probe site (see Fig 4.3 for an example of the improvements at one range-probe site location). Additional figures can be found in the supplementary materials (Chapter 4.6, Figures S4.3-4.21, p95-114).



**Figure 4.3.** Observed and adjusted/predicted average daily temperatures across at 25cm from a shaded range-probe at Finke Gorge National Park, NT, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted-blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.

#### 4.4.4 Risk of sex reversal

I used observed and adjusted temperatures to determine the relative risk of sex reversal across the time periods for which thermal loggers were deployed. Five range-probe locations had at least one risk window of 8 hours above  $T_{piv}$  (Table S4.6). The observed risk of sex reversal and the adjusted predicted risk showed a relatively high rank correlation ( $\rho = 0.837$ ) suggesting that the adjusted predicted risk will provide a good proxy for estimating relative sex reversal risk across the range of *P. vitticeps*. The accuracy of adjusted predictions varied among sites and across depths. Generally, deeper, shaded probes had more accurate predictions than shallow, open probes (Table 4.3).

**Table 4.2.** Table of estimates from generalised linear-mixed effect model for influence of parameters on frequency of risk windows from the adjusted range-probe data. Bolded p-values denote significance.

Fixed Effects	Estimate	SE	Z	P
Intercept	4.39	0.455	9.63	< .0001
Shaded Canopy	-1.14	0.0956	-11.90	< .0001
Depth	-0.0124	0.00111	-11.20	< .0001
Shaded Canopy -Depth Interaction	-0.0342	0.00451	-7.57	< .0001

When reviewing the observed data, north-central areas of the range of *P. vitticeps* (Bowra, Witjira, Innamincka, and Finke Gorge) had more sex reversal windows than the more southern areas (Ikara-Flinders, Fowler’s Gap, Yanga; Table S4.6). Open canopy locations are at higher risk of inducing sex reversal than shaded locations ( $p < .0001$ , Table S4.#). Shallow depths had significantly more windows than deeper ( $p < .0001$ ; Table S4.6). There was also a significant interaction between depth and canopy cover with deep-shaded probes having fewer or no windows ( $p < .0001$ ; Table S4.6).

Predicted windows of reversal for nest locations and associated random sites followed a similar trend to the probes. Canopy cover, depth, and their interaction all significantly

influenced the frequency of reversal windows ( $p < .0001$ , Table S4.9). Nest locations selected by female *P. vitticeps* had 1.28 times more sex-reversal windows than the associated random sites ( $p = .062$ ).

## 4.5 Discussion

Species with thermosensitive sex determination are at risk of skewed sex ratios if the available nest microhabitats chosen by mothers experience temperatures at or near their pivotal temperature for sex reversal. Here, I use observational temperature data from nests and from non-nest soils, as well as predicted soil temperatures, to assess the relative risk of sex reversal at point locations across the range of *P. vitticeps* both within and outside of the known sex reversal cluster. My observed data show that soil temperatures can vary greatly with depth and canopy cover. Additionally, I provide the first report of nest site selection in free-ranging *P. vitticeps* and show that nests laid early in the reproductive season (September–November) are cooler and rarely reach the temperatures needed to induce sex reversal. Furthermore, by integrating my observed microclimate data with predicted microclimate data from the NicheMapR Shiny app, I show that while generally accurate, the model was improved by my general correction. Once corrected, I assess the risk of sex reversal more accurately and show the utility of this tool across broad geographic, temporal, and microhabitat scales.

Although I observed only a small sample of nests within the sex reversal cluster, they are the first *P. vitticeps* nests to be fully described. The nest sites chosen by females early in the reproductive season (September – early November) were relatively shallow and temperatures were below  $T_{piv}$  and unlikely to induce sex reversal. However, sites chosen during late spring and summer were deeper but frequently exceeded  $T_{piv}$ . For example, Nest 2, which had the highest risk of reversal, was laid in early January at 41cm deep (at the bottom of the nest) and had an average nest temperature of 36.9°C (range 34–39°C). While none of the eggs hatched, presumably all ZZ offspring from this clutch would have been sex-reversed as the full duration of development would have exceeded  $T_{piv}$ .

The differences in depth chosen by females between the early and late timings may indicate a behavioural tactic to mitigate the temperature or other risks associated with later nests such as water availability (Plummer and Snell 1988; Ji and Braña 1999; Warner and

Andrews 2002). Additionally, females tended to choose nests with less canopy cover than other available locations in the immediate area, which in turn increases the temperature experienced by the nest and the likelihood of sex reversal in later season nests. This aligns with the nesting behaviours of *Amphibolurus muricatus* (another Australian Agamid lizard) that consistently chooses more open canopy nests that are warmer (Warner and Shine 2008b). These choices are likely to be beneficial to incubating embryos early as they will increase developmental rates and increase early hatching (Warner and Shine 2007; Noble et al. 2018). Regardless of the benefits of decreased incubation period, the late season embryos are indeed more at risk for sex reversal because of these choices particularly in open areas.

Doubtless, female nest site selection will play a pivotal role in the risk of sex reversal. However, local microclimate data is key to understanding the nesting options available to females and to predicting the future risk of sex reversal across a broad geographic scale under warming climate scenarios. Castelli et al. (2021) have suggested that the lack of reversal in the north and north-western regions cannot be due to plastic nesting behaviours alone. Although, my range-probe data are only singular points in the species' range of ~2.2 million km<sup>2</sup>, I show that microclimates in the presumed hottest areas of their range can vary drastically and can provide nesting environments that may not induce sex reversal. However, the models run by Castelli et al. (2021) were all estimated using an open canopy and assumed nest depths of 15cm (from observations of *P. minor* in Pianka 2005). My data suggests that their models could be expanded upon through the incorporation of variation in nest depth of *P. vitticeps* and their preference for some shade (mean 30.01%), which could provide a more holistic understanding of current presumed distribution of sex reversal.

Microclimate models will act as invaluable tools for future studies of the consequences of nesting behaviours particularly under climate scenarios to inform conservation management strategies (Kearney and Porter 2016; Carter et al. 2017; Briscoe et al. 2022, 2023; Meyer et al. 2023). However, there is a need for further validations when working with simplified models particularly when working with TSD or thermolabile species where very small variations in temperature could misinform predicted sex ratios. I show that my predicted soil temperatures were overall quite accurate but had lower correlation coefficients than other studies that used a more complex model of NicheMapR (Briscoe et al. 2022; Carter et al. 2016). Overall, the model frequently underpredicted soil temperatures, which could misinform the risk of sex reversal occurring at a location. For *P. vitticeps*, a difference as small as 2°C could push nests

past the  $T_{piv}$  and tip a population into loss of the W chromosome (Schwanz et al. 2020). Therefore, the application of a general correction was necessary to produce more accurate reversal risk assessments. My adjusted predictions better matched the observed data, with more accurate temperatures and an accurate relative risk ranking for the number of sex reversal windows.

Current records of sex reversal in wild populations of *P. vitticeps* show a partial mismatch between where sex reversal is predicted and it occurs (Holleley et al. 2015; Castelli, Georges, Cherryh, et al. 2021). The observed sex reversal cluster covers approximately 24% of their total range in the south-eastern range (Castelli, Georges, Cherryh, et al. 2021) and includes the two areas of highest observed risk from this study (Table S4.6) and three of the highest five predicted risk (Table S4.7). However, observed and predicted soil temperatures in the northern and north-western portions are still quite warm with many observed reversal windows (Table S4.6) and the second and third highest predicted risk (Table S4.7), yet there are no recorded instances of sex reversal in this region (Castelli, Georges, Cherryh, et al. 2021). This lack of sex reversal despite high soil temperatures could be attributed to poor animal sampling in this region. Thus, the sex reversal risk estimates can help identify regions of the range where greater sampling effort should be concentrated to provide a reliable record of the prevalence of sex reversal. Alternatively, if low sex reversal in these regions were true, the discrepancy could be explained by behavioural adaptations in nest site selection, phenological differences in reproduction, or local adaptations for a higher  $T_{piv}$  (Castelli, Georges, Cherryh, et al. 2021).

My results indicate there are clear opportunities for decreasing nest temperatures and reducing sex reversal risk via behavioural alteration of nesting site or nesting timing. The tendency of female *P. vitticeps* to choose more open nest sites may provide an explanation (in part) for the prevalence of sex reversal in this cluster. More open, shallow sites early in the reproductive season will convey the benefit of warmer nests (Noble et al. 2018), but the same open sites later in the reproductive season may induce sex reversal (Table. S4.8). My open sites within the sex reversal cluster had more or close to the same frequency of sex reversing windows as western open sites outside the cluster (Table S4.7). Furthermore, the shaded site at Finke Gorge, NT, actually had fewer windows than open sites within the cluster and shaded sites at both locations at Bowra, QLD (Table S4.7), which supports our finding that shaded sites outside the cluster may allow females to mitigate the risk of reversal. Other studies of



reptiles have shown that a variety of taxa indeed do utilise plastic behaviours and timing to ameliorate the extreme climatic variation (Doody et al. 2006; Schwanz and Janzen 2008; Telemeco et al. 2009; Refsnider 2016; Li et al. 2018; Pearson and Warner 2018; Carter et al. 2019; Tiatragul et al. 2020; Leivesley et al. 2022; Sullivan et al. 2022). Given the plastic behaviours of other reptiles, it is likely that *P. vitticeps* outside of the reversal cluster are exhibiting plastic responses to their environment, which warrants further sampling and study of individuals in this region.

Limited understanding of the nesting behaviours and phenology of *P. vitticeps* across their range makes understanding and predicting areas of sex reversal difficult. My data begin to bridge this gap but more intensive sampling of individuals outside of the sex reversal cluster and alongside extensive monitoring of females (both concordant and sex-reversed) to document more nesting behaviours would improve this understanding. Until these logistically strenuous field experiments can be conducted, microclimate models provide a robust proxy for informing the risk of sex reversal at point locations and across their range. Additionally, I offer that as our correction equation is only for soil temperatures, it can be used more broadly for other researchers to improve the predictive capacity of NicheMapR's Shiny app.

## 4.6 Supplementary Materials

### 4.6.1 Supplementary Tables

**Table S4.1.** Microhabitat variables for *P. vitticeps* nests and associated random sites.

Nest ID	Date	Female ID	Latitude	Longitude	Slope	Aspect	Depth Top	Depth Middle	Depth Bottom	% Canopy Openness
Nest1	27/11/2018	POV127	-27.99465	145.65863	54.7	224	18	-	-	70.23
Nest1 R1	27/11/2018	POV127	27.99468	145.65858	3.2	225	18	-	-	64.14
Nest1 R2	27/11/2018	POV127	27.99461	145.65863	4.9	329	18	-	-	78.56
Nest1 R3	27/11/2018	POV127	27.99461	145.6586	0.7	289	18	-	-	68.72
Nest2	3/01/2019	POV145	-27.99880	145.60475	26	320	30	35	41	87.04
Nest2 R1	3/01/2019	POV145	27.98877	145.60478	0	18	-	35	-	85.88
Nest2 R2	3/01/2019	POV145	27.98881	145.60477	0	120	-	35	-	84.55
Nest2 R3	3/01/2019	POV145	27.98881	145.60472	0	253	-	35	-	84.64
Nest3	3/09/2019	POV141	-27.96266	145.50679	32.9	81	8	10	12	73.38
Nest3 R1	3/09/2019	POV141	-27.96265	145.50682	168	7.5	-	10	-	69.22
Nest3 R2	3/09/2019	POV141	-27.96268	145.50674	238	25.3	-	10	-	58.93
Nest3 R3	3/09/2019	POV141	-27.96265	145.50681	171	8.6	-	10	-	55.4
Nest4	13/10/2019	POV153	-27.97043	145.51355	21.5	37	14	18	24.5	60.41
Nest4 R1	14/10/2019	POV154	-27.97048	145.51353	7.5	175	-	18	-	46.29
Nest4 R2	14/10/2019	POV154	-27.97047	145.51357	6.3	106	-	18	-	61.87
Nest4 R3	14/10/2019	POV154	-27.97044	145.51355	5.7	272	-	18	-	54.93
Nest5	6/11/2019	POV141	-27.96007	145.50609	14	162	13	14	15	58.52
Nest5 R1	6/11/2019	POV141	-27.96005	145.50607	4.8	176	-	14	-	56.47
Nest5 R2	6/11/2019	POV141	-27.96009	145.50607	4.9	331	-	14	-	52.58
Nest5 R3	6/11/2019	POV141	-27.96014	145.50612	9.3	11	-	14	-	57.62

**Table S4. 2.** Type III ANOVA table for comparisons of empirically recorded range-probe data. Bolded p-values denote significance.

Response	Sum Sq.	Mean Sq.	Num. DF	Den. DF	F Value	P
Canopy Cover (Shade vs. Open)	66699	22233.0	3	66083	458.691	<.0001
Depth	25699	25698.9	1	66082	530.197	<.0001
Depth : Canopy Cover Interaction	5346	1781.9	3	66083	36.763	<.0001

**Table S4. 3.** Correlation coefficients (r) and root mean square deviation (rmsd) of range-probes temperatures and NicheMapR modelled predictions from point locations across the range of *P. vitticeps*.

	Canopy Cover	0cm		10cm		30cm		50cm	
		r	rmsd	r	rmsd	r	rmsd	r	rmsd
Bowra Wildlife Sanct. – Front, QLD	Shade	0.0282	12.50	0.730	3.93	0.889	2.34	0.819	2.08
Bowra Wildlife Sanct. – Western Paddock, QLD	Open	-0.412	24.97	0.285	8.54	0.881	2.41	0.896	2.22
Bowra Wildlife Sanct. – Western Paddock, QLD	Shade	-0.187	16.23	0.456	8.13	-	-	0.815	2.16
Finke Gorge National Park, NT	Shade	-0.391	18.69	0.373	5.40	0.855	2.14	-	-
Fowler’s Gap Arid Zone Research, NSW	Open	-0.525	26.09	0.109	8.91	0.911	1.74	0.939	1.13
Fowler’s Gap Arid Zone Research, NSW	Shade	-0.184	15.27	-	-	0.941	1.31	0.919	1.20
Innamincka Regional Reserve, SA	Open (2)	-0.371	23.19	-	-	0.919	2.06	0.891	1.73
Witjira National Park, SA	Open	-0.571	29.27	0.00366	8.85	0.797	5.15	0.762	4.64
Witjira National Park, SA	Shade	-0.542	23.27	0.215	5.97	0.848	1.91	0.820	1.69
Yanga State Cons. Area, NSW	Shade	-0.0665	13.71	0.649	4.17	0.875	2.11	-	-

**Table S4. 4.** Correlation coefficients (r) of array-probes temperatures and NicheMapR modelled predictions from Fowler’s Gap, NSW. All probes were buried 15cm below the surface.

Centre	10		10		10		10		10		25		25		25		25					
	E	N	NE	NW	S	SE	SW	W	N	NE	NW	S	SE	SW	W	E	S	SE	SW	W	E	
<b>Array 1</b>	0.97	0.972	0.96	0.956	0.965	0.976	0.972	0.965	0.97	0.968	0.962	0.973	0.966	0.974	0.962	-						
<b>Array 2</b>	0.97	0.971	-	0.963	0.967	0.968	0.97	0.948	0.972	0.95	0.965	0.972	0.967	0.966	0.962	0.976						
<b>Array 3</b>	0.819	-	0.853	0.971	0.97	0.937	0.961	0.955	0.962	0.799	0.969	0.92	0.942	0.962	0.96	0.945						
<b>Array 4</b>	0.964	0.962	0.954	0.972	-	0.976	0.973	0.973	0.96	0.974	0.938	0.969	0.953	0.953	0.953	0.977						
<b>Array 5</b>	0.957	0.933	0.946	0.957	0.961	0.967	0.964	0.972	0.937	0.958	0.937	0.973	0.967	0.967	0.969	0.949						

**Table S4. 5.** Root-mean-squared deviation (*rmsd*) of array-probes temperatures and NicheMapR modelled predictions from Fowler's Gap, NSW. All probes were buried 15cm below the surface.

Centre	10 E	10 N	10 NE	10 NW	10 S	10 SE	10 SW	10 W	25 N	25 NE	25 NW	25 S	25 SE	25 SW	25 W	25 E
<b>Array 1</b>	2.25	2.53	1.77	1.77	1.79	1.7	2.25	2.06	2.0	1.57	1.5	1.74	1.31	1.4	1.91	-
<b>Array 2</b>	1.5	2.65	-	3.35	1.28	1.4	1.47	1.41	1.88	1.47	1.44	1.26	1.32	1.84	1.47	1.14
<b>Array 3</b>	10.6	-	9.51	1.27	2.09	3.6	2.22	1.73	11.5	2.06	1.77	3.23	3.04	2.76	2.78	2.18
<b>Array 4</b>	1.3	2.19	2.04	1.39	-	1.41	1.27	2.01	1.31	7.36	1.86	1.28	1.66	1.6	1.46	1.25
<b>Array 5</b>	3.02	3.78	3.09	2.96	2.79	2	1.76	3.48	2.85	3.47	1.75	2.29	2.38	2.35	2.29	3.45

**Table S4.6** Total observed and adjusted windows of sex reversal windows from range-probes from 15 October 2019 to 15 March 2020 and the ranked risk associated.

Locality	Canopy Cover	Total Observed Windows	Total Adjusted Window	Observed Risk	Predicted Risk
Innamincka Regional Reserve, SA	Open	146	187	1	1
Bowra Wildlife Sanctuary – Western Paddock, QLD	Open	114	126	2	2
Witjira National Park, SA	Open	47	14	3	3
Finke Gorge National Park, NT	Shade	17	6	4	5
Fowler’s Gap Arid Zone Research, NSW	Open	9	13	5	4

**Table S4.7.** Total predicted (adjusted) two-week windows of eight hours above T<sub>piv</sub> (>33°C) during 2019-2020 and cumulative risk of sex reversal across all locations where probes were deployed. Total windows are summed across 10, 15, 20, 30, and 50cm depths.

Locality	Canopy	Total Predicted Windows	Predicted Risk
Innamincka Regional Reserve, SA	Open	1008	1
Witjira National Park, SA	Open	925	2
Finke Gorge National Park, NT	Open	775	3
Bowra Wildlife Sanctuary – Western Paddock, QLD	Open	730	4
Bowra Wildlife Sanctuary – Front, QLD	Open	606	5
Innamincka Regional Reserve, SA	Open	439	6
Witjira National Park, SA	Shade	242	7
Fowler’s Gap Arid Zone Research, NSW	Open	204	8
Bowra Wildlife Sanctuary – Western Paddock, QLD	Shade	165	9
Bowra Wildlife Sanctuary – Front, QLD	Shade	71	10
Ikara-Flinders Ranges National Park, SA	Open	64	11
Finke Gorge National Park, NT	Shade	52	12
Yanga State Conservation Area, NSW	Open	31	13
Fowler’s Gap Arid Zone Research, NSW	Shade	5	14
Ikara-Flinders Ranges National Park, SA	Shade	0	15
Yanga State Conservation Area, NSW	Shade	0	15



**Table S4.8.** Relative risk of sex reversal by month. Total predicted (adjusted) two-week windows of eight hours above  $T_{piv}$  ( $>33^{\circ}\text{C}$ ) during 2019-2020 and cumulative risk of sex reversal across all locations where probes were deployed. Total windows are summed across 10, 15, 20, 30, and 50cm depths from all range-probe locations.

Month	Canopy	Total windows	Risk
January	Open	1408	1
December	Open	1165	2
February	Open	952	3
November	Open	815	4
March	Open	385	5
December	Shade	248	6
February	Shade	98	7
January	Shade	79	8
November	Shade	64	9
October	Open	55	10
March	Shade	46	11
April	Open	2	12

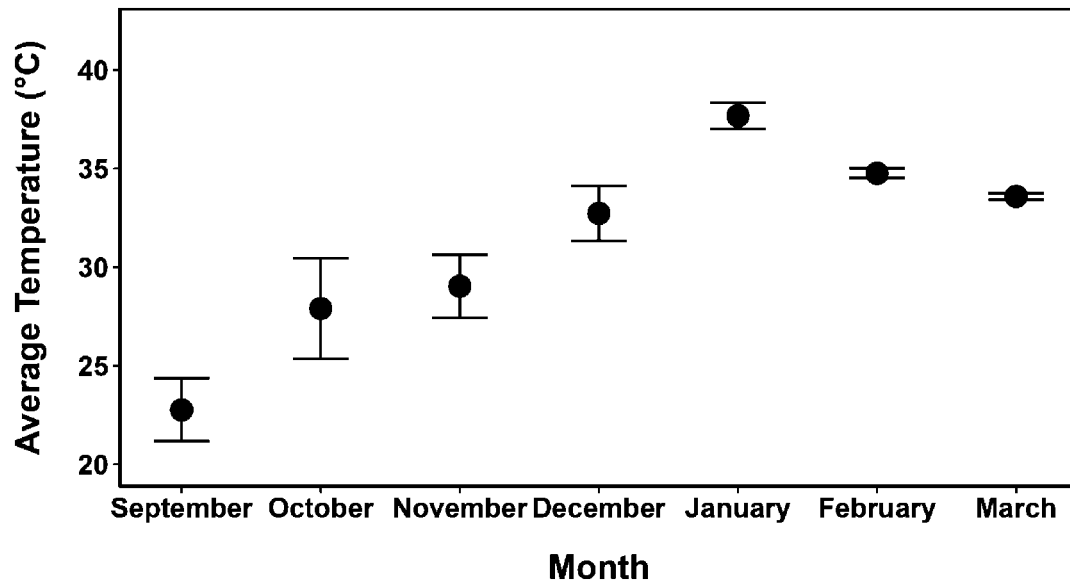
**Table S4.9** Total two-week windows of at least 8 hours over pivotal temperatures (>33°C) in 2018-2019 using adjusted predicted temperatures for found nests and associated random site locations ordered from highest to lowest window counts.

	Minimum Shade (%)	10cm	15cm	20cm	30cm	50cm
<b>Nest 2</b>	12.96	206	176	121	88	35
<b>Nest 3</b>	26.62	138	104	79	32	19
<b>Nest 2-R1</b>	14.12	121	117	96	81	35
<b>Nest 2-R2</b>	15.45	121	117	91	74	35
<b>Nest 2-R3</b>	15.36	121	117	91	74	35
<b>Nest 1-R2</b>	21.44	99	94	88	54	32
<b>Nest 5</b>	41.48	99	76	40	22	0
<b>Nest 1-R3</b>	31.28	79	76	40	30	19
<b>Nest 1</b>	29.77	79	76	40	30	19
<b>Nest 3-R1</b>	30.78	63	68	39	24	8
<b>Nest 1-R1</b>	35.86	54	48	38	24	9
<b>Nest 4-R2</b>	38.13	44	40	31	23	6
<b>Nest3-R2</b>	41.07	42	39	30	20	0
<b>Nest 4</b>	39.59	42	39	31	22	5
<b>Nest 5-R3</b>	42.38	42	39	30	20	0
<b>Nest 3-R3</b>	44.6	41	32	24	20	0
<b>Nest 4-R3</b>	45.07	41	32	30	20	0
<b>Nest 5-R1</b>	43.53	41	33	24	20	0
<b>Nest 5-R2</b>	47.42	39	32	24	19	0
<b>Nest 4-R1</b>	53.71	31	25	22	8	0

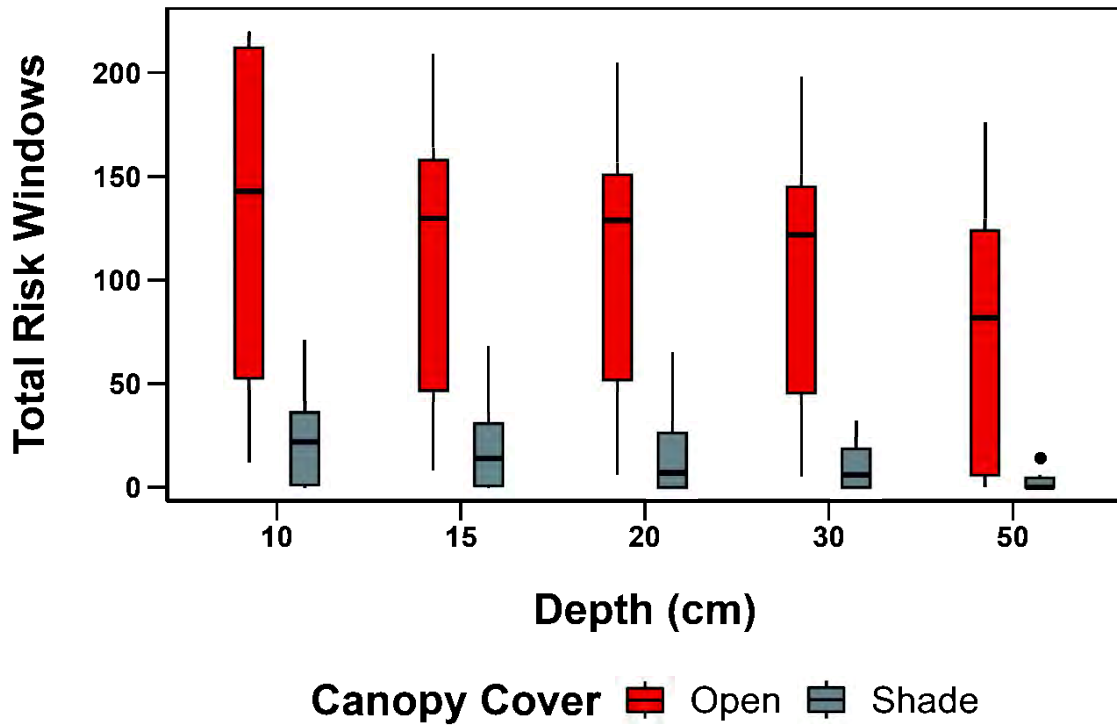
**Table S4.10.** Total predicted (adjusted) two-week windows of eight hours above  $T_{piv}$  ( $>33^{\circ}\text{C}$ ) during 2018-2019 and cumulative risk of sex reversal across all nests and associated random sites. Total windows are calculated from 10, 15, 20, 30, and 50cm depths.

Nest ID	Type	Minimum Shade (%)	Total Windows	Cumulative Risk
Nest 2	Nest Bottom	13.0	626	1
Nest 2	Random 1	14.1	450	4
Nest 2	Random 2	15.4	438	5
Nest 2	Random 3	15.4	438	6
Nest 3	Bottom	26.6	372	7
Nest 1	Random 2	21.4	367	10
Nest 1	Random 3	31.3	244	11
Nest 1	Nest Top	29.8	244	12
Nest 5	Nest Bottom	41.5	237	13
Nest 3	Random 1	30.8	202	16
Nest 1	Random 1	35.9	173	17
Nest 4	Random 2	38.1	144	18
Nest 4	Nest Bottom	39.6	139	19
Nest 3	Random 2	41.1	131	22
Nest 5	Random 3	42.4	131	23
Nest 4	Random 3	45.1	123	24
Nest 5	Random 1	43.5	118	25
Nest 3	Random 3	44.6	117	26
Nest 5	Random 2	47.4	114	27
Nest 4	Random 1	53.7	86	28

4.6.2 *Supplementary Figures*

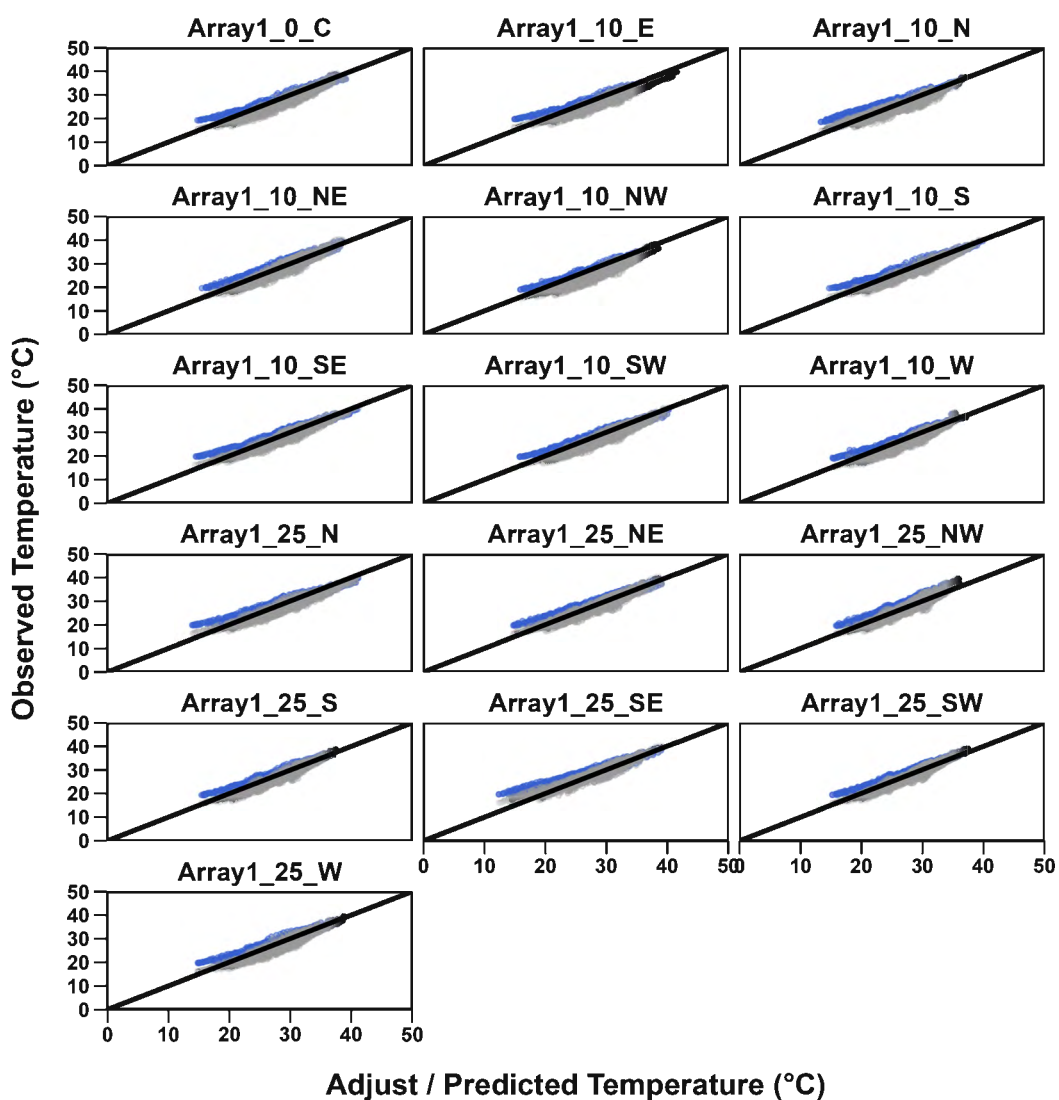


**Figure S4.1.** Average monthly temperatures across all nests at Bowra Sanctuary, QLD, Australia.



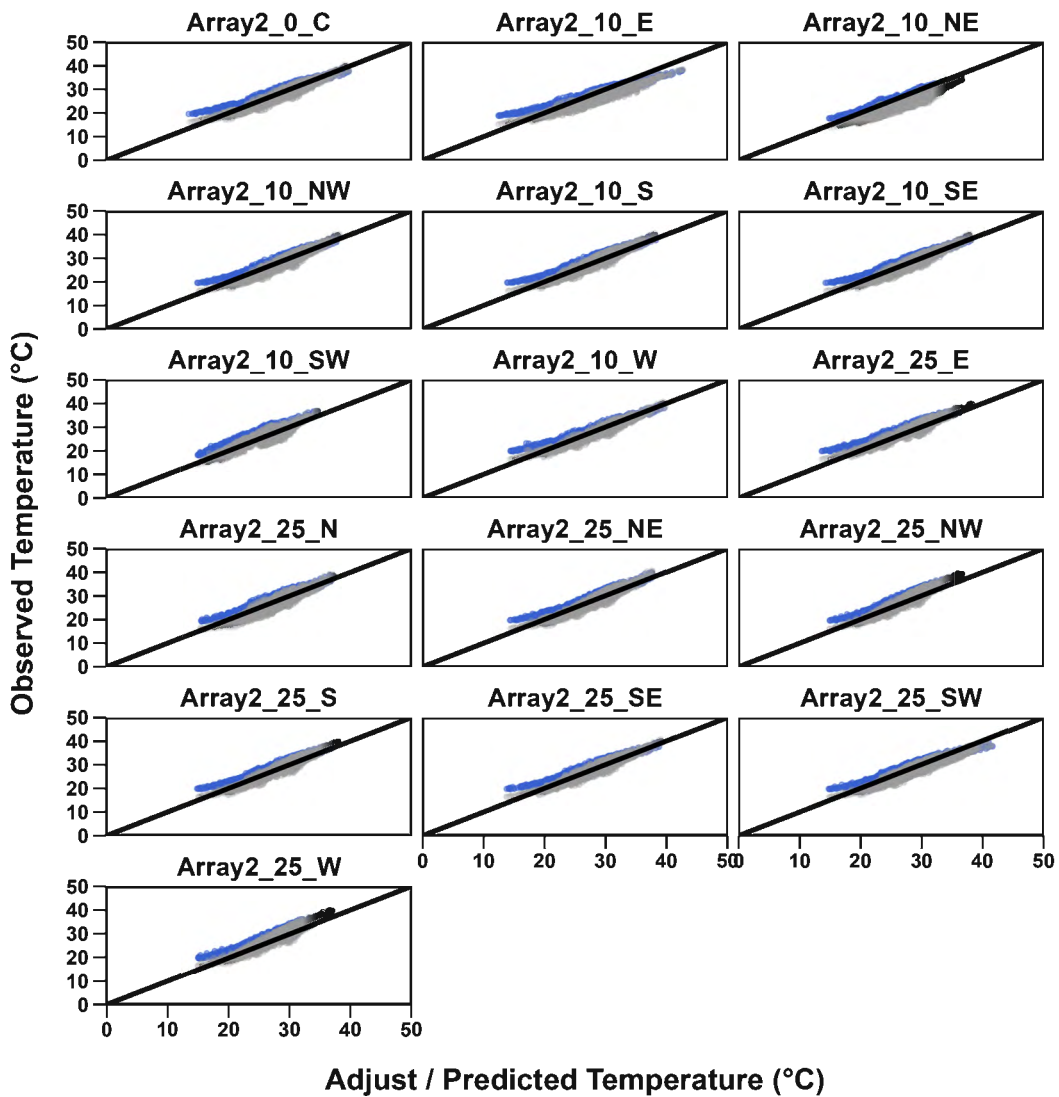
**Figure S4.2.** Frequency of risk windows of sex reversal by canopy cover and depth from the adjusted predictions of the 16 range-probe locations.

### Array 1, Fowler's Gap, NSW



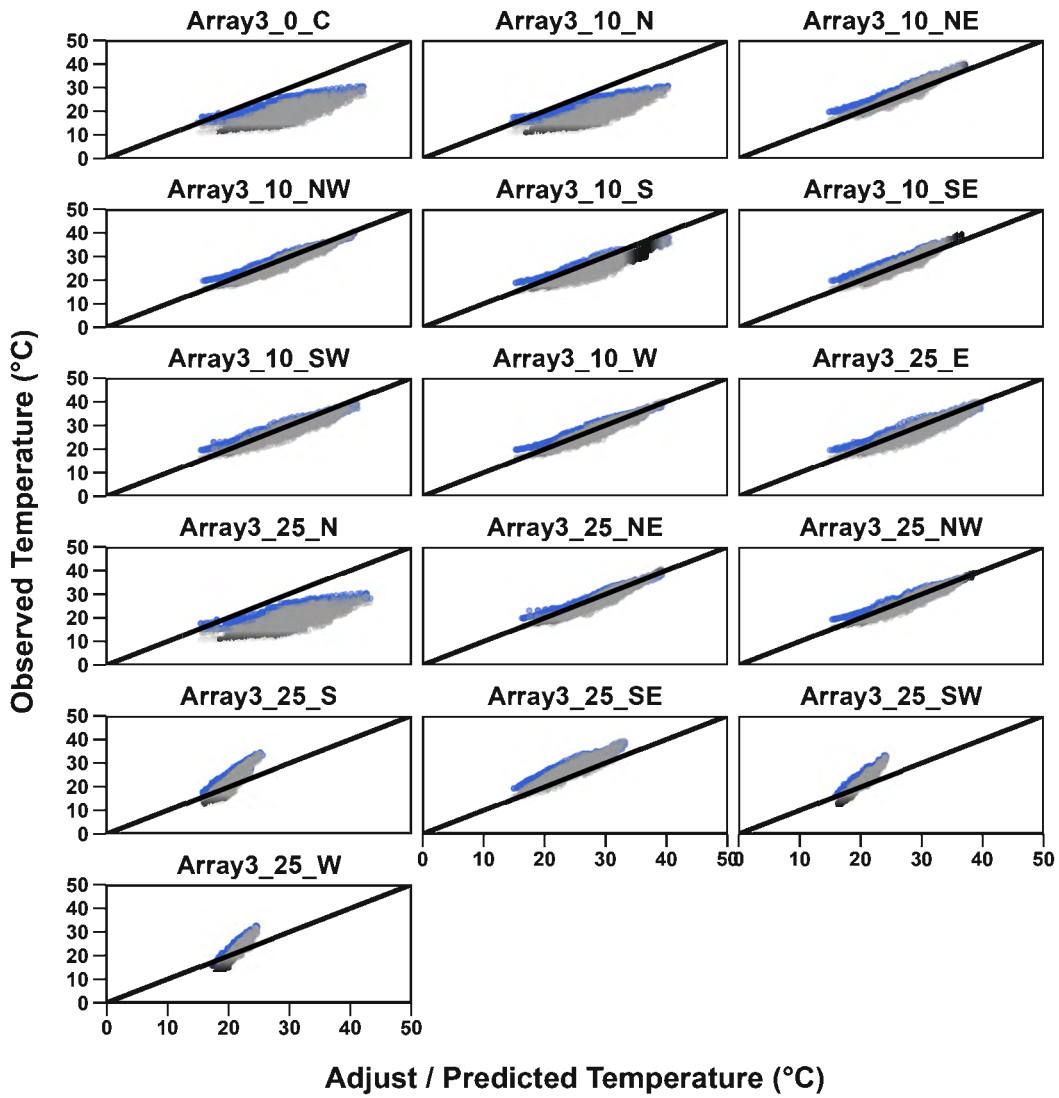
**Figure S4.3.** Fowler's Gap, NSW Array 1 relationships between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted. Panel headings denote distance and direction.

### Array 2, Fowler's Gap, NSW



**Figure S4.4.** Fowler's Gap, NSW Array 2 relationships between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted. Panel headings denote distance and direction.

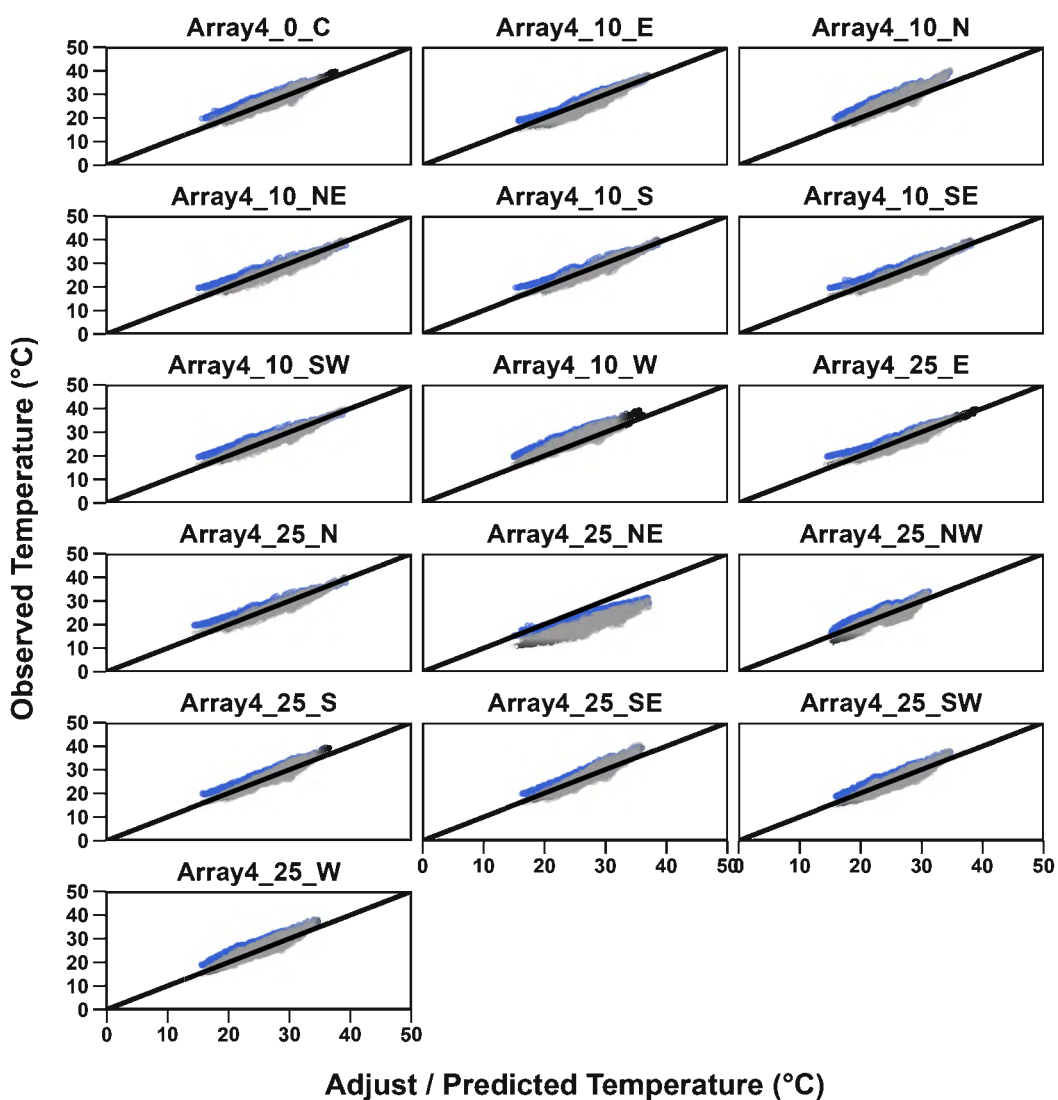
### Array 3, Fowler's Gap, NSW



**Figure S4.5.** Fowler's Gap, NSW Array 3 relationships between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted. Panel headings denote distance and direction.

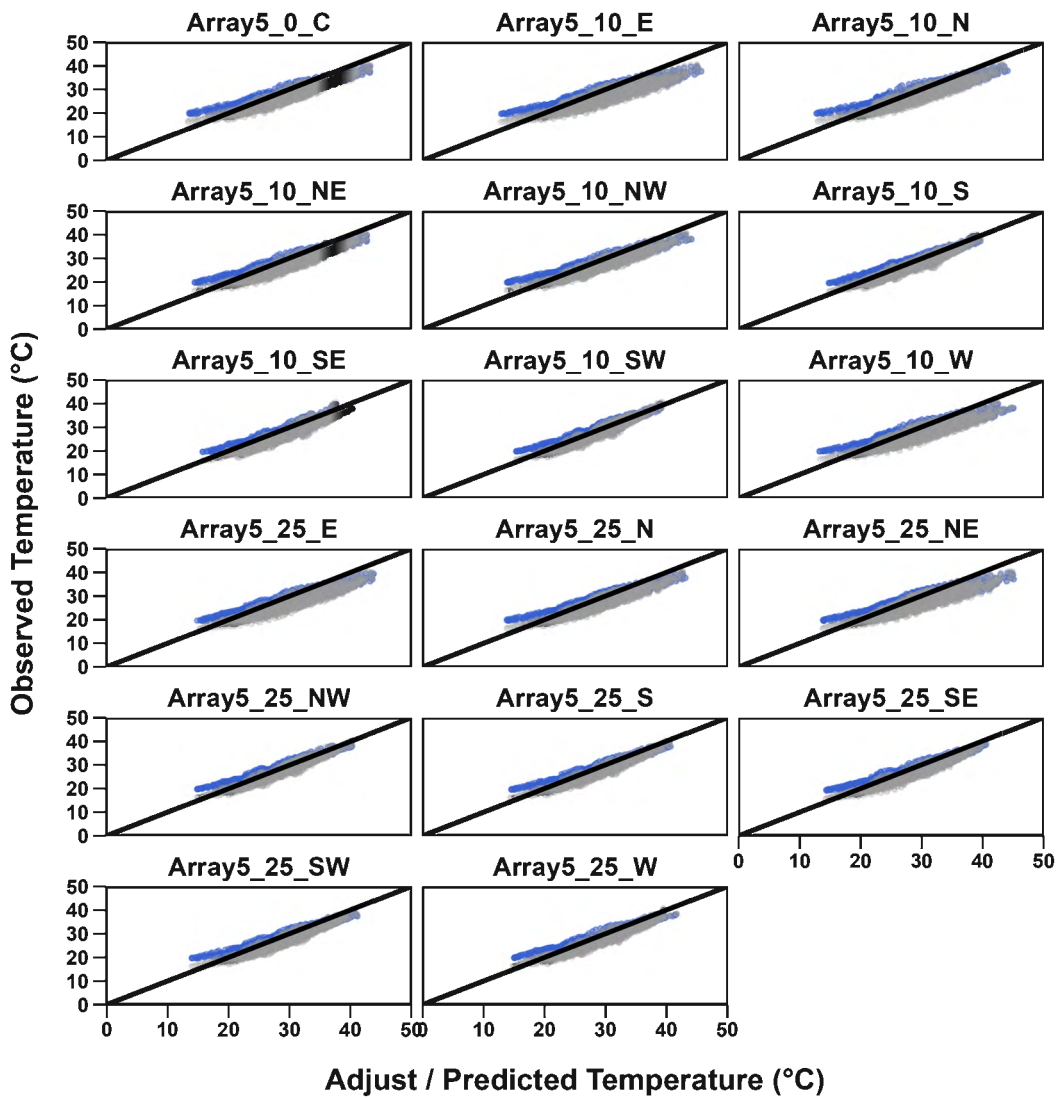


### Array 4, Fowler's Gap, NSW



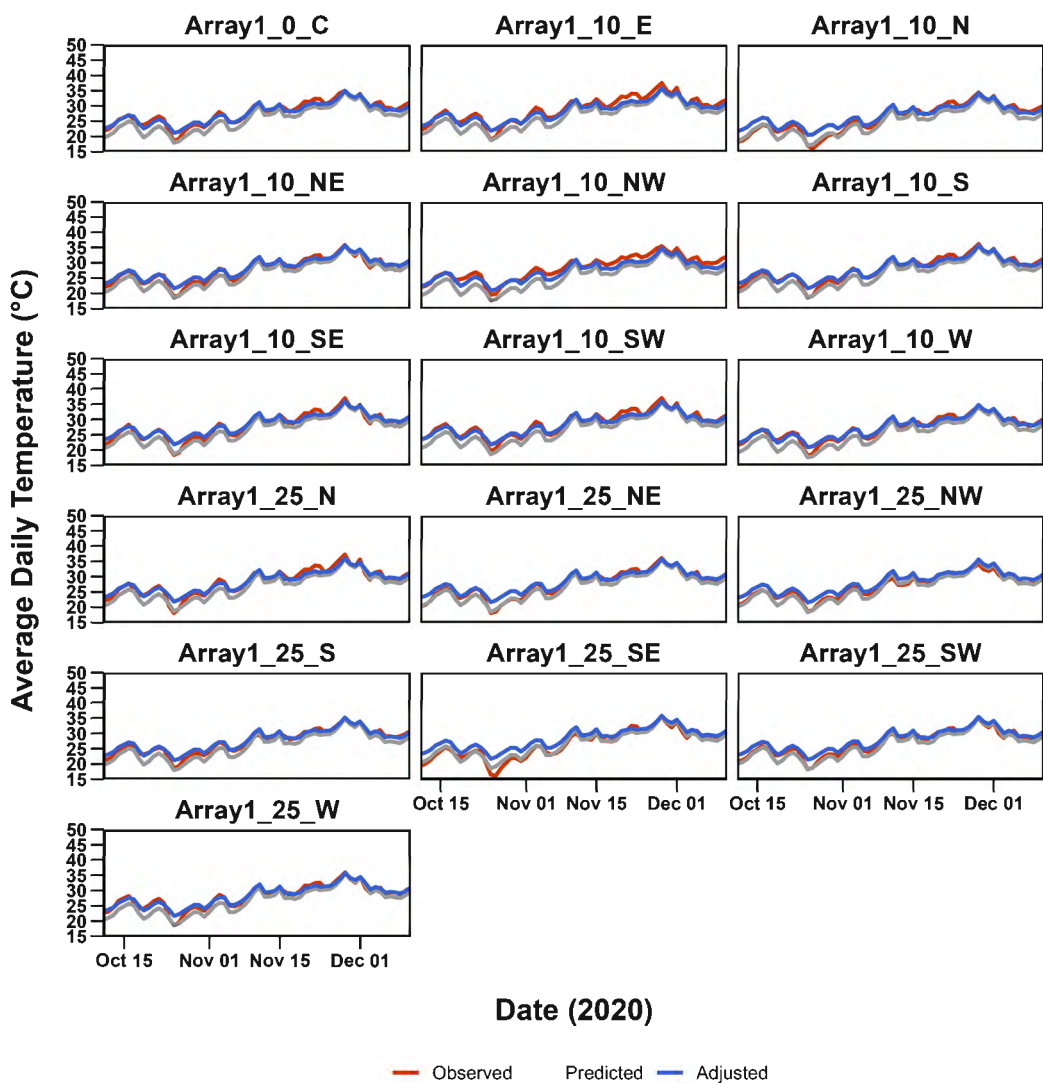
**Figure S4.6.** Fowler's Gap, NSW Array 4 relationships between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted. Panel headings denote distance and direction.

### Array 5, Fowler's Gap, NSW



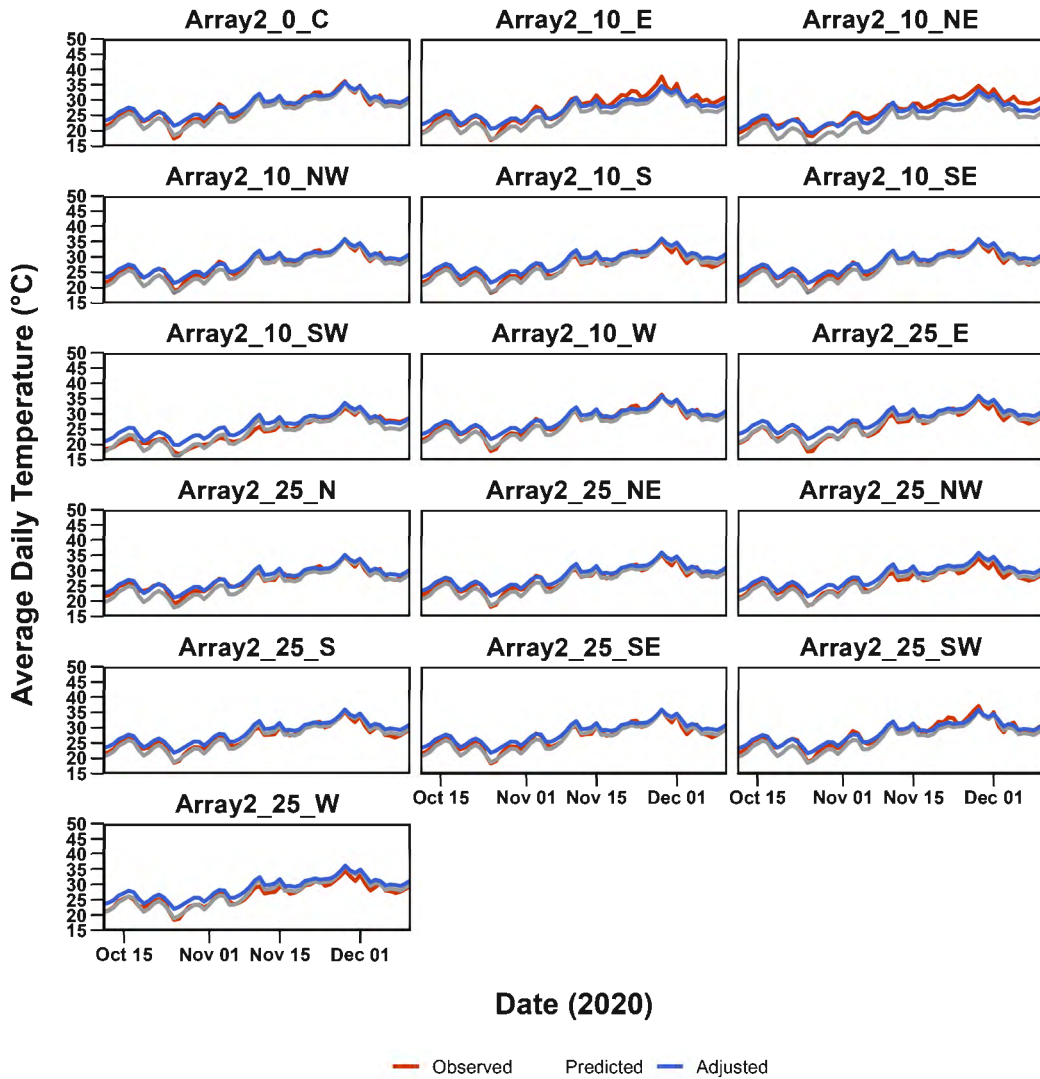
**Figure S4.7.** Fowler's Gap, NSW Array 5 relationships between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted. Panel headings denote distance and direction.

### Array 1, Fowler's Gap, NSW



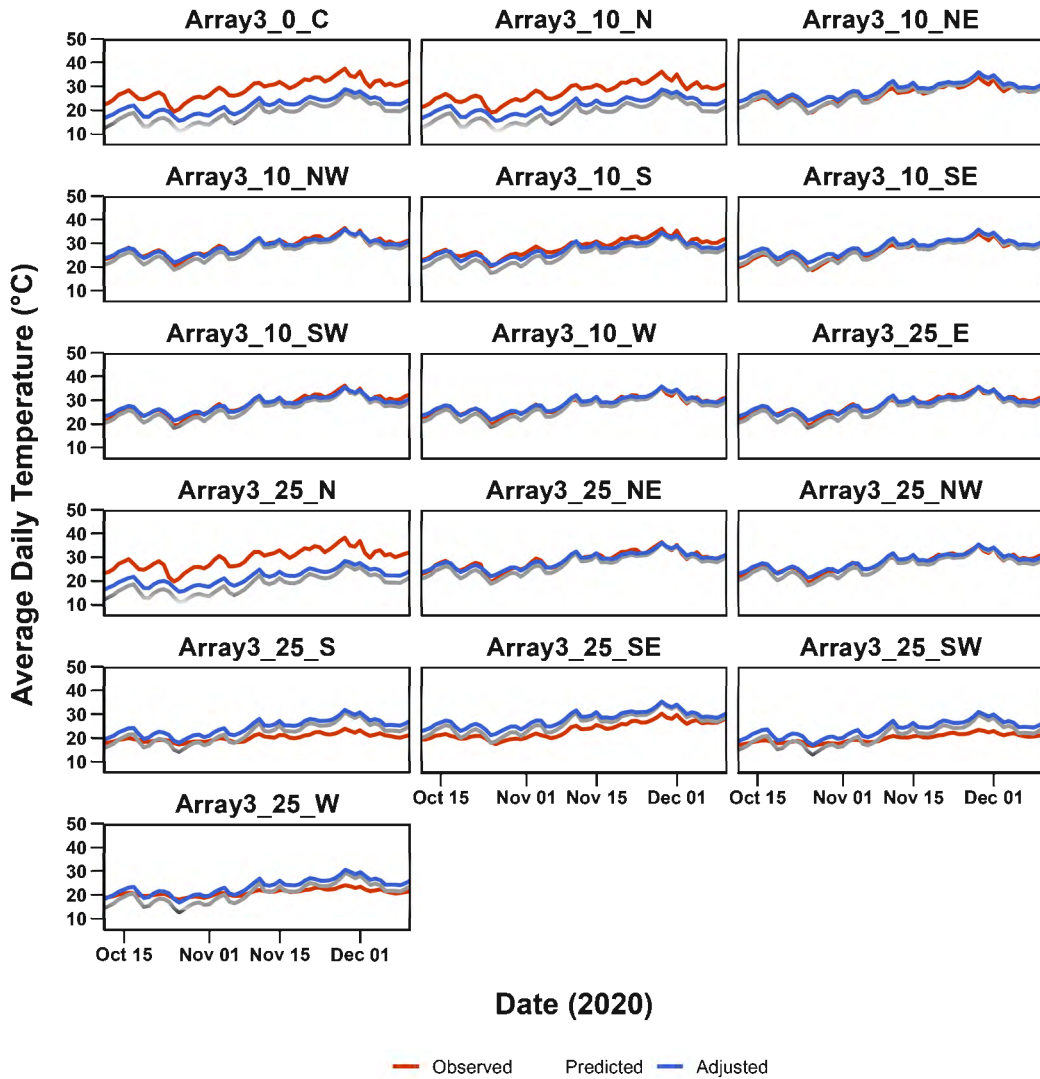
**Figure S4.8.** Fowler's Gap, NSW Array 1 mean daily temperatures across three months in 2020 (observed- black, adjusted predicted-blue, NicheMapR predicted-red).

### Array 2, Fowler's Gap, NSW



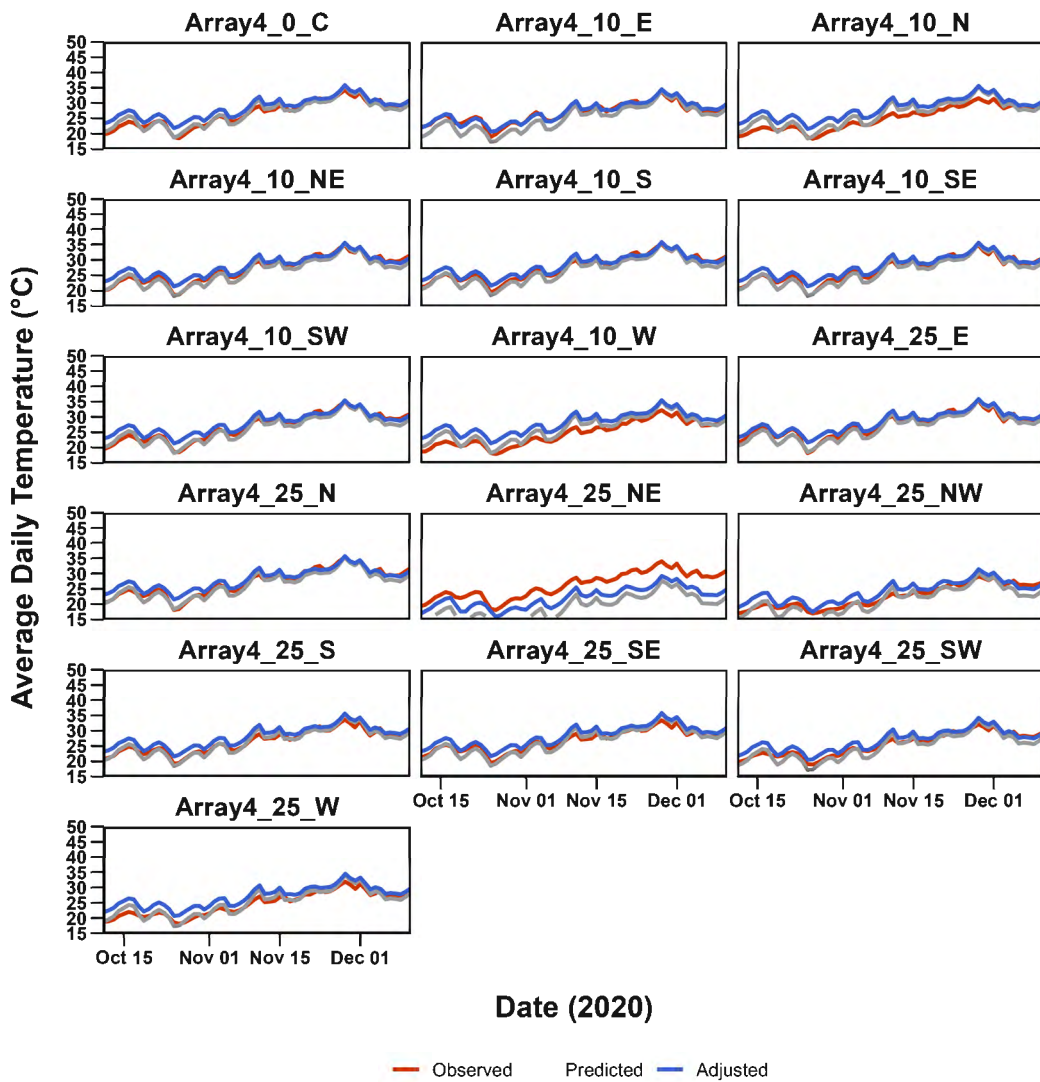
**Figure S4.9.** Fowler's Gap, NSW Array 2 mean daily temperatures across three months in 2020 (observed- black, adjusted predicted-blue, NicheMapR predicted-red). Panel headings denote distance and direction.

### Array 3, Fowler's Gap, NSW



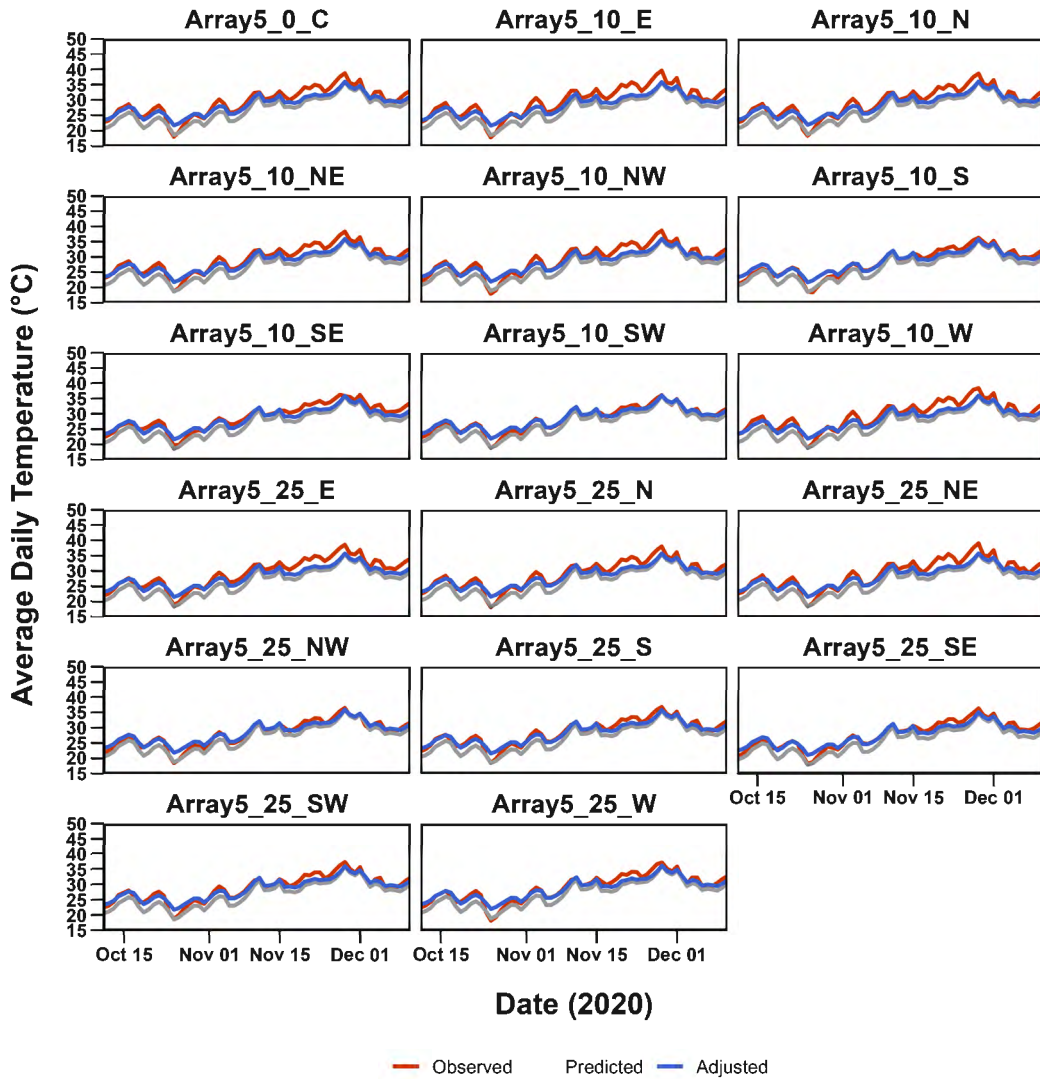
**Figure S4.10.** Fowler's Gap, NSW Array 3 mean daily temperatures across three months in 2020 (observed- black, adjusted predicted-blue, NicheMapR predicted-red). Panel headings denote distance and direction.

### Array 4, Fowler's Gap, NSW

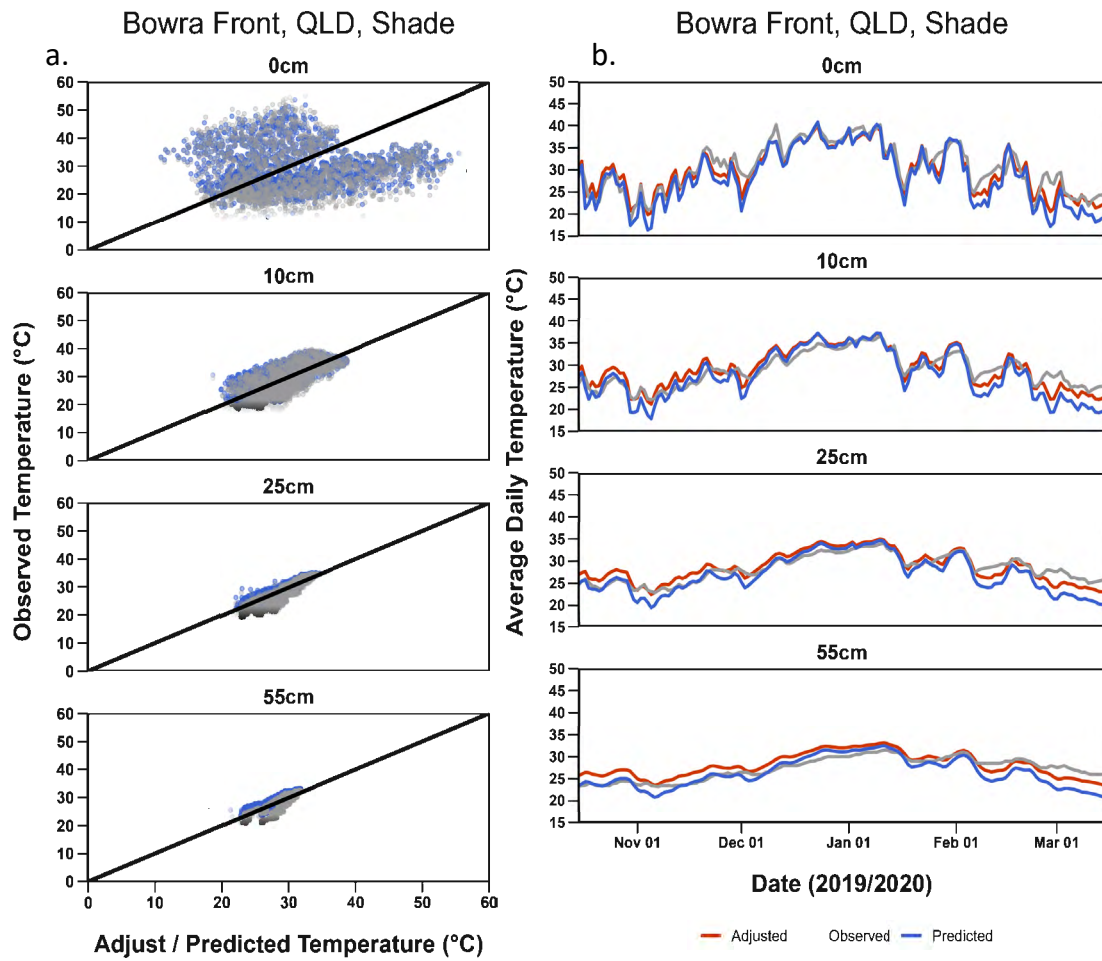


**Figure S4.11.** Fowler's Gap, NSW Array 4 mean daily temperatures across three months in 2020 (observed- black, adjusted predicted-blue, NicheMapR predicted-red). Panel headings denote distance and direction.

### Array 5, Fowler's Gap, NSW

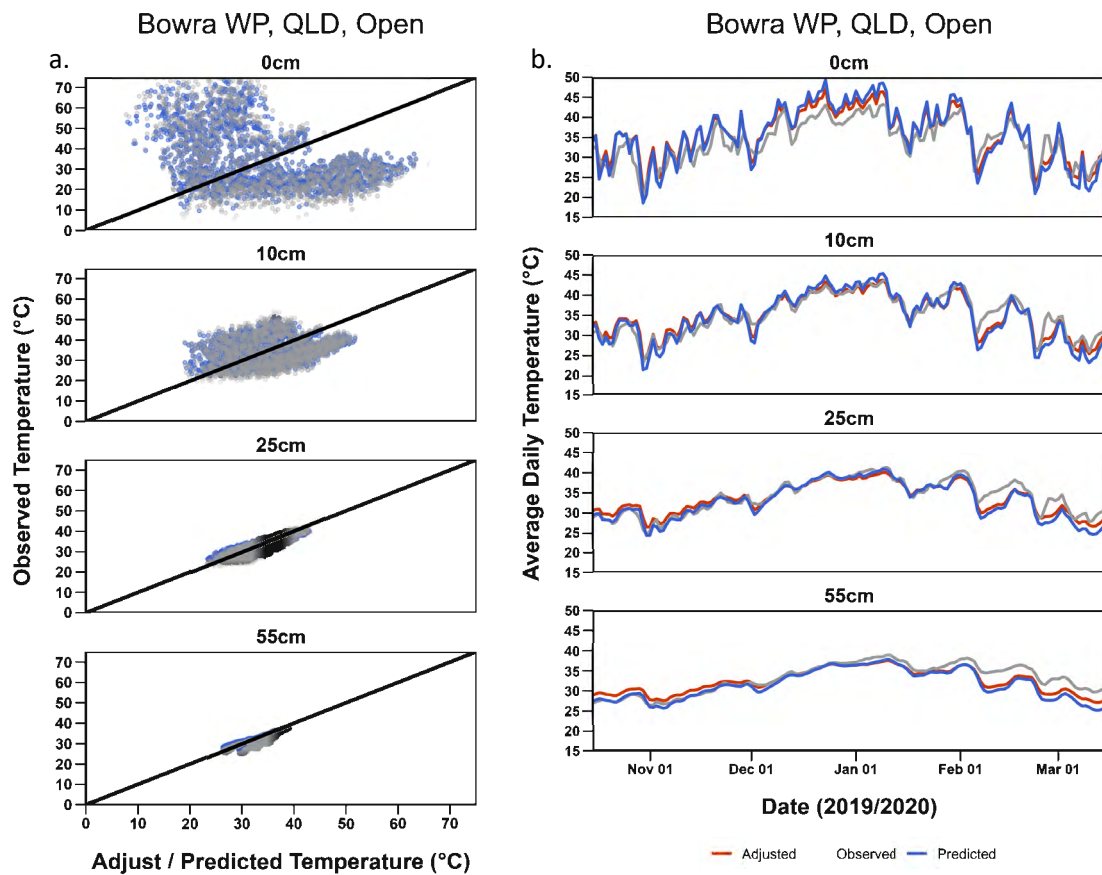


**Figure S4.12.** Fowler's Gap, NSW Array 5 mean daily temperatures across three months in 2020 (observed- black, adjusted predicted-blue, NicheMapR predicted-red). Panel headings denote distance and direction.

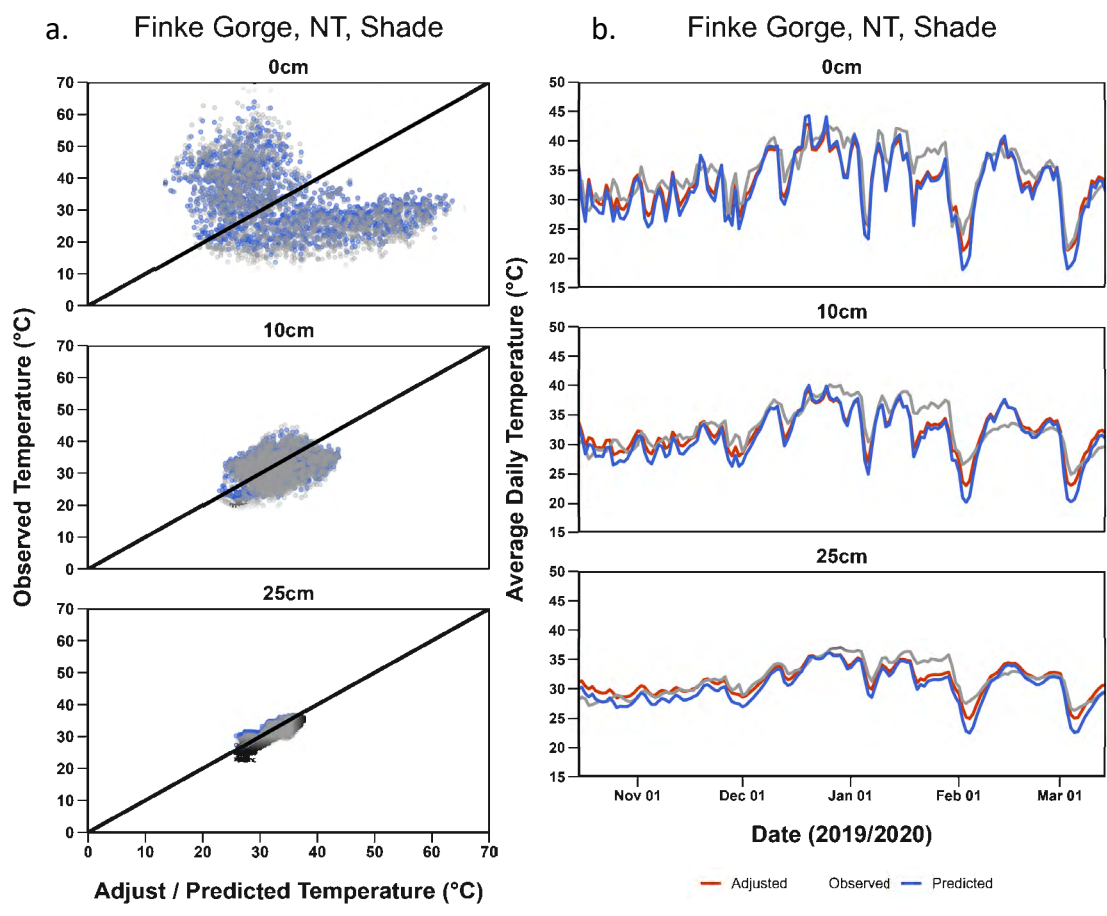


**Figure S4.13.** Observed and adjusted/predicted average daily temperatures across all depths from a shaded range-probe at Bowra Sanctuary (Front), QLD, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted- blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.

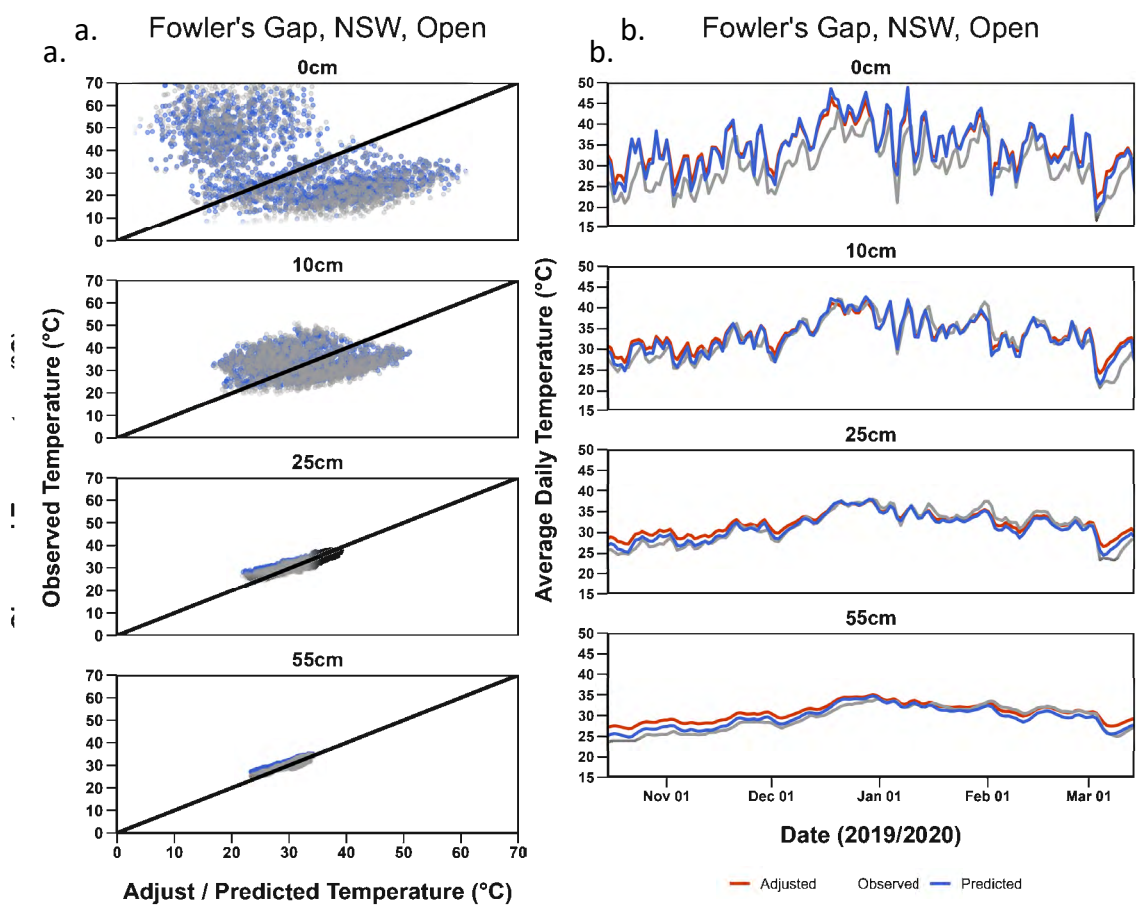




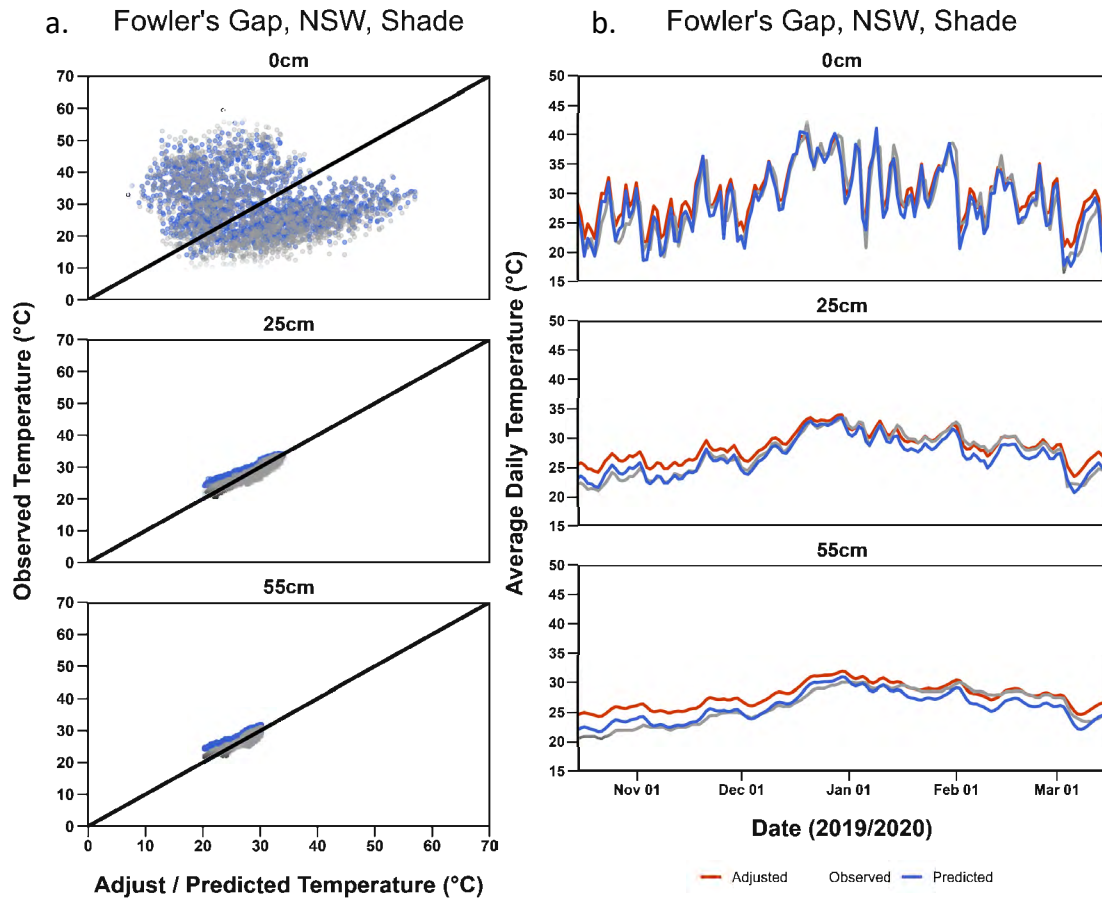
**Figure S4.14.** Observed and adjusted/predicted average daily temperatures across all depths from an open range-probe at Bowra Sanctuary (Western Paddock), QLD, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted-blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



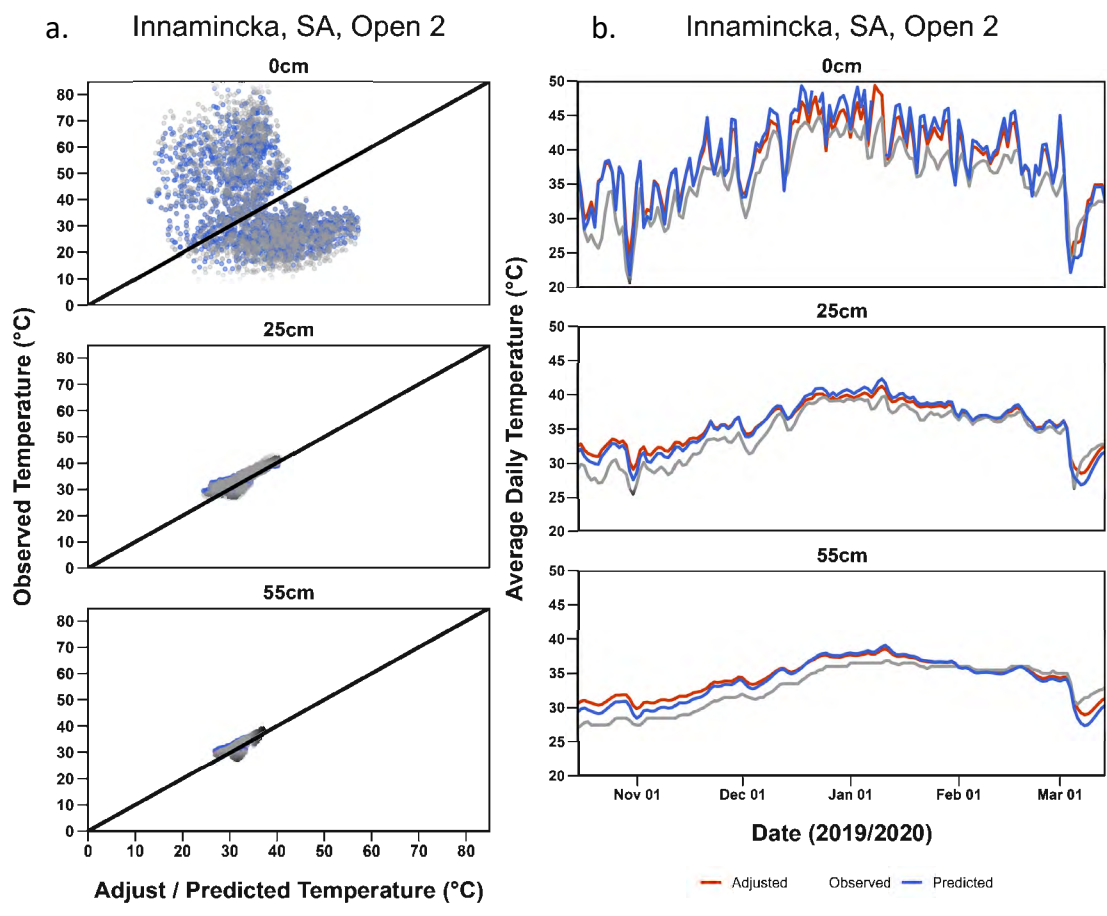
**Figure S4.15.** Observed and adjusted/predicted average daily temperatures across all depths from a shaded range-probe at Finke Gorge, NT, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted-blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



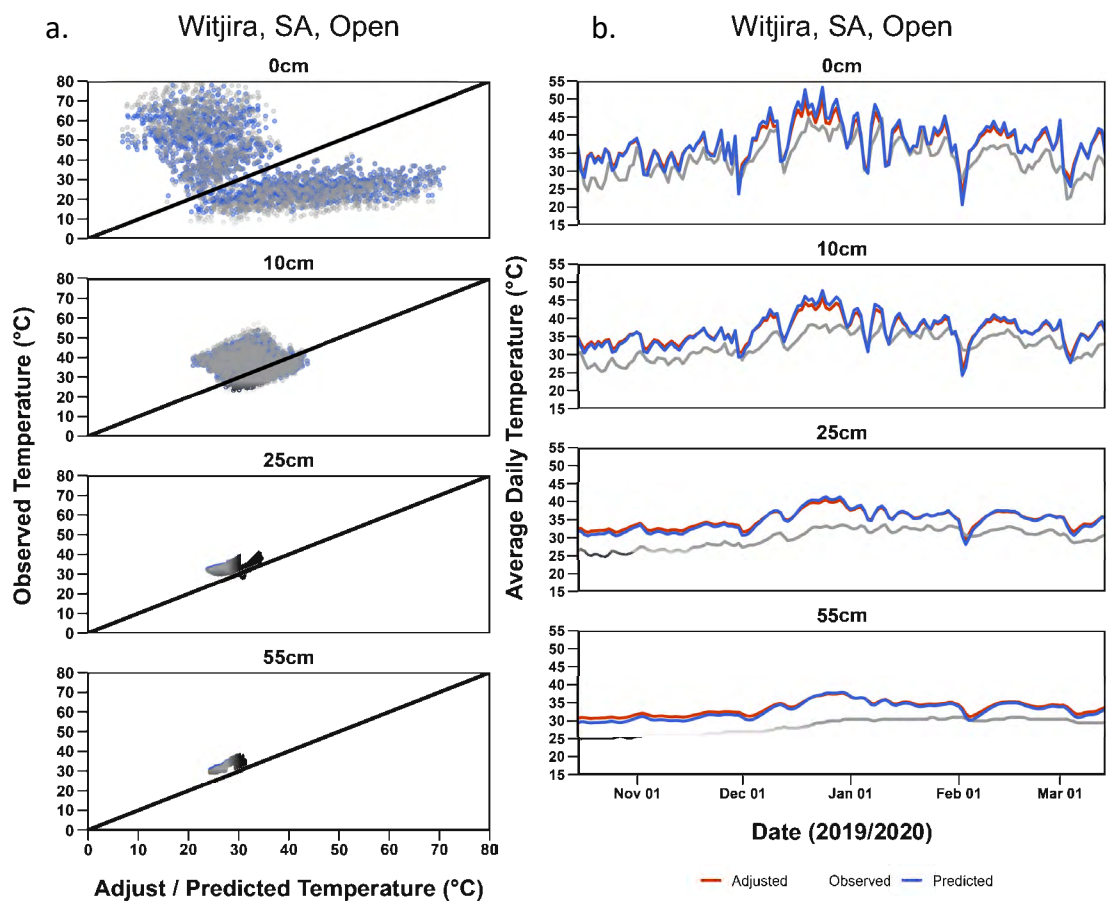
**Figure S4.16.** Observed and adjusted/predicted average daily temperatures across all depths from an open range-probe at Fowler's Gap, NSW, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted-blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



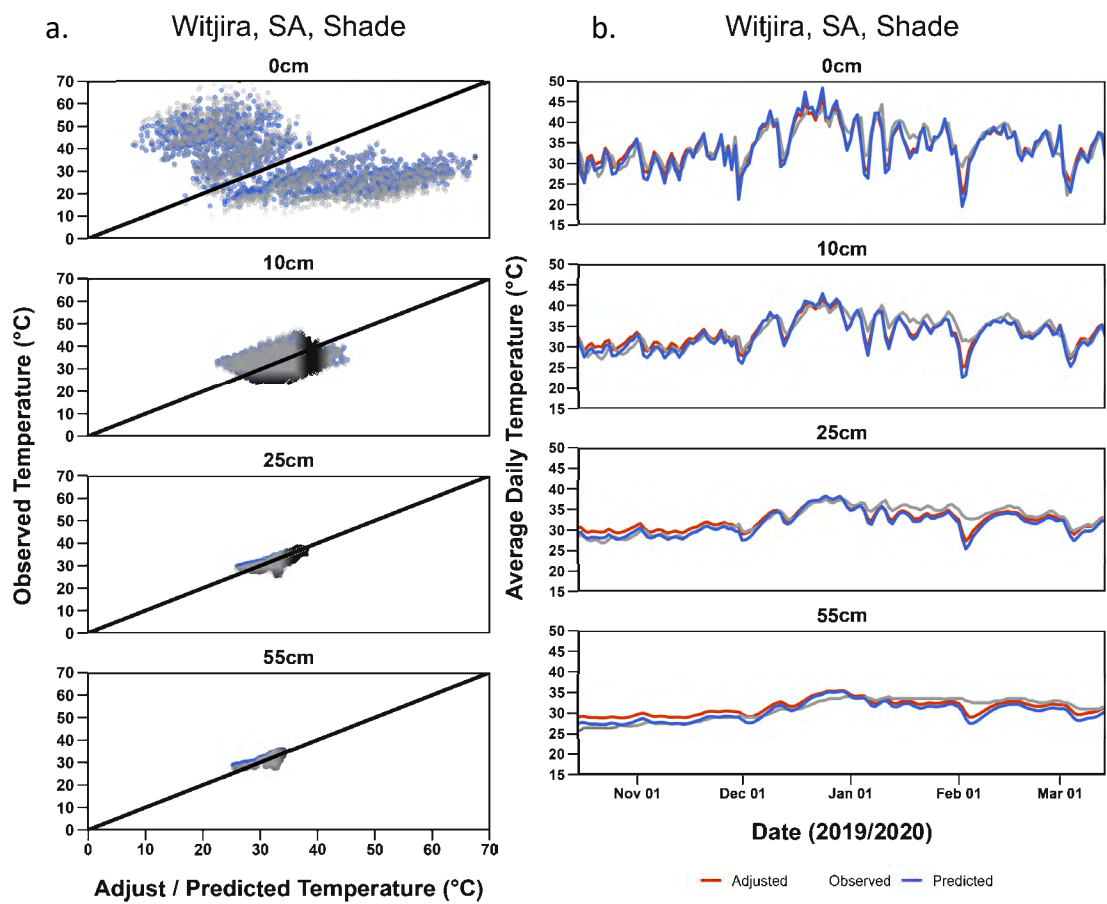
**Figure S4.17.** Observed and adjusted/predicted average daily temperatures across all depths from a shaded range-probe at Fowler's Gap, NSW, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted-blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



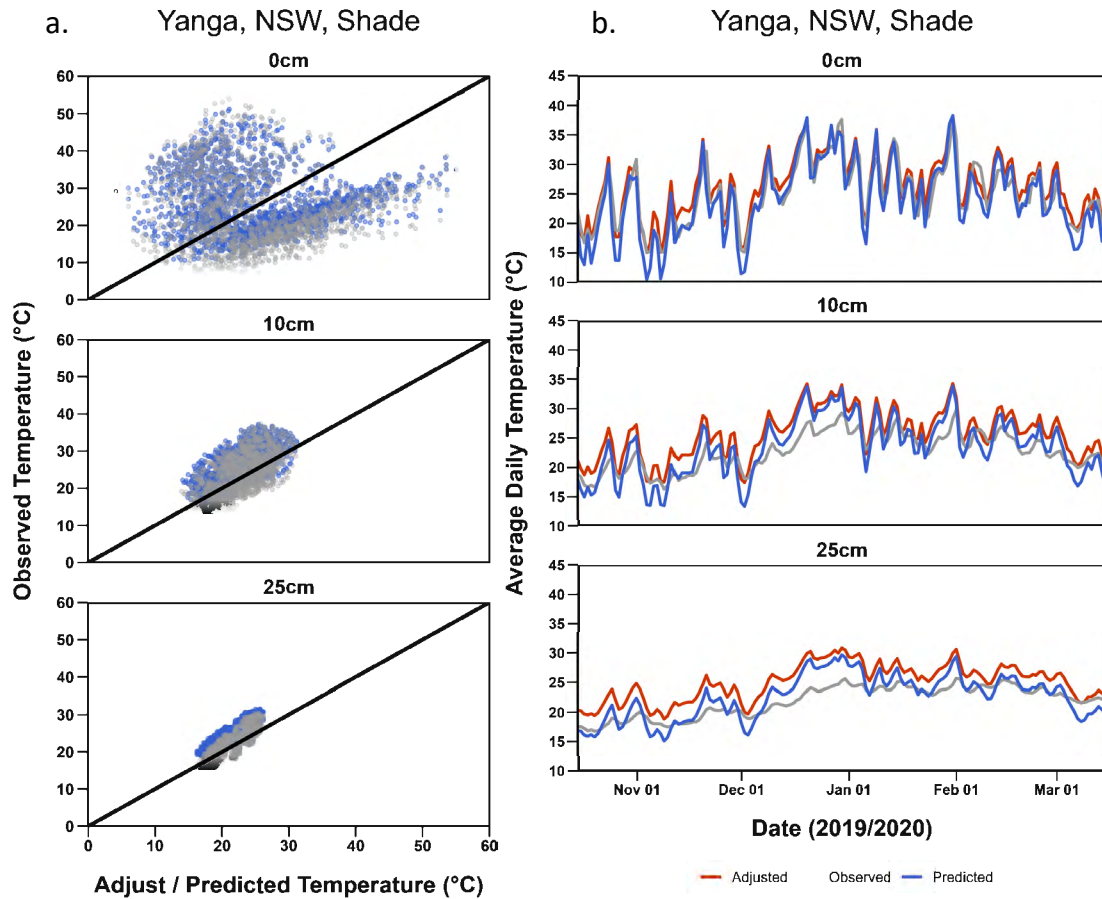
**Figure S4.18.** Observed and adjusted/predicted average daily temperatures across all depths from an open range-probe at Innamincka Regional Reserve, SA, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted- blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



**Figure S4.19.** Observed and adjusted/predicted average daily temperatures across all depths from an open range-probe at Witjira National Park, SA, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted- blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



**Figure S4.20.** Observed and adjusted/predicted average daily temperatures across all depths from a shade range-probe at Witjira National Park, SA, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted- blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.



**Figure S4.21.** Observed and adjusted/predicted average daily temperatures across all depths from a shade range-probe at Yanga State Conservation Area, NSW, Australia. **a.** Temperatures across six months in 2019-2020 (observed- black, adjusted predicted- blue, NicheMapR predicted- red). **b.** relationship between observed and predicted (grey) and observed and adjusted predicted (blue) 1:1 line plotted.





## Chapter 5. Synopsis



Female *P. vitticeps* nesting

## 5.1 Research summary

In this thesis, I address four primary aims in my three data chapters focusing on factors that may promote evolutionary transitions in modes of sex determination using the central bearded dragon (*P. vitticeps*). First, I examine the influence of sex reversal on fecundity utilising laboratory-based experiments and historical reproductive data of captive *P. vitticeps*. Then, I quantify the influence of sex reversal on their offspring's fitness-related traits utilising laboratory-based experiments. Next, I provide the first documentation of the nesting ecology of *P. vitticeps*. Finally, I also employed field-based studies and predictive modelling to quantify and predict available microclimate data across their distribution. Combined, I show that sex reversal alone is unlikely to drive this species towards a TSD system. Rather, the traits influenced by sex reversal may only allow for the perpetuation of thermolabile sex determination by providing minor advantages to offspring of sex-reversed females in early ontogeny.

Sex reversal in *P. vitticeps* has been previously shown to convey a large reproductive advantage over their concordant counterparts providing the potential to push populations toward TSD quickly under climate warming scenarios (Holleley et al. 2015; Schwanz et al. 2020). I show in Chapter 2 that the opposite is true, with fecundity of concordant females exceeding that of sex-reversed females. By expanding my data with five additional years of historical reproductive data, I show that the previous findings by Holleley et al. (2015) were driven by a single unusually productive sex-reversed female that laid 5 clutches and 105 eggs in a single breeding season although sex reversed females did produce larger eggs suggesting a trade-off between egg size and clutch size. Additionally, I show that, counter to other results of Holleley et al. (2015), embryos of sex-reversed females do not have a higher propensity for sex reversal and that ZZ embryos of concordant females have a lower  $T_{\text{piv}}$ . Together, these results shift our understanding of sex reversal in *P. vitticeps* and the trajectory of the species' sex determining system as they are not “super-females” but more likely evolutionarily stable intermediates – a finding that is consistent with the conclusions of Wild et al. (2022).

Prior to the research reported here, little was known about the effects of sex reversal on growth, survival, or performance during early ontogeny. These traits are important to the success of individuals and act as fitness-correlates of the maternal generation (Lovern and Jenssen 2001) and could help explain how transitions in sex determining systems occur. I also

explored how maternal sex genotype, incubation temperature, and offspring sex genotype influences morphology, growth, and performance (Chapter 3). I show that the production of larger eggs by sex-reversed females noted in Chapter 2 is translated into larger offspring with better body condition at hatching. This may afford them an advantage over the offspring of concordant mothers in the critical period post hatching albeit for only a short time. As with many other reptiles (Noble et al. 2018; Raynal et al. 2022), incubation temperature played an important role in performance traits. Warm incubated offspring (sex-reversed included) outperformed cool incubated offspring at ecologically relevant warm temperatures. This suggests that warm incubation may prime hatchlings for warm environments following the environmental matching hypothesis (Monaghan 2008). Environmental matching was also evident in cool incubated hatchlings which exhibited lower  $CT_{min}$  than those incubated at warmer temperatures. These results again show that sex reversal provides only small advantages to their offspring in early ontogeny and may be overwhelmed by the influence of temperature. Additionally, warm incubated concordant offspring will likely prove to be strong competitors as they experience the same post-hatching environment.

Although *P. vitticeps* is widely studied in laboratory settings, only anecdotal evidence of their nesting ecology exists outside of my study, albeit only five nests. While previous work has modelled the potential impacts of sex reversal (Holleley et al. 2015; Schwanz et al. 2020) and explored the potential underlying causes for the geographic distribution of sex reversal (Castelli, Georges, Cherryh, et al. 2021), I provide the first evidence of nesting characteristics in the wild for this species (Chapter 4). I found that concordant *P. vitticeps* preferentially choose more open-canopied nest sites that can increase the temperature of the nest past the point of sex reversal in the late reproductive season. This suggests that sex reversal is likely to occur more frequently during the late reproductive season, and as global climates continue to rise, sex reversal is likely to become more prevalent if females do not actively choose nests that will not exceed pivotal temperatures.

To provide a broader picture of the risk of sex reversal across the range of the species, I examined patterns of soil temperatures both empirically and via microhabitat modelling. The models frequently underpredicted the temperatures at the locations I measured, so I generated an improved general correction. While I used this correction to help better understand patterns of sex reversal, this is a general correction that can be applied to any predicted soil temperatures derived from the *micro\_aust* model within NicheMapR if the user has limited

input parameters. My adjusted predictions revealed times and microhabitats of highest risk. The highest risks of sex reversal were seen for open canopy microhabitats where shaded were lower. In rank order, summer (December-February), late spring (November), and early autumn (March) were the times with the highest risk of reversal. This shows that reproductive phenology and canopy cover are likely influencing the incidence of sex reversal within populations. In addition, shaded microhabitats in the north-western region of the species' distribution where high ambient temperatures suggest sex reversal should occur although it has not yet been documented, are cooler than open locations in areas within the sex reversal cluster. Based on the range of temperatures seen across microhabitats, it is clear that nesting female *P. vitticeps* have options in the thermal landscape to mitigate the risk of sex reversal. While Castelli, Georges, Cherryh, et al. (2021) suggest that those populations outside the sex reversal cluster have evolved a higher threshold for reversal, thus displaying no sex reversal, my findings show that sex reversal would be avoided if females in this region could simply nest earlier or choose shaded nest sites. This in turn may aid in the persistence of the W chromosome in populations under climate warming scenarios.

Overall, my research does not provide compelling evidence for a reproductive advantage in sex reversed females. Although, it is possible that offspring of sex-reversed females experience early advantages to that allows them to persist in populations, but the presence of cooler nest refugia early in the reproductive season may mitigate the chances of sex reversal in some populations. While climatic warming will likely increase the prevalence of sex-reversed females in wild populations in the future by increasing nest incubation temperatures, a low proportion (<50%) of sex-reversed females in a population is unlikely to drive the species towards an evolutionary transition in mode of sex determination (Schwanz et al. 2020). Transitions to TSD in *P. vitticeps* are more likely to occur if there is a high proportion of sex reversal, low migration, or a fitness advantage to sex-reversed females or their offspring (Schwanz et al. 2020). However, some populations appear to be buffered from sex chromosome loss as there has not been a substantial increase in documented cases of sex reversal over an 11 year period and suggests that there is sufficient migration (Wild et al. 2022). To add to previous findings, I found that sex-reversed females may have a fecundity disadvantage. My findings also highlight the potential of warm incubated concordant female juveniles to be equally as fast as sex-reversed juveniles of a similar age intimating they may provide strong competition in the post hatching environment (Chapter 3). While we can expect to see more sex reversal occurring in the future as the climate warms, we may also see

those adapted to the warmest areas of their range finding refugia for their nests mitigating against warmer nests (Chapter 4). Together, I show that at present, sex reversal is likely acting as a stable intermediate in populations allowing only for its persistence.

## 5.2 Future research directions

The presence of sex reversal in natural populations provides a myriad of opportunities to examine to the role of sex reversal and its impact on the ecology, physiology, and behaviour of females. There is increasing evidence that sex reversal is common in wild lizards than previously assumed (e.g. Wiggins et al. 2020; Dissanayake et al. 2021; Whiteley et al. 2021; Nemesházi et al. 2021; Hill et al. 2022; Hansson et al. 2023) but my research into *P. vitticeps* shows that we should reframe our understanding of sex reversal from that which produces hyper-fecund, “super-females” and to one that favours an intermediate and possibly stable state with small, but sufficient, advantages that allow them to persist. These advantages need be further explored particularly in free-ranging populations to test the laboratory data reported here. Moreover, research into the physiological differences (such as endogenous hormone levels) induced by sex reversal is lacking and should be investigated as they may provide explanations for some of the behaviours as described in Li et al. (2016). Below, I outline future research pathways to continue to investigate the influence of sex reversal and how my research can be further applied.

The larger eggs and larger hatchlings produced by sex-reversed females (Chapter 3) suggests that sex-reversed mothers differentially allocate resources to their offspring. The diet of mothers during gestation and vitellogenesis can play an essential role in the development of their offspring and provides key nutrients for investment into their offspring’s future (Deeming and Ferguson 1991; Warner et al. 2015; Warner and Lovern 2014; Warner and Shine 2008b). The amount of nutrients required to carry a developing embryo to term can be costly; therefore, oviparous mothers must allocate sufficient nutritional content for the embryo to develop fully (Van Dyke and Griffith 2018). If their developing embryos are supplied with more nutrients in their yolks, they may have a greater chance of survival. I suggest that yolk nutrient composition be examined first using laboratory collected eggs to determine if sex-reversed female provide higher quality nutrition to their developing offspring. Additional comparative research on the influence of yolk ablation on offspring

growth and performance could provide key insights into use of those resources provided by sex-reversed mothers as other studies in lizards have shown differential growth and survival after yolk ablation (Sinervo 1990; Warner and Lovern 2014). The diet of mothers may vary seasonally whereby females allocate yolk contents differently to produce larger eggs later in their reproductive season (Warner and Lovern 2014). These factors affecting yolk provisioning can play important roles in the overall survival and growth of their offspring post hatching (Warner and Lovern 2014; Warner et al. 2015). Varied diet studies in this species could provide crucial insight into differences in fecundity, nutrient provisioning, and offspring success particularly as this species inhabits regions known for its inter-annual extremes in rainfall which can quickly alter food availability (Dickman and Pavey 2022).

Longitudinal studies of captive *P. vitticeps* could aid in understanding the persistence of plastic traits across the lifetime of an individual and if these traits are heritable. Few studies have looked at how plastic traits induced by developmental temperature may persist (Noble et al. 2018). Furthermore, longitudinal studies can provide insight into the adaptive significance of sex reversal. As sex-reversed female are only produced at warm temperatures and perform better at warmer temperatures, this may suggest that warmer temperatures are more beneficial for sex-reversed females hinting that they may follow the Charnov-Bull Model of adaptive significance of TSD (Charnov and Bull 1977, 1989). This would require more extensive tests to measure fitness between temperature-induced sex reversal, hormonally-induced sex reversal (to produce sex-reversed females at low temperatures), and concordant individuals. While this is highly speculative, few studies have explored the adaptive significance of TSD since many of the species are long-lived (Warner and Shine 2008a), and *P. vitticeps* could provide an opportunity to study this in a species that has thermolabile determination.

Other phenotypic traits of sex reversal have been described, yet the underlying mechanisms have yet to be explored. Li et al. (2016) suggest that sex-reversed females are more male-like in their activity and willingness to explore. They hypothesised that this could be due to their male genotype, which could cause them to have a higher level of male hormones such as testosterone. Thus far, only the stress hormone, corticosterone, has been measured in *P. vitticeps* and no differences in basal corticosterone levels were detected between the three sexes (Castelli et al. 2021). Therefore, I suggest exploration of the basal endogenous sex-hormone levels such as testosterone and oestradiol in *P. vitticeps*. Understanding any differences in these hormones levels may help our understanding of their growth and behaviours of an individual. Furthermore, females deposit these hormones into

their eggs in variable concentrations across the reproductive season (Arcos 1972; Lovern and Wade 2001; Warner and Lovern 2014) and yolk hormones are known to influence traits of their offspring (Schwabl 1993; Gil et al. 1999; Lovern and Wade 2001). I suggest that yolk sex-steroid concentrations be studied in conjunction with maternal concentrations to understand if any differences may drive the behaviours seen in previous studies. Understanding these processes will provide further insight into how the differences between the two female genotypes may influence behaviours or physiological processes in *P. vitticeps*.

Further laboratory incubation studies should be explored using fluctuating temperatures from field derived temperatures as relatively few studies have utilised these techniques (Pearson and Warner 2018; Raynal et al. 2022). My studies only use two constant temperatures, which are ecologically relevant but not what would be experienced in nature. I suggest using fluctuating incubation regimes using data from nests or potential nests to explore the effects fluctuating temperatures may induce on plastic traits and understand what the sex ratios of field derived nests will yield as field incubated nests may not survive.

Nesting behaviours of *P. vitticeps* across their range will provide crucial data to understand if the females outside the sex reversal cluster are indeed choosing areas that mitigate the risk of sex reversal, are reproducing outside the range of reversal, or if they have evolved a higher threshold to reversal as suggested in Castelli, Georges, Cherryh, et al. (2021). As I have noted, gathering this data is challenging. Therefore, in the absence of field studies, I suggest expanding upon my work in Chapter 4 to include many points across the range in a similar way as done by Castelli, Georges, Cherryh, et al. (2021) while including the parameters of true nests such as varied depth and partial canopy cover. These data can be further used to inform models under climate warming scenarios, which could provide areas of increased risk.

### 5.3 Thesis conclusions

The research presented here highlights important advancements in how we view sex reversal in the context of evolutionary transitions. By revisiting historical data, a previous presumption of sex reversal inducing “super females” in *P. vitticeps* has been debunked. Tests of offspring phenotypes has shown that sex reversal does not provide much of an advantage past hatching. Finally, free-ranging females may be able to mitigate the risk of sex reversal. While there is



much to be studied around what may drive evolutionary transitions in modes of sex determination, the phenotypes of sex-reversed *P. vitticeps* are unlikely to provide the necessary push needed to fully shift this species to TSD.

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