The effect of incubation temperature on offspring phenotypes and survival of velvet gecko, *Amalosia lesueurii*

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Certificate of Original Authorship

I, M .G. T. H. Abayarathna declare that this thesis is submitted in fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the School of Life Sciences, Faculty of Science, at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualification at any other academic institution.

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Date: 30th July 2019

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My apologies if I have missed anyone!

Preface

The main body of this thesis consists of eight chapters, including five data chapters that I have submitted to journals, or which are ready to submit. Chapter 6 has been published in the journal *Biology Open*. I have therefore formatted each chapter according to the guidelines of the individual journals. This has caused some minor formatting differences between chapters and some unavoidable repetition in the species description, experimental methods and background information in some of the chapters.

At the beginning of each chapter, I have acknowledged all authors involved, in the same order as they appear in the manuscripts under review (or soon to be submitted). In all of the chapters, I am listed as the first author and was primarily responsible for conceiving, designing and implementing the research and writing the manuscripts.

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Abstract

Heatwaves are a regular occurrence in Australia, and climate modellers predict that they will increase in intensity and duration in future. Increases in summer temperatures could produce higher incubation temperatures inside lizard nests, which could influence key traits linked to fitness, such as body size, learning ability, and locomotor performance. Developmental plasticity could also buffer hatchling lizards from higher environmental temperatures by shifting critical thermal maxima upwards, enabling lizards to withstand higher temperatures. The velvet gecko *Amalosia lesueurii* is vulnerable to summer heatwaves because females oviposit in communal nests, so that changes in nest temperatures can affect a high proportion of progeny in local populations.

To investigate how incubation temperatures influence offspring traits, I incubated eggs under cold and warm conditions that mimicked thermal profiles inside currently used shaded (cold; mean = 23.3 °C; range = 17.5–30.5 °C) and sun-exposed nests (warm; mean = 25.4 °C; range = 16.5–35.5 °C) respectively. I then measured the morphology and locomotor performance of hatchlings, and assessed their survival via mark-recapture. I found that hatchlings from the cold-incubation were larger, and hatched later, than hatchlings from the warm-incubation treatment. However, neither incubation treatment nor body size affected survival in the field. To assess how heatwaves might affect hatchlings, I incubated eggs under current (mean = 24.3 °C, range 18.4–31.1 °C) and potential future ‘hot’ (mean = 28.9 °C, range 19.1–38.1 °C) nest temperatures. After the eggs hatched, I measured the morphology, thermal tolerance, thermal preference and learning ability of hatchlings, before releasing them at field sites. Future incubation temperatures produced smaller hatchlings that emerged from the eggs several weeks

earlier than the current-incubated lizards. In addition, future-incubated lizards displayed reduced cold tolerance, and were slower learners, than cold-incubated lizards.

Overall, my results show how developmental plasticity can shape the phenotypic traits of hatchling geckos. While incubation under current nest temperature regimes had little effect on offspring traits or survival, incubation under future temperatures produced smaller hatchlings with reduced cognitive abilities and cold tolerance. Some of these phenotypic differences persisted for six months, and have the potential to affect offspring survival in the field. However, future studies that link offspring traits to survival are necessary to elucidate the links between incubation temperature and offspring fitness. In particular, we need a better understanding of maternal nest-site decisions, particularly if we want to forecast how lizards will cope with changing environments in future.

Chapter 1 General Introduction

1.1 Climate change

Over geological time, the climate on earth has oscillated between periods of extreme cold, when glaciers covered much of the land, and warmer interglacial periods, when ice sheets shrunk and glaciers retreated. The earth is currently in the midst of a warm interglacial period, and climate scientists have suggested that average temperatures have increased by 0.6 °C globally in the past 100 years (Walther et al., 2002), and may increase further due to anthropogenic forces (Houghton, 2001, Coumou and Robinson, 2013, Coumou and Rahmstorf, 2012, Hansen et al., 2012). In addition, climate modellers have predicted that the duration and intensity of heatwaves will increase in the future (Cowan et al., 2014, Hansen et al., 2012) In the past, average global temperatures have been higher than current temperatures; for example, during the Medieval Warming, temperatures were 0.75 °C higher than those experienced today (Zachos et al., 2001). Although many species adapted to these past warming events, recent anthropogenic activities, such as urbanisation, land clearing and habitat fragmentation, may make it more difficult for species to adapt to the current levels of climate warming (Houghton, 1997).

1.2 Effect of climate change on reptiles

Reptiles are particularly sensitive to changing climates because the thermal environment strongly influences their physiology and behaviour (Huey et al., 2009). Most reptiles use complex behaviours, colour changes, or postural adjustments to maintain their T_b within their preferred temperature range (Avery et al., 1982). However, when these adjustments fail to keep temperatures within their preferred range, ectotherms must seek shelter in cooler environments (Bogert, 1949). Seeking shelter for prolonged periods can incur costs as it can preclude other activities linked to fitness such as reproduction, feeding, and territory defence. In desert regions of Mexico, reduction in activity due to increases in temperature are thought to have contributed to local extinctions of lizard populations (Sinervo et al., 2010b).

Thermal performance curves provide a framework for understanding the costs and benefits associated with thermoregulation. These curves show how physiological and behavioural functions change across different T_bs. Most curves are slightly right skewed, with an increase from the minimum critical temperature (CT_{min}) to the temperature at which ectotherms perform best (the optimum temperature or T_{opt}), before dropping to the critical thermal maximum (CT_{max}, Figure 1-1 (Huey et al., 2012). Most authors assume that there is a strong correlation between performance and fitness (Arnold, 1983, Garland Jr and Losos, 1994), so performance curves can provide information about the potential fitness of ectotherms at different temperatures.

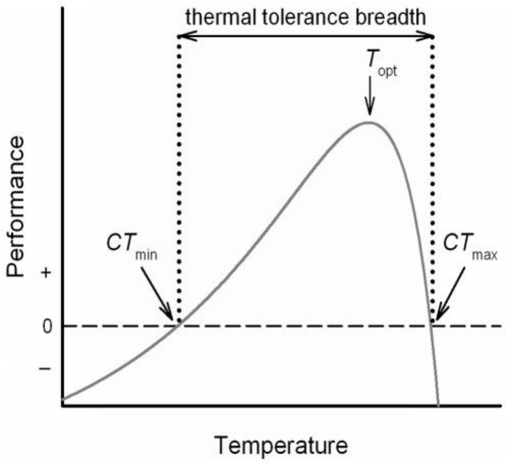

Figure 1-1 Effect of T_b on the performance (fitness) of an ectotherm (Huey and Stevenson, 1979, Huey et al., 2012)

Ectotherms, particularly lizards, are frequently used as model organisms for addressing questions about the evolution of physiological traits (Huey and Kingsolver, 1993, Angilletta, 2009). A thermal performance curve is an indicator of how changing T_b s influences the physiology and fitness of the ectothermic animals (Angilletta, 2009). Extremely low and high body temperatures can impair performance, and if the animal is incapacitated, these extreme temperatures are lethal. These endpoints are called critical temperatures; the critical thermal minimum (CT_{min}) and critical thermal maximum (CT_{max}) respectively. In between these limits, physiology and performance reaches its maximum level at some optimal temperature (T_o).

How temperature increases affect fitness will depends on how shifts in temperature affect T_b relative to T_o. If T_b is less than T_o in a habitat, then an increase in T_b induced by increases environmental temperatures will increase fitness. By contrast, if warming is modest, and T_b is similar to T_o, then there will be little impact on fitness. However, if increases in temperature force T_b to rise significantly above T_o, then warming can cause a sharp drop in fitness (Figure 1-2).

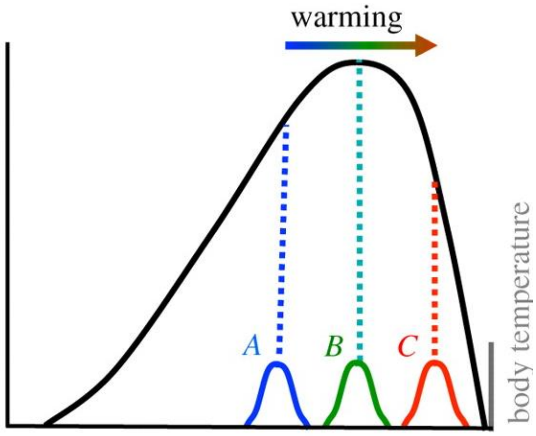

Figure 1-2 Shifts in the T_b of an ectotherm under climate warming, under the assumption that increases in ambient temperatures result in increases in T_b (Huey et al., 2012). A

modest increase in T_b (shift from A to B) results in an increase in fitness, whereas larger increases in temperature (shift from A to C) results in a loss of fitness.

The sensitivity of reptiles to climate warming will also depend on whether the species is thermal generalist or a thermal specialist. Thermal specialists perform well over a wide range of T_b s, whereas thermal specialists operate over a narrow range of T_bs (Huey et al., 2012). Thus, if we assume that climate warming results in an identical increase in body temperature from the dark blue line to the red line in Figure 3, then the fitness cost will be higher for the thermal specialists (Figure 1-3a) than for the thermal generalist (Figure 1-3b). Understanding the shape of the thermal performance curve can therefore be useful for predicting ectothermic species potentially at risk from climate warming (Gilbert and Miles, 2017, Huey et al., 2012).

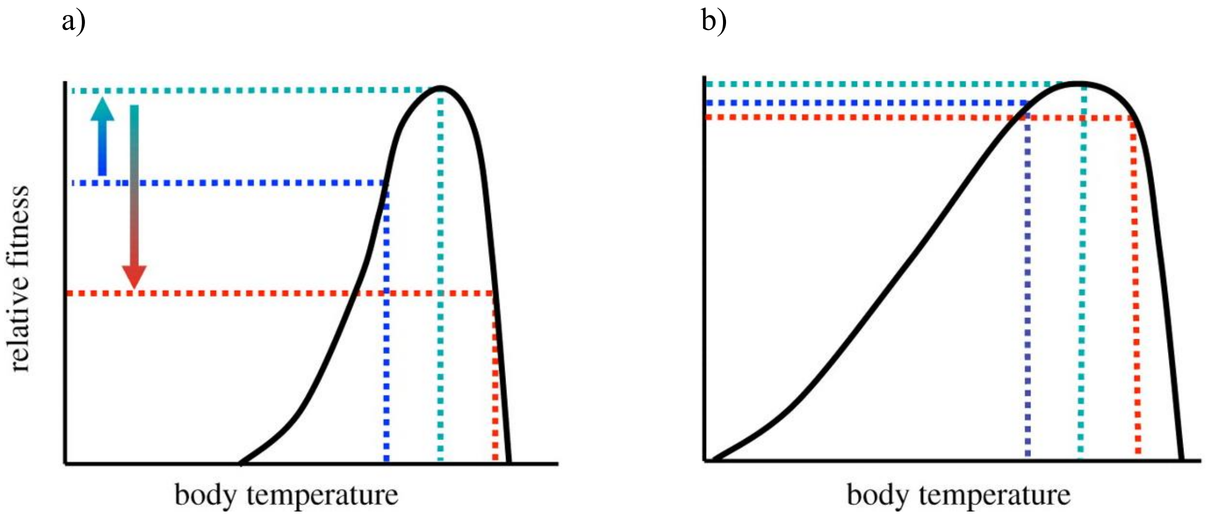

Figure 1-3 Thermal performance curves for thermal generalist and thermal specialist, The figure shows the hypothetical fitness cost for a given shift in T_b from the dark blue to the red line. Note that thermal specialists (a) suffer a larger fitness cost for a given increase in temperature compared the thermal generalist (b) (Huey et al., 2012)

A key gap in our knowledge is whether critical thermal limits, particularly CT_{max}, are fixed, or whether they are plastic traits that might allow lizards to adapt to higher temperatures. Phenotypic plasticity and genetic changes could help reptiles adapt to climate warming (Urban et al., 2014). Adaptive shifts in performance or thermal limits may also allow reptiles to cope with increasing temperatures (Angilletta Jr et al., 2002). Over time, natural selection may result in adaptive changes to key traits such as CT_{min} and CT_{max} (Kingsolver and Huey, 1998). One study proposed a model of evolutionary response to climate change based on phenotypic variation in CT_{max} in amphibians and reptiles. This model predicted that CT_{max} could increase by 3.2 °C in 50 years, which would enable some reptiles to cope with predicted increases in global temperatures (Skelly and Freidenburg, 2010). However Urban *et al* (2014) pointed out that this model ignored the potential limits of adapted genetic variation at extreme temperatures. Specifically, while CT_{min} is relatively plastic, CT_{max} is not, and there is little evidence that lizards can evolve higher CT_{max} in response to higher temperatures (Urban et al., 2014). Thus, thermal adaptations, particularly shifts in CT_{max}, may be inadequate to allow lizards to cope with both the speed and magnitude of climate warming, which, when coupled with habitat loss and fragmentation, makes ectotherms particularly vulnerable to extinction (Hoffmann and Sgrò, 2011).

1.3 Fate of early life stages towards climate warming

To date, most studies have focused on the effects of climate warming on the adult life stages of reptiles. However, to understand how warming may influence the persistence of lizard species, we need to understand how thermal changes influence vulnerable life stages, such as embryos (Levy et al., 2015). In most lizard species, females lay their eggs in soil nests in open areas, and abandon the eggs soon after oviposition. Consequently, the survival and development of embryos is influenced primarily by the temperature and humidity within the nest (Warner and Andrews, 2002, Deeming, 2004, Noble et al., 2018). Consequently, increases in air temperature, such as those associated with summer heatwaves, can cause increases in nest temperatures. If nest temperatures are too hot, eggs can desiccate, or embryos can die from thermal stress, resulting in nest failure (Angilletta et al., 2000, Telemeco et al., 2016, Telemeco et al., 2017, Levy et al., 2015).

While extreme temperatures can cause embryo death (Telemeco et al., 2017, Angilletta et al., 2013, Telemeco et al., 2016, Hall and Warner, 2018) more subtle increases in nest temperatures can have profound effects on the morphology and fitness of the resulting hatchlings (Noble et al., 2018). Numerous laboratory experiments have shown that incubation temperature affects the phenotypic traits of hatchling reptiles, including growth rate, body size and shape, locomotor performance, thermal preference, antipredator behaviours and sex (Elphick and Shine, 1998, Qualls and Andrews, 1999, Booth, 2006, Shine et al., 1997, Deeming, 2004). In many species of lizards, eggs incubated at high temperatures close to the upper thermal threshold for embryonic development produce smaller hatchlings because less yolk is converted to somatic tissue (Lin et al., 2010). For example, experiments on *Calotes versicolour* revealed that eggs incubated at moderate temperatures produced heavier hatchlings with longer bodies and tails than eggs incubated at higher temperatures (Ji et al., 2002).

In turn, incubation induced shifts in hatchling body size can influence the subsequent fitness of hatchlings. Several studies on reptiles have found links between hatchling body size and apparent survival (Ferguson and Fox, 1984, Janzen, 1993, Janzen et al., 2000, Sinervo and Adolph, 1989, Sinervo et al., 1992, Sorci and Clobert, 1999, Dayananda et al., 2017). In some lizard species, larger hatchlings may have higher survival than smaller hatchlings (Brana and Ji, 2000). Larger hatchlings might be more efficient foragers and be more successful in avoiding predators (Ji et al., 2002), two traits that could influence survival. Incubation temperatures can also affect the locomotor performance of hatchling lizards (Andrews, 2008, Elphick and Shine, 1998). In the scincid lizard, *Bassiana duperreyi*, hot incubated individuals ran faster than cold incubated hatchlings (Elphick and Shine, 1998). Similar results have been found in other species of lizards and snakes (Burger, 1989, Burger, 1991, Van Damme et al., 1992, Elphick and Shine, 1998). Hind limb length is correlated with the locomotor performance of some lizard species (Brana and Ji, 2000). Speed can affect predator avoidance and prey capture (Hertz et al., 1982, Huey and Dunham, 1987, Irschick and Losos, 1998, Christian and Tracy, 1981). Thus, incubation induced changes in morphology could potentially influence locomotor performance, which in turn can potentially affect survival (Christian and Tracy, 1981).

Incubation temperatures may not only influence the performance of offspring, but may also affect their cognitive abilities. The ability of an animal to acquire, process and store information from their environment through perception and learning can influence fitness, and as such, cognitive traits are likely to be subject to natural selection (Dukas, 2009). Recent studies on lizards have revealed that like other traits, cognitive abilities of hatchling are influenced by thermal regimes experienced during embryogenesis. For example, Amiel and Shine (2012) incubated eggs of *Bassiana duperreyi* under cold (diel cycle of 16 ± 7.5 °C) and hot (diel cycle of 22 ± 7.5 °C) incubation treatments to mimic thermal profiles of nests at low and high elevations. These researchers found that in a task where hatchlings had to locate a 'safe' shelter, the hot incubated hatchlings achieved higher learning scores than cold incubated hatchlings. These changes were thought to be brought about by incubation induced changes in brain development (Amiel et al., 2017). In *B. duperreyi*, the telencephalons of hatchlings from low-temperature incubation were larger than those of hot-incubated hatchlings. In other lizard species, incubation can also effect brain development (Coomber et al., 1997, Sakata et al., 2000). For example, in the leopard gecko, *Eublepharis macularius*, incubation temperature influenced the volume of the preoptic areas and the ventromedial hypothalamus (Coomber et al., 1997).

By modulating neuron development, incubation temperatures may potentially influence other hatchling traits, such as thermoregulatory set points. For example, hatchling Siamese crocodiles incubated at high temperatures had higher preferred T_bs than hatchlings incubated at low temperatures. The mechanism underlying this phenomenon is still unclear, but thermal effects on thermosensitive neurons or genes might influence the ability of hatchlings to control their body temperatures (Lang, 1987). As discussed earlier, selected body temperatures can influence hatchling traits linked to fitness including locomotor performance, growth rates, and survival (Noble et al., 2018).

1.4 Current knowledge gaps

Although there is a wealth of knowledge about how incubation temperatures influence the anatomical, physiological and behavioural characters of hatchling reptiles, we know little about how future increases in nest temperatures may influence those traits, nor how such traits will influence fitness (Noble et al., 2018). Another gap in our understanding is whether higher temperatures influence the thermal tolerance of hatchlings. Higher incubation temperatures could produce adaptive shifts that protect hatchlings from higher temperatures. For example, *Drosophila* larvae that were reared at higher temperatures have produced flies with higher heat tolerance (Heerwaarden et al., 2016). Whether similar adaptive changes in thermal tolerance could buffer embryos or hatchlings from increasing temperatures is unknown. Finally, the majority of incubation experiments on lizards have used constant temperatures, which are biologically unrealistic as temperatures within natural nests typically fluctuate widely by day (Elphick and Shine, 1998, Angilletta, 2009). Moreover, the magnitude of daily temperature fluctuations is more pronounced during heatwaves (Telemeco et al., 2009, Levy et al., 2015). Thus, to understand how climate warming may affect reptile species, we need to incubate eggs using fluctuating temperatures, and we need to simulate nest temperatures likely to be experienced by embryos in future (Noble et al., 2018, Pearson and Warner, 2018). Already, lizards have experienced local extinctions due to climate warming, and this group is expected to suffer more extinction in the future (Sinervo et al., 2010a). By understanding how embryos respond to increased nest temperatures, and how hatchlings cope with increased temperatures, we can improve our ability to predict how lizards will cope with future warming.

1.5 Study species: The Velvet gecko *Amalosia lesueurii*

Figure 1-5 an adult velvet gecko from Nowra field site (*Photo by Yingyod Lapwong*)

The velvet gecko, *Amalosia lesueurii* (Duméril and Bibron, 1836) is an ideal model organism to investigate how incubation temperatures influence the performance and survival of hatchling lizards. Velvet geckos are small nocturnal lizards (up to 65 mm snout–vent length) that inhabit sandstone rock outcrops from south eastern New South Wales to South Eastern Queensland (Cogger, 2014). Geckos thermoregulate during the day under small stones or inside rock crevices, and they forage at night (Webb and Shine, 1998, Schlesinger and Shine, 1994). Adult male geckos are territorial but adult females are frequently found occupying the same rock with juveniles (Schlesinger and Shine, 1994).

Female velvet geckos lay two eggs per clutch inside communal nests in exposed rock crevices (Webb et al., 2008). In the Nowra region, air temperatures were positively correlated with temperatures inside communal gecko nests (Dayananda et al., 2016). Females lay their eggs during spring (late October through early November) and the eggs have a long incubation period of up to 120 days (Doughty, 1997). Because of the long incubation period, and the high correlation between ambient temperatures and nest temperatures, increases in the duration of summer heatwaves predicted to occur in future (Cowan et al., 2014) could result in exposure of eggs to lethally high temperatures (Dayananda et al., 2016).

Interestingly, female geckos show nest site philopatry and return to the same nests to lay eggs (Webb et al., 2008). At sites in Morton National Park, females have laid eggs in the same sun-exposed nest sites for the last 26 years, suggesting that they lack plasticity in nesting behaviour. Thus, higher nests temperatures predicted to occur in future could affect the persistence of gecko populations. Using programmable incubators, Dayananda et al. (2016) incubated eggs to mimic nest temperatures under current ‘cold’ conditions (mean = 23.2 °C, range 10-33 °C) and future ‘hot’ conditions (mean = 27.0 °C, range 14-37 °C) likely to be experienced in 2050. The hot-incubated eggs hatched 24 days earlier than the cold incubated eggs, and hot-incubated hatchlings were smaller and lighter than cold-incubated hatchlings. In the field, cold-incubated hatchlings had higher survival than the hot-incubated hatchlings (Dayananda et al., 2016). However, it was not clear what mechanism was responsible for the lower survival of hot-incubated hatchlings. Potentially, variation in learning ability, body size, or thermal tolerance could have influenced the survival of the hatchlings. Currently, we do not know whether incubation at higher temperatures could lead to adaptive shifts in critical thermal maxima, which could potentially enable hatchlings to withstand higher temperatures in future.

1.6 Aims of the study

The broad aim of my study is to investigate how incubation temperatures influence the phenotypes and survival of hatchling velvet geckos. If predicted future warming occurs, and females continue to lay eggs in the same communal nests, then temperatures inside communal nests will become hotter in future (Dayananda et al., 2016) . To understand how such shifts in temperature might influence hatchling traits, I carried out a series of incubation experiments using 10 step programmable incubators in the laboratory. In year 1, I investigated how current incubation temperatures found in exposed ("cold") and shaded ("warm") gecko nests influence offspring phenotypes. In year 2, I investigated how current "cold" nest temperatures and future "hot" temperatures (predicted to occur in 2050) influence offspring traits. Each year, after the eggs had hatched, I measured a series of hatchling traits in the laboratory. I then individually marked the hatchlings, and released them at the field sites. To estimate hatchling growth rates and survival, I turned all rocks at the release sites once per month, and captured and measured all geckos found beneath rocks. I then analysed the mark-recapture data using Cormack-Jolly-Seber models in program MARK to investigate whether incubation temperatures, or hatchling traits, influenced the survival of hatchlings the wild.

In focussing on this goal, I outlined five aims that are more specific for my research.

1. How does the nest site choice of a female velvet gecko affect her offspring?
2. How do incubation temperatures and the location of origin affect the locomotion of hatchling velvet geckos?
3. Does incubation temperature modulate the thermal preference of hatchling velvet geckos?
4. How does incubation temperature modulate the hatchlings thermal tolerance?
5. Does incubation temperature affect the learning ability of hatchling velvet geckos?

1.7 Thesis structure

The thesis consists of a series of papers that I have submitted to journals for consideration for publication. I explored the effects of higher incubation temperature on the phenotypes and survival of hatchling velvet geckos. To align with this aim, I assessed the effects of incubation temperature on the morphology, locomotion, thermal preference, thermal tolerance and learning ability of hatchling velvet geckos, which I explain in detail in chapters two to seven.

Chapter 2 describes my study sites and some of the general methodologies I used to carry out the experiments.

Chapter 3 describes the effects of current nest temperatures ('cold' and 'warm') on the morphology of hatchling velvet geckos. It includes the results of the survival analysis that explains the effect of incubation temperature on the survival of hatchlings.

Chapter 4 describes the effects of current 'cold' and 'warm' nest temperatures on the locomotion of hatchling velvet geckos. I also investigate the interactions between the location of origin and incubation temperature on the hatchlings' locomotion.

Chapter 5 reveals that current 'cold' and future 'hot' nest temperatures have little effect on the thermal preference of hatchling velvet geckos.

Chapter 6 investigates the effect of current 'cold' and future 'hot' nest temperatures on the thermal tolerance of hatchling velvet geckos. I discuss how thermal environments during incubation influence both hot and cold tolerance of hatchlings.

Chapter 7 investigates the effect of current 'cold' and future 'hot' nest temperatures on the learning ability of hatchling velvet geckos.

In Chapter 8, I provide my general conclusions, and give a critical summary of my results and give some directions for future research.

1.8 Ethics and Permits

All the experiments presented in this thesis was conducted according to the code of practice for the care and use of animals for scientific purposes, and all procedures were approved by the University of Technology Sydney Animal Care and Ethics Committee (protocol # ACEC 2012000256). The research was supported by a grant from the University of Technology Sydney (to Jonathan K. Webb). All fieldwork was carried out according to the New South Wales National parks and Wildlife act under the scientific licence (SL 101013 to JKW)

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Chapter 2 General methodology

2.1 Study sites

My study sites were located in Dharawal National Park and on Crown Lands (climbing site) near Nowra (Figure 2-1). Sandstone rock outcrops in these areas provide thermally distinctive microhabitats for velvet geckos and other reptiles, including the vulnerable broad headed snake, *Hoplocephalus bungaroides* (Webb and Shine, 1998). Illegal removal of exfoliated rocks for landscaping purposes has reduced the availability of rocks for rock-dwelling reptiles and invertebrates. Each study site was restored with artificial rocks made from fibre-reinforced concrete that provide temperatures and crevices similar to those found under natural rocks (Figure 2-2) (Croak et al., 2010).

Dharawal National park is located 67 km south of Sydney. In this area, exposed rock outcrops are separated by mixed eucalyptus forest dominated by scribbly gum, *Eucalyptus haemastoma*, Sydney peppermint, *E. pipperita*, and red bloodwood *E. gummifera* (Figure 2-3) (Croak et al., 2013). My second study site in Nowra is located 160km south of Sydney. This site is on the western edge of a plateau that is bordered by steep cliffs. The study site is adjacent to the steep cliffs, and the vegetation consists of a mixed eucalypt forest dominated by red bloodwood *Eucalyptus gummifera*, Sydney peppermint *E. Pipperia*, blue leaf stringy bark *E. agglomerate* and turpentine *Syncarpia glomulifera* (Figure 2-4) (Webb et al., 2008).

Figure 2-1 Map showing the two field sites at Dharawal National Park and Nowra

Figure 2-2 Restored artificial rocks made up of fibre reinforced concrete in Dharawal national park.

Figure 2-3 Sandstone rock outcrops separated by mixed eucalyptus forest at Dharawal national park

Figure 2-4 Field site bordered by a steep cliff at Nowra

2.2 Gecko collection and husbandry

I collected a total of 48 and 35 gravid female velvet geckos from Dharawal national park and Nowra respectively from October to November 2015. In 2016, I collected 51 and 36 gravid females from each site respectively.

I transported the females to the University of Technology Sydney. I measured the snout vent length (SVL) and tail length (TL) of each female with a ruler (to nearest mm), and recorded their mass (to nearest 0.01g) with an electronic balance. I housed geckos individually inside ventilated plastic cages (Sistema NZ 2.0 L, 220 × 150 × 60 mm) in a room maintained at 22 °C with a 12:12 light cycle. Each cage contained a moist vermiculite substrate, a plastic shelter (half pipe, 80 × 40 mm) and a petri dish of water (Figure 2-5). I placed cages on wooden racks and placed one end of each cage on a timer-controlled heating cable set to 32 °C. Heating cables created a thermal gradient (22–32 °C) in the cages during the day. At night, cage temperatures fell to room temperature (23 °C). I fed geckos with crickets twice weekly and provided access to water *ad libitum.* I checked the cages twice daily for eggs. I remoistened the vermiculite in the morning and in the afternoon to prevent eggs desiccating (Pike et al., 2011). After they laid eggs (Figure 2-6), I recorded the mass of each female (to nearest 0.01 g) and released them at their exact site of capture.

Figure 2-5 Housing of gravid female geckos with vermiculite as the substrate

Figure 2-6 Female velvet gecko just after laying eggs inside the cage

2.3 Incubation experiments

After oviposition, I measured the maximum length and width of eggs with callipers (to nearest 0.01 mm) and mass (to nearest 0.01 g) with an electronic balance. I placed eggs in 100 mL glass jars filled with moist vermiculite (water potential of 200 K Pa). I covered all the jars with plastic food wrap to prevent eggs desiccating (Figure 2-7). One egg from each clutch of two eggs produced by each female was randomly allocated to each of two incubators (Panasonic MIR 154) (Figure 2-8).

Figure 2-7 Deposition of eggs in glass jars for incubation experiment

Figure 2-8 Incubating eggs inside the programmed incubator

The incubation temperatures were as follows in each experiment series.

In 2015-2016, I programmed the incubators to mimic nest temperatures currently observed in ‘cold’ shaded nests (cold: mean = 23.3 °C; 17.5–30.5 °C) and sun-exposed ‘warm’ nests (warm: mean = 25.4 °C; range = 16.5–35.5 °C) in the field. The mean temperature of the hot incubation treatment was 2.2 °C higher than the cold treatment.

In 2016-2017, I programmed the incubators to mimic nest temperatures currently observed in sun-exposed communal nests (mean = 24.3 °C, range 18.4 – 31.1 °C), while thermal cycles of the hot treatment (mean = 28.9 °C, range 19.1 °C – 38.1 °C) mimicked the potential future nest temperatures that might occur in 2050 under climate warming (Dowdy et al., 2015).

I based incubation temperatures on the temperatures that we have recorded inside communal nests near Nowra. Each incubator was programmed using 10-step functions to create a 24 hour curve to simulate the daily fluctuation in temperature. I programmed minimum and maximum temperatures to increase each month, as occurs in nature. For the 2015-2016 temperatures, I used nest temperatures obtained from five shaded nests and sun-exposed nests to provide mean thermal profiles for my cold and warm treatments, respectively. For the ‘future’ temperature treatment, I assumed that temperatures would be hotter in 2050, as predicted by climate modellers.

2.4 Hatchlings maintenance

After eggs hatched, I measured the snout-vent length (SVL) and tail length (TL) of each hatchling with a ruler (to nearest mm), and body mass (to nearest 0.01 g) with an electronic scale (Figure 2-9).

I housed each hatchling in a plastic container (Sistema NZ 2.0 L, 220 × 150 × 60 mm) with a tissue paper substrate, plastic half pipe, and a water dish. I placed all cages on heating racks in a room maintained at 23 °C with a 12:12 light cycle. One end of the cage was kept on a heating cable (timer controlled, 32 °C) that created a thermal gradient (22–32 °C) in the cages during the day. Cage temperature fell to room temperature at night (23 °C) (Figure 2-10). I fed hatchlings pinhead and small crickets twice weekly.

Figure 2-9 Emergence of hatchlings after incubation period

Figure 2-10 Hatchling maintenance on heating racks in the laboratory

2.5 Experiments to measure hatchling performance

I carried out experiments to test the effect of incubation temperature on locomotor performance, thermal tolerance, thermal preference and learning capacity of hatchlings in the laboratory, which is discussed in the next chapters. Then I gave a unique toe clip code for each hatchling and released them to the sites where their mothers were captured. I carried out mark-recapture studies in each month to collect survival data for hatchlings.

2.6 Mark recapture studies

I carried out mark-recapture studies once a month at both sites after releasing the hatchlings. On each visit, I carefully turned all rocks present on the study sites, captured any geckos under rocks, and recorded their toe-clips and SVL in data sheets. I also measured any new unmarked geckos, and gave them a unique toe-clip.

Figure 2-11 Collecting mark recapture data in the field

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Chapter 3 Consequences of oviposition site choice for geckos in changing environments

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3.1 Abstract

Understanding how variation in oviposition site choice influences offspring phenotypes and juvenile survival is crucial for predicting how populations will cope with future environmental changes. In south eastern Australia the velvet gecko (*Amalosia leseurii*) is potentially at risk of climate warming because females nest communally in rock crevices and nest temperatures are positively correlated with air temperatures. Thus, nest temperatures are likely to become hotter in future, particularly during prolonged summer heatwaves. Nonetheless, there is considerable variation in the thermal regimes of currently used nests, which might generate sufficient diversity in offspring phenotypes to buffer populations from future change. To assess the ecological consequences of oviposition site choice, we incubated geckos at two fluctuating temperature regimes that mimicked temperatures currently experienced inside currently used sun-exposed (warm: mean = 25.4 °C; range = 16.5–35.5 °C) and shaded (cold: mean = 23.3 °C; 17.5–30.5 °C) communal nests. In the laboratory, we measured the phenotypic traits of hatchlings and released them at two study sites and monitored their survival over 6 months. Cold-incubated hatchlings hatched 16 days later, on average, and had larger snout-vent lengths (SVL), than their warm-incubated clutchmates. Survival analyses showed that incubation treatment did not influence hatchling survival and there was little evidence that hatchling body size influenced survival as predicted by the 'bigger is better' hypothesis. Collectively, these findings suggest that a female gecko's current choice of nest site has little effect on her offspring's viability. Thus, even if mean nest temperatures increase in future, some currently used shaded nest sites will provide thermal regimes suitable for embryo development. Maternal variation in nest site choice, coupled with natural successional vegetation changes (emergent trees shading nests), may therefore help to buffer velvet gecko populations from future environmental change.

3.2 Introduction

A female's decision about where and when to oviposit can influence egg hatching success, the quality and sex of her offspring, and can also affect a female's lifetime reproductive success. Maternal oviposition site choices can thereby influence demographic processes that can affect the longer-term persistence of populations (Brown and Shine, 2004, Blouin-Demers et al., 2004). In reptiles, key evolutionary drivers of oviposition site choice include maximizing maternal survival, maximizing egg survival, enhancing offspring traits that influence juvenile survival, and providing suitable habitats for hatchlings (Refsnider and Janzen, 2010). Although a key assumption of life history theory is that females should choose oviposition sites that are optimal for offspring development (Resetarits, 1996), females may choose suboptimal sites to maximize their own survival at the expense of offspring quality. For example, during the nesting season, females of the freshwater turtle *Emydura macquarii* are vulnerable to predation by introduced red foxes. An experimental study showed that females adjusted their choice of egg laying sites depending on the level of predation risk. At sites where foxes were common, females laid their nests closer to the water, where eggs were more likely to be eaten by predators, or drown during floods. By contrast, at sites where foxes were removed, females laid their nests further from the water, at sites where nests were less likely to be destroyed by predators or impacted by floods (Spencer, 2002). Thus, maternal nest site choice may not always maximise offspring fitness.

Determining the consequences of maternal oviposition site choice is crucial for understanding how future environmental changes may affect the persistence of populations. Reptiles are threatened by anthropogenic changes such as habitat loss, fragmentation, and overharvesting (Webb et al., 2002, Wolf et al., 2013) and because they are ectothermic, they are also at risk from climate warming (Sinervo et al., 2010). Embryonic life stages are particularly sensitive to increasing temperatures because most reptiles lack parental care, and eggs within natural nests experience marked fluctuations in temperature during the incubation period (Deeming, 2004, Warner and Andrews, 2002, Shine et al., 2003, Hall and Warner, 2018). Therefore, a mothers' choice of nest site will dictate the thermal (and hydric) conditions experienced by her embryos (Packard and Packard, 1988). Incubation temperatures can affect embryo survival (Angilletta et al., 2013) and hatchling traits such as size, shape, sex, and behaviour (Amiel et al., 2016, Shine, 2004b, Shine, 2004a, Warner and Shine, 2011, Deeming, 2004). Because nests of many lizards will likely become hotter in future, either because of land use changes (urbanization) or climatic change (Hall and Warner, 2018), it is important to consider how natural variation in nest sites influences offspring phenotypes and survival (Levy et al., 2015, Telemeco et al., 2017). While we know much about incubation-induced phenotypic variation, we know less about how that variation influences hatchling survival in the wild (Noble et al., 2018, While et al., 2018).

Here we investigate the consequences of nest site selection for velvet geckos *Amalosia lesueurii.* Velvet geckos are vulnerable to increasing temperatures because they lay eggs in communal nests, and nest temperatures are positively correlated with air temperatures (Dayananda et al., 2016). Thus, nest temperatures are likely to shift upwards if the intensity and duration of summer heatwaves increases in the future. However, at small spatial scales (< 1 ha) there is considerable variation in the physical characteristics (nest depth, aspect, and canopy cover) and thermal profiles of communal nests (Pike et al., 2010). To simulate the effects of variation in nest site temperatures, we incubated eggs of the velvet gecko *Amalosia lesueurii* under fluctuating thermal regimes that mimic temperature profiles from currently used shaded (cold) and exposed (warm) nest sites. After the eggs hatched, we measured the morphology of hatchlings in the laboratory. We then individually marked the hatchlings, released them at field sites, and carried out a mark-recapture study to assess whether incubation environments or phenotypic traits influenced their survival in the wild.

3.3 Materials and Methods

3.3.1 Study Species

Lesueur's Velvet Gecko (*Amalosia lesueurii*) inhabits sandstone and granite rock outcrops in eastern Australia and occurs from southeastern New South Wales to southeastern Queensland (Wilson and Swan, 2013). Females lay their eggs inside communal nests in rock crevices, and nesting crevices have different physical and thermal attributes than non-used potential nest sites (Webb et al., 2008, Pike et al., 2011, Pike et al., 2010). Females oviposit two eggs per clutch in spring from late October to early November with an incubation period of 80-120 days (Doughty, 1997). Temperatures recorded inside nine communal nests from Morton National Park in 2006–2007 ranged from 9.5–44.5 °C (mean = 22.7 °C) during the incubation period (Dayananda et al., 2016).

3.3.2 Site descriptions and collection of adult females

We carried out a mark-recapture study of velvet geckos at two sites, one near Nowra, NSW, 170 km south of Sydney, and another in Dharawal National Park, 60 km south of Sydney, NSW. Both sites contained sun-exposed sandstone rock outcrops surrounded by dry sclerophyll forest. Both sites were restored in 2009 with 50 identical artificial rocks (512 mm long x 352 mm wide x 46 mm thick) constructed from fiber-reinforced cement that were placed in sun-exposed locations. These rocks provide thermal regimes and crevices that are very similar to those found under natural rocks (Croak et al., 2010), and were rapidly colonized by velvet geckos (Croak et al., 2013). We chose these habitat restoration sites because they support large populations of velvet geckos, and provide identical shelter sites for geckos, thereby reducing variation in shelter site, which could potentially influence survival of geckos.

In October 2015, we collected gravid females from the study sites by carefully turning all artificial and natural rocks. When we captured a gecko, we recorded the rock's unique number (that was painted on its underside) and its location (with a GPS) so that we could return the females to their exact site of capture. Females were transported to the University of Technology Sydney and were housed individually in clear ventilated containers (Sistema, NZ, 220 x 150 x 60 mm) with an identical shelter (plastic half pipe), and moist vermiculite as an oviposition site. Cages were placed in a constant temperature room (22 °C) with 12:12 light cycle. One end of each cage was placed on timer controlled heating racks to create a daytime thermal gradient of 22-32 °C. Geckos had access to water ad libitum and were fed crickets twice weekly. Females were held until they had laid eggs and were then released at the exact site of capture.

3.3.3 Egg incubation experiment

After oviposition eggs were weighed (to 0.01 g) and placed in 100 mL autoclaved glass jars containing autoclaved moist vermiculite. One egg from each clutch of two eggs was randomly selected and allocated to one of the two incubation treatments. Eggs were incubated inside two programmable temperature incubators (Panasonic MIR-154-PE, with 10 step functions) programmed to mimic fluctuating temperatures experienced inside current sun-exposed "warm" (mean = 25.4 °C; range = 16.5–35.5 °C) and shaded "cold" (mean = 23.3 °C; range = 17.5–30.5 °C) nest sites. Incubation treatment temperatures were based on field data collected from 9 communal nests in 2006-2007; these nests are still used as communal egg laying sites by female velvet geckos.

After eggs hatched, one of us (TA) weighed each hatchling (to 0.01 g), and measured their snout-vent length and tail length (SVL, TL, with a ruler, to nearest mm). Each hatchling was housed in a ventilated plastic cage (220 x 155 x 61 mm) with a paper substrate, water dish and a plastic shelter (PVC half pipe, 100 x 55 x 25 mm). Cages were placed on timer controlled heating racks to provide a thermal gradient during the daytime (22–32 °C) dropping to room temperature at night. Geckos were fed small crickets twice weekly and were released at the sites where their mothers were captured after experiments were completed.

3.3.4 Release and mark-recapture

Prior to release, we gave each gecko a unique toe-clip to allow subsequent identification. Previous studies indicate that toe-clipping causes minimal stress to lizards (Langkilde and Shine, 2006) and does not appear to affect the longer-term survival of hatchling velvet geckos (Webb, 2006, Croak et al., 2013). Each gecko was released at the site where its mother was captured. At each site, each gecko was placed underneath a vacant artificial rock to reduce variation in habitat structure and temperature that could potentially affect hatchling survival. Prior to release, we measured the temperatures of the rock substrate and the underside of each rock with an infrared thermometer (Cool Tech CT-663, spot diameter = 13 mm) to ensure that the rock temperatures were within the thermal tolerance limits of geckos. At our field site in Nowra, we released 37 warm-incubated hatchlings on 23 March 2016 and 34 cold-incubated hatchlings on 6 April 2016. At the field site in Dharawal, we released 14 warm incubated and 21 cold incubated hatchlings on the 6 April

2016. To estimate gecko survival, we visited each study site twice monthly from April 2016 to September 2016 and carefully turned all artificial rocks and natural rocks that could be safely lifted without causing a back injury. For each captured gecko, we recorded the rock number, and measured the gecko's SVL and TL, to the nearest mm (with a ruler), and recorded the toe-clip.

3.3.5 Statistical analyses

To investigate whether incubation temperature affected the morphology of hatchlings, we used two factor ANOVAs, with location and treatment as factors, and SVL, TL and mass as the dependent variables. To test whether incubation treatment or body size influenced gecko survival, we ran Cormack-Jolly Seber (CJS) models in Program MARK v 9.0 (White and Burnham, 1999). Data for each study site (Nowra and Dharawal) were analysed separately. For each site, we ran two analyses. The first analysis tested whether incubation treatment affected survival, and the second analysis tested whether incubation temperature and/or body size influenced survival. This step wise analysis was necessary because we first had to check whether the assumptions of mark-recapture were met, and MARK is unable to do this when covariates are present in the input file. For the first analysis, we included incubation treatment (cold versus warm) as a group factor in the input file, and ran several models to see whether survival and recapture rates were constant, group-dependent, or time-dependent. To verify that the CJS assumptions were met, we tested the goodness of fit of the most parameterized model in our candidate model set using a bootstrap GOF test. For the Dharawal data, the bootstrap GOF test showed that model assumptions were met ($p = 0.30$), with only minor over-dispersion (c-hat = 1.068). For the Nowra data, the bootstrap GOF test showed that while model assumptions were met ($p = 0.06$), there was more over-dispersion (c-hat = 1.66).

For the second analysis, we replaced the group coding in the input file with a covariate (1 or 0) to code for incubation treatment, and included the covariates SVL, TL and mass. By including covariates, we were able to test whether survival was influenced by incubation treatment, snout-vent length, tail length, or mass. We then ran a series of models in MARK to test the following *a priori* hypotheses: (1) survival is dependent on incubation treatment; (2) there is directional selection on snout-vent length, tail length, or mass (the "bigger is better" hypothesis). We also ran equivalent survival models, in which recapture probability was constant, time dependent, or was influenced by snout-vent length. We included these latter models to explore the possibility that size might influence recapture rates. Candidate models were ranked based on their AIC values and associated AIC weights (Burnham and Anderson, 1998). In general, models with delta AIC < 2.0 are considered to be supported by the data, while models with delta AIC > 4 have little support.

3.4 Results

3.4.1 Hatching success and incubation period

Hatching success did not differ between the two incubation treatments. In the warm incubation treatment, 54 eggs of 78 eggs hatched successfully, while 56 of 79 eggs hatched from the cold incubation treatment ($\chi^2 = 0.05$, p = 0.82, Table 3-1). Warm incubated eggs hatched from the 25th of January to the 18th of February 2016, while the cold incubated eggs hatched from the 2nd of February to the 4th of March 2016. On average, warm-incubated eggs hatched 15 days earlier (mean incubation period 86.24 ±8.67 days) than cold incubated eggs (mean incubation period 101.83 ± 8.25 days, $F_{1,108} = 92.52$, $p < 0.001$,Table 3-1)

3.4.2 Hatchling morphology

Incubation treatment had a significant effect on hatchling body size. Overall, cold incubated hatchlings had larger snout-vent lengths than warm incubated lizards ($F_{1, 105} = 9.06$, $P = 0.003$ Figure 3-1), and this difference occurred at both locations ($F_{1, 105} = 1.62$, $P = 0.21$; interaction $F_{1, 105} = 3.40$, $P = 0.07$). Incubation treatment did not affect hatchling tail length (incubation: $F_{1, 102} = 3.44$, $P = 0.06$), and this was true for both locations ($F_{1, 102} = 0.02$, $P = 0.89$, interaction: $F_{1, 102}= 0.99$, $P = 0.32$). Incubation treatment also had no effect on hatchling mass ($F_{1, 105} = 2.96$, $P = 0.09$; location $F_{1, 105} = 0.004$, $P = 0.95$, interaction $F_{1, 105} = 0.26$, $P = 0.62$, Table 3-1).

Table 3-1**:** The effect of cold vs. warm incubation treatment on hatching success, incubation period, hatchlings' snout-vent length, tail length and wet body mass. Last two columns represent the results of two way ANOVA, with incubation treatment and location as factors and the trait as dependent variable. Effect of location or interaction of location and incubation treatment was not significant for any of the traits.

	Cold incubation (n = 56)	Warm incubation (n=54)	F value	P value
Hatching success (%)	70.9	70.1		
Incubation period (days)	101.83 (8.25)	86.24 (8.67)	92.52	< 0.001
Snout-vent length (mm)	27.05 (1.94)	25.79 (1.70)	9.06	0.003
Tail length (mm)	22.23 (5.23)	21.01 (5.00)	3.44	0.06
Wet body mass (g)	0.48 (0.06)	0.45 (0.05)	2.96	0.09

3.4.3 Effects of incubation temperature on hatchling survival

For the Dharawal data set, the results of the CJS survival analyses showed that incubation temperature did not affect lizard survival, nor was there any support for the ‘bigger is better’ hypothesis. The top three models all had similar AICc weights (Table 3-2) so had equivalent support. All three models had constant recapture rates, but in the first model survival was influenced by tail length, in the second model survival was influenced by SVL, and in the third model survival was constant (Table 3-2). Notably, the influence of body size was not in the direction predicted by the ‘bigger is better hypothesis’, models one and two produced estimates of survival that decreased with increasing SVL or TL, but with large confidence intervals around the means (& Figure 3-3).Given these findings, we can reject the ‘bigger is better hypothesis’. None of the other models were well supported by the data (delta AICc > 2; Table 3-2). From the model with constant survival and recapture rates, estimates of mean monthly survival were 0.88 (SE = 0.03), while mean monthly recapture rates were 0.51 (SE = 05).

Table 3-2. Results of survival analyses used to compare rates of survival (s) and recapture (p) for warm-incubated and cold-incubated hatchlings from Dharawal. The candidate models were ranked based on their QAICc values and associated AICc weights; models with delta AICc < 2.0 have the greatest statistical support. The table also shows the model likelihood, number of parameters (N), and model deviance.

Model	AICc	Delta AICc	AICc Weights	Likelihood	N	Deviance
s (TL) p (constant)	229.1685	0	0.28773	1.0000	3	222.8894
s (SVL) p (constant)	229.5206	0.3521	0.24128	0.8386	3	223.2415
s (constant) p (constant)	229.6689	0.5004	0.22404	0.7787	2	225.5309
s (mass) p (constant)	230.674	1.5055	0.13554	0.4711	3	224.3949
s (incubation) p (constant)	231.6932	2.5247	0.08142	0.283	3	225.4141
s (constant) p (time)	234.0153	4.8468	0.0255	0.0886	8	216.2375
s (time) p (constant)	237.5642	8.3957	0.00432	0.015	8	219.7864
s (time) p (time)	243.9707	14.8022	0.00018	0.0006	13	213.1812

For the Nowra data set, there was no evidence that incubation temperature influenced survival (Table 3-3). The best-supported model was one in which survival rates varied with time, and recapture rates were constant. This model was 1.3 times better supported than the next three models, in which survival rates varied with SVL, TL, or was constant, and recapture rates were constant. Given that all three models had similar AICc weights, and thus, were similarly well supported, there was little support for the 'bigger is better hypothesis'. None of the other models were well supported by the data (delta AICc > 2; Table 3-3). From the model with constant survival and recapture rates, estimates of mean monthly survival were 0.64 (SE = 0.07), while mean monthly recapture rates were 0.36 (SE = 0.09)

Table 3-3: Results of survival analyses used to compare rates of survival (Phi) and recapture (p) for warm-incubated, cold-incubated and wild-born hatchling geckos that were captured at Nowra in 2016. The candidate models were ranked based on their QAICc values and associated AICc weights; models with delta AICc < 2.0 have the greatest statistical support. The table also shows the model likelihood, number of parameters (N), and model deviance.

Model	AICc	Delta AICc	AICc Weights	Likelihood	N	Deviance
s (time) p (constant)	137.9025	0.0000	0.2646	1.0000	7	122.7479
s (mass) p (constant)	138.5001	0.5976	0.19626	0.7417	3	132.2625
s (SVL) p (constant)	138.6047	0.7022	0.18626	0.7039	3	132.3670
s (constant) p (constant)	138.7366	0.8341	0.17437	0.6590	2	134.6189
s (incubation) p (constant)	139.9599	2.0574	0.09459	0.3575	3	133.7223
s (TL) p (constant)	140.6170	2.7145	0.0681	0.2574	3	134.3794
s (constant) p (time)	143.9622	6.0597	0.01279	0.0483	7	128.8076
s (time) p (time)	146.8386	8.9361	0.00303	0.0115	11	121.9999

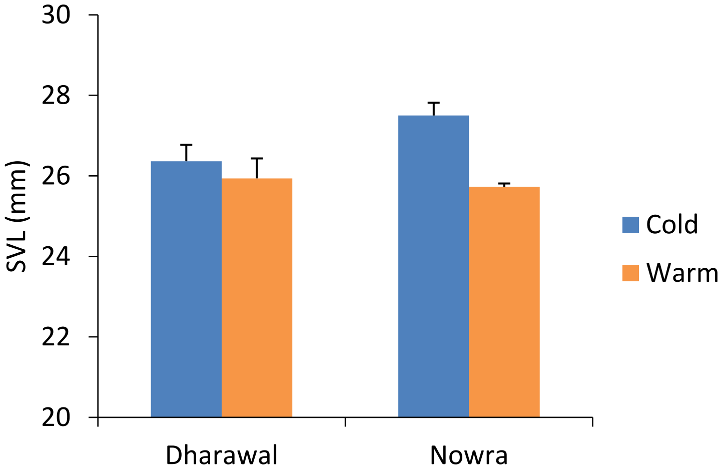

Figure 3-1 Snout – vent length of cold and warm incubated hatchlings from Dharawal and Nowra study sites

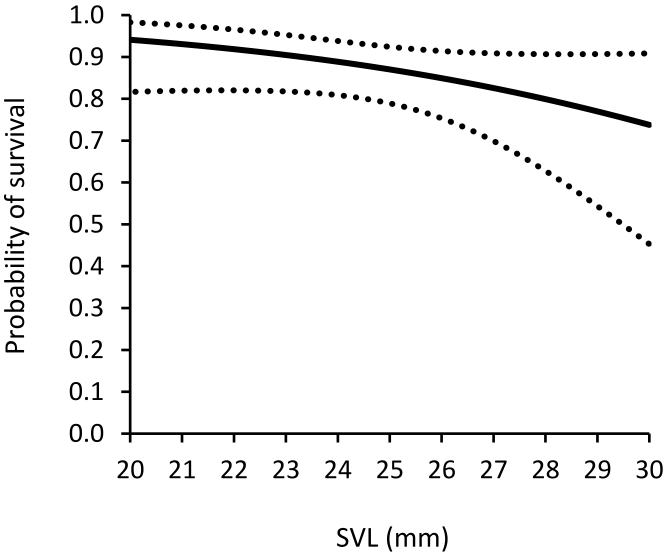

Figure 3-2 Relationship between snout – vent length of hatchling velvet geckos and their probability of survival over six months after releasing in Dharawal. Dotted lines show 95% Cls.

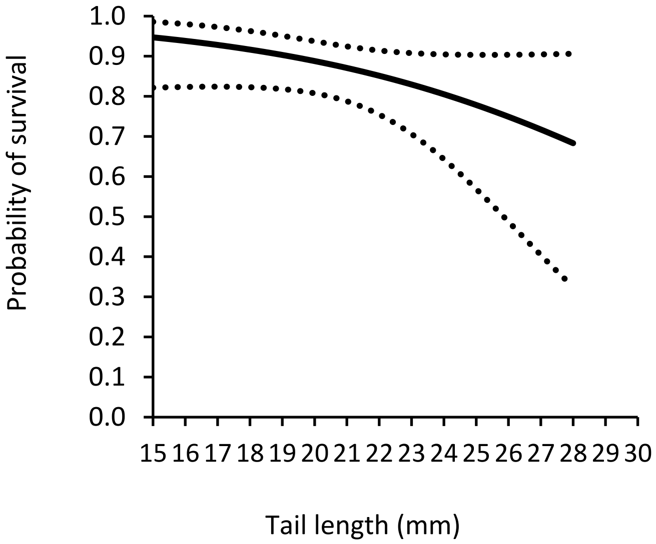

Figure 3-3 Relationship between tail length of hatchling velvet geckos and their probability of survival over six months after releasing in Dharawal. Dotted lines show 95% Cls.

3.5 Discussion

Variation in nest temperatures can engender considerable variation in the phenotypes of hatchling lizards (Deeming and Ferguson, 1991, Noble et al., 2018, While et al., 2018). By simulating thermal conditions inside sun-exposed and shaded nests, we found that incubation temperature did not affect hatching success, but warm incubated hatchlings emerged 16 days earlier than the cold incubated hatchlings. This finding is consistent with the results of previous experimental studies on lizards; in general, incubation under 'benign' temperatures typically experienced inside natural nests does not affect hatchling success, but higher temperature incubation results in shorter incubation periods (Noble et al., 2018, While et al., 2018).

Incubation periods are an important trait because they dictate the timing of birth, which in turn can influence the survival of hatchling lizards (Warner and Shine, 2007). In cold climates, earlier hatching is thought to be advantageous because hatchlings can acquire important resources such as food and space (Andrews, 2008), which can affect growth and/or fat storage, which may in turn influence overwinter survival (Shine, 1983, Bauwens, 1981). However, for some species of lizards, hatching later may be an advantage. For example, in *Sceloporus undulatus*, hatchlings which were hatched later had higher survival than lizards that were hatched earlier (Andrews et al., 2000). In our study species, hatchling geckos settle under small rocks near communal nests shortly after birth (Webb, 2006). Our results suggest that hatchlings from sun-exposed nests (warm treatment) will be hatch two weeks earlier than hatchlings from shaded nests (cold treatment). In hot years, hatching early may be a handicap for lizards if they emerge from nests during summer heatwaves, when rock temperatures are lethally high (Dayananda et al., 2016). During heatwaves, hatchlings would be forced to use cooler crevices, which might compromise their growth (Sinervo and Adolph, 1989), or make them more vulnerable to predators (Christian and Tracy, 1981). By contrast, hatchlings that emerge from shaded, cooler nest sites, may be able to avoid this thermally stressful time period (Dayananda et al., 2016).

Interestingly, cold incubated hatchlings were larger in snout-vent length than their warm incubated clutchmates. This result agrees with a recent study on the same species, which found that hatchlings from eggs incubated at high temperatures were smaller and lighter than hatchlings from eggs incubated at colder temperatures (Dayananda et al., 2016). This pattern is broadly consistent with previous studies on lizards, which in general, have found that hatchlings from higher temperature incubation tend to be smaller than hatchlings from colder incubation temperatures (While et al., 2018, Noble et al., 2018). The physiological mechanism responsible for this size difference appears to be linked to incubation period and yolk conversion. In most reptiles, lower incubation temperatures generate longer incubation periods that lead to increased conversion of yolk to tissue, which results in larger hatchlings (Booth, 2006). Furthermore, long incubation period allows embryos more time to continue differentiation and neuromuscular development, which in turn could influence their locomotor performance (Shine and Olsson, 2003).

To date, few studies have investigated how incubation temperatures affect the survival of hatchlings, as recapturing individually marked small lizards can be challenging for some species. One study on the North American lizard *Scleroporous undulatus* found that cold incubated lizards had higher survival than their hot incubated clutchmates (Andrews et al., 2000). However, our survival analyses provided no support for an effect of incubation temperature on the survival of hatchling geckos from either of our field sites (Table 3-2 and Table 3-3). By contrast, a recent study on *A. lesueurii* found that hot incubated hatchlings had lower survival than cold-incubated hatchlings over the first 10 months of life. Why do our results differ from this previous study? Notably, temperatures in the hot treatment of Dayananda et al. (2016) were higher (mean = 27.0 °C, range 14–37 °C) than the temperatures in our warm treatment (mean = 25.4 °C, range 17.5–35.5 °C). Moreover, Dayananda et al. (2016) reported strong differences between the mass of hot and cold incubated lizards, whereas we did not detect any significant effect on mass (although there was a trend for cold-incubated lizards to be slightly heavier, $P = 0.09$). Because survival rates of lizards can fluctuate temporally in response to changes in prey availability and weather (Sorci et al., 1996, Ferguson and Fox, 1984), it is difficult to directly compare the two studies. Nonetheless, our results suggest that incubation under current (presumably, non-stressful) thermal regimes appears to have little effect on offspring survival, at least in the year in which our study was conducted.

We also explored the ‘bigger is better’ hypothesis that predicts that larger offspring have higher survival than smaller conspecifics (Janzen, 1993). In lizards, a larger body size may enable individuals to capture larger prey, establish territories in better habitats, and outcompete conspecifics. For example, in side-blotched lizards (*Uta stansburiana*), larger juveniles had a survival advantage, and occupied better quality territories, than smaller conspecifics (Ferguson and Fox, 1984). In our study, we found no evidence for selection for larger snout-vent length, tail length, or mass. At Dharawal, there was weak evidence that snout-vent length and tail length influenced survival, albeit not in the direction predicted by the ‘bigger is better hypothesis’ (and Figure 3-3). This finding may reflect an absence of selection on body size, or temporal variation in selection regimes. For example, in side-blotched lizards *Uta stansburiana*, and grass lizards *Lacerta agilis*, selection on body size varied among years, with larger hatchlings having a survival advantage in some years, but not others (Ferguson and Fox, 1984, Olsson and Madsen, 2001). In both of these studies, larger juveniles accrued a survival advantage during poor years, when prey availability was low, and presumably, predation on lizards was higher. In many lizards, the tail is used as a fat storage organ, so individuals with larger tails may have higher survival, presumably because they are more resilient to food shortages (Sorci and Clobert, 1999). However, as for SVL, tail length did not influence survival rates in the direction predicted by the ‘bigger is better hypothesis’ (and Figure 3-3). Nonetheless, our results are not unique in this respect, and are consistent with previous studies on our study species (Dayananda et al., 2017) and other lizards (Warner and Shine, 2007).

To date, few studies have incubated eggs under conditions likely to be experienced inside lizard nests in future, which makes it difficult to predict how lizard populations will adapt to climate warming (Levy et al., 2015). In this study, we incubated eggs under thermal regimes that mimicked nest temperatures inside currently used shaded nests (cold treatment) and sun-exposed nests (warm treatment). Despite the 2.1 °C difference between the mean temperatures in our experimental treatments, we found no evidence that incubation temperatures influenced offspring survival. This result is reassuring, because even if global temperatures increase by 2-3 °C in future, temperatures within shaded nest sites will remain suitable for offspring development. This is an important finding, because a previous study which assumed that there was no maternal variation in nest-site choice (i.e. females only lay eggs inside sun-exposed nests) predicted that velvet gecko populations would decline under climate warming (Dayananda et al., 2016). Our results provide a more optimistic scenario, at least for the two populations that we studied. However, we lack information on the physical and thermal characteristics of velvet gecko nests in other locations, nor do we know if this species nests communally throughout its range. Furthermore, we do not know how warming will influence the humidity within nests, and whether such changes might affect hatching success. Future research is needed to estimate whether maternal variation in nest site selection occurs throughout the species' range and whether such variation can buffer the species from future change (Doody, 2009). Finally, although our research focused on the consequences of nest site selection for velvet geckos, it has conservation implications for the endangered broad-headed snake (*Hoplocephalus bungaroides*), a species that feeds mostly on juvenile geckos (Webb and Shine, 1998). Local extinction of the gecko population could adversely affect the viability of snake populations, so understanding how geckos are likely to respond to future changes will be crucial for managing and conserving populations of both predators and prey.

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Chapter 4 Population differences in developmental plasticity of locomotor performance in velvet geckos

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Manuscript in preparation

4.1 Abstract

Many lizards lay eggs inside shallow nests where embryos often experience high temperatures during summer heatwaves. Increases in the intensity and duration of summer heatwaves in the future may therefore result in higher incubation temperatures, yet it is unclear how lizards will cope with these changes. Increases in developmental temperatures could produce adaptive shifts by enhancing offspring performance at higher temperatures, although non-adaptive shifts are also possible. To investigate how developmental temperature affects locomotor performance, we incubated eggs of the velvet gecko *Amalosia lesueurii* from a southern (Nowra) and northern (Dharawal) population under two fluctuating temperature regimes to mimic temperatures inside currently used shaded "cold" (mean = 23.3 °C, range 17.5–30.0 °C) and sun-exposed "warm" (mean = 25.4 °C, range 16.5–35.5 °C) communal nests. We measured the running performance of hatchlings at 25 °C, 30 °C, and 35 °C and recorded their propensity to stop during runs. We found strong inter-population differences in the degree to which developmental temperatures influenced locomotor performance. In the northern population, incubation temperature did not influence sprint speed or speed over 1m, and lizards ran fastest at 35 °C. In the southern population, warm-incubated hatchlings sprinted faster than cold-hatchlings at 35 °C, but not at other temperatures. For speed over 1m, warm-incubated lizards ran faster than cold-incubated lizards at all three test temperatures. This difference in speed was due to differences in behaviour; cold-incubated hatchlings made more stops during runs over 1 m than warm-incubated lizards. Our results provide little support for the predictive anticipatory hypothesis, and suggest that developmental effects are complex and vary geographically.

4.2 Introduction

Lizards are threatened by anthropogenic activities including habitat fragmentation and land clearing, collection for the pet trade, pollution, the introduction of invasive species, and changing climates (Webb et al., 2015, Auliya et al., 2016, Bohm et al., 2013, Gibbons et al., 2000). Because lizards are ectothermic, changes in temperature can have profound effects on physiological and behavioural traits that are linked to fitness (Huey, 1982, Angilletta, 2009). Recent increases in global temperatures are thought to have contributed to local extinctions of lizards (Sinervo et al., 2010), and not surprisingly, recent studies have focused on forecasting species responses to climate warming (Huey et al., 2012, Telemeco et al., 2017, Levy et al., 2015).

Thermal performance curves provide a useful framework for understanding how shifts in environmental temperatures may influence lizard T_bs, organismal performance and fitness (Angilletta, 2009). Typically, curves are asymmetrical, and the maximum performance occurs at the optimal body temperature (T_o) before dropping markedly thereafter at higher T_bs. The two endpoints of the curve, where very low and very high temperatures induce a loss of righting ability, are termed the critical thermal minimum and critical thermal maxima, respectively.

To predict how increases in temperature will affect lizards, we need to know how warming influences T_b relative to T_o, and whether thermal performance curves can shift in response to changing temperatures (Huey et al., 2012). In this context, locomotor performance has received extensive study because it is relatively straight forward to measure, and is thought to be tightly linked to fitness (Arnold, 1983, Le Galliard et al., 2004, Irschick et al., 2008).

Field studies on squamates have demonstrated that variation in locomotor performance can influence survival in lizards (Christian, 1981) and snakes (Jayne and Bennett, 1990). Two recent studies have also provided evidence that performance curves can evolve rapidly in adult lizards. In a study on *Anolis sagrei*, researchers transplanted 98 adult males from a cooler forest site to a warmer coastal site on the island of Eleuthera, while 108 males from another island (Kidd Cay) served as a reference population. After three months, the researchers resampled lizards on both islands, and found strong directional selection on T_o for sprint speed at the transplant site, but not at the reference site (Logan et al., 2014). In another study on *Urosaurus ornatus* from an arid grassland in southeastern Arizona, researchers found significant positive selection for higher preferred T_bs, and higher sprint speed at the mean preferred T_b of 36 °C (Gilbert and Miles, 2017). These elegant field studies demonstrated that selection favoured lizards that maximised performance at higher temperatures.

While adult lizards may adapt to higher environmental temperatures via selection on performance curves, we know less about how other life stages will cope with rising temperatures (Levy et al., 2015). In many lizard species, eggs and embryos may experience higher temperatures in future (Telemeco et al., 2017, Dayananda et al., 2016). Theoretically, developmental plasticity may enable populations to adapt to higher temperatures. According to the ‘predictive anticipatory hypothesis’ (PAH), if environmental cues experienced during development provide an accurate forecast of conditions likely to be experienced during later life, developmental plasticity should produce phenotypes better suited to future conditions (Bateson, 2007, Bateson et al., 2014). That is, offspring that experience higher developmental temperatures should perform better at higher temperatures than offspring that experience lower developmental temperatures (Le Roy et al., 2017). Studies on invertebrates have provided some support the PAH (Angilletta, 2009). For example, in *Drosophila melanogaster*, flies reared under high incubation temperatures had higher performance at higher temperatures compared to flies reared at lower temperatures (Gilchrist et al., 1997). However, studies on vertebrates have provided mixed support for the PAH (Uller et al., 2013, Esquerre et al., 2014, Le Roy et al., 2017). Furthermore, most studies on reptiles have focused on single populations, and there is little information on between-population variation in thermal reaction norms (Noble et al., 2018).

In this study, we investigated the effects of population of origin and developmental temperatures on the locomotor performance of hatchling velvet geckos *Amalosia lesueurii*. Females of this rock-dwelling gekkonid species lay their eggs in communal nests in sun-exposed crevices, and nest temperatures are positively correlated with air temperatures (Dayananda et al., 2016). Thus, nests are likely to become hotter in future if the frequency and duration of summer heatwaves increases. Furthermore, there is substantial small-scale variation in nest site characteristics, such that nests from a local area can differ substantially in both mean and maximum temperatures (Chapter 3). To explore how variation in nest site temperatures might influence hatchling performance, we incubated eggs under two thermal treatments to mimic temperatures experienced inside sun-exposed and shaded communal nests. To explore whether geographic variation influenced developmental plasticity, we incubated eggs from a southern population (Nowra) and a more northerly population (Dharawal).

4.3 Materials and Methods

4.3.1 Gecko collection and husbandry

During late spring, 2015, we collected 83 gravid female velvet geckos from rock outcrops in Dharawal national park (n = 48) 67 km south of Sydney and Nowra (n = 35) 160 km south of Sydney. We transported female geckos to the University of Technology Sydney where we measured their snout vent length (SVL) tail length (TL) (to nearest mm), and mass (to nearest 0.01 g). We housed them individually inside ventilated plastic cages (Sistema NZ 2.0 L, 220 × 150 × 60 mm) in a room maintained at 22 °C with a 12:12 light cycle. Each cage contained a moist vermiculite substrate, a plastic shelter (half pipe, 80 × 40 mm) and a petri dish of water. We placed the cages on wooden racks and one end of each cage was placed on a timer-controlled heating cable set to 32 °C. Heating cables created a thermal gradient (22–32 °C) in the cages during the day. At night, cage temperatures fell to room temperature (22 °C). We fed geckos with crickets twice weekly and had access to water *ad libitum.* We checked the cages twice daily for eggs. We remoistened the vermiculite in the morning and in the afternoon to prevent eggs desiccating (Pike et al., 2011). After they laid eggs, we recorded the mass of each female (to nearest 0.01 g) and released them at their exact site of capture.

4.3.2 Incubation experiments

After oviposition, one of us (TA) measured the maximum length and width of eggs with Vernier callipers (to nearest 0.01 mm) and mass (to nearest 0.01 g) with an electronic balance. We placed eggs in 100 mL glass jars filled with moist vermiculite (water potential of 200 K Pa). We covered all the jars with plastic food wrap to prevent eggs desiccating. We randomly allocated one egg from each clutch of two eggs produced by each female to each of two incubators (Panasonic MIR 154, ten step programs). Each incubator was programmed to mimic nest temperatures currently observed in shaded 'cold' nests (mean = 23.3 °C, range 17.5–30.0 °C) and sun-exposed "warm" (mean = 25.4 °C, range 16.5–35.5 °C) in the field (Dayananda et al., 2016) (Figure 4-1)

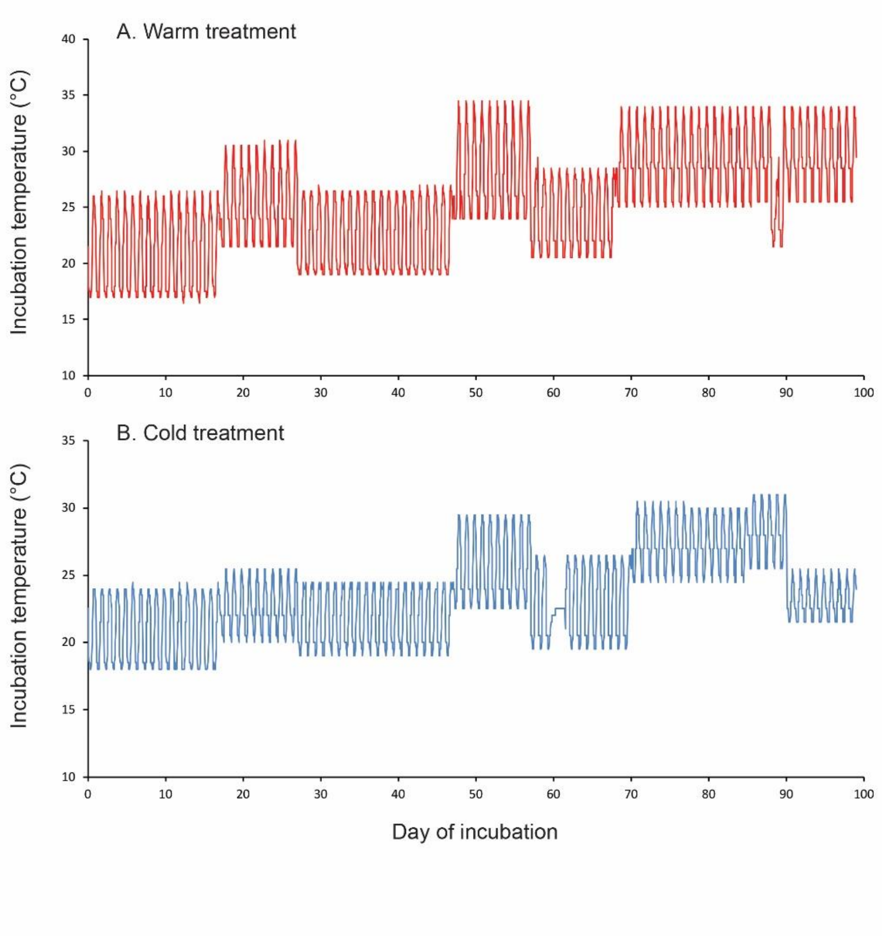

Figure 4-1 Thermal profiles of the warm (A) and cold (B) incubation treatments.

4.3.3 Hatchling maintenance

After eggs hatched, we measured the snout-vent length (SVL) and tail length (TL) of each hatchling with a ruler (to nearest mm), and body mass (to nearest 0.01 g) with an electronic balance. We housed each hatchling in a plastic container (Sistema NZ 2.0 L, 220 × 150 × 60 mm) with a paper substrate, plastic half pipe (40 mm diameter, 80 mm long), and a water dish. We placed the cages on heating racks in a room maintained at 22

°C with a 12:12 light cycle. One end of each cage was placed over a heating cable (timer controlled, 32 °C) that created a thermal gradient (23 – 32 °C) in the cages during the day. Cage temperature fell to room temperature at night (22 °C). We fed hatchlings with pinhead crickets twice weekly.

4.3.4 Measurement of locomotor performance

We measured the locomotor performance of two to three week old hatchlings at three test temperatures (25, 30 and 35 °C) over three consecutive days in a temperature controlled room. Test temperatures were achieved by keeping the animal in an incubator (Panasonic MIR-154-PE) at the relevant temperature, 30 minutes prior to testing (Li et al., 2013). To measure locomotor performance, we chased each hatchling along a wooden racetrack (1 m long x 8 cm wide x 8 cm tall, with a marine carpet floor marked at 0.25 m increments) with an artist's paintbrush. Trials were carried out between 10 am and 1 pm, and were videotaped with a video camera (Panasonic HC-V110) placed directly above the racetrack. Each hatchling was run three times with 15 minutes rest between each trial (Shine, 2001). We recorded the hatchling's T_b with an IR thermometer (Cool Tech, CT 663, USA spot diameter = 13 mm) before and after each trial. During the rest period between trials, we placed the hatchlings inside the incubator so that they would maintain their T_b at the designated test temperature. We analysed the video tape using frame-by-frame playback (25 frames per second) with a video editing program (Cyber link Power Director 10, New Taipei City, Taiwan). For each trial, we recorded the hatchling's burst speed (fastest speed over 0.25 m), speed over 1.0 m, and the number of stops made during the 1.0 m run. For each individual lizard, the maximum burst speed (0.25) and maximum speed over 1 m, and the number of stops made by hatchlings over the fastest 1 m was used as dependent variables in the statistical analyses.

4.3.5 Statistical analysis

Because we tested the running speed of each hatchling at three different T_bs (25, 30 and 35 °C) we used repeated measures ANOVA to investigate the effect of incubation treatment (cold *versus* warm) and T_b on locomotor performance. Because we were primarily interested in testing the hypotheses about the patterns of developmental plasticity at each location (rather than any effect of location), we ran separate analyses for each location. Data were checked to see if it met the assumptions of normality prior and sphericity prior to statistical analysis. We used SPSS 25 (www.ibm.com) to analyse all data.

4.4 Results

4.4.1 Burst speed

For Dharawal lizards, the data for burst speed violated the assumption of sphericity (Mauchley's test: $\chi^2 = 8.52$, $P = 0.014$, epsilon = 0.815), so we used the Greenhouse-Geisser correction for F tests. Incubation treatment did not affect burst speed ($F_{1, 34} = 1.45$, $P = 0.46$), but lizards ran faster at higher T_bs ($F_{1.6, 55.4} = 23.0$, $P = 0.0001$). The interaction between T_b and incubation treatment was not significant ($F_{1.6, 55.4} = 0.86$, $P = 0.41$); that is, lizards from both treatment groups ran at similar speeds when tested at the same T_b (Figure 4-2 A).

For Nowra lizards, the data violated the assumption of sphericity (Mauchley's test: $\chi^2 = 7.69$, $P = 0.021$, epsilon = 0.894), so we used the Greenhouse-Geisser correction for F tests. There was a significant effect of incubation treatment on burst speed ($F_{1, 62} = 4.07$, $P = 0.046$), and lizards also ran faster at higher T_bs ($F_{1.8, 110.9} = 31.5$, $P = 0.0001$). The interaction between T_b and incubation treatment was also significant ($F_{1.8, 110.9} = 4.86$, P

= 0.012). That is, when tested at 35 °C, warm-incubated lizards ran faster than cold-incubated lizards (Figure 4-2 B).

4.4.2 Speed over 1 m

For Dharawal lizards, the assumption of sphericity was met (Mauchley's test: $\chi^2 = 2.80$, P = 0.25). Incubation treatment did not affect speed ($F_{1, 34} = 1.68$, P = 0.20), but lizards ran faster at higher T_bs ($F_{2, 68} = 8.82$, P = 0.0001). The interaction between T_b and incubation treatment was not significant ($F_{2, 68} = 2.40$, P = 0.10); that is, lizards from both treatment groups ran at similar speeds at the same T_b (Figure 4-3 A).

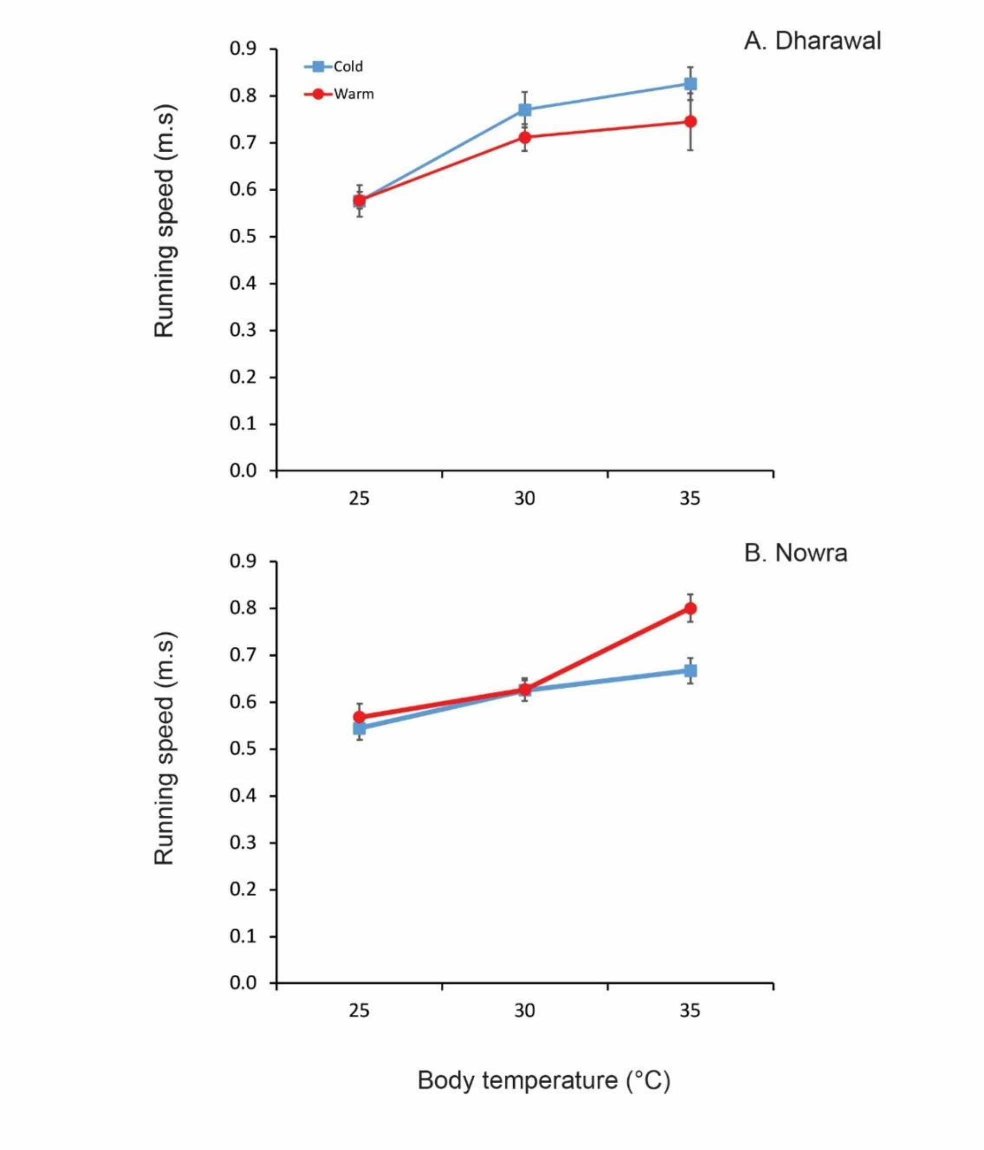

Figure 4-2 Running (burst) speed of cold and warm incubated hatchling lizards at three T_bs from a population in Dharawal National Park (A) and Nowra (B). Error bars denote standard errors.

For Nowra lizards, the data met the assumption of sphericity (Mauchley's test: χ^2 = 1.10, P = 0.58). There was a significant effect of incubation treatment on burst speed ($F_{1,62}$ = 9.03, P = 0.004), and lizards also ran faster at higher T_bs ($F_{2,124}$ = 28.9, P = 0.0001). The interaction between T_b and incubation treatment was not significant ($F_{2,124}$

= 1.09, P = 0.34). That is, warm-incubated lizards ran faster than cold-incubated lizards at all T_bs (Figure 4-3 B).

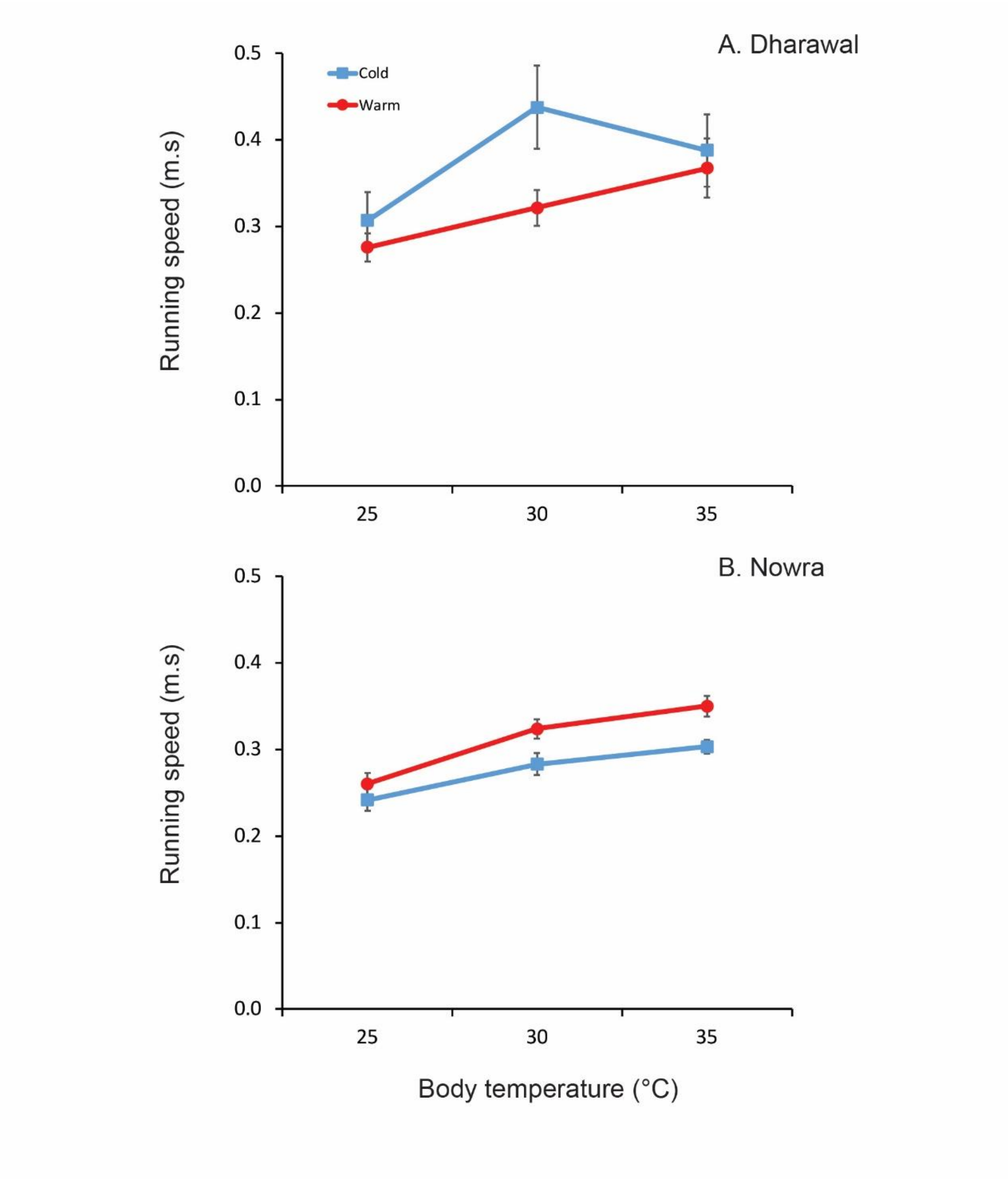

Figure 4-3 Running speed of cold and warm incubated hatchling lizards over 1 m at three T_bs from a population in Dharawal National Park (A) and Nowra (B). Error bars denote standard errors.

4.4.3 Number of stops made over 1 m

For Dharawal lizards, the assumption of sphericity was met (Mauchley's test: $\chi^2 = 1.35$, $P = 0.51$). Incubation treatment did not influence the number of stops ($F_{1, 34} = 0.15$, $P = 0.70$), nor did T_b influence the number of stops that lizards made down the racetrack ($F_{2, 68} = 2.23$, $P = 0.11$). The interaction between T_b and incubation treatment was not significant ($F_{2, 68} = 0.37$, $P = 0.70$). In other words, the number of stops made by hatchlings on the racetrack was not affected by incubation treatment or T_b (Figure 4-4 A).

For Nowra lizards, the assumption of sphericity was met (Mauchley's test: $\chi^2 = 1.88$, $P = 0.39$). There was a significant effect of incubation treatment on the number of stops made by hatchlings ($F_{1,62} = 24.18$, $P = 0.0001$). T_b also influenced the number of stops ($F_{2, 124} = 3.45$, $P = 0.035$), but post-hoc tests were not significant. The interaction between T_b and incubation treatment was not significant ($F_{2, 124} = 0.02$, $P = 0.98$); overall, cold-incubated lizards made significantly more stops than warm-incubated lizards at all three test temperatures (Figure 4-4 B).

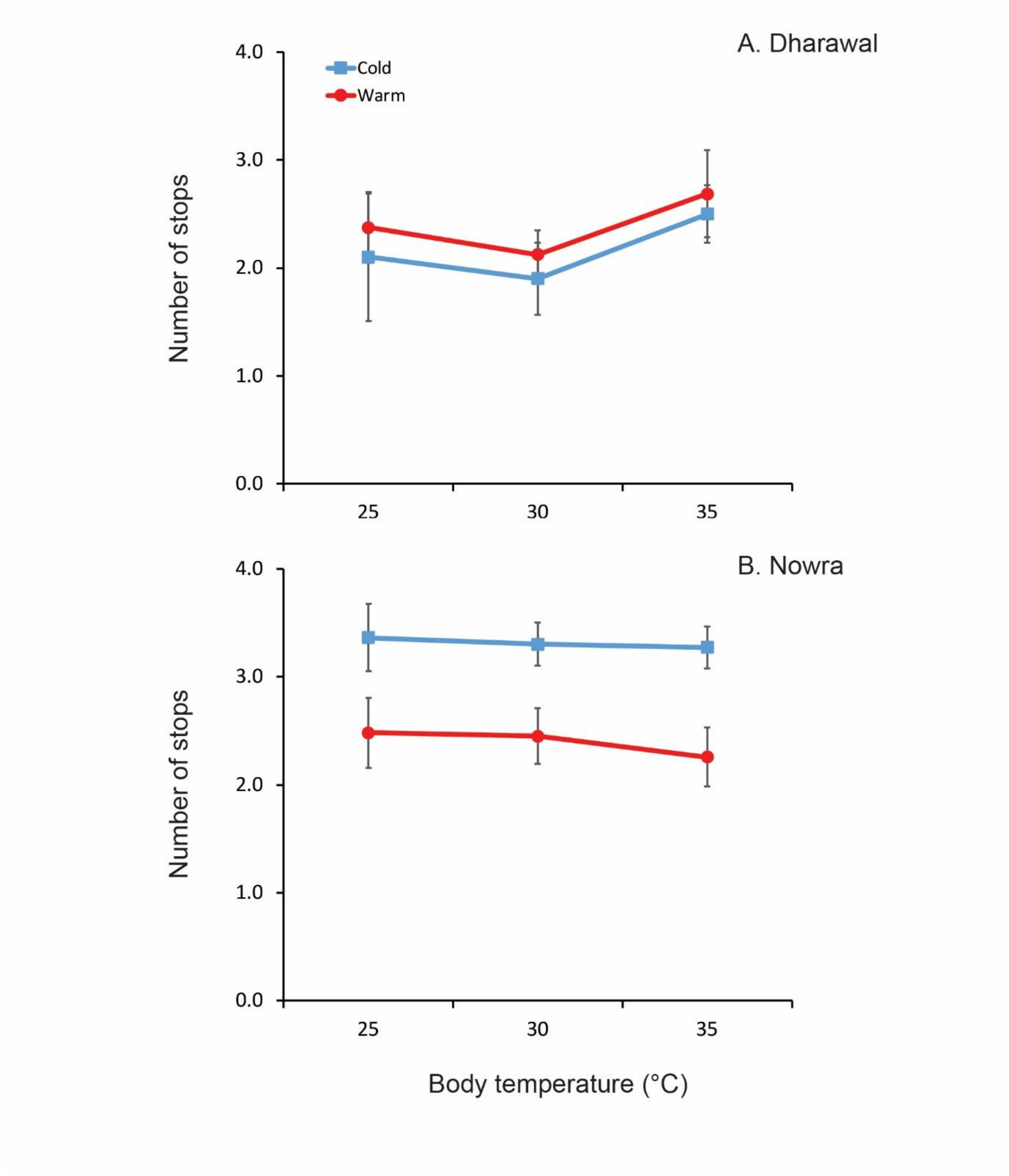

Figure 4-4 The number of stops made in the fastest 1 m run by cold and warm-incubated hatchlings from a population in Dharawal National Park (A) and Nowra (B). Error bars denote standard errors.

4.5 Discussion

We found strong inter-population differences in the effects of developmental temperature on hatchling running speed and behaviour. In the northern population (Dharawal), incubation temperature did not affect hatchling burst speed. At both incubation temperatures, hatchlings ran faster at 35 °C and slower at 25 °C. This result does not support the predictive anticipatory hypothesis which posits that hot-incubated lizards should run faster at higher temperatures (Bateson et al., 2014). Instead, our results support the developmental buffering hypothesis (Huey and Berrigan, 1996), suggesting that performance in this population was unaffected by developmental temperature, at least at these two experimental incubation regimes. Likewise, hatchling speed over 1 m, and behaviour along the racetrack, were not affected by developmental temperatures (Figure 4-2 and Figure 4-3). By contrast, in the southern population (Nowra), incubation temperature affected hatchling burst speeds, but only at 35 °C, and not at other test temperatures (Figure 4-2B). For speed over 1 m, warm-incubated lizards ran faster than cold-incubated lizards at all test temperatures (Figure 4-3B). This difference in speed was likely due to incubation-induced shifts in behaviour; that is, cold-incubated hatchlings made more stops on the race-track than warm-incubated lizards, and hence, took longer to complete the run (Figure 4-4 B).

Overall, our results provide limited support for the idea that incubation environments can 'match' offspring to future environments. The only pattern which supported this hypothesis was that warm-incubated Nowra lizards ran faster over 1 m than cold-incubated lizards. However, the ecological significance of performance over 1 m is unclear, as the hatchlings stopped frequently during runs, and so may rely on immobility (rather than speed per se) to reduce the risk of predation. This raises the question of whether laboratory estimates of sprint speed are a biologically meaningful proxy for fitness (Irschick et al., 2005). In some lizard species, individuals may not run at their full capacity along racetracks in the laboratory (Irschick et al., 2005). To determine the usefulness of laboratory estimates of speed, future studies could use high speed videography to estimate the running speed and behaviour of hatchlings in the wild, and of the same individuals in the laboratory.

The patterns of developmental plasticity in velvet geckos suggests that any conclusions about the effects of developmental temperature on locomotion in lizards may be premature (Noble et al., 2018). Most previous studies on lizards have used individuals from a single population, and have used constant incubation temperatures. Not surprisingly, some studies have reported no effects of incubation temperature on locomotion, whereas other studies have found strong effects. For example, a study on jacky dragons *Amphibolurus muricatus* incubated eggs at constant temperatures (25 °C, 28 °C and 32 °C) and estimated hatchling speeds at 20 °C and 30 °C. In that study, hatchlings ran fastest at the higher test temperature, but incubation treatment did not influence speed (Esquerre et al., 2014). By contrast, in a study on three-lined sun skinks *Bassiana duperryi*, hatchlings from 'hot' (27 ± 4 °C) temperature incubation ran faster than hatchlings from 'cold' incubation (20 ± 4 °C) at four test temperatures (15, 20, 25 and 30 °C), and this difference persisted into later life (Elphick and Shine, 1998). In another study on western fence lizards *Sceloporus occidentalis* from four populations, there was no effect on developmental temperature on sprint speed, but there were significant between population differences (Buckley et al., 2010). Thus, as we found in our study, population-level effects likely mediate the effects of incubation temperatures on performance in lizards (Noble et al., 2018), and further studies are necessary to measure the extent of this variation.

Because we used only two incubation treatments, we could not assess whether there was an optimal developmental temperature for sprint speed in hatchling geckos. However, we can compare our data to a recent study on velvet geckos in which eggs were incubated eggs at ‘current’ (mean = 23.2 °C, range 10–33 °C) and future nest temperatures (mean = 27.0 °C, range 14–37 °C). In that study, future-incubated hatchlings ran more slowly than current-incubated hatchlings at four test temperatures (20, 25, 30 and 35 °C, (Dayananda et al., 2017)). When we compared the sprint speeds of Nowra lizards to this previous study, we found that hatchlings from our warm treatment (mean = 25.4 °C) ran faster than hatchlings from the future (mean = 27.0 °C) or current (mean = 23.2 °C). This suggests that the optimal developmental temperature for the southern population is close to 25.4 °C, which interestingly, is very similar to the mean temperature of communal sun-exposed nests in that region (Dayananda et al., 2016). Future experimental incubation experiments are needed to formally test this conclusion.

What are the ecological implications of our study? Our warm incubation treatment mimicked the temperatures currently experienced inside sun-exposed nest sites in the southern population (Nowra). Thus, at Nowra, hatchlings from sun-exposed nests may be faster runners than hatchlings that emerge from cooler shaded nests. Previous studies on lizards have shown positive correlations between speed and survival (Miles, 2004, Husak, 2006), suggesting that ‘faster’ geckos ought to have higher survival. However, a recent study on hatchling *A. lesueurii* found no link between survival and speed (Dayananda et al., 2017). Thus, while it is tempting to suggest an adaptive interpretation for our experimental results, we have little evidence that being faster necessarily confers a survival advantage to hatchling geckos. Furthermore, in Chapter 3, I showed that incubation treatment did not influence hatchling survival, suggesting that maternal nest site choice had little effect on offspring viability. Thus, although females could manipulate offspring phenotypes, and 'match' offspring to higher environmental temperatures by laying their eggs in hotter nests, there is little evidence that doing so produces 'fitter' hatchlings. Likewise, a recent review of studies on a range of organisms also found weak support for anticipatory matching of offspring phenotypes (Uller et al., 2013).

In conclusion, we found that incubation temperatures affected sprint speed in hatchling geckos from the southern population, but not the northern population. More broadly, researchers seeking to use performance curves to predict the impacts of future warming on ectotherms (Huey et al., 2012) will need to consider geographic variation in developmental plasticity. Given that most organisms have broad geographic distributions, this represents a challenging task, and highlights the difficulty we face for forecasting the persistence of populations in the face of environmental change.

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Chapter 5 Does incubation temperature modulate the thermal preference of hatchling velvet geckos?

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5.1 Abstract

Developmental plasticity is an important source of variation for phenotypic traits of hatchling reptiles. In many species of lizards, temperatures experienced during embryogenesis can influence key traits that are linked to fitness, such as body size, learning ability, and locomotor performance. However, other hatchling traits, such as preferred T_bs, may be less labile, and may have little effect on hatchling fitness, particularly if post-hatching environmental sources of variation outweigh developmental effects. To investigate this question, we incubated eggs of velvet geckos *Amalosia lesueurii* under two fluctuating temperature treatments. The cold treatment mimicked temperatures of currently used communal nests (mean = 24.3 °C, range 18.4 – 31.1 °C), while the hot treatment (mean = 28.9 °C, range 19.1 – 38.1 °C) mimicked 2050 temperatures that might occur under future warming. We placed hatchlings inside a thermal gradient, and measured their preferred T_bs after they had access to food, and after they had fasted for five days. Incubation temperatures did not influence the thermal preference of hatchling velvet geckos. However, the hatchlings feeding status significantly affected their preferred T_bs. Hatchlings maintained higher T_bs after feeding (mean = 30.6 °C, range 23.5 –35.5 °C) than when they had fasted for 5 d (mean = 25.8 °C, range 20.0 – 32.0 °C). Thus, we found no evidence for developmental plasticity in preferred T_bs. Instead, reversible changes in hatchling preferred T_bs were influenced by food availability.

Key words: developmental plasticity; preferred T_b; thermoregulation; lizard; heatwave

5.2 Introduction

Developmental plasticity, the changes in the phenotype induced by the environment experienced by the developing embryo, is an important source of variation for many organismal traits that can influence individual fitness (West-Eberhardt, 2003). In most oviparous reptiles, females abandon their eggs soon after laying (Reynolds et al., 2002). In the absence of parental care, the thermal and hydric conditions inside reptile nests can vary markedly throughout the incubation period, and temperatures often fluctuate widely on a daily basis (Shine and Harlow, 1996, Andrews and Warner, 2002). In the last few decades, a large body of experimental research has demonstrated that incubation temperatures can influence a multitude of offspring traits, including sex, morphology, behaviour, performance, and cognitive abilities (Deeming, 2004, Noble et al., 2018, Deeming and Ferguson, 1991, While et al., 2018). Some of these developmental effects can be long lasting, and can influence the growth and survival of offspring (Dayananda et al., 2016, Andrews et al., 2000, Qualls and Andrews, 1999) and may influence lifetime reproductive success (Warner and Shine, 2008). Thus, an understanding of developmental plasticity can provide insights into how reptiles may cope with changing environments (Carlo et al., 2018, Angilletta, 2009, Mitchell et al., 2008).

Most research on thermal developmental plasticity has focused on how incubation temperatures affect morphological traits, physiology, sex ratios and incubation duration (While et al., 2018). By contrast, few studies have investigated whether incubation temperatures influence the thermal preferences or thermal tolerances of hatchlings (Blumberg et al., 2002, Du et al., 2010, Lang, 1987, Dayananda et al., 2017). Most lizards maintain their T_b within a preferred range by carefully selecting suitable microhabitats, altering their behaviour, or by adjusting their posture, shape, or colour (Huey, 1982). In turn, selected T_bs influence the physiology, behaviour, performance, activity budgets, and growth of individuals, which can influence their survival and reproduction (Huey, 1982, Angilletta, 2009). Thus, incubation-induced plasticity in preferred T_bs may have important fitness for hatchling lizards.

Experimental studies on lizards have found that incubation temperatures may affect the thermoregulatory behaviour of hatchlings of some species, but not others (Du et al., 2010). Moreover, it is unclear whether incubation induced shifts in thermal preferences are ecologically relevant, particularly if the effects are short lived or are masked by interactions with the post-hatching environment (Andrews et al., 2000, Buckley et al., 2007). For example, small differences in preferred T_bs of newly hatched lizards might have little effect on subsequent growth or survival if hatchlings alter their preferred T_bs in response to food availability. In some lizards, individuals may elevate their preferred T_bs after feeding, or may select cooler T_bs when food is scarce (Brown and Griffin, 2005). Hence, to understand the ecological significance of incubation-induced shifts in preferred T_bs, we also need to assess whether environmental variation influences the preferred T_bs of hatchlings.

In this study, we investigated whether incubation temperature influences the thermal preferences of hatchling velvet geckos *Amalosia lesueurii*. This species lays eggs communally in exposed rock crevices (Webb et al., 2008). During the incubation period, nest temperatures are highly correlated with air temperatures, and are therefore likely to increase during summer heatwaves (Dayananda et al., 2016). A recent experimental study found that hatchlings from hot-incubation treatments mimicking potential future nest temperatures had lower thermal tolerance than hatchlings from cold-incubation treatments that mimicked current nest temperatures (Dayananda et al., 2017). However, the authors did not investigate whether incubation temperatures also affected the thermal preference of hatchlings. To address this knowledge gap, we incubated velvet gecko eggs under two different temperature regimes that mimicked current temperatures experienced by geckos in the natural nests (cold) and future temperatures that geckos might experience during hot summers in future. We then measured the preferred T_bs of hatchlings in a thermal gradient when they had fed and when they had fasted.

5.3 Methods

5.3.1 Study species

The velvet gecko, *Amalosia lesueurii,* is a small (up to 65 mm snout to vent length), nocturnal lizard that inhabits sandstone rock outcrops from south eastern New South Wales to south-eastern Queensland (Cogger, 2014). By day the geckos thermoregulate under small, sun-exposed stones (Schlesinger and Shine, 1994, Webb et al., 2008). At dusk, they venture from their rocks or crevices to forage in leaf litter (Cogger, 2014). Female velvet geckos lay eggs in communal nests located in rock crevices in late spring, and the eggs hatch from February to March (Webb et al., 2008). After emergence, hatchlings settle under small stones located near the communal nests, and they spend the first 8 months of life sheltering beneath one or two rocks (Webb, 2006). Annual observations of communal nests at three study sites in Morton National Park, NSW, have revealed that gravid geckos have laid eggs inside the same communal nests since 1992 (Webb, unpublished data). Thus, temperatures inside communal nests may increase in the future if the frequency and duration of summer heatwaves increases.

5.3.2 Collection of females

In October and November 2016, we collected 51 gravid velvet geckos from a study site in Dharawal National Park, 67 km south of Sydney, NSW, Australia. We transported them to the University of Technology Sydney, where we measured their snout vent length (SVL) and tail length (TL) with a ruler (to nearest mm), and recorded their mass (to nearest 0.01 g) with an electronic balance. We housed geckos individually inside ventilated plastic cages (Sistema NZ 2.0 L, 220 × 150 × 60 mm) in a room maintained at 23 °C with a 12:12 light cycle. Each cage contained a moist vermiculite substrate (for egg laying), a plastic shelter (half pipe, 80 mm × 40 mm) and a water dish. One end of each cage was placed on a timer-controlled heating cable set to 32 °C to create a thermal gradient (23 – 32 °C) in the cages during the day. At night, cage temperatures fell to room temperature (23 °C). We fed females crickets twice weekly and gave them access to water *ad libitum*. We checked the cages for newly laid eggs in the morning and afternoon, and remoistened the vermiculite twice daily to prevent eggs from desiccating. After females laid eggs, we recorded their mass (to nearest 0.01 g) and released them at their exact site of capture.

5.3.3 Egg incubation experiment

After oviposition, we measured the maximum length and width (to nearest 0.01 mm) of each egg with vernier callipers and recorded its mass (to nearest 0.01 g) with an electronic balance. We placed eggs singly inside 100 mL glass jars filled with moist vermiculite (water potential of 200 KPa) and covered each jar with plastic food wrap to prevent the eggs from desiccating. We randomly allocated one egg from each clutch of two eggs produced by each female to each of two programmable incubators (Panasonic MIR 154, 10 step functions). We programmed one incubator to mimic the thermal regimes of currently used exposed communal nests (cold: mean = 24.3 °C, range 18.4 – 31.1 °C), and the second incubator was programmed to mimic future nest temperatures (hot: mean = 28.9 °C, range 19.1 – 38.1 °C). Temperatures in the hot treatment mimicked the potential nest temperatures that might occur in 2050 under climate warming (Dowdy et al., 2015). We incubated 47 eggs in the hot incubation treatment and 50 eggs in the cold incubation treatment.

5.3.4 Hatchling husbandry and measurement of preferred T_bs

After hatching, we housed hatchlings individually in plastic containers (Sistema NZ 2.0 L, 220 × 150 × 60 mm) with a paper substrate, a plastic half pipe and a water dish. We placed the hatchling cages on heating racks as described previously for females. We fed hatchlings with five pinhead crickets twice weekly, and cleaned their cages at weekly intervals. We recorded the T_bs of 22 four-week old hatchlings (10 hot-incubated and 12 cold-incubated hatchlings) inside a thermal gradient. The thermal gradient consisted of a wooden enclosure (1.5 m long × 0.5 m wide × 0.5 m high) with a mesh lid and a clear glass front at one end. We partitioned the enclosure into 8 lanes, each 1.5 m long and 6 cm wide, each of which contained a 1.4 m long white plastic half pipe as a shelter, with a water dish in the middle. To create the thermal gradient, we placed the cage on a wooden base that contained heating cables at one end, and plastic tubes connected to a water bath (Haake F3 K Circulating Water Bath) carrying chilled water (5 °C) at the other end. Two 250-watt infrared lamps provided additional heating at the hot end. The substrate temperatures within the thermal gradient ranged from 10 °C to 40 °C. To measure the substrate temperature within the gradient, we placed miniature data loggers (Thermochron i-buttons, factory calibrated and accurate to ± 0.1 °C) along the floor of each lane. The data loggers recorded the temperature every 60 minutes.

To measure the preferred T_b of the hatchlings, we placed each hatchling in the middle of each lane of the thermal gradient at 0900 h. After 1 hour of acclimation, we observed the location of each hatchling though the front glass wall, and recorded the numbers of the data loggers nearest to the lizard. If we could not see the hatchling, we confirmed its position by gently lifting the half pipe without disturbing the animal. In such cases, we recorded the lizard's T_b with an infrared thermometer (Cool Tech, CT663, spot diameter = 13 mm). We repeated this procedure every hour from 1000 to 1700 hours. We used substrate temperature as a proxy for lizard T_b (Buckley et al., 2007, Goodman and Walguarnery, 2007) because the hatchlings small body size (SVL < 30 mm, mass < 0.55 g) precluded the use of cloacal probes. In addition, the T_b of small lizards can change rapidly within seconds of handling, so aside from the risk of injuring the lizard, cloacal probes may not provide accurate estimates of hatchling T_bs. In addition, the capture of lizards could affect their subsequent behaviour within the thermal gradient, which could affect their T_b. Although our method was crude, substrate temperatures recorded from data loggers near lizards were positively correlated with lizard temperatures that were measured with the IR thermometer ($r^2 = 0.94$, $P < 0.001$).

To assess whether feeding influenced the T_b of hatchling geckos, we tested lizards under their normal feeding regime. For the fasted treatment, lizards were not fed for five days prior to placement in the thermal gradient, which allowed us to compare our results with other studies on lizards (Brown and Griffin, 2005). Hence the experiment was a 2 × 2 factorial design with two temperatures by two feeding status.

5.3.5 Statistical analyses

We used two-way repeated measure ANOVAs to determine whether incubation temperature or feeding status influenced the mean selected T_bs of hatchling velvet geckos. Although we used a split-clutch design, and placed one egg from each clutch of two eggs into each incubator, hatching success was low, and only two hatchlings had the same mother. For this reason, we did not include maternal ID as a factor in our analyses. We ran statistical tests in SPSS version 23.

5.4 Results

Hatching success did not differ between the incubation treatments; 12 of 47 cold-incubated eggs hatched and 10 of 50 hot-incubated eggs hatched ($\chi^2 = 0.42$, $P = 0.51$). However, the hatching success was very low with compared to the previous study. There was no significant effect of incubation treatment on the mean temperatures selected by hatchlings ($F_{1, 20} = 0.126$, $P = 0.727$, Figure 5-1). However, recently fed animals maintained higher temperatures than unfed animals ($F_{1, 20} = 206.78$, $P < 0.01$). In both fed and fasted hatchlings, T_bs varied significantly with hour of day ($F_{7, 140} = 20.73$, $P < 0.01$, Figure 5-1).

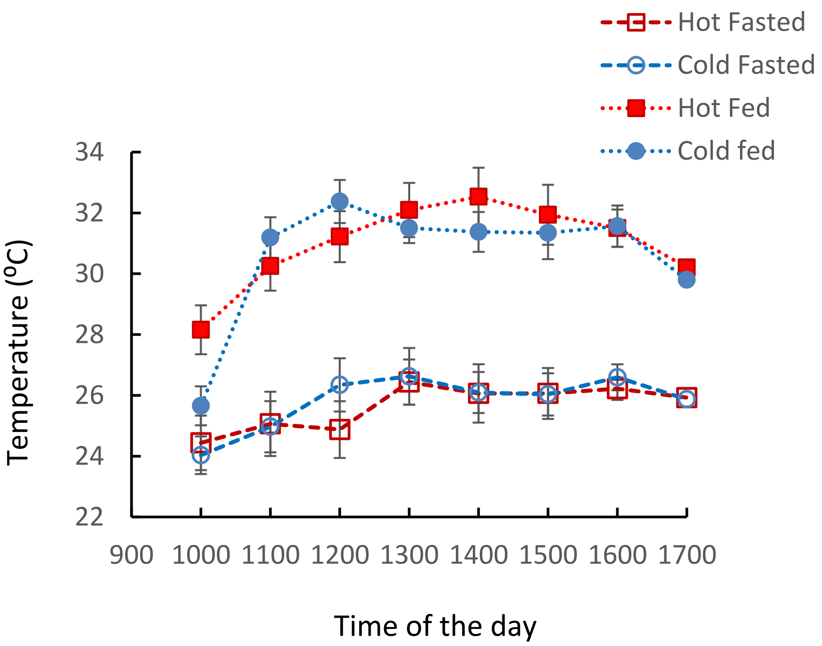

Figure 5-1. Mean T_bs of cold-incubated and hot-incubated hatchling velvet geckos that were placed inside a thermal gradient between 0900 h and 1500 h. Figure shows temperature profiles of recently fed lizards and lizards that were fasted for 5 days. Error bars denote standard errors.

5.5 Discussion

We found no evidence that incubation temperatures affected the thermal preferences of four-week old hatchlings. Mean selected T_bs of cold- and hot-incubated hatchlings were very similar, as was the precision of thermoregulation. These findings agree with previous studies on other lizard species. For example, incubation temperature did not affect the selected T_bs of hatchling veiled chameleons *Chamaeleo calyptratus* (Andrews, 2008), western fence lizards *Sceloporus occidentalis* (Buckley et al., 2007), three lined skinks *Bassiana duperreyi* (Du et al., 2010), or Cuban rock iguanas *Cyclura nubila* (Alberts et al., 1997). By contrast, other studies have found that incubation temperatures can influence the thermoregulatory behaviour of hatchlings. For example, in the Madagascar ground gecko, *Paroedura pictus*, hatchlings from hot incubation temperatures had higher dorsal temperatures prior to crossing between the cold and hot sides of a thermal shuttle apparatus (Blumberg et al., 2002). In *Sceloporus virgatus*, hatchlings from cold temperature incubation (15 – 25 °C) selected higher T_bs, and maintained T_bs more precisely than hatchlings from hot temperature (20 –30 °C) incubation (Qualls and Andrews, 1999). In a study on jacky dragons using constant temperature incubation, hatchlings from 28.1 °C treatment had lower T_bs after two hours in a thermal gradient than hatchlings from 25 °C or 32 °C treatments (Esquerre et al., 2014). This result suggests that like other traits, reaction norms for thermal preferences may be non-linear (Noble et al., 2018). Hence, we cannot rule out the possibility that intermediate incubation temperatures might affect preferred T_bs of velvet geckos. Future studies, using intermediate temperatures, would help resolve this issue.

Ultimately, the biological relevance of incubation-induced shifts in preferred T_bs will depend on the magnitude and duration of such effects relative to other sources of environmental variation. Notably, several studies have shown that incubation-induced shifts in T_b are transitory, so are unlikely to influence traits linked to fitness (Buckley et al., 2007, Goodman and Walguarnery, 2007). In the present study, hatchlings maintained significantly higher T_bs after feeding (fed: mean T_b = 30.6 °C; fasted mean T_b = 25.8 °C), demonstrating that food availability has large effects on hatchling T_bs. Thermophilic responses to feeding are widespread in snakes (Blouin-Demers and Weatherhead, 2001) but are less common in lizards (Wall and Shine, 2008, Schuler et al., 2011). Notably, the 4.8 °C increase in mean T_b of hatchling geckos after feeding is similar to that reported for snakes in thermal gradients (typically, increases of 2 to 6 °C, (Slip and Shine, 1988, Tsai and Tu, 2005, Lysenko and Gillis, 1980), and is higher than the modest increases (typically, < 2 °C), reported for lizards such as *Heloderma suspectum* (Gienger et al., 2013) and *Anolis carolinensis* (Brown and Griffin, 2005). Future studies on hatchlings of other lizard species in this respect, particularly geckos, would help to evaluate the generality of our results.

Why do fasted hatchlings select lower T_bs than recently fed individuals? After feeding, selection of higher T_bs likely maximises digestive efficiency and rates of energy assimilation (Beaupre et al., 1993, Harlow et al., 1976). However, because metabolic rates scale with T_b, maintenance of high T_bs increases energy expenditure (Angilletta, 2009). Therefore, in the absence of food, hatchlings may select lower T_bs to reduce energy expenditure. Conserving energy might be particularly important for hatchlings, as they may lack sufficient energy reserves in their tails to survive long periods in the absence of food (Greer, 1989). Ultimately, shifts in T_b in response to food availability may represent a trade-off between energy conservation versus maintenance of other fitness related behaviours (Huey, 1982). For example, adults of Yarrow's spiny lizard *Sceloporus jarrovi* that were deprived of food for 5 days maintained high T_bs, presumably so they could maximise important fitness related behaviours such as territory defence (Schuler et al., 2011). In the wild, hatchling velvet geckos congregate under rocks near communal nest sites, and hatchlings often share rocks with conspecifics during the first few months of life (Webb, 2006), so territory defence may be unimportant during this period.

Irrespective of feeding status, hatchling geckos displayed strong diel variation in preferred T_bs. Hatchlings selected low T_bs in the morning, and thereafter they raised their T_b and maintained elevated temperatures throughout the afternoon (Figure 5-1). Similar diel patterns of thermoregulation were reported for individuals of two gecko species, *Eublepharis macularius* and *Oedura marmorata*, that were fasted for 3 d before being placed in a thermal gradient (Angilletta et al., 1999). Similarly, individuals of the gecko *Tarentola mauritanica* increased their T_bs during the day (Gill, 1994). The underlying cause for this pattern of thermoregulation in geckos is not known, but we note that T_bs of hatchling *A. lesueurii* follow the same pattern as rock temperatures; i.e. delayed heating, reaching a peak in early afternoon (Webb and Shine, 1998). Potentially, this pattern might represent an entrained circadian rhythm for activity or thermoregulation (Refinetti and Susalka, 1997, Tawa et al., 2014). Because hatchling geckos commence foraging shortly after dusk, maintaining high T_bs around dusk would aid in prey capture and potentially, escape from predators (Christian and Tracy, 1981). As for diurnal lizards, maintenance of high T_bs during daylight hours would facilitate physiological processes such as digestion, growth and ecdysis (Huey, 1982, Angilletta et al., 1999)

In conclusion, we found no effects of incubation temperature on the thermal preference of hatchling velvet geckos. However, there was a strong effect of feeding status on the hatchlings thermal preference, suggesting that food availability may influence thermoregulation by hatchlings in the wild. To evaluate the role of thermal developmental plasticity on the thermal preferences of hatchling lizards, future studies should calculate the duration of such effects, and their magnitude relative to plasticity caused by the post-hatching environment.

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Chapter 6 Higher incubation temperatures produce long lasting upward shifts in cold tolerance but not heat tolerance of hatchling geckos

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Higher incubation temperatures produce long-lasting upward shifts in cold tolerance, but not heat tolerance, of hatchling geckos

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Key words: Heat wave, developmental plasticity, critical thermal limits, thermal tolerance, lizard

SUMMARY STATEMENT

Hatchling geckos from current temperature incubation tolerated cold better than hatchlings from future temperature incubation. The developmental shift in cold tolerance persisted for six months, and could affect hatchling survival.

6.1 Abstract

Heatwaves are a regular occurrence in Australia, and are predicted to increase in intensity and duration in the future. These changes may elevate temperatures inside lizard nests, shortening the incubation period, so that hatchlings are more likely to emerge during heatwaves. Potentially, developmental plasticity or heat hardening could buffer hatchings from future warming. For example, higher incubation temperatures could shift critical thermal maxima upwards, enabling lizards to withstand higher temperatures. To investigate whether developmental plasticity affects hatchling thermal tolerance, we incubated eggs of the velvet gecko *Amalosia lesueurii* under two fluctuating incubation treatments to mimic current (mean = 24.3 °C, range 18.4 – 31.1 °C) and future 'hot' (mean = 28.9 °C, range 19.1 – 38.1 °C) nest temperatures. We maintained the hatchlings under identical conditions, and measured their thermal tolerance (CT_{max}) at age 14 d and 42 d. We then released hatchlings at field sites, and recaptured individually marked lizards at age six months, to determine whether incubation induced shifts in thermal tolerance were transitory or long-lasting. We found that at age 14 d, hatchlings from hot-temperature incubation had higher CT_{max} (mean = 39.96 ± 0.25 °C [SD]) than hatchlings from current-temperature incubation (mean = 39.70 ± 0.36 °C [SD]). Hatchlings from the current-incubation treatment also had significantly higher heat hardening capacity (mean = 0.79 ± 0.37 °C [SD]) than hatchlings from hot-temperature incubation treatment (mean = 0.47 ± 0.17 °C [SD]). However, both of these incubation-induced effects did not persist into later life. By contrast, incubation treatment had significant and long-lasting effects on the cold tolerance of hatchlings. At age 14 d, current-incubated hatchlings tolerated colder temperatures (CT_{min} = 11.24 ± 0.41 °C [SD]) than hot-incubated hatchlings (CT_{min} = 14.11 ± 0.25 °C [SD]). This significant difference in cold tolerance persisted into the juvenile life stage, and was present in 6 month old lizards that we recaptured from field sites. This

finding indicates that upward shifts in cold tolerance caused by higher incubation temperatures might affect overwinter survival of lizards, but field studies linking fitness to thermal tolerance are necessary to test this idea. Overall, our results suggest that developmental plasticity for heat tolerance is unlikely to buffer lizard populations from increasing temperatures.

6.2 Introduction

An understanding of how organisms cope with heatwaves can help to predict how future climatic changes may affect populations. Heatwaves are predicted to increase in intensity and duration in the future, and can have major effects on populations via direct mortality (Welbergen et al., 2008), or through more complex interactions with early life stages. Lizards are particularly sensitive to acute temperatures because their physiology, behaviour and locomotor performance is strongly dependent on T_bs (Huey, 1982). Although juvenile and adult lizards can avoid extreme temperatures by selecting appropriate microhabitats (Huey, 1982), sessile life stages (eggs) are particularly vulnerable to exposure to extreme temperatures because embryos cannot thermoregulate (Telemeco et al., 2016). In most lizard species, females lay eggs in shallow underground nests where the developing embryos can experience thermal spikes during extreme heat events (Shine et al., 2003, Telemeco et al., 2009). While chronic exposure to high temperatures (typically > 42 °C) can result in embryo mortality (Levy et al., 2015, Angilletta et al., 2013), the effects of exposure to high, but not lethally high temperatures, on embryos and offspring have received less study.

Phenotypic plasticity may reduce the vulnerability of early life stages to extreme heat events. Maternal plasticity in nest site selection (choosing shadier nests) or the timing of oviposition (nesting earlier) could reduce the exposure of developing embryos to high temperatures (Urban et al., 2014). Even if females only partially compensate for increases in nest temperatures, developmental plasticity and acclimation may also affect the physiological traits of offspring in ways that increase fitness. For example, in several species of ectotherms, exposure of embryos to higher developmental temperatures may confer higher thermal tolerance in later life stages (Slotsbo et al., 2016, van Heerwaarden et al., 2016). Most of these studies have involved *Drosophila*, but concordant results have been demonstrated for other taxa (Sgro et al., 2016), suggesting that developmental plasticity for thermal tolerance may be widespread. Over shorter time periods, heat hardening, the process whereby individuals increase their heat tolerance after brief exposure to high temperatures, may provide fitness benefits to ectotherms during summer heatwaves (Hoffmann et al., 2003). For example, in *Drosophila melanogaster* heat hardened flies that were released during hot weather had significantly higher rates of recapture than control flies, suggesting that heat hardening conferred an advantage during hot conditions (Loeschcke and Hoffmann, 2007).

While developmental plasticity and heat hardening may help to buffer lizard populations from heatwaves, few studies have investigated how incubation temperatures influence the thermal tolerance or heat hardening capacity of hatchlings (Noble et al., 2018, While et al., 2018, Llewelyn et al., 2018). Moreover, it is not clear whether such effects, if present, persist into later life. For example, lizards may show ontogenetic shifts in thermal tolerance, and can exhibit longer-term acclimation to the changing environments in the field (Bowler, 2005). Such acclimation might swamp any effects of developmentally induced changes in thermal tolerance. To address these knowledge gaps, we investigated whether exposure to higher developmental temperatures likely to be experienced in the future affected the thermal tolerance of hatchling lizards. We also asked whether developmental temperatures influenced the heat hardening responses of hatchlings. To determine whether developmental effects were transient or long lasting, we measured the thermal tolerance of hatchlings within two weeks (14 days) of birth, and after six weeks (42 days), before releasing them to field sites. To assess whether incubation-induced changes in thermal tolerance persisted into later life, we recaptured lizards from the field four months after release (at age six months), brought them back to the lab, and measured their thermal tolerance.

6.3 Materials and methods

6.3.1 Collection and maintenance of pregnant females

Gravid velvet geckos were collected from rock outcrops near Nowra, approximately 170 km south of Sydney, and Dharawal National Park, approximately 70 km south of Sydney, in late spring 2016. Females were transported to the University of Technology Sydney. Upon arrival, one of us (TA) measured their snout vent length (SVL) and tail length (TL) with a ruler (to nearest mm), and recorded their mass (to nearest 0.01 g) with an electronic balance. The females were housed individually inside identical plastic cages (Sistema NZ 2.0 L, 220 × 150 × 60 mm, with ventilated lid) in a constant temperature room (23 °C) with a 12:12 light cycle. Each cage contained a white plastic half-pipe shelter (80 mm × 40 mm) and a water dish, with a layer of moist vermiculite to prevent eggs from desiccating. Cages were placed on timer-controlled heating cables set to 32 °C which created a thermal gradient (23–32 °C) inside the cages during daylight hours, falling to 23 °C at night. All geckos had a constant supply of drinking water and were fed crickets twice weekly. Each morning and afternoon, one of us (TA) checked all the cages for newly oviposited eggs, and sprayed the vermiculite to maintain a moist substrate. After females laid eggs, we recorded their body mass, and released them at their exact site of capture during suitable weather conditions.

6.3.2 Egg incubation experiment

On the day of egg laying, one of us (TA) placed each egg singly inside a 100 mL glass jar filled with moist vermiculite (water potential of 200 KPa), and was covered with plastic food wrap to reduce water loss. Nearly all females laid two eggs, so we placed one egg from each clutch into the 'current' incubator, and the other into the 'hot' incubator (Panasonic MIR 154, 10 step functions). Both incubators were programmed to mimic the cycling temperatures seen inside natural nests at our study sites, but with short heatwaves to simulate a hot summer (Figure 6-1). Temperature profiles of the 'current' treatment (mean = 24.3 °C, range 18.4 – 31.1 °C, SD = 3.2 °C) were similar to those recorded inside sun-exposed communal nests (Dayananda et al., 2016), while thermal cycles of the 'hot' treatment (mean = 28.9 °C, range 19.1 °C – 38.1 °C, SD = 4.3 °C) simulated the potential future nest temperatures that could occur in 2050 according to climate models that predict increases in air temperature between 2.9 and 4.6 °C in southeast Australia (Dowdy et al., 2015) (Figure 6-1). We incubated 84 eggs in the current incubation treatment and 81 eggs in the hot incubation treatment.

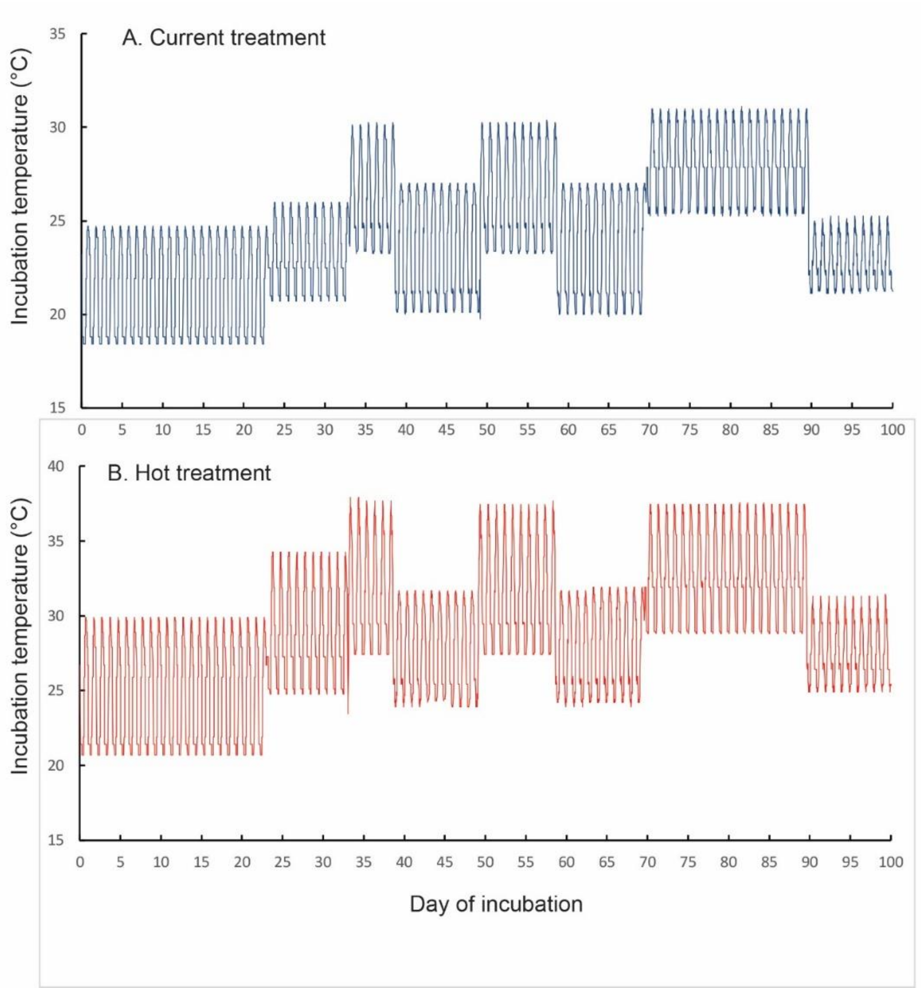

Figure 6-1 Temperature profiles experienced by velvet gecko eggs in the (A) current and (B) hot incubation treatments.

6.3.3 Maintenance of hatchling geckos

Once hatchlings emerged from eggs, one of us (TA) measured their snout-vent length (SVL) with a ruler (to nearest mm), and body mass with an electronic scale (to nearest 0.01 g). Each hatchling was housed individually as described above for females, except that the cages were lined with a paper substrate and lacked vermiculite. We fed hatchlings with five pinhead crickets (*Gryllus assimilis*) twice weekly, and cleaned their cages at weekly intervals. All hatchlings were maintained under the same conditions in captivity for 6 weeks, after which they were released at their mothers' site of capture. All procedures were approved by the UTS Animal Care and Ethics Committee (protocol

#2012000256) and a NSW National Parks and Wildlife Service scientific licence (SL101013 to JKW).

6.3.4 Measurements of hatchling thermal tolerance

The same researcher (TA) measured the thermal tolerance of 51 hatchlings (34 current incubated lizards, 17 hot incubated lizards) using the methods of Phillips et al. (2016). To measure the thermal tolerance, each lizard was placed inside a standard 50 mL plastic vial (115 mm long, 30 mm diameter) that was fitted with a removable screw cap. The vials were placed inside an incubator (Panasonic MIR 154, 10 step functions) for 30 minutes at 22 °C to ensure that all lizards had the same starting T_b (Terblanche et al., 2007). To commence the experiment, the vial containing a lizard was removed from the incubator, and the screw cap was replaced with a cap fitted with a thermistor thermocouple passing through its centre. The other end of the thermocouple was attached to an electronic thermometer (OMEGA 450 ATH Thermistor Thermometer 2252 Ω @25 °C, accuracy 0.01 °C). The thermistor was positioned so that it measured the air temperature within the tube, rather than cloacal T_b. Because the hatchlings were very small (< 0.3 g), insertion of the thermistor into the lizard's cloaca would have injured the lizards and would have prevented them from righting themselves. Given the lizards' small size, the tube temperature would provide a very close approximation of the lizard's internal temperature (Phillips et al., 2016). To commence each trial, we submerged the tube in a water bath (Thermoline) and increased the temperature gradually at a rate of 0.25 °C per minute. Once the temperature reached 36 °C, we checked the lizards righting response every 10 s by turning them upside down by rotating the tube. The temperature at which the lizard could not right itself was deemed the CT_{max}. The same procedure was used to measure CT_{min}, except that we cooled lizards from 22 °C, and commenced rotating the tube to measure their righting response once they reached 18 °C. All trials were carried out when hatchlings were one to two weeks old between 1000 h and 1500 h. On day 1, we measured CT_{max}, and on day 2, we measured CT_{min}, so that lizards had 24 h to recover between trials. Hatchlings were maintained in captivity as described above, and all lizards (except two that died in captivity) were retested at age 6 weeks.

6.3.5 Measuring heat hardening capacity

We measured heat hardening of 16 current-incubated and 16 hot-incubated hatchlings at age 6 weeks. Heat hardening has not been measured in our study species previously so we first measured the time course for hardening. To do this, the same researcher (TA) measured the CT_{max} of a subset of lizards, as described above, and retested each individual after a periods of one to six hours. Because it can be detrimental to expose the same animal to multiple high temperatures, each individual was only tested for one time interval. The resultant curve of the change in CT_{max} (Δ CT_{max}) versus time showed that maximum heat hardening occurred after four hours (Figure 6-2). Thereafter, we recorded the second measurement of CT_{max} of each lizard four hours after the first measurement.

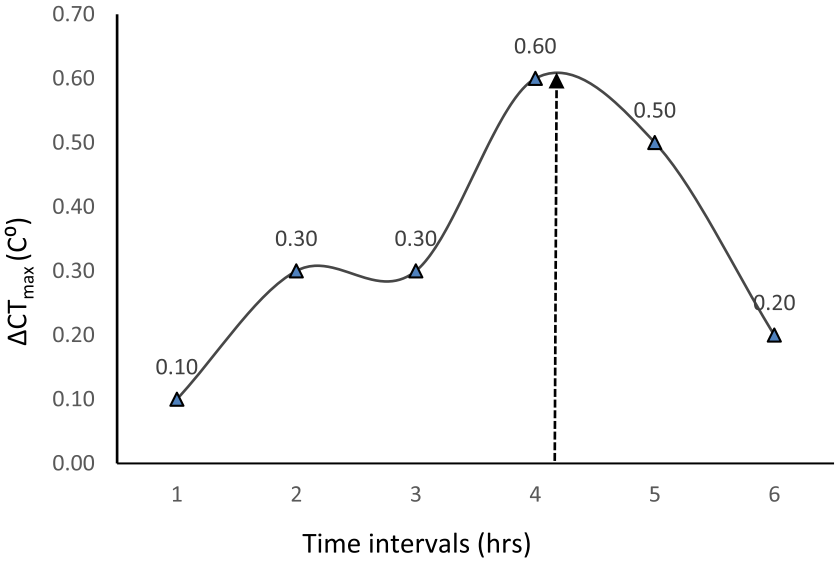

Figure 6-2 Heat hardening (delta CT$_{max}$) as a function of the number of hours after the first exposure to the high temperature that resulted in a loss of righting response in hatchling velvet geckos.

6.3.6 Persistence of incubation induced changes in thermal tolerance

To estimate whether thermal tolerance changed with age under laboratory conditions, the same researcher (TA) retested 49 lizards (33 current-incubated, 16 hot incubated) at age six weeks. All lizards were raised under the same environmental conditions (see above) in the laboratory prior to testing. After testing was completed, we individually marked each lizard with a unique toe-clip, and released them at their mother's site of capture. In mid-winter (July), we systematically searched under all the rocks at our field sites and checked the toe-clips of all geckos captured. The hatchlings from our incubation experiment that we recaptured were brought to the laboratory, and housed as described previously. We measured the thermal tolerance and heat hardening of these lizards within one day of capture using the methods described above. After testing was complete, the lizards were returned to their exact site of capture.

6.4 Statistical analyses

A chi-square test was used to determine whether hatchling success varied between incubation treatments. Two factor ANOVAs were used to determine whether incubation period or body size SVL, mass) differed between sites or treatments. We used linear mixed effects models to estimate whether CT_{max}, CT_{min} and heat hardening differed between hot-incubated and current-incubated lizards. Each model had either CT_{max}, CT_{min} or heat hardening as the response variable. Incubation temperature was a fixed explanatory variable (current, hot) and both location (Dharawal National Park, Nowra) and mother identity were included as random control variables. Mother identity was nested within location in the models. Heat hardening was $\log_e$-transformed prior to analyses to meet assumptions of normality and homogeneity of variance in the model residuals. Satterthwaite approximations were used to calculate degrees of freedom for t-tests in the mixed models and P values were calculated using the adjusted degrees of freedom. Statistical analyses were performed using the package lmerTest (Kuznetsova et al., 2017) in R 3.1.3 (https://www.r-project.org/).

6.5 Results

6.5.1 Effects of incubation temperature on hatching success, incubation period and body size

Hatching success was higher in the current-treatment (34 of 84 eggs hatched) than the hot-treatment (18 of 81 eggs hatched, Pearson chi-square = 6.37, P = 0.12). Incubation treatment also affected the incubation period; hot-incubated lizards were hatched, on average, 26 d earlier (mean incubation period = 65.4 d, range 61–70 d) than current-incubated lizards (mean incubation period = 91.6 d, range 73–101 d; two-factor ANOVA: incubation treatment $F_{1,48} = 181.6$, P = 0.0001; location $F_{1,48} = 0.008$, P = 0.93, interaction

$F_{1,48} = 1.49$, $P = 0.23$). Hot-incubated lizards were also smaller in snout-vent length (mean = 21.4 ± 1.82 mm [SD]) than current-incubated lizards (mean = 25.4 ± 2.07 mm [SD]; ANOVA: incubation treatment $F_{1,48} = 48.6$, $P = 0.001$; location $F_{1,48} = 1.76$, $P = 0.19$, interaction $F_{1,48} = 0.78$, $P = 0.38$), and were also lighter (mean = 0.31 ± 0.06 g [SD]) than current-incubated lizards (mean = 0.40 ± 0.06 g [SD]. ANOVA: incubation $F_{1,48} = 40.73$, $P = 0.0001$; location $F_{1,48} = 0.15$, $P = 0.70$, interaction $F_{1,48} = 0.25$, $P = 0.62$).

6.5.2 Effects of incubation temperature on thermal tolerance

Hot-incubated hatchlings had a higher CT_{max} than current-incubated hatchlings (39.96 °C *versus* 39.70 °C; $t_{23.27} = 3.12$, $P = 0.005$; Figure 6-3A). Random factors in the model were associated with very little (mother identity within location: variance ± SD = 0.04 ± 0.20) to none (location) of the variation in CT_{max}. Hot-incubated lizards also had a higher CT_{min} than current-incubated lizards (14.11 °C *versus* 11.24 °C; $t_{32.22} = 27.59$, $P < 0.0001$, Figure 6-3B). Random factors in the model explained very little (mother identity within location: variance ± SD = 0.02 ± 0.15) to none (location) of the variation in CT_{min}.

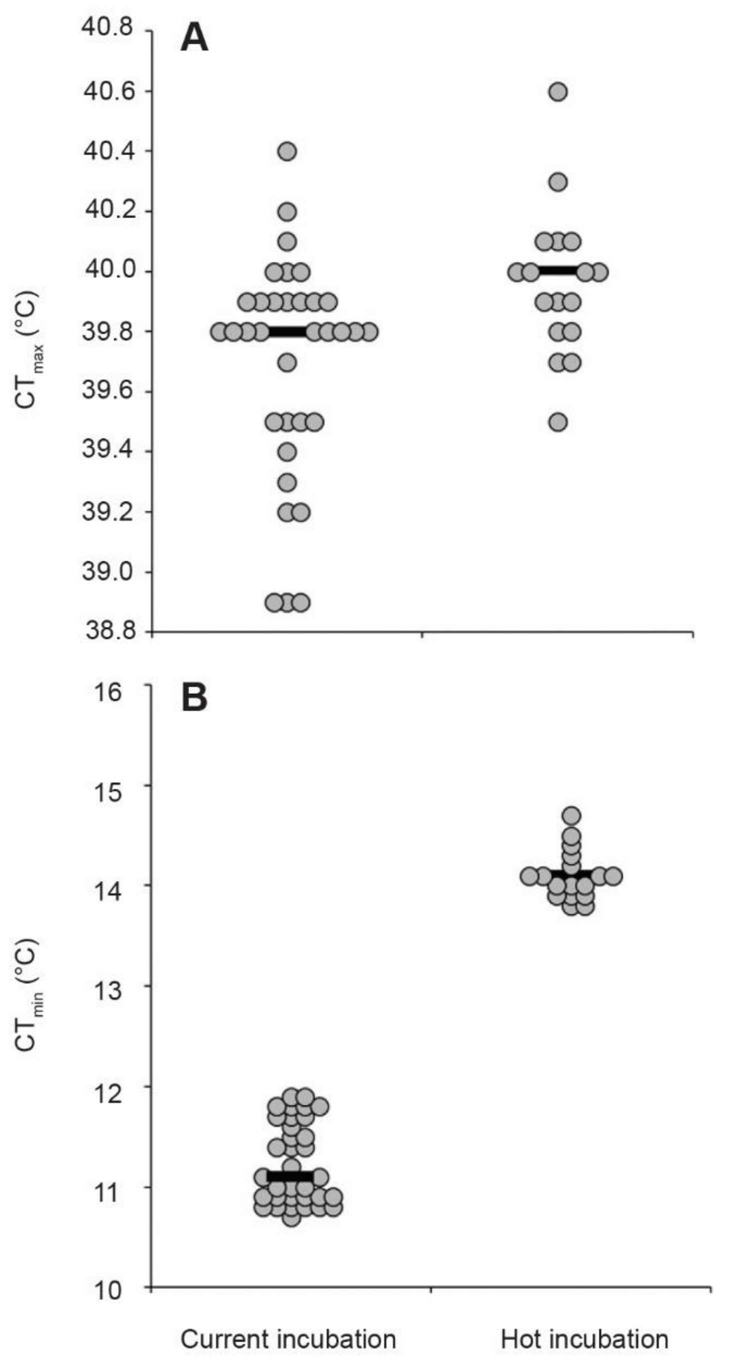

Figure 6-3 Dot plots of (A) maximum critical thermal tolerance (CT$_{max}$) and (B) minimum critical thermal tolerance (CT$_{min}$) of 14 d old hatchling geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity

6.5.3 Effects of incubation temperature on heat hardening

Hot-incubated hatchlings also demonstrated reduced heat hardening compared with current-incubated hatchlings (0.47 °C *versus* 0.79 °C; $t_{29.58} = 3.41$, P = 0.002; Figure 6-4). Random factors in the model were associated with very little (location: variance ± SD = 0.01 ± 0.09) to none (mother identity within location) of the variation in $\Delta\ CT_{max}$. We found no significant correlation between initial CT_{max} and $\Delta\ CT_{max}$ (Pearson correlation r = -0.54, P = 0.77).

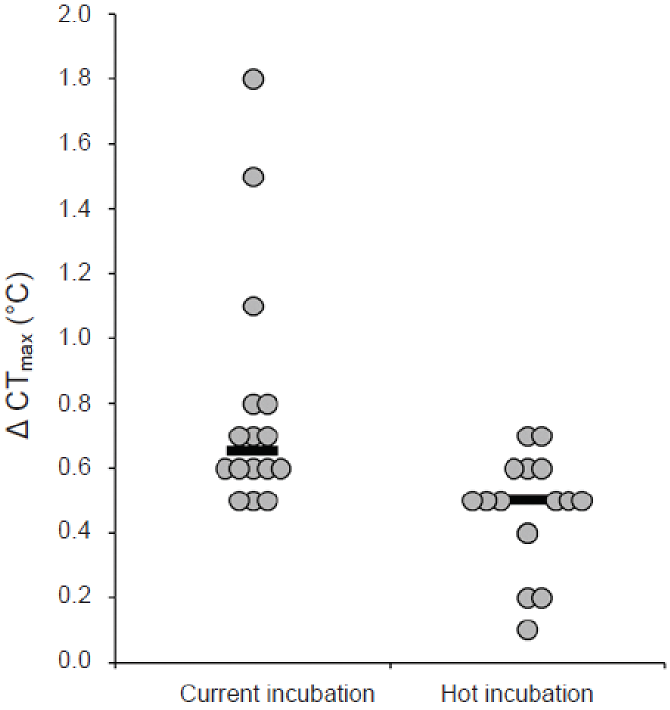

Figure 6-4 Heat hardening dot plots for six week old hatchling velvet geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity.

6.5.4 Persistence of incubation induced shifts in thermal tolerance

At age six weeks, there was no difference in the CT_{max} of hot-incubated or current-incubated lizards (40.02 °C *versus* 39.88 °C; $t_{46.92} = 1.51$, $P = 0.14$), but current-incubated lizards had significantly lower CT_{min} (11.28 °C) than hot-incubated lizards (14.58 °C) ($t_{46.48} = 30.22$, $P < 0.0001$, Figure 6-5 A). In mid-July, we systematically searched our field sites for individually marked lizards. We recaptured three current-incubated lizards from Nowra, and seven current-incubated and five hot-incubated lizards from Dharawal. Given the low sample size for Nowra, we could only analyse data for lizards from Dharawal. For these juveniles, we found no significant difference in CT_{max} of current-incubated (mean = 39.9 ± 0.21 °C [SD]) or hot-incubated (mean = 40.2 ± 0.21 °C [SD]) lizards at age six months (ANOVA: $F_{1, 10} = 3.5$, $P = 0.09$). Likewise, incubation treatment did not influence heat hardening capacity of current-incubated (mean = 0.51 ± 0.146 °C [SD]) or hot-incubated lizards (mean = 0.30 ± 0.152 °C [SD]; ANOVA: $F_{1, 10} = 0.09$, $P = 0.07$). However, current-incubated lizards had lower CT_{min} (mean =10.24 ± 0.22 °C [SD]) than hot-incubated lizards (mean = 12.56 ± 0.46 °C [SD], $F_{1, 10} =144.47$, $P <0.0001$, Figure 6-5 B).

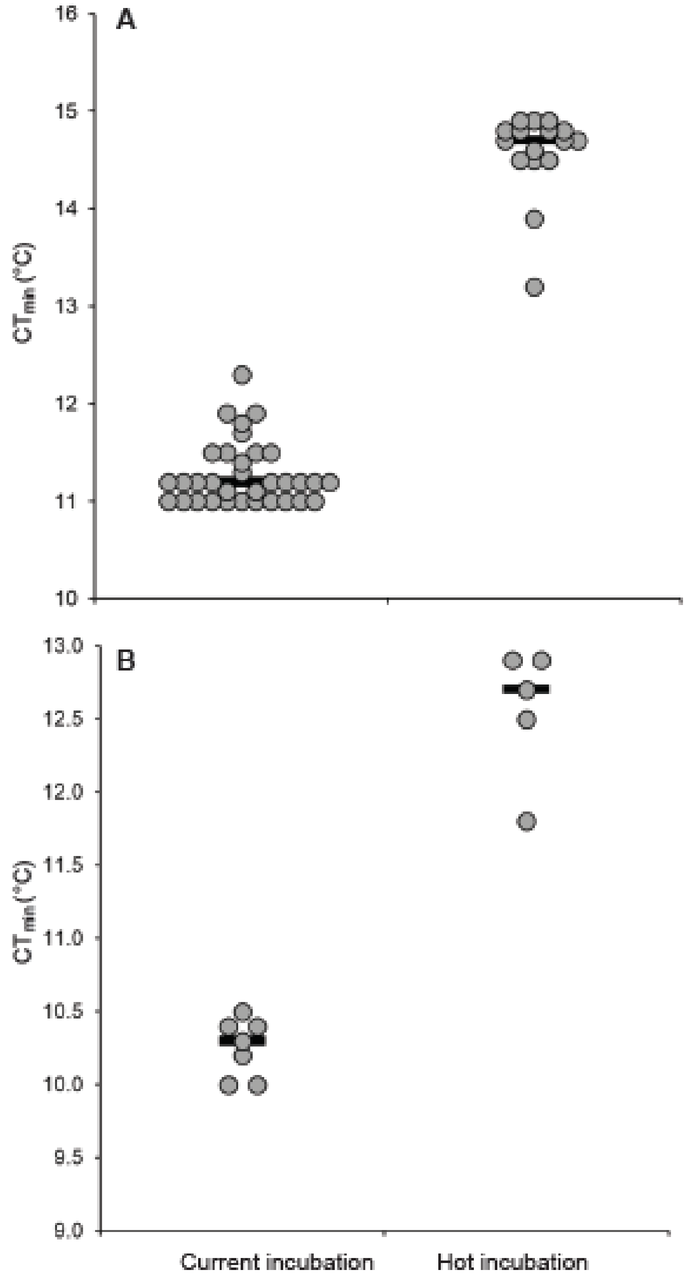

Figure 6-5 Dot plots of the critical thermal minima (CT_{min}) of (A) 6 week old and (B) 6 month old hatchling geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity.

6.6 Discussion

Developmental plasticity coupled with short-term heat hardening could potentially buffer lizards from the effects of summer heatwaves. In this study, we subjected developing embryos of the velvet gecko to thermal profiles that mimicked temperatures of currently used natural nests (current incubation treatment), and temperatures likely to occur during summer heatwaves in 2050 (hot incubation treatment). We found that hot-incubated hatchlings had significantly higher CT_{max} than current-incubated hatchlings, demonstrating that exposure to higher developmental temperatures shifted thermal tolerance upwards. This finding agrees with results from studies on *Drosophila* which found that flies reared at higher temperatures had higher heat tolerance than flies reared at lower temperatures (Hoffmann et al., 2003, Heerwaarden et al., 2016, Slotsbo et al., 2016). However, in the *Drosophila* studies, developmental plasticity resulted in increases in heat tolerance of up to 1 °C. By contrast, developmental shifts in heat tolerance in velvet geckos were small, and thus, may confer little benefit to individuals.

Nonetheless, six week old geckos exhibited clear heat hardening responses 4 hours after exposure to high temperatures, with some individuals increasing their heat tolerance by up to 1.8 °C (Figure 6-4). Current-incubated geckos had significantly higher hardening capacity (mean = 0.79 ± 0.09 °C) than hot-incubated geckos (mean = 0.47 ± 0.04 °C). To date, few studies have measured heat hardening in lizards (Llewelyn et al., 2018, Phillips et al., 2016). In the tropical sun skink *Lampropholis coggeri,* the average hardening capacity was 0.42 °C, with some individuals displaying upward shifts in heat tolerance of 2.6 °C (Phillips et al., 2016). These authors also found an inverse relationship between initial CT_{max} and heat hardening, whereby skinks with higher initial heat tolerance had a lower heat hardening response than skinks with lower initial heat tolerance. This negative correlation between heat tolerance and heat hardening has been recorded for other ectotherms, including *Drosophila* (Sørensen et al., 2001, Zatsepina et al., 2001, Berrigan and Hoffmann, 1998) and porcelain crabs (Stillman, 2003). By contrast, we found no relationship between the initial CT_{max} and hardening in 6 week old hatchlings. Nonetheless, the magnitude of the heat hardening response that we observed in velvet geckos is very similar to that reported for skinks, and suggests that like skinks, geckos have limited ability to shift their CT_{max} upwards (Phillips et al., 2016).

Interestingly, incubation under higher temperatures resulted in a significant upward shift in cold tolerance of hatchlings (Figure 6-3B); at age two weeks, the CT_{min} of hot-incubated hatchlings was 3.3 °C higher than the CT_{min} of current-incubated hatchlings. This finding mirrors the results of experimental studies on *Drosophila*. For example, in *D. melanogaster*, flies which developed at 15 °C had a 4 °C lower CT_{min} than flies which developed at 25 °C (Slotsbo et al., 2016). Similar patterns have been reported for other species of *Drosophila* reviewed in (Hoffmann et al., 2003). While there are fewer comparable studies on lizards, a recent study on the rainforest sunskink, *Lampropholis coggeri* found that hatchlings from cool incubation (constant 23 °C) had significantly lower CT_{min} at age 1 month than hatchlings from warm (constant 26 °C) incubation (Llewelyn et al., 2018). One question that arises from our study is whether the shift in cold tolerance was triggered by differences in the mean, variance, or maximum temperature, since minimum temperatures in each treatment differed by only 0.7 °C. In other organisms, both mean and variance in developmental temperatures can contribute to differences in cold tolerance. For example, a study on *D. melanogaster* reared flies under a warm constant environment (25 °C), a warm variable environment (25 ± 4 °C [SD]), and a cool variable environment (18 ± 4 °C [SD]). Heat tolerance of flies was unaffected by developmental temperatures, whereas chill coma recovery was longest for warm constant flies and shortest for cold variable flies (Cooper et al., 2012). However, additional studies are necessary to estimate the generality of these patterns, and to elucidate the molecular pathways underpinning changes in cold tolerance.

Theoretically, developmental plasticity should result in traits that are irreversible, or at least, longer lasting than those induced via short-term heat hardening or acclimation (Piersma and Drent, 2003). To date, only one previous study on lizards has examined whether developmental plasticity for thermal tolerance persists into later life (Llewelyn et al., 2018). In a study on rainforest sunskinks, egg incubation temperature had a significant effect on the CT_{min} of hatchlings, but this difference was absent when the individuals were retested as adults (Llewelyn et al., 2018). In our study, developmental plasticity for heat tolerance was short-lived; when we retested hatchlings after six weeks, there was no difference in the CT_{max} of lizards from the two incubation treatments. By contrast, developmental plasticity for cold tolerance persisted into later life, and was still apparent after six months in the juveniles that we recaptured from our field sites. Although lizards from both incubation treatments displayed acclimation to field conditions, and shifted cold tolerance downwards, CT_{min} was still 2.32 °C lower, on average, in lizards from the current-incubation treatment (Figure 6-5). This pattern agrees with the results from similar studies on insects, which have found that developmental plasticity for cold tolerance is only partly reversible. For example, a study on *D. melanogaster* found that flies reared at 25 °C and acclimated to 15 °C as adults were able to shift their cold tolerance downwards, but still had a higher CT_{min} than 15 °C reared flies after 24 days (Slotsbo et al., 2016). However, the results for the persistence of cold tolerance may be affected with “missing fraction”. The hatchlings with some traits may not be recaptured because they die. So that, there may be a bias of collecting CT_{min} data with the hatchlings that recovered with some phenotypic traits.

The ecological consequences of developmental shifts in thermal tolerance remains poorly studied, and further research is needed to assess likely effects on survival and demography. In this study hot-incubated eggs hatched, on average, 26 days earlier than current-incubated eggs. Thus, if nest temperatures increase in future, hatchlings will be hatch during mid-summer, when temperatures on rock outcrops can be lethally high during heatwaves (Dayananda et al., 2016). Whether the small developmentally induced shifts in CT_{max} and heat hardening that we observed in the laboratory could buffer hatchlings from higher environmental temperatures requires further study. Notably, the developmental shift in CT_{max} was transient, and may therefore have little effect on survival or activity budgets. Furthermore, in most lizard species studied to date, increases in incubation temperatures tend to produce smaller hatchlings (While et al., 2018), a pattern that we also observed in this study. Therefore, developmental shifts in heat tolerance may not outweigh potential survival costs associated with a smaller body size (Qualls and Andrews, 1999, Dayananda et al., 2017, Andrews et al., 2000). Given that developmental shifts in cold tolerance were less reversible than heat tolerance, it is possible that increases in nest temperatures may produce lizards less able to cope with cold winter temperatures. For example, a study on *Anolis cristatellus* found significant downward shifts in CT_{min} between introduced and source populations, suggesting that selection has acted on this trait in natural populations (Leal and Gunderson, 2012). In our study species, winter rock temperatures routinely fall to 2.5 °C in Nowra and 3 °C in Dharawal (Webb, unpublished data), so that lizards with lower cold tolerance may be more likely to survive cold snaps, or could have enhanced activity levels during winter.

Future studies examining links between cold tolerance, heat tolerance and survival would help evaluate the demographic consequences of developmentally induced shifts in thermal tolerance.

In conclusion, we used a fluctuating temperature incubation experiment to examine the potential for developmental plasticity to produce upward shifts in the heat tolerance of hatchling velvet geckos. After maintaining hatchlings under identical conditions for six weeks, we found that the small increase in heat tolerance acquired from hot-temperature incubation was short-lived. Importantly, heat hardening capacity was greater in current-incubated than hot-incubated lizards, so that at six weeks of age, the capacity to withstand high temperatures was similar in both treatment groups. Strikingly, developmental shifts in cold tolerance were not reversible, and although both hot and current-incubated hatchlings showed similar acclimation responses in the field, six month old current-incubated lizards still had lower cold tolerance than hot-incubated lizards. Overall, our results add to the mounting body of evidence suggesting that there is little scope for developmental plasticity to buffer lizards from climate warming.

6.7 Acknowledgements

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Competing interests

The authors declare no competing or financial interests

Author contributions

TA and JKW conceived and designed the experiments, and collected geckos; TA maintained the gecko colony, incubated eggs, and carried out all of the experiments on hatchlings; BM analysed the data and drew figures; all authors contributed to the writing and revision of the manuscript.

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Data Availability

Data files (Microsoft Excel) are available in the Supplementary Information.

Figure captions

Fig.6.1. Temperature profiles experienced by velvet gecko eggs in the (A) current and (B) hot incubation treatments. Both incubators were programmed to mimic the cycling temperatures that occur inside natural gecko nests, with intermittent summer heatwaves followed by cooler weather. The current treatment mimicked temperatures recorded inside sun-exposed communal nests, while the hot treatment mimicked temperatures that might occur in 2050 under climate warming.

Fig. 6.2. Heat hardening (delta CT_{max}) as a function of the number of hours after the first exposure to the high temperature that resulted in a loss of righting response in hatchling velvet geckos.

Fig. 6.3. Dot plots of (A) maximum critical thermal tolerance (CT_{max}) and (B) minimum critical thermal tolerance (CT_{min}) of 14 d old hatchling geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity.

Fig. 6.4. Heat hardening dot plots for six week old hatchling velvet geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity.

Fig. 6.5. Dot plots of the critical thermal minima (CT_{min}) of (A) 6 week old and (B) 6 month old hatchling geckos from current and hot incubation treatments. Black lines show medians and repeat values are jittered for clarity.

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Chapter 7 Effects of incubation temperatures on learning abilities of hatchling velvet geckos

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Conflict of interest

Theja Abayarathna and Jonathan Webb declare that they have no conflict of interest

Key words: reptile cognition, developmental plasticity, heatwave, Y-maze, learning

7.1 Abstract

Stressful environments in utero can have a profound influence on cognitive functions and learning ability. In lizards, thermal environments experienced by embryos can shape a range of traits, including sex, body size, and locomotor performance, which may influence fitness. Recent studies suggest that incubation temperatures may also influence brain development and learning ability of some lizard species. Therefore, predicted increases in nest temperatures of lizards may not only affect hatchling morphology and performance, but could also affect their learning ability. To assess how incubation temperatures influence cognitive abilities of hatchlings, we incubated eggs of the velvet gecko *Amalosia lesueurii* under two fluctuating temperature regimes to mimic thermal profiles of currently used sun-exposed communal nests (warm: mean = 24.3 °C, range 18.4–31.1 °C) and thermal profiles likely to be experienced in 2050 (hot: mean = 28.9 °C, range 19.1–38.1 °C). At age three to four weeks, we measured the ability of hatchlings to locate an open shelter in a Y-maze choice test. Both hot and warm-incubated hatchlings successfully learned the task, but hatchlings from the warm-temperature treatment learned the task faster, and made more successful escapes than hot-incubated hatchlings. These patterns were consistent for hatchlings from two geographic locations, suggesting that thermally stressful conditions *in utero* may alter the learning abilities of hatchling lizards. Because learning ability can affect the survival of hatchling velvet geckos, future increase in nest temperatures may have wide reaching impacts on populations.

7.2 Introduction

Cognition, the process by which animals acquire, process, store and recall information in order to make behavioural decisions (Shettleworth, 2001, Rowe and Healy, 2014), is an important trait that can affect how individuals cope with novel or changing environments (Sol et al., 2008, Sol et al., 2005, Wright et al., 2010). In the face of rapid change, the ability to rapidly learn new behaviours may enable individuals to avoid novel predators, track shifting resources, exploit novel foods, and cope with complex environments (Dukas, 2004). Thus, individuals with enhanced cognitive abilities are predicted to have higher fitness, although demonstrating this linkage in wild populations is difficult (Morand-Ferron et al., 2016).

While flexible behaviours may help individuals to cope with environmental challenges, changing environments can in turn modify cognitive abilities via developmental plasticity, and such shifts are not always adaptive (Chevin et al., 2010, West-Eberhardt, 2003). For example, stressful conditions experienced *in utero* can have detrimental effects on brain development, memory, spatial learning, and cognitive function in a wide range of species (Markham and Koenig, 2011, McEwen et al., 2012, Schoech et al., 2011). While most of this research has focused primarily on mammals, there is a growing body of evidence that environments experienced by early life stages of ectotherms can influence the cognitive abilities of later life stages (Groh et al., 2004, Tautz et al., 2003, Arenas and Farina, 2008). Given the rapid pace of current anthropogenic change, an understanding of how changes in developmental environments will effect cognitive traits of ectotherms, and the ecological consequences of such changes, may enable more accurate forecasts of species persistence under future warming (Carlo et al., 2018, Levy et al., 2015) .

Lizards are excellent model organisms for investigating how developmental environments influence the cognitive abilities of offspring (Noble et al., 2018). Most lizards lay eggs inside shallow nests, and embryos may experience thermal spikes during summer heatwaves (Hall and Warner, 2018, Shine and Elphick, 2001). In years with prolonged and more intense heatwaves, it is likely that the nests of some lizard species will experience higher-than-average temperatures (Angilletta et al., 2013, Telemeco et al., 2017). While there is a wealth of studies demonstrating that incubation temperatures can have strong and often persistent effects on the morphology, behaviour, and locomotor performance of neonatal reptiles (Deeming and Ferguson, 1991, Deeming, 2004, While et al., 2018, Noble et al., 2018), relatively few studies have examined how incubation temperature affects learning behaviour. Thus, we lack a robust understanding of how changes in nest temperatures will affect the cognitive abilities of lizards (Clark et al., 2014).

Several recent studies on lizards have begun to address this knowledge gap. A study on hatchling three-lined skinks (*Bassiana duperreyi*) found that lizards from warm-temperature incubation (diel cycle of 22 ± 7.5 °C) made fewer mistakes in a task where they had to locate an open shelter whilst being chased than lizards from cold-temperature (16 ± 7.5 °C) incubation (Amiel and Shine, 2012). In a follow up study, in which the skinks had to locate a food reward in a Y-maze equipped with local cues, lizards from hot-temperature incubation (24 ± 7.5 °C) were faster learners, and made fewer mistakes, than lizards from cold-temperature incubation (Amiel et al., 2014). Using the same incubation treatments, researchers also found that only the hot incubated skinks could complete an elementary learning task (Clark et al., 2014). In bearded dragons, egg incubation temperatures affected the social learning ability of adults. After watching a demonstrator open a door, lizards from a cold-temperature incubation treatment (27 ± 3.0

°C) opened the door faster than lizards from a hot-temperature (30 ± 3.0 °C) treatment (Siviter et al., 2017). In a recent study on velvet geckos *Amalosia lesueurii*, hatchlings were chased with a paintbrush until they located an open shelter. Hatchlings from hot-temperature incubation (27 °C, range 14–37 °C) made more mistakes, and were slower learners, than cold-incubated (23.2 °C, range 10–33 °C) hatchlings (Dayananda and Webb, 2017).

While the above studies demonstrate that lizards are adept learners, the discordant effects of incubation temperature on lizard learning behaviour (hotter temperatures produce 'better' learners in some species, but not in others) hampers our ability to predict how higher nest temperatures may influence the cognitive abilities of hatchlings. Furthermore, most studies have used individuals from a single population, so it is unclear whether the observed patterns necessarily translate to other populations within the species' geographic ranges. To address these knowledge gaps, we investigated whether incubation temperatures affect the cognitive abilities of hatchling velvet geckos *Amalosia lesueurii* from two populations; one from Nowra, near the most southern part of its geographic range in New South Wales, and another from Dharawal National Park, 100 km further north. To facilitate comparisons with previous studies on lizards, we scored the learning ability of hatchlings in a Y-maze, a well-established method in both the animal behaviour and psychological literature (Burghardt, 1977, Macphail, 1982, Wilkinson and Huber, 2012).

7.3 Materials and methods

7.3.1 Collection and maintenance of pregnant females

We collected gravid female velvet geckos from two study sites located near Nowra, approximately 170 km south of Sydney, and Dharawal National Park, approximately 70 km south of Sydney, in late spring 2016. Females were transported to the University of Technology Sydney and were housed inside standard plastic cages (Sistema NZ 2.0 L, 220 × 150 × 60 mm, with ventilated lid) in a constant temperature room (23 °C) with a 12:12 light cycle. Each cage contained a white plastic half-pipe shelter (80 mm × 40 mm) and a water dish, with a layer of moist vermiculite to prevent eggs from desiccating. Cages were placed on timer-controlled heating cables set to 32 °C, which created a thermal gradient (23–32 °C) inside the cages during daylight hours, falling to 23 °C at night. All geckos had a constant supply of drinking water and were fed crickets twice weekly. Each morning and afternoon, one of us (TA) checked all the cages for newly oviposited eggs, and sprayed the vermiculite to maintain a moist substrate. After females laid eggs, we recorded their body mass, and released them at their exact site of capture during suitable weather conditions.

7.3.2 Egg incubation experiment

On the day of egg laying, one of us (TA) recorded the mass of each egg (to nearest 0.01 g) and measured the maximum length and width of each (to nearest 0.01 mm) with Vernier callipers. Each egg was placed singly inside a 100 mL glass jar filled with moist vermiculite (water potential of 200 KPa), and was covered with plastic food wrap to reduce water loss. Nearly all females laid two eggs, so we placed one egg from each clutch into the 'warm' incubator, and the other into the 'hot' incubator (Panasonic MIR

154, 10 step functions). Both incubators were programmed to mimic the cycling temperatures seen inside natural nests at our study sites, with intermittent heatwaves. Temperature cycles in the warm treatment mimicked those recorded inside currently used sun-exposed communal nests (mean = 24.3 °C, range 18.4 – 31.1 °C), while thermal cycles of the hot treatment (mean = 28.9 °C, range 19.1 °C – 38.1 °C) mimicked the potential future nest temperatures that might occur in 2050 under climate warming (Dowdy et al., 2015). We incubated 84 eggs in the warm incubation treatment and 81 eggs in the hot incubation treatment.

7.3.3 Maintenance of hatchling geckos

Once hatchlings emerged, one of us (TA) measured their snout-vent length (SVL), tail length (TL) with a ruler (to nearest mm), and body mass with an electronic scale (to nearest 0.01 g). Each hatchling was housed individually as described above for females, except that the cages were lined with a paper substrate and lacked vermiculite. We fed hatchlings with five pinhead crickets twice weekly, and cleaned their cages at weekly intervals.

7.3.4 Learning task

We tested the learning ability of 16 hot-incubated hatchlings (Dharawal n = 10, Nowra n = 6) and 33 warm-incubated hatchlings (Dharawal = 13, Nowra n = 20). Hatchlings were tested at age three to four weeks (mean age = 27 d ± 7.2 SD) from 14 February to 24 March 2017. We used a Y maze constructed of opaque plastic arms (33 × 10 × 5 cm) to test the lizard's ability to learn the location of an open shelter during a simulated predatory attack (by a paintbrush). We used this approach to ensure that lizards were sufficiently motivated to learn the task (Burghardt, 1977). Each arm contained an identical shelter constructed of a white PVC half pipe (65 x 55 x 25 mm, Figure 7-1). One shelter was closed, while the other was open; both shelters were positioned such that lizards could not see the entrance to the shelter until they had reached it. Because lizards may use position or local cues to navigate (Day et al., 1999), we provided local visual cues by placing coloured paper flooring inside each arm. Geckos have excellent colour vision, but lack the ability to see red; thus, the floor colours were either solid blue, or blue with orange stripes. To control for colour and positional biases, we randomly allocated geckos into four groups, and each group had a different position and colour combination (Table 7-1). These combinations remained constant for each individual throughout the trials. Lizards also had access to external landmark cues (objects in the room) which remained constant throughout the trials. While lizards could have used any combination of cues to locate the open shelter, we were not interested in which cues they uses; rather, we were interested whether incubation temperature affected the ability of lizards to locate a novel shelter in a biologically relevant task that simulated a predatory attack. In this situation, lizards that failed to learn the location of the ‘safe’ refuge would be exposed to predators, and thus, more likely to be killed.

We ran two trials per day for five consecutive days for each hatchling (total of 10 trials). To begin the test we placed the hatchling at the base of the empty arm under a plastic cover. One of us (TA) then removed the cover, and gently tapped the lizard on the tail with a paintbrush to simulate a predatory attack. This was repeated whenever the hatchling stopped running. Hatchlings that could not locate the correct shelter within 2 minutes were directed to the correct shelter with the paint brush, and were allowed to hide under the shelter for 30 seconds before they were removed from the maze. For each trial, we recorded: (1) time taken to reach the open shelter; (2) number of mistakes (times that an incorrect arm was chosen). To facilitate comparisons with previous studies, hatchlings that located the correct shelter within 30 s were considered to have made a successful escape (Amiel and Shine, 2012). We summed the total number of successful escapes over the 10 trials to calculate a learning score for each individual. After completing the trials we released the hatchlings to the sites where their mothers captured.

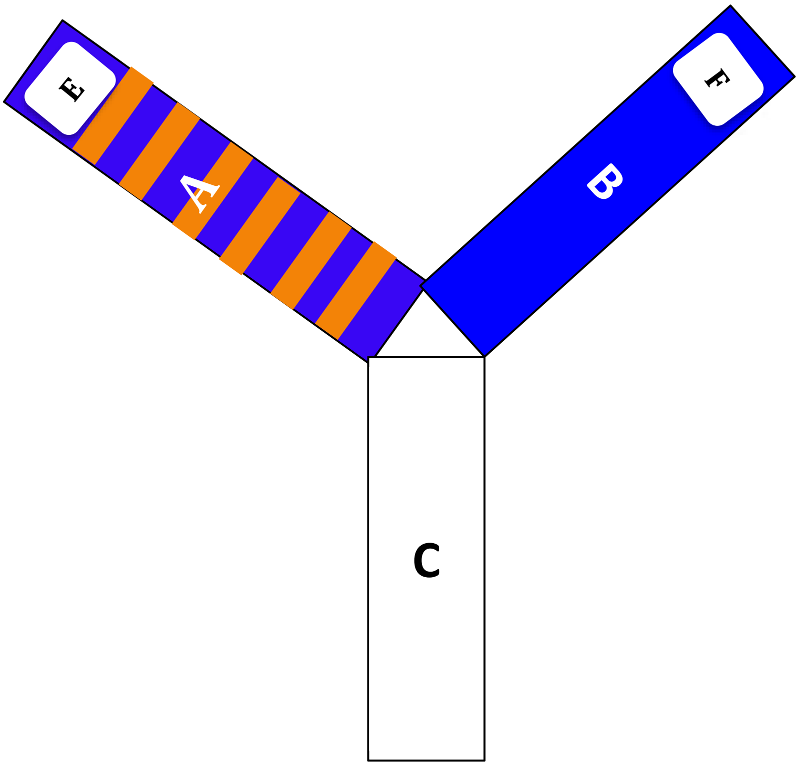

Figure 7-1 Y-maze used for the learning trials of hatchling geckos. The position of the open shelter (E), and closed shelter (F), and the coloured floor of the two arms, remained constant for each individual throughout the trials. Each gecko was allocated to one of four groups, and each group had a different combination for the position of the open shelter (left or right), or solid blue floor (left or right), as explained in Table 1.

Table 7-1 Position of the open and closed shelters and the composition of the colour for the base allocated for four different groups of hatchlings.

	Open shelter	Closed shelter	Striped floor	Solid floor
Group 1	Left	Right	Left	Right
Group 2	Right	Left	Left	Right
Group 3	Left	Right	Right	Left
Group 4	Right	Left	Right	Left

7.3.5 Statistical analysis

To determine if local cues (colour pattern and directional combinations, Table 7-1) influenced learning ability, we first analysed the data with one-way ANOVA, with group as the factor, and time to complete the task and the number of mistakes as the dependent variables. We then carried out a two factor repeated measures ANOVAs to test whether incubation treatment and the location of origin influenced the time to reach the correct shelter and the number of mistakes. The number of successful escapes was analysed using a two factor ANOVA, with incubation treatment and location as fixed factors. Prior to statistical analysis, we checked the data to see whether the assumptions of normality and heterogeneity of variance were met.

7.4 Results

Local cues (Table 7-1) did not influence the time taken to locate the correct shelter ($F_{3,45}$ = 0.82, p = 0.49) or the number of mistakes ($F_{3,45}$ = 0.50, p = 0.68). We then examined the effects of incubation temperature and location on the time taken to complete the task using a repeated measures ANOVA. For this analysis, Mauchley's test indicated that the assumption of sphericity was violated ($\chi^2_{(44)}$ =116.02, p <0.001), so the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon = 0.6$). The analysis revealed a significant effect of time ($F_{5.4,\ 199.2} = 33.82$, $p < 0.0001$), and incubation treatment ($F_{1,37} = 4.828$, $p = 0.034$), but no effect of location ($F_{1,37} = 0.455$, $p = 0.504$, interaction $F_{1,37} = 2.891$, $p = 0.097$). These results showed that hatchlings learned the task, but hatchlings from the warm-incubation treatment learned faster than hatchlings from the hot-incubation treatment (Figure 7-2). Warm-incubated hatchlings also made more successful escapes (mean = 7.0 ± 1.46) than hot-incubated hatchlings (mean = 5.25 ± 1.0, two factor ANOVA: incubation $F_{1,45} = 17.982$, $p < 0.01$, location $F_{1,45} = 0.222$, $p = 0.640$, interaction $F_{1,45} = 0.22$, $p = 0.64$).

We then analysed the number of mistakes made per trial using a two factor repeated measures ANOVA. For this analysis, Mauchley's test indicated that the assumption of sphericity was violated ($\chi^2_{(44)} = 262.31$, $p < 0.001$), so the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\epsilon = 0.47$). The analysis revealed a significant effect of time ($F_{4.2,\ 190.3} = 33.37$, $p < 0.0001$), but there was no significant effect of incubation treatment ($F_{1,45} = 0.82$, $p = 0.37$), and no effect of location ($F_{1,45} = 0.417$, $p = 0.68$, interaction $F_{1,45} = 1.56$, $p = 0.22$). Plots of mistakes versus trial number showed that hatchlings from both incubation treatments made fewer mistakes over time, again indicating that they learned the task (Figure 7-3).

To further explore the effect of incubation treatment on learning ability, we plotted the total time from all 10 trials versus the total number of mistakes. There was a significant positive linear relationship between the number of mistakes and time ($r = 0.72$, $n = 49$, $p < 0.0001$); as might be expected, geckos that made more mistakes took longer to complete the task. An analysis of covariance (ANCOVA), with the number of mistakes as a covariate, and time as the dependent variable, showed that the slopes of the relationship between mistakes and time were similar across incubation treatments ($F_{1, 45} = 0.30$, $p = 0.59$; i.e. slopes were parallel). However, when the number of mistakes was held constant, the warm-incubated hatchlings completed the task faster than the hot-incubated hatchlings ($F_{1, 46} = 12.45$, $p = 0.001$, Figure 7-4).

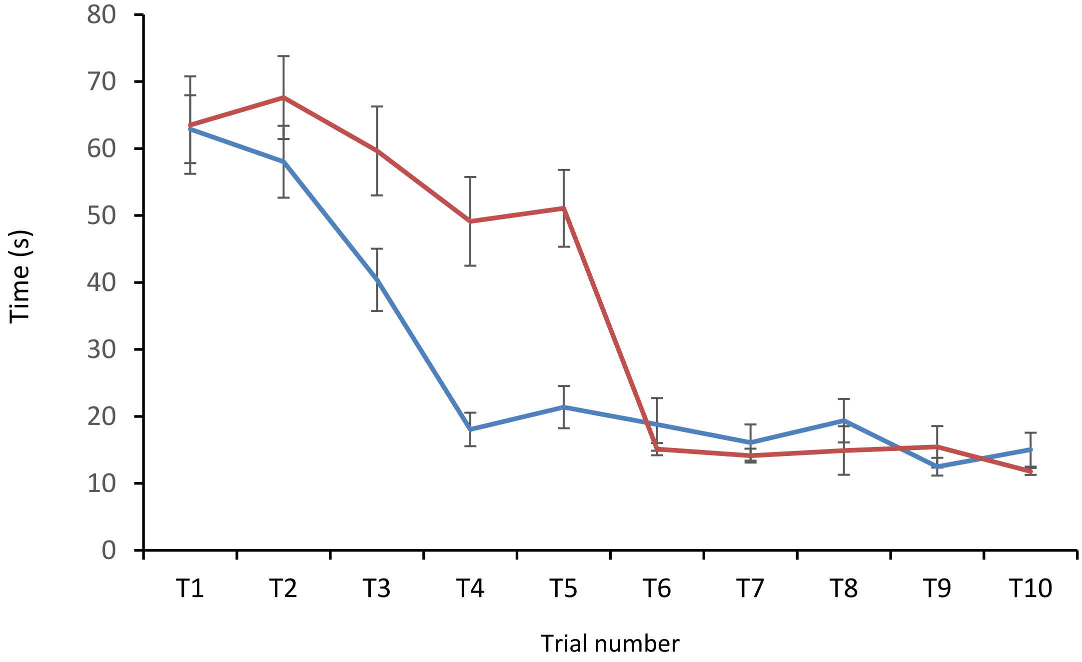

Figure 7-2 Mean time taken by warm-incubated (blue line) and hot-incubated (orange line) hatchling velvet geckos to enter the open shelter in each of 10 trials in a Y-maze learning task. Error bars denote the standard errors.

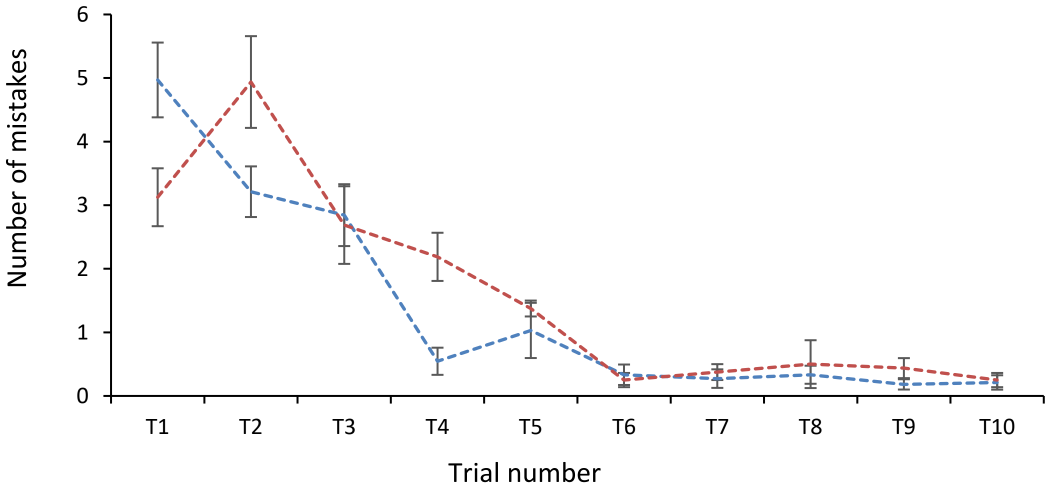

Figure 7-3 Mean number of mistakes made by warm-incubated (blue line) and hot-incubated (orange line) hatchling velvet geckos in each of 10 trials in a Y-maze learning task. Error bars denote the standard errors.

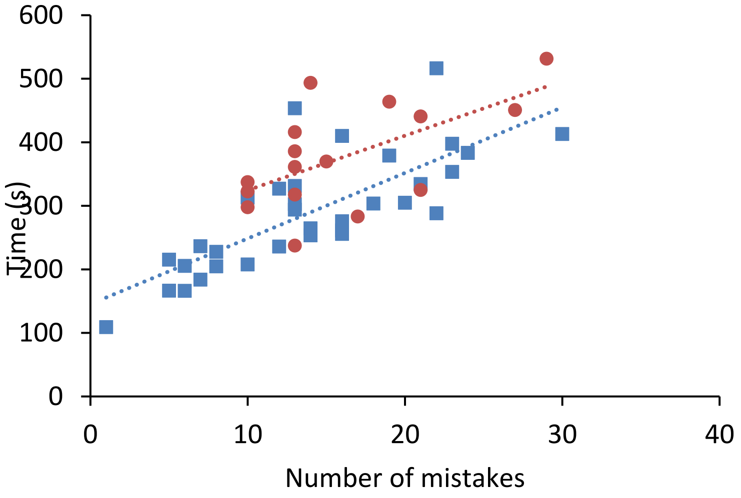

Figure 7-4 Relationship between the number of mistakes and time to complete the learning task (data summed for 10 trials) for warm-incubated (blue squares) and hot-incubated (orange circles) hatchling velvet geckos. Each individual is represented by a single data point.

7.5 Discussion

Heatwaves are a common occurrence in Australia, but their intensity and duration is predicted to increase in coming decades (Cowan et al., 2014, Anderson et al., 2018, Kang and Eltahir, 2018) and will likely elevate temperatures inside nest of many lizards, including our study species (Dayananda and Webb, 2017). To understand how increases in incubation temperature may affect the cognitive abilities of hatchling velvet geckos we measured their ability to locate a safe refuge in a Y-maze. Warm-incubated hatchlings learned to locate an open refuge in a Y maze faster, and made more successful escapes, than did hot-incubated hatchlings. These results confirm that higher incubation temperatures produce geckos with poorer learning abilities. Importantly, our results extend on previous work by showing that this pattern was consistent for two populations: one from the southern edge of the species' geographic range (near Nowra), and another approximately 100 km north (Dharawal).

Collectively, our results, and those of a previous study, provide strong evidence that above-average incubation temperatures produce smaller hatchlings with poorer cognitive abilities (Dayananda and Webb, 2017). By contrast, the work on three-lined skinks found that lizards from hot-temperature incubation were better learners (Amiel et al., 2014, Amiel and Shine, 2012, Clark et al., 2014). However, closer examination shows that the 'hot' temperatures used in those experiments (22 ± 7.5 °C and 24 ± 5 °C) were similar to temperatures of our warm treatment (24.3 °C, range 18.4 – 31.1 °C). Thus, the 'hot' temperatures of the three lined skink studies were within the natural range of nest temperatures, and are unlikely to be thermally stressful to embryos. It may be that reaction norms for cognitive functions are not linear, but rather, may show inverse U-shaped curves with temperature (Noble et al., 2018). To clarify the generality of our findings, additional studies on lizards, using higher than normal incubation temperatures, are necessary.

What mechanisms might explain the differences in learning ability in hatchlings from the two incubation treatments? In vertebrates, a large proportion of brain development occurs during embryogenesis, making it highly susceptible to changes in the incubation environment (Purves and Lichtman, 1985). In reptiles, incubation temperature may affect several biochemical pathways that influence brain development. In leopard geckos, a species with temperature dependent sex determination (TSD), incubation temperatures directly affected the volumes of the preoptic area and ventromedial nucleus of the hypothalamus, while an individual's sex influenced their metabolic capacity (Coomber et al., 1997, Crews et al., 1997, Sakata et al., 2000). Follow up work on this species showed that incubation temperatures induced differential expression of several genes involved in neural and gonadal differentiation (Pallotta et al., 2017). In three-lined skinks (*B. duperreyi*) lizards from a cold incubation treatment (18 ± 5 °C) had larger telencephalons than lizards from a hot incubation treatment (24 ± 5 °C) irrespective to the body size, while incubation temperature also produced stark differences in neuronal density in the medial cortices (Amiel et al., 2017). While the molecular pathways responsible for these incubation-induced changes in brain structure in lizards have not been elucidated, it is clear that incubation temperatures can influence the structure and activity of parts of the brain involved in learning and social behaviours (Sakata and Crews, 2004, Sakata et al., 2005).

Although our primary goal was to explore how incubation temperature affected the learning ability of hatchlings, we were also able to explore, to some degree, whether local cues influenced learning. We provided hatchlings with salient local cues consisting of a colour cue and a contrast cue, using blue colours that geckos can see, whilst keeping positional cues constant, and vice versa. Had geckos used positional cues (i.e. remember left, or right), then we would have expected to see differences across groups in terms of mistakes or speed. However, no such effects were evident, but it could be that geckos used distal cues to navigate (since these were constant), or a combination of positional, local, and distal cues. Future studies, using a more elegant design, such as a Morris Water Maze, could help to answer the question of how lizards navigate (LaDage et al., 2012).

In conclusion, our results show that higher incubation temperatures that are likely to occur inside natural nests during prolonged summer heatwaves (Dayananda et al., 2016), produced poorer learners. While we do not know whether this incubation-induced effect is long-lasting, a previous mark-recapture study at one of our sites (Dharawal) showed that learning scores of hatchlings obtained in the laboratory were correlated with their subsequent survival in the field during their first year of life (Dayananda and Webb, 2017). However, learning scores encapsulate several skills associated with spatial learning (speed, accuracy, memory), which makes it difficult to assess which aspects of cognition are subject to natural selection (Rowe and Healy, 2014). Although current research on this question has focused mostly on birds and mammals (Morand-Ferron et al., 2016), many lizard species have high recapture rates (While et al., 2018), making them excellent model organisms for clarifying how developmental plasticity shapes cognitive traits, and whether those traits are subject to natural selection in the wild.

Compliance with ethical standards

Funding: The project was supported by a UTS postgraduate research support grant to TA, and a UTS research grant to JKW.

Ethical approval: All applicable international, national and institutional guidelines for the care and use of animals were followed. The procedures described herein were approved by the UTS Animal Care and Ethics Committee (protocol #2012000256 to JKW). The collection of geckos from the study sites was approved by the NSW National Parks and Wildlife Service (scientific licence SL101013 to JKW).

Supplementary Information

Table 1. Raw data for times taken by each gecko in each treatment group to successfully complete the learning task in each of 10 trials in a Y-maze. Cells with '120' indicate that the individual failed to complete the task within two minutes.

ID	INC	LOC	t1	t2	t3	t4	t5	t6	t7	t8	t9	t10
26	W	D	52.3	66.0	9.5	6.8	69.9	7.2	10.2	10.6	12.6	10.7
40	W	D	113.9	48.6	52.9	12.7	7.5	9.6	12.8	15.9	40.5	12.6
22	W	D	54.7	110.1	14.8	12.3	15.8	101.2	29.1	14.0	12.5	88.8
47	W	D	105.0	84.1	53.2	19.3	6.3	72.9	12.6	8.8	12.3	9.1
42	W	D	88.3	58.3	56.9	15.6	14.3	6.2	9.9	11.2	9.2	32.8
21	W	D	108.5	84.1	48.8	16.8	12.1	84.7	6.3	12.9	29.3	6.5
30	W	D	120.0	54.8	34.8	9.2	12.8	18.3	12.9	89.8	11.4	15.0
50	W	D	53.8	16.2	17.7	6.2	11.3	9.3	16.4	54.9	6.9	12.8
46	W	D	48.5	48.3	45.3	16.5	12.9	16.1	15.3	12.8	9.5	11.3
48	W	D	111.0	33.5	25.4	12.5	14.2	25.1	10.5	16.0	6.5	9.8
12	W	D	18.5	62.9	12.7	14.3	9.5	7.8	9.5	11.8	12.4	6.9
13	W	D	120.0	66.1	16.9	18.4	15.4	14.5	15.1	5.1	10.9	11.7
9	W	D	97.5	108.5	15.4	16.6	14.8	10.6	12.4	14.1	12.9	10.2
74	W	N	82.4	18.9	14.6	40.5	20.5	12.3	12.2	12.7	6.5	6.9
77	W	N	14.3	12.6	9.0	10.6	7.6	11.9	10.4	11.5	10.0	11.3
84	W	N	17.0	65.3	9.5	12.3	14.8	10.0	9.2	18.9	12.2	14.7
71	W	N	68.5	22.5	84.5	11.3	14.4	10.6	10.4	11.8	7.9	11.6
73	W	N	45.2	15.4	17.5	20.8	11.9	12.1	15.3	6.7	9.4	12.3
81	W	N	38.5	15.9	98.8	9.6	9.6	6.2	6.9	10.9	6.5	12.5
63	W	N	54.6	25.1	48.9	12.5	36.3	12.3	14.6	10.7	6.6	14.3
56	W	N	82.5	24.3	54.8	18.3	12.3	10.9	12.7	37.0	11.0	11.9
60	W	N	23.5	72.2	36.6	9.5	15.2	11.3	8.3	11.5	9.2	10.3
80	W	N	120.0	50.5	64.5	66.0	19.9	15.8	32.5	19.6	9.9	14.4
83	W	N	25.6	21.0	120.0	7.6	58.6	10.3	8.2	17.5	12.5	6.8
52	W	N	83.8	36.6	48.7	12.5	42.5	6.4	85.4	14.3	12.7	10.5
72	W	N	108.3	83.2	48.8	36.9	13.0	7.9	6.2	9.6	7.3	10.2
59	W	N	113.8	108.6	116.9	52.8	12.7	8.2	9.9	59.8	8.3	25.5
62	W	N	6.3	84.0	47.6	6.3	9.5	9.9	11.9	6.9	10.5	11.9
61	W	N	52.4	85.6	40.6	12.4	14.9	12.1	10.9	7.3	14.5	10.6
53	W	N	56.9	72.8	60.9	14.9	36.6	11.9	15.8	12.7	10.5	11.9
66	W	N	43.2	77.9	53.3	48.5	11.9	6.3	56.2	53.3	34.3	12.7

86	W	N	18.9	112.0	18.0	5.8	71.6	30.7	9.3	18.3	12.5	6.5
87	W	N	98.9	69.3	14.8	9.8	55.2	20.0	11.9	10.2	12.8	31.3
50 II	H	D	7.0	96.9	114.9	5.7	48.3	15.9	19.2	6.3	12.2	11.0
40	H	D	65.2	60.7	49.7	38.9	32.3	12.2	9.6	10.6	7.9	10.9
22	H	D	16.8	51.6	49.5	25.9	25.7	20.7	15.8	12.2	8.9	10.3
26	H	D	54.7	120.0	103.7	40.1	50.0	16.9	18.5	14.3	9.9	12.5
50	H	D	42.9	71.1	108.9	37.8	108.9	6.9	8.7	10.2	9.7	10.8
21	H	D	97.1	49.0	42.2	36.4	30.7	15.5	9.2	12.9	15.3	9.3
47	H	D	48.6	116.0	120.0	77.6	45.8	13.3	14.3	68.2	14.9	13.0
25	H	D	54.2	44.4	39.1	31.7	35.4	20.8	18.1	12.7	13.7	13.0
17	H	D	103.1	120.0	53.2	44.3	59.7	16.7	15.8	15.3	12.3	10.3
16	H	D	70.7	61.3	37.4	38.2	31.4	18.1	20.5	16.8	17.8	12.8
69	H	N	83.8	62.1	59.3	48.8	53.4	13.0	12.8	9.5	12.3	14.9
84	H	N	109.0	72.2	43.7	89.7	59.8	18.2	18.3	12.3	60.9	9.5
81	H	N	83.8	23.1	36.5	112.1	46.6	15.3	12.3	5.9	11.3	14.3
59	H	N	66.9	75.9	48.2	120.0	97.1	10.5	7.9	12.3	12.4	12.7
67	H	N	75.8	104.3	54.6	54.6	37.6	15.0	10.2	9.9	15.6	8.3
77	H	N	36.5	58.0	53.9	54.9	54.5	12.9	15.3	9.2	12.2	15.2

Table 2. Raw data for the number of mistakes made by each gecko in each treatment group in each of 10 trials carried out in a Y-maze.

ID	INC	LOC	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10
26	W	D	2	4	0	0	10	0	0	0	0	0
40	W	D	6	1	3	0	0	0	0	0	2	0
22	W	D	3	3	0	0	0	3	1	0	0	3
47	W	D	8	7	4	1	0	4	0	0	0	0
42	W	D	5	2	4	0	0	0	0	0	0	2
21	W	D	8	4	2	0	0	1	0	0	1	0
30	W	D	12	3	2	0	0	0	0	2	0	0
50	W	D	3	0	1	0	0	0	0	2	0	0
46	W	D	3	2	2	0	0	0	0	0	0	0
48	W	D	11	2	1	0	0	0	0	0	0	0
12	W	D	0	5	0	0	0	0	0	0	1	0
13	W	D	7	4	1	1	0	0	0	0	0	0
9	W	D	6	4	0	0	0	0	0	0	0	0
74	W	N	6	0	0	2	0	0	0	0	0	0
77	W	N	0	0	0	0	0	0	0	0	1	0
84	W	N	0	7	0	0	0	0	0	0	0	0
71	W	N	8	0	6	0	0	0	0	0	0	0
73	W	N	3	0	1	1	0	0	0	0	0	0
81	W	N	1	0	4	0	0	0	0	0	0	0
63	W	N	6	1	4	0	1	0	0	0	0	0
56	W	N	7	1	5	1	0	0	0	2	0	0
60	W	N	2	6	2	0	0	0	0	0	0	0

80	W	N	11	3	8	6	0	0	2	0	0	0
83	W	N	3	2	11	0	6	0	0	0	0	0
52	W	N	8	4	5	0	2	0	4	0	0	0
72	W	N	5	4	3	1	0	0	0	0	0	0
59	W	N	5	4	6	3	0	0	0	3	0	1
62	W	N	0	4	4	0	0	0	0	0	0	0
61	W	N	5	5	6	0	0	0	0	0	0	0
53	W	N	6	5	7	0	2	0	0	0	0	0
66	W	N	5	6	5	2	0	0	2	2	1	0
86	W	N	0	8	0	0	8	2	0	0	0	0
87	W	N	9	5	0	0	5	1	0	0	0	1
50 II	H	D	0	4	3	0	2	0	1	0	0	0
40	H	D	3	3	2	1	1	0	0	0	0	0
22	H	D	0	7	2	1	1	1	1	0	0	0
26	H	D	3	12	1	2	1	0	1	0	0	1
50	H	D	2	5	2	2	2	0	0	0	0	0
21	H	D	3	4	1	2	1	1	0	0	1	0
47	H	D	2	5	11	3	1	0	0	6	1	0
25	H	D	4	5	2	2	1	1	1	0	0	1
17	H	D	6	11	4	2	2	0	1	1	0	0
16	H	D	5	4	4	2	1	1	1	1	1	1
69	H	N	5	3	3	2	2	0	0	0	0	0
84	H	N	2	3	3	2	2	0	0	0	2	0
81	H	N	6	1	1	3	2	0	0	0	0	0
59	H	N	4	4	1	7	1	0	0	0	1	1
67	H	N	2	5	1	3	1	0	0	0	1	0
77	H	N	3	3	2	1	1	0	0	0	0	0

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Chapter 8 General Discussion

8.1 Introduction

Heatwaves are a common occurrence in Australia (Perkins-Kirkpatrick et al., 2016). Climate modellers have predicted that the frequency and duration of heat waves will increase in the future due to anthropogenic activities (Hansen et al., 2012, Cowan et al., 2014). Heatwaves pose physiological challenges for many animals, and can cause increases in mortality (Jones et al., 2018, Le Nohaic et al., 2017, Welbergen et al., 2008). Lizards populations are also vulnerable to thermal stress (Sinervo et al., 2010). Although adult and juvenile lizards can use behavioural mechanisms to avoid lethally high temperatures, embryonic reptiles cannot avoid thermal extremes. In the absence of maternal plasticity in nest site selection, the nests of many lizard species may get hotter in future, exposing embryos to higher temperatures (Telemeco et al., 2017, Levy et al., 2015).

How will future increases in nest temperatures affect lizard populations? Numerous laboratory studies on lizards have shown that incubation temperatures can affect multitude of traits such as body size, behaviour, locomotor performance, and cognition (Deeming, 2004, Noble et al., 2018, While et al., 2018). However, most of these studies have used either constant temperature incubation, or have used incubation temperatures within the current range of nest temperatures (While et al., 2018). These methodological issues make it difficult to predict how future incubation temperatures may affect lizards. Moreover, despite extensive research, there are several key knowledge gaps (Noble et al., 2018). First, it is unclear whether incubation-induced phenotypic plasticity is transient or longer-lasting. Second, although we know that higher incubation temperatures will shorten incubation periods, so that hatchlings will emerge earlier in summer, we do not know how this shift will affect hatchling survival. In this respect, adaptive shifts in thermal physiology (i.e. increases in preferred temperatures or heat tolerance) might buffer hatchlings from higher temperatures. However, higher incubation temperatures might also impair hatchling cognitive abilities, so adaptive shifts in one aspect of an organism's physiology should be evaluated in light of potential costs. Finally, there is a paucity of information on whether developmentally plastic traits influence offspring survival in the wild.

To address some of these knowledge gaps, I used egg incubation experiments to assess how fluctuating incubation temperatures affect the morphology, behaviour and physiology of hatchlings of the velvet gecko *Amalosia lesueurii.* In my first experiment, I incubated eggs under 'cold' (mean = 23.3 °C; 17.5–30.5 °C) and 'warm' (mean = 25.4 °C; range = 16.5–35.5 °C) incubation treatments that mimicked thermal regimes in shady and sun-exposed nests currently used by velvet geckos. I found that cold-incubated eggs hatched later than hot-incubated eggs, and produced larger hatchlings (Chapter 3). The results agree with previous studies of velvet geckos (Dayananda et al., 2016) and fits the pattern seen in many other reptiles (Noble et al., 2018). However, few studies have tested the 'bigger is better hypothesis' which posits that larger hatchlings should have higher survival (Ferguson and Fox, 1984, Miles, 2004, Olsson and Madsen, 2001). The results of survival analyses in MARK did not support this hypothesis, suggesting that a females' current choice of nest site had little influence on the viability of her offspring. This finding is important, because it suggests that even if temperatures increase by 2 °C in future, as predicted by climate modellers (Coumou and Robinson, 2013, Cowan et al., 2014, Hansen et al., 2012), shaded nest sites, which are currently 2.2 °C cooler than sun-exposed nests will provide suitable thermal regimes suitable for embryo development. Thus, maternal variation in nest site choice (Doody et al., 2006) coupled with natural successional vegetation changes (emergent trees shading nests), may therefore help to buffer velvet gecko populations from future environmental change.

However, this optimistic outlook assumes that nest-site temperatures are uniform across the species range. My nest sites were located in Nowra, in the southern part of the species' range, so this assumption is probably unrealistic. Future studies to document nest-site selection across the species' range, similar to the studies which have been carried out on water dragons *Intellagama lesueurii* (Doody et al., 2006), would help to answer this question. Another obvious question is whether the developmentally-induced shifts in morphology that I documented occur in other populations. In fact, given that I detected geographic variation in the thermal sensitivity of locomotion to developmental temperatures (Chapter 4), this assumption deserves further study. Another question that I did not consider was how humidity within natural nests might change in response to temperature. For example, increased temperatures might cause decreases in humidity that could increase desiccation rates of eggs, potentially contributing to egg the mortality. Future studies could deploy humidity sensors inside natural nests (shaded nests and sun-exposed nests) during the incubation period. This data would provide a better understanding of fluctuations in temperature and humidity in natural nest sites.

To explore whether thermal performance curves might shift in response to changing temperatures, I incubated eggs from two regions under 'cold' (mean = 23.3 °C; 17.5–30.5 °C) and 'warm' (mean = 25.4 °C; range = 16.5–35.5 °C) incubation treatments (above) measured the locomotor performance of hatchlings from two regions (Chapter 4). At Nowra, warm incubated hatchlings ran faster than cold incubated hatchlings. At Dharawal (100 km north of Nowra) incubation temperature had no effect on hatchling running speed. These findings underscore that complex responses to incubation temperatures are possible, and that patterns can be dependent on location of origin (Gibert et al., 2001).

Given that current incubation temperatures can have strong but complex effects on hatchling morphology and speed, how might future incubation temperatures influence hatchling traits? To answer this question, I incubated eggs under fluctuating temperature treatments to mimic the thermal profiles of currently-used sun-exposed nests ('warm': mean = 24.3 °C, range 18.4 – 31.1 °C) and the temperatures that might occur in nests in 2050 under climate warming ('hot': mean = 28.9 °C, range 19.1 °C – 38.1 °C). In Chapter 5, I showed that incubation temperatures did not influence preferred T_bs of hatchlings. In fact, feeding status had the greatest effect on T_bs, with geckos showing post-feeding increases in temperature similar to those seen in snakes (Peterson et al., 1993). Future studies should consider how resource variation affects T_bs of hatchling lizards.

Although incubation temperature did not affect the preferred T_bs of hatchlings, it markedly altered their thermal tolerance. Hot incubation temperatures produced hatchlings with higher heat tolerance, while warm incubation temperatures produced hatchlings with higher heat hardening capacity (Chapter 6). However, both of these effects were transitory, and did not persist into later life. By contrast, plasticity for cold-tolerance persisted into later life. Most strikingly, warm-incubated hatchlings tolerated colder temperatures (11.24 °C) than hot-incubated hatchlings (14.11 °C). This effect persisted until at least 6 months of age. Thus, shifts in cold tolerance wrought by increases in incubation temperature have the potential to affect overwinter survival in geckos. For example, field studies on the lizard *Anolis cristatellus* showed that populations in Miami had lower cold tolerance than the source population in Puerto Rico, whereas heat tolerance was similar in both populations, suggesting that selection had acted on cold tolerance rather than heat tolerance (Leal and Gunderson, 2012). Future studies to investigate whether cold tolerance influences over winter survival in hatchling geckos would be valuable. More generally, a better understanding of the strength of natural selection on thermal traits in lizards would increase our understanding of how future changes may affect populations (Gilbert and Miles, 2017).

In Chapter 7, I asked whether incubation temperatures influenced the learning abilities of hatchling lizards. I scored the ability of warm and hot-incubated hatchlings to locate an open shelter in a Y-maze. I found that both warm and hot hatchlings could learn the task, but warm incubated hatchlings were faster learners, and made more successful escapes than hot-incubated hatchlings. The pattern was similar for both geographic locations. Recently, my colleagues showed that learning scores measured in the laboratory were positively correlated with survival of hatchling geckos (Dayananda and Webb, 2017). Thus, it seems likely that hotter nests may produce hatchlings with poorer learning ability, and with lower survival rates. This is an area that clearly warrants more detailed studies.

8.2 Future studies

Although I have answered some important questions in this thesis, there are many unanswered questions. Despite the wealth of studies on thermal developmental plasticity in lizards, we lack an understanding of the genetic, epigenetic and hormonal mechanisms responsible for generating this plasticity (While et al., 2018). In this respect, future studies could focus on the molecular pathways responsible for the observed phenotypic plasticity. Some recent studies on lizards have made some progress in this respect. For example, studies on wall lizards *Podarcis muralis* found that embryos from stressfully low incubation temperatures (15 °C) expressed 20% less RNA than embryos from warmer (24 °C) incubation temperatures. Furthermore, the researchers found changes in the expression of genes involved in chromatin remodelling, suggesting that epigenetic mechanisms could be responsible for incubation induced changes in hatchling traits (Feiner et al., 2018). Indeed, there is a growing body of evidence that environmental stress experienced by embryos can produce substantial changes in phenotypes that persist for many generations (Crews, 2011, McGowan et al., 2008). The causes and consequences of such epigenetic effects for lizards will be an exciting area of research in the future.

Another broad question that deserves further study is what is the nature of trade-offs between traits that are influenced by developmental temperatures? For example, although hatchlings from warm-temperature incubation had higher heat hardening capacity, there might be costs associated with mounting a heat hardening response. For example, a study on *Drosophila* showed that heat hardened flies were more likely to be recaptured during hot weather, but not during cold weather, suggesting that costs and benefits depended on the environmental context (Loeschcke and Hoffmann, 2007). Similarly, hatchling geckos that develop in cool nest sites might have enhanced cognitive ability at the expense of other traits that influence fitness. For example, there may be energetic or physiological costs associated with maintaining and developing the neural architecture that promotes enhanced cognitive functioning (Buchanan et al. 2013, Maille and Schradin 2017). Few studies have examined whether such costs occur in reptiles. Likewise, the question of whether cognitive differences persist into later life, and ultimately influence reproductive success, awaits further study (While et al., 2018).

8.3 Conclusion

In my experimental study, I have provided new insights into the role of incubation environments in shaping the phenotypic traits of hatchling velvet geckos. Velvet geckos inhabit a thermally challenging environment that is likely to experience strong and frequent heat waves in future (Cowan et al., 2014, Hansen et al., 2012), resulting in higher nest temperatures (Dayananda et al., 2016). By subjecting eggs to current nest temperatures, and temperatures likely to be experienced in the future, I aimed to estimate the vulnerability of velvet geckos to climate warming. Hatchlings from higher incubation temperatures were smaller, hatched earlier, and were slower learners. In addition, higher developmental temperatures also influenced thermal tolerance, in ways that could potentially influence fitness. Future studies are necessary to measure whether these morphological, behavioural and physiological traits influence fitness. I have also identified some empirical gaps worthy of future research, which will help to tease apart the mechanistic underpinnings of plasticity, and provide a better understanding of how plasticity may buffer ectotherms from future environmental changes. While previous authors have speculated that lizards may fare poorly under warming climates (Sinervo et al., 2010) my results suggest a more optimistic future for velvet geckos. Indeed, small scale spatial variation in the thermal profiles of natural nests, coupled with successional vegetation changes, may generate sufficient variation in offspring phenotypes to ensure that populations persist under changing climates.

8.4 References

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