

RESEARCH ARTICLE

Hotter nests produce hatchling lizards with lower thermal tolerance

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ABSTRACT

In many regions, the frequency and duration of summer heatwaves is predicted to increase in future. Hotter summers could result in higher temperatures inside lizard nests, potentially exposing embryos to thermally stressful conditions during development. Potentially, developmentally plastic shifts in thermal tolerance could allow lizards to adapt to climate warming. To determine how higher nest temperatures affect the thermal tolerance of hatchling geckos, we incubated eggs of the rock-dwelling velvet gecko, *Amalosia lesueurii*, at two fluctuating temperature regimes to mimic current nest temperatures (mean 23.2°C, range 10–33°C, 'cold') and future nest temperatures (mean 27.0°C, range 14–37°C, 'hot'). Hatchlings from the hot incubation group hatched 27 days earlier and had a lower critical thermal maximum (CT_{max} 38.7°C) and a higher critical thermal minimum (CT_{min} 6.2°C) than hatchlings from cold incubation group (40.2 and 5.7°C, respectively). In the field, hatchlings typically settle under rocks near communal nests. During the hatching period, rock temperatures ranged from 13 to 59°C, and regularly exceeded the CT_{max} of both hot- and cold-incubated hatchlings. Because rock temperatures were so high, the heat tolerance of lizards had little effect on their ability to exploit rocks as retreat sites. Instead, the timing of hatching dictated whether lizards could exploit rocks as retreat sites; that is, cold-incubated lizards that hatched later encountered less thermally stressful environments than earlier hatching hot-incubated lizards. In conclusion, we found no evidence that CT_{max} can shift upwards in response to higher incubation temperatures, suggesting that hotter summers may increase the vulnerability of lizards to climate warming.

KEY WORDS: Climate warming, Developmental plasticity, Incubation temperature, *Amalosia lesueurii*, Critical thermal limits

INTRODUCTION

Climate warming poses a significant challenge to organisms across the globe (Pounds et al., 1999; Thomas et al., 2004; Walther et al., 2002). Reptiles are particularly vulnerable to climate warming because environmental temperatures directly affect their physiology and behaviour (Araújo et al., 2006; Huey et al., 2012; Sinervo et al., 2010; Tewksbury et al., 2008). Predicting the vulnerability of ectotherms to climate warming requires knowledge of how increases in temperature will affect subsequent life-history stages (Kingsolver et al., 2011; Levy et al., 2015; Radchuk et al., 2013). Unlike adult lizards, developing lizard embryos cannot thermoregulate (Telemeco et al., 2016b) and, consequently, they may experience thermally stressful temperatures in natural nests during summer

(Shine and Elphick, 2001). Heatwaves have become more common in recent decades (Hansen et al., 2012), and are predicted to increase in frequency in the future (Cowan et al., 2014). Thus, embryos of some lizard species may experience periods of acute thermal stress during development in the future (Levy et al., 2015; Telemeco et al., 2016a).

Studies using constant temperature or fluctuating temperature during incubation have shown that incubation temperature can influence several fitness-related traits of hatchlings including morphology (sex, body size, shape), locomotor performance, sex and learning ability (Amiel and Shine, 2012; Angilletta et al., 2002; Booth, 2006; Deeming, 2004; Doody et al., 2004; Janzen, 1994). Nearly all of the above studies have used incubation temperatures within the current temperature range found within natural nests, so we know very little about how embryos will respond to thermally stressful conditions that may be experienced in the future (Angilletta et al., 2013; Levy et al., 2015). Potentially, developmentally plastic shifts in thermal tolerance could allow lizards to rapidly adapt to climate warming (Hoffmann and Sgrò, 2011; Urban et al., 2014). For example, in *Drosophila*, larvae that underwent development at higher constant or fluctuating temperatures had higher heat tolerance as adults (Hoffmann et al., 2003; van Heerwaarden et al., 2016). In water fleas *Daphnia magna*, populations that were raised in outdoor tanks at temperatures 4°C higher than ambient rapidly evolved higher thermal tolerance (Geerts et al., 2015). It is less clear whether lizards can mount similar adaptive responses. To date, no studies have investigated how higher developmental temperatures likely to be experienced under future climates influence the thermal tolerance of hatchling lizards.

Compared with other lizards, geckos (family Gekkonidae) have an unusually low critical thermal maximum (CT_{max} ; mean±s.d. 40.8±2.2°C versus 44.9–47.1°C for the Agamidae, Iguanidae, Crotaphytidae and Teiidae; see Clusella-Trullas et al., 2011; Clusella-Trullas and Chown, 2014), and they also maintain lower preferred body temperatures (Angilletta and Werner, 1998). Although most geckos are nocturnal, some species thermoregulate during the day by selecting thermally suitable shelter sites (Greer, 1989; Webb and Shine, 1998). Potentially, the lower CT_{max} and thermal preference of geckos may increase their vulnerability to climate warming, especially for species that inhabit thermally challenging environments (e.g. exposed rock outcrops). However, there should be strong selection acting on upper thermal limits in species that inhabit hotter environments. For example, the upper thermal limits of the widespread lizard *Sceloporus undulatus* were 2.9°C higher in the population that experienced the highest temperatures, suggesting that CT_{max} can evolve upwards (Buckley et al., 2015). Likewise, CT_{max} of populations of the rainforest sun skink, *Lampropholis coggeri*, ranged from 36.6 to 41.3°C, suggesting that the skinks have adjusted to current climates via plastic or evolved responses (Llewelyn et al., 2016).

In this study, we investigated whether incubation temperatures affect the thermal tolerance limits of hatchling velvet geckos, *Amalosia lesueurii*. We incubated eggs under two fluctuating

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temperature regimes to mimic current temperatures experienced by eggs inside natural nests and future temperatures that eggs may experience under climate warming. In the field, we measured the operative environmental temperatures in microhabitats used by geckos to investigate whether hatchlings are likely to experience temperatures near their CT_{max} , and if so, whether plastic responses in CT_{max} could buffer lizards from lethally high temperatures.

MATERIALS AND METHODS

Study species

The velvet gecko, *Amalosia lesueurii* Duméril & Bibron 1836, is a small (up to 65 mm snout–vent length) nocturnal lizard that occurs in sandstone rock outcrops from south-eastern NSW to south-eastern QLD, Australia (Cogger, 2000). Velvet geckos forage at night and do not bask. During the daytime, the geckos thermoregulate by moving into crevices beneath sun-exposed rocks (Schlesinger and Shine, 1994; Webb et al., 2008). Female velvet geckos do not dig nests but lay their parchment-shelled eggs (two per clutch) in communal nests inside rock crevices or underneath rocks during late spring (Doughty, 1997; Webb et al., 2008). Eggs hatch in late summer and autumn (February–March), and hatchlings establish territories under thin sun-exposed rocks near communal nests. During their first year of life, hatchlings use one or two rocks as shelter sites, and rarely disperse more than 30 m. In Morton National Park (see below), near the most southerly range of this species, nest temperatures inside communal nests are positively correlated ($r^2=0.74$) with maximum daily air temperatures (Dayananda et al., 2016). Observations of communal nest sites at three study sites in the park revealed that females have laid eggs inside the same communal nests over the last 25 years (J.K.W., unpublished data), suggesting that there is little plasticity in female nesting behaviour. Thus, future increases in air temperature coupled with an increase in the frequency and duration of summer heatwaves (Cowan et al., 2014) are likely to produce hotter nests (Dayananda et al., 2016).

We collected gravid velvet geckos from two habitat restoration sites, one in Dharawal National Park, 50 km south of Sydney ($n=23$), and another near Morton National Park, 170 km south of Sydney ($n=20$), during October 2014. They were transported to the University of Technology Sydney, where they were individually housed in ventilated rectangular plastic cages (Sistema NZ 2.0 l, 220×150×60 mm), in a room (22°C) with a 12 h:12 h light:dark cycle. Each cage contained an identical plastic tube (PVC, 80×40 mm) cut in half lengthwise for a shelter, a water dish and a substrate of moist vermiculite. Cages were placed on wooden racks,

with one end of the cage sitting on a timer-controlled heating cable (32°C) to create a thermal gradient of 22–32°C during the day, dropping to room temperature at night (22°C). Geckos were fed with live crickets twice weekly and had access to water *ad libitum*. We released the geckos at their exact site of capture after they had oviposited.

After oviposition, each egg was weighed (to the nearest 0.01 g) and placed in a 100 ml glass jar containing moist vermiculite (water potential of 200 kPa) covered with plastic food wrap to prevent desiccation. The two eggs from each clutch were randomly allocated to either incubation treatment. We incubated eggs inside incubators (Panasonic MIR-154-PE) that were programmed to produce fluctuating temperatures that mimic current nest temperatures recorded inside natural nests (mean 23.2°C, range 10–33°C, ‘cold’) and future conditions (mean 27.0°C, range 14–37°C, ‘hot’; Fig. 1). Current temperatures were based on measurements taken from miniature data loggers that were placed in nine communal nests during the summer of 2006–2007. Previously, we showed that maximum daily air temperatures accurately predict temperatures inside communal nests (Dayananda et al., 2016). Thus, the hot incubator mimicked thermal regimes likely to occur inside nests in 2050, assuming that air temperatures increase by 3–4°C, as predicted by the climate models (Dowdy et al., 2015). After eggs hatched, we maintained hatchlings inside a temperature-controlled room (22°C; overhead lights 12 h:12 h cycle). Each hatchling was placed in a separate ventilated plastic cage (Sistema 2.0 l, 220×150×60 mm) with a paper substrate, a plastic shelter (80×40 mm opaque plastic tube cut in half lengthwise) and a water dish. One end of each cage was placed on a timer-controlled heating cable that created a thermal gradient inside the cage (22–32°C) during the day (08:00 h–16:00 h), dropping to room temperature at night (22°C). Hatchlings were fed small crickets twice weekly and upon completion of laboratory trials they were released at the original capture site of their mother.

Measurements of hatchling CT_{max} and critical thermal minimum (CT_{min})

CT_{max} and CT_{min} constitute the endpoints of thermal performance curves which are generally associated with the loss of righting response (Lutterschmidt and Hutchison, 1997). Thus, we used the loss of righting response to determine the thermal tolerance of individuals (Huey, 1982). We measured the thermal tolerance of 45 hatchlings aged 7–10 days. Each individual was tested for CT_{max} and CT_{min} unless it dropped its tail between trials. Final sample sizes were 41 for CT_{max} trials and 45 for CT_{min} trials.

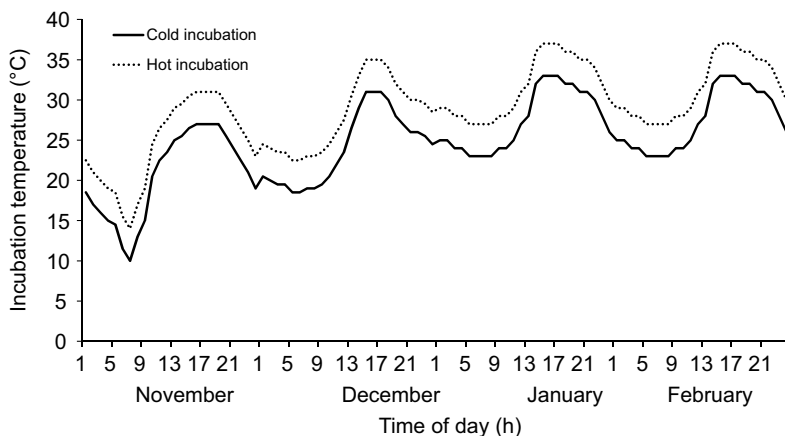


Fig. 1. Fluctuating thermal regimes used to incubate eggs in the cold and hot treatments, designed to simulate current and future nest temperatures. Each 1–24 h period was repeated during the month indicated (November to February). Note that October temperatures were identical to those of November.

To carry out trials, we placed each test subject inside a plastic container (Sistema NZ 2.0 l, 220×150×60 mm, with ventilated lid) inside a programmable incubator (Panasonic MIR-154-PE) during the daytime (11:00 h–15:00 h). We cooled hatchlings for CT_{min} tests and heated them for CT_{max} tests, at a rate of $0.25^{\circ}\text{C min}^{-1}$ from their designated acclimation body temperatures (22°C). We observed the behaviour of each individual lizard through the incubator window. In CT_{max} trials, once the temperature reached 36°C , we opened the glass door of the incubator every minute, opened the lid of the container and gently turned the lizard upside down with an artist's paintbrush. The body temperature associated with a transient loss of the righting response was deemed the endpoint for CT_{max} . In CT_{min} trials, we tested the righting response every minute for temperatures below 10°C . We tested 21 and 22 hot-incubated hatchlings for CT_{max} and CT_{min} , respectively, and 20 and 23 cold-incubated hatchlings for CT_{max} and CT_{min} , respectively.

No hatchlings were harmed during testing, and after testing in the laboratory was completed, they were individually marked and released at the capture site of the mother (Dayananda et al., 2016). All procedures were approved by the University of Technology Sydney Animal Care and Ethics Committee (protocol ACEC 2012000256), and were conducted under a permit from the NSW National Parks and Wildlife Service (SL 101013 to J.K.W.).

Measuring environmental operative temperatures

To quantify the thermal environment available to hatchling geckos during summer, we deployed miniature data loggers (Thermochron iButton, Dallas Semiconductor, Dallas, TX, USA; diameter 15 mm, height 6 mm) in microhabitats known to be used by geckos (i.e. inside crevices, underneath rocks and underneath boulders) at two study sites located in Dharawal National Park and near Morton National Park. The sites were located on ridge tops and were characterised by large swathes of exposed sandstone rock juxtaposed with eucalypts and low heath. At both sites, humans had removed loose surface rocks for landscaping urban gardens. In 2009, the sites were restored with artificial rocks that were placed in sun-exposed locations. The artificial rocks have thermal profiles very similar to those of natural rocks, and have been rapidly colonised by velvet geckos (Croak et al., 2010, 2013). At each study site, we placed data loggers beneath natural rocks (Dharawal: $n=6$, Morton: $n=9$), artificial rocks ($n=8$ and 9) and boulders ($n=5$ and 4), and inside rock crevices ($n=8$ and 11). The data loggers recorded temperatures every 30 min from 23 January until 6 March 2014.

Statistical analyses

We used general linear mixed model analyses to determine whether CT_{max} and CT_{min} differed between hot- and cold-incubated lizards. In each model, with either CT_{max} or CT_{min} as the response variable, incubation temperature was a fixed explanatory variable (hot, cold) and both location (Dharawal National Park, Morton National Park) and mother identity were included as random control variables. Mother identity was nested within location in the models. Satterthwaite approximations were used to calculate degrees of freedom for t -tests in the mixed models. Model residuals were inspected to ensure homogeneity of variances and normality. To determine whether hatchling geckos may experience lethally high temperatures in the field, we calculated: (1) the number of hours that rock temperatures exceeded the CT_{max} of hot- and cold-incubated lizards, and (2) the proportion of rocks that exceeded the CT_{max} of hot- and cold-incubated lizards. We incorporated hatching date to calculate these metrics for each study site. Statistical analyses were

performed using R 3.1.3 (<https://www.r-project.org/>) and SPSS 23.0 (www.ibm.com/) software.

RESULTS

CT_{max} and CT_{min}

Hot-incubated eggs hatched from 10 January 2015 to 7 February 2015 (median date 1 February 2015). Cold-incubated eggs hatched from 11 February 2015 to 11 March 2015 (median date 24 February 2015). Hot-incubated hatchlings had a lower CT_{max} than cold-incubated hatchlings (38.7 versus 40.2°C ; $t_{18,24}=9.82$, $P<0.001$; Fig. 2A). Random factors in the model were associated with very little (mother identity within location: variance±s.d. 0.02 ± 0.15) to none (location) of the variation in CT_{max} . Hot-incubated lizards were found to have a higher CT_{min} than cold-incubated lizards

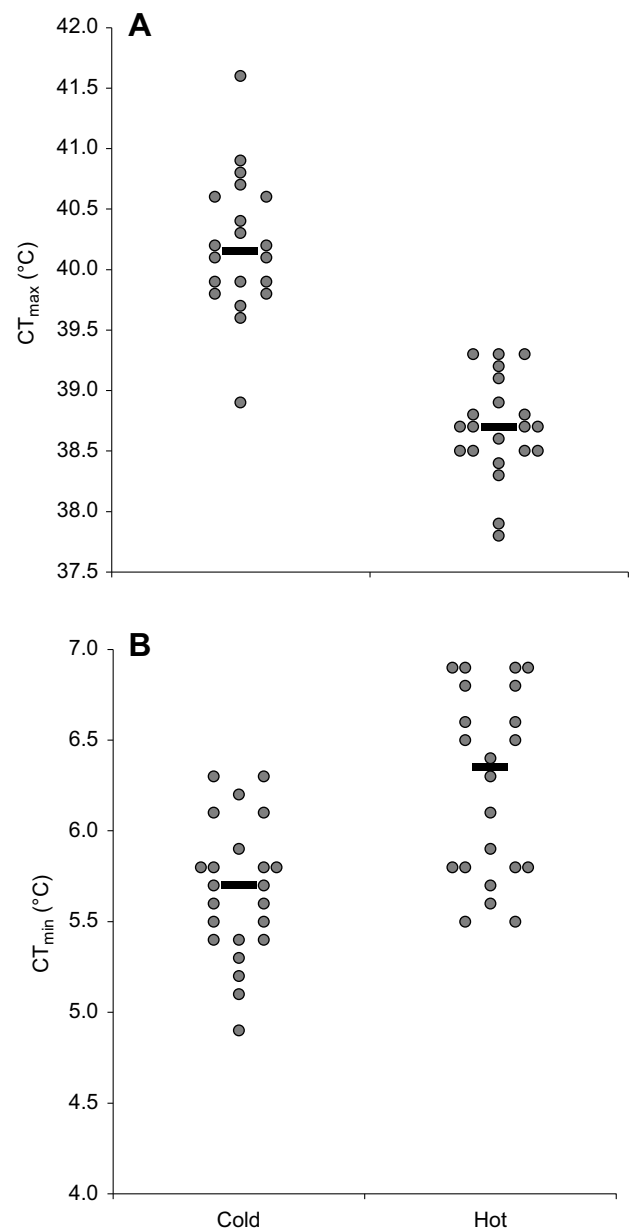


Fig. 2. Critical thermal maximum and minimum scatterplots for 1 week old hatchling geckos from cold and hot incubation treatments. (A) Critical thermal maximum (CT_{max}). (B) Critical thermal minimum (CT_{min}). Black lines show medians and repeat values are jittered for clarity.

(6.2 versus 5.7°C; treatment: $t_{29,10}=4.55$, $P<0.001$; Fig. 2B). Random factors in the model explained very little (mother identity within location: variance±s.d. 0.05 ± 0.23) to none (location) of the variation in CT_{min} .

Operative environmental temperatures at field sites

During the egg-hatching period, temperatures underneath rocks often reached 50°C, far exceeding the CT_{max} of hatchlings, whereas temperatures inside crevices never exceeded lizard CT_{max} (Fig. 3). The timing of hatching, rather than incubation-derived differences in heat tolerance, determined whether geckos could exploit rocks without overheating. If lizards hatched at the same time, temperatures under rocks would exceed the CT_{max} of hot- and cold-incubated lizards for 2.48 versus 2.00 h daily at Dharawal National Park, and 2.07 versus 1.57 h daily at Morton National Park. When we included median hatching date in the analysis, rocks would exceed the CT_{max} of hot- and cold-incubated lizards for 2.48 versus 0.29 h daily at Dharawal National Park ($t_{1,42}=3.98$, $P<0.001$), and 2.07 versus 0.05 h daily at Morton National Park ($t_{1,42}=3.94$, $P<0.001$). The timing of hatching also influenced the number of days that hatchlings would experience lethally high rock temperatures. At Dharawal National Park, hot-incubated lizards (which hatched earlier) experienced 26 days when rock temperatures exceeded their CT_{max} whereas cold-incubated lizards (which hatched later) experienced only 9 such days (Fig. 4A). At

Morton National Park, hot-incubated lizards were exposed to 16 days when rock temperatures exceeded their CT_{max} whereas cold-incubated lizards were exposed to only 2 such days (Fig. 4B).

DISCUSSION

Recent studies have highlighted the importance of understanding how heat stress influences early life stages in order to predict population-level consequences of warming (Levy et al., 2016, 2015). In lizards, the embryos of some species may experience thermally stressful temperatures during summer heatwaves (Levy et al., 2015), which are predicted to occur more frequently in future (Cowan et al., 2014; Hansen et al., 2012). In our study species, there is a strong positive correlation between maximum daily air temperatures and nest temperatures, so heatwaves may cause higher temperatures inside nests (Dayananda et al., 2016). Potentially, exposure to higher temperatures during development may confer higher heat tolerance to later life stages (Hoffmann et al., 2003; van Heerwaarden et al., 2016). Developmental plasticity, in conjunction with short-term heat hardening, could therefore buffer lizards from the effects of increasing temperatures (Chevin et al., 2010).

Contrary to predictions, hatchlings from the hot-incubation treatment had lower heat tolerance than hatchlings from the cold-incubation treatment (Fig. 2A). Thus, exposure to stressful but non-lethal temperatures (37°C) during development did not produce an

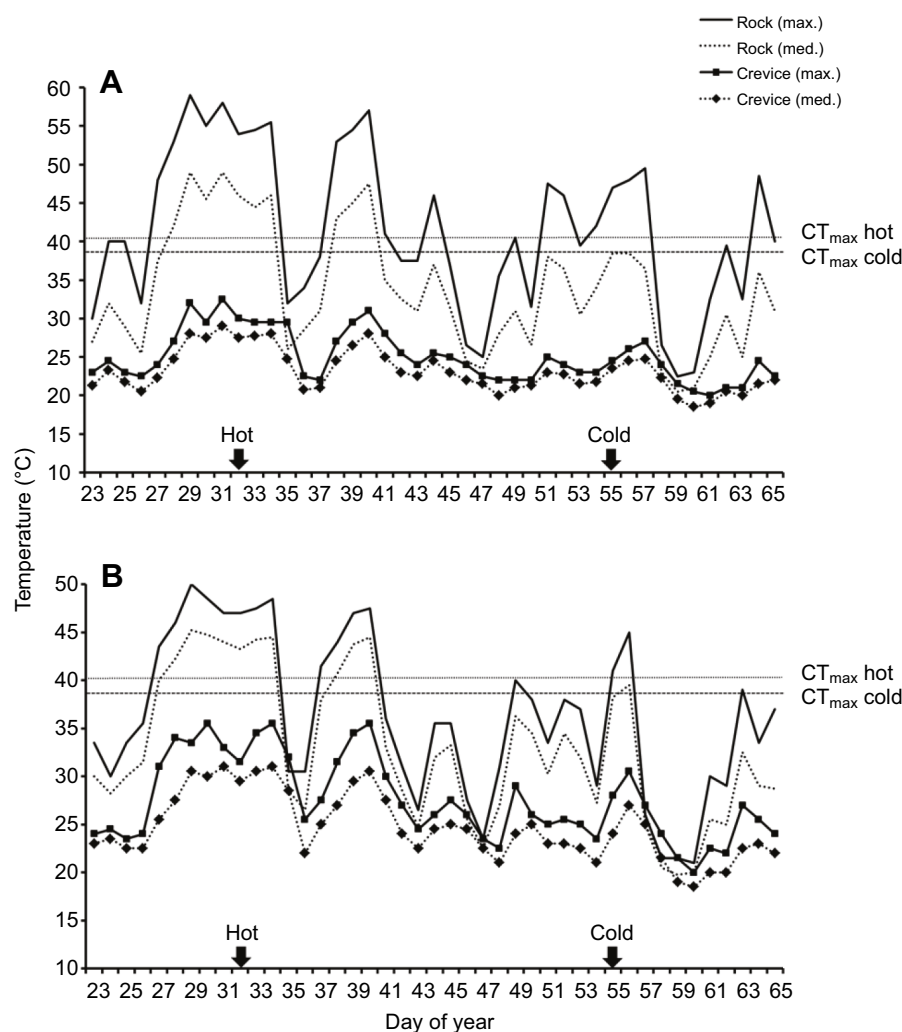


Fig. 3. Operative environmental temperatures recorded under rocks and inside crevices at study sites between 23 January and 6 March 2014. (A) Dharawal National Park (19 rocks, 8 crevices). (B) Morton National Park (22 rocks, 11 crevices). The solid lines show maximum temperatures for each habitat type and dotted/dashed lines show median temperatures for each habitat type. The horizontal dotted lines depict the CT_{max} of hot- and cold-incubated hatchlings. Arrows show median hatching dates of hot- and cold-incubated lizards. Note that rock temperatures often exceeded 45°C in summer.

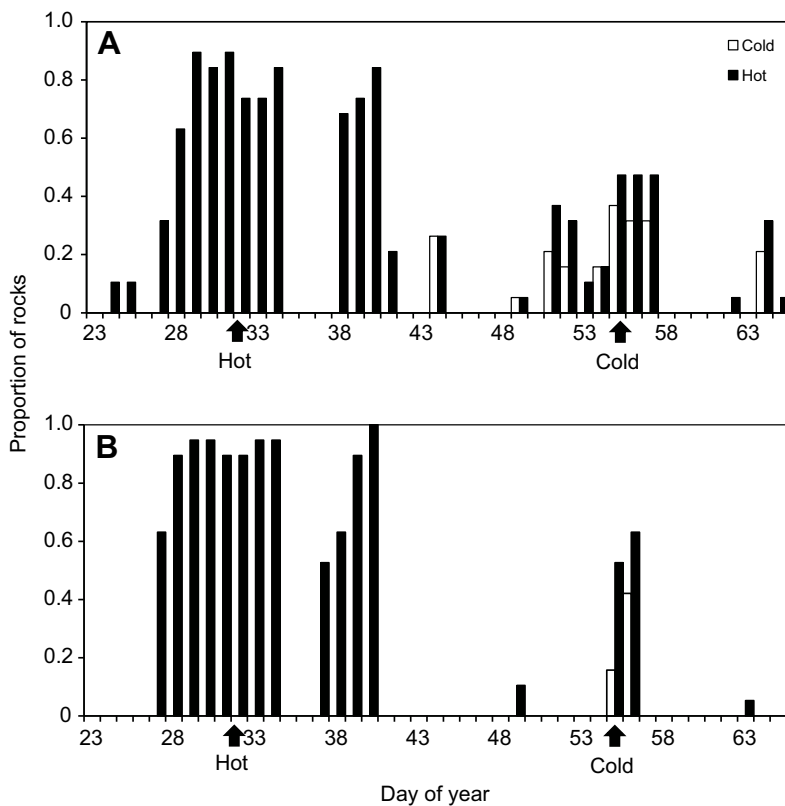


Fig. 4. Proportion of surface rocks at study sites that exceeded the CT_{max} of hot- and cold-incubated hatchling geckos during the period 23 January to 6 March 2014. (A) Dharawal National Park (19 rocks). (B) Morton National Park (22 rocks). Arrows show the median times of hatching of hot- and cold-incubated lizards.

upwards shift in the CT_{max} of hatchlings. This result was unexpected but mirrors recent findings for other lizards. For example, in *Sceloporus undulatus*, embryos were exposed to a diel cycle that peaked at either 35 or 40°C for 2 days before being exposed to a diel cycle that peaked at 42°C followed by a diel cycle that peaked at 44°C. Measurements of cardiac activity revealed that all embryos died when exposed to 44°C, which suggests that the embryos lacked a heat hardening response that could buffer them from high temperatures (Levy et al., 2015). Likewise, a recent study on *Sceloporus tristichus* found that embryos from heat-stressed females were less likely to survive prolonged (7 day) exposure to high temperatures, suggesting that embryos lacked the plasticity necessary to survive exposure to higher incubation temperatures (Telemeco et al., 2016a).

It is unclear why hatchling geckos from the high temperature incubation had lower heat tolerance than their cold-incubated litter mates. However, heat shock proteins (HSPs), which act as molecular chaperones, and which are upregulated when organisms are exposed to high temperatures, may play a role here (Feder and Hofmann, 1999; Sørensen, 2010). HSPs can have positive and negative effects on fitness (Feder and Hofmann, 1999). For example, in *Drosophila*, moderate levels of Hsp70 can promote thermotolerance, whereas high levels of Hsp70 decrease thermotolerance of adults (Krebs and Feder, 1998). High levels of HSPs can also affect embryo survival. For example, *Drosophila* larvae with additional copies of Hsp70 genes had slower development and higher mortality than larvae without extra copies (Krebs and Feder, 1997). High concentrations of HSPs may interfere with cell functions, while production and degradation of HSPs might consume a large portion of energy at the expense of other molecular functions (Feder and Hofmann, 1999).

To our knowledge, only one study has examined the effects of HSPs on heat tolerance in reptiles. In soft-shell turtles, *Pelodiscus*

sinensis, the overexpression of HSP70 in embryos resulted in increased heat tolerance of embryos, but the resultant hatchlings had decreased heat tolerance relative to the sham controls (Gao et al., 2014). These results support the idea that there are costs involved with the production of high levels of HSPs during embryogenesis (Feder and Hofmann, 1999). Future studies to investigate the role of HSPs in lizards would be worthwhile.

The CT_{max} of velvet geckos reported in our study falls within the range reported for other gekkonids (mean±s.d. 40.8±2.2°C; Clusella-Trullas and Chown, 2014). However, the CT_{min} of hot-incubated (6.2°C) and cold-incubated (5.7°C) lizards was lower than the CT_{min} reported for other geckos (mean±s.d. 10.6±1.7°C; Clusella-Trullas and Chown, 2014). Velvet geckos clearly have a broad thermal tolerance, as would be predicted for a species that lives in rock outcrops where diurnal temperatures can fluctuate markedly (Huey and Kingsolver, 1989); in winter, rock temperatures can range between 5 and 25°C (Webb et al., 2005), while in summer, temperatures can range between 14 and 59°C (this study).

Whether incubation-induced changes in heat tolerance are biologically relevant will depend on whether hatchlings encounter thermally stressful microhabitats. Previous studies demonstrated that shortly after hatching, velvet geckos establish territories underneath rocks near natal nest sites (Webb, 2006). We found that during heat waves, temperatures under rocks exceeded 50°C, well above the CT_{max} of both hot- and cold-incubated lizards. Thus, developmental shifts in heat tolerance would not buffer lizards from high temperatures. Instead, the timing of hatching dictated whether lizards were likely to encounter lethally high temperatures under rocks. Because they hatched later in summer, cold-incubated lizards could exploit rocks on most days. By contrast, hot-incubated lizards hatched earlier in summer, and so could only use rocks occasionally (Fig. 4). During heatwaves, hatchlings from hotter nests would be

forced to seek refuge in cooler crevices, or risk death from overheating. Potentially, hatchlings fleeing from rocks might be more vulnerable to avian predation (Christian and Tracy, 1981; Webb and Whiting, 2005), while geckos sheltering inside cooler crevices might incur growth costs (Sinervo and Adolph, 1989). In support of this, hot-incubated hatchlings from Dharawal National Park had lower growth rates and survival than cold-incubated lizards (Dayananda et al., 2016, 2017a).

Finally, we acknowledge several limitations of our study. First, although we focused on heat tolerance, differences in cold tolerance might also affect survival (Leal and Gunderson, 2012). Second, we measured the thermal tolerance of hatchlings aged 7–10 days, so we do not know whether the observed patterns are transitory or longer lasting (e.g. Buckley et al., 2007). In the field, hatchlings may show ontogenetic shifts in heat tolerance, or may exhibit heat hardening or acclimation in response to higher environmental temperatures (Bowler, 2005). For example, in the spiny lizard *Sceloporus jarrovi*, juveniles had a higher CT_{max} than adults (Gilbert and Lattanzio, 2016). In the rainforest skink *Lampropholis coggeri*, individuals that were briefly exposed to high temperature showed rapid heat hardening, with some individuals increasing their heat tolerance by up to 2.6°C (Phillips et al., 2016). Whether hatchling geckos are able to mount such heat-hardening responses is unknown, but deserves further study.

In conclusion, our results underscore the importance of studying the effects of heat stress on early life stages. Incubation of eggs at high temperatures that mimicked potential future nest temperatures did not enhance the heat tolerance of hatchlings. This finding suggests that there is limited developmental plasticity for heat tolerance in our study species, supporting the idea that physiological constraints may prevent CT_{max} from evolving upwards (Araújo et al., 2013; Clusella-Trullas and Chown, 2014). Future experiments, using biologically relevant cycling temperatures, in other lizard taxa are necessary to test the generality of our findings. Clearly, consideration should be given to all life stages (embryos, hatchlings and adults) when assessing the potential vulnerability of species to future warming.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.K.W.; Formal analysis: B.M.; Investigation: B.D., J.K.W.; Resources: J.K.W.; Writing - original draft: B.D.; Writing - review & editing: B.M., J.K.W.; Supervision: J.K.W.; Project administration: J.K.W.

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Data availability

Data have been deposited in the Dryad Digital Repository (Dayananda et al., 2017b): <http://dx.doi.org/10.5061/dryad.dp1fh>

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