**Maternal investment and early thermal environment interact to affect antipredator responses in two Australian lizard species**

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Abstract:

Keywords:

**Introduction**

Gradual and sudden changes in temperature due to anthropogenic activities pose a challenge to organisms, having important consequences on phenotype and fitness. Ectotherms are especially vulnerable to altered thermal conditions, and exposure to increased temperatures during early development have been shown to lead to plastic responses in the expression of phenotypic traits, including morphology, physiology and behaviour, that can impact fitness in a range of taxonomic groups (Valenzuela and Lance 2004; Dang et al. 2015; Dayananda and Webb 2017; Ślipiński et al. 2021; Raynal et al. 2022). While research has focused mainly on the effects of early life temperature on individual development, little work has addressed the multifaceted role that environments play across generations. Environmental stress (e.g., changes in thermal or resource conditions) experienced by mothers is expected to cascade to affect offspring through maternal effects, and this may interact in complex ways with environments experienced by offspring.

Maternal effects are important contributors to offspring phenotypic variation (Bernardo 1996; Wolf and Wade 2009; Noble et al. 2014), and may moderate the effects of the environment experienced during development. Mothers can adjust their reproductive behaviour or differentially invest in energy and resources that are deposited in eggs in response to changes in the environmental conditions they experience (Rutstein et al. 2005; Huang et al. 2013; Carter et al. 2018). For example, nutrient deprived mothers alter their reproductive allocation compared to mothers fed with normal quality food, leading to changes in offspring phenotype and sex (Warner et al. 2007). Similarly, maternal diet can affect hormone deposition in eggs, which is known to influence hatching success, and offspring phenotype and fitness (e.g., Rosenfeld and Roberts 2004; Warner and Lovern 2014; Rutstein et al. 2005; Huang et al. 2013; Carter et al. 2018). Therefore, maternal effects can exacerbate, dampen or counteract negative phenotypic or fitness effects of early life environments experienced by offspring, and may play an important role in explaining the diversity of phenotypic responses observed within and across species (e.g., Noble et al. 2018).

Maternal and offspring environments that impact upon behaviour are likely to have important consequences in shaping how organisms interact with their environment. Such behavioural changes can have a direct link to fitness. This is the case of antipredator responses, including predator escape and refuge seeking, since the inability to escape from predatory attacks are inevitably associated with mortality. Thermal developmental conditions and maternal effects are known to affect antipredator strategies. For instance, exposure to high temperatures during early development (e.g., eggs or juveniles) has been shown to affect sprint velocity and a range of antipredator behaviours such as predator avoidance and hiding time (Brodie and Russell 1999; Webb et al. 2001; Dalesman and Rundle 2010; McDonald and Schwanz 2018). Similarly, maternal effects can pre-adapt offspring responses to the prevalent predatory conditions by changing egg composition (Sharda et al. 2021). Such effects are likely mediated by changes in morphology (e.g., size, body condition etc), which in turn can influence the antipredator strategies of individuals (Räsänen et al. 2005; Lancaster et al. 2010; Mcghee et al. 2012). Clearly, thermal conditions and maternal effects independently have the potential to influence a range of fitness-related behaviours, however, whether the two interact to shape offspring responses remains unknown.

Whether and to what extent maternal investment and thermal developmental conditions interact to affect offspring performance and behaviour could be expected to be associated with specific evolutionary trajectories and life-history traits (e.g., Manenti et al. 2021, but see Overgaard et al. 2014). To test this, we explore how development temperatures interact with changes in maternal resource investment to shape integrated sets of morphological and behavioural traits related to antipredator responses in two closely related lizard species with different life-histories, *Lampropholis delicata* and *L. guichenoti*. We apply ‘phenotypic engineering’ methods (Sinervo XX) to manipulate both maternal investment in eggs and offspring temperature in a fully factorial design. We predicted that: 1) embryos experiencing high temperatures early in development would be smaller in size with longer tails and have reduced performance (e.g., (Tiatragul et al. 2017; Sanger et al. 2018). As a result, we expect them to be risk adverse relative to embryos experiencing cold temperatures; 2) lower maternal investment in eggs should exacerbate the effects of temperature; 3) *Lampropholis delicata* will be more strongly impacted by temperature and reduced maternal investment because of the smaller egg size and larger clutches compared to *L. guichenoti (REFS)*.

**Methods**

*Study species and housing*

We used two sympatric skink species. The delicate skink (*Lampropholis delicata*) is a small lizard (max. SVL 51 mm) native to south-eastern Australia (Wilson and Swan 2010). Females lay a single clutch of 3-6 small eggs each year. It has been widely used in experiments to explore how different environments affect the morphology and performance of individuals (Bilcke et al. 2006; Downes and Hoefer 2007). In addition, it is an important invasive species in some islands (Chapple et al. 2014; Chapple 2016), which suggests that the species has the potential to respond and become used to novel environmental conditions. The garden skink (*Lampropholis guichenoti*) is a small lizard (max. SVL 48 mm) and widespread across south-eastern Australia (Wilson and Swan 2010). Females lay one or two clutches of 2-4 large eggs a year. The garden skink has been used in previous studies exploring the effect of rearing temperatures and humidity conditions on offspring phenotypic plasticity (Qualls and Shine 1998; Booth et al. 2000).

We captured gravid females *L. delicata* and *L. guichenoti* in semiurban parks in Sydney (Australia). Animals were brought to the laboratory at The Australian National University (ANU, Canberra) where they were housed in single-species groups of five in indoor terraria (1-2 males: 3-4 females – width x length: 40x55 cm) to allow them to lay eggs. Terraria were filled with approximately 8 cm deep of soil, refuge, a water container, and a container full of vermiculite for egg laying. Terraria were heated by a lamp and a has a UV lamp for UVA/UVB exposure. The heat lamp was situated at one end to ensure a temperature gradient. Lights were set to a photoperiod of 12:12 h (light/dark). Animals were provided with water every day (both spraying the soil and filling the water container) and with crickets dusted in calcium and multivitamin every second day. Females were kept in the laboratory for around two weeks for egg-laying and were then released at their capture locations.

*Experimental design*

To explore how incubation temperature and maternal investment interact to affect performance and antipredator behaviour of juveniles, we designed a fully-factorial experiment where eggs of the two skink species were exposed to two levels of temperature [cold (23 + 3° C) or hot (28 + 3° C)] and yolk removal (yolk content reduced -R - or a sham-control - C). Incubation temperatures were selected to mimic those extreme temperatures measured in natural nests of *L. delicata* (Cheetham et al. 2011).

Enclosures were checked daily for eggs. We randomly allocated one egg from a clutch to each of our four treatments: 23°C sham-control eggs (23:C); 23°C yolk reduced eggs (23:R); 28°C sham-control eggs (28:C); and 28°C yolk reduced eggs (28:R). Our design was a partial split-clutch design as it was not possible to allocate eggs from a given clutch to each of the four treatments (i.e., a split-clutch design) given the small clutch sizes of some individuals. Eggs allocated to the yolk reduced treatment were weighed (to the nearest mg), and then pierced with a sterilised needle to extract part of the yolk. Eggs were weighted again, and the difference in weight pre- and post-extraction was used as an approximation of the percentage of yolk extracted. Following methods in Sinervo, 1990 (Sinervo 1990) we aimed for around 15% yolk removal (mean =- SD = 12.49% + 2.64). Control eggs were weighted and pierced with a needle, but we did not remove any yolk. Eggs were then placed in a container filled with four grams of vermiculite dampened with 12 g of water and covered with Wad Wrap cling-wrap to avoid dehydration. Each egg was then placed in an incubator at the corresponding temperature.

*Measures of morphological traits, performance, and antipredator behaviour*

We checked the eggs every day for hatchlings and each hatched lizard was individually housed in terraria (20 x 35 cm) heated by a heat cord and under UV. Enclosures contained paper as substrate, a water container, and a refuge. All the animals were housed in the same laboratory conditions. Juveniles were feed every second day with crickets.

Juveniles were measured and their behaviour tested when they were 3-5 weeks old. We ensured that there was equal representation from each of the four treatment combinations and species (e.g., eggs in cold treatment had longer incubation times) during each measurement session (groups of 48 per day, see below). Body size and mass can influence performance and behaviour (Huey and Hertz 1984; Baxter-Gilbert et al. 2018). As such, we measured weight, snout-vent length, tail length and total length at each measurement to control for these during the analysis. Given the small size of hatchlings, we weighed lizards (to the nearest mg) using an Ohaus scale and took a photo of each lizards ventrum. From this photo we later measured the snout-vent length (SVL) as the distance (in mm) from the snout to the cloaca, and the tail length as the distance from the cloaca to the tip of the tail using imageJ (Abràmoff et al. 2004). Total length was the sum of these two.

We set up 12 CCTV cameras with each recording four individual terraria simultaneously. Each week we selected 48 juveniles from the correct age window – a mix of the two species and four treatment combinations. We weighted and photographed the animals, and then placed them randomly across the shelves to avoid any biases associated with the location in the laboratory. The terraria for the assays were opaque to avoid lizards viewing each other which could influence their behaviour. All enclosures had a refuge and a water container. The heat lamp was placed on one side of the enclosure to ensure a thermal gradient of at least 6 degrees between the lamp and the refuge. The same group of animals were housed in these terraria during the 6 days of the trials.

Over the six days animals were under cameras we took three measurements of performance and antipredator behaviour for each animal with one day between subsequent measurements. More specifically, we collected the following behavioural variables:

1. *Running velocity (in seconds)*: We measured running performance as the total time needed to run the one-metre-long straight racetrack. We also recorded burst speed as the fast 25 cm section (the racetrack had a detector and time tracker every 25cm). For analysis, we used the total time used to cover the full one meter and the fastest time taken to run a 25 cm interval. Fifteen minutes before each performance measure, lizards were placed in an incubator at 28°C to ensure constant body temperature across lizards. After the trial, each animal was placed back in their corresponding terraria. We repeated the running trial 3 times for each individual, on alternate days.
2. *Activity*: We measured the distance travelled (in cm) as a proxy for the activity level of individuals. To record activity, we removed the refuge and the water container from the terraria to avoid animals hiding. We then switched on the camera and left the animals to behave and move freely in their terrariums for 20 minutes. The distance covered was later calculated using the software EthoVision XT (vers. 12.0).
3. *Antipredator behaviour*. Immediately after the activity trial, we replaced the refuge. After 30 minutes, we simulated a predatory attack. To do this, we approached the terraria and tapped the animals with a painting brush near the tail until they took refuge. The same person (JZ) performed all the predatory attacks. The only identification in the terraria was the randomly allocated ID number provided to the eggs at the beginning of the experiment such that JZ was blind to the treatment. After the simulated attack, we recorded each lizard’s response for 90 minutes. From the videos we calculated (1) the time (in seconds) each lizard took to seek refuge since the first tape in the tail (hereafter “time to hide”) (2) the time between the moment the animal took refuge (time to hide) until the animal’s head appeared at the entrance and was clearly visible in the videos (hereafter “hiding time”, in seconds) and (3) the time elapsed since the animal took refuge (time to hide) to the moment the animal left the refuge to start their normal activity after the predatory attack (hereafter “time to activity”). We consider this to be the moment when the back forelimbs left the refuge. We repeated the activity and antipredator assays three times in alternate days from performance trails.

All trials took place between 9 and 12 am, during the period when activity was the highest. We recorded 22 individuals per species and treatment. We discarded from the analysis any individual that lost their tail during the experiment (n = 5 out of 176) to avoid any bias associated with impaired running ability. The final sample sizes were 22 for hot-control *L. delicata*, and 23:C and 28:R *L. guichenoti*, and 21 for the rest of the combinations.

*Statistical analysis*

We used Bayesian Multivariate Mixed Effects Model in the package brms to explore whether development temperature and maternal investment impacted morphology (tail length, SVL and weight), performance (running velocity and activity level) and antipredator behaviour, and also to estimate the correlations between the variables measured at the between- and within-individual level. We first check for normality of the data by visualizing the residuals of intercept only random effects model. To meet the assumptions of normality running velocity (both 25 cm burst and 1 m long) was log-transformed in the case of *L. delicata*, and both running velocity and antipredator behaviour in the case of *L. guichenoti*.

We then ran two separate multivariate mixed models, one with morphological traits, and the other with performance and antipredator behaviour variables as response variables. Both models had a random intercept, and the interaction between development temperature and maternal investment was added as explanatory variable for each of the measured traits. In the morphology model, clutch was added as random factor. Due to the approach used during the experiment and our aim of testing animals from all treatments simultaneously to avoid any bias (see above), individuals from the hot temperature were older (they had shorter egg development times). To correct for this age effect on morphology, we added the age scaled to mean of 0 (using the function *scale*) as a covariate in the model. In the behaviour and performance multivariate model, individual and clutch identity were added as random factors and scaled SVL as covariate to control for any potential effect of body size on the traits measured. When the 2-way interaction between temperature and maternal treatments was non-significant, we repeated the models without the interaction to interpret the main effects. Both species were analysed separately. Most of the performance and antipredator variables showed high repeatability between the three different measures taken (Table S1).

**Results**

*Early thermal environment and maternal investment interact to affect morphology in both L guichenoti and L delicata*

We found a statistically significant two-way interaction between development temperature and maternal yolk investment in both SVL and tail length of both lizard species (*L. delicata*: SVL: estimate + SE = -0.85 + 0.35, 95% CI = -1.55– -0.16; tail length: estimate + SE = -4.21 + 0.82, 95% CI = -5.82– -2.62; *L. guichenoti*: SVL: estimate + SE = 1.18 + 0.35, 95% CI = 0.49–1.87; tail length: estimate + SE = -1.33 + 0.54, 95% CI = -2.38– -0.28, Tables S). In *L. delicata*, while individuals from the maternal control treatment had similar size and tail length when reared at hot or cold temperatures, in the yolk reduced treatment, individuals reared at hot temperatures were larger than those from cold temperature (SVL: HPDI = 0.516 – 1.769; tail length: HPDI = 3.787 – 6.593, Fig 1a,b). In *L. guichenoti*, instead, juveniles reared at hot temperatures were in general larger, but the effect was higher in the control yolk treatment than in the yolk reduced one (Fig 1d,f).

In addition, in both species, there was a positive correlation between SVL, tail length and body mass, with individuals with larger bodies also being heavier and having longer tails (Supp Info Table S2).

*Maternal investment and temperature impact performance in L guichenoti but not in L delicata.*

After controlling for body size, we found a two-way interaction between maternal yolk investment and development temperature in the 25cm burst of juveniles of *L. guichenoti* (estimate + SE = -0.41 + 0.20, 95% CI = -0.81– -0.01, Fig 2d, Table S4), however analyses of posteriors did not yield any significant comparison, so we interpret this interaction with caution.

In *L. delicata*, we found that juveniles from the yolk removed treatment took longer to run the 1m long track than those from yolk control treatment (estimate + SE = -0.27 + 0.1, 95% CI = -0.46– -0.08, Fig 2a). However, we did not find any effect of development temperature, maternal investment or the 2-way interaction between them in neither the amount of time needed to cover 25 cm or the distance travelled (Fig2a-c, Table S3).

In both species, trials where individuals had a faster burst speed also took shorter to travel the full 1m rack (*L. delicata*: estimate + SE = 0.42 + 0.06, 95% CI = 0.29–0.54, Table S3; *L. guichenoti*: estimate + SE = 0.51 + 0.06, 95% CI = 0.4–0.62, Table S4).

*Antipredator behaviour is affected by early thermal environment and maternal investment in L. delicata but not in L. guichenoti*

The time to activity after a simulated predatory attack was affected by the 2-way interaction between development temperature and maternal yolk investment in *L. delicata* (estimate + SE = -1008.81 + 503.67, 95% CI = -2023.9– -22.82, Table S3). While in the yolk control treatment individuals reared at both hot and cold temperatures showed similar time to activity, in the yolk reduced treatment individuals reared at hot temperatures took longer to be active (Figure 3b). However, the comparison of the posteriors was non-significant (HPDI: -9.024 – 26.115), so we interpret this 2-way interaction with caution. Antipredator response of *L. guichenoti* (Table S3) was not influenced by early developmental temperature, maternal investment or the 2-way interaction between these factors after correcting for body size (Fig3c,d).

In *L.delicata* in addition, we found a correlation at the between-individual level between the distance travelled and time to activity, so that more active individuals took in general shorter to be active after a simulated predatory attack (estimate + SE = -0.2 + 0.09, 95% CI = -0.37- -0.01). However, we did not find any correlation between running performance and anti-predator behaviour in neither of the two species (Table S4), indicating that individuals who ran slower on average did not also exhibit risk-adverse behaviours.

**Discussion**

In ectotherms, both thermal conditions experienced during development and maternal resource investment in eggs (in terms or yolk and hormone quantity) can impact population viability via their effects in the expression of offspring phenotypic traits, physiology, and behaviour. Climate change and other anthropogenic factors are likely to simultaneously alter not only temperature but also mother food availability and stress levels. Hence, it is critical to explore whether developmental temperature and maternal egg investment interact to shape plastic responses in offspring, and to what extent mothers can buffer the potential negative consequences of increased temperatures. Here we explored whether cold and hot incubation temperatures lead to different responses in morphology, performance and antipredatory behaviour in two sympatric skink species (*L. delicata* and *L. guichenoti*), and whether a controlled manipulation in the amount of yolk in eggs moderated such responses. We did not find any interaction between temperature and maternal investment in any of the traits measured, but both factors independently shaped species-specific plastic responses in some of the variables.

We found that, after controlling for age, juveniles of *L. guichenoti* emerging from eggs incubated at the hot temperature were larger and heavier than those reared at cold temperatures. However, an *ad hoc* analysis showed us that such effect of incubation temperature was not present the day of hatch (e.g., we did not find a significant effect of development temperature on recently hatched animals) and instead, the difference in body size between juveniles coming from cold and hot incubation regimes appeared during the 3-5 weeks elapsed between the hatch and the behavioural tests. Our results suggest that rather than a change in metabolic and developmental rates during embryo growth, incubation temperature programmed a post-hatching metabolic and growth physiology, potentially affecting feeding and growing rates, as observed in previous studies in lizards exposed to high temperatures (Singh et al. 2020). Other refs

In agreement with other studies (e.g., (Warner and Lovern 2014) our *ad hoc* analysis also showed that recently born juveniles were in general smaller in the yolk reduced treatment than in the control, however, in our study, maternal investment effects on body size disappeared by the age of the behavioural tests. This suggests that individuals can to some extent compensate the poor start in life by accelerating their growing rates during the first few weeks, probably by increasing their feeding rates. This is key, since in juvenile lizards, larger body sizes have been generally associated with better survival (refs). Yet, such compensation in body size might be associated with trade-offs in other fitness-associated traits, such as the observed slower size-corrected running speed of *L. delicata* juveniles hatched from yolk-reduced compared to those from control eggs. This result suggests that even though the animals from poor development conditions are able to morphologically catch up with individuals with more beneficial early-life conditions, this might come with a cost regarding performance. For instance, in the lizard *Sceloporus undulatus*, clutches with individuals with fast grow rates but slow runners had lower survival rates than slow growing but fast runners (Warner and Andrews 2002). However, we don’t know whether the reduction in running speed detected in *L. delicata* is below the threshold that might influence prey capture ability or vulnerability to predators (Shine 2003), and hence whether the reduced running ability observed might constrain survival. For instance, despite the highly exploratory behaviour of the species, individuals spend most of their time sheltered and near the refuge (Chapple et al. 2011), which would likely allow them to scape a predatory attack even under limited speed. In addition, the fact that the antipredatory behaviour was not similarly affected by the maternal investment treatment, and that within-individual responses did not show any correlation between performance and antipredatory responses, shows that individuals did not adjust their antipredatory responses to balance the potentially higher vulnerability. This finding therefore suggests that the observed reduction in running speed might not impose a survival cost.

Interestingly, we did not find any effect of egg development temperature on performance or antipredatory behaviour. This result agrees with some previous studies in reptiles that did not find any effect of incubation temperature in running speed and in the response to predators (refs), while contrasts with others that found, for instance, that higher development temperatures lead to both faster and slower runners (reviewed in (Booth 2006), including in *L. delicata* (Downes and Shine). One potential explanation for the lack of effect of incubation temperature in juvenile performance and behaviour in our experiment is the age at which animals were tested. Previous studies have found an impact of development temperature on a range of morphological and behavioural traits on young individuals, while the effect disappears as the individuals age (Pearson and Warner 2016; McDonald and Schwanz 2018). However, this is not always the case, and previous evidence also shows that for some species and for specific conditions, the effects of the development environment can be long-lasting (Elphick and Shine 2006; Trnik et al. 2011). Another alternative explanation is the thermal regime used, which is within the natural range of nest temperature of the species tested (Cheetham et al. 2011). The direction and extent in the expression of plastic changes in traits and behaviours, and the potential maladaptive consequences of such responses, depend on the thermal regime embryos are exposed to (e.g., (Van Damme et al. 1992; Braña and Ji 2000)). Our species are likely to be adapted to the temperatures used in our study, since they overlap with those experienced by our species in the wild, and therefore could explain the lack of stronger plastic responses. However, with anthropogenic climate change, the temperatures experienced in the nest are likely to increase, therefore, it is critical to explore how extreme thermal incubation conditions on it’s own and in interaction with different treatments of maternal investment will affect hatchling performance and survival.

Maybe add a short paragraph on species differences? Although not much to say.

Add somehow Beltran et al Efect of early thermal environment on the morphology and performance of a lizard species with bimodal reproduction somewhere

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**Author contribution:** MI-C and DN conceived and designed the experiment; MI-C, OZ and KD collected the data; MI-C analysed the data and drafted the first version of the manuscript; all authors contributed to the draft and gave final approval for publication.

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**Figures**

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Figure 1. Effect of temperature (cold vs hot) and maternal resource investment (yolk reduced, control) in morphological traits of *L. delicata* (a, b and c) and *L. guichenoti* (d, e and f).

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Figure 2. Effect of temperature (cold vs hot) and maternal resource investment (yolk reduced, control) in lizard performance: running velocity (25 cm and 1 m) and distance moved. *L. delicata* panels a, b and c, and *L. guichenoti* panels d, e and f.

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Figure 3. Effect of temperature (cold vs hot) and maternal resource investment (yolk reduced, control) in lizard antipredatory behaviour: time hiding (time, in seconds, since the lizard hided in the refuge until the appearance of the head) and time to active (time until the animal left the refuge and started being active again. *L. delicata* panels a and b, and *L. guichenoti* panels c and d.