Early environmental conditions do not impact associative learning in two species of skink

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## Abstract

Acquiring new information through learning is essential for animals to adjust their behaviour adaptively. However, animals’ abilities to learn can be constrained by the conditions experienced during early stages of development, when the brain is especially susceptible to environmental inputs. Temperature is a pervasive mechanism of phenotypic plasticity in ectotherms, exerting direct effects on growth, metabolism, or learning. In vertebrates, abrupt thermal changes can trigger an allostatic response, which is mediated by glucocorticoids - stress-related hormones - that can be transmitted to the offspring, potentially impacting their learning abilities. Thus, glucocorticoids and the thermal environment are predicted to have interactive effects on the development of learning functions in animals. Here, we investigated the combined consequences of corticosterone (CORT) and temperature elevations on learning in two species of lizards, *Lampropholis delicata* and *L. guichenoti*. We manipulated prenatal CORT levels and incubation temperature in a 2x2 factorial design, and then subjected juveniles to a colour-associative learning task. In the learning task lizards had to associate the attainability of a food item with the colour of the feeder. We perfomed 35 trials per individual and recorded whether lizards made the correct choice or not. In our analyses, we compared the rate of behavioural change over time (learning slopes) between treatments. We anticipated that elevated CORT and low temperatures would impair lizards’ performance. However, our results show that associative learning skills in these two species is robust to prenatal temperature and CORT. We also found a significant effect of the colour employed as the correct choice in our tests for *L. delicata*, underscoring the need to carefully select the color used in cognitive tests involving visual stimuli.

## Introduction

Cognition is defined as the processes by which animals gather, preserve, and use information from their environment through perception, learning, memory, and decision making ([Shettleworth 2010](#ref-shettleworth)). These cognitive processes are fundamental to foraging, mate selection, antipredatory strategies, and social behaviours, all of which are crucial for survival and reproduction ([Dukas 2004](#ref-dukas_evolutionary_2004)). Particularly, learning - the acquisition of new information ([Dukas 2004](#ref-dukas_evolutionary_2004)) - is essential for coping with environmental changes by enabling individuals to create new associations between events ([Dukas 2004](#ref-dukas_evolutionary_2004); [Leal and Powell 2012](#ref-leal_behavioural_2012); [Buchanan et al. 2013](#ref-buchanan_condition_2013)). Allowing animals to adequate their behavior to their conditions implies that learning can have cascading effects on population dynamics by influencing survival rates or reproductive success. However, the capacity to acquire information varies among individuals, shaped by factors such as age, sex, gut microbiota, and the developmental environment ([Szuran et al. 1994](#ref-szuran_water_1994); [Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Amiel and Shine 2012](#ref-amiel_hotter_2012); [Amiel et al. 2014](#ref-amiel_egg_2014); [Carazo et al. 2014](#ref-carazo_sex_2014); [Noble et al. 2014](#ref-noble_age-dependent_2014); [Alemohammad et al. 2022](#ref-alemohammad_2022_microbiota_learning)). These variations can further affect population dynamics, as differences in learning ability may lead to unequal responses to environmental pressures. Therefore, understanding the factors that shape learning is crucial to predict how populations will respond to novel circumstances.

Developing conditions are especially relevant in shaping learning abilities because the brain is highly susceptible to environmental inputs during early stages of development ([Zhu et al. 2004](#ref-zhu_prenatal_2004)). Impacts on brain development can have long-lasting effects on cognitive abilities, potentially influencing an individual’s ability to learn and adapt to new environments ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Amiel and Shine 2012](#ref-amiel_hotter_2012); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). For instance, prenatal glucocorticoids (GCs) - hormones related to organisms’ response to allostasis ([Sapolsky et al. 2000](#ref-sapolsky_how_2000)) - and prenatal thermal environment, are predicted to be important factors shaping learning abilities (see [Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Amiel and Shine 2012](#ref-amiel_hotter_2012); [Crino et al. 2014a](#ref-crino_corticosterone_2014); [Amiel et al. 2014](#ref-amiel_egg_2014); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). Elevating GCs during early stages of development is typically associated with learning impairments in different taxa ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Eaton et al. 2015](#ref-eaton2015mild); [Farrell et al. 2015](#ref-farrell_developmental_2015-learn)). However, factors like sex or the nature of the learning task can affect the direction and magnitude of these effects ([Szuran et al. 1994](#ref-szuran_water_1994); [Crino et al. 2014b](#ref-crino_corticosterone_2014-learn); [Farrell et al. 2015](#ref-farrell_developmental_2015-learn); [Bebus et al. 2016](#ref-bebus_associative_2016)). In ectotherms, temperature is a pervasive mechanism of phenotypic plasticity, influencing a suite broad spectrum of traits, including growth, metabolism, or learning abilities ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Amiel et al. 2014](#ref-amiel_egg_2014); [Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Noble et al. 2018](#ref-noble_developmental_2018); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). High incubation temperatures have been linked with faster learning rates in skinks ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Clark et al. 2014](#ref-clark_colour_2014); [Amiel et al. 2014](#ref-amiel_egg_2014)), while for velvet geckos, being incubated at temperatures beyond their natural thermal range reduce their performance in learning tasks compared to those incubated at colder temperatures ([Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). Learning effects of prenatal GCs and temperature are both related to alterations in neurogenesis, neural structure, and brain metabolic activity ([Coomber et al. 1997](#ref-coomber_independent_1997); [Sakata et al. 2000](#ref-sakata_neural_2000); [Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Du et al. 2009](#ref-du_dynamic_2009); [Amiel et al. 2017](#ref-amiel_effects_2017); [Beltrán et al. 2021](#ref-beltran_are_2021)), suggesting that both factors could be impacting similar neurological mechanisms to influence learning abilities. Furthermore, GCs can play an pivotal role in determining vertebrate responses to elevated temperatures ([Crino et al. 2023](#ref-Crino_2023)) potentially fostering natural interactions between temperature and GCs. This implies that the effects of GCs and temperature on learning could be interdependent, with the impact of one factor being modulated by the other. Nevertheless, the interactive effects of GCs and temperature on learning abilities remain unexplored.

In this study, we investigated the combined effects of prenatal GCs and thermal environment on learning in two species of skinks, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). We manipulated corticosterone (CORT) - the main GC in reptiles ([Crino et al. 2023](#ref-Crino_2023)) - levels in the eggs and then incubated them at two different temperatures in a 2X2 factorial design. Post-incubation, juveniles were subjected to a colour-associative task to assess their learning abilities. We hypothesise that prenatal CORT levels and the thermal environment will induce sustained effects on the brain’s physiology, ultimately impacting learning skills ([Zhu et al. 2004](#ref-zhu_prenatal_2004); [Amiel and Shine 2012](#ref-amiel_hotter_2012); [Eaton et al. 2015](#ref-eaton2015mild); [Amiel et al. 2017](#ref-amiel_effects_2017)). We predicted that individuals exposed to high levels of CORT or low temperatures would perform less proficiently than control individuals or those incubated at higher temperatures. Additionally, we predicted that incubation at high temperatures will mitigate the impact of CORT on skink performance, while cold incubation temperatures are expected to enhance the detrimental effects of CORT on learning. This may occur for two reasons that are not mutually exclusive: first, an increase in temperature leads to an overall rise in neural density ([Amiel et al. 2017](#ref-amiel_effects_2017)), thereby counteracting the impact of CORT (see [Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Eaton et al. 2015](#ref-eaton2015mild)); and second, the elevated metabolic rate associated with higher temperatures can accelerate CORT metabolism, resulting in embryos being exposed to CORT for a shorter time.

## Methods

#### Subjects

*L. guichenoti* and *L. delicata* are small (∼35–55 mm snout-vent length (SVL)), oviparous, and generalist skinks that usually share the same habitat in suburban areas throughout south-eastern Australia ([Chapple et al. 2011](#ref-chapple_know_2011)). Both species have similar breeding periods, but with some differences in reproductive output: while *L. delicata* lays 1 to 6 eggs in only one clutch per season, *L. guichenoti* clutches are smaller (1-5 eggs per clutch) and they usually lay two clutches per season ([Chapple et al. 2011](#ref-chapple_know_2011), [2014](#ref-chapple_biology_2014)). Previous studies exploring behavioral differences between the two species have found *L. delicata* to be more exploratory than *L. guichenoti* ([Chapple et al. 2011](#ref-chapple_know_2011)). However, no differences in learning were observed between the skinks in an associative learning task ([Bezzina et al. 2014](#ref-bezzina2014does)).

#### Husbandry

*Breeding colony* – We tested juveniles coming from a breeding colony established in the lab since 2019. There was a total of 270 and 180 adults of *L. delicata* and *L. guichenoti* respectively, housed in big containers (41.5 L x 30.5 W x 21 H cm) with six lizards (2 males and 4 females) per enclosure. Enclosures are provided with non-stick matting, shelter, and several small water dishes. Water is given daily, and they are fed approximately 40 mid-size crickets (*Acheta domestica*) per enclosure three days a week. Crickets are dusted with calcium weekly and multivitamins and calcium biweekly. To ensure a temperature gradient, we employed a heat chord and a heat lamp following a 12 h light:12 h dark cycle. Room temperatures were set to 22-24 °C, and the warm side of enclosures is usually at 32 °C.

*Eggs collection and incubation* – Between mid-October 2022 to the end of February 2023, we provided females with a place to lay the eggs by means of small boxes (12.5 L x 8.3 W x 5 H cm) with moist vermiculite inside, that were placed in one extreme of the communal enclosures (see above). We checked for the presence of eggs in the boxes three days a week. After collection, we measured length and width of eggs with a digital caliper to the nearest 0.1 mm, mass using a digital scale (OHAUS, Model spx123; ± 0.001g), and assigned individual IDs to the clutch and each egg. Eggs were treated with CORT or vehicle (see CORT and Temperature manipulation below) and were placed in individual cups (80 mL) with moist vermiculite (12 parts water to 4 parts vermiculite). The cups were covered with cling wrap to retain moisture and left in LATWIT 2X5D-R1160 incubators at two different temperatures (see CORT and Temperature manipulation below) until hatching. Eggs were then checked three times a week for hatchlings.

*Hatchlings* – After hatchling, we measured SVL and Tail Length (TL) with a ruler to the nearest mm and mass (g) with a digital scale(OHAUS, Model spx123;± 0.001). We then placed hatchlings in individual enclosures (18.7L x 13.2W x 6.3H cm) and provided them with non-stick matting, shelter, and a small water dish. During this period, they were sprayed with water every day and received 3-6 small *A. domestic* crickets three times a week. All care otherwise follows similar protocols to adults (see above).  
 Two weeks prior to the training phase (see details below), lizards were moved to the experimental arena for acclimatisation. The arenas were individual medium size (41 L x 29.7 W x 22 H cm) plastic containers with a shelter (9 L x 6 W x 1.5 H cm) on one of the sides and a water dish on the other. These new enclosures were placed in two rooms in 7 different racks that were monitored by 7 different CCTV systems (device model DVR-HP210475) that allowed us to record their behaviour during the experiment (see details below). Although the conditions in the new room were identical to the main room, the number of lizards per species and treatment in each rack was counterbalanced to control for any potential effect of the room or the position of the lizard in the rack. During acclimatisation and all the experiment, lizards were fed with only one cricket per day dusted with calcium and multivitamin (see protocol below), and water was supplied *ad libitum*. We provided a temperature gradient by means of a heat cord and heat lamps in a 12 h light: 12 h dark cycle.

#### CORT and Temperature manipulation

To empirically test the effect of early environment we manipulated CORT concentration in eggs and incubated them under one of two temperature regimes (‘Cold’ - 23ºC ± 3ºC or ‘Hot’ - 30ºC ± 3ºC) in a 2x2 factorial design ([Fig. 1](#fig-Methods) A). We first allocated eggs to one of two different treatments: CORT treatment, where eggs were topically supplied with 5µL of CORT dissolved in 70% Ethanol and 30% DMSO (vehicle) at a final (10 pg CORT/mL) concentration (CORT treatment); and a Control treatment, where eggs received an equal volume of the vehicle. We selected these doses based on previous studies publishing yolk CORT concentrations in other oviparous reptiles ([Lovern and Adams 2008](#ref-lovern2008effects); [Hanover et al. 2019](#ref-hanover2019corticosterone)) and our own unpublished data. CORT concentration represents 2 standard deviations above the mean natural concentration. After the treatment, the eggs were incubated in one of the two previously mentioned temperature regimes (‘Cold’ or ‘Hot’) until hatching. The number of eggs per clutch assigned to each hormone and temperature treatment was counterbalanced in both species.

#### Learning

To estimate learning skills, we tested skinks’ ability to locate a food reward in a colour-associative learning task ([Fig. 1](#fig-Methods) C, D). First, we performed a training phase where lizards had to learn to eat from white 3D-printed PLA ramps (9 L x 4 W x 5 H cm) identical to the ones from the experiment except for the colour (see below). We divided this training phase into three stages: the first stage where lizards had to eat a small frozen cricket (*A. domestica*) from an opaque petri dish (3D x 1.6H cm) placed in the middle of their enclosure ([Fig. 1](#fig-Methods) C, Stage 1); the second stage where the petri dish with the cricket was placed on top of the white 3D printed ramps ([Fig. 1](#fig-Methods) C, Stage 2); and the third stage where the cricket was left inside a well (3D x 1.75H cm) on the top of the ramp ([Fig. 1](#fig-Methods) C, Stage 3). Every trial began when we left the feeding block (petri dish, ramp, or both) inside the enclosure and finished one hour later when we took it away. At the end of each trial, we recorded whether the cricket had been consumed or not. Trial was considered successful if the lizard could locate and consume the reward, while completion of each stage required the lizards to eat the crickets in at least 5 out of 6 trials to ensure lizards’ consistency. This phase lasted 38 days until all the lizards could eat from the ramp; only in one case we decided not to use the lizard because its behaviour was not consistent over the course of the training phase.

During the associative learning phase, we trained lizards to associate colour with a food reward ([Fig. 1](#fig-Methods) D). This test was like the third stage of the training phase, but here lizards were presented with three feeders that differed in colour. We placed the food reward (small, frozen, *A. domestica* crickets) inside the wells of the three feeders, covering two of the crickets with 3D-printed lids (3D x 0.5H cm) so prey was only accessible in “the correct” ramp. The food reward was placed inside all three wells to avoid lizards using prey chemical cues, and the lids had a series of small holes on the top to allow the release of those chemicals. The colours of the feeders were green, red, and blue, as previous studies demonstrate that squamates can discriminate between these colours ([Baden and Osorio 2019](#ref-Baden_Osorio_2019_Vert_vision)). To control for potential colour preference that could bias our results, we split the subjects into two groups counterbalanced by treatment and species: in one group, the correct choice (i.e., the ramp with the non-covered frozen cricket) was the blue one, while for the other group, we assigned red as correct. In all trials, the position of the feeders was changed randomly to ensure subjects were using colour rather than spatial cues for the association. Lizards were tested in this task once a day for 35 days.

The full task took place between the 6th of March until the 17th of May 2023, and tests were performed between 11 to 12 am when the lizards were active. Trials in the learning phase were recorded with CCTV systems always using the same camera per individual. We recorded whether the animal chose the correct ramp first or not. We considered that a choice was made if the head of the lizard was inside the well of one of the ramps. We considered a trial failed if there was no choice in one hour of recordingcored those trials as NA. We excluded from our analyses those individuals with more than 15 trials failed (i.e. they did not make a choice), and we considered the first trial for each individual to be the first one where the a choice was made.

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| Fig 1— Experimental design of the experiment. Panel A shows the early environment manipulation. In panel B, the measurements of the 3D-printed ramps employed in the habituation and learning tasks. Panel C shows the habituation process with the three different stages. And in panel D, the associative task. White lids in \*\*D\*\* show the ramps where the food reward was not attainable. |

#### Statistical analyses

We performed the analyses for species separately. Because preliminary analyses showed a significant effect of the colour assigned, we decided to split the data by colour (blue or red). Therefore, we run a total of four different Bayesian multilevel models using the brm function from the brms package (Bürkner, [2017](#ref-burkner2017brms)) in Quarto (Allaire et al., [2022](#ref-Allaire_Quarto_2022)). We ran four parallel MCMC chains of 3000 iterations for each model, with a warmup period of 1000 iterations.

We employed correct choice [correct (1) or not (0)] as the response variable, and trial, incubation temperature (Cold versus Hot), hormone (CORT versus Control), and the three-way interaction as fixed factors. The error structure was modeled using a Bernoulli distribution with a logit link function (family = Bernoulli(link = ‘logit’)). We included a random intercept and slope (trial) for each lizard in our models. Furthermore, even if our split clutch design should control for potential effects of paternity, we also incorporated the clutch identity as a random factor. Because eggs were collected during half of the breeding season and both species do not lay more than two clutches ([Chapple et al. 2011](#ref-chapple_know_2011), [2014](#ref-chapple_biology_2014)), each clutch likely comes from a unique mother, and so, clutch identity captures potential maternal effects. Learning in lizards can be age-dependent ([Noble et al. 2014](#ref-noble_age-dependent_2014)), and given that incubation temperature will affect hatching time, we explored the effects of age on learning. Lizards’ age at the beginning of the experiment ranged from 41 to 148 days old in *L. delicata* and 48 to 132 in *L. guichenoti*. However, when this variable was included in the models we did not find any significant effect of age (see Supplementary material). As such, we present models without age as a fixed effect.

We used the posterior distributions of parameters from these models to test for differences in learning rate between treatments and species. Learning reates were estiamated as the choice-trial slope, obtained for each treatment by using the ‘trial’ estimates and its interaction with hormone and temperature. Slope estimates greater than zero were considered as evidence of learning, while those less or equal to zero were not. We employed the posterior predictive Markov chain Monte Carlo (pmcmc) to test the hypothesis that slopes and slopes contrasts were different from zero. We considered statistical significance if pmcmc < 0.05.

## Results

Originally, we started with 96 lizards, 48 individuals per species and 12 per treatment per species. However, due to natural mortality (n = 11), no completion of the training stage (n = 1), or no motivation during the learning tasks (n = 3), we ended up with a total of 81 lizards. Final sample sizes per treatment and species are listed in [Fig. 2](#fig-deli) and [Fig. 3](#fig-guich) (figures for both species with the raw data are included in the Supplementary Material). Mean slopes per treatment for both species are provided in Table 1 in Supplementary Material.

We found that that slopes for learning were lower when the blue feeders were the correct choice compared to slopes of individuals assigned to the group ‘Red’ for *L. delicata* (‘Blue’ mean learning slope = 0.023, 95 CI = [-0.041 , 0.090]; ‘Red’ mean learning slope = 0.085, 95% CI = [0.030 , 0.143]; slope contrast: ‘Blue’ learning slope - ‘Red’learning slope = -0.062, p~mcmc 0.12), but not for *L. guichenoti* (’Blue’ mean learning slope = 0.063, 95% CI = [-0.030 , 0.149]; ‘Red’ mean learning slope = 0.103, 95 CI = [0.032 , 0.180]; ‘Blue’ learning slope - ’Red’learning slope = -0.041, p~mcmc 0.46). However, further analyses using the first trial indicated a potential bias towards blue in the initial choice (see Supplementary Material) that could affect the estimated slopes. As such, we analysed the differences between treatments for each group separately.

For those *L. delicata* assigned to group ‘Red’, we did not find any significant differences between hormones (Control - CORT = -0.110, pmcmc < 0.05), temperature (Hot - Cold = -0.004, pmcmc format\_p(pmcmc(c(dar\_ControlHot, dar\_CORTHot) - c(dar\_ControlCold, dar\_CORTCold)), 3)), or the interaction ([(Control-Hot - CORT-Hot) - (Control-Cold - CORT-Cold)] = 0.061, pmcmc 0.17) ([Fig. 2](#fig-deli) A, B). Similarly, those assigned to ‘Blue’ were not afected by CORT (Control - CORT = -0.083, pmcmc 0.06), temperature (Hot - Cold = 0.040, pmcmc format\_p(pmcmc(c(dab\_ControlHot, dab\_CORTHot) - c(dab\_ControlCold, dab\_CORTCold)), 3)), or the interaction ([(Control-Hot - CORT-Hot) - (Control-Cold - CORT-Cold)] = 0.041, pmcmc 0.41) ([Fig. 2](#fig-deli) C, D).

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| Fig 2— Results for Lampropholis delicata for both colour groups ‘Red’ (A,B) and ‘Blue’ (C, D). Panels A and C show the predicted probability of choosing the correct feeder first over trials. The lines represent the mean predicted probability of choosing the correct feeder first on each trial, and the shaded areas indicate the standard deviation of the mean; both were obtained by using the slope and intercept estimates from the posterior distributions. The different colours indicate the different treatments. Panels B and D show the distribution of the estimates of slopes per each treatment. The x-axis represents the slope estimate, and in the y-axis are the density of the estimates. The different colours indicate the different treatments. Points and bars represent the mean and standard deviation of the mean of the estimates, respectively. |

In *L. guichenoti*, we did not find any significant differences between hormones (Control - CORT = -0.078, pmcmc 0.18), temperature (Hot - Cold = -0.032, pmcmc format\_p(pmcmc(c(gar\_ControlHot, gar\_CORTHot) - c(gar\_ControlCold, gar\_CORTCold)), 3)), or the interaction ([(Control-Hot - CORT-Hot) - (Control-Cold - CORT-Cold)] = 0.026, pmcmc 0.66) for those assigned to the ‘Red’ group ([Fig. 3](#fig-guich) A, B); and we did not find any significant effect of hormone (Control - CORT = -0.134, pmcmc < 0.05), temperature (Hot - Cold = 0.040, pmcmc format\_p(pmcmc(c(gab\_ControlHot, gab\_CORTHot) - c(gab\_ControlCold, gab\_CORTCold)), 3)), or the interaction ([(Control-Hot - CORT-Hot) - (Control-Cold - CORT-Cold)] = 0.016, pmcmc 0.79) for those assigned to ‘Blue’ ([Fig. 3](#fig-guich) C, D).

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| Fig 3— Results for Lampropholis guichenoti for both colour groups ‘Red’ (A,B) and ‘Blue’ (C, D). Panels A and C show the predicted probability of choosing the correct feeder first over trials. The lines represent the mean predicted probability of choosing the correct feeder first on each trial, and the shaded areas indicate the standard deviation of the mean; both were obtained by using the slope and intercept estimates from the posterior distributions. The different colours indicate the different treatments. Panels B and D show the distribution of the estimates of slopes per each treatment. The x-axis represents the slope estimate, and in the y-axis are the density of the estimates. The different colours indicate the different treatments. Points and bars represent the mean and standard deviation of the mean of the estimates, respectively. |

Finally, we also did not find any significant differences when we compared the estimated slopes between species for ‘Red’ group(*L. delicata* mean learning slope = 0.077, 95% CI = [0.026 , 0.135]; *L. guichenoti* mean learning slope = 0.092, 95% CI = [0.023 , 0.171]; *L. delicata* - *L. guichenoti* = -0.016, pmcmc 0.72) or the ‘Blue’ one (*L. delicata* mean learning slope = 0.023, 95% CI = [-0.041 , 0.090]; *L. guichenoti* mean learning slope = 0.063, 95% CI = [-0.030 , 0.149]; *L. delicata* - *L. guichenoti* = -0.040, pmcmc 0.40).

## Discussion

The scope of the present study was to investigate the effects of early environment on lizards’ learning using two closely related species, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). In our experiment, we manipulated CORT levels and temperature during incubation, and we then subjected hatched animals to a colour-associative learning task using food as a reward. We expected prenatal CORT and thermal environment to have combined effects on lizards’ performance in the test. However, we did not find any significant effects of the experimental conditions. We found that most individuals from both species learned to associate a colour with a food reward and that the learning process occurred at the same rate regardless of the early environment, suggesting that learning abilities in these skinks are robust to prenatal CORT and temperature. In addition, we found that the learning rates varied depending on the colour assigned to the correct choice, at least for *L. delicata*, with lizards assigned to blue feeders performing worse than the red group.

#### Effect of colour on learning

The colour of the ramp associated with the attainable cricket (i.e. the correct choice) influences learning slopes in *L. delicata*, but not *L. guichenoti*. For *L. delicata*, performance was proficient when the reward was linked to red feeders, but learning slopes for lizards assigned to the blue condition did not differ from zero. In contrast, *L. guichenoti* showed similar results in both colour scenarios. This indicates that *L. delicata* were not able to associate the blue ramp with the reward. However, our results could be consequence of an initial bias in lizards’ choice. For example show that, overall, the estimated probability of choosing the correct ramp in the first trial was higher than expected by chance for those animals assigned to the blue condition (See SI). Moreover, in those groups where we found that the learning slopes did not differ significantly from zero (see [Fig. 2](#fig-deli)), we also observed the highest probability of selecting blue ramps in the first trial (see Supplementary Material).

Because there was a possibility that this bias towards blue feeders was a byproduct of the habituation process, we compared the light spectrum and the perceived chromatic differences between the ramps used in the associative task and the ones used during habituation (see Supplementary Material). Since these results show no similarities between the white and blue ramps, we consider the preference towards blue to not be a derivate from our experimental design. Rather, it seems likely that lizards innately prefer the blue feeders. The species confidence hypothesis posits that animals should bias their attention towards colours they are familiar with ([Putman et al. 2017](#ref-putman2017fear)). For instance, dark and light blue T-shirts were associated with lower flight initiation distances and higher capture rates in Western fence lizards where blue is used in intraspecific communication ([Putman et al. 2017](#ref-putman2017fear)). However, to the best of our knowledge, noone has reported a colour bias in *L. delicata* or *L. guichenoti* before; and blue colouration is not considered to be involved in intraspecific communication in these species Chapple et al. ([2014](#ref-chapple_biology_2014)). Still, we recommend to select carefully the colours employed in associative tasks when relying on visual stimuli. Despite this, when lizards assigned to blue or red were analysed separately, the between treatments comparissons remain the same in both conditions and species.

#### Effect of prenatal CORT on learning

Prenatal CORT was expected to have a negative effect on learning, declining CORT-treated lizards performance in the learning task ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Eaton et al. 2015](#ref-eaton2015mild); [Farrell et al. 2015](#ref-farrell_developmental_2015-learn); [Bebus et al. 2016](#ref-bebus_associative_2016)). However, we did not find any significant difference between hormone treatments in none of the two species. Our results are consistent with other experiments that showed no effect of prenatal GCs on learning abilities ([Szuran et al. 1994](#ref-szuran_water_1994); [Bebus et al. 2016](#ref-bebus_associative_2016)). In this sense, GCs are known to have hormetic effects, with low or short exposure to GCs improving performance in learning tasks, and high levels impairing proficiency ([Du et al. 2009](#ref-du_dynamic_2009); [McEwen 2012](#ref-mcewen_brain_2012)). When using the same dose and application methods in eggs of *L. delicata*, Crino et al. ([2024](#ref-crino2024eggs)) found CORT treatment had sustained effects on growth, mitochondrial activity, and baseline CORT levels; and their findings also aligned with the predictions expected under high GCs elevations. This suggests that the absence of effects on learning abilities are not due to methodological caveats, but rather to *L. delicata* and *L. guichenoti*’s brain being robust to CORT elevations during development. However, since dose effects can differ between tissues ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000)), we cannot dismiss the possibility that our concentrations failed to elicit changes to the brain that would result in impaired learning.

Conversely, the absence of significant effects of prenatal CORT on learning could be due to the cognitive task employed, as some studies show that the impact of GCs can vary between brain regions ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000)). GCs action involve genomic and nongenomic mechanisms that implicate different types of receptors that can be distributed unevenly in the various regions of the brain ([McEwen 2012](#ref-mcewen_brain_2012)), and may be related to the learning performance in distinct tasks. For instance, in European starlings, males stressed during early stages of development performed worse in a visual associative learning task, but show no differences with control birds when the stimuli tested was acoustic ([Farrell et al. 2015](#ref-farrell_developmental_2015-learn)). Similarly, Scrub-jays that have lower CORT levels as nestlings performed better on an associative learning test as adults but not on a reversal-learning tasks ([Bebus et al. 2016](#ref-bebus_associative_2016)). We must acknowledge the possibility that prenatal CORT does not affect the brain regions involved in coding associative learning in these species, and future studies should focus on examining the effect of early-life stress on different cognitive domains.

#### Effect of incubation temperature on learning

We also expected hot-incubated lizards to perform better in the associative learning task since most of the studies demonstrate enhanced learning abilities when eggs are incubated at higher temperatures ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Clark et al. 2014](#ref-clark_colour_2014); [Amiel et al. 2014](#ref-amiel_egg_2014)). Furthermore, the opposite pattern was found only in studies that employed temperatures far above the natural thermal range of the species in their hot treatments ([Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)), and it is unclear how such conditions relate to wild environments. In *L. delicata* and *L. guichenoti*, associative learning appears to be robust to incubation temperature. Our results, therefore, do not align with the outcome observed our experiments. However, the effect of prenatal temperature on cognition, and brain physiology and structure has been investigated only in a small number of species (see [Coomber et al. 1997](#ref-coomber_independent_1997); [Sakata et al. 2000](#ref-sakata_neural_2000); [Amiel and Shine 2012](#ref-amiel_hotter_2012); [Clark et al. 2014](#ref-clark_colour_2014); [Amiel et al. 2014](#ref-amiel_egg_2014); [Amiel et al. 2017](#ref-amiel_effects_2017); [Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)), limiting our understanding on how thermal early environment can affect cognitive abilities.

In our analyses, we did not include sex as a factor for several reasons: first, lizads were tested before sexual maduration; and second, previous studies have found weak evidence for sex-dependent learning in reptiles ([Szabo 2019](#ref-szabo_sex_meta)). In fact, incubation temperature was not sex-depedent when learning abilities were tested in the Eastern three-lined skink (*Bassiana dupeyerri*) ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Clark et al. 2014](#ref-clark_colour_2014)). However, there is previous evidence for interactive effects of incubation temperature and sex on brain physiology in other species of reptiles ([Coomber et al. 1997](#ref-coomber_independent_1997); [Sakata et al. 2000](#ref-sakata_neural_2000)). Furthermore, in *B. dupeyerri* exposure to cold temperatures during incubation can induce sex-reversal, where individuals under cold incubation temepratures develop male phenotypes while retaining female genotypes ([Dissanayake et al. 2021](#ref-dissanayake2021effects)). Such effects were not easily accounted for in these experiments making it challenging to understand whether sex or temperature was the cause of learning differences ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Clark et al. 2014](#ref-clark_colour_2014); [Amiel et al. 2014](#ref-amiel_egg_2014)). Similarly, not accounting for sex in our analyses could be masking potential interactions between temperature and sex in shaping learning abilities. Future research should investigate the potential interactive impacts of sex and incubation temperature on cognitive abilities. Nevertheless, conducting such studies poses challenges due to the substantial sample sizes necessary.

#### Learning rates between species

We did not see any significant difference in learning rates when both species were compared. This result supports previous studies that found similar learning abilities in *L. delicata* and *L. guichenoti* ([Bezzina et al. 2014](#ref-bezzina2014does)). In the experiment conducted by Bezzina et al. ([2014](#ref-bezzina2014does)), both species failed to complete the associative learning task under the authors’ criterion, while in our experiment both species completed the task, exhibiting similar learning rates. Complexity, experimental design, or the criterion employed to define learning could be tha major cause of the discrepancies between ours and Bezzina et al. ([2014](#ref-bezzina2014does)). Developing common strategies and approaches to assess learning in animals could help understand how learning abilities are shaped in different taxa or under different environments.

#### Conclusion

Our results revealed that lizards completed the associative learning task at similar rates, with no significant differences observed between species or treatments. This outcome contrasts with our initial predictions, suggesting that the learning abilities of both species are resilient to the early environmental influences examined. Our findings indicate that the learning skills of these lizards may be more robust than anticipated under varying early life conditions. We also found significant effects of the colour employed in the task on learning rates in *L. delicata*, but not in *L. guichenoti*. These results seem to be a consequence of an innate colour bias and highlight the importance of carefully selecting the colour employed when testing cognition using visual stimuli.

Future research should continue exploring the potential effects of prenatal corticosterone (CORT) and temperature on cognitive function, but with a focus on interactions between early environmental factors and sex, as well as the specific nature of the cognitive tasks employed. Furthermore, it is crucial to explore how these treatments influence brain function at a neurological level. Investigating these aspects will help us understand these species' cognitive and physiological mechanisms underpinning adaptability and offer insights into how early developmental factors shape long-term cognitive outcomes.

## Data accessibility

All data, data description, and R code are available in public repository <https://github.com/Pablo-Recio/CORT_Temp_learning>.

## Satements and declarations

#### Competing Interests

We declare we have no competing interests #### Conflict of Interest We declare we have no conflict of interest #### Ethical Approval All experimental procedures and laboratory housing complied with Australian law and were approved by the Australian National University Animal Experimentation Ethics Committee (A2022\_33).

## Authors’ contributions

P.R.: conceptualization, methodology, data collection, data curation, formal analysis, writing—original draft, writing—review and editing; D.C.L.: data collection, writing—review and editing; O.C.: conceptualization, methodology, writing—review and editing; K.H.W.: conceptualization, writing—review and editing; C.F.: conceptualization, methodology, funding acquisition, writing—review and editing; B.M: data collection, writing—review and editing; A.Y.P.: data collection, writing—review and editing; D.N.: conceptualization, methodology, funding acquisition, project administration, resources, supervision, writing—review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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# Suplementary Material

#### Estimates of reversal learning slopes for all the different treatments per each task, specie, and group.

Table 1. Estimates of learning slopes for all the different treatments per each task, specie, and group. Mean shows the arithmetic means of the estimates obtained from the posteriors of the model, and 95% CI indicates the 95% confidence interval of the mean. All pmcmc tested the hypothesis that the mean equals zero. In bold, those values that are significant (pmcmc <0.05).

|  | | Red | | | Blue | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Specie | Treatment | Mean | 95% CI | pmcmc | Mean | 95% CI | pmcmc |
| *L. delicata* | CORT-Cold | **0.101** | **[0.055 , 0.152]** | **< 0.001** | 0.013 | [-0.035 , 0.064] | 0.60 |
|  | Control-Cold | **0.056** | **[0.018 , 0.096]** | **< 0.05** | -0.007 | [-0.056 , 0.042] | 0.77 |
|  | CORT-Hot | **0.066** | **[0.023 , 0.110]** | **< 0.05** | 0.032 | [-0.013 , 0.083] | 0.17 |
|  | Control-Hot | **0.082** | **[0.037 , 0.131]** | **< 0.001** | **0.053** | **[0.002 , 0.107]** | **< 0.05** |
| *L. guichenoti* | CORT-Cold | **0.119** | **[0.054 , 0.190]** | **< 0.05** | **0.070** | **[0.012 , 0.129]** | **< 0.05** |
|  | Control-Cold | **0.097** | **[0.032 , 0.171]** | **< 0.05** | 0.015 | [-0.058 , 0.091] | 0.65 |
|  | CORT-Hot | **0.074** | **[0.014 , 0.139]** | **< 0.05** | **0.102** | **[0.036 , 0.177]** | **< 0.05** |
|  | Control-Hot | **0.078** | **[0.016 , 0.145]** | **< 0.05** | **0.064** | **[0.004 , 0.125]** | **< 0.05** |

#### Colour preference

To test if lizards were biased towards the assigned colour as our preliminary analyses suggested, we employed the intercepts from our posterior distributions. We first estimated the predicted probability of choosing the correct feeder first in the first trial, by using the formula:

Second, we tested the hypothesis that the estimated probability was higher than 0.33 (the probability expected by chance of choosing the correct feeder) using pmcmc. If the estimated probability is above 0.33. we consider it as an indication that there was a preference towards that colour that could be affecting learning slopes. The results per treatment are summarized in Table 2.

Table 2. Probability of choosing the correct feeder in the first trial when the correct feeder was blue (Prob Blue) or red (Prob Red) for each species and each treatment. pmcmc tested the hypothesis that the probability is >0.33. In bold, those values that are significant (pmcmc <0.05)

| Specie | Treatment | Prob Blue | p-value Blue | Prob Red | p-value Red |
| --- | --- | --- | --- | --- | --- |
| *L. delicata* | CORT-Cold | **0.633** | **< 0.05** | 0.142 | 0.98 |
|  | Control-Cold | **0.752** | **< 0.001** | 0.294 | 0.67 |
|  | CORT-Hot | **0.553** | **< 0.05** | 0.340 | 0.51 |
|  | Control-Hot | 0.502 | 0.08 | 0.317 | 0.58 |
| *L. guichenoti* | CORT-Cold | 0.355 | 0.46 | 0.072 | 1.00 |
|  | Control-Cold | **0.614** | **< 0.05** | 0.191 | 0.91 |
|  | CORT-Hot | 0.482 | 0.14 | 0.235 | 0.83 |
|  | Control-Hot | 0.498 | 0.10 | 0.283 | 0.69 |

On average, we found that, for both species, the proportion of correct choices in the first trial was significantly above chance when the correct feeder was blue for *L. delicata* (mean Prob choice = 0.610, pmcmc < 0.05) but not for *L. guichenoti* (mean Prob choice = 0.487, pmcmc 0.18). When the correct choice was red, it was not significant for neither species (*L. delicata*: mean Prob choice = 0.273, pmcmc 0.68; *L. guichenoti*: mean Prob choice = 0.273, pmcmc 0.68).

#### Light spectrum

To test if the bias towards blue was something acquired during training, we compared the light spectrum of the ramps used in the associative task and the white ones used during habituation. We took three measurements of ten feeders per colour with a spectophotometer, and then analysed the spectrum and the perceived differences in colour using the package pavo. The spetrum of each type of feeder are shown in [Fig. 4](#fig-spectrum); the perceived chromatic constrasts between feeders are shown in [Fig. 5](#fig-perceived1) and [Fig. 6](#fig-perceived2).

|  |
| --- |
| Fig 4— Light spectrum of the ramps used in the associative task and the white ones used during habituation. The different colours represent the different ramps. |

|  |
| --- |
| Fig 5— Perceived chromatic contrasts between feeders. |

|  |
| --- |
| Fig 6— Perceived achromatic contrasts between feeders. |

#### Checking the models plots

Model formula for task is:  
Choice ~ Trial*cort*temp + (1 + Trial|lizard\_id)  
Plots for the different models of the associative task:  
1.- *L. delicata*  
1.a.- Red

Estimate Est.Error Q2.5 Q97.5  
R2 0.1695644 0.02196313 0.1255778 0.2110525

|  |
| --- |
|  |
|  |
|  |

1.b.- Blue

Estimate Est.Error Q2.5 Q97.5  
R2 0.07757601 0.02088415 0.03926128 0.1200952

|  |
| --- |
|  |
|  |
|  |

2.- *L. guichenoti*  
2.a.- Red

Estimate Est.Error Q2.5 Q97.5  
R2 0.1858621 0.02466494 0.1359576 0.2330882

|  |
| --- |
|  |
|  |
|  |

2.b.- Blue

Estimate Est.Error Q2.5 Q97.5  
R2 0.1367205 0.02551682 0.08796974 0.1865906

|  |
| --- |
|  |
|  |
|  |

#### Models with age included

Table 3. Results for *L. delicata* assigned to red feeders when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | -2.16 | 0.66 | -3.49 | -0.89 | 1.00 | 3402.24 | 3887.20 |
| age.start | 0.00 | 0.02 | -0.03 | 0.04 | 1.00 | 5418.98 | 5776.45 |
| trial\_associative | 0.10 | 0.02 | 0.06 | 0.15 | 1.00 | 3692.82 | 4207.48 |
| cortControl | 1.09 | 0.86 | -0.60 | 2.83 | 1.00 | 3201.98 | 3890.41 |
| tempHot | 1.28 | 0.93 | -0.47 | 3.15 | 1.00 | 2934.34 | 3471.54 |
| trial\_associative:cortControl | -0.05 | 0.03 | -0.11 | 0.01 | 1.00 | 3572.26 | 4634.15 |
| trial\_associative:tempHot | -0.04 | 0.03 | -0.10 | 0.03 | 1.00 | 3574.66 | 4356.48 |
| cortControl:tempHot | -1.26 | 1.21 | -3.73 | 1.08 | 1.00 | 3074.88 | 4021.52 |
| trial\_associative:cortControl:tempHot | 0.06 | 0.04 | -0.03 | 0.15 | 1.00 | 3628.87 | 4183.50 |

Table 4. Results for *L. delicata* assigned to blue feeders when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | 0.50 | 0.58 | -0.64 | 1.64 | 1.00 | 3044.27 | 4311.87 |
| age.start | -0.00 | 0.01 | -0.01 | 0.01 | 1.00 | 5860.40 | 5710.61 |
| trial\_associative | 0.01 | 0.02 | -0.04 | 0.06 | 1.00 | 3068.62 | 4168.88 |
| cortControl | 0.70 | 0.82 | -0.88 | 2.34 | 1.00 | 2870.11 | 3815.82 |
| tempHot | -0.34 | 0.79 | -1.93 | 1.22 | 1.00 | 2598.17 | 3477.53 |
| trial\_associative:cortControl | -0.02 | 0.04 | -0.09 | 0.05 | 1.00 | 2915.98 | 3893.50 |
| trial\_associative:tempHot | 0.02 | 0.03 | -0.05 | 0.09 | 1.00 | 2593.93 | 3764.25 |
| cortControl:tempHot | -0.98 | 1.13 | -3.23 | 1.25 | 1.00 | 2537.70 | 3924.03 |
| trial\_associative:cortControl:tempHot | 0.04 | 0.05 | -0.06 | 0.14 | 1.00 | 2642.40 | 4091.36 |

Table 5. Results for *L. guichenoti* assigned to red feeders when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | -3.06 | 0.84 | -4.87 | -1.55 | 1.00 | 2641.91 | 2963.70 |
| age.start | -0.00 | 0.02 | -0.04 | 0.03 | 1.00 | 5914.10 | 5309.44 |
| trial\_associative | 0.12 | 0.04 | 0.06 | 0.20 | 1.00 | 2411.12 | 2897.00 |
| cortControl | 1.28 | 1.15 | -0.97 | 3.62 | 1.00 | 2479.35 | 3043.41 |
| tempHot | 1.65 | 1.14 | -0.57 | 3.97 | 1.00 | 2336.65 | 2802.60 |
| trial\_associative:cortControl | -0.02 | 0.05 | -0.13 | 0.08 | 1.00 | 2444.48 | 3222.97 |
| trial\_associative:tempHot | -0.05 | 0.05 | -0.15 | 0.04 | 1.00 | 2253.20 | 2466.35 |
| cortControl:tempHot | -1.03 | 1.53 | -4.17 | 1.91 | 1.00 | 2353.37 | 3274.21 |
| trial\_associative:cortControl:tempHot | 0.03 | 0.07 | -0.11 | 0.17 | 1.00 | 2319.80 | 3341.04 |

Table 6. Results for *L. guichenoti* assigned to blue feeders when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | -0.61 | 0.73 | -2.07 | 0.85 | 1.00 | 4476.16 | 4928.86 |
| age.start | 0.01 | 0.01 | -0.02 | 0.03 | 1.00 | 5957.56 | 5181.70 |
| trial\_associative | 0.07 | 0.03 | 0.01 | 0.13 | 1.00 | 4004.25 | 4029.03 |
| cortControl | 1.32 | 1.00 | -0.70 | 3.29 | 1.00 | 3729.95 | 4199.07 |
| tempHot | 0.38 | 0.94 | -1.61 | 2.22 | 1.00 | 4055.41 | 4399.38 |
| trial\_associative:cortControl | -0.05 | 0.05 | -0.14 | 0.04 | 1.00 | 3437.88 | 3686.90 |
| trial\_associative:tempHot | 0.03 | 0.04 | -0.05 | 0.12 | 1.00 | 3667.39 | 4011.10 |
| cortControl:tempHot | -1.23 | 1.34 | -3.86 | 1.52 | 1.00 | 3524.34 | 4053.85 |
| trial\_associative:cortControl:tempHot | 0.02 | 0.06 | -0.12 | 0.14 | 1.00 | 3340.33 | 4029.47 |