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

## 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 underpin animal foraging, mate choice, antipredatory strategies and social behaviours that are crucial for survival and reproduction ([Dukas 2004](#ref-dukas_evolutionary_2004)). Learning - the acquisition of new information ([Dukas 2004](#ref-dukas_evolutionary_2004)) - is fundamental 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)). However, in nature, there is individual variation in the capacity to acquire new information, depending on factors like age, sex, gut microbiota, or the environment where organisms develop ([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)). The latter can be particularly relevant since the brain is especially susceptible to environmental conditions during early stages of development ([Zhu et al. 2004](#ref-zhu_prenatal_2004)).Understanding how the environment shapes learning processes is essential due to its profound impact on animals’ fitness and adaptability to environmental changes.

In this context, the prenatal environment emerges as a relevant key of animals’ cognitive skills, shaping their capacity for learning and adaptation. Prenatal glucocorticoids (GCs) - hormones related to organisms’ response to stress ([Sapolsky et al. 2000](#ref-sapolsky_how_2000)) - and prenatal thermal environment are known to influence learning abilities in different taxa (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)). For instance, some studies have demonstrated that prenatal stress and high prenatal GC levels impair learning ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Farrell et al. 2015](#ref-farrell_developmental_2015-learn)), while others showed diverse effects depending on factors like sex or the nature of the learning task ([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)). Similarly, some experiments have shown significant impacts of prenatal temperature on learning in ectotherms ([Amiel and Shine 2012](#ref-amiel_hotter_2012); [Amiel et al. 2014](#ref-amiel_egg_2014); [Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). In fact, 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 velvet geckos incubated at temperatures over their natural range learn slower than those incubated within the natural thermal limits ([Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)). In this vein, the effects of prenatal temperature and GCs are linked to similar alterations in neural structure and 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)), which suggests that bot factors may interact to shape learning ([Noble et al. 2018](#ref-noble_developmental_2018)). 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. However, our understanding of how these two factors interact remains incomplete.

In this study, we aim to explore the interactive effects between prenatal GCs and the prenatal thermal environment on learning. We utilized two species of skinks, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*), as model species. We experimentally increased corticosterone (CORT) - the main GC in reptiles ([Crino et al. 2023](#ref-Crino_2023)) - in the eggs and then incubated them at two different temperatures in a 2X2 factorial design. Post-incubation, the juveniles were subjectd to a colour-associative task to assess their learning abilities. Our hypothesis posits that changes in CORT levels and temperature during early development will induce sustained effects on brain’s physiology that will ultimately impact learning skills. We predicted that individuals exposed to high levels of CORT or low temperatures will perform less proficiently compared to 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)); 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. Finally, we expect that the effects of the treatments will be similar in both species, as both species share similar life history traits and are closely related ([Chapple et al. 2011](#ref-chapple_know_2011), [2014](#ref-chapple_biology_2014)), and other cognitive studies have not found any difference between species when tested in an associative learning task ([Bezzina et al. 2014](#ref-bezzina2014does)).

## 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)). Also, some sudies show behavioural differences between both skinks; *L. delicata* is bolder and tends to explore more than *L. guichenoti* ([Chapple et al. 2011](#ref-chapple_know_2011)). These differences have been associated with *L. delicata*’s success as an invassive species ([Chapple et al. 2011](#ref-chapple_know_2011); [Bezzina et al. 2014](#ref-bezzina2014does)), but not with their ability to perform an associatve 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 is 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 approx. 40 mid-size crickets (*Acheta domestica*) per enclosure three days a week. Crickets are dusted with calcium weekly and multivitamin and calcium biweekly. To ensure a temperature gradient, we employ a heat chord and a heat lamp following a 12 h light:12 h dark cycle. Room temperatures are set to 22-24 Celsius, and warm side of enclosures is usually at 32 Celsius.  
*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 and weight them with a (OHAUS, Model spx123) digital scale ± 0.001g error. Then 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.  
*Hatchlings* – Eggs in the incubator were checked three times a week for hatchlings. After hatchling, we measured juveniles’ SVL and Tail Length (TL) with a rule to the nearest mm and weighted them with a (OHAUS, Model spx123) digital scale ± 0.001g error. We then placed hatchlings in individual enclosures (18.7L x 13.2W x 6.3H cm) and provided them with non-stick matting and a small water dish. During this period, they were sprayed water every day and received 3-6 small *A. domestica* crickets three times a week. All care otherwise follows similar protocols to adults (see above).  
Two weeks before we started the training phase (see below), lizards were moved to the experimental arena for acclimatation. 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 extremes and a water dish on the other. These new enclosures were placed in two rooms in 7 different racks associated to 7 different CCTV systems (device model DVR-HP210475) that allowed us to record their behaviour during the experiment (see details below). The number of lizards per species and treatment in each rack was counterbalanced to control for any effect of the room or the position of the lizard in the rack. During acclimatation 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. The rooms temperature was set to between 22-24 Celsius.

#### CORT and Temperature manipulation

To test empirically 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. CORT concentration employed in the CORT treatment represents 2 standard deviations above the mean natural concentration obtained in eggs from both species (non-published data). Then, 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 were counterbalanced in both species.

#### Learning

To estimate learning skills, we tested skinks’ ability to locate a food reward in a series of behavioural tasks ([Fig. 1](#fig-Methods) B) (see [Leal and Powell 2012](#ref-leal_behavioural_2012); [Clark et al. 2014](#ref-clark_colour_2014)). 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: in the first stage, 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) B, Stage 1); in the second stage, the petri dish with the cricket was placed on top of the white 3D printed ramps ([Fig. 1](#fig-Methods) B, Stage 2); and finally, the cricket was left inside a well (3D x 1.75H cm) on the top of the ramp in the third and last stage (Fig. [Fig. 1](#fig-Methods) B, 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. This phase lasted 38 days until all the lizards were able to 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.  
For the learning phase, we trained lizards to associate between colour and a food reward (‘Associative task’ in Fig. [Fig. 1](#fig-Methods) B). The test was like the third stage of the training phase, but here lizards were presented with three feeders that differed in the 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 in all three wells to avoid lizard 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 experiment was done daily between the 6th of March until the 17th of June 2023, 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. Videos were analysed manually using a standard video player (IINA) by PR, who recorded whether the first choice made by each subject was the correct feeder or not. A choice was considered to be made if the head of the lizard was inside the well of one of the ramps. PR was blinded to the treatments of the lizards during the analyses of the video. We considered a trial failed if there was no choice in one hour of recording and those trials were considered as ‘non data’ in the analyses. 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 to be the first one where the individual made a choice.

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| Fig 1— Experimental design of early environment manipulation (**A**) and learning tasks (**B**). Stages 1-3 indicate the different phases of the habituation process. In the associative and reversal tasks, white lids 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). As such, 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 their interaction as fixed factors. We included a random intercept and slope (trial) for each lizard in our models. We also incorporated the clutch identity as a random factor. Since learning in lizards can be age-dependent ([Noble et al. 2014](#ref-noble_age-dependent_2014)), and given that incubation temperature can 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 slopes were obtained using the ‘trial’ estimates and its interaction with hormone and temperature treatments. Slope estimates greater than zero were considered as evidence of learning, while those less or equal to zero were not. We calculated the probability that slopes or contrasts between slopes differed from zero (pmcmc) using the posterior distributions. We considered statistical significance if pmcmc < 0.05.

## Results

Originally, we started with 96 lizards, 48 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\_deli?**](#ref-fig_deli)) and [Fig. 3](#fig-guich).

We found that the estimated learning slopes 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]; ‘Blue’ learning slope - ‘Red’learning slope = -0.062, p~mcmc = 0.125), 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 = 0.458). However, further analyses using the first trial indicated a potential bias towards blue in the initial choice (see Supplementary Material) that could be affecting the estimated slopes. As such, we analysed the between treatments differences 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 = 0.168) ([Fig. 2](#fig-deli) A, B). Similarly, those assigned to ‘Blue’ were not afected by CORT (Control - CORT = -0.083, pmcmc = 0.061), 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 = 0.406) ([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 = 0.181), 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 = 0.656) 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 = 0.788) 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 = 0.723) 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 = 0.403).

## Discussion

The scope of the present study was to investigate the effects of early environment on lizards’ cognition using two closely related species, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). We expected early environment to influence cognition in both skinks. However, we did not find any significant effects of the experimental conditions on lizards’ performance in an associative learning task. We found that both *L. delicata* and *L. guichenoti* learned to associate a colour with a food reward, at least for those assigned to the red feeders, and that the learning process ocurred at the same rate regardless of the early environment manipulation. These results suggest that learning abilities in these species are robust to prenatal CORT and thermal environment.

The colour of the correct choice had a significant effect on the estimated learning slopes in *L. delicata*. When the correct choice was red lizards learned faster. Furthermore, the learning slope in some of the ‘Blue’ assigned lizards did not differ from zero. This could indicate that *L. delicata* did not learn to associate the blue ramp with the reward. However, the analyses presented in the supplemenatry material show that there was an initial preference towards blue feeders that can be masking our results. Indeed, the estimated probability of choosing the correct ramp in the first trial was higher than expected by chance when the correct choice was blue. 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 to select 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). Given that but we did no see any relevant similarity 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, and empirical studies show that, 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 employing visual stimuli. Despite this, when lizards assigned to blue or red were analysed separately, the between treatments comparissons remain the same in both species. Furthermore, the trends we observed were consistent between species or colour assigned. As such, we conclude that, overall, lizards learned at the same rate regardless of the early environment manipulation.

We predicted prenatal CORT to have a negative effect on learning, as previous studies have shown ([Lemaire et al. 2000](#ref-lemaire_prenatal_2000); [Zhu et al. 2004](#ref-zhu_prenatal_2004); [Farrell et al. 2015](#ref-farrell_developmental_2015-learn); [Bebus et al. 2016](#ref-bebus_associative_2016)). However, our results are consistent with other experiments that revealed no effect of prenatal GCs on learning abilities ([Szuran et al. 1994](#ref-szuran_water_1994); [Bebus et al. 2016](#ref-bebus_associative_2016)). GCs are known to have hormetic effects, with low levels of GCs being beneficial for learning and memory, and high levels being detrimental ([Du et al. 2009](#ref-du_dynamic_2009); [McEwen 2012](#ref-mcewen_brain_2012)). In a previous study, we found that the same dose of CORT affected mortality and hatch size in both species [**WAIT FOR REFERENCE**]. 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, while Scrub-jays that had lower CORT levels as nestlings performed better on an associative learning test as adults, but not on a reversal learning task ([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. Future studies should focus on examining the effect of early-life stress on different cognitive domains.

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, those authors that found the opposite pattern ([Dayananda and Webb 2017](#ref-dayananda_incubation_2017); [Abayarathna and Webb 2020](#ref-abayarathna_effects_2020)) employed temperatures far above the natural thermal range of the species in their hot treatments and it is unclear how such conditions relate to wild environments. In lizards, higher incubation temperatures are associated with higher neural density in the dorsal and the medial cortex ([Amiel et al. 2017](#ref-amiel_effects_2017)). Both brain regions have been linked to learning in reptiles ([Day et al. 2001](#ref-day2001effects); [López et al. 2003](#ref-lopez2003spatial); [Amiel et al. 2017](#ref-amiel_effects_2017)), and, in consequence, we predicted performance in the task to be affected by the prenatal thermal environment. However, the effect of prenatal temperature in cognition, and brain physiology and structure has been investigated 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 *L. delicata* and *L. guichenoti*, associative learning appears to be robust to incubation temperature. 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 ([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 - developing male phenotypes while retaining female genotype ([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)). Future investigations 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.

Finally, we did not see any significant difference in learning rates when both species were compared. This result is in line with previous studies that found no differences in learning abilities between *L. delicata* and *L. guichenoti* ([Bezzina et al. 2014](#ref-bezzina2014does)). In the experiment conducted by Bezzina et al. ([2014](#ref-bezzina2014does)) the authors did not find any evidence of learning in neither *L. delicata* or *L. guichenoti*. The learning task conducted in the present experiment was very similar, but we found a different outcome, since both species learned at the same rate. The differences between studies could be due to the different experimental designs or the complexity of stimuli discrimination.

In conclusion, our results indicate that lizards completed the associative learning task at similar rates, with no significant differences between species or treatments. This outcome contrasts with our predictions and suggest that learning skills in both species are robust to the impact of early environment (at least for temperature and GCs). Future research should examine the possible impact of prenatal CORT and temperature on cognitive function, taking into account variables such as the early environment-sex interaction, or the nature of the cognitive task. Additionally, it is essential to assess the effects of these treatments on brain function. Exploring these areas will enhance our comprehension of the cognitive and physiological mechanisms involved in the adaptability of both species.

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# Suplementary Material

#### 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 1.

Table 1. 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** | **= 0.978** |
|  | Control-Cold | **0.752** | **< 0.001** | **0.294** | **= 0.672** |
|  | CORT-Hot | **0.553** | **< 0.05** | **0.340** | **= 0.505** |
|  | Control-Hot | **0.502** | **= 0.078** | **0.317** | **= 0.581** |
| *L. guichenoti* | CORT-Cold | **0.355** | **= 0.458** | **0.072** | **= 0.999** |
|  | Control-Cold | **0.614** | **< 0.05** | **0.191** | **= 0.908** |
|  | CORT-Hot | **0.482** | **= 0.135** | **0.235** | **= 0.831** |
|  | Control-Hot | **0.498** | **= 0.100** | **0.283** | **= 0.693** |

**?(caption)**

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 = 0.183). When the correct choice was red, it was not significant for neither species (*L. delicata*: mean Prob choice = 0.273, pmcmc = 0.684; *L. guichenoti*: mean Prob choice = 0.273, pmcmc = 0.684).

#### 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).

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| 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. |

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| Fig 5— Perceived chromatic contrasts between feeders. |

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| 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

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1.b.- Blue

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

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

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

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2.b.- Blue

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

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#### 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 |

**?(caption)**

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 |

**?(caption)**

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 |

**?(caption)**

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 |

**?(caption)**