Early environmental conditions do not impact behavioural flexibility in an invasive and non-invasive lizard species

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## Abstract

Behavioural flexibility, the ability to adjust behaviour adaptively in response to internal or external changes, is expected to be crucial for animals adapting to environmental fluctuations. However, the conditions experienced during early development can profoundly impact behavioural flexibility making it unclear how populations will respond to novel circumstances. Stressful situations faced by the parents can have a direct impact on offspring cognition through the transmission of glucocorticoids - stress-related hormones that affect offspring cognition. At the same time, stressful conditions can influence parental behaviour during nesting and consequently the thermal developmental conditions offspring experience. Here, we investigated the interactive effects of prenatal corticosterone (CORT) and temperature on behavioural flexibility in two lizard species, *Lampropholis delicata* and *L. guichenoti*. We manipulated prenatal CORT levels and incubation temperature in a 2x2 factorial design, and then assessed behavioural flexibility through a reversal learning task. We hypothesized prenatal CORT and cold temperatures would impair performance in the reversal task. Given *L. delicata*’s success as an invasive species, we expected this species to behave more flexibly and be less affected by early environmental conditions. Contrary to our expectations, behavioural flexibility appears to be robust to prenatal temperature and CORT in both species. The lack of difference in reversal learning between *L. delicata* and *L. guichenoti* suggests that novel environments are unlikely to influence flexible behavioural learning and that behavioural flexibility itself is unlikely to explain differences in invasion success between these species.

## Introduction

Behavioural flexibility describes the ability of individuals to adjust behaviour adaptively in response to changes in the internal or external environment ([Brown & Tait, 2010](#ref-brown2010behavioral)). It comprises a suite of behaviours, including problem-solving or innovation ([Brown & Tait, 2010](#ref-brown2010behavioral)), that can be beneficial for adapting to novel circumstances ([Szabo et al., 2020](#ref-szabo2020can); [Szulkin et al., 2020](#ref-szulkin2020urban)). Species with greater behavioural flexibility are expected to be more resilient to environmental changes because behaving flexibly is predicted to be crucial for exploiting new resources and facing new challenges ([Chapple et al., 2012](#ref-chapple2012can); [Szabo et al., 2020](#ref-szabo2020can); [Szulkin et al., 2020](#ref-szulkin2020urban); [Wright et al., 2010](#ref-wright2010behavioral)). For example, successful colonisation of new environments by birds is related to higher frequency of foraging innovations ([Sol & Lefebvre, 2000](#ref-sol2000behavioural)). Although there is limited understanding of the physiological mechanisms involved in animals’ ability to behave flexibly, relative brain size has been shown to be a good predictor of behavioural flexibility in mammals, birds, reptiles and amphibians ([Amiel et al., 2011](#ref-amiel_smart_2011); [Sol et al., 2008](#ref-sol2008brain); [Sol & Lefebvre, 2000](#ref-sol2000behavioural)). This suggests that behavioural flexibility is likely to be underpinned by complex neural mechanisms, and that alterations in these mechanisms may impact animals’ ability to respond to novel situations. The brain is considered especially sensitive to environmental change during the early stages of development ([Zhu et al., 2004](#ref-zhu_prenatal_2004)), creating uncertainty about how the conditions faced during early life might impact behavioural flexibility.

The conditions in which organisms develop can be altered by the circumstances faced by their parents. For instance, exposing three-spined stickleback (*Gasterosteus aculeatus*) females to a model predator during ovulation, affected their offspring’s tendency to use social cues ([Feng et al., 2015](#ref-feng2015effect)). Under stressful situations, animal sensory systems detect and transmit environmental signals to instigate adaptive physiological and behavioural adjustments – responses that are mediated by glucocorticoids (GCs) ([Sapolsky et al., 2000](#ref-sapolsky_how_2000)). Glucocorticoids can be transmitted to offspring and influence their phenotype and development through transgenerational effects ([Crino et al., 2023](#ref-Crino_2023)). In addition to the environments experienced by parents, offspring also experience potentially stressful environmental conditions that can interact with or amplify parental effects. For example, human disturbance alters snapping turtles’ nest-site choice, ultimately impacting the temperature at which eggs are incubated ([Kolbe & Janzen, 2002](#ref-kolbe2002impact)). Animals’ responses to abrupt temperature changes are mediated by GCs ([Crino et al., 2023](#ref-Crino_2023)), which can influence animals’ decision-making while nesting ([Kolbe & Janzen, 2002](#ref-kolbe2002impact)). As a result, GCs transmission and early thermal environment are expected to interact and shape offspring traits. Both GCs and temperature are known to exert potent effects on brain development ([Amiel et al., 2017](#ref-amiel_effects_2017); [Coomber et al., 1997](#ref-coomber_independent_1997); [Jonson et al., 1976](#ref-jonson1976effect); [Zhu et al., 2004](#ref-zhu_prenatal_2004)) and cognition ([Clark et al., 2014](#ref-clark_colour_2014); [Lui et al., 2017](#ref-lui2017chronic)), but little is known about how they may interact to affect behavioural flexibility. Since behaving flexibly is essential for dealing with novel environments, understanding how early developmental environments collectively impact cognition is crucial for predicting the consequences of environmental change.

Here, we studied the effects of prenatal temperature and corticosterone (CORT) (the main GC in reptiles) ([Crino et al., 2023](#ref-Crino_2023)), on behavioural flexibility in two species of reptiles, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). Both species have similar life-histories and overlapping distributions ([Chapple et al., 2011](#ref-chapple_know_2011), [2014](#ref-chapple_biology_2014)), but only *Lampropholis delicata* has been successful in colonising different areas around the globe ([Baker, 1979](#ref-baker1979rainbow); [Chapple, Miller, et al., 2013](#ref-chapple2013divergent); [Chapple et al., 2015](#ref-chapple2015deliinvLHI)). Previous studies exploring behavioural differences between the two species have found *L. delicata* to be more exploratory than *L. guichenoti* ([Chapple et al., 2011](#ref-chapple_know_2011)), but no differences in learning were observed between the skinks in an associative learning task ([Bezzina et al., 2014](#ref-bezzina2014does)). Disparities in behavioural flexibility may be driving the differences in invasion success between both skinks, a prediction supported in other invasive species ([Amiel et al., 2011](#ref-amiel_smart_2011); [Chapple et al., 2012](#ref-chapple2012can); [Sol et al., 2008](#ref-sol2008brain); [Sol & Lefebvre, 2000](#ref-sol2000behavioural); [Wright et al., 2010](#ref-wright2010behavioral)). However, the relative impact of early environments on behavioural flexibility remains unexplored. Early environments are known to affect suites of morphological and behavioural traits differently in each species ([Carrasco et al., 2024](#ref-carrasco2023maternal); [Kar et al., 2023](#ref-kar2023heritability)). We manipulated CORT and incubation temperature in both species and then subjected hatchlings to a reversal learning task. Reversal learning is a widely employed tool to measure behavioural flexibility ([Gapp et al., 2014](#ref-gapp2014early); [Hurtubise & Howland, 2017](#ref-hurtubise2017effects)), as it assesses an individual’s ability to reverse a previously learnt behaviour providing researchers with a clear indicator of their ability to adjust to new conditions ([Brown & Tait, 2010](#ref-brown2010behavioral)).

We predicted that both cold incubation temperatures and CORT treatment will impair behavioural flexibility ([Clark et al., 2014](#ref-clark_colour_2014); [Lui et al., 2017](#ref-lui2017chronic)), and the combination of both treatments to have the most detrimental effect on behavioural flexibility. Since invasive species are expected to behave more flexibly ([Amiel et al., 2011](#ref-amiel_smart_2011); [Chapple et al., 2012](#ref-chapple2012can); [Sol et al., 2008](#ref-sol2008brain); [Sol & Lefebvre, 2000](#ref-sol2000behavioural); [Wright et al., 2010](#ref-wright2010behavioral)), we predicted that *L. delicata*, on average, will perform more proficiently in the task compared to *L. guichenoti* and that behavioural flexibility in *L. delicata* will be more robust to early environmental conditions.

## Methods

#### Husbandry

*Breeding colony* – We tested juveniles originating from a breeding colony established in the lab since 2019. A total of 270 and 180 adults of *L. delicata* and *L. guichenoti*, respectively, were established in containers (41.5 L x 30.5 W x 21 H cm) with six lizards (2 males and 4 females) per enclosure. Enclosures had non-stick matting, a shelter, and several small water dishes. Water was given daily, and they were fed approx. 40 mid-size crickets (*Acheta domestica*) per enclosure three days a week. Crickets were dusted with calcium weekly and multivitamin and calcium biweekly. Using a heat chord and a heat lamp we created a temperature gradient and kept lights on a 12 h light:12 h dark cycle. UVA/UVB lighting was also provided. Room temperatures were set to 22-24ºC, and the warm side of enclosures reached 32ºC.

*Eggs collection and incubation* – Between mid-October 2022 to the end of February 2023, we provided females with a place to lay eggs by means of small boxes (12.5 L x 8.3 W x 5 H cm) with moist vermiculite inside that were placed on one side of the communal enclosures (see above). We checked for the presence of eggs in the boxes three days a week. After collection, we measured the length and width of eggs with a digital caliper to the nearest 0.1 mm and weighed them with a digital scale ± 0.001 g error (OHAUS, Model spx123). We also assigned a unique number to the clutch and each egg. Eggs were then 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 ruler to the nearest mm and weighed them with a digital scale ± 0.001 g error. We then placed hatchlings in individual enclosures (18.7L x 13.2W x 6.3H cm) and provided them with non-stick matting, a shelter, and a small water dish. During this period, they were sprayed with water every day and received 3-6 small *A. domestica* crickets three times a week. All care otherwise followed similar protocols to adults (see above). Two weeks before starting the training phase (see below), lizards were moved to an 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 across 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). Conditions in these rooms where identical to the main room, but 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 on the rack. During acclimatisation and throughout 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 temperature of both rooms was set to between 22-24 ºC.

#### Manipulating Early Thermal and CORT Environments

To empirically test the effect of early environment we manipulated CORT concentration in eggs and incubated them under one of two temperature regimes (Cold – 23 ºC ± 3 ºC or Hot – 30 ºC ± 3 ºC) in a 2x2 factorial design ([Fig. 1](#fig-Methods) A). We first allocated eggs into one of two different treatments: in the CORT treatment, eggs were topically supplied with 5µL of CORT dissolved in 100% Ethanol (vehicle) at a final concentration of 10 pg CORT/mL; in the Control treatment eggs received an equal volume of the vehicle. This method has been validated before in *L. delicata* ([O. Crino et al., 2024](#ref-crino2024eggs)), increasing CORT concentration in eggs by approximately 2 standard deviations above the mean natural concentration. After the hormone treatment, eggs were incubated in one of the two previously mentioned temperature regimes (Cold or Hot) until hatching. These temperatures represent the upper and lower limit of the natural incubation temperatures ([Cheetham et al., 2011](#ref-cheetham2011embryonic); [Qualls & Shine, 2000](#ref-qualls2000post)).

We counterbalanced the number of eggs assigned to each hormone and temperature treatment using a partial split-clutch design. We assigned at least one egg per clutch to each treatment, and the remaining eggs were randomly assigned to one of the treatments. When the eggs per clutch was less than four eggs, we assigned each egg randomly to one of the treatments. The final total number of clutches per species is included in Results.

#### Quantifying Behavioural Flexibility

The learning test was divided in three main phases: a habituation phase where lizards had to learn to eat a frozen-thawed cricket from white 3D-printed polylactic acid (PLA) ramps (9 L x 4 W x 5 H cm); a colour association task where lizards had to associate the food reward with one of two colours (red or blue) ([Fig. 1](#fig-Methods) Associative task); and finally, to quantify behavioural flexibility, we reversed the previously trained colour (red or blue) so that the lizards needed to learn to associate the food with the opposite colour ([Fig. 1](#fig-Methods) Reversal task).

The habituation phase was divided into three stages: in the first stage, the reward, a small, frozen cricket (*A. domestica*) was placed in an opaque petri dish (3 D x 1.6 H cm) ([Fig. 1](#fig-Methods) Habituation phase, 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) Habituation phase, Stage 2); and in the third stage, the cricket was left inside a well (3D x 1.75H cm) on top of the ramp (Fig. [Fig. 1](#fig-Methods) Habituation phase, Stage 3). Trials in every stage lasted one hour, beginning when the feeding block (petri dish, ramp, or both) was placed inside the enclosure and finishing when we took the feeding block away. We recorded whether the cricket had been consumed or not and we considered a stage as completed if the lizards ate the crickets in at least 5 out of 6 trials. This phase lasted 38 days, and only in one case a lizard did not progress to the next phases because it did not pass the aqcuisition criteria.

In the colour association (Associative task in [Fig. 1](#fig-Methods)) and reversal task (Reversal task in [Fig. 1](#fig-Methods) B), we used three ramps that were identical in shape and size but different in the colour. The colours of these ramps were green, red, and blue, as previous studies demonstrate that squamates can discriminate between these colours ([Baden & Osorio, 2019](#ref-Baden_Osorio_2019_Vert_vision)). However, to control for potential colour biases, we assigned one group of lizards within each treatment to associate food in the blue ramp followed by the red ramp in the reversal, while in the other group the order was reversed. As in the last stage of the habituation phase, we placed the cricket inside the wells of the ramps, but then two of them were covered with 3D-printed lids (3 D x 0.5 H cm) so prey was only accessible on one ramp. The food reward was placed on all three ramps to avoid lizards using prey chemical cues that were released in the closed wells through a series of small holes on the top of the lids. 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 the association task once a day for 35 days and once a day for 40 days in the reversal.

The full experiment was performed between the 6th of March until the 26th of June 2023. Tests were made between 11 to 12 am, when the lizards were active. Trials in the learning phases (colour associative task and reversal tasks) were recorded with different 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 recording. Those trials were scored as NA. Here, we analysed the data of the reversal task as a measure of behavioural flexibility ([Brown & Tait, 2010](#ref-brown2010behavioral)).

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#### Statistical analyses

We performed the analyses for each species separately. We fitted two different Bayesian multilevel models using the brm function from the *brms* package ([Bürkner, 2017](#ref-burkner2017brms)) using an R extension (version 2.8.2) ([R Core Team, 2021](#ref-R)). We ran four parallel MCMC chains of 3000 iterations for each model, with a warmup period of 1000 iterations. We modelled correct choices [correct (1) or not (0)] as the response variable, and trial, hormone (CORT versus Control), incubation temperature (Cold versus Hot), along with the three-way interaction between trial, hormone, and temperature. If early environments impact learning then we would predict that the rate of learning (i.e., trial slope) varies by treatment as captured by the interactions. The error structure was modelled 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. We also incorporated the clutch identity as a random factor. *L. delicata* lays one clutch per year, while *L. guichenoti* lays two ([Chapple et al., 2011](#ref-chapple_know_2011), [2014](#ref-chapple_biology_2014)). Since eggs were collected during half of the breeding season, clutches likely come from different mothers. Additionally, previous research has shown that clutches are generally sired by a single male, but sperm storage can occur ([Kar et al., 2023](#ref-kar2023heritability)). Given our partial split-clutch design, and the fact that maternal effects are expected to be stronger than paternal effects in these species, including the clutch as a random factor should account for the effects of parental identity.

Learning can also be age-dependent (see [Noble et al., 2014](#ref-noble_age-dependent_2014)), and given that lizards incubated at different temperatures hatch at different times we explored the effects of age on learning. Indeed, 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. pmcmc test the hypothesis that slopes and slopes contrasts are different from zero. We considered an effect statistically significant if pmcmc < 0.05.

#### Ethical note

We collected subjects from a breeding colony kept in the lab since 2019. Both the breeding animals and the ones used in this experiment were provided humane laboratory housing, with thermoregulatory opportunities, light (UV and heat) and moderate levels of humidity (see above for details). Lizards in the colony received food *ad libitum* while the subjects in the experiment were fed one cricket daily during the experiment. All lizards were handled minimally to avoid stress. Experimental procedures and laboratory housing complied with Australian law and were approved by the Australian National University Animal Experimentation Ethics Committee (A2022\_33). Animals born in captivity cannot be re-released into the wild as stringent biosafety protocols across the states prohibit this. It is also not possible to rehome such a large number of animals. Therefore, upon completion of experimental trials, lizards were kept in their enclosure until they were humanely euthanized following approved procedures. To euthanise animals, we use an injection of sodium pentobarbital (i.e., Lethabarb) (diluted 1:4 ringers solution) at 60-100mg/kg, injected intrapleuroperitoneally. We monitored the animals to ensure there was no irritation from the agent as indicated by distressed animals. Gently pinching the toes was used to confirm the absence of a response before the animal was disposed of.  
Crickets were maintained in big communal enclosures (68.5 L x 49 W x 39 H cm) in the same room where the big colony is, and at the same temperature and light conditions. They were provided vegetables and water ad libitum, and they were given several egg carton shelters to refuge. All lizards were fed alive crickets except during the experiment, when we used crickets frozen at -21 ºC for 24 hours.

## Results

We started with 96 lizards, 48 per species and 12 per treatment per species. However, our final sample size was 81 due to natural mortality (n = 11), failure to complete the training stage (n = 1), or because the number of failed tasks (see above) was higher than 15 (n = 3). The final sample sizes per treatment and species are listed in [Fig. 2](#fig-results). These animals came from a total of 36 clutches in *L. delicata* and 33 in *L. guichenoti*.

Both species learnt across all treatments ([Fig. 2](#fig-results) B, D), but we did not find any difference between treatments or species in the rate of learning [mean slopes (denoted as β throughout) per treatment for both species provided in Table 1 in Supplementary Material]. For *L. delicata*, hormone treatment (βControl - βCORT = 0.005, 95% CI = [-0.034, 0.043], pmcmc = 0.873), incubation temperature (βHot - βCold = -0.001, 95% CI = [-0.039, 0.038], pmcmc = 0.989), or the interaction ([(βControl-Hot - βCORT-Hot) - (βControl-Cold - βCORT-Cold)] = -0.034, 95%CI = [-0.070, 0.002], pmcmc = 0.062) did not affect learning (see [Fig. 2](#fig-results) A, B). We similarly found no significant effects of hormone (βControl - βCORT = 0.005, 95% CI = [-0.034, 0.046], pmcmc = 0.810), incubation temperature (βHot - βCold = 0.009, 95% CI = [-0.033, 0.048], pmcmc = 0.629), or the interaction ([(βControl-Hot - βCORT-Hot) - (βControl-Cold - βCORT-Cold)] = -0.016, 95%CI = [-0.069, 0.035], pmcmc = 0.529) on learning in *L. guichenoti* (see [Fig. 2](#fig-results) C, D).

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Finally, when all individuals from all the treatments were pooled together and species were compared, there were no significant differences in the estimated slopes (mean of learning slope in *L. delicata* = 0.051, 95% CI = [0.027, 0.075]; mean learning of slope in *L. guichenoti* = 0.059, 95% CI = [0.030, 0.088]; β*L. delicata* - β*L. guichenoti* = -0.008, 95% CI = [-0.043, 0.026], pmcmc = 0.636). Figures for both species with the raw data were included in the Supplementary Material.

## Discussion

This study aimed to determine whether prenatal temperature and GCs impact behavioural flexibility in two closely related species, the delicate skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). We expected the early environment to influence behavioural flexibility in both species. We also predicted that *L. delicata* would be more flexible than *L. guichenoti* and less affected by prenatal conditions. Nevertheless, we did not find significant differences between treatments or species when we compared their performance in a reversal task. Our results show that, on average, all the lizards from the four treatments and the two species learnt the reversal task - indicated by estimated slopes that are significantly different from zero - and that they learn, on average, at the same rate. Furthermore, these results were consistent when only those individuals who performed better in the previous associative task were included (see Supplementary material).

Behavioural flexibility appears to be robust to early exposure to maternal GCs in both *L. delicata* and *L. guichenoti*. We predicted that prenatal CORT treatment would decrease reversal learning abilities. As such, our results contrast with previous studies that show high GCs levels impaired performance in different types of learning tests ([Farrell et al., 2016](#ref-farrell_developmental_2016); [Lemaire et al., 2000](#ref-lemaire_prenatal_2000); [Zhu et al., 2004](#ref-zhu_prenatal_2004)). Our results are, however, consistent with other experiments where GCs did not impact learning abilities ([Bebus et al., 2016](#ref-bebus_associative_2016); [Szuran et al., 1994](#ref-szuran_water_1994)). One possible reason for why GCs did not affect learning in our study may be because GCs can have hormetic effects on brain structure and learning. For example, in Sprague–Dawley rats, exposure to high doses of CORT, but not low doses, reduced the density of immature neurons and cell proliferation in the dentate gyrus ([Brummelte & Galea, 2010](#ref-DOSEneurobrummelte2010chronic)). It is possible such dose-dependent effects exist and our concentrations failed to elicit changes in the brain that would result in impaired behavioural flexibility. We find this possibility unlikely since, in a previous experiment, we observed that a similar dose affected a multitude of other traits including growth and baseline CORT levels in *L. delicata* ([O. Crino et al., 2024](#ref-crino2024eggs)). Nonetheless, we cannot dismiss this possibility because dose effects can differ between tissues ([Lemaire et al., 2000](#ref-lemaire_prenatal_2000)). Conversely, the effects of GCs early in development may vary depending on the cognitive task, as evidenced by studies showing variations in the impact of prenatal GCs on different brain regions ([Lemaire et al., 2000](#ref-lemaire_prenatal_2000)). Prenatal GCs can have programmatic effects on HPA axis sensitivity, potentially impacting the lizards’ sensitivity to stressors ([Crino et al., 2014](#ref-crino_corticosterone_2014)). This altered stress response may influence learning dynamics through hormetic effects of CORT ([Du et al., 2009](#ref-du_dynamic_2009)), particularly in stressful contexts of which our experiment was not ([Taborsky et al., 2021](#ref-taborsky2021towards)). These hypotheses are not mutually exclusive and could be tested in future experiments using multiple doses of prenatal CORT treatment, assessing learning using multiple cognitive tasks, and testing the effect of stressors during the learning process.

More striking is the absence of an effect of incubation temperature on behavioural flexibility because most studies have reported better performance in different associative learning and motor tasks when incubated at higher temperatures ([Amiel et al., 2014](#ref-amiel_egg_2014); [Amiel & Shine, 2012](#ref-amiel_hotter_2012); [Clark et al., 2014](#ref-clark_colour_2014)). However, studies that have reported cold-incubated lizards to learning faster ([Abayarathna & Webb, 2020](#ref-abayarathna_effects_2020); [Dayananda & Webb, 2017](#ref-dayananda_incubation_2017)) used incubation temperatures far beyond the natural range in their hot treatments. As such, it is unclear how such conditions relate to wild environments. Our results suggest that behavioural flexibility is robust to temperature in both species of skinks at least in this study. It could be argued that the differences in our incubation temperatures were not extreme enough to elicit significant differences in learning performance. However, the mean difference between the temperatures used in our experiment were similar to those in previous studies ([Amiel et al., 2014](#ref-amiel_egg_2014); [Amiel & Shine, 2012](#ref-amiel_hotter_2012); [Clark et al., 2014](#ref-clark_colour_2014)), and are aligned with natural variation at the extremes of incubation conditions in the wild ([Cheetham et al., 2011](#ref-cheetham2011embryonic); [Qualls & Shine, 2000](#ref-qualls2000post)). In lizards, impacts on learning from early thermal environments are associated with changes in neural density ([Amiel et al., 2017](#ref-amiel_effects_2017)). However, several studies have shown that the effects of temperature on brain structure and physiology are region dependent ([Amiel et al., 2017](#ref-amiel_effects_2017); [Coomber et al., 1997](#ref-coomber_independent_1997); [Sakata et al., 2000](#ref-sakata_neural_2000)). It is possible that the temperatures used in our experiment did not affect the brain regions involved in coding reversal learning. Future studies should explore the effects of the thermal environment on brain structure and function in these species to understand the mechanisms underlying the effects of temperature on learning. Furthermore, testing learning across different cognitive tasks could provide a more comprehensive understanding of the influence of temperature on cognition. In our analyses, sex was not included as a factor because lizards were not considered sexually mature, and because previous studies have found weak evidence for sex-dependent learning in many cognitive domains ([Szabo et al., 2019](#ref-szabo2019sex)). Indeed, the impacts of temperature on learning was not sex-depedent for the Eastern three-lined skink (*Bassiana dupeyerri*) ([Amiel & Shine, 2012](#ref-amiel_hotter_2012); [Clark et al., 2014](#ref-clark_colour_2014)). However, some studies find 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)). In addition, *B. dupeyerri* are known to sex-reverse - some individuals can have female chromosomes but male phenotypes - under cold incubation temperatures ([Dissanayake et al., 2021](#ref-dissanayake2021effects)). Such effects were not easily accounted for in these experiments making it challenging to understand if sex or temperature were the major drivers of learning differences ([Amiel et al., 2014](#ref-amiel_egg_2014); [Amiel & Shine, 2012](#ref-amiel_hotter_2012); [Clark et al., 2014](#ref-clark_colour_2014)). Further research should focus on the possible interactive effects of sex and incubation temperature on cognitive performance. However, such studies will be challenging given the large sample sizes required to execute these studies rigorously.

Despite sharing life-history traits, only *L. delicata* has proven successful in colonising various regions globally ([Baker, 1979](#ref-baker1979rainbow); [Chapple, Miller, et al., 2013](#ref-chapple2013divergent); [Chapple et al., 2015](#ref-chapple2015deliinvLHI)), and we predicted this may relate to higher behavioural flexibility. Furthermore, we expected behavioural flexibility in *L. delicata* to be less susceptible to the effect of early environment since this trait can be adaptive in several stages of invasion involving more than one generation. Regardless, we did not find differences in reversal learning between *L. delicata* and *L. guichenoti* as expected. Bezzina et al. ([2014](#ref-bezzina2014does)) also found no differences in learning between the two skinks in an associative learning task. Our results align with the latter study and suggest that behavioural flexibility does not differ between these species either. While it will be important to explore replicate invasion events (both successful and unsuccessful) such information is seldom available. Nonetheless, there is precedence in the literature to expect differences between these two species ([Chapple et al., 2011](#ref-chapple_know_2011); [Chapple, Whitaker, et al., 2013](#ref-chapple2013biosecurity)). For example, previous studies have shown that *L. delicata* more readily explores novel environments than *L. guichenoti*, which can be advantageous during colonisation ([Chapple et al., 2011](#ref-chapple_know_2011)). Our results along with others’ ([Bezzina et al., 2014](#ref-bezzina2014does)) suggest that other aspects of their biology are more relevant for *L. delicata*’s success as invaders than learning and behavioural flexibility. Differences in exploratory behaviour are likely to be the key factor explaining why *L. delicata* and not *L. guichenoti* is better at colonising new areas ([Chapple et al., 2011](#ref-chapple_know_2011)).

We acknowledge that sample size may be limiting our ability to detect the small effect sizes in our experiment given the uncertainty around slope contrasts between treatments (see 95% CI of contrasts in results). We analysed the behaviour of 81 individuals coming from a total of 36 unique clutches in *L. delicata* and 33 in *L. guichenoti*, yet it is challenging to do more animals than this given the logistical constraints associated with running such detailed cognitive trials. However, it is noteworthy that our effect sizes are very small and likely of little biological significance. For example, slopes of CORT-treated animals is 100.094% the slope of Controls in *L. delicata* and 96.865% in *L. guichenoti*, while the slope in hot-incubated lizards is 111.582% the slope of those incubated at cold temperatures in *L. delicata* and 127.384% in *L. guichenoti*.

In conclusion, our results indicate that lizards performed the reversal learning task, a measure of behavioural flexibility, at similar rates, with no discernible differences between treatments or species. These findings contrast with our initial predictions and suggest that behavioural flexibility in both species is robust to insults (at least from temperature and GCs) during early stages of development. Furthermore, our results indicate that other aspects of the biology of *L. delicata* may be more influential for their success as invaders than behavioural flexibility alone. Nevertheless, future studies should investigate the potential influence of prenatal CORT and temperature on cognition, considering other factors such as the type of cognitive task. It is also imperative to explore the effects of these treatments on brain function comprehensively. Additionally, further research is essential to determine which aspects of *L. delicata*’s biology contribute more significantly to their success as invaders than behavioral flexibility alone. Investigating these areas will provide a better understanding of the cognitive and biological mechanisms underlying the adaptability and invasive potential of both species.

## Data accessibility

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

## Declaration of AI use

We declare Chat GPT was used for questions related to coding and data analyses. All other parts of the manuscript were written by the authors and those parts where AI was used were checked and modified accordingly by the authors.

## 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.

## Conflict of interest declaration

We declare we have no competing interests.

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Fig 1— Experimental design of early environment manipulation and learning tasks. Panel A represents the early environment manipulation for both species. Panel B shows the habituation phase with the respective three different stages. And panel C represents the associative and reversal tasks; white lids show the ramps where the food reward was not accessible.

Fig 2— Results for *L. delicata* [(a), (b)] and *L. guichenoti* [(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.

# Suplementary Material

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

Table 1. Estimates of Reversal learning slope for all the different treatments per each task, specie, and group.

| Specie | Treatment | Mean | 95% CI | pmcmc |
| --- | --- | --- | --- | --- |
| *L. delicata* | CORT-Cold (n = 11) | **0.041** | **0.023 , 0.058** | **< 0.001** |
| N = 47 | Control-Cold (n = 12) | **0.062** | **0.044 , 0.081** | **< 0.001** |
| Obs = 1880 | CORT-Hot (n = 12) | **0.057** | **0.039 , 0.075** | **< 0.001** |
|  | Control-Hot (n = 12) | **0.045** | **0.026 , 0.063** | **< 0.001** |
| *L. guichenoti* | CORT-Cold (n = 10) | **0.048** | **0.024 , 0.073** | **< 0.001** |
| N = 37 | Control-Cold (n = 7) | **0.061** | **0.032 , 0.093** | **< 0.001** |
| Obs = 1480 | CORT-Hot (n = 10) | **0.065** | **0.041 , 0.090** | **< 0.001** |
|  | Control-Hot (n = 10) | **0.062** | **0.038 , 0.088** | **< 0.001** |

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).

Table 2. Estimates of Reversal learning slope for all the different treatments per each task, species, and group.

| Specie | Treatment | Mean | 95% CI | pmcmc |
| --- | --- | --- | --- | --- |
| *L. delicata* | CORT-Cold (n = 4) | **0.052** | **0.021 , 0.084** | **< 0.05** |
| N = 19 | Control-Cold (n = 5) | **0.067** | **0.038 , 0.096** | **< 0.001** |
| Obs = 760 | CORT-Hot (n = 3) | **0.073** | **0.036 , 0.113** | **< 0.001** |
|  | Control-Hot (n = 7) | **0.046** | **0.023 , 0.070** | **< 0.001** |
| *L. guichenoti* | CORT-Cold (n = 3) | **0.068** | **0.012 , 0.124** | **< 0.05** |
| N = 18 | Control-Cold (n = 3) | **0.086** | **0.032 , 0.147** | **< 0.05** |
| Obs = 720 | CORT-Hot (n = 5) | **0.072** | **0.030 , 0.115** | **< 0.05** |
|  | Control-Hot (n = 7) | **0.066** | **0.031 , 0.104** | **< 0.001** |

Here we included only those individuals who made the right choice in 8 out of the last 10 trials in the previous associative task. 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).

#### Figures with raw data

|  |
| --- |
| Probability of choosing correctly over trials for each of the treatments and species. The dots represent the proportion of individuals per trial that chose correctly while the lines show the mean predicted probability of choosing correctly, estimated using the posteriors of our model. |

#### Results using a standard learning criterion of 5 consecutive correct choices

Table 3. Number of individuals per treatment and species that reached a learning criterion of 5 consecutive correct choices (n lizards), and the average of trials taken (Trial) together with the standard deviation (sd).

| Specie | Treatment | n lizards | Mean | sd |
| --- | --- | --- | --- | --- |
| *L. delicata* | Control-Cold | 9 | 25.89 | 5.84 |
|  | CORT-Cold | 6 | 21.17 | 8.80 |
|  | Control-Hot | 2 | 33.50 | 9.19 |
|  | CORT-Hot | 4 | 24.25 | 13.20 |
| *L. guichenoti* | Control-Cold | 3 | 28.00 | 5.20 |
|  | CORT-Cold | 4 | 26.00 | 12.36 |
|  | Control-Hot | 8 | 22.62 | 9.47 |
|  | CORT-Hot | 6 | 28.33 | 9.69 |

#### Checking the main models plots

Model formula for the reversal task is:

Choice ~ trial\_reversal*cort*temp + (1 + trial\_reversal|lizard\_id)

Plots for the different models of the reversal task:  
1.- *L. delicata*

Estimate Est.Error Q2.5 Q97.5  
R2 0.1040341 0.01243751 0.07984544 0.1290352

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2.- *L. guichenoti*

Estimate Est.Error Q2.5 Q97.5  
R2 0.1464713 0.0153245 0.1158186 0.1763292

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#### Checking the effect of age on the learning rate in the associative task.

Model formula: Choice ~ age.start + trial\_associative \* cort \* temp + (1 + trial\_associative|lizard\_id) + (1|clutch)

Table 4. Results for *L. delicata* when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | -0.68 | 0.23 | -1.14 | -0.22 | 1.00 | 3099.14 | 5364.68 |
| age.start | 0.00 | 0.00 | -0.00 | 0.01 | 1.00 | 7524.39 | 6689.19 |
| trial\_reversal | 0.04 | 0.01 | 0.02 | 0.06 | 1.00 | 3033.03 | 5261.65 |
| cortControl | -0.34 | 0.32 | -0.99 | 0.30 | 1.00 | 2845.28 | 4978.49 |
| tempHot | -0.69 | 0.33 | -1.33 | -0.05 | 1.00 | 2868.81 | 4595.09 |
| trial\_reversal:cortControl | 0.02 | 0.01 | -0.00 | 0.05 | 1.00 | 2939.03 | 4497.28 |
| trial\_reversal:tempHot | 0.02 | 0.01 | -0.01 | 0.04 | 1.00 | 2759.31 | 4331.14 |
| cortControl:tempHot | 0.64 | 0.46 | -0.24 | 1.56 | 1.00 | 2786.33 | 4315.03 |
| trial\_reversal:cortControl:tempHot | -0.03 | 0.02 | -0.07 | 0.00 | 1.00 | 2849.12 | 4135.58 |

Table 5. Results for *L. guichenoti* when the model included the age:

| Predictors | Estimate | Est.Error | l-95% CI | u-95% CI | Rhat | Bulk\_ESS | Tail\_ESS |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Intercept | -0.94 | 0.32 | -1.59 | -0.32 | 1.00 | 4613.34 | 5435.92 |
| age.start | 0.01 | 0.01 | -0.01 | 0.02 | 1.00 | 7490.80 | 6179.03 |
| trial\_reversal | 0.05 | 0.01 | 0.02 | 0.07 | 1.00 | 4170.12 | 4876.09 |
| cortControl | -0.01 | 0.46 | -0.92 | 0.92 | 1.00 | 4008.70 | 4602.77 |
| tempHot | -0.33 | 0.45 | -1.19 | 0.57 | 1.00 | 4124.09 | 4611.95 |
| trial\_reversal:cortControl | 0.01 | 0.02 | -0.02 | 0.05 | 1.00 | 3933.61 | 4885.22 |
| trial\_reversal:tempHot | 0.02 | 0.02 | -0.02 | 0.05 | 1.00 | 4123.86 | 4555.44 |
| cortControl:tempHot | 0.35 | 0.63 | -0.93 | 1.58 | 1.00 | 3791.58 | 4842.61 |
| trial\_reversal:cortControl:tempHot | -0.02 | 0.03 | -0.07 | 0.03 | 1.00 | 3623.25 | 4622.64 |

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| Fig 3— Distribution of the age of the lizards by treatment and species |