

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

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23 Abstract

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

43 Introduction

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

61 The conditions in which organisms develop can be altered by the circumstances faced by their
62 parents. For instance, exposing three-spined stickleback (*Gasterosteus aculeatus*) females to a
63 model predator during ovulation, affected their offspring's tendency to use social cues (Feng et
64 al., 2015). Under stressful situations, animal sensory systems detect and transmit environmental
65 signals to instigate adaptive physiological and behavioural adjustments – responses that are

66 mediated by glucocorticoids (GCs) (Sapolsky et al., 2000). Glucocorticoids can be transmitted to
67 offspring and influence their phenotype and development through transgenerational effects
68 (Crino et al., 2023). In addition to the environments experienced by parents, offspring also
69 experience potentially stressful environmental conditions that can interact with or amplify
70 parental effects. For example, human disturbance alters snapping turtles' nest-site choice,
71 ultimately impacting the temperature at which eggs are incubated (Kolbe & Janzen, 2002).
72 Animals' responses to abrupt temperature changes are mediated by GCs (Crino et al., 2023),
73 which can influence animals' decision-making while nesting (Kolbe & Janzen, 2002). As a
74 result, GCs transmission and early thermal environment are expected to interact and shape
75 offspring traits. Both GCs and temperature are known to exert potent effects on brain
76 development (Amiel et al., 2017; Coomber et al., 1997; Jonson et al., 1976; Zhu et al., 2004) and
77 cognition (Clark et al., 2014; Lui et al., 2017), but little is known about how they may interact to
78 affect behavioural flexibility. Since behaving flexibly is essential for dealing with novel
79 environments, understanding how early developmental environments collectively impact
80 cognition is crucial for predicting the consequences of environmental change.

81 Here, we studied the effects of prenatal temperature and corticosterone (CORT) (the main GC in
82 reptiles) (Crino et al., 2023), on behavioural flexibility in two species of reptiles, the delicate
83 skink (*Lampropholis delicata*) and the common garden skink (*L. guichenoti*). Both species have
84 similar life-histories and overlapping distributions (Chapple et al., 2011, 2014), but only
85 *Lampropholis delicata* has been successful in colonising different areas around the globe (Baker,
86 1979; Chapple, Miller, et al., 2013; Chapple et al., 2015). Previous studies exploring behavioural
87 differences between the two species have found *L. delicata* to be more exploratory than *L.*
88 *guichenoti* (Chapple et al., 2011), but no differences in learning were observed between the
89 skinks in an associative learning task (Bezzina et al., 2014). Disparities in behavioural flexibility
90 may be driving the differences in invasion success between both skinks, a prediction supported
91 in other invasive species (Amiel et al., 2011; Chapple et al., 2012; Sol et al., 2008; Sol &
92 Lefebvre, 2000; Wright et al., 2010). However, the relative impact of early environments on
93 behavioural flexibility remains unexplored. Early environments are known to affect suites of
94 morphological and behavioural traits differently in each species (Carrasco et al., 2024; Kar et al.,
95 2023). We manipulated CORT and incubation temperature in both species and then subjected
96 hatchlings to a reversal learning task. Reversal learning is a widely employed tool to measure
97 behavioural flexibility (Gapp et al., 2014; Hurtubise & Howland, 2017), as it assesses an
98 individual's ability to reverse a previously learnt behaviour providing researchers with a clear
99 indicator of their ability to adjust to new conditions (Brown & Tait, 2010).

100 We predicted that both cold incubation temperatures and CORT treatment will impair
101 behavioural flexibility (Clark et al., 2014; Lui et al., 2017), and the combination of both
102 treatments to have the most detrimental effect on behavioural flexibility. Since invasive species
103 are expected to behave more flexibly (Amiel et al., 2011; Chapple et al., 2012; Sol et al., 2008;
104 Sol & Lefebvre, 2000; Wright et al., 2010), we predicted that *L. delicata*, on average, will
105 perform more proficiently in the task compared to *L. guichenoti* and that behavioural flexibility
106 in *L. delicata* will be more robust to early environmental conditions.

107 **Methods**

108 *Husbandry*

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

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

129 *Hatchlings* – Eggs in the incubator were checked three times a week for hatchlings. After
130 hatchling, we measured juveniles' SVL and Tail Length (TL) with a ruler to the nearest mm and
131 weighed them with a digital scale ± 0.001 g error. We then placed hatchlings in individual
132 enclosures (18.7L x 13.2W x 6.3H cm) and provided them with non-stick matting, a shelter, and
133 a small water dish. During this period, they were sprayed with water every day and received 3-6
134 small *A. domestica* crickets three times a week. All care otherwise followed similar protocols to
135 adults (see above). Two weeks before starting the training phase (see below), lizards were moved
136 to an experimental arena for acclimatisation. The arenas were individual medium size (41 L x
137 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
138 and a water dish on the other. These new enclosures were placed in two rooms across 7 different
139 racks that were monitored by 7 different CCTV systems (device model DVR-HP210475) that
140 allowed us to record their behaviour during the experiment (see details below). Conditions in
141 these rooms were identical to the main room, but the number of lizards per species and
142 treatment in each rack was counterbalanced to control for any potential effect of the room or the
143 position of the lizard on the rack. During acclimatisation and throughout the experiment, lizards
144 were fed with only one cricket per day dusted with calcium and multivitamin (see protocol
145 below), and water was supplied *ad libitum*. We provided a temperature gradient by means of a
146 heat cord and heat lamps in a 12 h light: 12 h dark cycle. The temperature of both rooms was set
147 to between 22-24 °C.

148 *Manipulating Early Thermal and CORT Environments*

149 To empirically test the effect of early environment we manipulated CORT concentration in eggs
150 and incubated them under one of two temperature regimes (Cold – 23 °C ± 3 °C or Hot – 30 °C ±
151 3 °C) in a 2x2 factorial design (Fig. 1 A). We first allocated eggs into one of two different
152 treatments: in the CORT treatment, eggs were topically supplied with 5µL of CORT dissolved in
153 100% Ethanol (vehicle) at a final concentration of 10 pg CORT/mL; in the Control treatment
154 eggs received an equal volume of the vehicle. This method has been validated before in *L.*
155 *delicata* (O. Crino et al., 2024), increasing CORT concentration in eggs by approximately 2
156 standard deviations above the mean natural concentration. After the hormone treatment, eggs
157 were incubated in one of the two previously mentioned temperature regimes (Cold or Hot) until
158 hatching. These temperatures represent the upper and lower limit of the natural incubation
159 temperatures (Cheetham et al., 2011; Qualls & Shine, 2000).

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

165 *Quantifying Behavioural Flexibility*

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

172 The habituation phase was divided into three stages: in the first stage, the reward, a small, frozen
173 cricket (*A. domestica*) was placed in an opaque petri dish (3 D x 1.6 H cm) (Fig. 1 Habituation
174 phase, Stage 1); in the second stage, the petri dish with the cricket was placed on top of the white
175 3D printed ramps (Fig. 1 Habituation phase, Stage 2); and in the third stage, the cricket was left
176 inside a well (3D x 1.75H cm) on top of the ramp (Fig. Fig. 1 Habituation phase, Stage 3). Trials
177 in every stage lasted one hour, beginning when the feeding block (petri dish, ramp, or both) was
178 placed inside the enclosure and finishing when we took the feeding block away. We recorded
179 whether the cricket had been consumed or not and we considered a stage as completed if the
180 lizards ate the crickets in at least 5 out of 6 trials. This phase lasted 38 days, and only in one case
181 a lizard did not progress to the next phases because it did not pass the acquisition criteria.

182 In the colour association (Associative task in Fig. 1) and reversal task (Reversal task in Fig. 1 B),
183 we used three ramps that were identical in shape and size but different in the colour. The colours
184 of these ramps were green, red, and blue, as previous studies demonstrate that squamates can
185 discriminate between these colours (Baden & Osorio, 2019). However, to control for potential
186 colour biases, we assigned one group of lizards within each treatment to associate food in the
187 blue ramp followed by the red ramp in the reversal, while in the other group the order was
188 reversed. As in the last stage of the habituation phase, we placed the cricket inside the wells of
189 the ramps, but then two of them were covered with 3D-printed lids (3 D x 0.5 H cm) so prey was

190 only accessible on one ramp. The food reward was placed on all three ramps to avoid lizards
191 using prey chemical cues that were released in the closed wells through a series of small holes on
192 the top of the lids. In all trials, the position of the feeders was changed randomly to ensure
193 subjects were using colour rather than spatial cues for the association. Lizards were tested in the
194 association task once a day for 35 days and once a day for 40 days in the reversal.

195 The full experiment was performed between the 6th of March until the 26th of June 2023. Tests
196 were made between 11 to 12 am, when the lizards were active. Trials in the learning phases
197 (colour associative task and reversal tasks) were recorded with different CCTV systems always
198 using the same camera per individual. We recorded whether the animal chose the correct ramp
199 first or not. We considered that a choice was made if the head of the lizard was inside the well of
200 one of the ramps. We considered a trial failed if there was no choice in one hour of recording.
201 Those trials were scored as NA. Here, we analysed the data of the reversal task as a measure of
202 behavioural flexibility (Brown & Tait, 2010).

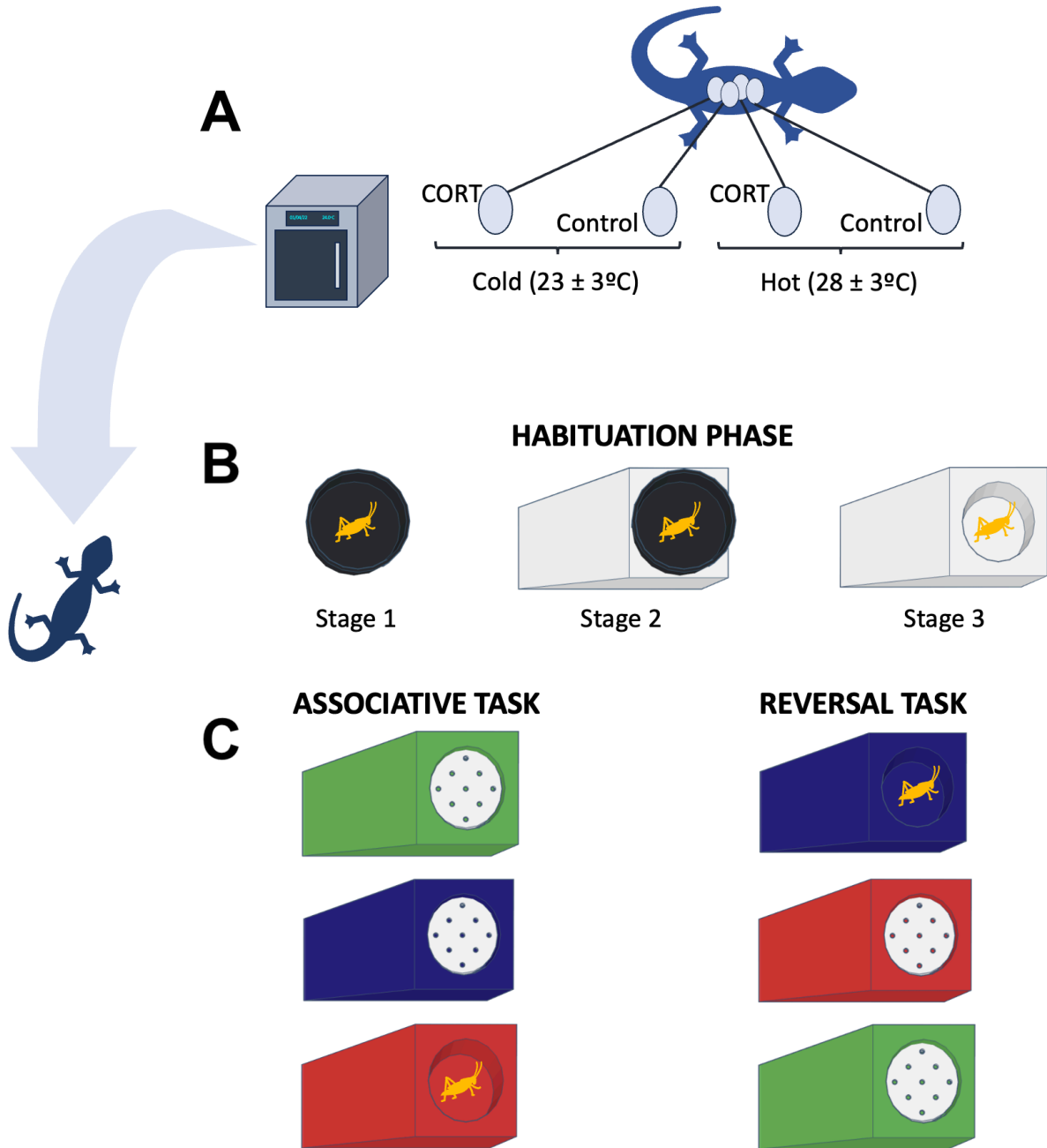


Fig 1— Experimental design of early environment manipulation and learning tasks. Panel A represents the early environment manipulation for both species. Panel B shows the habituation phase with the respective three different stages. And panel C represents the associative and reversal tasks; white lids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

203 *Statistical analyses*

204 We performed the analyses for each species separately. We fitted two different Bayesian
 205 multilevel models using the *brms* function from the *brms* package (Bürkner, 2017) using an R
 206 extension (version 2.8.2) (R Core Team, 2021). We ran four parallel MCMC chains of 3000

207 iterations for each model, with a warmup period of 1000 iterations. We modelled correct choices
208 [correct (1) or not (0)] as the response variable, and trial, hormone (CORT versus Control),
209 incubation temperature (Cold versus Hot), along with the three-way interaction between trial,
210 hormone, and temperature. If early environments impact learning then we would predict that the
211 rate of learning (i.e., trial slope) varies by treatment as captured by the interactions. The error
212 structure was modelled using a Bernoulli distribution with a logit link function (family =
213 Bernoulli(link = 'logit')). We included a random intercept and slope (trial) for each lizard in our
214 models. We also incorporated the clutch identity as a random factor. *L. delicata* lays one clutch
215 per year, while *L. guichenoti* lays two (Chapple et al., 2011, 2014). Since eggs were collected
216 during half of the breeding season, clutches likely come from different mothers. Additionally,
217 previous research has shown that clutches are generally sired by a single male, but sperm storage
218 can occur (Kar et al., 2023). Given our partial split-clutch design, and the fact that maternal
219 effects are expected to be stronger than paternal effects in these species, including the clutch as a
220 random factor should account for the effects of parental identity.

221 Learning can also be age-dependent (see Noble et al., 2014), and given that lizards incubated at
222 different temperatures hatch at different times we explored the effects of age on learning. Indeed,
223 lizards' age at the beginning of the experiment ranged from 41 to 148 days old in *L. delicata* and
224 48 to 132 in *L. guichenoti*. However, when this variable was included in the models we did not
225 find any significant effect of age (see Supplementary Material). As such, we present models
226 without age as a fixed effect.

227 We used the posterior distributions of parameters from these models to test for differences in
228 learning rate between treatments and species. Learning slopes were obtained using the 'trial'
229 estimates and its interaction with hormone and temperature treatments. Slope estimates greater
230 than zero were considered as evidence of learning, while those less or equal to zero were not.
231 p_{mcmc} test the hypothesis that slopes and slopes contrasts are different from zero. We considered
232 an effect statistically significant if $p_{\text{mcmc}} < 0.05$.

233 *Ethical note*

234 We collected subjects from a breeding colony kept in the lab since 2019. Both the breeding
235 animals and the ones used in this experiment were provided humane laboratory housing, with
236 thermoregulatory opportunities, light (UV and heat) and moderate levels of humidity (see above
237 for details). Lizards in the colony received food *ad libitum* while the subjects in the experiment
238 were fed one cricket daily during the experiment. All lizards were handled minimally to avoid
239 stress. Experimental procedures and laboratory housing complied with Australian law and were
240 approved by the Australian National University Animal Experimentation Ethics Committee
241 (A2022_33). Animals born in captivity cannot be re-released into the wild as stringent biosafety
242 protocols across the states prohibit this. It is also not possible to rehome such a large number of
243 animals. Therefore, upon completion of experimental trials, lizards were kept in their enclosure
244 until they were humanely euthanized following approved procedures. To euthanise animals, we
245 use an injection of sodium pentobarbital (i.e., Lethabarb) (diluted 1:4 ringers solution) at 60-
246 100mg/kg, injected intrapleuroperitoneally. We monitored the animals to ensure there was no
247 irritation from the agent as indicated by distressed animals. Gently pinching the toes was used to
248 confirm the absence of a response before the animal was disposed of.

249 Crickets were maintained in big communal enclosures (68.5 L x 49 W x 39 H cm) in the same
250 room where the big colony is, and at the same temperature and light conditions. They were

251 provided vegetables and water ad libitum, and they were given several egg carton shelters to
252 refuge. All lizards were fed alive crickets except during the experiment, when we used crickets
253 frozen at -21 °C for 24 hours.

254 **Results**

255 We started with 96 lizards, 48 per species and 12 per treatment per species. However, our final
256 sample size was 84 due to natural mortality (n = 11) or failure to complete the training stage (n =
257 1). The final sample sizes per treatment and species are listed in Fig. 2. These animals came from
258 a total of 36 clutches in *L. delicata* and 33 in *L. guichenoti*.

259 Both species learnt across all treatments (Fig. 2 B, D), but we did not find any difference
260 between treatments or species in the rate of learning [mean slopes (denoted as β throughout) per
261 treatment for both species provided in Table 1 in Supplementary Material]. For *L. delicata*,
262 hormone treatment ($\beta_{\text{Control}} - \beta_{\text{CORT}} = 0.005$, 95% CI = [-0.034, 0.043], $p_{\text{mcmc}} = 0.873$), incubation
263 temperature ($\beta_{\text{Hot}} - \beta_{\text{Cold}} = -0.001$, 95% CI = [-0.039, 0.038], $p_{\text{mcmc}} = 0.989$), or the interaction
264 ($[(\beta_{\text{Control-Hot}} - \beta_{\text{CORT-Hot}}) - (\beta_{\text{Control-Cold}} - \beta_{\text{CORT-Cold}})] = -0.034$, 95%CI = [-0.070, 0.002], $p_{\text{mcmc}} =$
265 0.062) did not affect learning (see Fig. 2 A, B). We similarly found no significant effects of
266 hormone ($\beta_{\text{Control}} - \beta_{\text{CORT}} = 0.005$, 95% CI = [-0.034, 0.046], $p_{\text{mcmc}} = 0.810$), incubation
267 temperature ($\beta_{\text{Hot}} - \beta_{\text{Cold}} = 0.009$, 95% CI = [-0.033, 0.048], $p_{\text{mcmc}} = 0.629$), or the interaction
268 ($[(\beta_{\text{Control-Hot}} - \beta_{\text{CORT-Hot}}) - (\beta_{\text{Control-Cold}} - \beta_{\text{CORT-Cold}})] = -0.016$, 95%CI = [-0.069, 0.035], $p_{\text{mcmc}} =$
269 0.529) on learning in *L. guichenoti* (see Fig. 2 C, D).

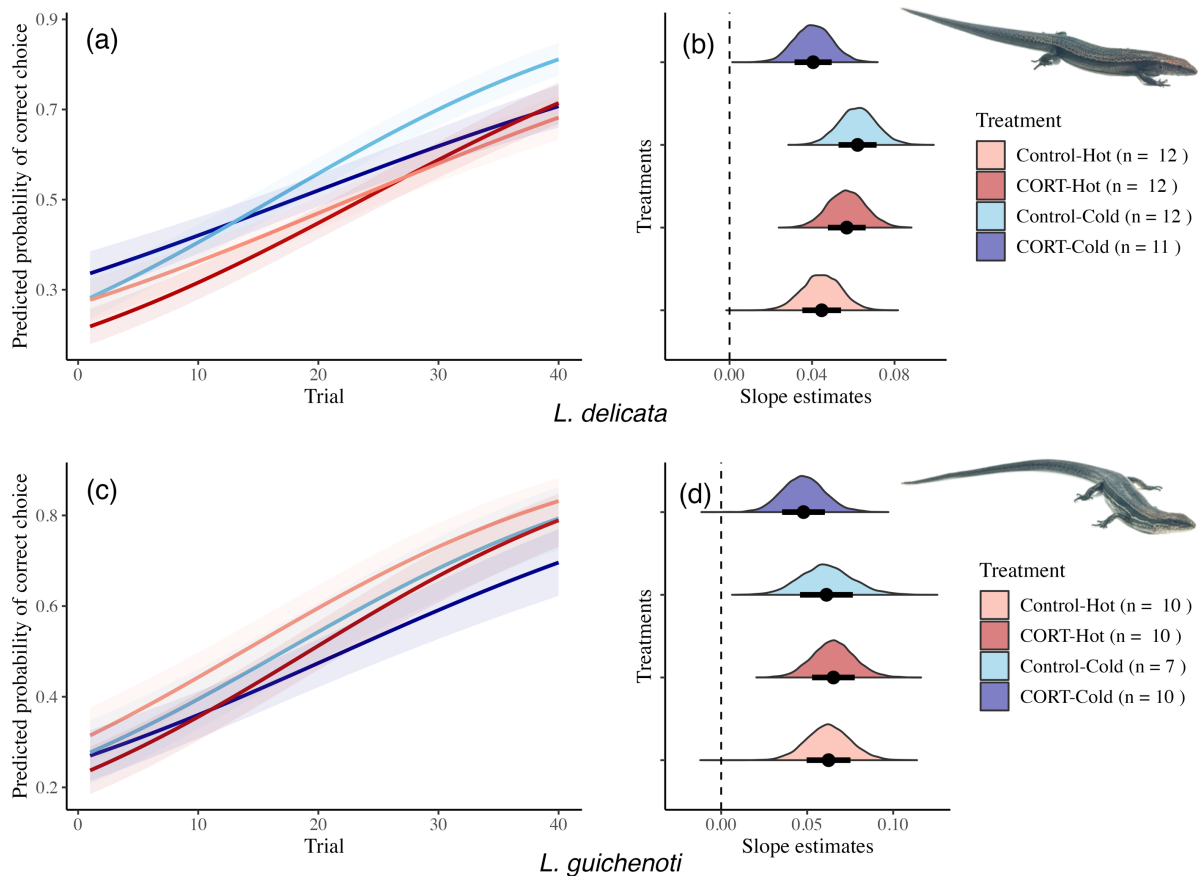


Fig 2— Results for *L. delicata* [(a), (b)] and *L. guichenoti* [(c), (d)]. Panels (a) and (c) show the predicted probability of choosing the correct feeder first over trials. The lines represent the mean predicted probability of choosing the correct feeder first on each trial, and the shaded areas indicate 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.

270 Finally, when all individuals from all the treatments were pooled together and species were
 271 compared, there were no significant differences in the estimated slopes (mean of learning slope
 272 in *L. delicata* = 0.051, 95% CI = [0.027, 0.075]; mean learning of slope in *L. guichenoti* = 0.059,
 273 95% CI = [0.030, 0.088]; $\beta_{L. delicata} - \beta_{L. guichenoti} = -0.008$, 95% CI = [-0.043, 0.026], $p_{\text{mcmc}} =$
 274 0.636). Figures for both species with the raw data were included in the Supplementary Material.

275 Discussion

276 This study aimed to determine whether prenatal temperature and GCs impact behavioural
 277 flexibility in two closely related species, the delicate skink (*Lampropholis delicata*) and the
 278 common garden skink (*L. guichenoti*). We expected the early environment to influence
 279 behavioural flexibility in both species. We also predicted that *L. delicata* would be more flexible

280 than *L. guichenoti* and less affected by prenatal conditions. Nevertheless, we did not find
281 significant differences between treatments or species when we compared their performance in a
282 reversal task. Our results show that, on average, all the lizards from the four treatments and the
283 two species learnt the reversal task - indicated by estimated slopes that are significantly different
284 from zero - and that they learn, on average, at the same rate. Furthermore, these results were
285 consistent when only those individuals who performed better in the previous associative task
286 were included (see Supplementary material).

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

311 More striking is the absence of an effect of incubation temperature on behavioural flexibility
312 because most studies have reported better performance in different associative learning and
313 motor tasks when incubated at higher temperatures (Amiel et al., 2014; Amiel & Shine, 2012;
314 Clark et al., 2014). However, studies that have reported cold-incubated lizards to learning faster
315 (Abayarathna & Webb, 2020; Dayananda & Webb, 2017) used incubation temperatures far
316 beyond the natural range in their hot treatments. As such, it is unclear how such conditions relate
317 to wild environments. Our results suggest that behavioural flexibility is robust to temperature in
318 both species of skinks at least in this study. It could be argued that the differences in our
319 incubation temperatures were not extreme enough to elicit significant differences in learning
320 performance. However, the mean difference between the temperatures used in our experiment
321 were similar to those in previous studies (Amiel et al., 2014; Amiel & Shine, 2012; Clark et al.,
322 2014), and are aligned with natural variation at the extremes of incubation conditions in the wild
323 (Cheetham et al., 2011; Qualls & Shine, 2000). In lizards, impacts on learning from early thermal
324 environments are associated with changes in neural density (Amiel et al., 2017). However,

325 several studies have shown that the effects of temperature on brain structure and physiology are
326 region dependent (Amiel et al., 2017; Coomber et al., 1997; Sakata et al., 2000). It is possible
327 that the temperatures used in our experiment did not affect the brain regions involved in coding
328 reversal learning. Future studies should explore the effects of the thermal environment on brain
329 structure and function in these species to understand the mechanisms underlying the effects of
330 temperature on learning. Furthermore, testing learning across different cognitive tasks could
331 provide a more comprehensive understanding of the influence of temperature on cognition. In
332 our analyses, sex was not included as a factor because lizards were not considered sexually
333 mature, and because previous studies have found weak evidence for sex-dependent learning in
334 many cognitive domains (Szabo et al., 2019). Indeed, the impacts of temperature on learning was
335 not sex-dependent for the Eastern three-lined skink (*Bassiana dupeyerrii*) (Amiel & Shine, 2012;
336 Clark et al., 2014). However, some studies find evidence for interactive effects of incubation
337 temperature and sex on brain physiology in other species of reptiles (Coomber et al., 1997;
338 Sakata et al., 2000). In addition, *B. dupeyerrii* are known to sex-reverse - some individuals can
339 have female chromosomes but male phenotypes - under cold incubation temperatures
340 (Dissanayake et al., 2021). Such effects were not easily accounted for in these experiments
341 making it challenging to understand if sex or temperature were the major drivers of learning
342 differences (Amiel et al., 2014; Amiel & Shine, 2012; Clark et al., 2014). Further research
343 should focus on the possible interactive effects of sex and incubation temperature on cognitive
344 performance. However, such studies will be challenging given the large sample sizes required to
345 execute these studies rigorously.

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

364 We acknowledge that sample size may be limiting our ability to detect the small effect sizes in
365 our experiment given the uncertainty around slope contrasts between treatments (see 95% CI of
366 contrasts in results). We analysed the behaviour of 81 individuals coming from a total of 36
367 unique clutches in *L. delicata* and 33 in *L. guichenoti*, yet it is challenging to do more animals
368 than this given the logistical constraints associated with running such detailed cognitive trials.
369 However, it is noteworthy that our effect sizes are very small and likely of little biological

370 significance. For example, slopes of CORT-treated animals is 100.094% the slope of Controls in
371 *L. delicata* and 96.865% in *L. guichenoti*, while the slope in hot-incubated lizards is 111.582%
372 the slope of those incubated at cold temperatures in *L. delicata* and 127.384% in *L. guichenoti*.

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

386 **Data accessibility**

387 All data, data description, and R code are available in public repository [https://github.com/Pablo-](https://github.com/Pablo-Recio/CORT_Temp_Behavioural_flexibility)
388 [Recio/CORT_Temp_Behavioural_flexibility](https://github.com/Pablo-Recio/CORT_Temp_Behavioural_flexibility).

389 **Declaration of AI use**

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

393 **Authors' contributions**

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

403 **Conflict of interest declaration**

404 We declare we have no competing interests.

405 **Funding**

406 This work was supported by a National Australian University fellowship (P.R.), the Australian
407 Research Council (grant no. DP210101152) to D.N. and C.F., and the ACT Herpetological
408 Association grant to P.R.

409 **Acknowledgements**

410 We thank the help and assistance of our lab technicians Benjamin Durant and Michelle Stephens
411 for taking care of the lizards. We are also grateful to ACTHA for the grant for the 3D printed
412 feeders, and we also thank ANU MakerSpace, where we designed and built the prototypes of the
413 3D printed feeders. Finally, we wish to acknowledge the anonymous reviewers for their valuable
414 feedback on the manuscript.

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577

578 **Supplementary Material**

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

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

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

583

584 Mean shows the arithmetic means of the estimates obtained from the posteriors of the model, and
 585 95% CI indicates the 95% confidence interval of the mean. All pmcmc tested the hypothesis that
 586 the mean equals zero. In bold, those values that are significant ($p_{\text{pmcmc}} < 0.05$).

587

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

Specie	Treatment	Mean	95% CI	pmcmc
<i>L. delicata</i>	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			
<i>L. guichenoti</i>	CORT-Hot (n = 3)	0.073	0.036 , 0.113	< 0.001
	Control-Hot (n = 7)	0.046	0.023 , 0.070	< 0.001
	CORT-Cold (n = 3)	0.068	0.012 , 0.124	< 0.05
	N = 18			
<i>L. guichenoti</i>	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

590

591 Here we included only those individuals who made the right choice in 8 out of the last 10 trials
 592 in the previous associative task. Mean shows the arithmetic means of the estimates obtained from
 593 the posteriors of the model, and 95% CI indicates the 95% confidence interval of the mean. All
 594 pmcmc tested the hypothesis that the mean equals zero. In bold, those values that are significant
 595 (pmcmc <0.05).

596

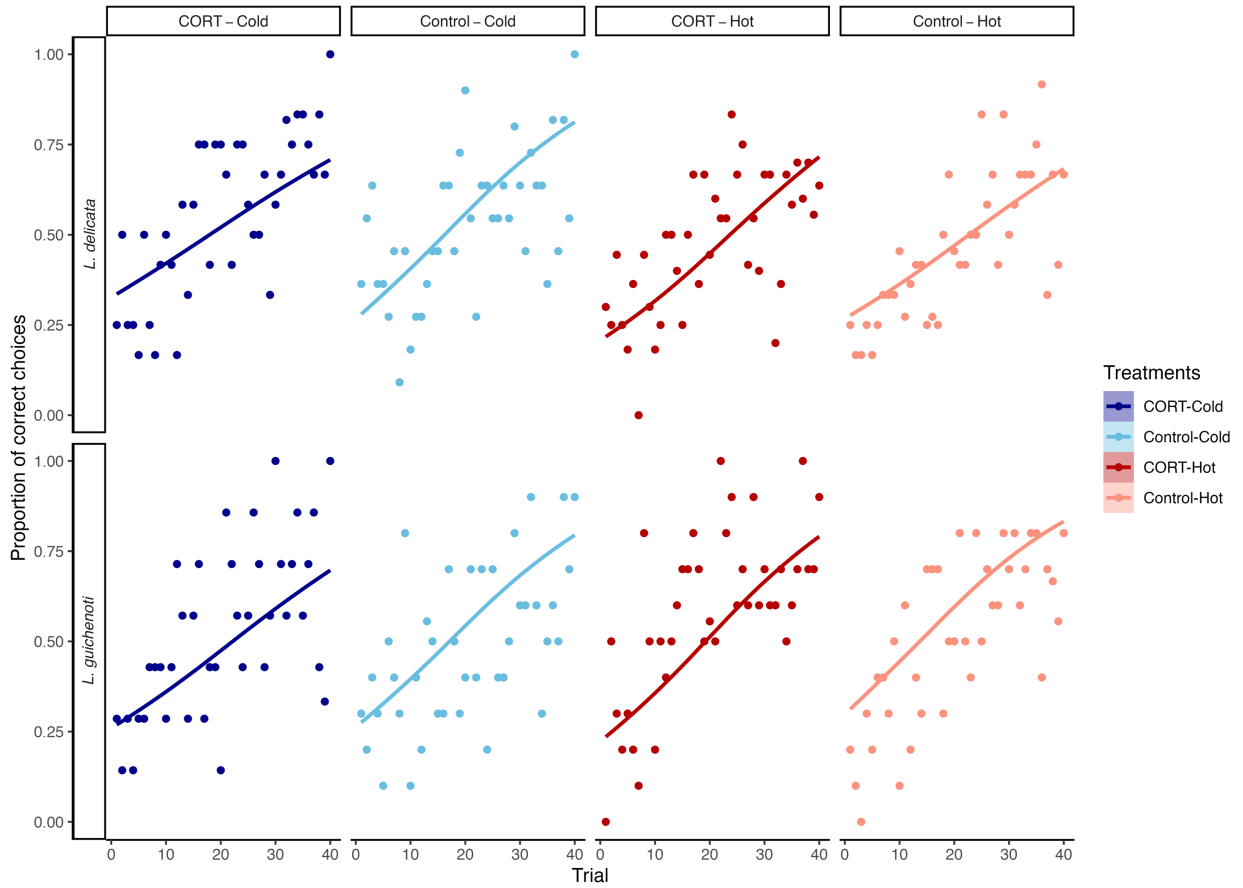


Fig 3— Probability of choosing correctly over trials for each of the treatments and species. The dots represent the proportion of individuals per trial that chose correctly while the lines show the mean predicted probability of choosing correctly, estimated using the posteriors of our model.

599 *Results using a standard learning criterion of 5 consecutive correct choices*

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

Specie	Treatment	n lizards	Mean	sd
<i>L. delicata</i>	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
<i>L. guichenoti</i>	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

603

604 *Checking the main models plots*

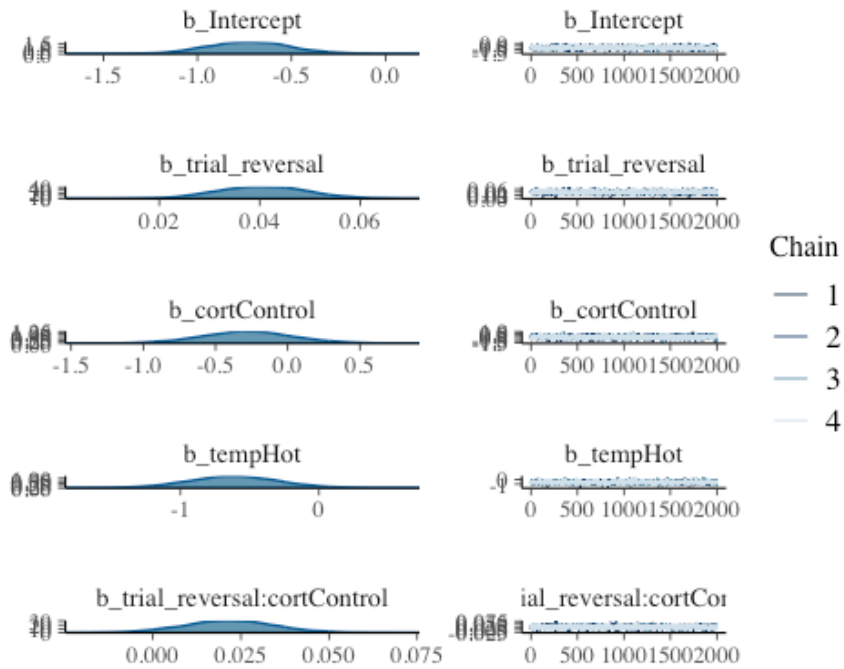
605 Model formula for the reversal task is:

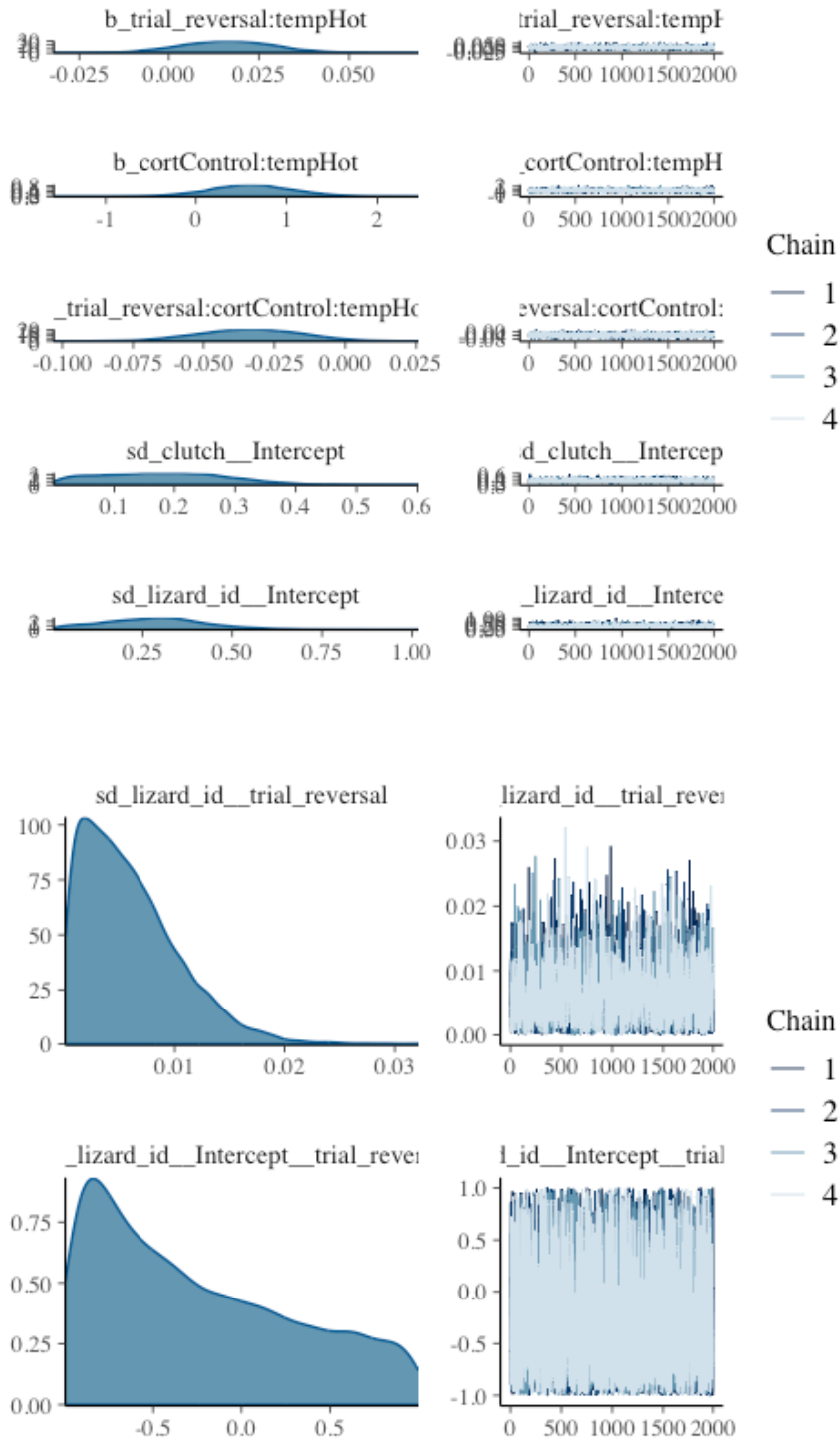
606 $\text{Choice} \sim \text{trial_reversal} \text{corttemp} + (1 + \text{trial_reversal} | \text{lizard_id})$

607 Plots for the different models of the reversal task:

608 1.- *L. delicata*

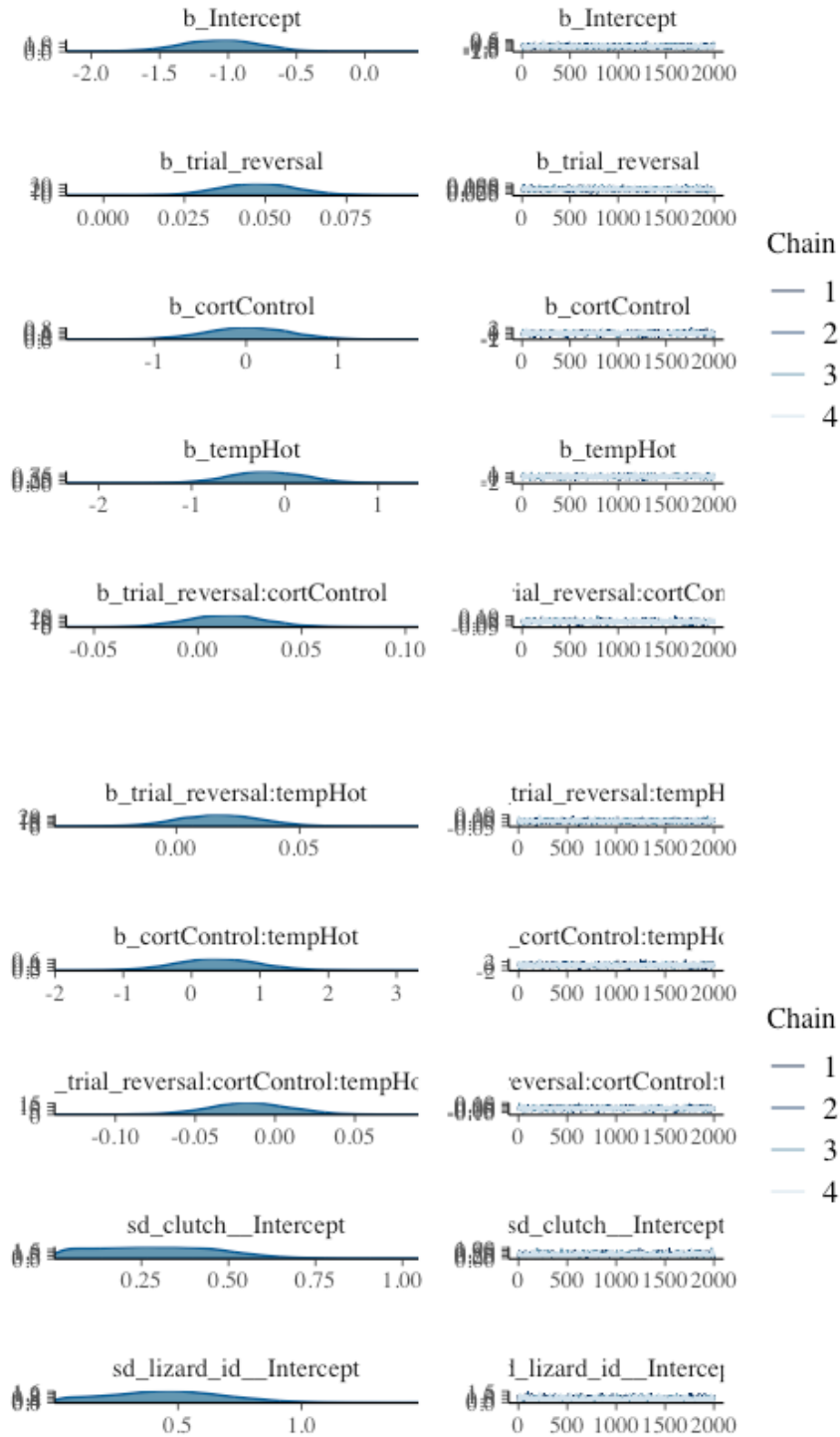
	Estimate	Est.Error	Q2.5	Q97.5
609 R2	0.1040341	0.01243751	0.07984544	0.1290352

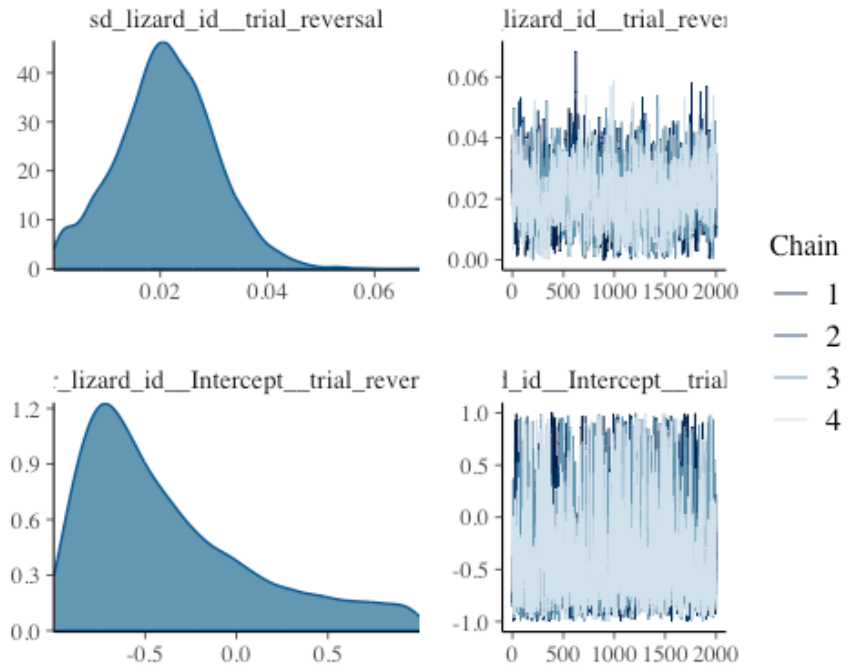




612 2.- *L. guichenoti*

	Estimate	Est.Error	Q2.5	Q97.5
613				
614	R2	0.1464713	0.0153245	0.1158186 0.1763292





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

617 Model formula: Choice ~ age.start + trial_associative * cort * temp + (1 +
618 trial_associative|lizard_id) + (1|clutch)

619 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_rever sal	0.04	0.01	0.02	0.06	1.00	3033.03	5261.65
cortContro l	-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_rever sal:cortCo ntrol	0.02	0.01	-0.00	0.05	1.00	2939.03	4497.28
trial_rever sal:tempH ot	0.02	0.01	-0.01	0.04	1.00	2759.31	4331.14
cortContro l:tempHot	0.64	0.46	-0.24	1.56	1.00	2786.33	4315.03
trial_rever sal:cortCo ntrol:temp Hot	-0.03	0.02	-0.07	0.00	1.00	2849.12	4135.58

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622 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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624

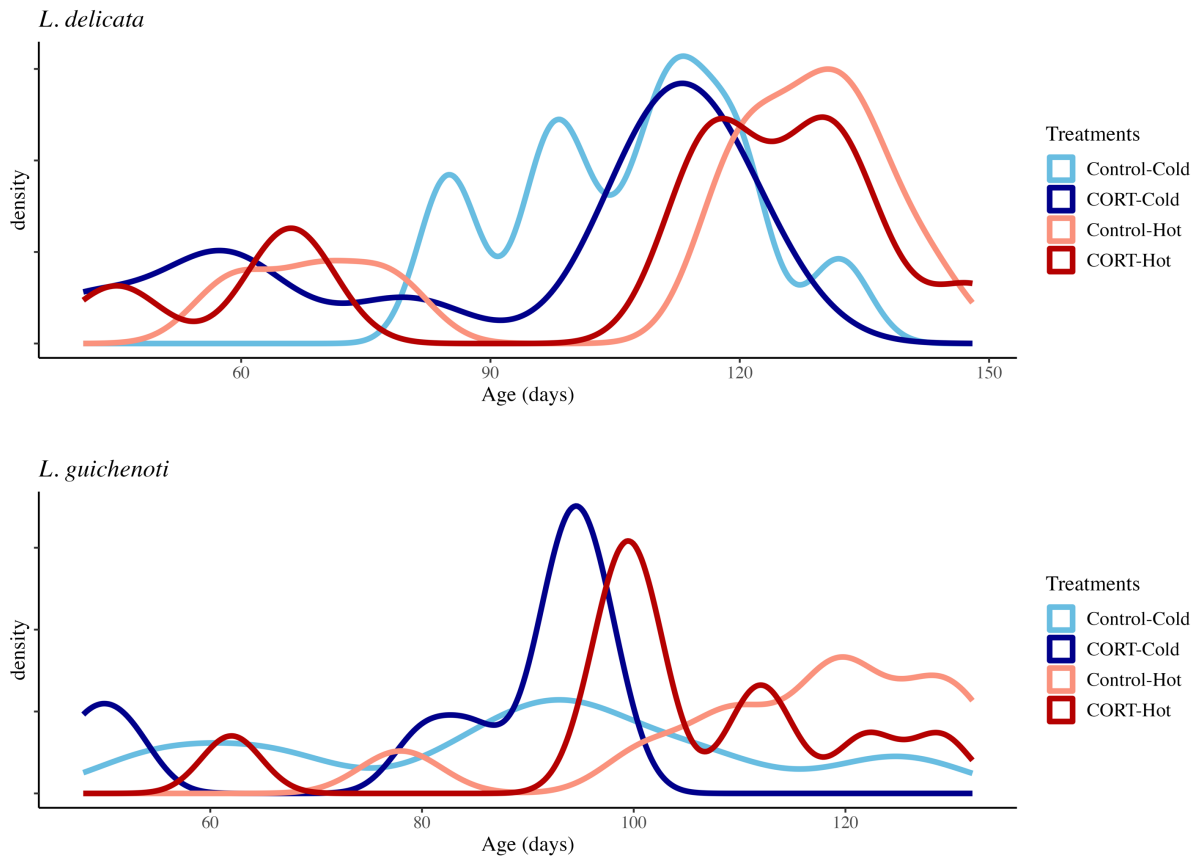


Fig 4— Distribution of the age of the lizards by treatment and species