## Methods

#### Subjects

We employed juveniles from two species of skinks in our experiments. One species was the common garden skink (*L. guichenoti*), and the other the delicate skink (*Lampropholis delicata*). Both species are small (∼35–55 mm snout-vent length (SVL)), generalist, and oviparous skinks that can be found in suburban areas in south-eastern Australia (Chapple et al. 2011). Breeding periods are similar, but they differ in their reproductive output: L. guichenoti lays 1-5 3ggs per clutch, twice per season, while *L. delicata* lays 1 to 6 eggs in only one clutch per season (Chapple et al. 2011; 2014). This difference in reproductive output through the breeding season may involve different degree of sensitivity to maternal effects and its interaction with other aspects of early environment.

#### Husbandry

Breeding colony – Juveniles tested came from a breeding colony established in the lab since 2019. In the colony, there are 270 adults of *L. delicata* and 180 *L.* guichenoti, housed in big containers (41.5 L x 30.5 W x 21 H cm) with 2 males and 4 females of the same species per enclosure. Enclosures are provided with non-stick matting, shelter, and several small water dishes. We give water daily and feed them with approx. 40 mid-size crickets (*Acheta domestica*) per enclosure three days a week. Crickets are dusted with calcium once a week, and with multivitamin powder biweekly. Rooms temperature are set to 22-24 Celsius, but we ensured a temperature gradient in each enclosure by means of a heat chord and a heat lamp that follow a 12 h light:12 h dark cycle; warm side of enclosures is usually at 32 Celsius.

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

Hatchlings – Eggs in the incubator were checked three times a week for hatchlings. Snout-Vent Length (SVL) and Tail Length (TL) of newborns was recorded immediately after hatching with a rule to the nearest mm and then weighted with a (OHAUS, Model spx123) digital scale ± 0.001g error. Hatchlings were housed in individual enclosures (18.7L x 13.2W x 6.3H cm) provided 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).

Four months before the experiments (see below) lizards were moved to different individual enclosures as they were part of different learning and personality studies; but those experiments ended three weeks before the beginning of the present one. These enclosures were medium size (41 L x 29.7 W x 22 H cm) plastic containers that we provided 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 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. 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. The day before starting the present experiment (Day 0 in Fig. 1B) we returned the lizards back to the small enclosures (18.7L x 13.2W x 6.3H cm), which were kept inside the medium size ones in the same position and conditions as before the beginning of the present study. During learning and personality experiments, lizards were fed with only one cricket per day dusted with calcium and multivitamin, but three weeks before the experiments we provided crickets *ad libitum*; water was always supplied daily. The rooms temperature was set to between 22-24 Celsius and temperature gradient inside the enclosures was obtained by means of a heat cord and heat lamps in a 12 h light: 12 h dark cycle.

#### 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. 1a). Eggs were allocated to treated or control groups randomly. In the treated group, 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). Eggs in the control group 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). After the hormone/vehicle treatment, we incubated the eggs in one of the two previously mentioned temperature regimes (‘Cold’ or ‘Hot’) until hatching. We counterbalance the number of eggs per clutch assigned to each hormone and temperature treatment in both species.

Diagram

Description automatically generated

**Fig. 1.** Design of the experimental manipulation of early environment

#### Stressor experiment

HPA reactivity. I will test HPA sensitivity by measuring CORT secretion both before and after a stressful situation (Louvra et al., 2009; Crino et al., 2014b). First we measured them and collect faeces, and leave them in their new enclosures (Day 0). Then we stressed the lizards by simulating a predatory attack once a day for 8 days. This predatory attack was simulated by chasing, and/or tapping then in the dorsum or the head with a thick brush for one minute. We made this process between 11am to 1pm. The days 1 and 8 we recorded lizards’ activity for one hour. On Day 9 we collect faeces and the measurements again and then euthanised the animals to obtain blood samples for CORT analysis.

#### CORT Analyses

Faeces were dried in a freeze drier (BRAND and MODEL) at 0.01(BLABLA) pressure and -80 degrees C for more than 12 hours, and then stored at -80 degrees C until their analysis.

Blood was immediately After obtaining blood samples, I will centrifuge to obtain plasma, that will be kept at -20oC until assay until analyses. Plasma corticosterone levels will be determined with a radioimmunoassay kit (see Louvra et al., 2009; Crino et al., 2014b).

HYPOTHESES:

| **Pattern** | **Interpretation** |
| --- | --- |
| Latency to move ↓, latency to shelter ↔ | Reduced stress reactivity |
| Latency to move ↓, latency to shelter ↓ | Learning / improved coping |
| Latency to move ↑ | Increased allostatic load |
| Latency to move ↔, latency to shelter ↓ | Strategic escape / shelter-seeking behaviour |
| Emergence ↓ over time | Persistent stress perception or overload |
| Emergence ↑ over time | Habituation or confidence |

| **Term** | **Meaning if ingestion is centered** |
| --- | --- |

|  |  |
| --- | --- |
| Intercept | Mass change at *average* ingestion |

|  |  |
| --- | --- |
| Slope (per treatment) | Efficiency: how much mass gained per extra cricket |

|  |  |
| --- | --- |
| Negative slope | Poor assimilation; possible stress or inefficiency |

|  |  |
| --- | --- |
| Significant interaction | Treatment affects how ingestion translates into mass gain |