**Quantifying Maternal Metabolic Hormone Variation in Skink Eggs Across Two Australian Species**

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# **Abstract**

Maternal effects occur when the mother’s environment and phenotype affect offspring phenotype. For example, metabolic hormones including corticosterone (CORT) and thyroxine (T4) can transfer from egg-carrying mothers to offspring during parturition, affecting development. Increased CORT concentration during stress impacts developmental and metabolic processes, and decreases T4 concentration. Australian native species *Lampropholis delicata* and *Lampropholis guichenoti* are oviparous (egg-laying) lizards, allowing maternal hormone transfer to be investigated. Currently, it is unknown how maternal CORT and T4 transfer into egg yolk, and what the baseline variation is. I aimed to measure CORT and T4 concentrations in *L. delicata* and *L. guichenoti* eggs to quantify between and within clutch variation, compare hormone concentrations across species and identify the relationship of CORT and T4. For both species, I found evidence of between clutch variation for CORT and no variation between clutches for T4. I found within clutch variation for both species in CORT and T4. There was no difference in CORT and T4 maternal hormone concentrations across *L. delicata* and *L. guichenoti*, and I found no relationship of CORT and T4. There is evidence of maternal hormone transfer and maternal effects; however, the mechanism of transfer is unknown. Factors such as maternal hormone differences and environment, egg retention, and unequal and incomplete maternal hormone transfer could influence maternal hormone transfer, but this remains to be investigated.

# **Introduction**

Hormones are chemical messengers secreted into the bloodstream by endocrine glands, such as the thyroid or adrenal glands, in response to environmental stimuli, such as stress. Hormones affect bodily functions including metabolism and development (Hiller-Sturmhöfel and Bartke, 1998). During parturition in oviparous (egg-laying) lizards, circulating maternal metabolic hormones can transfer to the offspring via the yolk, consequently affecting development as the yolk is a nutrient source (Itonaga et al., 2011; Rafferty and Reina, 2012; Stewart and Thompson, 2017). Yolk metabolism and transport to the developing embryo occurs via dense vascular tissue. During yolk formation, the ovum (large cell with yolk-rich cytoplasm) enters the oviduct. After fertilisation, calcium is secreted to form the eggshell as the now-zygote travels through the oviduct (Stewart and Thompson, 2017).

In lizards, maternal CORT could transfer into egg yolk via P-glycoproteins on yolk surface, or simultaneously with yolk deposition as it collects in the lipid-rich yolk due to its lipophilic nature (Cohen and Wade, 2010; Itonaga et al., 2011; Miltiadous and Buchanan, 2021). It is unknown how T4 transfers into the yolk, but thyroid hormone receptors and transporters are suggested (Ruuskanen and Hsu, 2018). Maternal hormone transfer is considered a maternal effect, which is how the mother’s phenotype and environment affects offspring phenotype (Rafferty and Reina, 2012; Ruuskanen and Hsu, 2018). This can prepare offspring for the environmental conditions the mother is experiencing, which can increase maternal and offspring fitness (Itonaga et al., 2011; Miltiadous et al., 2019). Majority of studies investigating maternal effects overlook the developmental period inside the mother; instead focussing on development between oviposition (laying) and hatching (Rafferty and Reina, 2012). This study provides an insight into maternal effects on offspring development inside the mother. Additionally, maternal hormone variation between and within females, and in eggs in a natural range remain unknown (Ruuskanen and Hsu, 2018). Measuring egg maternal hormone concentrations in this study contributes to filling this knowledge gap.

Metabolic hormones, such as corticosterone (CORT) and thyroid hormone, can transfer from mother to offspring during parturition. CORT is a glucocorticoid (a class of steroid hormones) which is equivalent to human cortisol and is a major stress steroid produced in non-human mammals and lizards (Bonier et al., 2009). CORT is secreted from the adrenal glands, and is produced when a neuroendocrine pathway, the hypothalamic-pituitary-adrenal (HPA) axis, is stimulated by stress, affecting metabolism and behaviour (Bonier et al., 2009; Helmreich et al., 2005). When carrying eggs, elevated maternal CORT can increase prenatal CORT and affect offspring development (Figure 1).

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Figure 1 – Summary of stress stimulating the hypothalamic-pituitary-adrenal (HPA) axis to increase CORT concentration in mothers (black). The effect of elevated maternal CORT on offspring phenotype is shown in red.

Thyroid hormones are derived from the amino acid tyrosine, and consist of thyroxine (T4), and the more active form Triiodothyronine (T3) (Hiller-Sturmhöfel and Bartke, 1998). To use thyroid hormone, animals convert T4 to T3 with deiodinase enzymes (Helmreich et al., 2005). Consequently, 90% of thyroid hormone produced in the thyroid gland is T4 (Hiller-Sturmhöfel and Bartke, 1998). T4 is secreted from the thyroid gland due to stimulation of a neuroendocrine pathway, the Hypothalamic-Pituitary-Thyroid (HPT) axis, to alter metabolism (Helmreich et al., 2005). Stress decreases T4 concentrations, which consequently, decreases metabolism (Helmreich et al., 2005). CORT is suggested to prevent T4 production by decreasing thyrotropin-releasing hormone (Helmreich et al., 2005; Ranabir and Reetu, 2011). Thus, in egg-carrying mothers, stress can decrease T4, potentially decreasing prenatal T4 and affecting offspring development (Figure 2).

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Figure 2 – Summary of stress suppressing the hypothalamic-pituitary-thyroid (HPT) axis, decreasing T4 concentration in mothers (black). CORT inhibits thyrotropin-releasing hormone (TRH), consequently inhibiting thyroid releasing hormone, causing HPT axis suppression and T4 decrease. The effect of decreased maternal T4 on offspring phenotype is shown in red.

I investigated hormone-mediated maternal effects in two Australian native skink species – *Lampropholis delicata* and *Lampropholis guichenoti*. Both species are oviparous and have adult snout-vent lengths of around 35-51 mm (Chapple et al., 2011a; Chapple et al., 2011b; Prosser et al., 2006). *L. delicata* are found in Eastern Australia and inhabit moist areas, including rainforest, heaths, woodlands, wet sclerophyll forests and suburban gardens. *L. delicata* reproduce in the spring and summer, producing a single clutch per year of around 2-6 eggs (Chapple et al., 2011b). *L. guichenoti* are found in South-Eastern Australia and preferentially inhabit dry habitats including woodlands and dry sclerophyll forests, and are also found in moister habitats such as wet sclerophyll and subtropical rainforests. Additionally, these lizards are found in suburban gardens. *L. guichenoti* reproduce during summer to autumn, producing two clutches of 1-5 eggs (Chapple et al., 2011a; Prosser et al., 2006).

Furthermore, measuring maternal hormone concentrations in lizards has applications for invasive species management by investigating how mothers respond and adapt to novel environments, and how this affects offspring. *L. delicata*, for example, is the only invasive Australian-native species overseas in New Zealand, Hawaiian Islands, and Lord Howe Island after its introduction in the 1980s (Chapple et al., 2014). Studying environmental effects on mothers can inform offspring survival success, allowing implementation of appropriate control measures to limit potentially adverse effects on native wildlife. Additionally, understanding maternal effects on offspring is essential for conservation efforts to recover critically endangered oviparous species (Rafferty and Reina, 2012). Understanding natural history allows optimal stress-free maternal conditions for the greatest offspring survival (Rafferty and Reina, 2012).

In this study, I measured CORT and T4 hormone concentrations in clutches of *L. delicata* and *L. guichenoti* to investigate four aims:

1. Quantify maternal CORT and T4 variation between clutches of *L. delicata* and *L. guichenoti*;
2. Quantify maternal CORT and T4 variation within clutches of *L. delicata* and *L. guichenoti*;
3. Compare hormone concentrations across species;
4. Identify the relationship of CORT and T4.

I hypothesised hormone concentrations would vary between clutches due to differences between mothers and environmental conditions. In contrast, I hypothesised hormone concentrations would not vary within clutches as eggs are laid at the same time and exposed to identical conditions. I also hypothesised species would differ in the concentration of hormones due to egg laying life history differences. Finally, I hypothesised T4 concentrations would be negatively associated with CORT concentrations due to the suppressive effects of CORT on thyroid hormone production (Chapple et al., 2011a; Chapple et al., 2011b; Helmreich et al., 2005; Prosser et al., 2006; Ranabir and Reetu, 2011; Ruuskanen and Hsu, 2018).

# **Materials and Methods**

## **Lizard husbandry**

Six adults (four females, two males) of each *L. delicata* and *L. guichenoti* were housed in separate plastic enclosures (40 × 29.5 × 20.5 cm), containing substrate (non-stick mat), shelter (bark and halved PVC pipes), water, UVA/UVB lighting and 20W heat lamp per enclosure (Zhang et al., 2023). The lizards were fed calcium and vitamin-dusted *Acheta domesticus* every second day (Zhang et al., 2023). Opaque plastic boxes (egg boxes) with dimensions 12.5 × 8.5 × 5 cm contained moistened vermiculite and were present in each enclosure for egg oviposition. Water was refilled and egg boxes misted daily to maintain a humid environment (Kar et al., 2022).

## **Egg collection and dissection**

I collected clutches of *L. delicata* and *L. guichenoti* from respective lizard enclosures on Monday and Wednesday each week for 9 weeks over November 2022 to February 2023. I collected a total of 21 clutches each for *L. delicata* and *L. guichenoti*, for a total of 146 eggs (79 for *L. delicata* and 67 for *L. guichenoti*). Eggs were incubated in plastic cups with moist vermiculite at 28°C for 24 hours to allow yolk and albumin separation.

Eggs were dissected the day following collection. Egg mass was measured with a high precision balance (to 0.001 mg) immediately after egg removal from the cup to minimise evaporative-induced mass decrease. Length and width were measured with a dial calliper, estimating the hundredth of a millimetre. To dissect eggs, an incision was made on the albumin side (observed as the more transparent side) with a razor blade on the long side of the egg. The incision was lengthened with dissection scissors and yolk scooped out with a small spatula. The embryo and major blood vessels were removed with forceps and excess albumin was removed. The yolk was scraped into an Eppendorf tube, and yolk mass was weighed on the high precision balance (to 0.001 mg). Doubly distilled water was added and homogenised with the yolk, then stored at -20°C until extracted.

## **Hormone extraction and assays**

To accurately measure thyroid hormone concentration in yolks, T4 concentration was measured as it is the dominant thyroid hormone in vertebrates (Hiller-Sturmhöfel and Bartke, 1998). T4 and CORT were extracted from samples using Solid Phase Extraction (SPE) based on methods by Miltiadous and Buchanan (2021). This technique allows analytes (such as hormones) to be extracted from complex mixtures (Buszewski and Szultka, 2012). Yolk samples were thawed at room temperature. Silica-bonded C18 vacuum columns (United Chemical Technologies, CEC18156) were mounted onto a vacuum manifold and washed with doubly distilled water. Yolk sample was loaded into the column and drawn through slowly, allowing hormones to bind to the column substrate. The column was washed with 40% methanol to remove lipids (hormones remain bound due to strong polar bonds to column substrate). The flow through liquid was discarded. 100% methanol was added and set aside to soak for 2 minutes, allowing steroid release from column substrate. Columns were eluted, ensuring all liquid was drawn out. Samples were then dried under nitrogen with a microplate evaporator (Organomation MICROVAP) at 37°C until fully evaporated, and stored at -20°C overnight. To measure extraction efficiency, 2-3 thawed non-experimental yolk samples were combined. This new sample was centrifuged at 4000 rpm for 2 minutes. Supernatant was collected and aliquots of sample were created. One aliquot was spiked with CORT standard (100 000 pg/mL) supplied with Arbor Assay Corticosterone Enzyme Immunoassay Kit (Catalog number K014-H5). For T4, one aliquot was spiked with T4 standard (1000 ng/mL) supplied with the Thyroxine (T4) Enzyme Immunoassay Kit (Catalog number K050-H5). Samples were extracted as earlier. Extraction efficiency was calculated as:

Extraction efficiency for CORT was 56.30% and 8.07% for T4 samples. During extraction, 18 eggs were lost due to not running in the silica-bonded column (0 for *L. delicata* and 18 for *L. guichenoti*).

After extraction, CORT and T4 concentrations were determined with an Enzyme Immunoassay (EIA) following protocols from the Arbor Assay Thyroxine (T4) and Corticosterone Enzyme Immunoassay Kits (Catalog number K050-H5 and K014-H5 respectively). In total, 21 clutches were tested for CORT for each species, and 16 clutches for T4 for each species, as I decided to measure T4 concentration after previously assaying 5 clutches. CORT inter-assay standard (Green Top) allows CORT EIA plate comparison and was made to a concentration of 500 pg/mL using the CORT supplied standard (100 000 pg/mL). T4 inter-assay standard (Red Top) allows T4 EIA plate comparison and was made to a concentration of 5 ng/mL using the T4 supplied standard (1000 ng/mL). Additional Green and Red Top aliquots were stored at -20°C for future plates.

Samples were suspended in assay buffer, then agitated for 20 minutes. T4 standards were prepared and plated in triplicate with samples, non-specific binding and maximum binding wells as per the Arbor Assays Thyroxine (T4) Enzyme Immunoassay Kit protocol, with the addition of the Red Top sample. T4 antibody and conjugate were added as per the Arbor Assays Thyroxine (T4) Enzyme Immunoassay Kit protocol and shaken at 750 rpm for 1 hour at room temperature (20°C), allowing complexes to form with the T4 antigens present in the sample.

The CORT EIA plate was prepared by re-suspending samples (already measured for T4) in assay buffer. CORT standards were prepared and plated in triplicate with samples, non-specific binding and maximum binding wells as per the Arbor Assays Corticosterone Enzyme Immunoassay Kit protocol, with the addition of the Green Top sample. CORT antibody and conjugate were added as per the Arbor Assays Corticosterone Enzyme Immunoassay Kit protocol and shaken at 750 rpm for 1 hour at room temperature (20°C), allowing complexes to form with the CORT antigens present in the sample.

After the 1-hour incubation, contents of the T4 plate were aspirated, and wells rinsed with wash buffer. The plate was dried by tapping on Kim Wipes and checked for dryness and bubbles. TMB substrate was added to each well and incubated at room temperature (20°C) for 30 minutes, allowing reaction with bound conjugate to form a coloured product.

After the 30-minute incubation, Stop Solution (1M HCL) was added to each well in the T4 plate and set aside. After the CORT plate 1-hour incubation, contents of the plate were aspirated, and wells rinsed with wash buffer. The plate was dried by tapping on Kim Wipes. TMB substrate was added to each well and incubated at room temperature (20°C) for 30 minutes, allowing reaction with bound conjugate to form a coloured product.

T4 plate absorbance was read in a plate reader (BMG Labtech microplate reader) at 450 nm. A linear regression was fitted to the standard curve in the MARS microplate reader data analysis software to calculate the concentration of T4 in the samples, as T4 concentration was too small for detection using a four-parameter fit. A well was removed from triplicates with high % Coefficient of Variation (%CV) to reduce intra-assay variation. To ensure the highest accuracy, wells removed from standard curve triplicates were done to ensure the Red Top wells had a concentration closest to 5 ng/mL.

After the CORT 30-minute incubation, Stop Solution (1M HCL) was added to each well in the plate. CORT plate absorbance was then read in the plate reader (BMG Labtech microplate reader) at 450 nm. A four-parameter fit was fitted to the standard curve in the MARS microplate reader data analysis software to calculate the concentration of CORT in the samples. A well was removed from triplicates with high %CV to reduce intra-assay variation. To ensure the highest accuracy, wells removed from standard curve triplicates were done to ensure the Green Top wells had a concentration closest to 500 pg/mL. For CORT, intra- and inter-plate variation was 5.72% and 29.04% respectively. For T4, intra- and inter-plate variation was 6.90% and 9.56% respectively.

Final CORT and T4 concentration were determined in pg/mg from the raw EIA concentration (in pg/mL for CORT, ng/mL for T4) by accounting for yolk mass (in mg), resuspended volume (in µL) and dilution factor using the formula (with final T4 concentration converted from ng/mg to pg/mg):

Hormone concentration =

## **Statistical analysis**

A sample had a double yolk and was removed from analysis as it is a biological anomaly and would confound clutch variation.

I performed all statistical analyses in R studio using R version 4.2.2. Upon initial analysis, 70% of total variation in CORT and 25% of total variation in T4 was due to differences between EIA plates, confounding clutch variation determination. As this variation is not biologically relevant, I used a conditional repeatability approach, and included EIA plate variation as a fixed factor in the repeatability models. Between and within clutch variation is calculated from variation excluding plate variation.

### Between clutch variation

I estimated between clutch variation for CORT and T4 for each of *L. delicata* and *L. guichenoti* by estimating between plate (), between clutch () and within clutch () variance parameters. I then calculated between clutch Intra-class correlation () using the following formula with the rptR package in R as described by Nakagawa and Schielzeth (2010):

Plate variance was included as a fixed effect in the model to control for this variable.

### Within clutch variation

Between plate (), between clutch () and within clutch () variance parameters estimates from between clutch variation from CORT/T4 and *L. delicata*/*L. guichenoti* were used to calculate within clutch Intra-class correlations () using the following formula with the rptR package in R as described by Nakagawa and Schielzeth (2010):

Plate variance was included as a fixed effect in the model to control for this variable.

### Species comparison

*L. delicata* and *L. guichenoti* concentrations of CORT and T4 were compared using a linear model, accounting for egg mass as a fixed effect.

### CORT and T4 relationship

A linear regression was used to compare CORT and T4 concentrations for each egg. A linear model was used to determine coefficient and significance.

# **Results**

## ***Lampropholis delicata***

I obtained an average of 3.76 eggs per clutch (21 clutches in total) for *L. delicata* (SD = 0.7) with a minimum of 2 and a maximum of 5 eggs for each clutch. The average log CORT concentration was 0.68 (SD = 1.08) with a minimum of -1.51 and a maximum of 3.38 (Table 1). Overall, there was evidence of differences between clutch repeatability in CORT concentrations (R = 0.36, 95% CI: 0.07 to 0.6; Figure 3), suggesting approximately 35.64% of the variation was the result of differences between mothers (Table 2). Additionally, there was evidence of repeatability difference within clutches in CORT concentrations (R = 0.64, 95% CI: 0.4 to 0.93; Figure 3), suggesting around 64.36% of the variation was due to egg hormone deposition differences in respective clutches (Table 2).

The average log T4 concentration for *L. delicata* was -1 (SD = 0.51) with a minimum of -1.85 and a maximum of 0.53 (Table 3). For T4 concentration, there was no strong evidence for between clutch repeatability difference (R = 0.14, 95% CI: 0 to 0.43); Figure 4). This suggests approximately 14.34% of T4 variation was due to differences between mothers (Table 2). Additionally, there was evidence of repeatability difference within clutches in T4 concentrations (R = 0.86, 95% CI: 0.57 to 1; Figure 4), indicating about 85.66% of the variation was due to egg hormone deposition differences in respective clutches (Table 2).

Table 1 – Summary statistics including mean, standard deviation (SD), minimum (min), maximum (max), number of eggs (n) and clutches for log CORT concentration (pg/mg yolk) in L. delicata and L. guichenoti eggs.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Species** | **Mean** | **SD** | **Min** | **Max** | **n** | **Clutches** |
| *L. delicata* | 0.684 | 1.08 | -1.51 | 3.38 | 76 | 21 |
| *L. guichenoti* | 0.687 | 0.933 | -1.24 | 2.53 | 51 | 21 |

Table 2 – Between and within clutch variation for CORT and T4 for L. delicata and L. guichenoti. Values are the amount of variation explained by that variable as a percentage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Species** | **CORT between** | **CORT within** | **T4 between** | **T4 within** |
| *L. delicata* | 35.64% | 64.36% | 14.34% | 85.66% |
| *L. guichenoti* | 42.51% | 57.49% | 33.82% | 66.18% |

Table 3 – Summary statistics including mean, standard deviation (SD), minimum (min), maximum (max), number of eggs (n) and clutches for log T4 concentration (pg/mg yolk) in L. delicata and L. guichenoti eggs.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Species** | **Mean** | **SD** | **Min** | **Max** | **n** | **Clutches** |
| *L. delicata* | -1.00 | 0.508 | -1.85 | 0.528 | 60 | 16 |
| *L. guichenoti* | -1.19 | 0.529 | -2.50 | 0.01 | 36 | 16 |

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Figure 3 – Log CORT concentration (pg/mg yolk) in egg yolks for clutches of *L. delicata* (n = 21) and *L. guichenoti* (n = 21). Each box represents a unique clutch, where *L. delicata* clutches are shown in shades of warmer colours (red, orange and yellow), and *L. guichenoti* clutches are shown in shades of cooler colours (blue and purple).

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Figure 4 – Log T4 concentration (pg/mg yolk) in egg yolks for clutches of *L. delicata* (n = 16) and *L. guichenoti* (n = 16). Each box represents a unique clutch, where *L. delicata* clutches are shown in shades of pink, and *L. guichenoti* clutches are shown in shades of green.

## ***Lampropholis guichenoti***

I obtained an average of 3.33 eggs per clutch (21 clutches in total) for *L. guichenoti* (SD = 0.58) with a minimum of 3 and a maximum of 5 eggs for each clutch. The average log CORT concentration was 0.69 (SD = 0.93) with a minimum of -1.24 and a maximum of 2.53 (Table 1). Overall, there was evidence of between clutch repeatability differences in CORT concentrations (R = 0.43, 95% CI: 0 to 0.69; Figure 3), suggesting around 42.51% of the variation was the result of differences between mothers (Table 2). Additionally, there was evidence of repeatability difference within clutches in CORT concentrations (R = 0.57, 95% CI: 0.31 to 1; Figure 3), indicating approximately 57.49% of the variation was due to egg hormone deposition differences in respective clutches (Table 2).

The average log T4 concentration for *L. guichenoti* was -1.19 (SD = 0.53) with a minimum of -2.5 and a maximum of 0.01 (Table 3). For T4 concentration, there was no strong evidence for differences between clutch repeatability (R = 0.34, 95% CI: 0 to 0.72); Figure 4). This suggests approximately 33.82% of T4 variation was due to differences between mothers (Table 2). Additionally, there was evidence of repeatability differences within clutches in T4 concentrations (R = 0.66, 95% CI: 0.28 to 1; Figure 4), suggesting approximately 66.18% of the variation was due to egg hormone deposition differences in respective clutches (Table 2).

## **Species comparisons**

*L. delicata* had higher CORT concentrations, however there were no significant differences in CORT concentrations between species (mean difference = -0.13, 95% CI: -0.21 to 0.11, p = 0.45) when controlling for egg mass. *L. delicata* had higher T4 concentrations when accounting for egg mass, however this difference in T4 concentration between species was not significant (mean difference = -0.14, 95% CI: -0.18 to 0.09, p = 0.32).

## **CORT and T4 relationship**

There was no significant relationship of CORT and T4 (Beta = 0.06, SE = 0.21, p = 0.77; Figure 5), suggesting no correlation of these hormones.

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Figure 5 – Relationship of log CORT concentration (pg/mg yolk) and log T4 concentration (pg/mg yolk) in egg yolks. Raw data is shown in black (n = 96), the linear regression line is represented in purple, and 95% confidence interval is shaded in grey. The coefficient is 0.06 with a p-value of 0.77.

# **Discussion**

I found support for maternal CORT transfer, as there was between clutch variation in CORT concentrations. I found weaker support for maternal T4 transfer due to high within clutch variation. There was no difference in hormone concentrations across *L. delicata* and *L. guichenoti*. Finally, I discovered no support for a relationship of CORT and T4 in yolks.

## **Between clutch variation**

For both *L. delicata* and *L. guichenoti*, egg yolks varied only in CORT between clutches, suggesting mothers may vary in endogenous CORT concentrations or the amount of CORT they transfer to their eggs. The exact mechanisms for maternal hormone transfer in oviparous lizards are unknown, as is the sources for between-female hormone variation (Miltiadous and Buchanan, 2021; Ruuskanen and Hsu, 2018). It is evident maternal hormone transfer can occur passively in two species of birds (*Tyto alba* and *Coturnix japonica*); maternal CORT passively transferred from plasma to egg, as elevated egg CORT was proportional to elevated maternal CORT (Miltiadous et al., 2019). However, this is likely species-dependent, highlighting the need for future research into maternal hormone transfer (Miltiadous et al., 2019).

Between clutch variation could be due to differences in maternal hormone concentration, environment, and egg retention time. Mothers with differing concentrations of CORT at the time of ovulation could influence the amount of CORT deposited in the clutches, as a mother with higher CORT could produce a clutch with higher CORT (Miltiadous and Buchanan, 2021). Differences in the mother’s environment, such as changes in food and water availability, temperature and predators can affect maternal CORT concentration and consequently affect the amount of hormone deposited into clutches (Jones and Guillette Jr, 1982). The time taken to develop an egg could also contribute to between clutch variation, as mothers can have varying times between ovulation and laying, and varying eggshell thickness (Radder et al., 2008; Rafferty and Reina, 2012). Longer egg retention in mothers has been associated with reduced eggshell thickness, consequently increasing the exposure time of eggs to maternal CORT and likelihood of transfer (Rafferty and Reina, 2012). In this study, between clutch variation in CORT concentrations suggests maternal effects could be mediated by CORT, suggesting a potential pathway through which mothers can transmit environmental information to their offspring (Macleod et al., 2021).

## **Within clutch variation**

Within clutches, eggs varied in CORT and T4 concentrations for *L. delicata* and *L. guichenoti*. Sources of within-female hormone variation are unknown (Ruuskanen and Hsu, 2018). The observed variation in hormone concentrations within clutches could be due to unequal and incomplete hormone transfer. Unequally transferring hormones could cause eggs within clutches to have different hormone concentrations. This may be due to the number of eggs present in the mother’s ovaries, as more eggs increases likelihood of unequal hormone transfer, and both species produce clutches with multiple eggs (Chapple et al., 2011a; Chapple et al., 2011b; Jones et al., 1979; Prosser et al., 2006). Also, differences in ovary properties such as permeability to hormones may influence the amount of hormone transferred (Griffith et al., 2016). Unequal hormone transfer may also be due to egg laying order, as hormone concentrations may increase or decrease with laying (Ruuskanen and Hsu, 2018). Incomplete hormone transfer could influence within clutch variation by causing eggs within clutches to have different hormone concentrations. This could be due to transfer barriers such as hormone-metabolising enzymes in the mothers, which can reduce maternal hormone transfer (Itonaga et al., 2011). This could protect developing offspring from receiving excessive maternal hormones to reduce adverse effects (Itonaga et al., 2011). Additionally, it has been suggested endodermal cells in the egg acquire nutrients from the yolk non-specifically before being transported into the embryonic vascular system (Stewart and Thompson, 2017). This implies incomplete transfer, as some maternal hormone may not completely transfer, causing differences between eggs in the clutch.

Thus, CORT and T4 concentrations vary within clutches, indicating hormone deposition differences. While this does suggest maternal effects, it is not strong evidence, especially seen with T4 because there is no variation between clutches, yet variation within clutches, suggesting mothers may not have a direct effect on offspring hormone concentration. Thus, variation seen within clutches may not be due to differences in the mother’s environment and phenotype. Conversely, within clutch variation could be due to adaptive functions to adjust sibling competition, but this remains to be studied (Ruuskanen and Hsu, 2018).

## **Species comparisons**

I found *L. delicata* and *L. guichenoti* were similar in maternal hormone transfer. This suggests both species have similar maternal effects for CORT and T4 on offspring, despite having different egg laying life histories. This similarity could be due to their overlapping species distribution (both found in Eastern Australia) and similar habitats. This suggests *L. delicata* and *L. guichenoti* have similar survival strategies to increase offspring survival, as they are preparing offspring for a similar maternal environment (Chapple et al., 2011a; Chapple et al., 2011b; Prosser et al., 2006). Despite having similar hormone concentrations, sensitivity to hormones can be species-specific (Ruuskanen and Hsu, 2018). Different distribution and availability of hormone transporters and receptors, and deiodinases for T4, can influence hormone sensitivity (Ruuskanen and Hsu, 2018). Additionally, maternal hormone uptake by embryos may not be passive, as the actions of hormones in embryos are dependent on cellular uptake via transporters and intracellular metabolism (Ruuskanen and Hsu, 2018). This suggests that despite having similar hormone concentrations, physiological processes may differ between *L. delicata* and *L. guichenoti*.

## **CORT and T4 relationship**

I found no evidence of a CORT and T4 relationship. Maternal T4 had lower concentration in the yolks, suggesting mothers may transfer less T4 than CORT to their eggs, preventing accurate associations between CORT and T4 to be determined. Alternatively, as I measured hormones under normal, baseline conditions, higher normal CORT concentrations may be insufficient to decrease T4 concentrations as predicted during stress (Helmreich et al., 2005; Ranabir and Reetu, 2011). Additionally, the concentration of T4 in the yolks may have been too small to detect accurately with EIA.

## **Limitations**

Several factors may have affected the results, including hormone extraction efficiency, EIA plate variation, hormone detection ability and the assumption of passive hormone transfer. Neither CORT nor T4 were completely extracted from the yolk samples. This is likely due to the nature of yolks being bulky, potentially preventing complete elution through the column. Despite this, the SPE technique used in this study is considered an effective technique to extract yolks (Buszewski and Szultka, 2012; Miltiadous and Buchanan, 2021). High plate variation initially prevented accurate analysis of clutch variation as it contributed to a large proportion of the total variation, causing clutch variation to be considerably smaller. However, once this confounding variable was included as a fixed effect in the model, clutch variation was able to be partitioned from the total variation and analysed in isolation. This highlights the importance of controlling for confounding variables during analysis to avoid misinterpretation and misleading conclusions, as not controlling for this variable would have changed the conclusions. Low T4 concentration also may have affected the results, as a linear regression rather than a four-parameter fit was used for the standard curve to determine concentration. However, this allowed measurement of low T4 concentrated yolks, increasing the statistical power of the study. Finally, assuming passive maternal transfer to yolks is misleading, as the mechanism of maternal hormone transfer in lizards is unknown, highlighting the need for future research into this mechanism (Miltiadous and Buchanan, 2021).

## **Future directions**

It is evident from my results that lizard maternal metabolic hormones, particularly CORT, are transferred to egg yolks. Currently, the mechanisms of maternal hormone transfer between and within clutches is unknown (Miltiadous and Buchanan, 2021; Ruuskanen and Hsu, 2018). Additionally, the influence of stress on metabolic hormones in egg-carrying mothers and the effect on maternal hormone transfer is unknown (Ruuskanen and Hsu, 2018). I have proposed potential factors influencing maternal hormone transfer – maternal hormone differences and environment, egg retention, and unequal and incomplete maternal hormone transfer. Thus, to further our understanding of maternal hormone transfer, a future study could measure circulating maternal hormone concentrations in stressed egg-carrying mothers, and measure resulting hormone concentrations in clutches. Additionally, these values can be compared to the baseline values measured in this study to determine changes in clutch variation, providing an insight into maternal hormone transfer during stress.

## **Conclusion**

To conclude, there was evidence of between clutch variation for CORT for both *L. delicata* and *L. guichenoti* and no between clutch variation between clutches for T4 for both species. There was variation within clutches for both CORT and T4 (both species). Finally, there was no difference in hormone concentrations across species, and no relationship of CORT and T4. The evident clutch variation suggests maternal effects due to maternal hormone transfer; however, the mechanism for this transfer and the influence of stress remains to be investigated.

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