**Quantifying thyroxine (T4) from *Lampropholis delicata* egg yolk**

**Project goal:** manipulate T4 within a biologically meaningful range

**Summary of problems:**

Dosing - I initially tried dissolving T4 in 100% EtOH but a visible solute remained undissolved. Next, I tried 100% DMSO which completely dissolved T4 powder but did not absorb into the eggs within 24 hours (beads of solution remained on the eggshells). Also, apparently 100% DMSO would probably be lethal to the lizards. It was possible to dissolve T4 into a 5% DMSO/95% EtOH solution. This was used for the trial doses.

Extractions – Identical extraction methods were used as I used to extract CORT. The only change was I used 100% EtOH for the second wash in an effort to increase extraction efficiency. Extraction efficiency is higher for T4 than it was for CORT (39.6%) so I think I will use 100% EtOH in CORT extractions in the future (compared to 90%).

EIA’s – Although a preliminary dilution curve suggested that reconstituting samples in 300ul of buffer for low doses and control and 600ul of buffer for high doses was ideal, the majority of samples fell off the low end of the standard curve when using these doses. For the first plate of samples (Plate 2), I re-assayed the samples the same day using the lowest dilution (reconstituted in 300ul of buffer) and added four additional low standards. This enabled me to get values for most of the samples, but the lowest standards on the standard curve are not on the linear portion suggesting that pushing the curve this low is not ideal. For the next plate (Plate 4), I tried reducing the amount of buffer I used to reconstitute the samples to 100ul. 7 of 19 samples still fell off the curve. For the final plate (Plate 5), I reconstituted samples in 50ul but this seems like the least amount of buffer that should be used because the buffer needs to be able to coat the sides of the test tube when vortexing to wash any hormone solution off the sides. Using 50ul, only 2 samples fell off the low end of the curve.

Intraassay error was incredibly high in general and particularly for plate 3. Potentially intraassay variation is higher because lower volumes of samples and standards are used for the T4 EIA compared to the CORT EIA (10ul instead of 50ul). I have ordered Eppendorf pipette tips specifically for Eppendorf pipettes and smaller volumes (0.5 – 20ul). Hopefully, this will reduce the error in the future. For now, stats could be run including all values, but really no value with an error above ~15% should be used. This will eliminate n=10 data points.

**Methods**

Dosing

T3 (Sigma T2877) and T4 (Sigma T2376) hormones were dissolved in 100% DMSO and then diluted to 5% DMSO using EtOH. We tested two hormone doses (low and high) for each thyroid hormone. Initial doses were estimated from Brasford et al. (2004) who measured whole animal T3 and T4 levels from fence lizards within 24 hours of hatching. We estimated a range of hormone values in eggs using average egg mass (0.157 g) and assuming a similar concentration of thyroid hormones in eggs as in fence lizard hatchlings. For T3, we estimated a range of 1.346 – 1.553 ng/mg. For T4, we estimated a range of 1.382 – 2.575 ng/mg. Low doses for each hormone were determined by taking adding the difference in the high and low values of the range to the low value of the range and high doses were calculated by adding double the difference to the lowest value. The resulting doses were as follows: low T3 (41.76 ng/mL), high T3 (83.52 ng/mL), low T4 (238.6 ng/mL), and high T4 (477.3ng/mL). Eggs were dosed topically with 5µl of one of the four hormone solutions or a control solution (5% DMSO/95% EtOH).

Egg dissection

Eggs were dissected 24 hours ± 2 hours after dosing. Egg shells were incised with a razor blade and opened with surgical scissors. The egg yolk was separated from the albumin and embryo and patted dry with Kim Wipes. The egg yolks were weighed to the nearest 0.001mg prior to vortexing them diluting them in 1mL of ddH20. The resulting suspension was stored at -20°C until assayed.

Hormone Extraction

Frozen egg yolks were thawed at room temperature prior to extraction. Extractions were done with vacuum filtration. Silica bonded C18 vacuum columns (Clean-Up C18 500 mg/ 6 mL; Cat No. CUC18156, UCT) were prepped by adding 10mL of MilliQ H2O (5mL + 5mL) and drawing through the liquid. Each sample was vortexed and then added to the column. An additional 1mL of dd H20 was added to each tube and then added to the column (to rinse the tube). Each column was washed with 5mL of 40% methanol to remove lipids (weak polar bonds in 40% methanol, will wash out lipids, but not disrupt the strong polar bonds between the steroids and column substrate). All flow through liquid up until the completion of this step is discarded. 5mL 90% (or 100%) methanol solution is added to each column and allowed to soak for 2 minutes. Elute the columns into a glass collection tube (this flow through liquid contains the steroids of interest). Allow column to run completely dry. Dry the tubes under nitrogen at 37 ºC, until fully evaporated.

EIAs