**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 to increase extraction efficiency. Extraction efficiency is higher for T4 than it was for CORT (22.9%) so I think I will use 100% EtOH in CORT extractions in the future (compared to 90% EtOH used in the second wash).

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, most 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 sample dilution (reconstituted in 300ul of buffer) and added four additional low standards (lowest at 0.02 ng/ml). 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. This worked well as only two samples fell off the bottom of the curve, 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

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 in analyses. This will eliminate n=10 data points. The high intraassay variation also means that samples should not be run in duplicate for T4 assays. Interassay variation was excellent at 5.51% and in general yellow top (made to 5.0 ng/ml) always ran right around 5.0 ng/ml (5.5 – 6.3ng/ml).

**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. 5mL 90% (or 100%) methanol solution was added to each column and allowed to soak for 2 minutes and then eluted until the column ran dry. The samples were then dried under nitrogen at 37 ºC, until fully evaporated. They were store at -20°C until assayed.

To measure extraction efficiency, two yolk samples were pooled. They were centrifuged to remove albumin and any ‘chunks.’ Two 500ul aliquots were isolated (without disrupting the pellet). One sample was spiked with 5ul of the stock T4 solution that came with the kit (1,000 ng/ml) for a total of 5ng of T4 added to samples (5ul \* 1000ng/ml). Samples were then extracted and spiked versus not spiked samples were compared to measure extraction efficiency [(spiked – unspiked)/5ng] \* 100 = percent extraction efficiency.

EIAs

Assays were run following instructions detailed in the Arbor Assay kits (K050-H5). Slight modifications as follows: 1) plates were mixed on an orbital shaker at 700 rpm; 2) 250 ul of wash buffer (x4 washes) was used instead of 300ul to avoid contaminating the multichannel pipette.

To test assay parallelism, a yolk sample was serially diluted six times as follows: 1) reconstituted in 300ul of buffer, 2) 150 of (1) + 150 ul of buffer, 3) 150ul of (2) + 150 ul of buffer, 4) 150 ul of (3) + 150 ul of buffer, 5) 150 ul of (4) + 150 ul of buffer, 6) 150ul of (5) + 150 ul of buffer. Dilution (1) fell off the upper end of the curve. These values were assayed and compared to the standard curve. T4 values were log transformed prior to analysis.

Para\_Test <- lm(LogT4 ~ DilutionFactor + Type, data = T4Para)

anova(Para\_Test)

summary(Para\_Test)

p=0.23, F1,8= 1.65

Non significance indicates that the lines are parallel and extraction methods were sufficient in removing any lipids or other substances that may interfere with the EIA.

Stats

Average extraction efficiency was 22.86%. Intrassay variation = 14.91% (including all very high values). Interassay variation = 5.51%. Assay parallelism = p=0.23, F1,8= 1.65

Stats were run with high CV values (CV>.15; n=12) removed and with all values. Most accurate to run stats without high values for which there is no affect of treatment on T4 levels (P=0.65, F2,27=0.43).

Control: mean = 0.005915ng/ml +/-0.01078 ng/ml

Low: mean = 0.0026665 ng/ml +/- 0.0033446 ng/ml

High: mean = 0.0020758 ng/ml +/- 0.0033327 ng/ml



Summary: Either doses were not high enough or the solvent was not sufficient to facilitate transfer to T4 across the egg shell into the yolk.