Protocol for measuring mitochondrial function and CORT from pilot *delicata* – by OC 18/11/2022

**Background:** High CORT treatment reduces body size in hatchling and juvenile skinks.

**Hypotheses:** We will test two mechanisms to explain differences in body size.

1) Treatment with CORT *in ovo* programs HPA axis such that lizards exposed to the high CORT treatment have higher levels of baseline CORT at hatching and as juveniles. For these effects to be programmatic, we should be able to detect them into adulthood.

2) Developmental CORT treatment reduces the efficiency of liver mitochondria. The liver has an important role in gluconeogenesis. Decreased efficiency of liver mitochondrial function would explain why lizards exposed to CORT *in ovo* hatch at a smaller body size.

These hypotheses are potentially related. For example, if developmental CORT treatment has programmatic effects on HPA axis and there is a correlation between baseline CORT and liver mitochondrial function, this would explain the continued effects of developmental treatment on body size (i.e., they grow slower and potentially achieve a smaller adult body size). However, if there are no effects of developmental treatment on adult baseline CORT, but there are effects on liver mitochondrial function (for example), this suggests that there are programmatic effects of developmental treatment on mitochondria, but that these effects are not being regulated indirectly through changes in the HPA axis.

**Project objectives:**

**1) Measure baseline CORT levels – priority**

**2) Measure mitochondrial function in liver tissue – priority**

**3) Measure body size and mass** – **priority;** we need a final body size measurement which will give us n= 4 measurements from hatching onward. Surely it’s best to do these on live lizards as it reflects how we’ve measured morphometrics previously.

**4) Measure mitochondrial function in whole red blood cells –** will only be addressed if there is enough time to isolate cells from liver and whole red blood before tissues start to die. Liver mitochondria die faster than mitochondria in whole red blood.

**5) Measure hemoglobin** (some studies have shown interactions between hemoglobin and mitochondrial function in whole red blood cells) – will only be addressed if there is enough blood for objectives 1 and 4.

**6) Dissect out brains –** This will not generate data for this project but will allow Pablo the opportunity to practice brain dissections for his project and to collect tissues to be used to optimize methods.

**7) Sex lizards** – we need to sex all the lizards for analyses. Apparently, this can be done after they are euthanized, and tissues are removed.

**Methods**

Body measurements, euthanasia, and tissue collection

* Start a timer as soon as an enclosure is disturbed.
* Measure SVL, tail, and body mass
* Euthanize lizard by decapitation
* Trunk blood will be collected into heparinized microcapillary tubes. We will note the time as soon as the blood has been collected. The blood will be put into Eppendorf tubes and kept on ice until processing (see below). Ideally, we will collect ~100ul of blood.
* If there is extra blood, we will measure hemoglobin (see below)
* The head goes to Pablo who will follow his own protocol for dissection and processing.
* The liver will be removed, rinsed with ice cold phosphate buffer saline (PBS; pH=7.4?) and processed (see below). We will record the time when the liver is removed
* Lizards will then be sexed by either palpitation or dissection to determine the presence or absence of hemipenes

Blood processing (CORT and mitochondrial measurements)

1. Centrifuge blood at 3000g for 10 minutes to separate plasma from red blood cells.
2. Remove plasma, put in new Eppendorf tube, and store at -20°C. This plasma will be used to measure baseline CORT levels.
3. Transfer 20µl of red blood cells to a new tube containing 200 µl of ice-cold phosphate buffer saline (PBS; pH=7.4?). To transfer red blood cells, cut off the tip of a pipette tip for a 100 µl pipette and then pipette as per normal. This prevents red blood cells from being ruptured by the pipette tip. Pipette from the bottom of the blood cell pellet to avoid white blood cells which should be located on the top. Homogenize sample gently by taking up solution and ejecting it back into the tube using the pipette
4. Wash red blood cells by centrifuging at 600 g for 5 minutes to pellet the cells. Discard the supernatant.
5. Resuspend the pellet in 200 µl of ice-cold PBS and store at 4°C until use in mitochondrial assays.

Hemoglobin measurements

1. Calibrate the hemoglobin meter before use
2. Using a capillary tube, collect a small amount of blood and expel a drop of it onto a hard surface (I use the waterproof side of the package from each cuvette)
3. Wick whole blood into cuvette (specific to meter).
4. Read cuvette (fast) and record value.

Liver processing

1. Remove whole liver from lizard and place in Eppendorf tube on ice.
2. Wash once in 1 mL ice cold solution containing 0.22 *M* mannitol, 0.07 *M* sucrose, 0.02 *M* HEPES, 2 m*M* Tris-HCL, pH 7.2, and 1 m*M* EDTA (*Media A*). Wash an additional three times with 1 mL of *Media A* + 0.4% BSA (bovine serum albumin; does it have to be fatty acid free?). BSA will help remove the blood and connective tissue. Livers will be washed by pipetting ice cold solutions into the Eppendorf tube and then removing each wash before adding another one (this potentially minimizing movement of the liver tissue).
3. Liver tissue will be transferred (how?) to a prechilled glass homogenizer and homogenized in 1mL of media (as above) using a teflon pestle using three gentle strokes. The homogenate will be filtered through a 100 µm cell strainer (disposable). The cell strainers fit onto small glass culture tubes. After the straining the homogenate will be transferred to Eppendorf tubes (doubtful we have easy access to a centrifuge with a rotor for glass culture tubes).
4. Centrifuge at 3000 g for 1.5 minutes at 4°C. Decant supernatant (this is what we want) and centrifuge at 17,500 g for 4.5 minutes at 4°C. Remove and dispose of supernatant. Resuspend pellet in 1mL of *Media B* (0.22 *M* mannitol, 0.07 *M* of sucrose, 0.01 *M* Tris-HCL, pH 7.23, and 1 m*M* of EDTA with gentle stirring (from a pipette tip?).
5. Resuspended samples will be kept on ice until assayed.

Measuring mitochondria function using the Seahorse

***The evening before the assay/day of tissue collection*** – hydrate the Seahorse XFe96 sensor cartridge

* 200 µl of sterile water to each well on the calibrant plate
* There must be no air bubbles. To dislodge air bubbles, gently raise and lower the sensor cartridge into the water in the calibrant plate several times.
* Incubate the plate and cartridge overnight in a humidified incubator at 28°C at atmospheric CO2. Include a 50mL aliquot of the XF calibrant in the incubator (why?)

*Preparing compound stock solutions*

* Resuspend all compounds in prepared assay medium (does this include supplemental compounds e.g., sucrose, etc?)
* Oligomycin – add 630 µl of media to stock tube for 100 µM stock concentration
* FCCP – add 720 µl of media to stock tube for 100 µM stock concentration
* Rot/antimycin A – add 540 µl of media to stock tube for 50 µM concentration

*Plating the drugs on the drug plate*

* Prepare 50 ml of media with 250 ul of glucose (5mM; or sucrose?) and 250 ul of glutamine (1 mM) and anything else? Check what’s added to media for liver assays.
* Port 1:
* Port 2:
* Port 3:
* Port 4:

*Preparing the culture plate with tissue homogenates*

* Determine cell density of each sample using hemocytometer. 10 µl aliquots, stained with trypan blue to determine live versus dead cells.
* Optimal seeding density will be determined during a trial run. Assuming an optimal density of 2x104 cells per well.
* Insert formula for calculating dilution from hemocytometer counts here.
* Spin samples at 1,500 g for 10 minutes, take off media, and resuspend each sample in the appropriate volume of media calculated above to get correct dilution for every sample
* Add 100 µl of tissue suspension to each designated well in the PVC coated culture plate. This will be three wells (samples run in triplicate). Sample order will be recorded on 96 well data sheet.
* Fill blank (background) wells with 100 µl of media.
* Centrifuge plate at 800 g for 3 minutes on a low brake centrifuge setting (es:3)
* Top up each well with 75 µl of media for a total volume of 175 µl

Measuring plasma CORT (EIAs)

1. Ideally CORT levels will be measured in triplicate. 50µl aliquots are required per replicate for assay. Therefore, 200 µl of sample needs to be available. Baseline CORT levels are likely low and a dilution of 1:10 has been used in other species (e.g., *Zootoca vivipara*; Voituron et al. 2022). Following this, plasma samples will be diluted as 20 µl of plasma + 180 µl of assay buffer. (Dilutions will be optimized prior to assays using standard techniques. If a 1:10 dilution is too dilute OR if it is not possible to get at least 50 µl of whole blood (assuming a 50% hematocrit), then samples can be run in duplicate (diluted to a volume of 150 µl).
2. Standard EIA methods will be followed.

Division of labor

* Dan – catching, measuring, and euthanizing lizards. Will collect trunk blood following decapitation.
* Dalton – initial blood processing. Will blow out capillary tubes in Eppendorf tubes.
* Pablo – will dissect out brains
* Ondi – will dissect out livers and make homogenates
* Kris – will keep track of time and morphometric data and confirm the sex of lizards following liver dissection