**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.

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

**5) 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.

**6) 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 5.

**7) 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 (non-mito measurements).

**Methods**

1. Body measurements, euthanasia, and tissue collection

* Start a timer as soon as an enclosure is disturbed.
* Inject animal with sodium pentobarbital (record time of injection)
* 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 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.

2. 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 (or on wet ice) until mitochondrial assays.

3. 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.
4. Read cuvette (takes about 30 seconds) and record value.

4. Liver processing – making homogenates and isolating cells (all this done on ice, cold temps, and using ice-cold buffers)

1. Remove whole liver from lizard.
2. Rinse with ice-cold phosphate-buffered saline (1X); does not have to be sterile.
   1. For 500 mL of 10X PBS: 8.9 g of Na2HPO4\*2H20 (100mM) + 1.2 g of KH2PO4 (18mM) + 40g of NaCl (1.37M) + 1 g KCl (27mM).
   2. For 1 L of 1X PBS: 100 ml of 10x PBS + 900ml of ddH2O
3. May have to mince before homogenizing. Use razor blade on plastic weigh boat on ice.
4. Homogenize in dounce homogenizer using mitochondrial isolation buffer (MIB).
   1. MIB = 200 mM sucrose, 10 mM Tris/MOPS [3-(N-morpholino) propanesulfonic acid; pH 7.4], and 1mM EGTA/Tris)
      1. 1M Tris/MOPS: 12.1 g of Tris base in 70 mL of ddH2O. Add dry MOPS to get pH to 7.4; Adjust volume to 100mL. Filter sterilize and store at 4°C. 10mM Tris/MOPS = 10 mL of 1M Tris/MOPS + 90 mL of ddH2O. Filter sterilize and store at 4
      2. 0.2 M EGTA/Tris by adding 3.8 g EGTA to 10 mL of ddH2O. Add 1M Tris/MOPS until dissolved, ~30-40µl. Adjust to 50mL. Filter and store at room temperature.
   2. Rinse homogenizer with ice cold PBS 3x before use
   3. Add liver and ice cold MIB to homogenizer (amount? Need enough to fill to certain point on homogenizer…so will vary based on mass of liver. Don’t want to use too much or will overflow homogenizer).
   4. With homogenizer on ice, use three hand gentle hand passes to homogenize (upward motion more important than downward motion).
   5. Transfer homogenate to clear 15 mL falcon tubes
   6. Centrifuge tubes at 750 x g, 4°C for 10 minutes
   7. Save the supernatant into a clean 15 mL tube (mitochondria are in the supernantant)
   8. Centrifuge at 10,000 x g, 4°C for 5 minutes. Take off and dispose of supernatant (mitochondria are now in pellet)
   9. Resuspend in 500 µl of MIB.
5. Or, homogenize in 100 µm cell strainers.
6. Resuspended samples will be kept on ice until assayed.

5. Measuring mitochondria function using the Seahorse

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

* Add 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 (assay temperature) at atmospheric CO2.
* Include a 50mL aliquot of the XF calibrant in the incubator (why?)

*Preparing medium*

* Prepare 10 mL of supplemented media (10 mL is enough for one plate). The additions to base media are what is recommended for work with liver cells.
* XF Base medium – 9.70 mL
* Glucose (1.0 M solution) – 100 µl
* Pyruvate (100 mM solution) – 100 µl
* L-glutamine (200 mM solution) – 100 µl
* Incubate at 28°C (assay temperature) in a sterile bottle.

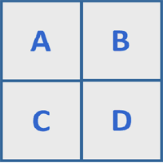
*Preparing the culture plate with tissue homogenates*

* Determine cell density of each sample using hemocytometer OR cell counter (to be determined). If using a 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 (it will be helpful to have a data sheet for calculated this with the cell counts for each sample and how much media needs to be added to each one because they will all be different).
* Add 100 µl of tissue suspension to each designated well in the PVL coated culture plate. This will be three wells (samples run in triplicate) so samples should be re-suspended at least 350 µl of media. Sample order will be recorded on 96 well data sheet.
* Fill blank (background) wells with 100 µl of media. There should be plenty of these (e.g., n = 3 in each corner of plate)
* Centrifuge plate at 800 g for 3 minutes on a low brake centrifuge setting (es:3). This sticks the cells to the bottom of the plate
* Top up each well with 75 µl of media for a total volume of 175 µl

*Preparing compound* ***stock*** *solutions*

* Resuspend all compounds in prepared assay medium
* 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

*Preparing compound* ***working*** *solutions – prepare solutions but do not load ports until getting to the JCSMR (volumes for each port listed here though)*

* Port A Oligomycin (final well concentration 1.0 µM)
  + 300 µl of stock solution + 2700 µl of media to make working solution
  + 20 µl of working solution added to each port
* Port B FCCP (final well concentration 0.5 µM)
  + 150 µl of stock solution + 2850 µl of media to make working solution
  + 22 µl of working solution added to each port
* Port C Rotenone/antimycin A (final well concentration 0.5 µM)
  + 300 µl of stock solution + 2700 µl of media to make working solution
  + 25 µl of working solution to each port
* Port D: N/A

*Before leaving for the JCSMR*

* Replace the water in the wells of the sensor cartridge with 200 µl of calibrant solution (do you just dump out the water?)
* Put the sensor cartridge back in the calibrant plate ensuring no air bubbles

*Loading working solutions into the ports of the sensor cartridge*

* Load working solutions into ports at the JCSMR (to avoid walking with loaded cartridge between labs)
* Use multi-channel pipette and reverse pipette to prevent air bubbles

*Running the assay*

* Set up file template ahead of time
* Place calibration plate with loaded sensor cartridge in instrument tray and click START. Calibration takes 15 – 30 minutes.
* After calibration, load cell culture microplate and click I’m Ready. Note: cell culture microplate will have to be kept at 28°C in the JCSMR during calibration.

6. Calculating protein content of wells

7. 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 diluted 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 makes 25 µl of plasma), then samples can be run in duplicate (diluted to a volume of 150 µl with 15 µl plasma + 135 µl assay buffer).
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