**Protocol for measuring mitochondrial function and CORT from pilot *delicata* – by OC 14/05/2023**

**Good resources here: https://www.agilent.com/en/product/cell-analysis/how-to-run-an-assayhttps://www.agilent.com/en/product/cell-analysis/how-to-run-an-assay**

**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**
2. **Measure mitochondrial function in liver tissue**
3. **Measure body size and mass;** we need a final body size measurement which will give us n= 4 measurements from hatching onward.
4. **Sex lizards;** we need to sex all the lizards for analyses. Apparently, this can be done after they are euthanized, and tissues are removed.

**Timeline:**

1. **Book Seahorse well in advance.**
2. **The day before the assay**
   1. Prepare PBS, isolation media, respiration media, and mitochondria buffer stock solutions if needed (except pyruvate which should be made the day of the assay).
   2. Hydrate Seahorse sensor cartridge the night before the assay (see below).
3. **The day of the assay**
   1. Change the temperature of the Seahorse to 30°C.
   2. Prepare mitochondria stress chemicals prior to euthanizing animals. Keep on ice or in refrigerator before use.
   3. Take respiration media out of freezer.
   4. After euthanizing animals, make sure the centrifuges has been turned on and is chilled to 4°C before processing tissue samples.
   5. Save aliquot of processed homogenate after processing samples (see below).
   6. Run Seahorse assay.
4. **After the assay**
   1. Bradford assay
   2. CORT assay

**Recipes:**

1. **Phosphate buffered saline (PBS) - make before tissue collection.**
   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
2. **Mitochondria buffer stock solutions - make before assays if needed.**
   1. Glucose (1.0 M solution) – To make 25 ml

Molecular weight of glucose (anhydrous) = 180.16 g/mol

Concentration = 1.0 mol/L \* 180.16 g/mol

1M concentration = 180.16 g/L = 180.16 mg/mL

*To make 25mL:*

* + - 180.16 mg/mL \* 25 mL = 4,504 mg = 4.50 g
    - Combine 4.50 g of glucose with 25 mL of ddH20
    - Aliquot in 500ul and store at -20C
  1. Sodium Pyruvate (100 mM solution) – To make 1 mL

**Make fresh day of use.**

Molecular weight of sodium pyruvate = 110.04 g/mol

Concentration = 1.0 mol/L \* 110.04 g/mol

1M concentration = 110.04 g/L

1mM concentration = 0.11004 g/L

100mM concentration = 0.11004 g/L \* 100 = 11.004 g/L = 11.004 mg/mL

Use correction of expected to actual:

11.004 mg / 1 mL ddH2O = actual mg / x mL of dd H2O

* 1. L-glutamine (100 mM solution) – To make 5 mL

**Note: concentration greater than 100 mM will not dissolve in water.**

Molecular weight of sodium pyruvate = 146.14 g/mol

Concentration = 1.0 mol/L \* 146.14 g/mol

1M concentration = 146.14 g/L

1mM concentration = 0.14614 g/L

100mM concentration = 0.14614 g/L \* 100 = 14.614 g/L = 14.614 mg/mL

*To make 5mL:*

* + - 14.614 mg/mL \* 5 mL = 73.07 mg
    - Combine 73.07 mg of L-glutamine with 5 mL of ddH20
    - Aliquot in 500ul and store at –20C

1. **Mito stress chemicals**
   1. **Stocks – make day of assay from Mito Stress kit or use stocks from Oroboros**
      1. Oligomycin – add 630 µl of respiration media to stock tube for **100 µM** **stock concentration.**
      2. FCCP – add 720 µl of respiration media to stock tube for **100 µM stock concentration.**
      3. Rot/antimycin A – add 540 µl of respiration media to stock tube for **50 µM concentration.**
   2. **Diluted stocks for assays – make day of assay.**

**Stocks are made to 10x to achieve desired final dilution in well.**

* + 1. Oligomycin – Testing four concentrations (1.5, uM, 2.0 uM, 2.5 uM, 3.0 uM; **use 125 uM stock solution in freezer**)
       1. 3.0 uM/**30 uM** = 1,200 ul of 125 uM to 3,880 ul media
       2. 2.5 uM/**25 uM** = 2,500 ul of 3.0 uM/30 uM + 500 ul media
       3. 2.0 uM/**20 uM** = 2,400 ul of 2.5 uM/25 uM + 600 ul media
       4. 1.5 uM/**15 uM** = 2,250 ul of 2.0 uM/20uM + 750 ul media
    2. FCCP – To test 0.5, 1.0, 1.5, and 2.0 uM
       1. 2.0/**20 uM** = 600 ul of 100 uM stock + 2,400 ul media.
       2. 1.5/**15 uM** = 2,250 ul of 2.0/20 uM + 750 ul media.
       3. 1.0/**10 uM** = 2,000 ul of 1.5/15 uM +1,000 ul media.
       4. 0.5/**5 uM** = 1,500 ul of 1.0/10 uM + 1,500 ul media.
    3. Rot/antimycin A – For final concentration of 0.5 uM/5 uM
       1. 400 ul of 50 uM stock solution + 3,600 ul media.
  1. **Mitochondria isolation media – pH to ~ 7.4 using KOH**

**Note: can be made with either HEPES or Tris. More studies seem to use HEPES, but our isolation media was effective when tested with delicata liver in the Oroboros so we will use that for the optimization. However, both recipes are provided here.**

Isolation media with Tris HCL:

1. Sucrose (250 mM)

Molecular weight = 342.3 g/mol

Concentration = 1.0 mol/L \* 342.3 g/mol

1M concentration = 342.3 g/L

1mM concentration = 0.3423 g/L

250mM concentration = 0.3423 g/L \* 250 = 85.575 g/L

1. EGTA (1 mM)

Molecular weight = 380.35 g/mol

Concentration = 1.0 mol/L \* 380.35 g/mol

1M concentration = 380.35 g/L

1mM concentration = 0. 38035 g/L

1. Tris HCL (20 mM) – Sigma Aldrich 3253

Molecular weight = 157.60 g/mol

Concentration = 1.0 mol/L \* 157.60 g/mol

1M concentration = 157.60 g/L

1mM concentration = 0.15760 g/L

20mM concentration = 20 \* 0.15760 g/L = 3.152 g/L

1. Directions for 500 mL:
   1. Sucrose: 42.7875 g +
   2. EGTA: 0.1902 g +
   3. Tris: 1.576 g +
   4. Dissolve in 500 mL of ddH2O. Use stir bar and may need to heat to get sucrose to go into solution.
   5. pH to ~7.5 while ice cold using KOH

Isolation media with HEPES:

* + 1. Sucrose (250 mM)

Molecular weight = 342.3 g/mol

Concentration = 1.0 mol/L \* 342.3 g/mol

1M concentration = 342.3 g/L

1mM concentration = 0.3423 g/L

250mM concentration = 0.3423 g/L \* 250 = 85.575 g/L

* + 1. EGTA (1 mM)

Molecular weight = 380.35 g/mol

Concentration = 1.0 mol/L \* 380.35 g/mol

1M concentration = 380.35 g/L

1mM concentration = 0. 38035 g/L

* + 1. HEPES (5 mM)

Molecular weight = 238.31 g/mol

Concentration = 1.0 mol/L \* 238.31 g/mol

1M concentration = 238.31 g/L

1mM concentration = 0. 23831 g/L

5mM concentration = 5 \* 0.23831 = 1.19155 g/L

* + 1. Directions for 500 mL:
       1. Sucrose: 42.7875 g +
       2. EGTA: 0.1902 g +
       3. HEPES: 0.5958 g +
       4. Dissolve in 500 mL of ddH2O. Use stir bar and may need to heat to get sucrose to go into solution.
       5. pH to 7.5 while ice cold using KOH.

1. Mitochondria respiration media – to make 50 mL; make day of assay.

**Check pH and adjust to ~7.4 with KOH if necessary**

* 1. DMEM – 48 mL
  2. Glucose (10 mM final) – 500 µl (1.0 M stock)
  3. Pyruvate (1 mM final) – 500 µl (100 mM solution)
  4. L-glutamine (2 mM final) – 1,000 ul (100uM solution)

**Methods**

1. Body measurements, euthanasia, and tissue collection

1. Start a timer as soon as an enclosure is disturbed.
2. Inject animal with Alfaxalone (record time of injection)
3. Measure SVL, tail, and body mass
4. Euthanize lizard by decapitation
5. 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).
6. The liver will be removed and processed (see below). We will record the time when the liver is removed.
7. Lizards will then be sexed by either palpitation or dissection to determine the presence or absence of hemipenes.

2. Blood processing (to obtain CORT for hormone assays)

1. Centrifuge blood at **7000 rpm for 7 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. 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 in 1 mL of ice-cold phosphate-buffered saline (1M) in Eppendorf tube by putting liver in tube and taking it out again with forceps. Repeat in fresh tube of PBS.
3. Place whole liver in test tube in 1 mL mitochondria isolation media on ice before transporting back to Robertson to process tissue.
4. Homogenize using a Potter Elvehjem Teflon pestle in test tube as follows:
5. Rinse pestle with ice cold PBS before use
6. With test tube on ice, use four hand gentle hand passes to homogenize.
7. Transfer homogenate to clean Eppendorf tube and keep on ice.
8. Centrifuge tubes at **750 x g, 4°C for 10 minutes.**
9. **Save the supernatan**t into a clean Eppendorf tube (mitochondria are in the supernatant).
10. Centrifuge tubes at **750 x g,** **4°C for 10 minutes.**
11. **Save the supernatan**t into a clean Eppendorf tube (mitochondria are in the supernatant).
12. **Centrifuge at 10,000 x g, 4°C for 10 minutes**. Take off and dispose of supernatant (**mitochondria are now in pellet**).
13. Resuspend in 1000 µl of respiration media using gentle up a down with pipette tip.
14. Keep on wet ice until use in Seahorse assay.

5. Measuring mitochondria function using the Seahorse

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

1. Add 200 µl of sterile water to each well on the calibrant plate.
2. 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.
3. Incubate the plate and cartridge overnight in a humidified incubator at 30°C (assay temperature) at atmospheric CO2.
4. Include a 50mL aliquot of the XF calibrant in the incubator.
5. Include a beaker of water to prevent water from calibrant plate from evaporating.

*Preparing mitochondria medium*

1. Prepare 50 mL of supplemented media (recipe above). The additions to base media are what is recommended for work with liver cells.
2. Incubate at 30°C (assay temperature) in a falcon tube.

*Preparing oligomycin*

*Preparing the tissue homogenate dilutions for Plate 1 of optimizations*

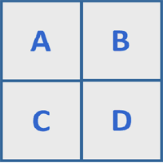
1. Need pooled samples to optimize oligomycin and tissue concentration.
   1. Starting dilution is whole liver homogenate resuspended in 1000 ul (Dilution 1); will need ~4000 ul for Dilution 1
   2. After preparing liver homogenate for 8 livers, combine into a 15 mL Falcon tube (this is **Dilution 1**). ~8 mL
   3. **Dilution 2**: 3.0 mL of Dilution 1 + 3.0 mL of mitochondria isolation media (1:2 dilution)
   4. **Dilution 3**: 3.0 mL of Dilution 1 + 3.0 mL of mitochondria isolation media (1:4 dilution)
2. Need pooled sample to optimize FCCP (20 samples, ~2000ul) assuming recommended oligomycin concentration of **2.5 uM** (based on Oroboros trial) and assuming **Dilution 1** concentration of tissue homogenate.

*Preparing the culture plate with tissue homogenates*

1. Add 100 µl of tissue suspension to each designated well in the PVL coated culture plate. Sample order will be recorded on 96 well data sheet.
2. **Save 200 ul aliquot of each sample for Bradford assay to quantify protein concentration. Store in Eppendorf tube at -20°C.**
3. Fill blank (background) wells with 100 µl of respiration media.
4. Centrifuge plate **at 2000 g at 4°C for 10 minutes on a low brake centrifuge setting** (es:3). This sticks the mitochondria to the bottom of the plate.
5. Top up each well with 80 µl of respiration media (30°C) for a total volume of 180 µl.

*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 (for all final well concentrations)
  + **20 µl of working solution added to each port**
* Port B FCCP (for all final well concentrations)
  + **22 µl of working solution added to each port**
* Port C Rotenone/antimycin A (final well concentration 0.5 µM)
  + **25 µl of working solution to each port**
* Port D: N/A



*Before leaving for the JCSMR*

1. Replace the water in the wells of the sensor cartridge with 200 µl of calibrant solution.
2. Put the sensor cartridge back in the calibrant plate ensuring no air bubbles.
3. Bring with you:
   1. Sample plate
   2. Sensor cartridge
   3. Working solutions: 4 of Oligomycin, 4 of FCCP, and 1 of Rotenone/antimycin A
   4. Reagent reservoirs: need 9
   5. 25 ul multi-channel pipette (borrow from Keogh group)
   6. Box of pipette tips.

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

1. Cell culture microplate will have to be kept at 28°C in the JCSMR during calibration.
2. Load working solutions into ports at the JCSMR (to avoid walking with loaded cartridge between labs)
3. Use multi-channel pipette and reverse pipette to prevent air bubbles.

*Running the assay*

1. Set up file template ahead of time.
2. Place calibration plate with loaded sensor cartridge in instrument tray and click START. Calibration takes 15 – 30 minutes.
3. Will need to manually enter plate ID.
4. After calibration, load cell culture microplate and click I’m Ready.