A501: Techniques in Reproductive Diversity
Hormone and DNA Labs

Thursday August 31: Introduction to extracting hormones and DNA from plasma, feces, & yolk

Please read the following (available from course website):

- Testosterone EIA Protocol
- DNA extraction protocol: Moore, D.D. and D. Dowhan. 2002. In: *Current protocols in molecular biology*. F. M. Ausubel, R. Brent; R. E. Kingston, D. D. Moore; J.G. Seidman, J. A. Smith, K. Struhl, eds. New York: John Wiley and Sons. Pp. 2.0.1-2.0.3, 2.1.1- 2.1.3, 2.1.9-2.1.10. [*not on website: will be handed out in class*]
- Clotfelter et al. 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? *Hormones and Behavior* 46: 171-178.
- Taberlet, P. and G. Luikart (1999). Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnaean Society* 68: 41-55.

Additional readings:

- Wingfield and Farner 1975. The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. *Steroids* 26: 311-327.

Tuesday September 5: Extractions

Extraction from plasma for testosterone EIA with Danielle Whittaker

Samples will already be measured out. Students will learn to perform steroid extractions using the diethyl ether method. Since each sample will need at least two extractions (three if female birds are used), each student will get multiple opportunities to practice snap-freezing and pouring off the supernatant.

1. Add 1 ml anhydrous diethyl ether to each sample using repeater pipette; vortex.
2. Let samples sit for 20 minutes for phase separation.
3. Snap freeze each sample and pour off supernatant into labeled test tube
4. Evaporate liquid in 40°C water bath under nitrogen gas
5. Repeat steps 1-4 twice
6. Reconstitute samples with 50µl 100% EtOH & vortex
7. Add 300µl Assay Buffer 3 & vortex
8. Cover with parafilm & refrigerate overnight

Demonstration: Egg yolk extraction [to coincide with a waiting time]

To save time, only the preparation of yolk samples will be done with the class.

Steps:

1. Separate yolk from the rest of the frozen egg.
2. Homogenize yolk
3. Add 5-15 mg of yolk to 1.5 ml Eppendorf tube
4. Add 500 µl distilled water and vortex until homogenized (use 2-3 glass beads).

After this, extraction of steroids is very similar to other methods so no need to repeat.

DNA extractions from blood with Amy Poehlman

DNA Extraction from blood - Part I / *(Done concurrently with Hormone plasma extraction for testosterone EIA)*

Last night, your bird blood samples were digested with Proteinase K, a proteolytic enzyme. This step removes many of the proteins present in the blood, and is the first step towards purifying nucleic acids. Today you will continue this purification:

1. To each tube of blood, add an equal volume of phenol:chloroform:isoamyl alcohol.
2. Invert 25 times, and then spin 6 min. at 12,000g.
3. Transfer upper layer to a new Eppendorf tube.
4. Repeat steps 1-3, twice more.
5. To each tube, add an equal volume of chloroform.
6. Invert 25 times, spin 6 min. at 12,000g.
7. Transfer upper layer to a new Eppendorf tube.
8. Add 0.1x volume of 3M Sodium acetate, and 2.5x volume of cold 100% Ethanol.
9. Invert slowly, 50 times.
10. Place samples in -20°C for at least 1 hour or until ready to complete extraction (in this case, Thursday).

Thursday Sept. 7: DNA Extraction -Part II/ *(Also, Introduction to measuring colorful phenotypes)*

Having extracted the DNA from your bird blood sample on Tuesday, you will now pellet the DNA, and clean it several times before resuspending it in a buffer. DNA in buffer can be stored at -20°C indefinitely, although repeated thawing and re-freezing will eventually damage the DNA beyond usefulness.

1. Spin DNA 30 min. at 12,000g.
2. Remove supernatant.
3. Add an equal volume of cold 70% ethanol.
4. Invert several times, spin 6 min. at 12,000g.
5. Remove ethanol.
6. Repeat steps 3 -5 once more.
7. Dry DNA pellet on bench (usually 15 min.-1hour)
8. Rehydrate DNA in 1X Tris-EDTA buffer.

ADDITIONAL INFORMATION AND READINGS
Steroid extractions from other sources

1. Fecal

This has the advantage of being less invasive, but requires more preliminary steps, like drying, weighing, and solubilizing samples in a standard solution prior to extraction. Also it may be necessary to extract several fractions, due to the breakdown of steroids in the digestive tract. It is typical to run fecal samples along with plasma samples, and also to measure gut passage times, if not known for your study species.

Reference:
Hirschenhauser, K,, E. Mostl, P. Peczely, B. Wallner, J. Dittami, and K. Kotrschal (2000). Seasonal relationships between plasma and fecal testosterone in response to GnRH in domestic ganders. *Gen. Comp. Endocrin.* 118:262-272.

2. Water from fish tanks, ponds, etc.

This again, is a less invasive method, but like the fecal extraction method, involves several lengthy preliminary steps, including filtering and concentrating water samples, as well as removal of polar molecules prior to extraction. In addition, it is necessary to extract several fractions, due to modification of steroids as they are excreted. Again, it is typical to validate the method by also running plasma samples.

Reference:
Dziewecyznski, T.L., A.C. Eklund, and W.J. Rowland (2006). Male 11-ketotestosterone levels change as a result of being watched in Siamese fighting fish, *Betta splendens*. *Gen. Comp. Endocrin*. 147(2):184-189.
-or-
Carlisle, S.L., S.K. Marxer-Miller, A.V.M. Canario, R.F. Oliveira, L. Carneiro, and M.S. Grober. 2000. Effects of 11-ketotestosterone on genital papilla morphology in the sex changing fish *Lythrypnus dalli*. *J. Fish Biology* 57: 445-456.

3. Egg yolks

Reference:
Lipar, J. L., E. D. Ketterson, V. Nolan, Jr., and J. M. Casto (1999). Egg yolk layers vary in the concentration of steroid hormones in two avian species. *General and Comparative Endocrinology* 115: 220-227.

ADDITIONAL INFORMATION AND READINGS
DNA extractions from other sources

1. Tissue

This is now as easy and reliable to extract from as blood, and has the advantage of sometimes being less invasive (bird feathers, mammalian hair or cheek swabs), but also may require longer digestion times, and more supplies, such as glass beads, pestles, or sonicators to grind tissue.

Reference:
Sambrook J. and D. Russell. *Molecular Cloning. A Laboratory Manual. 3rd edition*. Cold Spring Harbor, NY: Cold spring Harbor Laboratory Press; 2001, pp. 6.4- 6.11, 6.23-6.27, 6.28-6.30.

2. Fecal

This is attractive as a less invasive source of DNA, and so has been developed more recently as an alternative technique, however it has several disadvantages. The DNA isolated can be degraded, and usually long nuclear DNA sequences cannot be amplified. (However, if working with mammals, fecal matter has more DNA than shed or plucked hair, so it can be a good noninvasive choice.) Bile acids in the feces can inhibit PCR, but this can be overcome by adding Bovine Serum Albumin (BSA) to the PCR reaction. Also, a step can be included during the extraction process to remove PCR inhibitors (such as the Qiagen DNA Stool kit, which has a tablet called “inhibit-X”, or can also DIY with potato starch). Allelic dropout can be a big problem, so multiple PCR replications are necessary to verify genotypes.

Reference:
Fernando et al 2003. Reliable noninvasive genotyping: fantasy or reality? *Journal of Heredity* 94(2): 115-123.