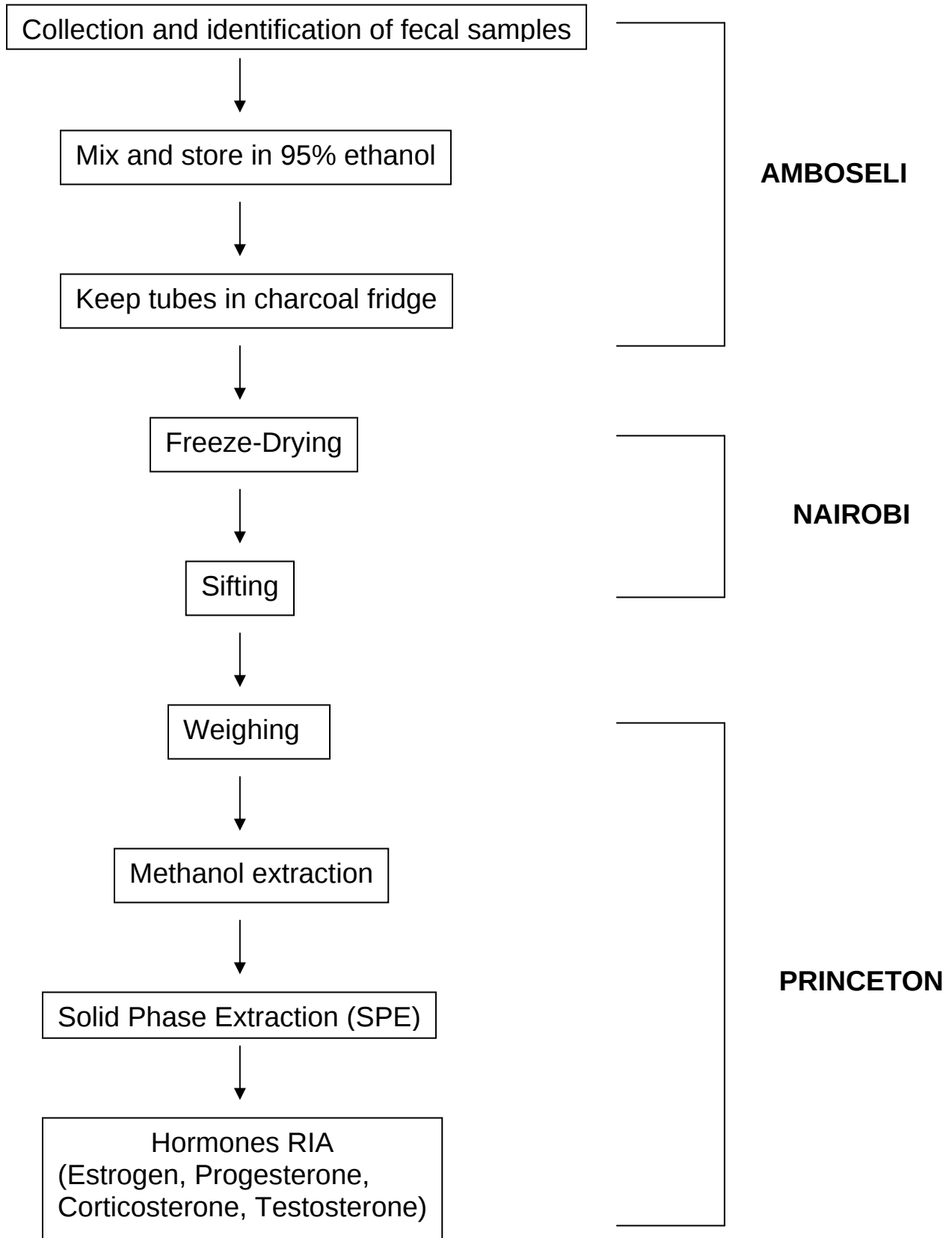


ALTMANN LABORATORY PROCEDURES FOR FECAL HORMONES

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FECAL SAMPLE COLLECTION

Samples are collected *ad libitum* from free-living baboons living in the Amboseli basin of Kenya. When a known animal is observed defecating, we collect the freshly-dropped sample within minutes of its deposit. The sample is well-mixed in a small waxed paper cup with a wooden stirrer and an aliquot is placed in a plastic 20ml vial (Evergreen Scientific 220-3519-080; caps 300-3532-G20) prefilled with 95% ethanol. We add feces until we get a 2.5:1 ratio of ethanol to feces. The vial is identified with the baboon's name (ID), the sample date, and time of collection. Samples are stored in a charcoal evaporation refrigerator, which keeps temperatures 15°C-25°C (~daily min, max), until samples can be shipped to Nairobi for further processing. A list of all samples, including the baboon id, the date and time of collection, and any additional notes about the sample, accompanies the samples to Nairobi.

FREEZE-DRYING

Samples are sent to the University of Nairobi every two weeks, where they are freeze-dried immediately.

Write the sample ID on the cap of each vial. Prepare and send to Princeton an excel sheet containing the sample ID, baboon's name (ID), collection date and time, freeze dried date and notes about the texture, quantity, or composition of the sample.

Evaporate ethanol:

Allow all ethanol to evaporate by removing the caps and placing the opened vials under a fume hood. Stir the samples regularly as the ethanol evaporates (about every 2 hours), as regular stirring facilitates complete and uniform drying of the sample. Continue with the hood drying until samples are completely dry (dry samples will be "crunchy" when stirred). Stir the vial thoroughly, especially at the bottom, to make sure that the whole sample is completely dry. It takes 24-40 hrs (depending primarily on the water content of the feces) for the ethanol to completely evaporate. Recap the vials and store samples at -20°C until ready for freeze drying.

Freeze dry samples:

Cut cotton gauze into squares (about 7 cm per side). Remove the cap from the sample and fasten the cotton gauze over the vials using a rubber band. Place the tubes in the freeze-drying flasks. Place the flasks in the freezer to cool them down (30 min, -20°C). Once samples are cold, place them into the freeze-drier.

- Temperature should be below -50°C
- Vacuum should be around 30 millitorrs
- Oil in pump must be checked before every use

Once samples are completely freeze-dried, cap the vials and store samples at -20°C until ready for sifting.

SIFTING

Note: Sifting should be done in a humid area to keep airborne fecal dust to a minimum. If necessary, run a humidifier to make the area locally humid.

1. Fold a piece of weighing paper (4" x 4") diagonally.
2. Sift the dried fecal sample through a mesh tea strainer (~40 mesh) onto the weighing paper.

NOTE: Use the end of a metal spatula to break-up and sift feces through strainer. The first particles that come through look like very dry, fine, dirt. Stop sifting when vegetative matter begins to come through. The purpose of sifting is to separate the feces from the vegetation, not to mash up the vegetation into fine pieces.

3. Transfer the fecal powder back into the collection plastic tube, and discard the vegetative matter.
4. Clean strainer and spatula with ethanol between each sample.
5. Store samples at -20°C until shipment to Princeton and upon arrival in Princeton place back in the freezer.

WEIGHING

Note: As for the sifting step, we recommend that sifting be done in a humid area to keep airborne fecal dust to a minimum.

We use a high precision balance Denver Instruments Co. A-250.

1. Label 16x100 mm test tubes with sample ID by using an alcohol resistant marker.
2. Crease weighing paper diagonally, place on balance, and press TARE.
3. Weigh 0.20 grams (between 0.2000 and 0.2050 grams) of fecal powder. If there is not enough sample to attain 0.20 g, then record the exact weight on the data sheet. Cap tubes loosely and place in rack.
4. If there is approximately 0.20 grams remaining, save in a tube labeled with sample number and date. If there is significantly less than 0.20 grams left over then add it to the tube marked "fecal pool" to be used as a standard or for various training exercises.
5. Discard the vial and weighing paper into the biohazard waste. Rinse the tea strainer and spatula with ethanol between each sample.
6. Store tubes and fecal powder at -20°C until ready for methanol extraction.

On the excel datasheet, record the date the sample was weighed, the sample's actual weight (particularly important when the sample is less than 0.2 g to correct the hormone concentration that is expressed in ng/g feces), and whether we have an extra tube of feces for that sample.

METHANOL EXTRACTION

1. Add 2 ml of 90% methanol using a repeater pipette to each tube that contains the sifted fecal sample.

2. Cap tightly and place on multi-pulse vortexer, and vortex for 30 minutes.
 - Motor speed =70
 - Pulser =60
3. Centrifuge at 1 g (2300 rpm) for 20 minutes at room temperature (~25°C)
4. Transfer supernatant from 16x100 mm tubes to 1.5 ml eppendorf tubes using Pasteur pipettes.
5. Centrifuge the eppendorf tubes at 2 g (3200 rpm) for 15 minutes.
6. Transfer supernatant to 2 ml microcentrifuge tubes and cap the tube (use a cap with an O-ring to avoid evaporation).
7. Place samples in storage boxes and store at -20°C until ready to assay.
8. Clearly label each box with the sample ID numbers.

SOLID PHASE EXTRACTION (SPE)

We use the Waters Oasis HLB cartridges (catalog # WAT 094226)
Remove samples from freezer and warm them up in 37°C water bath

Sample dilution:

1. Vortex tubes for about 10 sec
2. Place 800 µl of the methanol extract in 12x75 mm test tubes
3. Dry it down with pressurized air in 37°C water bath
4. Add 300 µl of 90% methanol
5. Vortex for about 30 sec
6. Add 700 µl of distilled water
7. Vortex again

Cartridge preparation:

1. Place cartridges (labeled with sample ID) on the cartridge support (a wooden support made in our lab containing 36 holes and a collection tray below it).
2. Wash the Oasis cartridges with 1 ml of 100% methanol (use Eppendorf Repeater pipette). Discard eluate.
3. Wash again with 1 ml of distilled water. Discard eluate.
4. Load diluted samples onto the cartridges. Discard eluate.
5. Rinse with 1 ml of 20% methanol. Discard eluate.

Sample elution:

1. Remove cartridges from wooden support and place into 16x100 mm test tubes.
2. Add 2 ml of 100% methanol. Let run through and collect eluate in tubes.
3. Dry with pressurized air in 37°C water bath.
4. Add 800 µL of 90% methanol. Vortex for 30 sec.
5. Transfer into 1.5 ml conical microcentrifuge tubes with O-ring cap.
6. Store samples at -20°C.

HORMONE ANALYSIS (using RadioImmunoAssay, RIA)

We used I¹²⁵ clinical diagnostic kits from various vendors. We have modified each kit to work with the baboon fecal samples.

ICN Corticosterone for Rats and Mice: Catalog #07-120102 (100 tube kit) or #07-120103 (200 tube kit). We use the kit at half the volumes suggested in the kit literature.

ICN Total Estrogens: Catalog # 07-140202 (100 tube kit), or #07-140205 (500 tube kit). We use a quarter of the volume suggested in the kit literature for the standards and half the volume for the tracer, first and second antibody. The samples are diluted to a 1:200 dilution in the assay buffer. Sample dilutions may need to be adjusted if the female is pregnant.

Progesterone: The tracer and standards are from Pantex (catalog # 137 TRA for tracer, catalog # 137 CAL for standards, catalog # 137 CAL0 for standard 0. The progesterone first antibody is the CL 425 (from C. Munro, UC Davis), diluted to 1:12000. And the second antibody is a Goat Anti-Mouse IgG (from Equitech-Bio catalog #GAMG-0100)

Diagnostic Systems Laboratories Testosterone: Catalog # DSL-4100. We use one fifth of the volume suggested for the tracer and one forth of the volume for the first and second antibody.

ICN Corticosterone RIA

1. Add standards and samples as follows:

	Tube number	Charcoal strip (µl)	Std or sample (µl)	Buffer (µl)	1st AB yellow (µl)	Tracer (blue) (µl)		2nd AB	
TC	1-2	-	-	400	-	100	Vortex, cover with parafilm and incubate at room T°C for 2 hr	-	Vortex
NSB	3-4	5, dry	-	150	-	100		250	
Bo	5-6	5, dry	-	50	100	100		250	Centrifuge at 4°C for 20 min at 3100 rpm
Sdt 1-6	7-18	5, dry	50	-	100	100		250	
Ctl Low	19-20	-	50	-	100	100		250	
Ctl High	21-22	-	50	-	100	100		250	
Pool Low	23-24	-	5, dry	50	100	100		250	
Pool Med	25-26	-	10, dry	50	100	100		250	
Pool High	27-28	-	15, dry	50	100	100		250	
Samples	29-150	-	10, dry	50	100	100		250	

NOTE: “dry” indicates tubes to be dried down first with pressurized air (in water bath)
Tubes 1-2 are not centrifuged. Cover samples with parafilm to avoid contamination.

2. After centrifugation place all tubes in a bucket of icy water.
3. Aspirate supernatant.
4. Load samples into gamma counter.

ICN Total Estrogens RIA

Sample dilution (1:20)

- Vortex samples (30 secs)
- Add 25 µl of sample
- Add 475 µl of buffer

1. Add standards and samples as follows:

	Tube number	Std or sample (µl)	1:100 methanol in Buffer (µl)	Buffer (µl)	1st AB (yellow) (µl)	Tracer (blue) (µl)		2nd AB	
TC	1-2	-	175	175	-	50	Vortex, cover with parafilm and incubate at room T°C for 90 min	-	Vortex and incubate 1 hr at room T°C
NSB	3-4	-	150	150	-	50		50	
Bo	5-6	-	125	125	50	50		50	
Sdt 1-6	7-18	125	125	-	50	50		50	
BioRad Low	19-20	250	-	-	50	50		50	Centrifuge at 4°C for 20 min at 3100 rpm
BioRad Med	21-22	250	-	-	50	50		50	
Biorad High	23-24	250	-	-	50	50		50	
Pool Low	25-26	10	-	240	50	50		50	
Pool High	27-28	20	-	230	50	50		50	
Samples	29-150	25	-	225	50	50		50	

2. Then proceed as indicated for corticosterone RIA.

Progesterone RIA

-Standarts + tracer (Pantex)

-1st AB: CL 425 (C. Munro, UC Davis)

-2nd AB: Goat Anti-Mouse IgG (Equitech-Bio GAMG-0100)

***Buffer preparation:**

-RIA buffer: PBS/BSA/Tween buffer (1 liter):

8g NaCl

0.2g KCl

1.44g Na₂HPO₄

0.24g NaH₂PO₄

Stir at low heat (Graduation 2 for heat on plate)

Once dissolved add:

1g BSA (Bovine Serum Albumin)

1g Tween 80

After complete dissolution, adjust pH to 7.5 (add NaOH if pH lower than 7.5)

-PEG buffer: 5% PEG in PBS buffer (1 liter):

8g NaCl

0.2g KCl

1.44g Na₂HPO₄

0.24g NaH₂PO₄

Stir at low heat (Graduation 2 for heat on plate)

Once dissolved add:

50 g PEG 8000 (Polyethylene Glycol)

After complete dissolution, adjust pH to 7.5 (add NaOH if pH lower than 7.5)

***Antibody and serum preparation:**

-1st AB (CL425)

d1/12000:

40µl 1st AB d1/10

48 ml PBS/BSA/Tween Buffer

- Normal mouse serum 1% (Sigma)

500µl serum

49.5ml PEG Buffer

-2nd AB: Goat- Anti-Mouse IgG (Equitech-Bio GAMG-0100)

d1/100:

10 ml 2nd AB

990 ml PEG Buffer

(if only for 1 RIA: 2 ml of 2nd AB in 198 ml PEG Buffer)

1. Add standards and samples as follows:

Pantex SDT	Tubes #	SPE pool (μl)	Sdt 1 or other (μl)	RIA Buffer (μl)	1st AB 1/12000 (μl)	Tracer Pantex (μl)	Overnight incubation at room T°C	Mouse serum (μl)	2 nd AB 1/100 (ml)	Incubate for 1hr at room T°C. Centrifuge at room T°C for 20min at 3100 rpm
TC	1-2	-	-	1250	-	200		-	-	
NSB	3-4	-	50	100	-	200		100	1	
B0 (Sdt0)	5-6	-	50	-	100	200		100	1	
Sdt 1	7-8	-	50	-	100	200		100	1	
Sdt 2	9-10	-	50	-	100	200		100	1	
Sdt 3	11-12	-	50	-	100	200		100	1	
Sdt 4	13-14	-	50	-	100	200		100	1	
Sdt 5	15-16	-	50	-	100	200		100	1	
Sdt 6	17-18	-	50	-	100	200		100	1	
Sdt 7	19-20	-	50	-	100	200		100	1	
Sdt 8	21-22	-	50	-	100	200		100	1	
SPE pool low	23-24	5, dry	50	-	100	200		100	1	
SPE pool low	25-26	10, dry	50	-	100	200		100	1	
SPE pool low	27-28	20, dry	50	-	100	200		100	1	
Samples	31-32	10, dry	50	-	100	200		100	1	

2. Then proceed as indicated for corticosterone RIA.

DSL testosterone RIA

Dilute 1st AB at 1:4 with PBS/BSA/Tween buffer (11ml 1st AB + 33ml Buffer)

1. Add standards and samples as follows:

	Tubes number	Std or CTL (μl)	SPE pool (μl)	PBS/BSA/ Tween Buffer (μl)	Sdt A (μl)	Tracer (μl)	1st AB d 1:4 (μl)	Vortex, cover with parafilm and incubate at room T°C overnight 12-24hrs	2 nd AB (μl)	Vortex Incubate 20 min at room T°C Centrifuge at 4°C for 20 min at 3100 rpm
TC	1-2	-	-	400	-	100	-		-	
NSB	3-4	-	-	100	50	100	-		250	
Bo (SdtA)	5-6	50	-	-	-	100	100		250	
Sdt B	7-8	50	-	-	-	100	100		250	
C	9-10	50	-	-	-	100	100		250	
D	11-12	50	-	-	-	100	100		250	
E	13-14	50	-	-	-	100	100		250	
F	15-16	50	-	-	-	100	100		250	
Ctl I	17-18	50	-	-	-	100	100		250	
Ctl II	19-20	50	-	-	-	100	100		250	
SPE pool	21-22	-	5, dry	-	50	100	100		250	
	23-24	-	10, dry	-	50	100	100		250	
	25-26	-	20, dry	-	50	100	100		250	
samples	27-150	-	10, dry	-	50	100	100		250	

2. Then proceed as indicated for corticosterone RIA.

CHARCOAL STRIP (Modified from Sousa and Ziegler (1998) Am. J. Primatol., 46: 105-117)
 Charcoal: Carbon decoloring alkanin Norit A, Fisher # C176

Wash charcoal:

1. Put charcoal in beaker, then add distilled water.
2. Let the charcoal settle for a few hours or longer, then pipette off the charcoal that floats to the top and pour off and discard most of the liquid.
3. Then pour the charcoal into an aluminum foil-lined box and spread the charcoal out under the hood to dry.

To charcoal strip:

1. Take 10 ml of pool fecal extract in 90% methanol. Add 250 mg of washed charcoal (the ratio should be 50 mg charcoal/2 ml suspension).
2. Stir slowly with stir bar for 1 hr at room temperature.
3. Centrifuge at 2 g for 10 minutes.
4. Pipette off liquid and push through a 0.2 μ m filter with a syringe; discard charcoal. The resultant liquid should be clear.
5. To double charcoal strip: Repeat steps 2 through 5.

Preparation of the SPE male/female pool

1. Place 0.2 g of the fecal pool in a 16x100 mm test tubes.
2. Do a methanol extraction (Follow the usual protocol)
3. Pipet 300 μ l methanol extract and 700 μ l water into a 12x75 mm test tube. Prepare as many tubes as needed until all methanol extract is used up.
4. Then conduct SPE, starting with cartridge preparation.
5. After final evaporation add 300 μ l of 90% methanol.
6. Vortex each sample well and then pool all the tubes together. Vortex again.
7. Place aliquots of 2 ml in microcentrifuge tubes and store at -20°C .

PBS/BSA/Tween Buffer (for Progesterone and Testosterone RIA)

To make 1 liter of buffer

1. Place 800 ml of distilled water into a beaker
2. Add the following:
 - 8 g NaCl (Sodium Chloride)
 - 0.2 g KCl (Potassium Chloride)
 - 1.44 g Na₂HPO₄ (dibasic sodium phosphate)
 - 0.24 g (monobasic sodium phosphate, MW = 120)
3. Mix on stir-plate at low heat.
4. When dissolved, add
 - 1 g of bovine serum albumin (BSA)
 - 1 g of Tween 80 (very thick, stick to the weighing cup, rinse with water and scrap the solid formed with a spatula)

5. Mix slowly until total dissolution.
6. Then adjust pH to 7.5
NOTE: If $\text{pH} < 7.5$, add a few drop of the sodium hydroxide solution (NaOH).
7. Transfer buffer into a graduated cylinder and fill to 1 liter with distilled water.
8. Transfer into a 1 liter glass bottle
9. Store in the refrigerator. Buffer should last 1-2 months. Check periodically for cloudiness.

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