Development of non-invasive reproductive monitoring techniques for endangered snow leopards and Amur leopards

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Abstract

Snow *(Panthera uncia)* and Amur leopards *(Panthera pardus orientalis)* are two of the most endangered large cats on Earth. Little is known about the reproductive physiology of these cats and a basic understanding of reproductive biology will aid in conservation efforts. Therefore, the objectives of this study were to optimize fecal steroid hormone extraction procedures and determine appropriate antibodies for enzyme immunoassays. For our first objective, we compared combinations of different fecal steroid hormone extraction procedures including, ethanol vs. methanol extraction and vortexing vs. rocking samples to determine which method extracted the greatest amount of steroid hormones. Results indicated that rocking fecal samples for 24 hr extracted less steroid hormone than vortexing. For fecal androgens, a 6.8-fold increase in hormone concentration was found when samples were extracted with methanol and vortexed for 20 minutes (M/V 20 min), compared to extraction with ethanol and vortexed for 20 minutes. Although results were not significant, the M/V 20 min extraction also yielded a greater amount of estrogens, compared to other extraction methods. For our second objective, we examined whether antibodies found to be appropriate for measuring fecal steroids in other felids were also suitable for snow and Amur leopards. As was found in other large cats, our preliminary results indicated that a broad scale testosterone antibody and an estrogen metabolite (E1G) antibody can be used to measure fecal androgens and estrogens in snow leopards, and may also be applicable to Amur leopards. Results from these studies will improve procedures to characterize the seasonal reproductive profiles of pubertal and adult female and male leopards.

Introduction

- Amur leopards (*Panthera pardus orientalis*, a subspecies of the leopard) and snow leopards (*Panthera uncia*) are both endangered big cats and are in direct risk of extinction.
- Little is known about the reproductive physiology of these felids and a fundamental understanding of reproductive biology will aid in conservation efforts.
- While little is known about the Amur leopard, a recent study examining fecal reproductive hormones was done in female Arabian leopards (*Panthera pardus nimr*) [1], another sub-species of the leopard that may be comparable to female Amur leopards.
- Limited research has been done examining reproductive hormones of female snow leopards [2, 3]. However, these studies used more invasive methods (serum) and/or radioimmunoassay on a small sample size.
- Although sperm morphology was characterized in captive male Arabian leopards [4], testosterone levels were not examined and testosterone has not been characterized in either male Amur or snow leopards.
- **Therefore, we wanted to non-invasively examine reproductive hormones in male and female Amur and snow leopards to provide a large and comprehensive data set by which other captive and/or wild animals can be easily compared to.**

Objectives

1. Determine the appropriate fecal steroid hormone extraction technique and the appropriate hormone antibodies for these leopards.
2. Apply the best fecal extraction technique to non-invasively characterize seasonal reproductive hormones in male and female snow and Amur leopards.

Materials and Methods

Objective 1:

- Steroids were extracted from fecals using either a wet or dry sample
- For dry extraction, samples were dried at 60 C for ~48 hrs and ground into a fine powder
- Exactly 0.25 g of wet or dry fecal were extracted using the following procedure:

A) Rocking overnight in	B) Vortexing in
1. 90% ethanol	1. 90% methanol for 10 or 20 min
2. 90% methanol	2. 90% ethanol for 10 or 20 min

- Samples were centrifuged at 2000 x g for 20 min at 4 C and extracted hormones in supernatant were removed
- Analyze extracted hormones via enzyme-immunoassay (EIA) to determine the best extraction method and to determine if antibodies appropriate for other leopard species are also appropriate for snow and Amur leopards

Objective 2:

- ***Dry Fecal Extraction:*** Vortex for 20 min in 90% methanol
- ***EIA:*** For each leopard type, monitor 10 male and 10 female leopards year-round using an estrogen metabolite (E1G) and a broad-scale progestin antibody (females), or a broad- scale testosterone antibody (males)

These data present the results of Objective 1 and preliminary results for Objective 2

Results

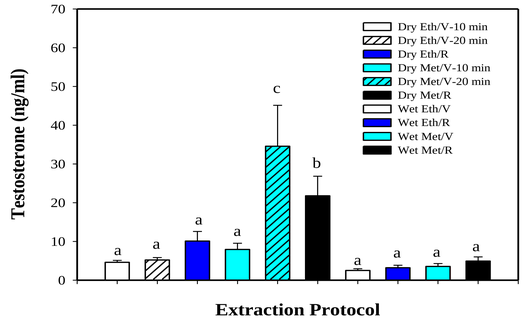

Figure 1. Comparison of fecal extraction techniques for testosterone in the adult male snow leopard (SB1661, DOB May 1992; Memphis Zoological Society). Data are means SEM of five samples. Values with different superscripts differ significantly (*P < 0.05* **).**

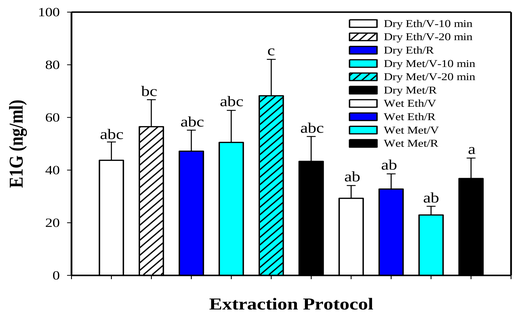

Figure 2. Comparison of fecal extraction techniques for estrogen metabolite, E1G, in the adult female snow leopard (SB1868, DOB May 1995; Memphis Zoological Society). Data are means SEM of seven samples. Values with different superscripts differ significantly (*P < 0.05* **).**

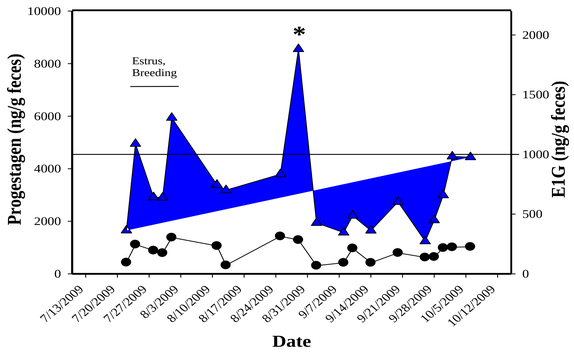

Figure 3. Concentrations of E1G and progestagens in female Amur leopard SB502 over time (DOB April 2000; Jacksonville Zoo and Gardens). SB502 was housed with a male and estrus/breeding behavior was observed on 7/29 through 7/31. No ovulation occurred during the months examined thus far. Peaks of E1G where no estrus/breeding behavior was observed are marked with an asterisk (*). The follicular cycle for July through August lasted 28 days. No follicular activity was observed for the month of September based on the levels of E1G.

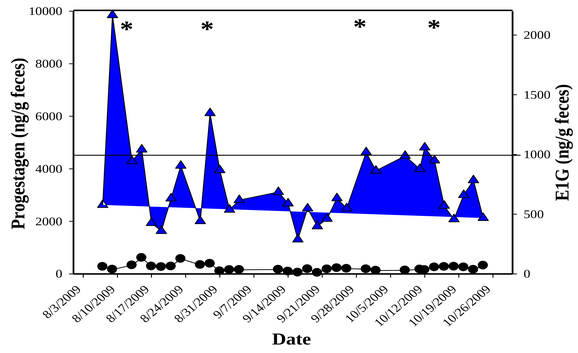

Figure 4. Concentrations of E1G and progestagens in female snow leopard SB2013 over time (DOB May 1997; Knoxville Zoological Gardens). SB2013 was housed with another female and no ovulation occurred during the months examined thus far. Peaks of E1G where no estrus/breeding behavior was observed are marked with an asterisk (*). The follicular cycle for the month of August lasted 14 days, while the follicular phase for the month of October lasted 12 days. No follicular activity was observed for the month of September based on the levels of E1G.

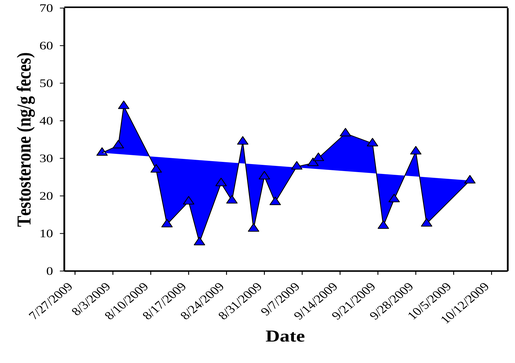

Figure 5. Concentrations of testosterone in male snow leopard SB2443 over time (DOB June 2003; Chicago Zoological Park). SB2443 was housed with a female. The average preliminary levels of testosterone for the summer months of August and September were 24 ng/g feces and 25 ng/g feces, respectively for SB2443.

Summary

- Vortexing for 20 min in 90% methanol obtains the highest proportion of bound testosterone in feces, and this method was one of the optimal methods for the estrogen metabolite E1G
- Specific antibodies against estrogen metabolites (E1G) and broad-scale progesterone (P4) antibodies accurately determine concentrations of fecal estrogens and progestins in female Amur and snow leopards
- Broad-scale testosterone antibodies accurately determine concentrations of fecal testosterone in male snow leopards (and male Amur leopards; results not-shown)

Future Directions

- Monitor animals year-round to determine the effect of season
- Compare pubertal to adult animals
- Include fecal samples from “more pure” captive Amur leopards housed in zoos from Europe and Russia
- Include fecal samples from 4 wild Amur leopards currently on a tracking study in Russia

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