Assessment of Breeding Difficulties in Captive Black-necked Stork (*Ephippiorhynchus asiaticus*) by Hormonal Study at Khao Kheow Open Zoo,Thailand

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1.Introduction

The Black-necked stork (*Ephippiorhynchus asiaticus*) is one of the tallest extant species of storks. Over the past 30 years, it originates from the Wet land in Southeast Asia (Except in Malaysia, Indonesia and Philippine), and partly of South Asia and also Australia. As the Black-necked stork is threatened in the wild, especially in several country of Southeast Asia such as Thailand. The greatest threat to the extinction in the wild of Thailand continues to be poaching and habitat losses from the fast development of social industry, it is important that a viable population is maintained in captivity. Before that, it is one main objective for The Zoological Park Organization of Thailand, under the royal patronage to conduct conservation breeding. This will help *ex situ* conservation, and also biodiversity conservation. Black-necked Storks are in our focus of this objective. However, breeding in captivity is relatively erratic. There are many factors that involving with unsuccessful breeding in captivity such as, infertility and non-paired breeding. In the present study describes a project initialed to establish basic reproductive parameters in this species, the diagnosis and monitoring of hormonal are two of the most important reproductive evaluation that can be made for the management of animals in breeding programs, with the ultimate aim of achieving better breeding success. Hormonal detection permits initiation of monitoring and health care for the animal individual. Knowledge of the reproductive status of each female allows animals managers to optimize production of a colony to ensure sustainable conservation of endangered species.

Enzyme immunoassays (EIAs) have been developed for evaluation of hormone metabolites in nondomestic species. These EIAs have the advantages of being less expensive to perform and do not require the use of controlled radioisotopes (Czekala *et al*., 1986)

Non-invasive methods using in quantification of steroid hormones in feces is one suitable way for captive wild animals (Rupert *et al*., 2005).This method can avoid traumatizing or stressing the animals from capture and can be explained in the principal of hormone metabolism. Hormones produced from endocrine glands or tissues will be excreted into blood stream to the target organs or tissues. Then, the hormones will pass through liver, where they were change to different metabolites. Metabolites then excreted into bile system and finally the bile with metabolites is mixed with feces and expelled out of the body. Metabolite levels can reflect Steroid hormones level. All the detectable steroids in feces are called “Fecal steroids” (Erich *et al*., 2005)

2. Objectives

2.1 To quantify levels of stress hormone in Black necked stork from fecal extraction method in order to use as a tool to evaluate cage management

2.2 To determine individual reproductive physiological status of each animal in different reproductive stages in captive male and females.

2.3 To quantify levels of nutrition requirement in Black neck storks on average years round

3. Methods

3.1 Materials (Essential Component of the EIA)

- Solid Phase: polystyrene microtiter plate (Nunc Immuno plate)
- Antibody: Polyclonal Antibody and Monoclonal Antibody
- Coating buffer: Carbonate/bicarbonate buffer pH 9.6
- Wash solution: Nacl and Tween 20 solution
- Enzyme conjugate (tracer): Horseradish peroxidase (HRP)
- Assay buffer: Phosphate or Tris buffers pH 7.0
- Standards: Known concentrations of hormone (Sigma Diagnostics)
- Substate: Chromagen (ABTS) and catalyst (Hydrogen peroxide) Substrate
- Reading: Spectrophotometer or Plate reader

3.2 Collection of Storks dropping:

Individual fecal samples 10-50 g were collected one to three times weekly for 12 to 24- month periods. Criterions for fecal collection are fresh, no material contamination, and homogeneous feces so the metabolite is eventually distributed in fecal sample. After collections, samples were frozen and stored at -20 °C until analysis.

3.3 Preservation with Freezing or Drying immediately:

Incase of drying process is delayed, we collect fecal sample in dark, close container to prevent from moisture and light. Then the box is kept at -20 degree Celsius, in order to prevent the sample from bacterial activities which can interfere with steroid levels

Drying of sample is done by lyophilizer or hot air oven until no change in weight of the sample is not detected. Hot air oven is set at 96 hours, at 60 degree Celsius. Dry sample is collected in close container with moisture absorbent substance, in the refrigerator. (Erich *et al*., 2005; Tony *et al*., 2005)

3.4 Fecal Extraction:

Extraction process is done by boiling 0.2 g of dry sample in 90% Alcohol. Then the sample is kept at -20 degree Celsius for the analytical process

3.5 Assay Protocols:

Hormonal assay is performed by Competitive ELISA (Enzyme Linked Immuno Sorbent Assay) according to Brown *et al* (2004)

ELISA or Enzyme-linked Immuno Sorbent Assay works on the principals of reaction between coated specific antibody, to the particular substance, and antigen in the sample solution. The plate is coated with antibody which will adhere with the antigen from the sample and also from the conjugated enzyme. Both type of antigen will compete for coated antibody on the plate. Then free antigen of both antigens will be washed out. The remaining antigen with enzyme will change substrate in gradient levels according to amount of enzyme attached on antigen and antibody. Quantification can be done by comparing with color produced by known amount of antigen, by photometer machine.

3.6 Data analysis

Mean data are presented as ±SEM. Definition of the reproductive status was based on fecal hormone profiles. For each individual, a non pregnant baseline progesterone value was calculated using an iterative process in which values that exceeded the mean plus 1.5 standard deviations (SD) were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD [Brown *et al*., 1994, 2001]

4. Result and Conclusions

The results of the Assessment of Breeding Difficulties in Captive Black-necked Stork (*Ephippiorhynchus asiaticus*) by Hormonal Study: we group animals into various groups for comparisons as;

Black-necked Stork females

All study females showed at least some evidence of ovarian activity. There was seasonality in ovarian activity because cycles were not observed every month of years, with observed only in Breeding season (October-November or January-February). Anestrous periods, ranging from as long as 8 to 10 consecutive months. One singleton female has successful to breed and given birth during a 2-year period. Others not success to breeding pair or not given birth, were observed in 3 females; however, one of these episodes occurred sickness and died in later. There was no age difference ($P>0.05$) between paired and non-paired females in this study population. The progesterone concentrations in breeding season of all females were significantly different between paired female and non-paired females, with paired females had higher than two non-paired females ($P<0.05$) but non-significant different in another time with non-breed season during a year period ($P>0.05$) (Fig. 1). For each female, a non-breeding (out of breeding time) Overall baseline concentrations of fecal progesterone averaged 77.8 ± 1.91 ng/g of dry feces, with peak concentrations ranging from approximately 12 to 180 ng/g

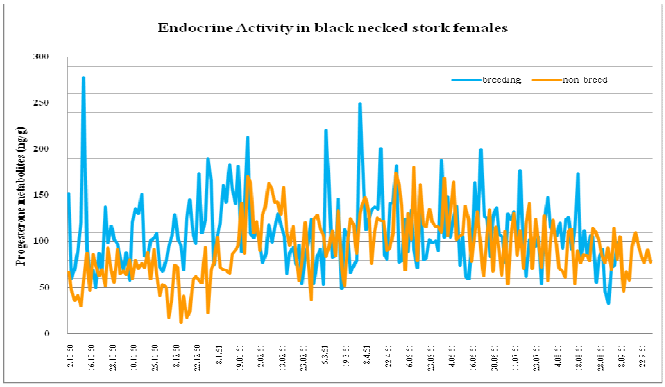

Fig.1. Fecal progesterone concentrations in an individual Black-necked Stork female throughout average one year round.

Fecal total 17-ß Estradiol metabolite concentrations in Black-necked stork females fluctuated markedly and were not useful for characterizing follicular activity, even when samples were collected three times per week (Fig. 2). All those compared mean average 17-ß Estradiol concentration in female between breeding paired and non-paired female group, there was non-significant different in paired and non-paired (non-breeding) group ($P > 0.05$), however 17-ß Estradiol level in paired female to be seem higher than in non-paired females. For fecal cortisol concentrations, when compare the overall cortisol concentrations of all females were significantly different between paired female and non-paired females, with paired female had lower than two non-paired females ($P < 0.05$) were 17.58±1.04 ng/g and 23.57±1.03 ng/g of dry feces respectively. Finally, in paired female at Khao Kheow Open Zoo can bred 2 times and lay several of eggs in single season from cage management.

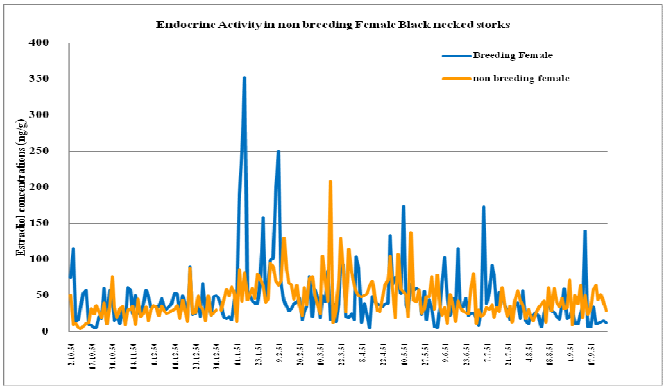

Fig.2. Fecal 17-ß Estradiol concentrations in an individual Black-necked Stork females throughout average one year round.

Black-necked Stork males

The results of the Testosterone in Black-necked stork males are depicted in Figures 3 a, b. In contrast to females, there was difference ($P<0.05$) between paired male and three non-paired males in this study population. The average age of paired male was greater ($P<0.05$) than that for the non-paired males. There was no evidence of seasonality in testicular activity. Overall mean fecal testosterone concentrations were higher ($P<0.05$) in the paired male (205.60±13.10 ng/g of dry feces); range, 21-877 ng/g) than in non-paired males (128.71±4.76 ng/g of dry feces); range, 8-1,042 ng/g). All this when compared in the same non-breeding group, there was no difference ($P>0.05$) in average fecal testosterone concentrations. The study also found that cortisol level was highest in breeding season for paired male. Another male was highest in the summer.

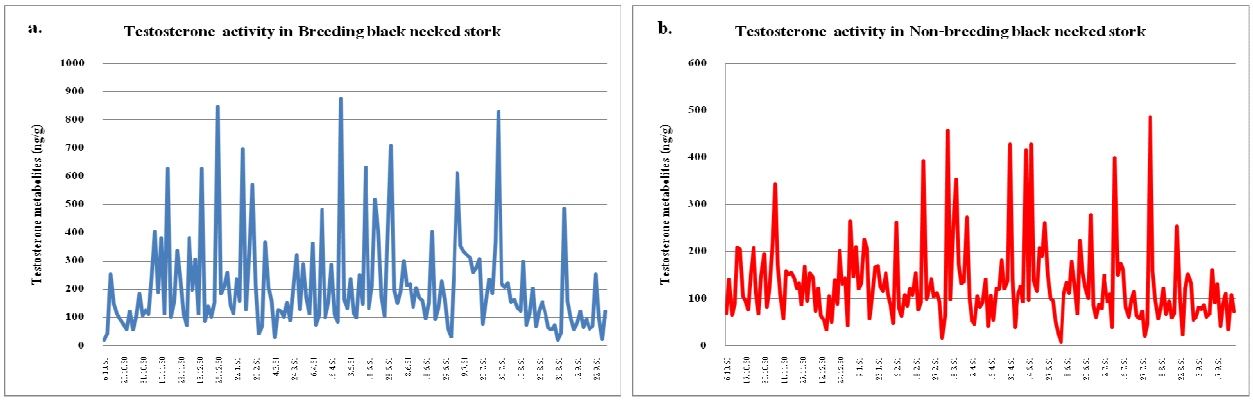

Fig. 3 a, b. Fecal Testosterone concentrations in an individual Black-necked Stork males throughout average one year round.

5. References

Brown, J.L., Wasser, S.K., Wildt, D.E. and Graham, L.H. 1994. **Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non-invasively in feces**. Biol Reprod. 51: 776-786.

Brown, J.L., Bellem, A.C., Michael, F., Wildt, D.E. and Roth, T.L. 2001. **Comparative Analysis of Gonadal and Adrenal Activity in The Black And White Rhinoceros in North America by Noninvasive Endocrine Monitoring**. Zoo Biology. 20: 483-486.

Brown, J.L., Walker, S. and Steinman, K. 2004. **Endocrine manual for the reproductive assessment of domestic and non-domestic species, 2nd edition,** Smithsonian institution. USA.

Czekala, N.M., Gallusser, S., Meier, M.E., Lasley, B.L. 1986. **The development and application of an enzyme immunoassay for urinary estrone conjugates**. Zoo Biology. 5: 1-16.

Erich Mostl, Sophi Rettenbacher, And Rupert Palme. 2005. **Measurement of Corticosterone Metabolites in Birds' Droppings: An Analytical Approach**. Ann. N.Y. Acad. Sci. 1046: 17–34.

Palme, R. 2005. **Measuring Fecal Steroids Guidelines for Practical Application.** Academy of science, New York.

Toni E. Ziegler, and Daniel J. Wittwer. 2005. **Fecal Steroid Research in the Field and Laboratory Improved Methods for Storage, Transport, Processing, and Analysis**. University of Wisconsin. American Journal of Primatology, 67 : 159-174.