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# Reproductive Assessment of the Great Hornbill (*Buceros bicornis*) by Fecal Hormone Analysis

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The population of great hornbills (*Buceros bicornis*) in the United States is rapidly aging, and captive breeding efforts have not met population managers' expectations for a sustainable captive group. Little is known about the reproductive physiology of these birds. This study reports the first data on the reproductive endocrinology of the great hornbill. The hormone profiles of the only pair of these birds that hatched a chick in the 1999–2000 breeding season are compared to the profiles of six other pairs of hornbills, from different institutions in the United States, that did not reproduce successfully that season. The study investigates the estradiol, corticosterone, and testosterone profiles of these seven pairs of birds, establishing a base of knowledge from which endocrine data may be used to improve the success of captive breeding programs. The estradiol profiles from this study indicate a difference in hormonal patterns between laying and non-laying female great hornbills. Egg-laying females had significantly higher estradiol concentrations during the breeding season than the non-laying females ($P < 0.003$). Testosterone concentrations of the males were not significantly different between the mates of egg-laying and non-egg-laying females. The corticosterone concentrations tended to be lower in the females that laid eggs vs. the non-egg-laying group. The males of the egg-laying pairs showed a significantly lower ($P < 0.036$) corticosterone concentration than the non-egg-laying male pairs. This, combined with the extremely low corticosterone levels (compared to the other birds in the study) of the pair of hornbills that hatched a chick in the 1999–2000 breeding season, suggests that adrenal activity may play a role in the reproductive failure of some captive great hornbills. Zoo Biol 22:135–145, 2003. © 2003 Wiley-Liss, Inc.

**Key words: steroid hormones; estradiol; corticosterone; testosterone**

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## INTRODUCTION

With its large size, striking black and white coloring, and casqued head, the great hornbill (*Buceros bicornis*) is one of the most charismatic birds exhibited by zoos around the world. As of 1996, there were 72 great hornbills in North American zoos (31 males, 39 females, and two juveniles of unknown gender)—more than any other species of Asian hornbill. The great hornbill is currently listed in Appendix 1 of CITES [CITES, 2001] and as vulnerable in the Red Data Book [IUCN, 1996]. Its habitat ranges from the Ghats region of southwestern India through southeast Asia and Malaysia, but the numbers of the free-ranging population are currently unknown [de Ruiter, 1998]. One threat to the long-term viability of the wild population is habitat destruction, via logging and agricultural development, since great hornbills have a lower reproduction rate in disturbed habitat. In addition, females and chicks sealed in nests are easy targets for poachers, and the captive population is gender-skewed toward females as a result.

Great hornbills are a monogamous, pair-bonded species. The female seals herself into a nest cavity (usually a hollowed-out tree) before laying her eggs, and remains there throughout the entire incubation period and for about a month after her chick hatches. During this time, she can undergo a complete molt and is dependent on her mate for food. The selectivity of great hornbills in choosing mates, and the strength of the pair-bond once formed have presented problems for captive breeding efforts.

The population of great hornbills living in U.S. zoos is rapidly aging, and captive reproduction efforts have not met a self-sustaining population goal (only 19 chicks have hatched in the last 10 years). A number of case reports have been published in the last 20 years describing successful breeding seasons at different institutions [Choy, 1978; de Ruiter, 1998; Golding and Williams, 1986; Bohmke, 1987]. However, no one captive breeding program has been consistently successful, and to date no critical variable that increases the chances of hatching a chick has been identified. In 1996 and again in 1998, the American Zoo and Aquarium Association (AZA) proposed that the hornbill be the subject of a concerted captive breeding effort, and directed zoos to develop artificial nest sites, record life history data (which is difficult to obtain in the field), and increase support for field conservation through exhibition and publication. To date, these projects have had very little success. In the 1999–2000 breeding season, a pair of birds at the San Diego Wild Animal Park (SDWAP) hatched and reared the only chick in the United States.

While the nesting behavior of the great hornbill has been well documented elsewhere [Kannan and James, 1997], little is known about their reproductive physiology. This study compares the testosterone, estradiol, and corticosterone levels in the pairs of birds that successfully and unsuccessfully reproduced in the 1999–2000 season. The aim of this study was to determine what constituted a baseline reproductive hormone profile for great hornbills (*Buceros bicornis*), and to determine how the profiles of nonreproducing pairs compared to that of the only pair in the United States that hatched a chick in the 1999–2000 mating season. This information may ultimately be used to increase the success of captive breeding programs by pairing great hornbills with individuals whose hormone profiles indicate they might successfully reproduce.

## MATERIALS AND METHODS

### Animals and Collection Procedures

Fecal samples were collected from seven pairs of great hornbills (*Buceros bicornis*) housed at four different U.S zoos: three at SDWAP, two at Zoo New England (ZNE) in Boston, one at the Denver Zoo, and one at the Kansas City Zoo (KCZ) (Table 1). Collections were made on an opportunistic basis over a period of 3–4 months (December 1999 to April 2000), with an average of 24 samples per bird. The sample collection was discontinued once the female was sealed in her nest, to minimize disturbance of the female. All fecal samples were placed in plastic cups, immediately frozen, and stored at –20°C until analysis.

Over the duration of the study, only one pair of birds (female 28-5 and male 28-6, housed at SDWAP) hatched and reared a chick. The other two females at SDWAP both laid eggs (females 195 and 24-37). Evidence of two eggs was found in the nest of female 24-37, and fragments of at least one and possibly two eggs were found in the nest of female 195. The remaining birds in the study did not lay eggs, although the female from the Denver Zoo (female 1530) exhibited breeding behaviors and was sealed into her nest box. The two pairs of great hornbills (females 93A016 and 93A019) at the ZNE showed some interest in the nesting box and displayed courtship behavior, but the females were never sealed in. The pair of birds (female 50 and male 264) from the KCZ did not mate or exhibit nesting behavior. The male had to be separated from the female immediately after the breeding season because of the extreme levels of aggression she exhibited towards him.

### Extraction and Processing

Fecal samples were lyophilized for 96 hr to reduce the variability in water content. The dried feces were crushed to a powder to ensure thorough uniformity and mixing. A 0.05 g sample of crushed feces was weighed out in a $16 \times 150$ mm test tube, mixed with 0.5 ml of phosphate buffer (pH 5), and vortexed for 1 min. The sample was then hydrolyzed, using 0.02 ml of $\beta$-glucuronidase/arylsulfatase enzyme (Boehringer Mannheim, Indianapolis, IN) with a 12-hr incubation at 37°C. After this incubation, testosterone $H^3$ recovery (3,000 cpm) was added to the samples to account for extraction loss, and the mixture was vortexed for 2 min. Five milliliters of anhydrous diethyl ether were then added to each tube, vortexed for 2 min, and flash-frozen in a methanol/dry ice bath. The supernatant was decanted into $12 \times 75$ mm test tubes and allowed to evaporate in a water bath (37°C). The ether extract was reconstituted in 1 ml of phosphate buffer (pH 7), vortexed, and stored in a refrigerator at 4°C.

### Assay Methods

#### *Estradiol*

To analyze fecal extracts of the female hornbill, an estradiol RIA was utilized as previously described [Medler and Lance, 1998]. The estradiol assay was a modification of an $^{125}I$ assay kit (Diagnostics Products Corp., Los Angeles, CA). Two microliters of the reconstituted fecal extract were combined with the estradiol antiserum, followed by a 2-hr incubation at room temperature. $^{125}I$-estradiol was

**TABLE 1. Individual reproductive and housing status of each hornbill**

| Indian hornbills | | | | | | |
|---|---|---|---|---|---|---|
| Females | Males | Institution | Reproductive status 2000 | Size of enclosure | Photoperiod | Exhibit status |
| 28-5 | 28-6 | SDWAP[a] | Hatched chick 3/15/00 | 1,600 sq ft<br>20 ft × 8 ft × 10 ft | Natural | Off |
| 24-37 | 24-35 | SDWAP[a] | Two eggs laid | 2,400 sq ft<br>20 ft × 12 ft × 10 ft | Natural | Off |
| 195 | 28-7 | SDWAP[a] | One egg laid, Chick vocalizations confirmed<br>No evidence of chick found in nest | 2,400 sq ft<br>20 ft × 12 ft × 10 ft | Natural | Off |
| 1530 | 1529 | Denver Zoo | Female sealed in nest No egg laid | 8,280 sq ft<br>24 ft × 15 ft × 23 ft | Natural | Off during breeding season |
| 93A019 | 97A465 | Franklin Park Zoo, Boston | Female showed some interest in nest box but did not seal herself into nest | 1,680 sq ft<br>20 ft × 12 ft × 7 ft | Natural via fiberglass windows | Off |
| 93A016 | 93A780 | Franklin Park Zoo, Boston | Female showed some interest in nest box but did not seal into nest | 4,320 sq ft<br>30 ft × 18 ft × 8 ft | Artificial 12/12 cycle | Off |
| 50 | 264 | Kansas City Zoo | Female showed no interest in nest box | 1,620 sq ft<br>12 ft × 15 ft × 9 ft | Natural, tentative data | Off, vehicle traffic |

[a]Avian Propagation Center at the San Diego Wild Animal Park.

then added and the samples were incubated for another hour at room temperature. An addition of 0.5 ml of dese charcoal dextran solution terminated the competitive reaction by separating bound from free hormones. After a 10-min incubation, the samples were centrifuged for 15 min at room temperature at 1,500 *g*. Then 700 μl of the supernatant were pipetted into $12 \times 75$ mm test tubes, and counted in a gamma counter.

Fecal extracts which were serially diluted (0–1:256) and analyzed in the estradiol assay resulted in a curve parallel to the estradiol standards (r=0.9755). The intra-assay coefficient of variation was 9% and 10% at 20% and 70% binding (n=18 and 20), respectively. The interassay coefficient of variation was 20% for all three controls provided. Assay sensitivity was calculated at 1.29 pg/tube.

High-pressure liquid chromatography (HPLC) separation of the fecal extract revealed the radioactive estradiol-17-beta elution was more polar by two tubes (2 minutes) than the immunoreactive peak. The exact structure of this estrogen compound was not further identified.

### *Testosterone*

Testosterone (T) RIAs were run on the samples from all study birds (male and female). Fifty microliters of fecal extract were assayed for each of the male hornbill samples. For the T RIA, antisera (produced against testosterone 19-carboxymethyl-ether; ICN Biomedicals, Costa Mesa, CA) was combined with 10,000 cpm $H^3$-T (ICN Biomedicals). The cross-reactivity of the antisera in this assay is: testosterone 100%, 5α-dihydrotestosterone 18.75%, 5α-androstane-3α, 17β-diol 3.00%, 5-androstene-3β, 17β-diol 1.00%; all others tested were $<1.00\%$. After an overnight incubation at 4°C, the competitive reaction was terminated by the addition of 0.5 ml of charcoal dextran solution. Following a 30-min incubation at 4°C, the samples were centrifuged for 15 min (4°C), and decanted into scintillation vials. Scintillation fluid (5 ml) was added to each of the vials and counted for 2 min in a Beckman liquid scintillation spectrometer. The same procedure was followed for the females' samples, except that 100 μl of fecal extract was assayed to account for their lower basal testosterone levels.

Fecal extracts that were serially diluted and analyzed in the assay gave a parallel displacement curve to the testosterone standard curve (r=0.992). The intra-assay coefficient of variation was 8% and 6% at 20% and 70% binding (n=7 and 7), respectively. The interassay coefficient of variation was 14% for 82 ng/tube and 11% for 49 ng/tube. The sensitivity was calculated at 8.11 pg/tube.

HPLC separation of the fecal extract showed antibody immunoreactivity consistent with the elution of the radioactive testosterone marker. This was the major immunoreactive peak.

### *Corticosterone*

Corticosterone (B) RIAs were run on all subjects in the study and the procedure did not differ between the genders. An assay kit prepared by ICN Biomedicals was used to analyze the fecal samples. An anticorticosterone antibody (produced against corticosterone-3-carboxymethlyoxime BSA) and a $^{125}$I-labeled corticosterone tracer was used in the procedure. Samples were vortexed for 30 sec and incubated at room temperature for 2 hr. A precipitant solution (0.5 ml) was added to each sample to terminate the reaction, and all tubes are vortexed and centrifuged (1,000 *g*) for 15

min. The supernatant was then decanted and the test tubes were placed in a gamma-counter to count the precipitate.

Fecal extracts that were serially diluted and analyzed in the assay corresponded in parallel to the corticosterone standard curve (r=0.986). Assay sensitivity was 13.9 pg/tube (calculated as mean pg/tube at 90% B/BO, n=10). The intra-assay coefficient of variation was 6% and 7% at 20% and 70% binding (n=18 and 18), respectively. The interassay coefficient of variation was 4% for 777 ng/tube and 6% for 131 ng/tube (n=5). The cross-reactivity of the assay system, as reported by the provider, was: corticosterone 100%, desoxycorticosterone 0.31%, testosterone 0.14%, aldosterone 0.03%, and cortisol 0.03%. All other steroids tested were below 0.02%.

HPLC separation of the fecal extract showed that radioactive corticosterone marker eluted one tube (1 min) prior to the major peak of immunoreactive corticosterone. It was not further identified.

## RESULTS

### Estradiol

There was no significant difference in average estradiol concentrations among egg and non-egg-laying birds during the study period (ANOVA, $F(1,5)=0.482$, $P=0.51$). However, females that produced eggs exhibited a rise in estradiol beginning at approximately 30 days prior to sealing in the nest cavity, with levels peaking four to 10 times above baseline at approximately 12 days prior to nest sealing (Fig. 1). This differed significantly (ANOVA $F(1,4)=37.46$, $P=0.003$) from the non-egg-laying females during the breeding season (January 15 to February 10) (Fig. 2).

Individual 28-5, the SDWAP female that hatched a chick, had a mean estradiol value of 76.75 ng/gm with a maximum value of 333 ng/gm at day –12 before nest sealing. This pattern was mirrored by the other two females that laid an egg: 24-7 and 195. Female 24-37 had a mean estradiol level of 41.46 ng/gm and had a gradual rise to 422 ng/gm (day –11). Female 195 showed the most dramatic elevation of estradiol levels. Her baseline value was 78.9 and peaked to 600 ng/gm (day –17). The female (1530) from the Denver Zoo that was sealed into a nest but did not lay an egg showed a rise in estradiol levels in the same time period; however, her levels were less than half those of the egg-laying birds.

### Testosterone

A one-way ANOVA showed no significant difference in mean testosterone levels between the mates of laying and non-laying females (ANOVA, $F(1,5)=0.002$, $P=0.9632$). Testosterone levels for the male hornbills in the study clustered in a range from 15–35 ng/gm with the mean value for all males except one (hornbill 1529) under 50 ng/gm (Fig. 3). Male 1529 had a mean testosterone value of 78 ng/gm, with a range of approximately 20–215 ng/gm. The three males from the SDWAP, paired with the egg-laying females, had at least three testosterone spikes of at least 50 ng/gm, with one of these reaching levels $>75$ ng/gm. The SDWAP male (28-6) that bred successfully in the 1999–2000 mating season showed a series of spikes that tripled his baseline values, as did the other two males (28-7 and 24-35) at the SDWAP, and one of the ZNE males (93A780). In contrast, a ZNE male (97A465) and the KCZ male

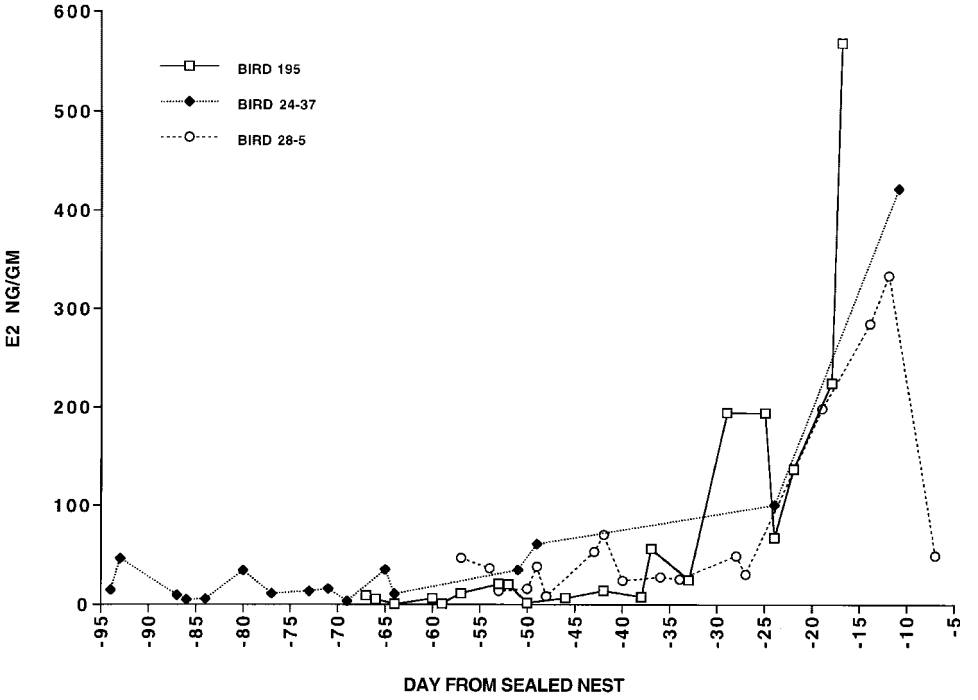


Fig. 1. Estradiol profiles of individual egg-laying female great hornbills.

(264) showed essentially flatline testosterone values and did not show the same series of testosterone spikes exhibited by the other males in the study.

The testosterone levels for the female hornbills in the study did not show any identifiable pattern.

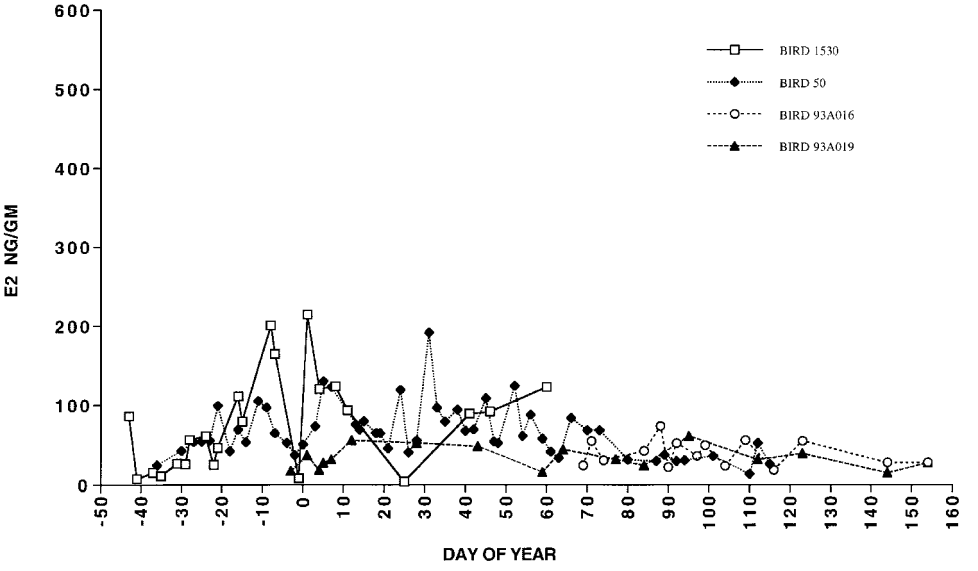


Fig. 2. Estradiol profiles of individual non-egg-laying female great hornbills.

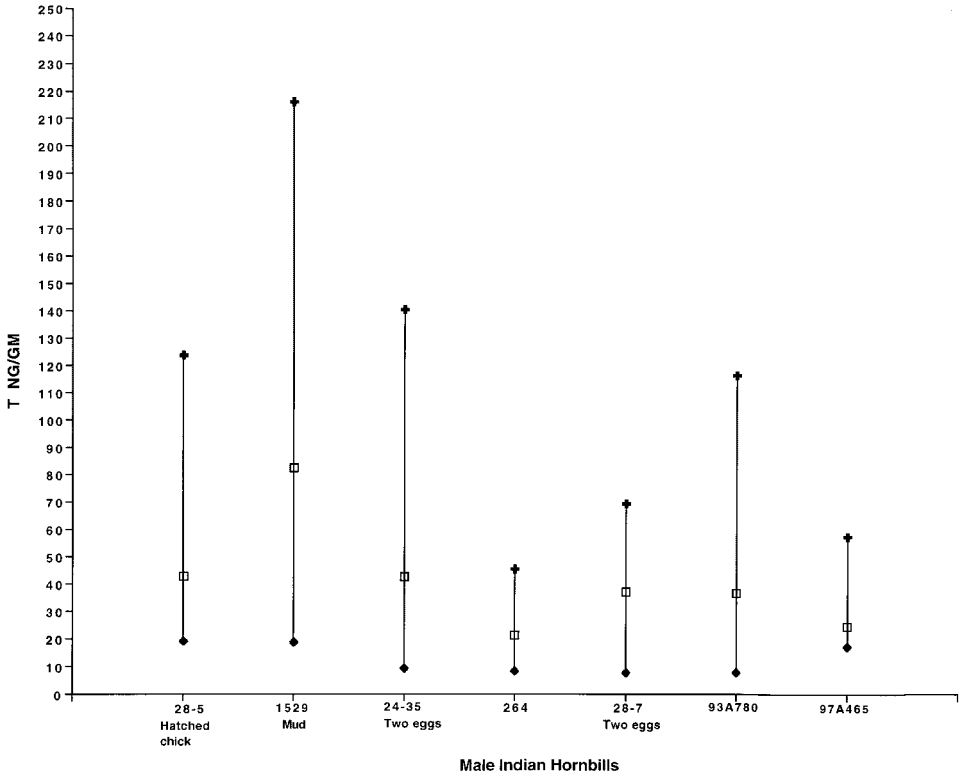


Fig. 3. Ranges of testosterone values of individual male great hornbills. Mean, open box; high value, cross; low value, diamond.

## Corticosterone

Two of the SDWAP females (28-5 and 24-37) had uniformly low corticosterone (means: 29.44 ng/gm and 28.19 ng/gm). In contrast, females 195, 93A016, and 93A019 had mean corticosterone values $>50$ ng/gm. However, a one-way ANOVA showed there was no significant difference among individuals at the four different institutions ($F_{(3,3)}=2.727$, $P=0.2159$). It is interesting to note that the corticoid concentrations fluctuated rapidly and dramatically in females that did not lay eggs—peaking and then falling back into normal range before peaking again. Female 93A019 registered the highest corticosterone level in the study, with a mean value of 251.83 ng/gm and a peak concentration of 850 ng/gm, 17 times the highest value measured in the female that reproduced.

When grouped according to reproductive status (hatched a chick: n=1; laid eggs, no chick: n=2; did not lay: n=4), the mean corticosterone values for the female hornbills increased with decreasing reproductive success. The female that hatched a chick had the lowest mean corticosterone, while the mean of the means for the egg-laying females was less than that for the non-egg-laying females (Fig. 4). While there was no significant difference between the corticosterone concentrations of females that laid an egg, hatched an egg, or were non-laying, there was a trend toward

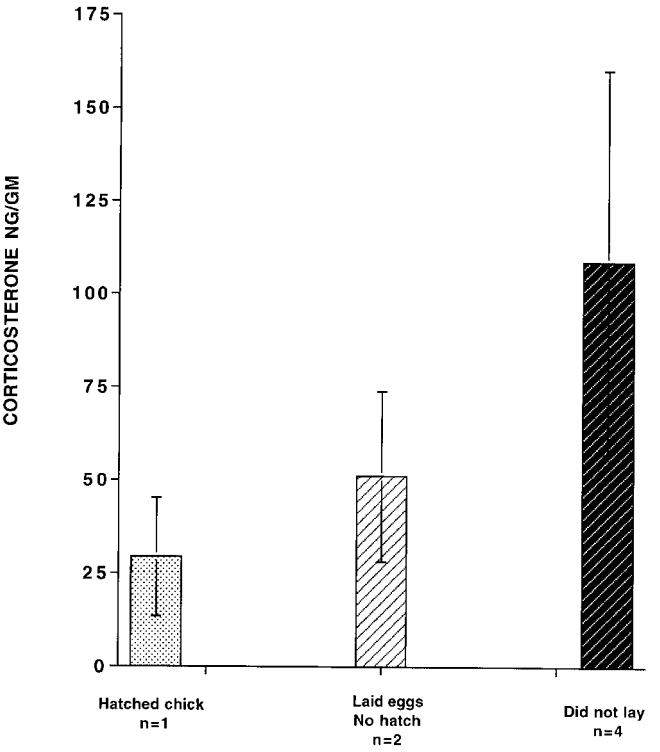


Fig. 4. Mean ± SEM corticosterone values of female great hornbills grouped by reproductive condition.

significance in the correlation of estradiol and corticosterone (Pearson r=0.647, $P$=0.116).

As with the females, the corticosterone levels of the males at SDWAP were lower than any other males in the study, with a baseline value of 25 ng/gm and no mean corticosterone value >50 ng/gm. However, there was no significant difference among the zoos and the corticosterone concentrations (F(3,3)=2.256, $P$=0.2607). Although the other males in the study (1529, 264, 93A780, and 97A465) showed baseline levels similar to those of the SDWAP males, they also had periods of elevated corticosterone (>100 ng/gm).

A one-way ANOVA comparing the mean corticosterone values of males with laying mates and those with non-laying mates was statistically significant F(1,6)= 8.087, $P$=0.036 (Fig. 5). A correlation between testosterone and corticosterone levels in the mates of laying and non-laying females was not statistically significant.

## DISCUSSION

The estradiol profile of the female hornbill that hatched a chick during the 1999–2000 breeding season was remarkably similar to those of the other two females in the study that laid eggs. All had dramatically elevated estradiol levels in the 3 weeks prior to being sealed into their nests, and significantly higher levels than

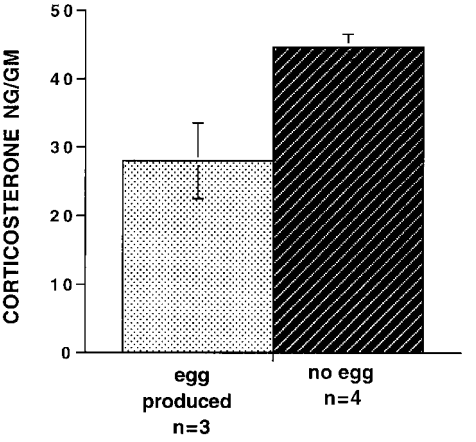


Fig. 5. Mean ± SEM corticosterone values of male great hornbills grouped by reproductive condition.

non-egg-laying females. The estrogen profile of the hornbill is prolonged compared with the fecal estrogen profile of the canary, in which estrogen elevation begins 10 days prior to egg laying [Sockman and Schwabl, 1999].

Apart from the three females at the SDWAP, only one other female, housed at the Denver Zoo, demonstrated courting/mating behavior or was sealed into her nest cavity. The estradiol profile of the female hornbill at the Denver Zoo did not show the dramatic elevation of the SDWAP females, but did have a moderate rise in estradiol levels before nesting. This is interpreted as a positive sign for her future breeding potential, and based on the results of this study she has been sent to the Audubon Zoo in New Orleans, where it is hoped the warmer climate and natural photoperiod will help lead to successful breeding in the future. None of the remaining three females in the study showed any noticeable rise in estradiol levels over the study period, and none displayed courtship or mating behavior.

The testosterone profiles of the males and females showed no significant results. The mean testosterone values of the mates of laying and non-laying birds were not significant, nor was the correlation between testosterone and corticosterone. Before any conclusions can be drawn about the testosterone profiles of great hornbills in relation to breeding biology, further studies, with concurrent data collection and greater sample size, are needed.

An interesting aspect of the current study is the corticosterone levels. Four of the six birds (28-5, 28-6, 24-37, and 28-7) had uniformly low corticosterone levels compared to the other hornbills, and the pair (28-5 and 28-6) that successfully hatched a chick had the lowest corticosterone levels of any birds in the study. In general, bird pairs that lay eggs have lower corticosterone concentrations than non-laying bird pairs, although there was no effect of sex by location. This indicates that increased corticoid levels may play some role in the reproductive failure of the great hornbills. While stress is routinely associated with elevated corticoid concentrations, there can be variable factors associated with these increased hormone levels. A correlation between estradiol and corticosterone for all females in the study showed

a high negative correlation (high corticosterone, low estradiol), nearing significance, which supports the idea that elevated corticoids may be associated with suppressed reproduction.

One of the SDWAP females (195) that laid eggs had elevated corticosterone, with levels in the range of the non-laying birds, and the mate of a female that laid eggs (24-35) also had elevated corticosterone. In addition, the KCZ male that received extreme aggression from his mate, necessitating their separation at the end of the study period, showed only moderately elevated corticosterone levels compared to other males in the study. Monitoring corticosterone levels and working to minimize any environmental stress experienced by great hornbills may be vital in efforts to increase their reproductive success in captivity.

## CONCLUSIONS

1. The estradiol profiles of female hornbills that laid eggs showed a dramatic rise prior to their being sealed into the nest, compared to the estradiol profile of the birds that did not lay an egg.

2. Corticosterone levels were uniformly lower in the pair of hornbills that hatched a chick in the 1999–2000 breeding season, and higher in non-laying pairs.

## ACKNOWLEDGMENTS

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