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Source: Journal of Wildlife Diseases, 34(4) : 715-721

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-34.4.715

Journal of Wildlife Diseases, 34(4), 1998, pp. 715–721

SERUM CORTICOSTERONE RESPONSE TO ADRENOCORTICOTROPIC HORMONE STIMULATION IN FLORIDA SANDHILL CRANES

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ABSTRACT: Florida sandhill cranes (*Grus canadensis pratensis*) were conditioned to confinement in an enclosure for 7 days, 6 hr a day. On day 8, cranes were catheterized and then confined in an enclosure. Venous blood (2 ml) was collected through the catheter and an attached IV line immediately before (−60 min) and 60 min after (0 min) confinement. Using a randomization table and a restricted cross-over experimental design, cranes were injected intravenously with either saline (control) or adrenocorticotropic hormone (ACTH; cosyntropin, Cortrosyn®; 0.25 mg). At 30, 60, 120, 180, 240 and 300 min after injection, blood samples were collected and assayed for corticosterone. The cranes receiving ACTH increased their serum corticosterone concentrations as much as fivefold above baseline concentrations. Serum corticosterone concentrations remained significantly elevated for approximately 60 min after ACTH stimulation. Physical restraint and catheterization caused an increase in serum corticosterone almost comparable to that induced by ACTH stimulation. In cranes injected with saline, serum corticosterone decreased within 1 hr after physical restraint and catheterization, and remained at lower levels throughout the remaining 5 hr of confinement.

Key words: ACTH, ACTH stimulation test, adrenocorticotropic hormone, corticosterone, *Grus canadensis pratensis*, sandhill cranes.

INTRODUCTION

The single best hormonal indicator of stress in birds is the glucocorticoid, corticosterone (Harvey et al., 1980, 1984; Harvey and Hall, 1990; Scanes et al., 1980; Wingfield et al., 1992). In birds, corticosterone significantly increases following a variety of stressful events (stressors) including crowding, extremes of temperature, treadmill exercise, starvation, and handling, restraint and bleeding (Harvey et al., 1980, 1984; LeMaho et al., 1992; Le Ninan et al., 1988; Okwusidi et al., 1991; Scanes et al., 1980; Siegel, 1980; Wingfield et al., 1992). Corticosterone could serve as a natural marker for monitoring stress in birds except that collecting blood for measuring serum corticosterone is itself a known stressor and limits the usefulness of serum corticosterone as a measure of stress. For this reason, studies of stress in birds have developed techniques to minimize or eliminate unwanted effects of experimental manipulations on serum corticosterone concentrations (Harvey et al., 1980; Le Maho et al., 1992).

In preparation for studies of acute and chronic stress in cranes, this study was undertaken to assess the effect of a method of confinement and blood collection on serum corticosterone, and to determine if adrenocorticotropic hormone (ACTH) stimulation reliably increases serum corticosterone concentration above baseline levels in sandhill cranes (*Grus canadensis*).

METHODS AND MATERIALS

This study was approved by the Institutional Animal Care and Use Committee of the International Crane Foundation (ICF; Baraboo, Wisconsin, USA). Ten captive-reared adult Florida sandhill cranes (*Grus canadensis pratensis*), housed at the International Crane Foundation (43°30′N, 89°42′W) were used in this study from 21 October 1996 through 21 January 1997. The cranes, 5 males and 5 fe-

males, had a median age of 21 yr (range 2 to 27 yr) and a median weight of 5.0 kg (range 4.2 to 6.0 kg). All cranes were healthy at the time of study as determined by physical examination, and hematological and biochemical profiles. The cranes were mated pairs used in ICF's captive breeding program; the pairings were maintained throughout the study.

Throughout the study the standard ICF feeding, watering and cleaning routines were completed between 0800 and 0900 hours. The cranes were given free access to food (Garver Crane Grower—Maintenance; Garver Feed and Supply Co., Inc., Madison, Wisconsin, USA) and water. The amount (grams) of food consumed by each pair of cranes was measured daily for the duration of the study. Each pair was housed so that they had free access to indoor and outdoor runs and an exercise yard. All cranes were exposed to natural day and night light cycles, and ambient temperatures. Day length ranged from 10 hr 53 min to 9 hr 31 min on 18 October 1996 and 21 January 1997, respectively. Outside temperatures ranged from 16 C to 1.5 C. Temperatures in the indoor runs ranged from 14 C to −3 C. The area in which the studies were conducted was lighted with fluorescent lights and the temperature in this area during the studies ranged from 11 to 16 C.

The order in which crane pairs were studied was randomized using a randomization table and a restricted cross-over design so that cranes were equally allocated by gender to one of two treatments as first treatment: a saline control group or an ACTH treatment group. This approach was used to create a balanced sample size to enable us to evaluate the within-crane effects of both treatments without serious confounding by treatment order.

Each pair of cranes was studied for 9 consecutive days—7 days for acclimation to confinement in a wooden enclosure and 1 day each for treatment with saline and ACTH. Each crane was acclimated to confinement in an enclosure for 6 hr per day (0900 to 1500 hours, Central Standard Time—CST) for 7 consecutive days. Each crane was captured by hand in an indoor run, weighed, and an intravenous line (Medex, Inc., Duluth Georgia, USA; length 152 cm) was taped to the right side of the neck approximately 4 cm from the angle of the jaw. The crane was placed in a wooden enclosure measuring 38 cm wide × 122 cm long × 114 cm deep. For air circulation and ventilation, both sides of each enclosure had openings 38 cm × 23 cm that were covered with wire mesh and sight barrier screening. The floor of each enclosure consisted of wire mesh (4 × 4, 23 gauge galvanized wire mesh) through which fecal matter could fall. The top of each enclosure consisted of 2 × 2 plastic mesh that was affixed to a centrally positioned piece of wood that extended the full length of the enclosure and that had a centrally located slot extending for most of its length; the free end of the intravenous line was passed through the slot to the outside of the enclosure. Approximately 300 g of food was made available to each crane in its enclosure. At the end of each day, both cranes were removed from their enclosures, the bandage and IV lines were removed, and the cranes were returned to their housing unit.

On days 8 and 9, the protocol, as outlined above, was followed except that each crane was catheterized with a catheter (18 gauge, 5.1 cm; Angiocath, Becton Dickinson, Sandy, Utah, USA) inserted into the right jugular vein. A jugular venous blood sample (2 ml) was collected (−60 min) after which an intravenous line (152 cm long) pre-filled with heparinized saline (heparin 4μ/ml; Elkins-Sinn, Inc., Cherry Hill, New Jersey, USA) was attached to the catheter. The catheter and IV line were used for collecting samples of jugular venous blood and for giving ACTH and saline treatments.

Blood samples (2 ml) were collected 60 min after the start of confinement (0 min), and at 30, 60, 120, 180, 240, and 300 min after injection of saline and ACTH. All blood samples were allowed to clot in test tubes and then were refrigerated until the end of the day when they were centrifuged, and the serum collected and stored at −20 C. Over the 2 days of the study, a total of 32 ml of whole blood was withdrawn from each crane (approximately 11% of blood volume).

On day 8, immediately after collecting the second blood sample (0 min), one crane was injected intravenously with Cortrosyn® (cosyntropin, 0.25 mg solubilized in 1 ml of normal saline; Organon Inc, West Orange, New Jersey, USA) that was flushed through the IV line and catheter with normal saline (2 ml) followed by a heparinized saline flush (1.5 ml). The other crane was injected intravenously with saline solution (0.9% sodium chloride) equal in volume to that of the ACTH injection and saline flush (3 ml) followed by heparinized saline (1.5 ml). The treatments were switched on day 9 so that each crane served as its own treatment control.

All serum samples were shipped on dry ice to the Center for the Reproduction of Endangered Species (CRES; Zoological Society of San Diego, San Diego, California, USA) for analysis of corticosterone concentrations which were determined by RIA using an antiserum to corticosterone-3-carboxymethyl-oxime:BSA (Radioassay systems Laboratories, Carson, Cal-

ifornia, USA), and tritiated corticosterone (NEN Dupont, Boston, Massachusetts, USA) which was competed against standard concentrations of corticosterone (Siga, St. Louis, Missouri, USA). Cross-reactivities of this antiserum are reported by the manufacturer as 100% corticosterone, 6.1% desoxycorticosterone, 0.29% progesterone, 0.19% cortisol, and other steroids <0.10%. Serum (0.05 ml) was extracted with 5 ml anhydrous diethyl ether. The extract was dried with filtered air, resolubilized in pH 7.0 phosphate buffer (0.5 ml), and then assayed. Charcoal dextran solution was used to separate bound and free label. Coefficient of variation for this assay was 9.7%.

A two-sided Wilcoxon signed-rank test was used to determine if serum corticosterone for the saline treatment group differed significantly from the ACTH treatment group at time −60 min. To address the question of whether there was any change in serum corticosterone after treatment with saline, each crane's serum corticosterone concentrations were regressed on time (actual minutes since saline injection). The slopes so derived were used in a one-sample, two-sided t-test to test if the mean slope equaled 0.0. A one-sided Wilcoxon's signed-rank test was used to test whether the serum corticosterone concentrations at 0 min were less than the concentrations at −60 min (within treatment). A non-parametric paired-data test was used because summary statistics of the data and visual inspection of histograms indicated that the data were right-skewed. The question of whether the changes from the serum corticosterone concentrations at 0 min to the serum corticosterone concentrations at 30, 60 and 120 min differed significantly between treatments was addressed first by calculating each crane's changes from 0 min (within treatment). Using Wilcoxon's two-sided signed-rank test, these changes then were compared between treatments, but still paired within crane. Statistical analyses were performed with a commercially available software program (STATISTIX for Windows; Version 1996; Analytical Software, Tallahassee, Florida, USA). Significance was assumed at $P \leq 0.05$ and trends were considered significant at $P \leq 0.10$.

RESULTS

Complete data describing the serum corticosterone response to ACTH and saline treatments were obtained from eight cranes (Table 1). Loss of serum corticosterone data at some time points for the eight cranes was due to catheter failures. One female was removed from the study 4 days into the acclimation period when she developed subcutaneous emphysema probably as a result of trauma to the head of her left humerus. Because of catheter failures, blood samples could not be drawn from one male during either treatment period. However, data from these two cranes are included in the analyses of food consumption because they remained paired with their respective mates throughout the study period, and it was not possible to distinguish food consumption by individual birds when they were outside of the enclosures.

The serum corticosterone concentrations for the saline and ACTH treatments differed significantly ($P = 0.04$; 2-tailed) at −60 min, but not at 0 min after the cranes were confined in the enclosures. There were no sex-related differences in corticosterone concentration within groups at −60 and 0 min (all $P \geq 0.11$), nor did treatment order (by day) affect serum corticosterone concentrations at −60 and 0 min (both $P \geq 0.37$). For both treatments, serum corticosterone concentration decreased significantly (both $P = 0.004$; 1-tailed) from −60 min to 0 min; the median decreases were 17.1 and 12.4 ng/ml for saline and ACTH, respectively. Following intravenous injection with saline, serum corticosterone did not vary significantly ($P = 0.44$ from the test for slope) for the remainder of the confinement period. After intravenous injection of ACTH, serum corticosterone concentrations were increased significantly at 30 and 60 min when compared to corticosterone concentrations at 0 min (Table 1), and when compared to the saline measurement at the comparable times (all $P \leq 0.02$ versus Saline; 1-tailed and adjusted for the values at 0 min). The peak corticosterone response to ACTH treatment occurred approximately 60 min after the injection of ACTH (Fig. 1).

Reaction to injection of ACTH was variable from bird-to-bird. The response of six cranes was unremarkable while two cranes regurgitated undigested food within 15

TABLE 1. Serum corticosterone concentrations (ng/ml) in Florida sandhill cranes before (−60 and 0 min) and after (30 to 300 min) treatment with saline and ACTH.

	Min							
	−60	0	30	60	120	180	240	300
Saline treatment (observed corticosteroid values)								
n	8	8	8	8	8	8	7	7
Minimum	7.8	1.1	1.0	2.3	2.3	2.1	1.9	1.7
Median	20.1	2.6	3.0	4.8	4.0	3.3	3.2	2.8
Maximum	25.2	6.4	7.4	7.2	6.9	7.6	9.7	5.2
ACTH treatment (observed corticosteroid values)								
n	8	8	7	6	6	6	6	5
Minimum	5.0	1.0	16.3	21.3	6.1	1.2	1.0	1.7
Median	13.8	1.9	21.4	25.2	10.7	1.5	1.8	3.4
Maximum	20.0	4.8	27.6	28.0	25.2	15.3	6.9	9.0
ACTH-associated changes in serum corticosterone compared to saline measurements at analogous times								
n	8	8	7	6	6	6	4	5
Minimum	−15.8	−5.4	12.7	19.0	0.0	−4.1	−2.7	−1.9
Median	−3.5	−0.6	18.2	20.4	5.7	−2.2	−0.6	0.6
Maximum	1.4	2.3	25.1	22.7	21.8	12.6	0.6	7.3
P[a]	0.04	0.23	0.02	0.04	0.06	0.40	0.58	0.79
ACTH-associated increase in serum corticosterone over measurement at 0 min on ACTH treatment day								
n	—	—	7	6	6	—	—	—
Minimum	—	—	14.9	20.3	5.1	—	—	—
Median	—	—	18.6	22.3	8.4	—	—	—
Maximum	—	—	25.1	27.0	22.4	—	—	—

[a] = H_0: increase = 0.0; H_A: increase ≠ 0.0.

min of receiving the ACTH. One of these two cranes made an uncharacteristic long low growl and briefly became agitated as indicated by feather ruffling and pacing in the enclosure. One other crane became agitated after receiving the ACTH. None of the three cranes were anemic. All signs of reaction to ACTH were gone by 20 min after injection of the drug. None of the birds reacted to injection with saline.

Behavioral responses to confinement in the enclosures varied from crane to crane. In general, over the course of the 7 day acclimation period, all cranes became calmer as evidenced by less pacing within the enclosures, decreased vocalization, and less pecking at the walls of the enclosures. However, two cranes (B2 and C1) paced incessantly during the initial days of the acclimation period, thus abrading their feet which bled. Their digits were bandaged and the wire mesh floor was covered with carpeting to protect them from further injury. On day 9, both cranes were anemic with hematocrits of 26% and 28% for crane B2 and crane C1, respectively. For five other cranes at the end of the study on the ninth day, the median hematocrit was 39% (range 38 to 48%). Corticosterone response of the anemic cranes to saline treatment did not differ significantly ($P \geq 0.86$) at 0 min compared to the other cranes, nor did the corticosterone response of the anemic cranes during the ACTH treatment period differ significantly ($P \geq 0.43$) at 0, 30 and 60 min compared to the other cranes.

For each pair of cranes, food consumption decreased significantly ($P = 0.04$) from a median of 495 g (range 437 to 575 g) during the day preceding confinement to a median of 95 g (range 0 to 118 g) during the first day of confinement. Over the 9 days of confinement, food consumption tended to increase but varied between crane pairs. There was a significant ($P =$

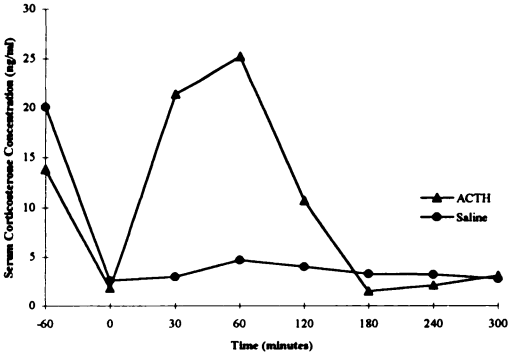

FIGURE 1. Median circulating serum corticosterone in Florida sandhill cranes at T-60 and T0 (n = 8 per period) and following intravenous injection with 0.9% saline or ACTH (cosyntropin) at T30 (saline, n = 8; ACTH, n = 7), T60 (saline, n = 8; ACTH, n = 6), T120 (saline, n = 8; ACTH, n = 6), T180 (saline, n = 8; ACTH, n = 6), T240 (saline, n = 7; ACTH, n = 5), and T300 (saline, n = 7; ACTH, n = 6).

0.03) change in body weight for the cranes as a group when body weights on day 1 were compared to body weights on day 9. Two cranes had no change in weight, and six cranes lost weight.

DISCUSSION

The higher corticosterone concentrations in the cranes at −60 min compared to concentrations at 0 min probably reflect the effects of handling, restraint and catheterization, all of which are known stressors for birds (Harvey et al., 1980, 1984; Scanes et al., 1980; Webb and Mashaly, 1984; Beuving and Vonder, 1986; LeMaho et al., 1992; Spellman et al., 1995). The metabolic half-life of corticosterone in ducks is about 10 min (Harvey et al., 1980) which, in the absence of additional stressors for these cranes, may explain the rapid decline in corticosterone concentration from −60 min to 0 min. Studies in geese (LeMaho et al., 1992) and black ducks (*Anas rubripes*) (Spellman et al., 1995) have shown that serum corticosterone returns to baseline levels within 30 to 90 min after exposing birds to stressors such as handling and injection with saline.

At −60 min there was a significant ($P \leq$ 0.04) difference in serum corticosterone concentrations between when the cranes were treated with saline and when they were treated with ACTH, but there is no readily apparent explanation for this difference. Treatment order (by day) and gender did not have a statistically significant effect on serum corticosterone concentrations, and there was no difference in corticosterone concentration between the two treatments at 0 min nor at 180, 240 and 300 min after saline and ACTH injections.

The lower corticosterone concentration at 0 min indicates that the cranes had adapted both to confinement and to catheterization. This conclusion is strengthened by the fact that serum corticosterone after saline treatment remained at this lower concentration and did not vary significantly throughout the remainder of the study period.

Avian ACTH contains 39 amino acids and differs from its mammalian homolog at positions 15, 27, 28, 29, 31 and 32 (Scanes, 1986). Cosyntropin is an open chain polypeptide that contains the first 24 of the 39 amino acids of naturally occurring ACTH, a feature that reduces its antigenicity while preserving its corticosteroidogenic activity (Package insert, Organon Inc., West Orange, New Jersey, USA). The dose of ACTH used in this study was selected based on data from other studies of birds (Lumeij et al., 1987; Spellman et al., 1995; Zenoble et al., 1985b), and took into consideration the weight of the cranes, the dose-dependent potential for allergic reactions, and the likelihood of achieving a measurable and significant serum corticosterone response. In these cranes, the intravenous administration of cosyntropin (0.25 mg) caused a fivefold increase in serum corticosterone by 60 min after injection with a return to approximately baseline concentrations 180 min after drug administration. In general, the pattern and magnitude of response to ACTH stimulation seen in these cranes is similar to that reported in other avian species, including chickens (Beuving and Von-

der, 1986), ducks (Harvey et al., 1980; Spellman et al., 1995), pigeons (Lumeij et al., 1987), psittacines (Lothrop et al., 1985; Zenoble et al., 1985a), and condors and eagles (Zenoble et al., 1985b). Depending on dose and route of administration, ACTH causes a peak corticosterone response within 30 to 60 min after injection with a return to baseline levels within 120 to 240 min after injection (Beuving and Vonder, 1986; Lothrop et al., 1985; Spellman et al., 1995).

Over the 2-day treatment period, a total of approximately 32 ml of whole blood was withdrawn from each crane, approximately 11% of their circulating blood volume (Morton et al., 1993). The smallest crane, a female, weighed 3.8 kg on days 8 and 9; the volume of blood withdrawn from her over the 2 days represented 14% of her estimated blood volume. This female also was anemic as a result of pacing in the enclosure and abrading her feet on the wire mesh flooring. The other anemic crane was a male that weighed 5.0 kg during the 2 days of the study. Despite their anemia and the volume of blood that was withdrawn over the 2 days, their serum corticosterone response to ACTH did not differ from those of the other cranes at any time (all $P \geq 0.41$; two-sided). In addition, within the saline treatment group, the amount of variation in serum corticosterone concentration in these two cranes was no different from that seen in the other cranes.

The order (by day) in which the two treatments were administered had no effect on serum corticosterone concentrations at −60 and 0 min. This suggests that there was no carry-over effect from ACTH stimulation. Naturally occurring ACTH, at least in ducks (Harvey et al., 1980), has a metabolic half-life of about 10 min. If the synthetic ACTH (cosyntropin) used in this study has a similarly short metabolic half-life, this could explain the lack of carry-over effect from one day to the next.

Food consumption in these cranes varied from pair-to-pair. In general, food consumption for all pairs decreased significantly during the first few days of confinement. Starvation is a known stressor for birds and it significantly increases the concentration of circulating serum corticosterone (Harvey et al., 1980; Harvey and Hall, 1990; Rees et al., 1985; Siegel, 1980). In addition, birds fed intermittently have chronically elevated corticosterone levels compared to birds fed *ad libitum* (Rees et al., 1985). The initial significant decrease in food consumption measured in these cranes probably reflects that confinement was an important stressor. The rate at which food consumption increased after the start of confinement probably indicates how well each pair adapted to confinement. Another possible cause of the reduced food consumption may relate to the fact that cranes eat during daylight hours (Meine and Archibald, 1996). As day length decreased throughout the period of study so too would the time available for eating when the cranes were not in the enclosures. Although food was made available to each crane while confined in an enclosure, the amount of food consumed varied from crane to crane and from day to day. However, the reduced food consumption did not affect the stability of the serum corticosterone concentration in the saline treatment period over 5 hr of confinement. In addition, ACTH stimulation caused a significant increase in circulating serum corticosterone, indicating that the cranes were able to respond to a novel stressor.

In summary, ACTH stimulation caused a significant increase in serum corticosterone in these sandhill cranes. The method of acclimating the cranes to confinement and confinement itself, resulted in stable concentrations of serum corticosterone over a 5 hr period as reflected by the saline treatment group. Furthermore, the confinement and blood collection methods used in this study made it possible to detect the effect of adrenocorticotropic hormone stimulation on serum corticosterone concentration. Thus, the techniques used

in this study for confining, acclimating and collecting blood are an effective means by which to study stress-related hormones, such as corticosterone, in individual cranes.

ACKNOWLEDGMENTS

This project was supported by a Conservation and Research Grant from the Zoological Foundation of San Diego, San Diego, California. The support and generous assistance of the International Crane Foundation and its staff are appreciated.

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Received for publication 27 August 1997.