1 Female gonadal hormones and reproductive behaviors as key determinants of
2 successful reproductive output of breeding Whooping Cranes (*Grus americana*)

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Abstract

Reproductive success of endangered whooping cranes (*Grus americana*) maintained *ex situ* is poor. As part of an effort to identify potential causes of poor reproductive success in a captive colony, we used non-invasive endocrine monitoring to assess gonadal and adrenal steroids of bird pairs with various reproductive outcomes and evaluated the relationships of hormones and behaviors to reproductive performance.

Overall, reproductively successful (i.e., egg laying) females had significantly higher mean estrogen levels but lower mean progestagen concentrations than did unsuccessful females. Other hormones, including glucocorticoids and androgens, were not significantly different between successful and unsuccessful individuals. Observations of specific behaviors such as unison calling, marching, and the number of copulation attempts, along with overall time spent performing reproductive behaviors, were significantly higher in successful pairs. Our findings indicate that overall reproductive performance of whooping crane pairs is linked to female gonadal hormone excretion and reproductive behaviors, but not to altered adrenal hormone production.

Keywords: Whooping Crane, Reproduction, Endocrinology, Glucocorticoids, Behavior, Egg Laying

1. Introduction

Whooping cranes (*Grus americana*) are listed as endangered in both the United States and Canada through the U.S. Endangered Species Act and the Canadian Species at Risk Act, respectively. Currently there are more than 400 cranes living in the wild in four distinct populations, three of which were established through the release of captive-bred birds (Butler *et al.*, 2013; Harrell, 2014; Moore *et al.*, 2012; Urbanek *et al.*, 2014). These reintroduced populations are not self-sustaining and lack positive growth rates (Moore *et al.*, 2012; Servant *et al.*, 2014). Due to low overall population size and continued threats to wild birds, a goal of the whooping crane recovery plan is to sustain a population of whooping cranes *ex situ*, for two purposes: first as a repository for retaining genetic diversity and insuring continued existence of the species, and second as a source of birds for reintroduction programs (CWS and USFWS, 2009).

Although an average of 19 chicks have been produced and released into reintroduced flocks each year, reproductive success in the captive flocks has been poor compared with wild populations (J. Chandler, Unpublished results; Kuyt, 1995), and is apparently limited by the rate of production of fertile eggs. At Patuxent Wildlife Research Center (PWRC), the largest captive breeding center, the average fertility rate is <60%, with 89% of fertile eggs successfully hatching (Smith *et al.*, 2011). By comparison, nests examined in the Aransas-Wood Buffalo population (AWBP) from 1967-1995 had an average fertility rate of 95%, and 73% of fertile eggs (conditional on the presence of a live embryo at the time of examination) hatched (Kuyt, 1995). Furthermore, captive birds at all centers experience delayed onset of reproduction, especially in females (7 years vs. 5 years *in situ*; Miranda *et al.*, 1996). Because a primary objective of the captive

breeding program is to maximize chick production for reintroductions, it is important to better understand basic reproductive biology of whooping cranes and to identify causes of poor reproduction in *ex situ* birds.

Whooping cranes are seasonal breeders (Archibald and Lewis, 1996; Gee and Rossman, 1996). At PWRC, the breeding season of whooping cranes begins in mid-March and continues through June, with the majority of eggs laid in April and May (Mirande et al., 1996). Reproductive behaviors are first observed 2 to 5 weeks before the onset of egg production (Swengel *et al.*, 1996).

To date, there is little information on whooping crane reproductive endocrinology. Studies in other avian species have shown that at the onset of the breeding season the hypothalamus is stimulated by increased daylight, triggering the release of gonadotropin-releasing hormone (GnRH; Leska and Dusza, 2007). Under the influence of GnRH, the anterior pituitary gland secretes follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate follicle development in females, spermatogenesis in males, and the production of gonadal hormones: **estrogen and progesterone in females and testosterone in males** (Leska and Dusza, 2007; Ottinger and Baskin, 1995). In female birds, small follicles produce estrogen, and as the follicles grow progesterone production begins, peaking just before ovulation (Liu *et al.*, 2001; Yang *et al.*, 1997). In males, the Leydig cells are stimulated by LH to produce testosterone (Leska and Dusza, 2007).

Increased adrenal glucocorticoid production has been shown to reduce synthesis and secretion of gonadotropins that, in turn, compromise reproduction (e.g., acyclicity, infertility, and high neonatal mortality) in an array of species, such as the white

rhinoceros (*Ceratotherium simum*; Carlstead and Brown, 2005) cheetah (*Acinonyx jubatus*; Jurke *et al.*, 1997) and maned wolf (*Chrysocyon brachyurus*; Reiter, 2012). In the cheetah, high levels of corticosteroid metabolites are associated with low ovarian activity (Jurke *et al.*, 1997). This relationship between elevated glucocorticoid concentrations (corticosterone in birds) and a change in the hypothalamo-pituitary-gonadal axis has also been documented in avian species. A study in wild white-crowned sparrows (*Zonotrichia leucophrys*) reported an increase in plasma corticosterone level along with a decline in LH and dihydrotestosterone when birds were moved into captivity as compared to initial levels observed at capture (Wingfield *et al.*, 1982). As birds acclimated to captivity, corticosterone levels decreased and LH and DHT levels returned to initial levels. Ouyang *et al.* (2011) showed that free living house sparrows (*Passer domesticus*) with elevated levels of corticosterone prior to the breeding season exhibited a delay in egg laying and produced fewer eggs and fledglings compared to individuals with low corticosterone levels. Corticosterone also has a large influence on parenting behaviors; studies of king penguins (*Aptenodytes patagonicus*; Groscolas *et al.*, 2008) and black-legged kittiwakes (*Rissa tridactyla*; Angelier *et al.*, 2009) showed that individuals with high glucocorticoid levels were more likely to abandon nests and eggs.

The objectives of the present study were to compare gonadal and adrenal hormones as well as reproductive behaviors between successful and unsuccessful whooping crane pairs. We hypothesized that low reproductive success in cranes housed at PWRC was caused by increased adrenal activity that, in turn, disrupted gonadal steroid excretion and decreased reproductive behaviors.

2. Methods

2.1. *Pair Selection*

Study pairs were selected from 17 available pairs maintained at PWRC. Pairs were chosen conditional on the flock manager's assessment that the pair had a suitable temperament: pairs were not considered if highly nervous or aggressive during the breeding season, or if they consistently broke their own eggs. Based on historical fertility, all suitable pairs were divided into one of three classes: class 1: 100-50% egg fertility; class 2: <50-0% egg fertility; class 3: non-layers. Two pairs were chosen randomly from each class for inclusion in the study. During the course of this study, a male in one of the class 2 pairs died (B16); therefore, a new pair of similar fertility was selected in Year 2 (S25; Table 1).

All pairs were housed in outdoor pens, ~13.7 m wide by 19.8 m long. Pairs were housed with or without flight netting based on wing condition, and with or without photoperiod lights, as summarized in Table 1. Cranes were fed a pelleted breeder diet (produced by Republic Mills, Okolona, OH; recipe provided by Swengel and Carpenter, 1996; any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government) in a gravity feeder and provided water *ad libitum*. All work associated with this study was performed with approval of Animal Care and Use Committees at USGS-PWRC (2009-08), Smithsonian Conservation Biology Institute (10-11), and University of Maryland (351893-2).

2.2. *Sample Collection*

Non-invasive monitoring was used to assess gonadal and adrenal fecal hormone metabolites during two breeding seasons for five pairs and one season each for two

additional pairs, totaling 12 reproductive cycles. Non-invasive hormone monitoring was utilized instead of blood sampling to prevent undue stress to the birds during a critical time while still enabling longitudinal sampling across the breeding season. Fecal samples were collected from each individual three times per week from February 1st, approximately 6 weeks before the start of the breeding season, through July 1st, approximately 6 weeks after the end of the breeding season. Chromic oxide (Cr_2O_3) or iron oxide (Fe_2O_3) in capsules was delivered by hand in lake smelt (*Osmerus mordax mordax*) the day before collection to identify an individual's fecal samples within a pair (i.e., male vs. female) by color (Brown *et al.*, 2014). Samples were collected at approximately the same time of day (mid-morning) using a 7.6x12.7 cm plastic zip-lock bag and a wooden tongue depressor, removing the white urate portion when possible (Hayward *et al.*, 2010) and then stored at -20°C until hormone extraction and analysis (see below). Samples were not collected if it was deemed too stressful or dangerous for the birds (e.g., just after the pair laid an egg or while the pair was incubating live eggs).

2.3. Hormone Extraction and Analysis

Hormone extraction was performed using previously described methods with slight modifications (Wasser and Hunt, 2005; Johnson *et al.* 2014). Crane fecal samples were freeze-dried and pulverized. For steroid hormone extraction, 0.2 ± 0.08 g of pulverized sample was mixed with 5 mL of 70% ethanol (EtOH; 70:30 deionized water), then vortexed for 30 s, and vigorously shaken for 30 min. The samples were then centrifuged (500g for 20 min), and the supernatant poured off into a clean glass tube. Another 5 mL of 70% EtOH was added to the fecal pellet and samples were again

vortexed and centrifuged. The supernatants were pooled and dried under an air stream. Once dried, the extract was resuspended in 1 mL of phosphate assay buffer (0.2 M NaH_2PO_4 , 0.2 M Na_2HPO_4 , 0.15 M NaCl; pH 7.0), vortexed, and stored at -20°C until hormone analysis. A mean extraction efficiency ($\pm$ SE; $84.7 \pm 6.5\%$ recovered hormone, total sample number = 1,301) was determined by assessing the recovery of radiolabeled ^3H -cortisol added before hormone extraction.

All samples were evaluated for gonadal (males: androgen; females: estrogen and progestagen) and adrenal (males and females: glucocorticoid) hormone metabolites. Concentrations of progestagen and androgen fecal metabolites were quantified using enzyme immunoassays. A monoclonal progesterone antibody (CL425) and polyclonal testosterone antibody (R156/7) were obtained from University of California, Davis, CA, USA. Samples diluted in buffer (CL425: 1:10-1:75 dilution; R156/7: 1:10-1:50) were run in duplicate according to assay specifications (Brown *et al.*, 2001; Wasser and Hunt, 2005). Estrogen metabolites were analyzed using a radioimmunoassay (RIA) for 17β -estradiol, and glucocorticoid metabolites were analyzed using a corticosterone RIA, both obtained from MP Biomedicals (Santa Ana, CA). Samples diluted with buffer (estradiol: neat to 1:2; corticosterone: 1:5 to 1:20) were run in duplicate according to assay specifications. All assays were validated by demonstrating: 1) parallelism between serial dilutions of whooping crane fecal extracts and the respective standard curves; 2) significant recovery of steroid standard added to fecal extracts after removal of endogenous hormone (determined by a high R value); and 3) biological relevance of hormone data. For parallelism tests, two-fold serial dilutions of samples were analyzed in each assay (neat to 1:256 for progestagens and androgens, and neat to 1:64 for

estrogens and glucocorticoids). Mass recovery tests were conducted by combining equal volumes of diluted fecal extract and known amounts of exogenous hormone and calculating the difference between the expected and observed concentrations of exogenous hormone (progestagens: $y = 0.63x - 0.12$, $r = 0.99$; androgens: $y = 1.19x + 21.40$, $r = 0.99$; estrogens: $y = 0.57x + 180.94$, $r = 0.98$; and glucocorticoids: $y = 3.17x + 33.23$, $r = 0.98$; Brown *et al.*, 1994; Scarlata *et al.*, 2011). The corticosterone assay was further validated by collecting fecal samples at nine time points over the course of 96 hr before and after a stressful event (i.e., restraint for 30 min during an annual health exam, $n = 4$), and demonstrating a significant increase in fecal glucocorticoid concentrations 24 hr later (Fig 1). For all assays, intra-assay coefficients of variation were $\leq 10\%$ and inter-assay coefficients of variation were $\leq 15\%$. Data are expressed as mass units of hormone per gram of feces.

2.4. Behavioral Analysis

Crane pairs were monitored daily through video recordings to assess reproductive behaviors during the breeding season. Camera systems were purchased from Vivotek (Portland, OR) and Super Circuits (Austin, TX). Behaviors were video-recorded for a total of 75 min beginning 15 min before sunrise and continuing for an hour after sunrise, which is considered to be the most reproductively active period (White, 2000). Sunrise times were determined for Laurel, MD using SunriseSunset database (Edwards, 2012). Behaviors were assessed using an ethogram modified from those previously described (Happ and Happ, 2011; Nelson *et al.*, 1995; White, 2000) and a continuous focal group sampling method (Mann, 1999). Specific reproductive behaviors (unison calling, marching, dancing, wing solicitation, mounting, and treading)

were recorded separately. Unison calling is an antiphonal duet where females produce two notes for each note of the males. Marching, or precopulatory behavior, is characterized by males and females strutting with stiff legs, necks outstretched, and bills raised in the air; this behavior can be performed in unison or individually. Dancing behavior involves jumping into the air, strutting, and conducting flight displays. Dancing may be followed by wing solicitation, during which the male stands with his back towards the female, neck stretched high, and both wings outstretched. If receptive, the female may wing solicit in return and then assume the mating position, during which she remains standing with head held out straight in front, wings outstretched, and back parallel to the ground. For copulation, the male mounts the female and balances on her back, called treading, and presses his cloaca against the female's, thereby depositing semen into the female's reproductive tract (Swengel et al. 1996, White 2000). Behaviors classified as 'Active, non-reproductive' included standing, walking, preening, and similar behaviors. Behaviors classified as 'Non-active' were sleeping, laying on ground, and hock sitting. 'Pair Together' was defined as one member of the pair being within three body lengths of the other. Time spent performing reproductive behaviors was calculated by day and by sex, as the ratio of Reproductive Time/Total Active Time. Time that an individual spent out of camera was discarded for analysis, thus proportions were calculated only using time a bird was visible.

2.5. Statistical Analysis

Hormone-specific means and baselines were calculated for each individual bird. The calculated baseline concentration represented an estimate of basal hormone metabolite excretion for a given bird that excluded increases in hormone output

associated with reproductive or stressful events (Scarlata *et al.*, 2013). To calculate baselines, we iteratively thinned the full dataset for a given bird/hormone combination. We thinned the data by calculating the means and SDs, then removed any points above the selected SD, and repeated this until no additional points could be removed using this rule (Brown *et al.*, 1994; Brown *et al.*, 2001). The baseline was defined as the mean of the remaining points. We considered 1.5, 2, and 2.5 SDs in this iterative process. Two SD was chosen for calculating baseline concentrations because this value produced a baseline that included the greatest average number of data points across all individuals and hormones, while creating reasonably normal distributions within each individual's data set (specifically, the baseline was approximately equal to the 50% quantile of the points used to calculate it). On average, $18 \pm 4.8\%$ of points were removed from each data set when calculating baselines for each of the hormones evaluated.

We classified each pair in each breeding season into laying ($n = 5$) and non-laying pairs ($n = 7$); hereafter, we refer to these as successful and unsuccessful pairs, respectively. To evaluate differences between successful and unsuccessful pairs, the overall hormonal profiles, hormone patterns associated with egg laying, and behavioral profiles of the groups were compared.

Breeding success was modeled as a function of seasonal hormone means, with separate analyses for each sex and each hormone. Logistic regression was performed using mixed models in the R language (R Core Team 2014) using the lme4 package (Bates *et al.* 2012). Success (whether an egg was produced by the pair) was the response variable, seasonal hormone mean was included as a fixed effect and pair was

initially included as a random effect (to account for repeated measures on individuals across years). For each analysis, AICc values were calculated for models with and without the random effect to determine if pair should be included in the model used for inference (Akaike 1973, Hurvich and Tsai 1989, Burnham and Anderson 2002).

Time spent performing reproductive behaviors served as the predictor variable in a logistic regression, again with separate analyses for males and females. In addition to overall behavior proportions, specific reproductive behaviors including time spent marching, unison calling, engaged in wing solicitation, and number of copulation attempts (i.e., specific reproductive behaviors) were examined as predictor variables. In each model, reproductive success served as the response variable. Similar to the hormone analysis, for each behavioral response variable AICc values were used to determine if pair should be included as a random effect in the models used for inference.

3. Results

Seven established breeding pairs were studied over two concurrent seasons, for a total of 12 male and 12 female reproductive cycles. Within these 12 cycles, five were successful, where the pair laid at least one egg, and seven were unsuccessful (Table 1). With the exception of progestagen, the model selection procedure resulted in exclusion of the random effect of pair from the model used for inference for all hormone and behavior predictor variables (Table 2). All means and SE presented below for hormones and behaviors are empirical means calculated from the raw data.

3.1. Seasonal hormone profiles in female and male whooping cranes

Mean estrogen metabolite concentration was significantly higher, based on the 95% confidence interval of the regression coefficient excluding 0, in successful females (488.1 ± 69.6 ng/g) than unsuccessful ones (221.6 ± 45.2 ng/g; Table 2; regression coefficient = 3.05, 95% CI = 0.42, 10.37). In each successful female cycle (Fig. 2A-2E) estrogen metabolite concentrations were elevated above the baseline value early in the breeding season and continued to fluctuate prior to egg laying. In cycles where eggs were pulled immediately after laying, and pairs were given artificial eggs to incubate (Fig. 2D and 2E), sample collection continued, and we observed that females maintained estrogen production until the end of the breeding season. Unsuccessful females had no consistent patterns of estrogen production, with some females producing very low concentrations throughout the entire breeding season (Fig. 3A and 3B), while others produced estrogens at elevated levels early in the season (Fig. 3C) or fluctuated throughout the season (Fig. 3D-3G). Two females that laid in the first season, but not in the second, are represented by Fig. 2A and 3F, and Fig. 2B and 3G, respectively.

Mean progestagen concentrations were lower in successful (75.2 ± 5.1 ng/g) compared to unsuccessful (87.2 ± 6.9 ng/g; Table 2; regression coefficient = -14.07; 95% CI = -171.41, -1.85) females. Individual profiles of successful females revealed a progestagen peak preceding each egg laying event (Fig. 2A-2E), although one female produced several progestagen peaks without subsequent egg laying (Fig. 2E). Elevated progestagen concentrations were also observed in unsuccessful females (Fig. 3A-3G).

There was no overall difference in mean androgen production (regression coefficient = -0.18; 95% CI = -3.50, 1.80; Table 2) between successful (186.1 ± 8.9

ng/g) and unsuccessful males (191.2 ± 17.6 ng/g). Androgen concentrations fluctuated between 50 and 400 ng/g of dry feces throughout the breeding season in all males. Periodic increases in glucocorticoid concentrations ($>$ baseline of 700 ng/g) were observed in successful and unsuccessful cycles for both sexes, with most occasions associated with potentially stressful management events (e.g., catching or holding birds; Fig. 1). Mean glucocorticoid production did not differ between successful and unsuccessful females (962.5 ± 41.5 ng/g vs. 926.5 ± 44.5 ng/g; regression coefficient = 0.49; 95% CI = -0.89, 5.12; Table 2) or males (852.4 ± 45.2 ng/g vs. 773.2 ± 29.0 ng/g; regression coefficient = 2.42; 95% CI = -2.43, 7.28; Table 2).

3.2. Reproductive behaviors

All behaviors previously reported as being associated with reproduction in cranes (Swengel *et al.*, 1996; White, 2000) were observed in the present study. Males typically initiated these behaviors by marching followed by wing solicitation, although in one pair the female initiated more frequently than the male. If a female was receptive to the male's solicitation, she would reciprocate the behaviors and allow him to walk behind her and mount. Typically if the female wing solicited, a copulation attempt followed, although in all pairs there were instances where the male hesitated and did not complete the mounting behavior. In one pair, the male was partially blind (in one eye), which may have caused him to not attempt copulations even when the female solicited.

During successful reproductive cycles, cranes displayed significantly higher proportions of reproductive behavior, as compared to unsuccessful counterparts in both males (0.04 ± 0.01 vs. 0.01 ± 0.002 ; regression coefficient: 1.090; 95% CI: 1.60, 30.99) and females (0.03 ± 0.004 vs. 0.01 ± 0.005 ; regression coefficient: 7.95; 95% CI: 0.21,

2.95; Table 2). There were also significant differences between successful and unsuccessful pairs in the time individuals spent performing specific reproductive behaviors, specifically marching (precopulatory behavior) in both sexes, unison calling in both sexes, copulation attempts, and amount of time spent together (Table 2). In general, an increase in reproductive behaviors, specifically copulations, was observed 3 days prior to an egg being laid (*data not shown*). Individual pairs who laid in year 1 but not in year 2 (S30 and S32, Table 1) displayed this same pattern: while in laying years, high levels of reproductive behaviors were exhibited mostly before egg laying events, but in non-laying years the behaviors were observed at low levels throughout the breeding season.

4. Discussion

Non-invasive assessment of hormone metabolites in fecal samples has been widely used to track gonadal and adrenal activity and has been used to investigate the relationship between endocrine hormones and behaviors in many wildlife species (Brown *et al.*, 2001; Brown, 2006; Carlstead and Brown, 2005; Scarlata *et al.*, 2011; Wielebnowski *et al.*, 2002). The present study utilized this technology to evaluate gonadal and adrenal hormone metabolites in captive whooping cranes. We have established, for the first time, hormonal profiles of male and female whooping cranes, and demonstrated divergence in the gonadal hormone production between females that laid eggs and those that did not. Estrogen production was significantly higher while progesterone production was significantly lower in successful females compared to unsuccessful ones. Additionally, the overall proportion of reproductive behaviors and the time spent performing specific reproductive behaviors were both higher in successful

than unsuccessful pairs. We found no differences between gonadal hormones in successful versus unsuccessful males. Finally, we determined that adrenal activity was not associated with reproductive success in males or females, as there was not a significant difference in mean concentrations of fecal glucocorticoids between reproductively successful and unsuccessful birds.

Visual inspection of time series plots of hormone levels in the period leading up to egg laying revealed that progesterone metabolites oscillated sharply and reached peak concentrations prior to egg production. This hormone pattern was similar to that reported in other bird species, including serum analysis of canaries (*Serinus canaria*; Sockman and Schwabl, 1999) and American kestrels (*Falco sparverius*; Rehder *et al.*, 1986), and fecal metabolite analysis of great hornbills (*Buceros bicornis*; Crofoot *et al.*, 2003) and brown kiwi (*Apteryx mantelli*; Jensen and Durrant, 2006). Our observation that several progesterone peaks were not followed by egg laying is consistent with that observed by Lagueet *et al.* (1975) in domestic fowl. Chickens in that study also displayed serum estradiol and progesterone peaks within hours of ovulation; however, ovulation did occur without a progesterone peak, and progesterone peaks were observed in the absence of ovulation events. Studies in domestic fowl have shown the necessity of early progesterone production to support LH release through a positive feedback loop prior to ovulation (Etches and Cunningham, 1976; Wilson and Sharp, 1976). Progesterone fluctuations without a subsequent ovulation, as observed in the present study, may participate in hypothalamus-priming events for further hormone release; thus, this relationship should be examined further in cranes.

Differences in estrogen production between successful and unsuccessful females were similar to those reported for the greater hornbill (Crofoot *et al.* 2003), in which successful females had significantly higher fecal estrogen concentrations. In other bird species, elevated estrogen concentrations are associated with follicular growth, stimulation of the oviduct in preparation for ovulation, and signaling the liver to uptake vitellogenin and calcium, which are crucial for egg shell production (Sockman and Schwabl 1999). Lagueet *al.* (1975) reported that chickens typically displayed estradiol and progesterone peaks in serum collected within hours of ovulation.

Unsuccessful females in this study had higher fecal progestagen concentrations than expected, given that mean values were higher than successful females, especially as there was no observed egg production. Females that displayed elevated progestagens without elevated estrogens may have experienced polycystic ovarian follicle (PCOF) syndrome, which would prevent egg production (Liu *et al.*, 2001). PCOF syndrome has been associated with low egg production in domestic hens and is characterized by high basal progesterone concentrations in serum, and is observed when large follicles remain attached to the ovary and become cystic. In those birds, large F1 follicles form, but without sufficient estradiol concentrations, ovulation does not occur and the follicles continue to produce progesterone (Liu and Bacon, 2004). If the follicles remain on the ovary they will continue to produce progestagens and block further ovarian activity (Liu *et al.*, 2001, Liu and Bacon, 2004). In birds, this condition is typically only diagnosed post-mortem, when multiple large, potentially cystic, follicles are found on the ovary. It may be possible to diagnose this condition endoscopically,

and if it proves to be a problem, future studies should focus on developing mitigating therapies.

In addition to differences in female hormone metabolite concentrations, we observed significant differences in reproductive behaviors between laying and non-laying pairs. Successful breeders displayed a higher proportion of reproductive behaviors when compared to unsuccessful ones. Behavioral observations showed that successful pairs performed more copulation attempts, unison calls, and marching(precopulatory) behavior. This was expected as these behaviors are integral to whooping crane pair bonds (Swengel *et al.*, 1996), and reproductive success is the ultimate result of a successful pairing. Frequent copulations are necessary to ensure that enough sperm are available for fertilization (Jones and Nicolich, 2001).

While male behaviors were significantly different in successful pairs, there were no differences in androgen concentrations between males of successful and unsuccessful pairs. This may be due to the lack of mating competitors in the captive environment. Beletsky *et al.* (1992) showed that male red-winged blackbirds (*Agelaius phoeniceus*) living in densely populated areas frequently experiencing territory challenges had higher serum testosterone and corticosterone concentrations than those living in areas of low population density. Similar findings have been reported in brown-headed cowbirds (*Molothrus ater*; Wingfield *et al.* 1987).

Differences in fecal glucocorticoid concentrations were not significant for males or females, which lends additional evidence that these pairs are stable and are all potentially reproductive; thus, low estrogen production observed in non-reproductive pairs was not associated with increased adrenal activity indicative of stress. Spikes in

glucocorticoid concentrations were observed in numerous individuals throughout the course of this study, the majority of which could be associated with husbandry or other disturbances, which is evidence that the adrenal glands of these individuals were capable of mounting an acute stress response (Wingfield and Sapolsky, 2003). However, it is possible that individuals may be producing higher glucocorticoid concentrations during the non-breeding season, which could impact subsequent breeding season success. This has been shown in the California spotted owl (*Strix occidentalis occidentalis*; Tempel and Gutierrez, 2004), where non-breeding owls had higher corticosterone levels at the onset of the breeding season. Therefore, future studies should include year-round monitoring of adrenal hormones to assess the influence of stress associated with captive management on reproductive success in this species. An additional consideration is that adrenal response can be modulated, with glucocorticoid production either being suppressed during the breeding season regardless of exposure to stress, or with acclimation to a specific stressor in which the glucocorticoid response ceases to appear but the physiological and mechanistic effects still manifest with the same ultimate results (Romero, 2004; Wingfield, 2005). Either mechanism may explain why a difference in glucocorticoid means between successful and unsuccessful birds was not detected.

5. Conclusions

In summary, our findings indicate that poor reproductive success in whooping crane pairs is associated with perturbations in gonadal function in female birds that in turn may compromise follicular development and ovulation. Our research also demonstrates that the deviation in gonadal function may not be associated with

increased adrenal activity, and indicates that current management practices are likely not causing undue levels of stress that are impeding reproduction in this species. Future studies should focus on identifying factors, such as aspects of the captive environment and husbandry practices that could be impacting hypothalamic-pituitary-gonadal function. We also plan on monitoring adrenal glucocorticoid activity during the non-breeding season and determining its effect on reproductive success. Knowledge gained from this research has improved our understanding of the reproductive endocrinology of whooping cranes and has laid the foundation for future research investigating the effects of management strategies on reproductive success in captive populations through the use of non-invasive hormone monitoring.

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Table 1: Summary of study whooping crane pairs during two consecutive breeding seasons at Patuxent Wildlife Research Center. Included are the total number of eggs laid and number of fertile eggs.

Pair Identification	Class ^a	2011		2012		Breeding Strategy ^c	Pen Type ^d
		# Eggs	# Fertile	# Eggs	# Fertile		
B12	1	1	1	1	0	Supplemental	Breeding
S30	1	2	2	0	0	AI	Breeding
B16	2	4	2	-	-	Natural	Breeding
S26	2	- ^b	-	0	0	Supplemental	Breeding-No Net
S32	2	3	0	0	0	Natural	Breeding
B7	3	0	0	0	0	Natural	Breeding-No Net
O5	3	0	0	0	0	Natural	Non-breeding-No Net

^aClass indicates the historic fertility of the pair, which was used to initially choose pairs for inclusion in the study. Class 1: 51-100% fertility, class 2: 0-50%, class 3: no previous eggs

^bDashes indicate when a pair was not included in the study.

^cBreeding Strategy indicates the degree of reproductive assistance a pair received. Levels include: naturally fertile (Natural), exclusively fertile through artificial insemination (AI), and Supplemental wherein artificial insemination was used to increase the natural fertility and probability of obtaining a fertile egg in a pair with low natural fertility.

^dPen Type indicates the type of pen in which the pair was housed. Breeding pens are pens located in breeding series near other breeding pairs, covered with flight netting, unless indicated otherwise, and lit by photoperiod lights during the laying season. Non-breeding pens are without photoperiod lights located outside of the breeding series, near few or no other breeding pairs.

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Table 2: Summary of analysis results for all hormones and behaviors examined in captive whooping cranes housed at Patuxent Wildlife Research Center during the 2011 and 2012 breeding season. Empirical means and standard errors (SE) are presented for both successful and unsuccessful pairs. Regression coefficients and 95% confidence intervals are included for each explanatory variable (either hormone or behavior). Bold indicates 95% confidence intervals that exclude 0, indicating a significant effect.

		Successful (Mean $\pm$ SE)	Unsuccessful (Mean $\pm$ SE)	ΔAICc^a	Regression Coefficient	95% Confidence Interval
Female	Estrogen	488.1 $\pm$ 69.6	221.6 $\pm$ 45.2	3.67	3.05	0.42, 10.37
	Progestagen	75.2 $\pm$ 5.1	87.2 $\pm$ 6.9	-2.54	-14.07	-171.41, -1.85
	Glucocorticoid	962.5 $\pm$ 41.5	926.5 $\pm$ 44.5	3.51	0.49	-0.89, 5.12
	Overall Behavior Proportions (% of total time)	0.03 $\pm$ 0.004	0.01 $\pm$ 0.005	3.66	7.95	0.21, 2.95
	Marching (hours)	77.05 $\pm$ 12.41	31.18 $\pm$ 12.42	3.67	0.38	0.19, 0.57
	Unison Calling (hours)	3.07 $\pm$ 1.01	0.33 $\pm$ 0.15	1.80	8.79	2.12, 15.45
	Dancing (hours)	5.15 $\pm$ 1.55	11.85 $\pm$ 4.35	3.57	-0.85	-4.07, 2.37
	Wing Solicitation (hours)	7.28 $\pm$ 1.26	4.93 $\pm$ 2.91	3.60	1.68	-6.44, 9.80
Male	Androgen	186.1 $\pm$ 8.9	191.2 $\pm$ 17.6	3.60	-0.18	-3.50, 1.80
	Glucocorticoid	852.4 $\pm$ 45.2	773.2 $\pm$ 29.0	2.37	2.42	-2.43, 7.28
	Overall Behavior Proportions (% of total time)	0.04 $\pm$ 0.01	0.01 $\pm$ 0.002	3.62	1.09	1.60, 30.99
	Marching (hours)	125.6 $\pm$ 20.31	30.03 $\pm$ 12.3	1.21	0.46	0.07, 0.84
	Unison Calling (hours)	3.93 $\pm$ 1.10	0.23 $\pm$ 0.12	1.43	6.70	1.84, 11.55
	Dancing (hours)	5.9 $\pm$ 2.09	7.8 $\pm$ 2.31	3.61	-1.03	-2.81, 0.74
	Wing Solicitation (hours)	2.27 $\pm$ 1.01	1.57 $\pm$ 0.79	3.67	0.85	-2.00, 3.70
Pair	Copulation Attempts	5.4 $\pm$ 1.17	1.42 $\pm$ 1.02	3.67	0.10	0.02, 0.18
	Time Together (hours)	44.81 $\pm$ 5.75	18.54 $\pm$ 6.04	0.76	-0.02	-0.23, -0.01

^a ΔAICc = $\text{AICc}(\text{model including a random effect of pair}) - \text{AICc}(\text{model not including a random effect of pair})$. Thus, because lower AICc values indicate greater support, a positive ΔAICc

- 32 indicates that a pair effect was not included in the model used for inference, and a negative
- 33 $\Delta AICc$ indicates that a pair effect was included in the model used for inference.
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2 Figure 1: Glucocorticoid biological validation for four adult whooping cranes. Fecal glucocorticoid
3 concentrations before and after an annual health exam, where birds were held for roughly 30 min,
4 received a physical exam, wormer, vaccines, and had blood drawn. An “M” indicates samples
5 collected from a male and an “F” indicates samples collected from a female. Results provide evidence
6 for the ability of the assay used to detect corticosterone response to stress.

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Figure 2: Individual gonadal hormone metabolite profiles during successful (egg laying) breeding seasons of female whooping cranes. Each panel represents a breeding season during which at least one egg was produced. Egg laying dates are denoted with a black arrow. Panels A-C represent females allowed to incubate their final clutch to full term. Samples were not collected while a pair was incubating live eggs. Panels D-E illustrate gonadal hormone profiles of the same female in 2 consecutive years; during both years the eggs were pulled immediately after they were laid and the pair was given dummy eggs to incubate to term. Baseline value of each hormone was determined through iterative thinning of the full dataset as described in the text.

Figure 3: Individual gonadal hormone metabolite profiles during unsuccessful (no eggs laid) breeding seasons of female whooping cranes. Each panel represents a breeding season during which no eggs were produced. Panels A-B and C-D, respectively, illustrate gonadal hormone profiles of the same female in consecutive years. Panel E indicates the female included in the study only the second year

29 (see Table 1). Panels F and G depict females that laid eggs in year 1 (Figure 2B and 2C respectively)
30 but not in year 2. Baseline value of each hormone was determined through iterative thinning of the full
31 dataset as described in the text.