ABSTRACT

Title of Document:	MULTIDISCIPLINARY APPROACH TO UNDERSTANDING THE POOR REPRODUCTION IN THE WHOOPING CRANE (*GRUS AMERICANA*)
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Reproductive success of the endangered whooping crane (*Grus americana*) maintained ex situ is suboptimal. The main goals of this multidisciplinary investigation are to advance the understanding of whooping crane reproduction and identify potential causes of poor reproductive success in a captive colony. The specific objectives include (Study 1) determining overall seminal characteristics and examine the influences of inbreeding, age, and stages of breeding season on seminal quality in captive birds and (Study 2) utilizing non-invasive endocrine monitoring to longitudinally assess gonadal and adrenal steroids of bird pairs with various reproductive outcomes and evaluating the relationship between hormones and crane behaviors. Our findings demonstrate that (1) there is no relationship between inbreeding coefficient or age and seminal quality of the whooping crane and (2) stage of breeding season impacted seminal output and characteristics. Overall estradiol concentrations and reproductive behaviors, but not glucocorticoid levels, were also key determinants to successful reproductive output.

MULTIDISCIPLINARY APPROACH TO UNDERSTANDING THE POOR
REPRODUCTION IN THE WHOOPING CRANE (*GRUS AMERICANA*)

By

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Chapter 1 Figure 2: A photograph of avian follicles displaying hierarchical development, is reprinted from Ansenberger, K., Y. Zhuge, J. Lagman, C. Richards, A. Barua, J. Bahr, and D. Hales. 2009. E-cadherin expression in ovarian cancer in the laying hen, *Gallus domesticus*, compared to human ovarian cancer,with permission from Dr. Hales and Elsevier Ltd.

For the cranes.

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Chapter 1: Literature Review

The Whooping Crane

The whooping crane (*Grus americana*) is listed as endangered by the

International Union for Conservation of Nature (IUCN 2011). The main threats to

wild whooping crane populations include habitat loss and modification (Archibald

and Mirande 1985, Ellis et al. 1992). Protected areas in the wintering ground in

southern Texas can sustain an estimated 500 birds and any population increase above

this number may force individuals to find other, possibly less suitable, habitats (Ellis

et al. 1992). In 1941, only 16 individuals remained in a single migratory population,

which traveled back and forth from Aransas National Wildlife Refuge in Texas to

Wood Buffalo National Park, located in Alberta and Northwest Territories, on its

yearly migration (Archibald and Lewis 1996). A non-migratory population existed in

Louisiana until 1950, when the last remaining individuals were brought into captivity

due to low population numbers (Glenn et al. 1999). Through *in situ* and *ex situ*

conservation efforts, the number of wild whooping cranes has increased to around

437 individuals as of March 2011 (CWS and USFWS 2009), which includes

individuals in the Wood Buffalo migratory population, as well as reintroduced non-

migratory populations in Florida and Louisiana and an Eastern migratory population

that breeds in Wisconsin. Currently, there are a total of 162 whooping cranes held in

captive breeding centers across North America. The largest captive population is

held at the USGS Patuxent Wildlife Research Center (PWCR) in Laurel, Maryland.

Birds produced in this facility are released into both the Eastern migratory and the

Louisiana non-migratory flocks (CWS and USFWS 2009).

In the past decade, PWRC has produced and released on average 18 birds into wild populations each year (J Chandler, pers. communication). However, reproductive performance of birds at this facility is suboptimal. The Wood Buffalo population exhibited an egg fertility rate of 95% and a known viability rate of 73% from 1967-1995 (Kuyt 1995). A viable egg was described as having a live embryo at the time of examination as determined by an egg immersion test. This population has remained self-sustaining without supplementation from captive sources (CWS and USFWS 2009). In contrast, PWRC had a 60% fertility rate in eggs laid from 2000 to 2010 with the aid of an extensive artificial insemination program (J Chandler, pers. communication). Furthermore, reproductive onset in captive populations appears to be delayed. Pair formation typically occurs when the birds are between 2 and 3 years of age, although older birds that were previously unpaired or that have lost their mates may pair again. In wild populations, reproductive onset (production of sperm and eggs) has occurred as early as 3 years of age, but typically occurs at 5 years of age in both males and females, roughly 2 years after initial pair formation occurs (Kuyt and Goossen 1987). However, captive females begin laying eggs closer to 7 years of age (Mirande and Archibald 1990, Mirande et al. 1996).

Herein, I present a review of literature review focused on topics relevant to factors that I considered in examining poor reproduction in captive whooping cranes at Patuxent Wildlife Research Center, including avian reproductive anatomy and reproductive endocrinology, factors influencing avian sperm production, sperm and egg production in whooping cranes, and reproductive behavior in whooping cranes.

This is followed by enumeration of the research objectives and hypotheses that I considered in this project. *Avian Reproductive Anatomy*

In most species of birds, females only retain the left side of the reproductive tract (Fig. 1A). Ova are released into the oviduct which consists of five regions (Gee and Russman 1996, Bakst and Akuffo 2008). The first region is the infundibulum, where fertilization occurs. The second region is the magnum where the albumen, or egg white, is added around the yolk. Shell membranes, which act as support and shock absorbers to the developing embryo once the egg is laid, are added to the ova in the third region, the isthmus. The next segment is the uterus, where an egg spends the most time and a calcified shell is added. The final part of the oviduct is the vagina which aids in egg expulsion through muscle contractions (Joyner 1990). The egg must enter the digestive tract through the urodeal-vaginal junction and then exit through the cloaca (Bakst and Akuffo 2008).

Sperm storage tubules [SST] are located within the vaginal wall. After copulation or artificial insemination, sperm is stored in the SST up to 100 days depending on species (Bakst and Akuffo 2008). In the female, multiple ova are recruited at the same time within the ovary. As ova develop in the ovary, more yolk is added within the peri-vitelline membrane (Joyner 1990). This structure is analogous to the zona pellucida found in mammals. Small follicles (F2-F4, Fig. 2) produce estradiol which aids the development of the larger (F1) follicles that produce progesterone. The F1 follicle ovulates first and the F2 follicles (the second largest follicles that have not yet fully developed) become ready for subsequent ovulations, becoming the new F1 follicles (Johnson et al. 1987). No corpora lutea are formed after ovulation, which

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allows birds to release multiple yolks within a few days of each other. Post ovulatory

follicles produce non-steroid hormones, especially prostaglandins, which aid in

oviposition and muscle contractions (Hertelendy et al. 1975, Joyner 1990). Once an

ovum is released, it enters the oviduct and is fertilized if sperm are present (Bakst and

Akuffo 2008). Fertilization is more likely to occur if sperm are already present in the

SST. This may be a mechanism for sperm competition, allowing for viable, fertile

sperm to be present when ovulation occurs, or a method for female selection,

allowing for potential post-copulatory or pre-fertilization selection to occur which is

mediated by unidentified mechanisms within the female reproductive tract (Birkhead

1998, Nicolich et al. 2001, Bakst and Akuffo 2008).

Avian male reproductive anatomy is similar to that of mammals. Males retain

both testes, although in some species one testis may be functional but reduced in size

compared to the other (Fig 1B). Birds have a vestigial epididymis, but sperm

maturation does not occur in this region. Rather, sperm reside in the ductus deferens

until they are mature and ready to be ejaculated (Joyner 1990). Male birds do not

possess the accessory sex glands observed in mammals; thus, the portion of the

ejaculate which maintains pH and provides energy is produced directly from the testis

(Anderson and Navara 2011). During ejaculation, sperm travels from the ductus

deferens and into the urodeum (Joyner 1990). Most bird species do not have a

copulatory organ, although some have a rudimentary phallus that deposits semen into

the female (Brennan et al. 2010). In species that do not have a copulatory organ, such

Figure 1: Reproductive anatomy of A) female and B) male cranes. Adapted from Gee and Russman 1996.

A) Female
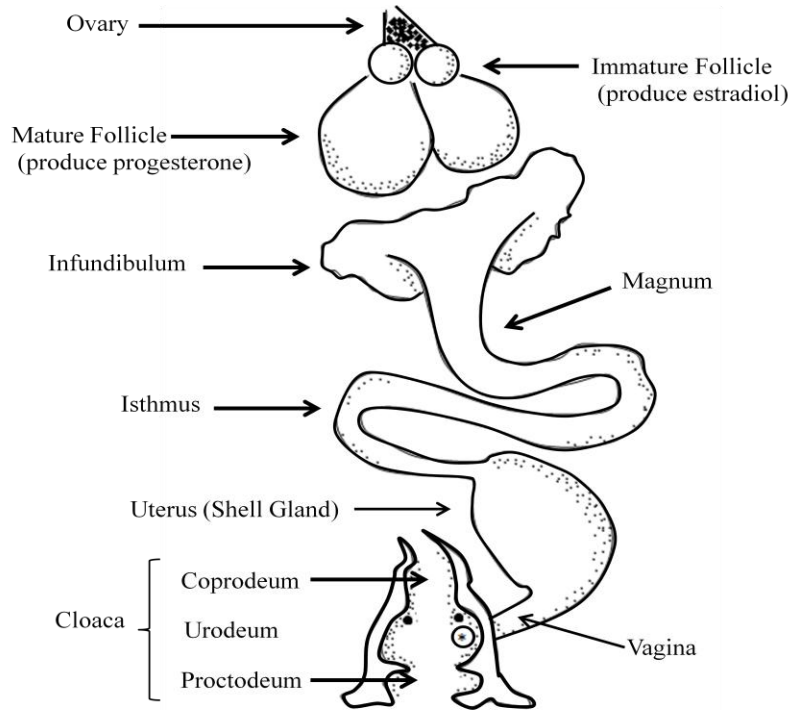
B) Male
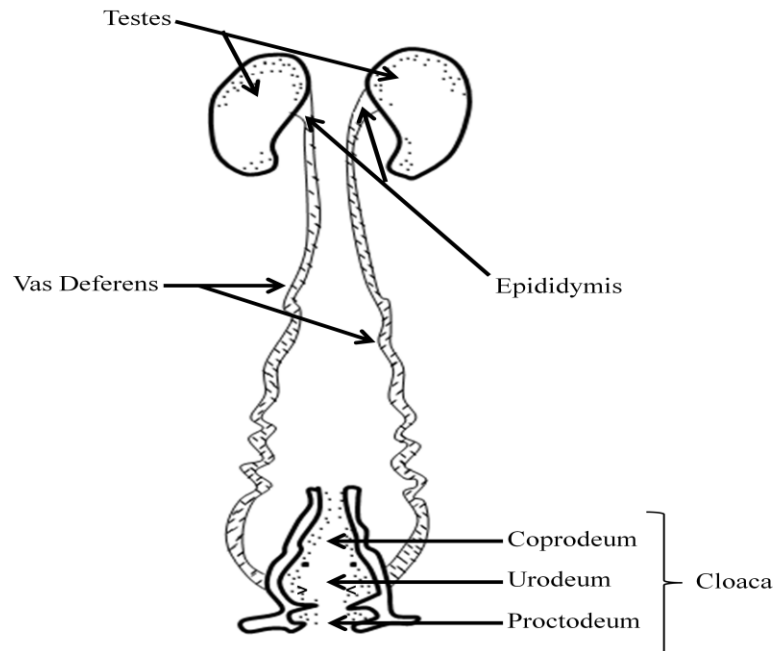
as cranes, the male must press the cloaca to that of the female for sperm deposition. Typically, the cloaca wall will invert to allow the papilla to come in contact with the female's cloaca (Joyner 1990, Bakst and Akuffo 2008).

Avian Reproductive Endocrinology

Avian reproduction is highly seasonal and controlled heavily by photo period (Robinson and Follett 1982, Dawson et al. 2001, Leska and Dusza 2007). The timing of the breeding season varies among species, and is typically dependent on the latitude of the species' habitat (Farner 1986). Equatorial birds tend to breed all year, but most frequently during the rainy season, when food is most abundant (Farner 1986, Archibald and Lewis 1996). As distance from the equator increases, the breeding season becomes narrower, with birds in northern regions farthest from the equator reproducing from mid April until June (Farner 1986). With each increasing degree of latitude (the equator is at 0^0 latitude), it can be expected that the breeding season will start 3 days later. Species at the highest latitudes lay eggs in late May or early June, when there are up to 20 hours of light per day. Because of the short spring at higher latitudes, wild cranes, like the whooping crane, breeding in these regions typically only lay one clutch, even if the first clutch is lost (Mirande et al. 1996). Some crane species will lay a second clutch depending on how much time is left in the breeding season and how long the pair incubated the first clutch.

The external environment influences the avian hypothalamo-pituitary-gonadal axis. External factors such as light and day length are translated through the retina in the eye to the hypothalamus which triggers the secretion of gonadotropin releasing

Figure 2: A photograph of avian follicles displaying hierarchical development. Small follicles (F2-4) produce estradiol which aids the development of the larger (F1) follicles that produce progesterone. The F1 follicle ovulates first and the F2 follicles become ready for subsequent ovulations.
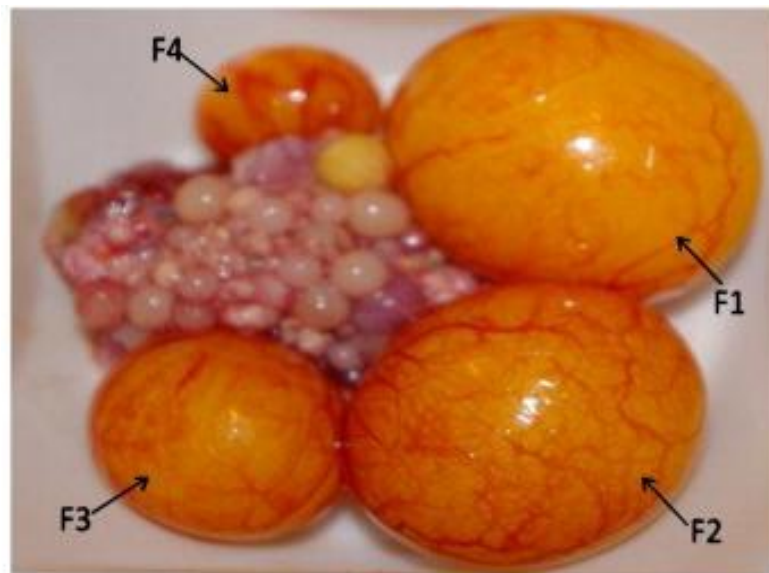
Reprinted from Ansenberger et al. 2009, with permission from Dr. Buck Hales,SIU School of Medicine, and Elsevier Ltd.

hormones (GnRHs) and vasoactive intestinal polypeptide (VIP; Leska and Dusza 2007; Fig.

3). In general, GnRH acts on the anterior pituitary to mediate the release of follicle

stimulating hormone (FSH) and luteinizing hormone (LH), that in turn regulate gonad

functions in both males and females (Liu et al. 2001). Nevertheless, there are many different

types of GnRHs, and their functions in birds are not entirely understood. It has been

suggested that GnRH, especially GnRH-1, regulates courtship and mating behavior (Ottinger

and Baskt 1995, Norris 2006). Studies have found that other environmental factors, rather

than just photoperiod, may mediate the onset of the breeding season (Dawson et al. 2001,

Leska and Dusza 2007). In mammals, melatonin is released from the pineal gland during

photoperiodic response to act upon the hypothalamus (Figure 3; Norris 2006). However,

in some birds, melatonin works in conjunction with metabolic responses to influence

reproduction via regulation of gonadotropin inhibiting hormone (GnIH) secretion,

that in turn down-regulates the release of gonadotropins from the pituitary (Leska

and Dusza 2007). This mechanism is believed to ensure that there are enough

resources available to sustain individuals throughout the breeding season and support

offspring.

In males, FSH induces testicular growth and the secretion of estradiol by the

Sertoli cells, which mediates the production of sperm through mitotic proliferation of

spermatogonia and by creating primary spermatocytes (Joyner 1990, Norris 2006;

Fig. 3). LH in males directs the secretion of androgens by the Leydig cells and

mediates spermiation, releasing mature sperm into the lumen of the seminiferous

tubule (Penfold et al. 2000; Fig. 3). High levels of 3β hydroxysteroid dehydrogenase

activity within the Leydig and Sertoli cells of the testis are indicative of steroid

synthesis, the process of converting cholesterol to the steroid hormones, including

Figure 3: Hormonal Control of Avian Reproduction. External cues stimulate the hypothalamus, triggering a hormone cascade in the pituitary and gonads that regulate gamete production and influence reproductive behaviors. Stress triggers a cascade which blocks the production of other pituitary hormones and slows reproduction. Red arrows indicate Hypothalamic-Pituitary-Adrenal Axis. Blue and pink arrows indicate the Hypothalamic-Pituitary-Gonadal Axis in males and females respectively. Green arrows indicate prolactin pathway. Each pathway plays a role in mediating reproduction in avian species.
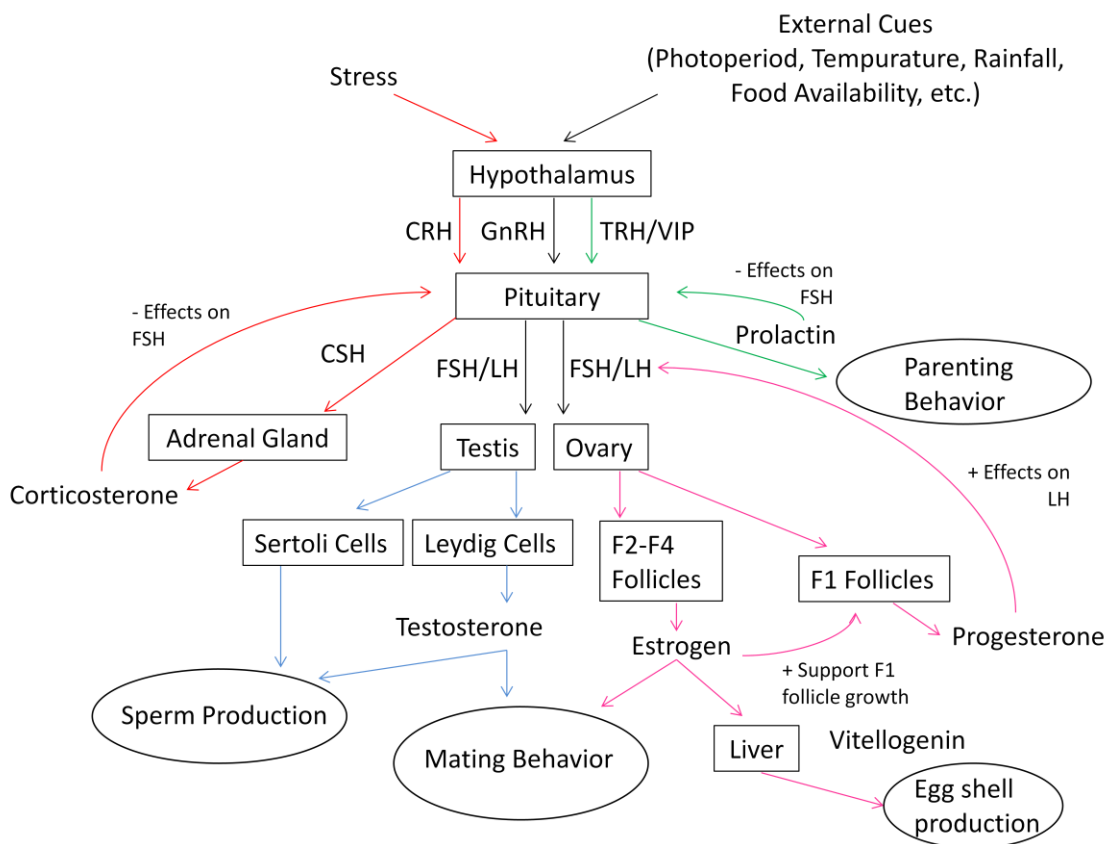
Abbreviations include:
CRH: Corticotropin Releasing Hormone
GnRH: Gonadotropin Releasing Hormone
TRH: Thyrotropin Releasing Hormone
VIP: Vasoactive Intestinal Polypeptide
CSH: Corticotropin Stimulating Hormone
FSH: Follicle Stimulating Hormone
LH: Luteinizing Hormone

testosterone that plays an important role in spermatogenesis (Norris 2006). Testosterone plays an important role in sperm production by influencing meiosis and maintaining spermatogenesis, as well as influencing secondary male sex characteristics and male reproductive behavior (Joyner 1990, Norris 2006).

In females, FSH simulates the growth of the ovary, the release of estrogen and recruitment of developing follicles (Johnson et al. 1987; Figure 3). Estrogen secreted from the follicle cells signals the liver to produce calcium binding vitellogenin, which is critical for production of egg shells (Norris 2006). Progesterone is produced by granulosa cells of the growing follicles within the female's ovary (Joyner 1990). Progesterone levels continue to increase as the ovum grows and signal LH secretion (Moudgal and Razdan 1985), which in turn up-regulates the conversion of androgen produced by the theca interna layer into estrogen and signals ovulation. LH secretion initiates when females nest and the concentration reaches peak levels at the onset of egg production and immediately declines after eggs are laid (Liu et al. 2001, Liu and Bacon 2005). After ovulation, progesterone levels rapidly decrease until the growth of a subsequent follicle (Liu et al. 2001).

Estrogen and testosterone are produced by the gonads during the reproductive season and act as a feedback regulation on the hypothalamus (Bluhm et al. 1983). Concentrations of these hormones rise at the onset of the breeding season and return to the baseline level after the breeding season is ended (Gee and Russman 1996). VIP also acts on the anterior pituitary and stimulates the secretion of prolactin. Prolactin mediates the transition from the breeding phase to the parental phase of reproduction. Prolactin levels begin to increase as nests are built and eggs are laid, and remain

elevated during the chick rearing period. In birds that have precocial young, prolactin levels return to the baseline much quicker than in birds that have altricial young that must be fed, guarded, and cared for over an extended period (Angelier and Chastel 2009). Prolactin has been thought to suppress reproduction by acting as an anti-gonadal agent and blocking the effect and/or release of gonadal hormones (Bluhm et al. 1983). Birds which are the best parents can be expected to have the highest levels of prolactin (Angelier and Chastel 2009).

Another hormone that has been shown to affect reproduction is corticosterone, which is produced by the adrenal gland of birds (Angelier and Chastel 2009). Although the secretion of corticosterone helps individuals cope with stressful situation, persistent elevation of this hormone (chronic stress) has been shown to suppress reproduction in an attempt to preserve body condition and maintain homeostasis. Ouyang et al. (2011) showed that individual birds with high levels of corticosterone prior to the breeding season have less reproductive success than those with low steroid levels. Specifically, the former exhibit a delay in egg laying, produce fewer eggs, hatch fewer eggs, and have fewer hatchlings that survive to fledging compared to the latter. Furthermore, Bluhm et al. (1983) reported that mallards that were rotated daily to new pens and housed with new conspecifics had much lower serum estradiol, LH, progesterone and prolactin levels than the control birds that were left with their established mate.

Factors Influencing Avian Sperm Production

To date, mechanisms influencing gamete production in birds are not well understood. It has been suggested that many external factors, including time of year,

photoperiod, resource availability, stress, and inbreeding, influence gamete production in birds (Mirande et al. 1996, Wingfield et al. 1997).

Low genetic diversity negatively affects sperm function and morphology in mammalian species that have undergone severe genetic bottlenecks (Cassinello et al. 2002, Asa et al. 2007, Fitzpatrick and Evans 2009). Low genetic diversity has also been shown to contribute to reproductive problems in birds, including soft eggs, low laying rates, low hatchability, and low individual fitness as observed in the Greater Prairie Chicken (*Tympanuchus cupido*) and other bird species (Briske and Montgomerie 1992, Mirande et al. 1996, Westemeier et al. 1998, Glenn et al. 1999, Briske and Machintosh 2004).

Because sperm must travel from the cloaca to the infundibulum in the female, strong motility is required for successful fertilization (Mirande et al. 1996). Some studies have shown that males of many avian species, including cranes and domestic fowls, housed with female conspecifics have decreased semen quality (Jones and Leighton 1987, Chen et al. 2001, Jones and Nicolich 2001). This is thought to be caused by the depletion of semen reserves through frequent copulations. Low quality semen, i.e., samples with lower sperm concentrations and higher instances of abnormalities, are frequently observed in naturally breeding males with high fertility rates, while males with high ranking semen
quality likely have low fertility rates because semen reserves are not being utilized and high quality sperm are remaining unused (Chen et al. 2001).

Sperm Production in the Whooping Crane

In captive whooping cranes, peak semen production has historically been between March 30th and April 26th (Mirande et al. 1996). Seminal volume varies greatly between individuals and can differ among ejaculates collected from the same male. Crane ejaculates may range in color from clear to milky white, but if contaminated (with urate or feces) samples may have a green, yellow, or brown tint. The consistency is typically slightly thicker than water. Average pH and osmolarity of the four crane species housed at Patuxent were reported by Gee et al. (1985). Whooping crane semen was found to have a pH around 8.0 ± 0.0 and an osmolarity of 270 ± 36 mOsm.

Egg Production in the Whooping Crane

The interval between ovulation and egg laying has been estimated to be about 3 to 4 days in the whooping crane (Mirande et al. 1996). Eggs are blue-green and mottled with brown spots. Whooping cranes lay clutches of sometimes one but more frequently two eggs. After the first egg is laid, the second egg can be expected 2 to 3 days later. Crane egg shells are relatively thick compared to other species, which is reflective of how much time the egg spends within the shell gland (crane: 45 hrs, chicken: 20 hrs; Mirande et al. 1996).

Whooping Crane Reproductive Behavior

The most common reproductive strategy (90%) found in birds is monogamy (Cech et al. 2009). This involves a pair of conspecific birds that inhabit and defend a breeding territory. A variation of this strategy involves the pair separating between breeding seasons.

Whooping cranes typically are socially monogamous and generally mate for life, although pairs have been known to separate if a pair is not reproductively successful (Swengel et al. 1996, Cech et al. 2009). Extra-pair copulations have also been reported in the wild (Dellinger et al. 2013). In cranes, pairs are formed around 2 years of age (Mirande and Archibald 1990). Pairing involves frequent unison calls and dancing. The stages of pairing include (1) standing side by side, (2) synchronized behaviors and (3) courtship dance. This set of interactions can take months to ensure appropriate pairing. If the courtship dance does not occur then a pair bond may not have formed. In unstable pairs, these behaviors could lead to aggression, especially if behaviors are not reciprocated by both members of the pair, and in extreme cases may result in one member of the pair killing the other. Many of the behaviors involved in a crane dance are the same as aggressive behaviors, and it is believed that sexual and aggressive behaviors are controlled by the same hormones (i.e., testosterone and estrogen). Therefore, it can be difficult to judge how members of a pair are reacting to each other (Swengel et al. 1996). When pairs are formed in captivity, if one member is too dominant, especially the female, the pair may never breed. Furthermore, if the pairs form too young, they are less likely to breed because they could see each other as siblings rather than mates (Derrickson and Carpenter 1987).

Once the breeding season begins, the pairs will copulate 2 to 5 weeks before the onset of egg production (Gee and Russman 1996). The mating ritual of the whooping crane is highly involved and each step of the process must be completed correctly in order to move on to the following steps. Breeding begins with unison calling. When both members of the pair are ready, the dance will begin. Elaborate

moves, including jumping into the air, strutting, and flight displays occur when the pair is getting ready to breed. When both members of the pair are engaged, the female assumes the mating position; she remains standing with head held out straight in front with wings outstretched. If the male is ready to breed, he will mount the female and balance on her back. If he misses or is not able to properly balance, the copulation will not continue. If the pair is able to balance, the male will press his cloaca against the female's, depositing semen into the female's reproductive tract. At any stage, if either member of the pair is not properly engaged, the entire process will stop and may be attempted again later (Swengel et al. 1996, White 2000). Crane pairs build a nest a few days before the first egg is laid. Wild nests are built from vegetation in shallow water and form a low but wide platform surrounded by a moat (Fig. 4; Archibald and Lewis 1996, Cech et al. 2009). Whooping cranes typically lay two eggs per clutch within two days of each other. Egg production is dependent on the quality of wintering habitat in migratory populations (Chavez-Ramirez and Wehtje 2012). Once the eggs are laid, both parents will incubate the clutch, taking turns and unison calling whenever they switch. Parents incubate eggs for 28 to 34 days, or until eggs hatch. If an egg does not hatch because it was infertile or damaged, the pair may continue to incubate the egg for up to 50 additional days (Archibald and Lewis 1996). Once the egg(s) hatch the parents will care for and guard the chicks. One chick is typically dominant, and if there is not enough food for both chicks the subordinate chick will not survive. Pairs that are not reproductively successful during a given year may adopt chicks from other pairs that cannot care for both chicks or from pairs that die while raising a chick. Raising chicks reinforces pair

bonds and increases chance of fertility for the next season; therefore, adopting another pair's chick allows non-reproductive pairs to have a better chance of successfully reproducing in the next breeding season. The chick will stay with the parents until the spring, after they have completed their first migration (Swengel et al. 1996).

Cranes go through a period of molt in order to restore feather quality every year, generally in late summer, either replacing all feathers or specific tracts targeting specific feathers (Gee and Russman 1996). In migratory populations, this occurs just before leaving for the wintering ground, which aids migratory flight (Archibald and Lewis 1996).

During the molt, birds lose their flight feathers and are temporarily flightless. Birds exhibiting molt tend to be much quieter to draw less attention. Molt and reproduction occur when food is most abundant (Dawson et al. 2001).

Hypotheses and Objectives

The overall goals of this project were to advance knowledge of reproductive biology of the whooping crane and identify potential underlying causes of poor reproduction in captive whooping cranes. Currently, the PWRC population consists of a total of 74 whooping cranes, forming 23 breeding pairs. Two of these pairs are consistently infertile (i.e., no production of fertile egg) and four other pairs have low fertility (30 -45%), which is variable from year to year. The hypotheses of this study were that reproduction in this captive colony is compromised by (1) low gene diversity and (2) abnormal gonadal functions associated with stress induced by suboptimal husbandry or captive environment.

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Figure 4: Whooping crane eggs in a nest at Patuxent Wildlife Research Center. Photo taken by Megan Brown 2012.

To test the first hypothesis, I assessed seminal traits in adult male whooping cranes and determined the influence of inbreeding coefficient on seminal quality. In this part of the study, I also determined the impact of age and time during breeding season on seminal traits.

For the second hypothesis, I utilized non-invasive hormone monitoring to characterize longitudinal profiles of testosterone, estrogen, progesterone and corticosterone for whooping crane pairs that fall into one of three categories; (1) good, (2) moderate, and (3) poor reproductive performance. Good reproductive performance indicates that the pair consistently produces fertile eggs every breeding season. Moderate reproductive performance indicates that the pair produces a variable number of eggs from year to year, some years producing no eggs for unknown reasons, as well as pairs that consistently produce fewer numbers of eggs than successful pairs. Pairs with poor reproductive performance do not ever produce eggs, or have a history of no fertility as a pair. I compared the levels of gonadal and adrenal hormone metabolites among the three groups. I collected behavioral data from these pairs and correlated reproductive behaviors with endocrine data, and attempted to identify key behaviors determining reproductive success. The findings of my research advance understanding about reproductive biology and the causes of poor reproduction in the whooping crane. This work should aid PWRC managers in modifying captive management to enhance reproduction of pairs.

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Chapter 2: Time of Season, but not Inbreeding or Age, Effect Semen Quality in the Whooping Crane (*Grus americana*)

Introduction

The whooping crane (*Grus americana*) is the only crane species endemic to North America. Because of habitat loss and overhunting, this species had been extirpated over large portions of its historic range and by 1941, only 16 whooping cranes remained in the Aransas-Wood Buffalo population (AWBP), which is the only whooping crane population that persisted in the wild (CWS and USFWS 2009). Through both *in situ* and *ex situ* conservation efforts, and protection under both the Endangered Species Act and the Canadian Species at Risk Act the number of whooping cranes has increased to roughly 500+ individuals as of 2011 (CWS and USFWS 2009). However wild cranes still face threats from habitat loss, poor habitat quality, and disturbance (Lewis 1997); while the AWBP has a positive growth rate, the growth rate is slow and several factors still threaten the species (CWS and USFWS 2009). Extant reintroduced whooping crane populations, as of 2013, include the Eastern Migratory Population, and two non-migratory populations, one in Florida and one in Louisiana. None of these currently has a positive growth rate in the absence of releases, and only the Eastern Migratory Population and the Louisiana Non-Migratory Population are expected to receive additional releases (Converse et al. 2011, Converse et al. In Press)

Captive propagation of whooping cranes began in 1967 when second or abandoned eggs were removed from wild nests in the AWBP (Kuyt 1996). Those eggs were sent to the Patuxent Wildlife Research Center (PWRC) in Laurel, Maryland to establish a captive flock. Since then, four more captive breeding centers

have been established throughout North America and as of 2011, 163 whooping cranes were housed in these five centers (CWS and USFWS 2009). Through the combined effort of these centers, 18 chicks, on average, are released into the reintroduced populations each year. Because all living whooping cranes are believed to be descended from eight founders (CWS and USFWS 2009), optimizing genetic management of the captive population across all captive centers is crucial.

Artificial insemination using fresh semen has been widely incorporated into the captive breeding program of the whooping crane. This technology allows females to be inseminated from donor males other than their social mate, thus maintaining genetic diversity while preserving established pairs, as well as permitting males and females from non- or poorly-reproducing social pairs to reproduce (Gee and Russman 1996). Some research has been done to establish cryopreservation techniques in sandhill cranes (*Grus canadensis*; Gee et al. 1985), and these protocols have been applied to the whooping crane to preserve semen samples (J Chandler, pers. comm.). However, to date whooping crane chicks have not been produced from cryo-preserved sperm. Therefore, an effective sperm cryopreservation protocol for this species needs to be established. Before this can be accomplished, it is essential to generate basic information on seminal characteristics within this species to see the effect that different cryopreservation protocols may have on these same characteristics. By identifying the effect that inbreeding, age, and season have on seminal characteristics in the whooping crane, the AI program can be utilized to its full potential, and new methods for recognizing the most important individuals to utilize and the best times to collect from them may be established.

Male whooping cranes reach sexual maturity around 2 years of age, and begin producing sperm regardless of pair status (Gee 1983, Gee and Russman 1996). Males continue to produce sperm for the majority of their lives (approximately 24 years in wild, but potentially much longer in captivity; Brinkley and Miller 1980, Gee and Russman 1996). Age and experience have been shown to increase the breeding success of male cranes (Gee 1983, Gee and Russman 1996, Chen et al. 2001), but it is unknown if this is due to a change in seminal quality with age or if it is due to some other factors. Whooping cranes breed seasonally under the influence of external cues, specifically photoperiod and temperature. In captivity, artificial lights are used to simulate a longer photoperiod to prompt the breeding season to begin earlier. At PWRC, cranes typically begin breeding in mid-March (a few weeks before the onset of egg laying) and breeding lasts until May (Gee 1983).

Inbreeding depression has been documented in a variety of species including the African lion (Wildt et al. 1987, Trinkel et al. 2010), Florida Panther (Barone et al. 1994), hihi (Brekke et al. 2010) and greater prairie chicken (Bouzat et al. 1998). One effect of inbreeding is decreased heterozygosity, which reduces a species' ability to deal with environmental stochasticity and increases the chance for extinction (Holt et al. 2003). Inbreeding depression is manifested in different ways depending on the species and the environment in which it lives. It has been shown that seminal quality is linked to the level of heterozygosity (Wildt et al. 1987, Cassinello et al. 2002, Gage et al. 2006, Ruiz-Lopez et al. 2010). Fitzpatrick and Evans (2009) compared ten endangered species to ten non-endangered species and found that heterozygosity was positively correlated with an individual's percentage of motile sperm and percentage

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of normal sperm in endangered species (including Florida panther, cheetah, black footed ferret, and giant panda). No such relationship was detected in non-endangered animals.

The objectives of this study were to characterize seminal characteristics of whooping cranes, determine the influences of age, time of season, and inbreeding on semen quality. I hypothesized that (1) bird age influences seminal quality; (2) sperm production would reach the peak level at the mid-breeding season and (3) there exists a negative correlation between inbreeding and seminal quality.

Methods

Semen Collection and Assessment

All individuals used in this study were housed at PWRC in Laurel, Maryland. Semen samples were collected from adult males (n = 29) by the established manual stimulation method (Gee 1983, Ellis et al. 1996, Chen et al. 2001) during early (April 1 and March 30), mid (April 28 and April 21), and late (May 12 and May 11) stages of 2 breeding seasons (2009 and 2010, respectively).

Samples were assessed for volume, concentration, pH, osmolarity, motility, progressive motility score, percent normal morphology (sperm with no visible anatomical defect), total sperm, total motile sperm, and total normal sperm. Sperm concentration was determined using a Neubauer hemocytometer (Mortimer 1994, Ellis et al. 1996, Penfold et al. 2001). The pH of each sample was assessed by using pH indicator strips (ColorpHast, EM Science, Gibbstown, NJ). Osmolarity was evaluated using a vapor pressure osmometer (Westcor Inc. Logan, UT). Motility was determined under a microscope as percent of total sperm that were motile (Ellis et al.

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1996, Penfold et al. 2001). Progressive Motility Score (PMS) was determined and scored on a range of 0 to 5, 0 having no forward movement, and 5 having rapid forward movement (Penfold et al. 2001). For morphology assessment, 2 to 5 □l samples were fixed with 0.3% gluteraldehyde. The fixed sample of each ejaculate was smeared on two slides, stained with eosin-nigrosin (Penfold et al. 2001), and 200 sperm were evaluated per slide. Morphological abnormalities included defects of the head, acrosome, mid-piece, and tail (Gee 1983, Penfold et al. 2001). For samples that had too small a volume to perform all analyses, priority was given to the evaluation of volume, concentration, motility, and morphology. Finally, total sperm per ejaculate (volume x concentration), total motile sperm per ejaculate (total sperm x motility), and total normal sperm per ejaculate (total sperm x percent normal morphology) were calculated.

Inbreeding Calculation

Inbreeding coefficients have been calculated for all members of the captive population. Microsatellite DNA genotypes across 12 loci were available as described in Jones et al. (2002). The program KINGROUP (Konovalov et al. 2004) was used to calculate pairwise kinship coefficients among the founder population using the Goodnight and Queller (1999) estimator. In addition to kinships, the program COANCESTRY (Wang 2011) was used to calculate Ritland's inbreeding coefficient (Ritland 1996) on the founders via the microsatellite DNA genotypes. The inbreeding and kinships among the founders were integrated into the captive genetic analysis using the captive genetic software PMx (Lacy et al. 2012) that now integrates the GENES algorithms used previously in this population. Inbreeding coefficients of

male breeders were calculated directly from their DNA profiles, or from subsequent pedigree analysis using the empirical kinships and inbreeding of their parents. These inbreeding coefficients enabled the inclusion of estimated relatedness values for existing male founders within this population.

Statistical Analysis

Separate linear mixed model sets were created for each of the different primary variables examined (inbreeding, age, time of season) and each of the response variables: ln(total sperm), percent normal sperm, percent motility, ln(total motile sperm), and ln(total normal sperm). Based on visual assessment of plots, total sperm per ejaculate, total normal sperm per ejaculate, and total motile sperm per ejaculate were log-transformed to improve normality.

In all models, individual served as a random effect; in this way we accounted for repeated samples from individuals. In addition, we considered other fixed variables in each of the model sets in addition to the main variable of interest (inbreeding, age, or time of season), because of their potential to influence the results of sampling. These included (1) if the male was housed with a female mate (Housing), (2) if the individual had experience with semen collection prior to this study (Experience), and (3) if the individual was currently being used as a semen donor during the study period (AI). We considered all combinations of these fixed effects (Housing, Experience, AI, and one of the main variables) to build the model sets, though the random effect of individual was included in all models. All analyses were run using the lme4 library in the R programming language (Bates et al. 2012).

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As per information-theoretic model selection and inference methods, Akaike's

information criterion (AIC) was calculated for each model (Burnham and Anderson

2004); the model within a model set with the lowest AIC value is thought to be the

most parsimonious model for the data. Akaike weights were calculated as

probabilities that each model is the best fit to the data using the following equation:

$$w_i = \frac{\exp(-\Delta_i/2)}{\sum_{r=1}^{R} \exp(-\Delta_r/2)}$$

Where w_i is the Akaike weight, Δ_i is the given model's AIC minus the minimum AIC

(AIC_{min}) of all models considered, and R equals total number of models. We also

calculated

relative importance values for each predictor variable within a model set by summing

the weights for all models containing that variable.

Results

There were large variations in volume, concentration, and total sperm output

among individual males (Table 1, Appendix A). Additionally, variation in seminal

output was observed within an individual male across multiple collections. Large

variation among males in percent motile sperm was also observed, with some samples

having no motile sperm and some as high as 95% motility. Normal sperm were

characterized as cells with uniform width and shape, intact acrosomes, and lacking

any defects to midpiece and tail (Fig. 1a and b). The most common abnormalities

were head size abnormalities, specifically macrocephalic (Fig. 1b) and microcephalic

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Table 1: Mean ± standard error of the means (SEM) seminal characteristics of whooping crane semen. Samples obtained in 2009-2010 breeding seasons (total = 88 ejaculates).

Seminal Characteristic	N	Mean ± SEM	Range
Volume (µl)	88	61.6 ± 7.3	0.15 – 350
pH	80	7.9 ± 0.1	6.5 - 9.5
Osmolarity (mOsm)	55	303.8 ± 8.2	104 – 445
Concentration (10^6/ml)	71	180.4 ± 43.9	0.4 – 2585
Total Sperm (x 10^6)	71	14.7 ± 4.43	0.0008 - 224.25
Motility (%)	80	44.6 ± 2.3	1 – 95
Progressive Movement Score	80	1.9 ± 0.05	1 – 3
% Normal Morphology	71	65.4 ± 2.3	12 – 94

Figure 1: Observed normal sperm (A) and sperm defects (B-F). Head defects included macrocephalic (B, arrow), and microcephalic (C), deformed head (D). The most common acrosomal defect was a missing acrosome (E) and the most common midpiece defect was a bent midpiece (F). Cells viewed under 1000x magnification.
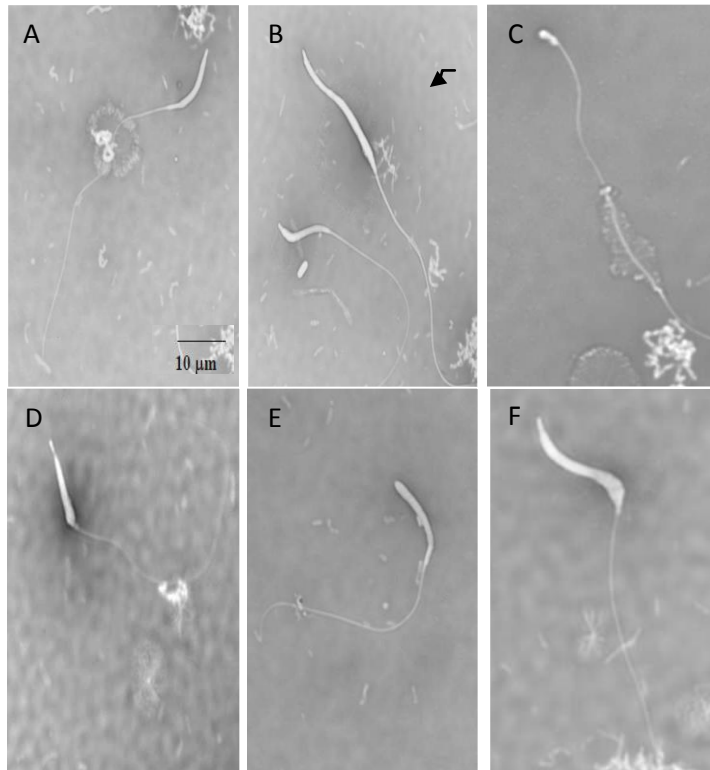
(Fig. 1c), as well as deformed heads (Fig. 1d); other morphological abnormalities observed included missing acrosomes (Fig. 1e) and bent mid-piece (Fig. 1f).

Top-ranked models, as well as each variable's relative importance, the effect estimate (from the top-ranked model in the set containing that variable), and 95% confidence intervals are presented for inbreeding (Table 2), age (Table 3), and time of season (Table 4). Values for all models examined, with corresponding AIC values, log(likelihood) values, and AIC weights are provided in Appendix A.

Inbreeding Effects

The top-ranked models for each measure of quality for the inbreeding model sets did not include the inbreeding effect; inbreeding had no observable impact on the five response variables examined (Table 2). The 95% confidence intervals for the estimated effect of inbreeding on each seminal characteristic included zero.

Age Effects

The top-ranked models selected for the age model sets for each measure of quality did not include the effect of age (Table 3). Thus males within the PWRC population showed little variation in semen quality as a function of age.

Time of Season Effects

Models selected for the time of season model sets indicated an effect of time of season on ln(total sperm per ejaculate), the percentage of sperm with normal morphology, and ln(total motile sperm per ejaculate; Table 4). Specifically, seminal

Table 2: Analysis results for the effect of inbreeding on seminal quality. Results include the models selected for each of the five response variables examined; the relative importance of the variables, the best effect size estimate for each variable, and the 95% confidence interval for the best estimate: ln(total sperm), percent normal sperm, percent motility, ln(total motile sperm), and ln(total normal sperm).

Response Variable Top Model	ln(Total Sperm per Ejaculate) ln(Total Sperm) = 1|Ind	Relative Importance[a]	Best Estimate[b]	95% CI (Lower, Upper)
	Inbreeding*	0.12	0.04	(-0.03 , 0.33)
	Experience*	0.43	0.43	(-0.15 , 1.01)
	Artificial Insemination*	0.25	0.17	(-0.44 , 0.78)
	Housing Situation*	0.27	-0.22	(-0.85 , 0.41)
Response Variable Top Model	Normal Sperm (%) Normal Sperm= 1|Ind			
	Inbreeding*	0.12	0.01	(-0.24 , 0.26)
	Experience*	0.47	-0.49	(-0.98 , 0.00)
	Artificial Insemination*	0.24	-0.33	(-0.82 , 0.16)
	Housing Situation*	0.20	-0.01	(-0.58 , 0.56)
Response Variable Top Model	Motility (%) Motility= 1|Ind + AI			
	Inbreeding*	0.25	0.19	(-0.03 , 0.41)
	Experience*	0.29	0.33	(-0.12 , 0.78)
	Artificial Insemination	0.48	0.42	(-0.03 , 0.87)
	Housing Situation*	0.31	-0.29	(-0.76 , 0.18)
Response Variable Top Model	ln(Total Motile Sperm per Ejaculate) ln(Total Motile Sperm) = 1|Ind			
	Inbreeding*	0.16	0.15	(-0.10 , 0.40)
	Experience*	0.39	0.43	(-0.12 , 0.98)
	Artificial Insemination*	0.39	0.42	(-0.15 , 0.97)
	Housing Situation*	0.31	-0.29	(-0.86 , 0.28)
Response Variable Top Model	ln(Total Normal Sperm per Ejaculate) ln(Total Normal Sperm) = 1|Ind			
	Inbreeding*	0.14	0.06	(-0.25 , 0.37)
	Experience*	0.34	0.34	(-0.29 , 0.97)
	Artificial Insemination*	0.26	0.19	(-0.44 , 0.82)
	Housing Situation*	0.25	-0.15	(-0.80 , 0.50)

a) Relative Importance of each variable is calculated by summing the AIC weights for all models in which the given variable is present;
b) The best estimate is the estimate of the effect from the most parsimonious model for the explanatory variable, which may not be the top-ranked model.
Variables marked with an * were not included in the selected model for the response variable.

Table 3: Analysis results for the effect of age on seminal quality. Results include the models selected for each of the five response variables examined; the relative importance of the variables, the best effect size estimate for each variable, and the 95% confidence interval for the best estimate: ln(total sperm), percent normal sperm, percent motility, ln(total motile sperm), and ln(total normal sperm).

Response Variable Top Model	ln(Total Sperm per Ejaculate) ln(Total Sperm) = 1|Ind	Relative Importance[a]	Best Estimate[b]	95% CI (Lower , Upper)
	Age*	0.04	0.03	(-0.01 , 0.07)
	Experience*	0.43	0.43	(-0.14 , 1.00)
	Artificial Insemination*	0.25	0.17	(-0.44 , 0.78)
	Housing Situation*	0.27	-0.22	(-0.85 , 0.41)
Response Variable Top Model	Normal Sperm (%) Normal Sperm= 1|Ind			
	Age*	0.03	-0.02	(-0.06 , 0.02)
	Experience*	0.47	-0.49	(-0.98 , 0.00)
	Artificial Insemination*	0.24	-0.33	(-0.82 , 0.16)
	Housing Situation*	0.20	-0.01	(-0.58 , 0.56)
Response Variable Top Model	Motility (%) Motility= 1|Ind + AI			
	Age*	0.04	0.02	(-0.06 , 0.10)
	Experience*	0.30	0.33	(-0.12 , 0.78)
	Artificial Insemination	0.48	0.42	(-0.03 , 0.87)
	Housing Situation*	0.32	-0.29	(-0.76 , 0.18)
Response Variable Top Model	ln(Total Motile Sperm per Ejaculate) ln(Total Motile Sperm) = 1|Ind			
	Age*	0.08	0.04	(0.00 , 0.08)
	Experience*	0.39	0.43	(-0.12 , 0.98)
	Artificial Insemination*	0.38	0.42	(-0.15 , 0.97)
	Housing Situation*	0.31	-0.29	(-0.86 , 0.28)
Response Variable Top Model	ln(Total Normal Sperm per Ejaculate) ln(Total Normal Sperm) = 1|Ind			
	Age*	0.05	0.03	(-0.01 , 0.07)
	Experience*	0.34	0.34	(-0.29 , 0.97)
	Artificial Insemination*	0.26	0.19	(-0.44 , 0.82)
	Housing Situation*	0.25	-0.15	(-0.80 , 0.50)

a) Relative Importance of each variable is calculated by summing the AIC weights for all models in which the given variable is present;
b) The best estimate is the estimate of the effect from the most parsimonious model for the explanatory variable, which may not be the top-ranked model.
Variables marked with an * were not included in the selected model for the response variable.

Table 4: Analysis results for the effect of time of season on seminal quality. Results include the models selected for each of the five response variables examined; the relative importance of the variables, the best effect size estimate for each variable, and the 95% confidence interval for the best estimate: ln(total sperm), percent normal sperm, percent motility, ln(total motile sperm), and ln(total normal sperm).

		Relative Importance[a]	Best Estimate[b]	95% CI (Lower , Upper)
Response Variable	ln(Total Sperm per Ejaculate)			
Top Model	ln(Total Sperm) = Date + 1|Ind			
	Date Intercept	0.49	-0.29	(-0.66 , 0.08)
	Date 2[c]	-	0.13	(-0.44 , 0.70)
	Date 3[d]	**-**	**0.54**	**(0.09 , 0.99)**
	Experience*	0.43	0.43	(-0.15 , 1.01)
	Artificial Insemination*	0.26	0.17	(-0.44 , 0.78)
	Housing Situation*	0.28	-0.22	(-0.85 , 0.41)
Response Variable	Normal Sperm (%)			
Top Model	Normal Sperm = Date + 1|Ind			
	Date **Intercept**	**0.81**	**0.36**	**(0.01 , 0.71)**
	Date 2	-	-0.55	(-1.12 , 0.02)
	Date 3	**-**	**-0.66**	**(-1.13 , -0.19)**
	Experience*	0.51	-0.49	(-0.98 , 0.00)
	Artificial Insemination*	0.29	-0.33	(-0.82 , 0.16)
	Housing Situation*	0.20	-0.01	(-0.58 , 0.56)
Response Variable	Motility (%)			
Top Model	Motility= 1|Ind + AI			
	Date* Intercept	0.14	-0.13	(-0.46 , 0.20)
	Date 2	-	0.39	(-0.26 , 1.04)
	Date 3	-	0.16	(-0.33 , 0.65)
	Experience*	0.30	0.33	(-0.12 , 0.78)
	Artificial Insemination	0.50	0.42	(-0.03 , 0.87)
	Housing Situation*	0.33	-0.29	(-0.76 , 0.18)
Response Variable	ln(Total Motile Sperm per Ejaculate)			
Top Model	ln(Total Motile Sperm)= Date + 1|Ind			
	Date Intercept	0.62	-0.33	(-0.72 , 0.06)
	Date 2	-	0.25	(-0.38 , 0.88)
	Date 3	**-**	**0.62**	**(0.13 , 1.11)**
	Experience*	0.37	0.43	(-0.12 , 0.98)
	Artificial Insemination*	0.42	0.42	(-0.15 , 0.97)
	Housing Situation*	0.34	-0.29	(-0.86 , 0.28)
Response Variable	ln(Total Normal Sperm per Ejaculate)			
Top Model	ln(Total Normal Sperm)= 1|Ind			
	Date* Intercept	0.16	-0.14	(-0.13 , 0.27)
	Date 2	-	-0.07	(-0.68 , 0.54)
	Date 3	-	0.33	(-0.18 , 0.84)
	Experience*	0.35	0.34	(-0.29 , 0.97)
	Artificial Insemination*	0.26	0.19	(-0.44 , 0.82)
	Housing Situation*	0.25	-0.15	(-0.80 , 0.50)

a) Relative Importance of each variable is calculated by summing the AIC weights for all models in which the given variable is present; b) The best estimate is the estimate of the effect from the most parsimonious model for the explanatory variable, which may not be the top-ranked model; c) Date 2 is calculated as the difference from the intercept or Date 1; d) Date 3 is calculated as the difference from the intercept

Variables marked with an * were not included in the selected model for the response variable.
Variables in bold have a confidence interval that does not overlap zero.

output increases throughout the season, with the late stage having the highest ln(total sperm per ejaculate). Percentage of sperm with normal morphology was the highest in the early breeding season, while normal morphology was the lowest in late season. Finally ln(total motile sperm) was the highest in late breeding season.

Discussion

The present study is the first to systemically assess seminal characteristics of whooping cranes, and examine the influences of inbreeding coefficient, age, and stages of breeding season on seminal quality in this species. Through my study, (1) I was unable to detect effects of inbreeding coefficient or age on seminal quality in whooping cranes and (2) I determined that stage of breeding season effects sperm output (total sperm per ejaculate) as well as specific sperm characteristics (percent normal morphology). The present study provides increased knowledge of sperm quality that is crucial for improving genetic management, knowing which birds to collect from and when, and developing cryopreservation protocols. These results also advance the basic knowledge of reproductive physiology in whooping cranes.

Average osmolarity and pH reflect values previously published for whooping cranes and other crane species (Gee et al. 1985). My data show that seminal characteristics of the whooping cranes are comparable to those reported for greater sandhill cranes (Chen et al. 2001).

Although low heterozygosity has been demonstrated to have detrimental effects on semen quality in many mammalian and avian species, we found no direct relationship between inbreeding coefficient and seminal characteristics. This may be caused by the overall high level of background inbreeding in whooping cranes, such

that the species has little remaining genetic variation (Fig. 2). Also, it is possible that the measure of inbreeding we used is not adequately sensitive, as values are based on information developed using only 12 microsatellites (Jones et al. 2002) and that high levels of of individual variation may mask effects of inbreeding (Holt et al. 2003). Finally, it is possible that whooping crane sperm characteristics are simply resistant to inbreeding effects.

If it was possible to compare seminal quality in whooping cranes that had not experienced the rapid population decline that the current population has experienced, effects similar to what has been observed in the greater prairie chicken (*Tympanuchus cupido*) might be seen. Bouzat et al. (1998) showed that in an individual population, a demographic bottleneck caused a marked decline in heterozygosity and reduced allele frequency, with fertility and hatching rates dropping from 93% to 56% compared to other populations that had not experienced the same bottleneck. As it is not possible to compare semen quality to pre-bottleneck whooping cranes, it would be interesting to see if these results were repeatable in other crane species that have larger populations in the wild, with small isolated populations in captivity serving as a model of a population bottleneck.

In the present study, we demonstrated that sperm output is influenced by the stage of breeding season; the lowest sperm output (total sperm) was obtained during early breeding season and sperm output increased with each subsequent collection date. These findings are not surprising as the majority of individual cranes do not begin breeding and finish breeding at the same time and thus more individuals would be at similar points of production at mid and late dates, and thus, more individuals

Figure 2: Normalized values for inbreeding coefficients of PWRC males as calculated by COANCESTRY program. Values are normalized to the mean and deviations above or below the mean are indicated by positive or negative values respectively. Each data point corresponds to one sampled male in the PWRC population.
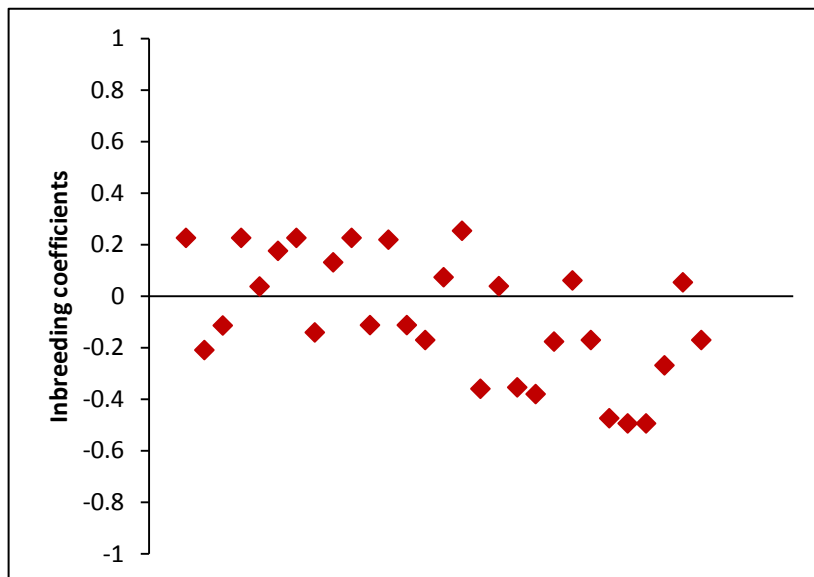
would be putting their energy toward gamete production during these dates. A similar pattern was reported in the Northern pintail duck, where high semen production was correlated with peak testosterone production in mid May, during the middle and end of the breeding season (Penfold et al. 2000). This pattern has been observed in other avian species as well, including blue rock pigeons (Sontakke 2004), and sharp tailed grouse (Tsuji et al. 2000).

In contrast to sperm output, we observed significant reductions in the proportion of structurally normal sperm during the mid and late breeding season compared to early breeding season. This may be driven by some males that were not using sperm as quickly as others. Jones and Leighton (1987) showed that breeding male turkeys, which were subject to frequent semen collections, had lower instances of sperm abnormalities; these authors also speculated that frequent collections/copulations may be necessary to ensure continued semen quality. Birkhead (1991) showed that Bengalese finches quickly deplete sperm reserves after just three copulations; this prevents the degradation of sperm that would occur if they remained in the seminal glomerula. Since some PWRC male are not paired or are prevented from breeding because of management reasons, they are not utilizing the sperm they are producing. With males that are used for AI but are also able to copulate naturally, there is no way to determine the number of copulations performed by each male, so there is no way to establish the sperm use of these males. Perhaps a decrease in percent normal sperm over the breeding season was observed because all males are producing sperm, but only some of them are able to use the sperm produced.

Many species exhibit consistent sperm motility across the breeding season (Spinks et al. 1997, Studen 1998, Sayyed et al. 2008). My results showed that motility did not change substantially across the breeding season. This pattern was also observed in mallard ducks examined by Stunden et al. (1998), where motility did not differ throughout the season. This pattern has also been shown in other non-avian species, including the common mole rat and the barbus fish, where volumes differed across the breeding season, but motility remained the same (Spinks et al. 1997, Sayyed et al. 2008). The only effect of age that was observed involved total motile sperm per ejaculate, with older males having more motile sperm per ejaculate than younger males. However, this may be a spurious effect caused by a few older males who happened to have high values.

Advances in the basic reproductive knowledge of a species held in captivity have enhanced the management of *ex situ* and *in situ* populations in many wildlife species. Although reproductive management has already played an important role in the recovery of whooping cranes during the past 40 years, the findings obtained from this research will lay a foundation for future research focused on improving genetic management of captive whooping cranes, through the further development of cryopreservation protocols and potentially changing collection protocols, and understanding the causes of poor reproductive success of the PWRC population.

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Chapter 3: Abnormal Gonadal Hormone Production is Associated with Decreased Reproductive Behaviors and Egg Production in Female Whooping Cranes

Introduction

The whooping crane (*Grus americana*) is listed as endangered by the International Union for Conservation of Nature (IUCN 2012). Currently, there are approximately 437 cranes living in the wild in four distinct populations, three of which were established through the release of captive born birds and do not yet have a positive growth rate (CWS and USFWS 2009). Due to the small size of the reintroduced and wild populations and continuing threats to wild birds, it is important to sustain a population of whooping cranes *ex situ*, largely 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.

Captive propagation of whooping cranes began in 1967. To create a captive population, one of two fertile eggs was collected from wild clutches at Wood Buffalo National Park in northern Canada and sent to Patuxent Wildlife Research Center (PWRC) in Laurel, MD (Kuyt 1996). These eggs were hatched and chicks were recruited into the *ex situ* breeding program. Since then, four more *ex situ* breeding centers have been established at the International Crane Foundation in Baraboo, Wisconsin; Audubon Zoo in New Orleans, Louisiana; San Antonio Zoo in San Antonio, Texas; and Calgary Zoo in Calgary, Alberta (CWS and USFWS 2009). Through a collaborative effort by all of the captive breeding centers, an average of 19 chicks has been released to the wild each year from 2001 to 2012 (J. Chandler, pers. communication). Although chicks have been produced and released each year, reproductive success in the captive population has been poor, based on comparisons

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with wild populations. In the Aransas-Wood Buffalo population (AWBP), nests examined from 1967-1995 had an average fertility rate of 95% and a known viability rate of 73% (Kuyt 1995). A viable egg was defined as one with a live embryo at the time of examination as determined by an egg immersion test. The AWBP has grown over the last 70 years without supplementation from captive sources (CWS and USFWS 2009). At PWRC, the largest captive breeding center, reproduction is characterized by low fertility (<60%) and delayed onset of reproduction, especially in females (7 years vs. 5 years *in situ*; Gee and Russman 1996). As a primary objective of the captive breeding centers is to maximize chick production for the reintroduction program, it is important to better understand basic reproductive biology of the whooping crane and to identify causes of poor reproduction in *ex situ* birds.

Whooping cranes are seasonal breeders (Archibald and Lewis et al. 1996), as are many other avian species (Cherel et al. 1994, Hirschenhauser et al. 1999, Penfold et al. 2000, Leska and Dusza 2007). Seasonality in birds is regulated by both photoperiod and temperature (Dawson et al. 2001), which initiate hormone production by the hypothalamus-pituitary-gonadal axis (Lui et al. 2001). Following the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released from the pituitary gland. These hormones regulate ovarian and testicular steroid secretions controlling gametogenesis.

In male birds, testosterone is the major androgen produced in the testes and is responsible for spermatogenesis (Penfold et al. 2000). In females, small follicles produce estrogen which acts as a positive feedback to the pituitary for the release of

FSH (Yang et al. 1997, Liu et al. 2001). As the follicles grow, progesterone production begins, and at the final stage of follicle development this steroid hormone stimulates the release of the preovulatory LH surge resulting in ovulation. A study utilizing non-invasive fecal hormone monitoring has shown that gonadal hormone metabolites gradually increase 10 days prior to the onset of egg laying and reach the peak level one day before ovulation in the Atlantic canary, *Serinus canaria* (Sockman and Schwabl 1999). After ovulation, estradiol immediately declines and reaches initial baseline levels within 3 days, whereas progesterone remains elevated for 2 days post-egg laying before returning to the initial low levels.

Stress has previously been indicated as a major cause of poor reproductive success in wildlife maintained *ex situ* (Beletsky et al. 1992, Zeigler and Snowdon 2000, Wielebnowski et al. 2002, Carlstead and Brown 2005, Angelier and Chastel 2009). In birds, corticosterone is produced by the adrenal medulla in response to variable physiological and environmental stimuli (Norris 2006). Although the secretion of corticosterone helps individuals cope with stressful situations, persistent elevation of this hormone (chronic stress) suppresses reproduction in an attempt to preserve body condition and maintain homeostasis (Landys et al. 2006, Angelier and Chastel 2009). Ouyang et al. (2011) showed that individual birds with high levels of corticosterone prior to the breeding season exhibited a delay in egg laying and produced fewer eggs and fledglings compared to individuals having low corticosterone levels. Corticosterone also has a large influence on both reproductive and parenting behaviors, as studies showing birds with high corticoid levels are more likely to abandon nests and eggs (Groscolas et al. 2008, Angelier et al. 2009).

The relationship between reproductive behaviors and hormones produced during the breeding season has been well established in a variety of avian species (Birkhead et al. 1987, Silver and Ball 1989, Beletsky et al. 1992, Monfort et al. 1993, Sockman and Schwabl 1999, Angelier et al. 2006, Angelier et al. 2009, Lobato et al. 2010, Ouyang et al. 2011). Reproductive behaviors in male quail have been shown to be androgen dependent, while female quail are influenced by both androgens and estrogen (Adkins and Adler 1972). However this link has not previously been explored in the whooping crane.

The role of hormones and the effect of stress on reproduction have not been previously studied in whooping cranes; however, their reproductive behaviors have been well documented. Reproductive behaviors in whooping cranes normally begin 2 to 5 weeks before the onset of egg production (Ellis et al. 1996). Breeding season courtship behaviors between established breeding pairs are highly elaborate and are performed in a specific order prior to copulation (Swengle et al. 1996, White 2000). While cranes have been seen copulating throughout the day, research has shown that the hour after sunrise is the most reproductively active time; copulation attempts are observed during this period more frequently than the rest of the day (White 2000). Any deviations in the courtship ritual or fewer instances of these behaviors may indicate breeding pair incompatibility or potentially represent decreased gonadal steroid production.

The purpose of this research was to determine possible causes for poor reproduction in whooping cranes and to develop a tool to assess how management decisions may influence reproductive success, based on proximal effects of

management on hormonal patterns. The hypothesis was that decreased reproductive

outcome is caused by increased adrenal activity, which would perturb gonadal

function and cause decreased gonadal steroid production as well as decreased

reproductive behavior performance. To date, whooping crane reproductive

endocrinology has not been researched; therefore, the first goal in this study was to

utilize non-invasive hormone assessment to longitudinally monitor gonadal hormones

of male and female whooping cranes. I planned to use this assessment to further

determine the relationship between gonadal and adrenal excretion and reproductive

behavior in successfully breeding vs. unsuccessful pairs.

Methods

Pair Selection

Twelve breeding pairs within the captive population at PWRC were divided

into three sampling strata based on their past fertility; (Class 1) Good ($n = 5$), pairs

that consistently produce fertile eggs every breeding season; (Class 2) Moderate ($n =$

5), pairs that produce a variable number of eggs from year to year, or consistently

produce fewer eggs than successful pairs and (Class 3) Poor ($n = 2$), pairs that have

never produced eggs or have a history of high infertility as a pair. Six pairs, two from

each class, were selected using a random number generator. During the course of this

study, a male in a class 2 pair died; therefore, a new pair of similar fertility was

selected in Year 2 (Table 1).

Of the pairs selected, there were three captive breeding strategies represented;

(1) Natural ($n = 4$): natural copulation was possible within the pair; (2) AI ($n = 1$): the

male was unable to copulate either because of a physical defect, injury, or wing-

clipping to prevent escape from pens without top nets, and the female was artificially inseminated with semen from her social mate or a donor male. Males in this category were also prevented from copulating if insemination from a donor male was desired; (3) Both ($n = 2$): natural copulation was allowed but the female was supplemented though AI with semen from her established mate or a donor male (Table 1).

Pairs were also housed in one of three types of pens. Breeding pens are defined as having overhead flight netting and photoperiod lights mimicking natural breeding season photoperiod. Four pairs were housed in breeding pens. Two pairs were housed in pens which had photoperiod lights but lacked flight netting, while one pair was housed in a pen lacking both photoperiod lights and flight netting during the course of the study (Table 1).

Sample Collection

Non-invasive monitoring of fecal hormone metabolites was used to assess gonadal and adrenal functions during two breeding seasons (2011 and 2012). Fecal samples were collected from each member of each pair 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) and Iron Oxide (Fe_2O_3) in capsules were delivered in lake smelt (*Osmerus mordax mordax*), as per normal husbandry practices, the day before collection in order to identify individual (e.g., male vs. female) fecal samples by color (Brown et al. In review, Appendix B). Samples were collected omitting the urate portion when possible, as it has been shown to be extremely dense and lacking in hormones, and could therefore bias results (Hayward et al. 2010). The collected samples

Table 1: Summary of egg production in the studied bird pairs during the 2011 and 2012 breeding seasons. A summary of reproductive output, breeding strategy, and housing type of all pairs utilized in this study. Dashes indicate when a pair was not included within the study.

Pair Identification	Class	2011		2012		Breeding Strategy	Pen Type
		# Eggs	# Fertile	# Eggs	# Fertile		
B12	1	1	1	1	0	Both	Breeding
S30	1	2	2	0	0	AI	Breeding
B16	2	4	2	-	-	Natural	Breeding
S26	2	-	-	0	0	Both	No Net
S32	2	3	0	0	0	Natural	Breeding
B7	3	0	0	0	0	Natural	No Net
O5	3	0	0	0	0	Natural	No Lights or Net

were labeled with individual ID, sex, and date of collection, and were stored in plastic

bags at -20° C until hormone extraction and analysis. Samples were not collected

when it was deemed too stressful or dangerous for the birds (e.g., after having just

laid an egg or while incubating eggs). Necessity of omitting sample collection was

determined by the PWRC flock manager and crane crew.

Hormone Extraction and Assay Analysis

Hormone extraction was performed using previously described methods

(Bishop and Hall 1991; Sockman and Schwabl 1999, Stanley et al. 2007) with slight

modifications. Briefly, we examined free hormones rather than conjugated hormones

that are bound as steroid glucuronide (Bishop and Hall 1991). Crane fecal samples

were freeze-dried and pulverized. For steroid hormone extraction, 0.2 ± 0.02 g of

pulverized sample was mixed with 5 mL of 70% ethanol (EtOH) and 30% distilled

water, then vortexed for 30 s, and vigorously shaken for 1 h (Glas-Col Large Capacity

Mixer). The samples were then centrifuged (500g for 20 min), and the supernatant

poured off into a clean glass tube. Another 5 mL of 70% ethanol 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 a phosphate buffered solution, vortexed, sonicated, and stored at -20°C until

hormone analysis (Brown 2008). When necessary based on a sample's mean binding

within the assay, samples were diluted with a phosphate buffered solution (estradiol:

1:1 to 1:2; corticosterone: 1:5 to 1:20; testosterone: 1:10 to 1:50; progesterone: 1:10

to 1:75). A mean extraction efficiency (84.7%, $n = 1,301$ samples) was determined

by assessing the recovery of radiolabeled ($[^3H]$) cortisol added before hormone extraction.

As these methods had not been previously validated in the whooping crane, we performed standard validations for each assay by: (1) demonstrating the efficiency and specificity of antibodies depicted as parallel displacement between the assay's standard curve and a serially diluted, pooled fecal extract; and (2) measuring the accuracy of the assay through percent recovery of exogenous hormone added to a pooled fecal extract (Brown 2008, Appendix C). A biological validation of the corticosterone assay was also performed to ensure that assessed hormone concentrations reflect adrenal responses to stressful events. Adult birds were housed individually and fecal samples were collected at intervals over the course of 96 hrs before and after a stressful event (annual health exams). Results from this validation illustrated that the peak fecal corticosterone concentration could be observed 24 h after a stressful event (Fig. 1).

All samples were evaluated for gonadal (males: testosterone; females: estrogen and progestagen) and adrenal (males and females: corticosterone) hormone metabolites. Concentrations of progestagen and androgen metabolites were quantified using enzyme immunoassays. Antibodies for pregnane (monoclonal pregnane CL425) and testosterone (polyclonal R156/7) were obtained from Coralie Munro (University of California, Davis, CA, USA). Samples were run in duplicate according to assay specifications (Brown 2008). CL425 is cross reactive 100% with 4-pregnen-3,20-dione (progesterone). It also binds highly to six other progesterone metabolites: 4-pregnen-3α-ol-20-one (188%), 4-pregnen-3β-ol-20-one (172%), 4-

Figure 1: Corticosterone biological stress validation for four adult whooping cranes. A biological stress validation was performed on four adult whooping cranes. Fecal samples were collected at specific time intervals before and after time zero, which indicates stressful event (health exam).
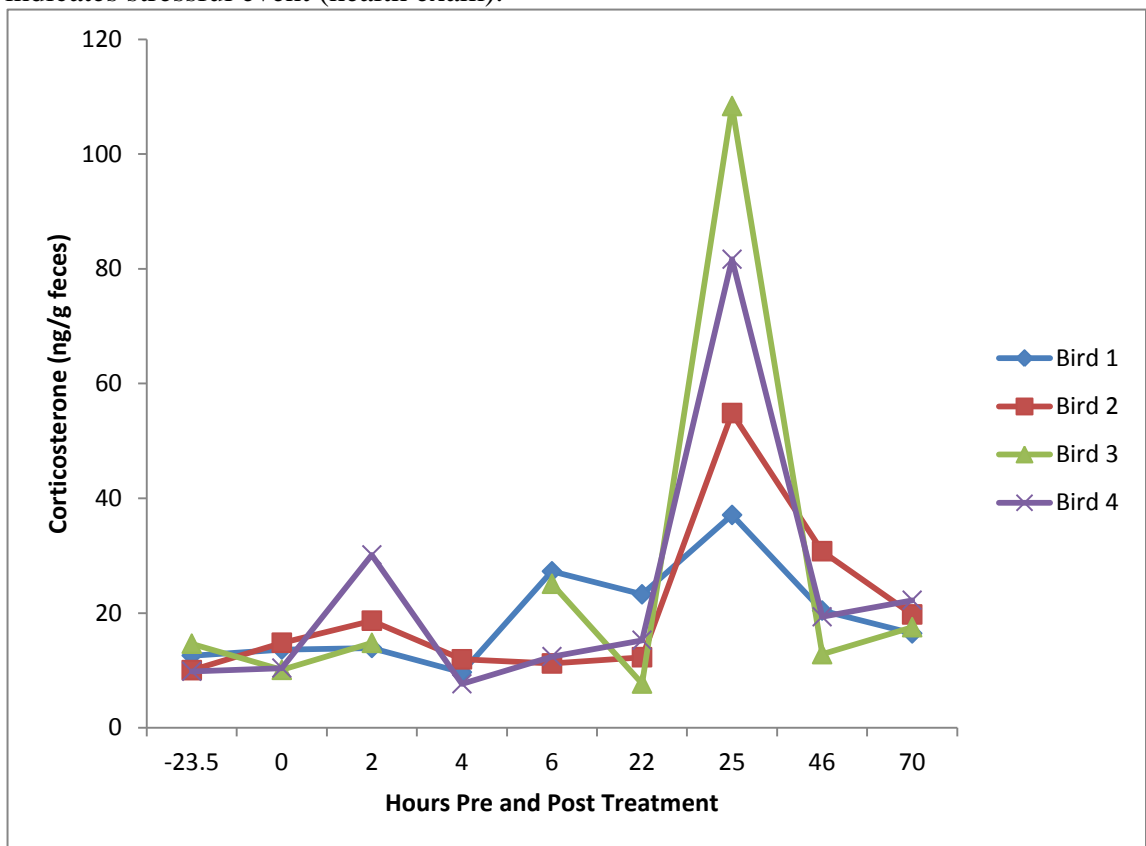
pregnen-11α-ol-3,20-dione (147%), 5α-pregnan-3β-ol-20-one (94%), 5α-pregnan-3α-ol-20-one (64%), 5α-pregnan-3,20-dione (55%). All other metabolites cross-reacting with the antibody were below appreciable levels. The testosterone antibody is cross reactive 100% with testosterone and 57, 37% with 5α-dihydrotestosterone and all other metabolites recognized by the assay are below recognizable levels. An inter assay variation of 10% was maintained for both assays and an intra assay variation of 13% (n = 31 assays) and 15% (n = 44 assays) was maintained for pregnane and testosterone, respectively.

Estradiol metabolites were analyzed using radio immunoassay (RIA) for 17 β estradiol, and corticosterone metabolites were analyzed using a corticosterone RIA kit, both obtained from MP Biomedicals (Santa Ana, CA). Samples were run in duplicate according to assay specifications. The estradiol antibody cross-reacts 100% with estradiol-17 β, and 20% with estrone. All other metabolites were below recognizable levels. Corticosterone antibody cross-reacts 100% with corticosterone, and all other metabolites were below detectable levels. An inter assay variation of 10% was maintained for both assays, while an intra assay variation of 13% (n = five assays) and 16% (n = 13 assays) was maintained for estradiol and corticosterone, respectively. For both EIAs and RIAs, data were expressed as mass units of hormone per gram of feces. Means and standard deviations were calculated for each hormone. Baseline values for each individual in each season were calculated using an iterative process where values equaling the mean plus 1.5 standard deviations were excluded, and then the process was repeated.

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). Crane behaviors were video-recorded for a total of 75 min beginning 15 min before sunrise and continuing for an hour after sunrise. Sunrise times were determined for Laurel, MD using SunriseSunset database (Edwards 2012). Behaviors were assessed using an ethogram modified from those previously described in Nelson et al. 1995, White 2000, and Happ and Happ 2011 (Appendix C). Continuous focal group sampling was utilized, as pairs were watched together and exact times and durations of behaviors were recorded for both males and females (Mann 1999). Specific reproductive behaviors (marching, unison calling, wing solicitation, mounting, and treading) were each recorded separately. Standing, walking, preening, and other non-reproductive behaviors were all included under 'Active, non-reproductive' whereas behaviors such as sleeping, laying on ground, and hock sitting were identified as 'non-active' behaviors. Time spent out of view was recorded as 'out of camera'. Time that the pair spent together or apart was also recorded. 'Pair Together' was defined as one member of the pair being within three body lengths of the other.

Statistical Analysis

To evaluate the differences between successful and unsuccessful pairs (with determination of success specific to a given breeding season, and where a successful pair is a pair that lays at least 1 egg), the overall hormonal profiles, hormone patterns associated with egg laying, and behavioral profiles of both groups were compared.

For purposes of analysis we did not analyze pairs based on their original sampling strata (good, moderate, poor); in fact we found that there was not a clear association between the sampling stratum of a pair and egg production during each breeding season. Instead, we classified each pair in each breeding season (total of 12 hormone and behavioral profiles) into laying ($n = 5$) and non-laying pairs ($n = 7$); hereafter, we refer to this as reproductive outcome.

Hormone profiles were compared by calculating the difference in means, standard deviations, baselines, and number of days above baseline levels for each hormone. PROC Mixed and Fischer's Least Significant Difference test (SAS Institute Inc., Cary, NC) were used for data analysis to compare seasonal reproductive hormone values, where egg laying (whether or not the pair laid an egg during a season) was the main fixed effect, and bird pair served as random effect. We also examined how hormonal concentrations changed approaching an egg laying event. Longitudinal hormone profiles of gonadal steroids starting from the beginning of sample collection (Feb 1) were aligned with the day of egg laying (D0) for each female. On average, the interval between the onset of sample collection and the presence of the first egg was 75 days, thus, the same time frame was evaluated for non-laying seasons. PROC Mixed was used to compare daily hormone values as a function of reproductive outcome, again with pair serving as a random effect. A date by success interaction was examined to determine if hormone levels increased during the time approaching an egg laying event.

The daily proportion of time spent performing reproductive behaviors as compared to total active time was calculated as:

$$\frac{\text{Reproductive time}}{(\text{Reproductive time} + \text{Active non-reproductive time})}$$

Analysis of this proportion as a function of different reproductive outcomes within the pair and for each sex were performed using PROC Mixed, again with pair as a random effect, and Fisher's Least Significant Difference test was used for all pairwise comparisons. Specific reproductive behaviors were compared between reproductive outcomes. PROC Corr was then used to perform correlations between mean hormone concentrations and mean behavior proportion.

Because productivity was at a record low for PWRC during the 2012 season (J. Chandler, pers. communication), the effect of temperature on egg production was examined. Monthly averages for January through May for the past decade were obtained from Weather Explorer (WeatherSource 2012). Correlation analysis was used to determine differences between average monthly temperatures and the number of eggs laid at PWRC in a given year. I chose an overall alpha of 0.10 for all statistical analysis to protect against type II error due to the low number of experimental units.

Results

Egg production varied among pairs and between the 2 years within the same breeding pair (Table 1). In 2011, four pairs produced a total of 11 eggs, five of which were fertile. The two pairs that did not produce eggs were originally classified as Class 3 pairs (poor reproducers). The percentage of fertile eggs produced by each

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pair varied; however, fertility values corresponded to assigned reproductive class. Specifically, a total of four eggs were produced from Class 1 pairs, three of which (75%) were fertile, whereas seven eggs were laid by Class 2 pairs, with only two (29%) being fertile. Of the pairs studied in 2012, only one

pair (Class 1) produced a single egg of unknown fertility as the egg was broken by the pair. No other pairs produced an egg in 2012.

Female Endocrinology

Large variations within an individual's hormonal concentrations and patterns were observed, with some individuals fluctuating greatly between measurements without an obvious biological cause (Fig. 2). Specifically, some females exhibited progesterone peaks early and late in the season which did not accompany an oviposition.

Three patterns of gonadal hormone profiles were observed in females. The first pattern observed in seasons in which birds produced eggs was characterized by cyclical waves of gonadal hormones (estrogen and progestagen) associated with egg production (estradiol: mean = 587.9 ± 644.7 ng/g dried feces; baseline = 40.3; progesterone: mean = 82.3 ± 60.8 ng/g dried feces; baseline = 40.0). In these profiles, gonadal hormone gradually increased and reached the peak level one day prior to the onset of egg laying. After the clutch was laid, the hormone concentration decreased and remained at the baseline level until the females were ready to lay the subsequent clutch of eggs (Fig 2A). The second pattern observed in non-reproductive seasons was characterized by a rather steady level of gonadal hormones throughout the breeding season (Fig 2B estradiol: mean = 416.7 ± 447.9 ng/g dried feces;

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Figure 2: Representative profiles of female steriod hormone production in laying and non-laying pairs. Representative longitudinal hormone profile of three female steroid excretion patterns: A) an egg laying female exhibiting cyclical waves of estrogen and progestagen metabolites, B) a non-egg laying female exhibiting consistent concentration of gonadal steroids and C) a non-egg laying female showing consistently low estrogen throughout the sample collection period. Black vertical lines indicate egg laying dates for figure 2A. Dates = 4/2/2011, 4/17/2011, 5/5/2011, and 5/9/2011.
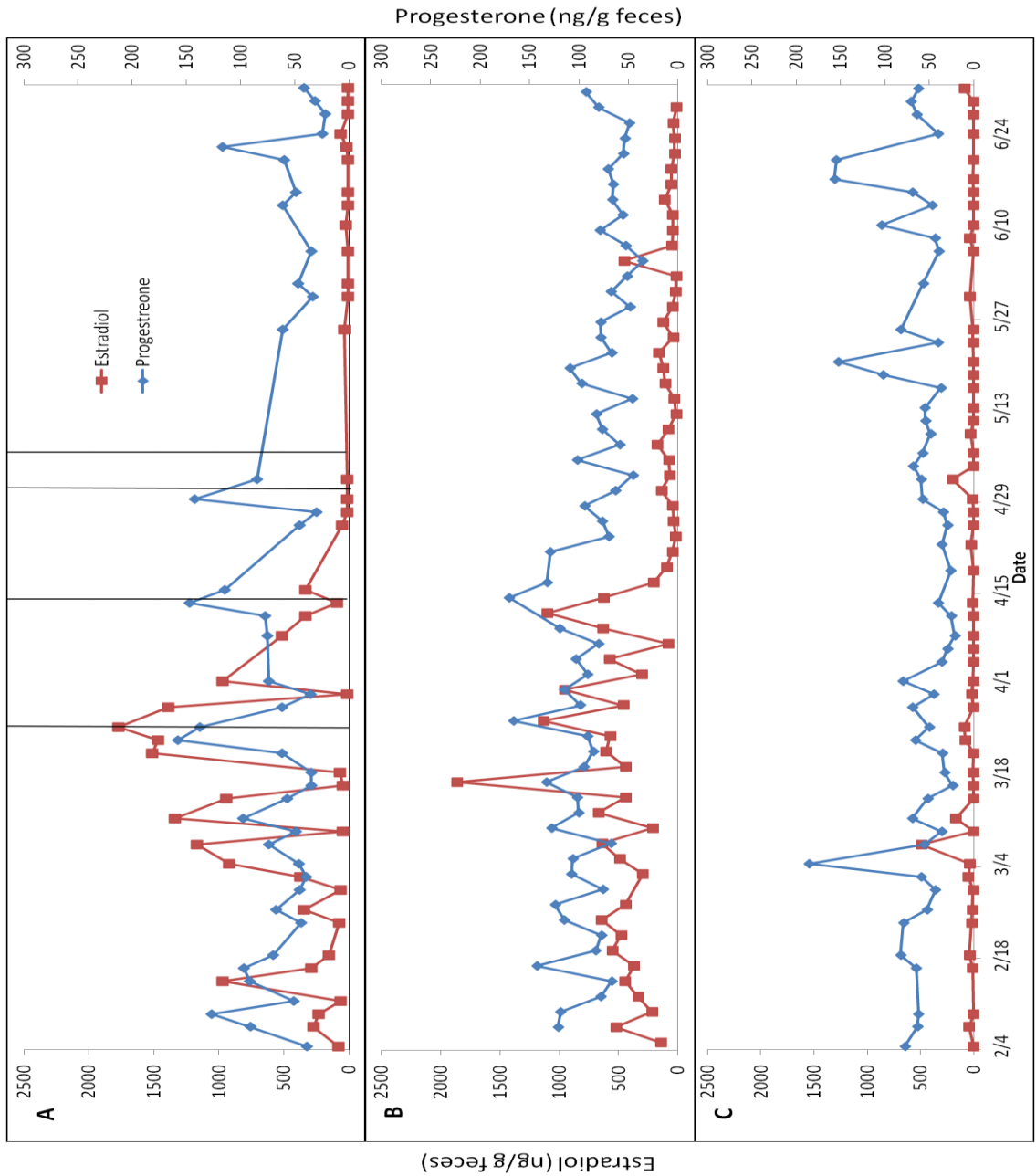
baseline = 26.9; progesterone: mean = 96.7 ± 53.2 ng/g dried feces; baseline = 47.6). The

third pattern seen in one female across both years, also associated with a non productive

breeding season, was characterized by low excretion of estrogen (Fig. 2C estradiol: mean

= 40.4 ± 86.9 ng/g dried feces; baseline = 6.2; progesterone: mean = 64.4 ± 26.7 ng/g

dried feces; baseline = 50.8). There were no differences in the pattern of corticosterone

among birds with different reproductive outcomes. All females exhibit periodic peaks of

adrenal throughout the breeding season, the majority of which could be linked to

management events, i.e. birds were moved or disturbance occurred nearby.

When looking specifically at egg laying events, estradiol concentrations for laying

seasons significantly increased in the days leading up to the egg laying date compared to

hormone concentrations of the same date in birds experiencing a non-laying breeding

season (Fig. 3A, p = 0.0449). There was also a significant interaction between date and

egg production in estradiol level (p = 0.0776), which indicates the rise in estradiol values

in laying females. Specifically, in seasons in which an egg was laid, there was an initial

rise in estradiol around 38 days prior to the onset of egg laying and the hormone

concentration remained elevated until oviposition. However, in breeding seasons where

no egg was laid, estradiol concentration remained consistent throughout the 75-day

period (Fig. 3A). There was no significant difference in progesterone levels between

laying and non-laying groups (Fig. 3B, p > 0.10).

Means, standard deviations, baselines, and number of days above baseline were

calculated for each hormone based on reproductive outcome (Table 2). Overall seasonal

mean estradiol for laying females was significantly higher than the non-laying group (p =

0.0630). Progesterone values showed significantly higher values for seasonal baseline in

Figure 3: Estradiol concentrations increase in laying females as compared to non-laying females approaching an egg laying event. Mean ± SEM (A) estradiol and (B) progestagen of from laying (n = 5) and non-laying (n = 7) seasons for the first 75 days of sample collection. Trend lines indicate the change in overall hormone concentration during the indicated dates.

Table 2: Female gonadal and adrenal steroid concentrations between laying and non-laying pairs. Mean ± SE, baseline ± SE hormone concentrations, and days above the baseline level ± SE of gonadal and adrenal steroids in females whooping cranes. Letter superscripts designate significant different value among reproductive classes at $\alpha < 0.10$ based on Fischer LSD.

Hormone	Female Estradiol			Female Progesterone			Female Corticosterone		
Parameter	Mean ± STD (ng/g feces)	Baseline ± STD	Days Above Baseline	Mean ± STD	Baseline ± STD	Days Above Baseline	Mean ± STD	Baseline ± STD	Days Above Baseline
Egg laying pairs	601.1[a] ± 585.7	114.2 ± 100.7	38[b] ± 2.2	80.3 ± 57.6	49.2[b] ± 11.7	34[b] ± 4.7	964.6 ± 440.2	689.7 ± 59.7	34[b] ± 4.3
Non-egg laying pairs	318.8[b] ± 308.8	120.1 ± 120.2	44[a] ± 5.5	88.0 ± 41.9	66.3[a] ± 21.9	41[a] ± 5.3	922.2 ± 450.8	707.2 ± 79.3	40[a] ± 4.1

non-laying females than laying females (p = 0.0780). Seasonal progesterone mean trended toward significance (p = 0.1130), again with non-laying females showing higher values than laying females. Interestingly, the numbers of days during which both estradiol and progesterone were elevated above the baseline level in non-laying seasons were more than in laying seasons (p = 0.0927 and 0.0780 respectively). Correlation analysis revealed that mean progestagen concentration was closely associated with mean estradiol excretion (p = 0.0424, r = 0.59). There were no significant differences in corticosterone means between laying and non-laying seasons, however again corticosterone values were elevated above baseline more days in non-laying females than in laying females (p = 0.0613).

Male Endocrinology

Unlike what was seen in females, there were no differences in mean, standard deviation, or baseline concentrations of gonadal hormone among male reproductive outcomes (Fig. 4, Table 3). However, the number of days above testosterone baseline was significantly higher in unsuccessful males compared to successful males (Table 3). However, similarly to females, no difference was observed between corticosterone values.

Behavior

Successful and non successful outcomes were compared based on proportion of reproductive behavior. Overall successful pairs showed a significantly higher proportion of reproductive behaviors than unsuccessful pairs (p = 0.0089; Fig. 5). When each sex was examined individually, successful males and females each
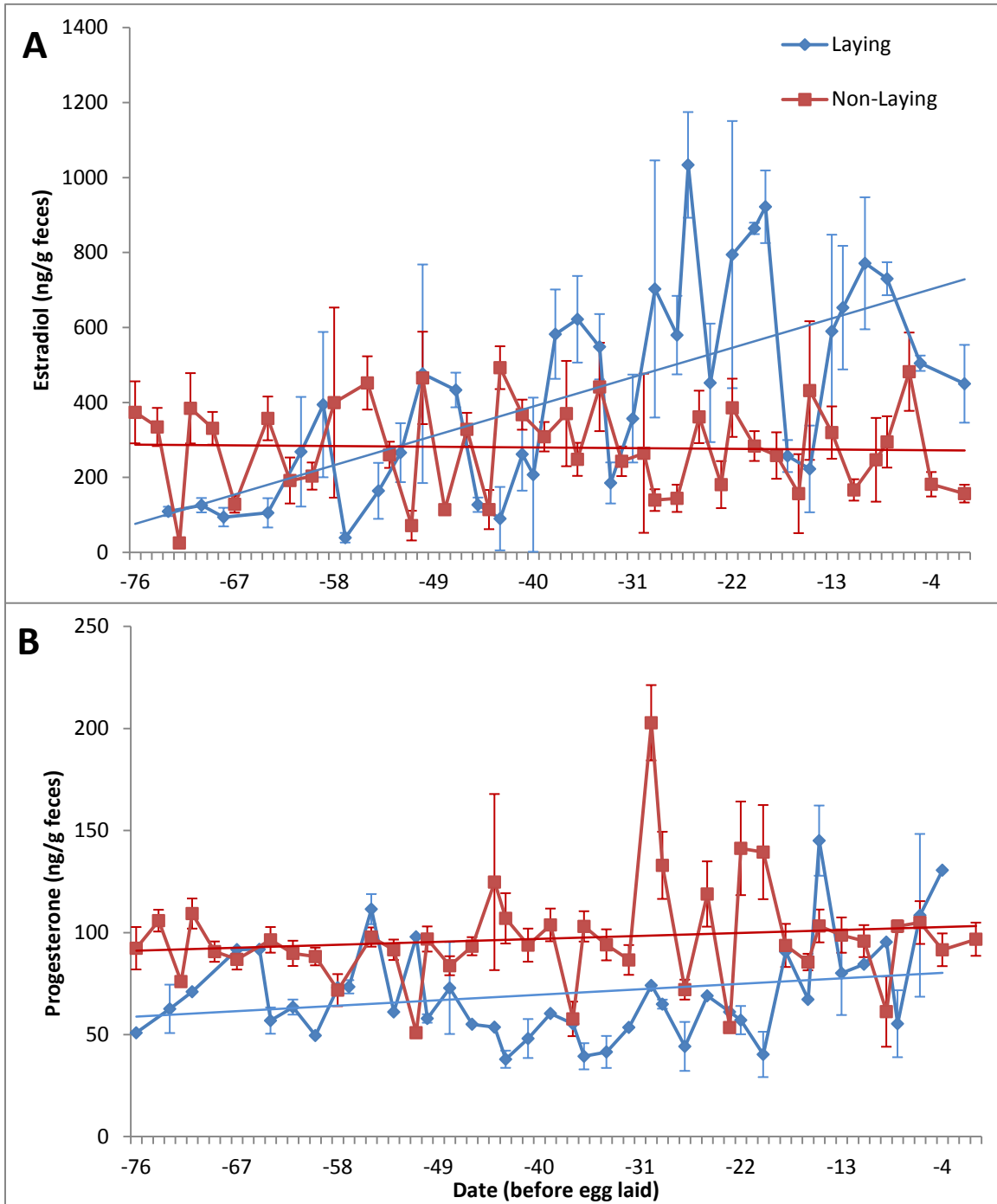
Table 3: Male gonadal and adrenal steroid concentrations between laying and non-laying pairs. Mean ± SE, baseline ± SE hormone concentrations, and days above the baseline level ± SE of gonadal and adrenal steroids in males whooping cranes. Letter superscripts designate significant different value among reproductive classes at $\alpha < 0.10$ based on Fischer LSD.

Hormone	Male Testosterone			Male Corticosterone		
Parameter	Mean ± STD (ng/g feces)	Baseline ± STD	Days Above Baseline	Mean ± STD	Baseline ± STD	Days Above Baseline
Egg laying pairs	179.6 ± 92.1	141.6[a] ± 16.4	32[b] ± 6.0	830.1 ± 333.7	714.2 ± 72.5	32 ± 4.8
Non-egg laying pairs	202.9 ± 110.7	116.9[b] ± 17.8	43[a] ± 5.6	777.8 ± 321.4	661.1 ± 50.9	37 ± 5.4

Figure 4: Representative profile of male steriod hormone production In laying and non-laying pairs. Representative longitudinal hormone profiles of two adult male whooping cranes: A) laying pair and B) non-laying pair. Black vertical lines indicate egg laying dates for figure 5A. Dates = 4/14/2011 and 5/4/2011.
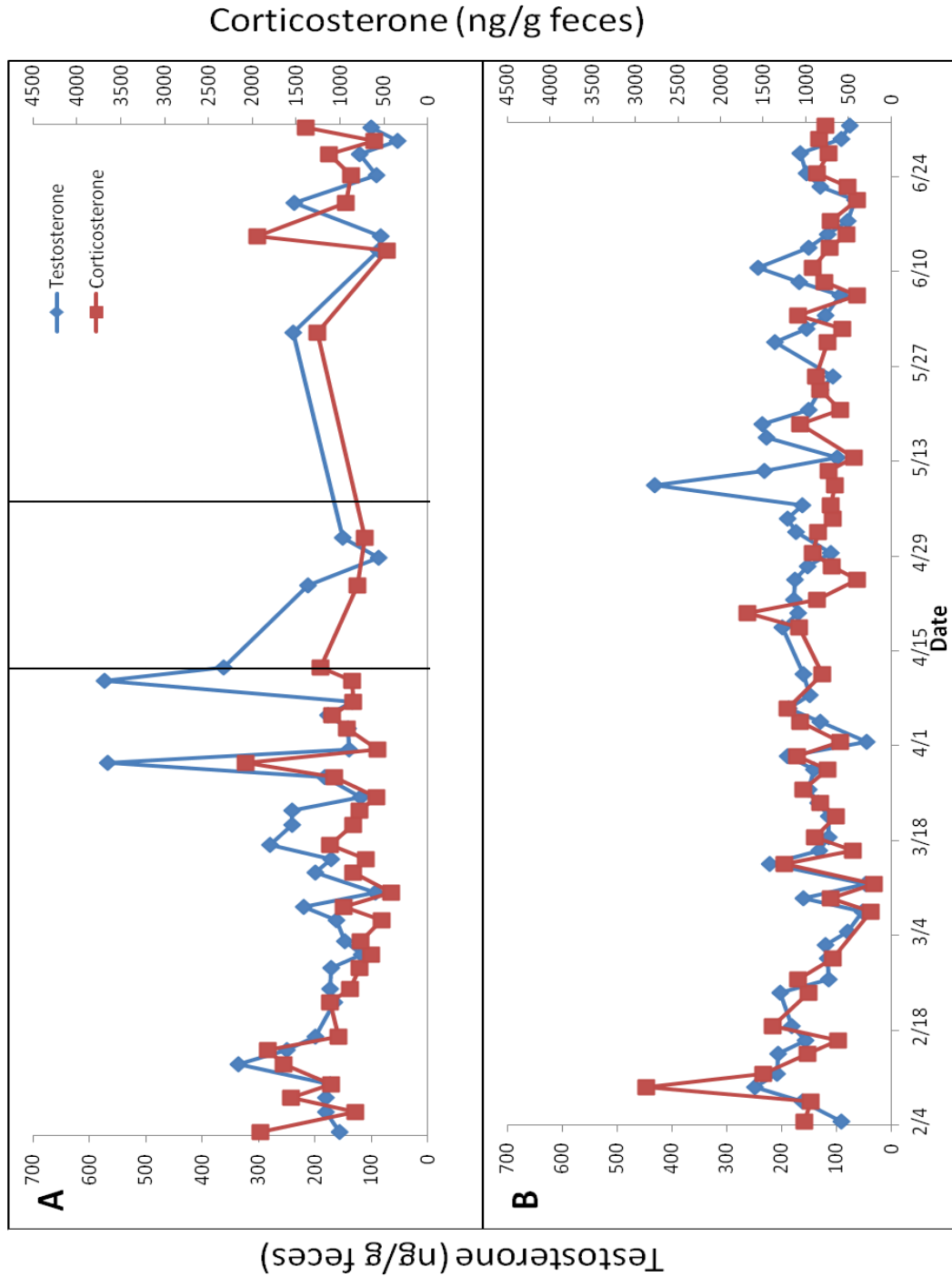
Figure 5: Proportion of reproductive behaviors of successful vs. unsuccessful pairs. Daily proportion of reproductive behaviors were compared between successful and unsuccessful pairs. Overall successful pairs had a higher proportion than unsuccessful. Individual sexes were examined and successful males were shown to have significantly higher proportions (p = 0.0073), while successful females trended towards significance (p = 0.1159).
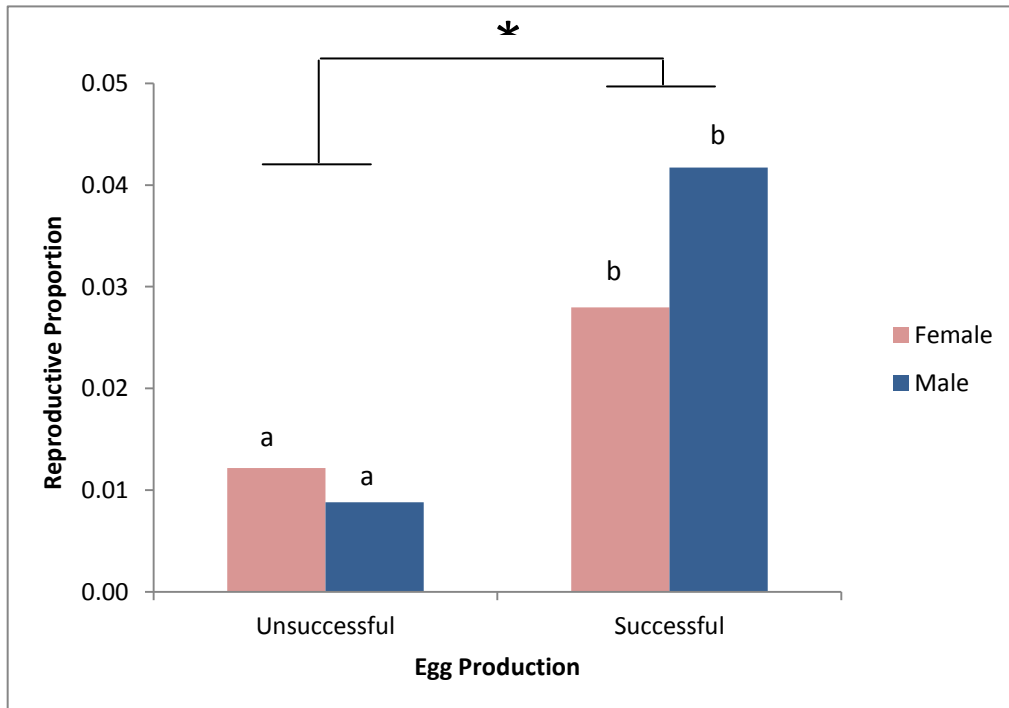
Figure 6: Reproductive behavior increases approaching an egg laying event in laying pairs. Proportion of time exhibiting reproductive behaviors across the breeding season. Representative graphs of A) egg laying pair, B) non-egg laying pair exhibiting reproductive behaviors, C) non- egg laying pair with few behaviors. Black vertical lines indicate egg laying dates for 6A: 4/2/2011, 4/17/2011, 5/5/2011, 5/9/2011.
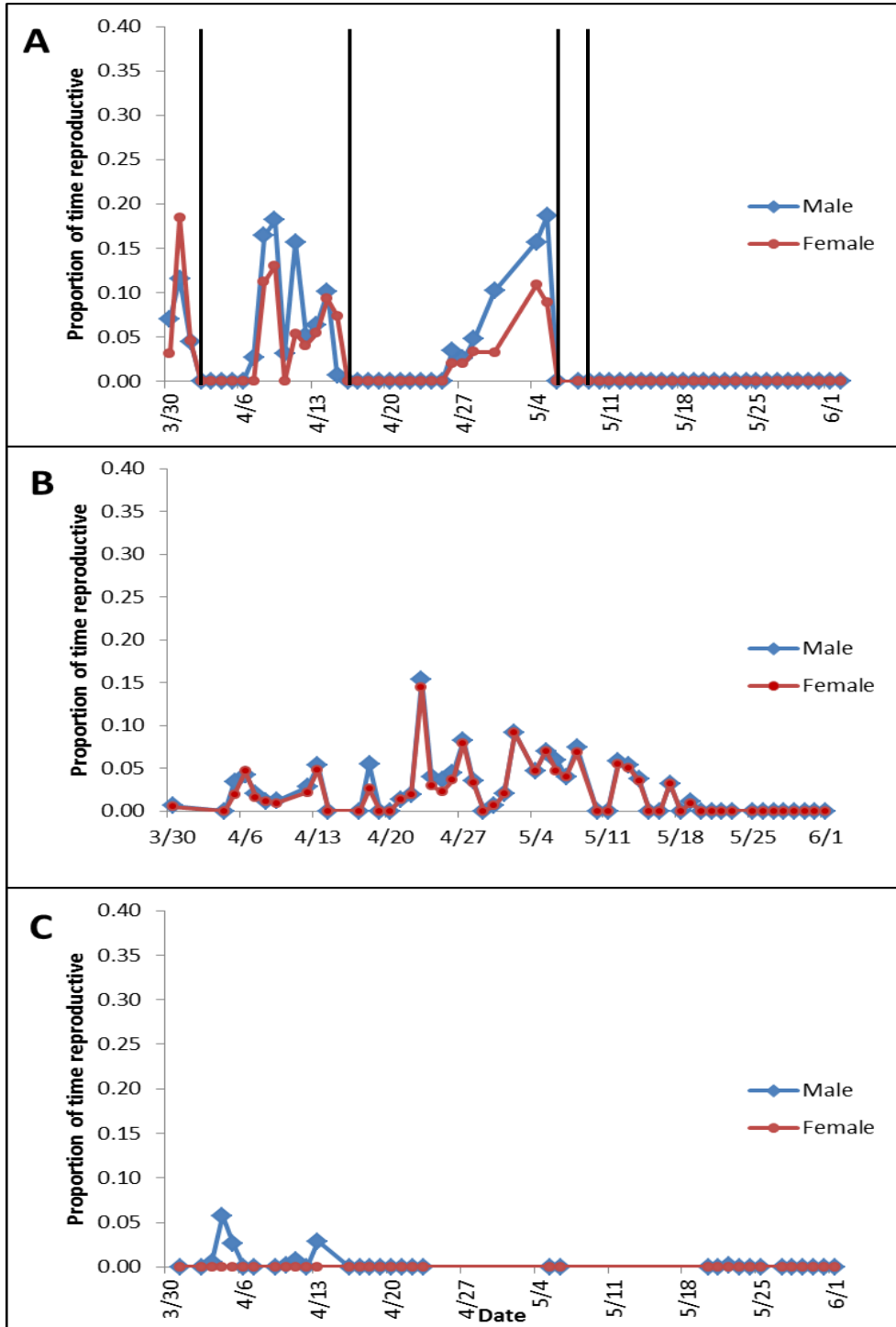
showed significantly higher proportion of reproductive behaviors than did unsuccessful males and females ($p < 0.0001$ and $p < 0.0001$; Fig. 5). Examining time spent in individual reproductive behaviors, successful pairs showed significantly more time performing unison calls (male: $p = 0.0251$; female: $p = 0.0251$), marching, or precopulation behavior (male: $p = 0.0031$; female: $p = 0.0428$) and showed significantly more copulation attempts ($p = 0.0045$) compared to unsuccessful pairs. Interestingly, unsuccessful pairs spent significantly more time together than successful pairs ($p = 0.0411$).

I also observed that reproductive behaviors typically increased before the onset of egg laying (Fig. 6a), while pairs that did not lay eggs showed a consistent level of reproductivebehaviors throughout the breeding season (Fig. 6b), or an overall low proportion of reproductive behaviors (Fig. 6c). There was evidence that reproductive behaviors were linked with egg production. In pairs that laid eggs during 2011 season but not in 2012, reproductive behaviors drastically reduced in the non-reproductive year (Fig. 7). Furthermore, the cyclical pattern of reproductive behaviors was not observed in the latter year. When behavior before and after an egg laying event was examined, an increase in time spent exhibiting reproductive behavior was observed peaking 3 days before egg laying, and then reproductive behavior rapidly declined and remained below baseline levels (Fig. 8). When female behaviors and estradiol concentrations are graphed together, the same temporal pattern is exhibited. A sustained increase in estradiol was observed, followed by a gradual rise in behaviors which peaked three days before oviposition (Fig. 9A). The

Figure 7: Reproductive behavior is different between laying and non-laying seasons within one pair. Proportions of reproductive behavior for a bird pair (S31, class 2) observed during breeding season 2011 (A) and 2012 (B) Black vertical lines indicate egg laying dates in 2011: 4/17/2011, 5/1/2011, and 5/6/2011.
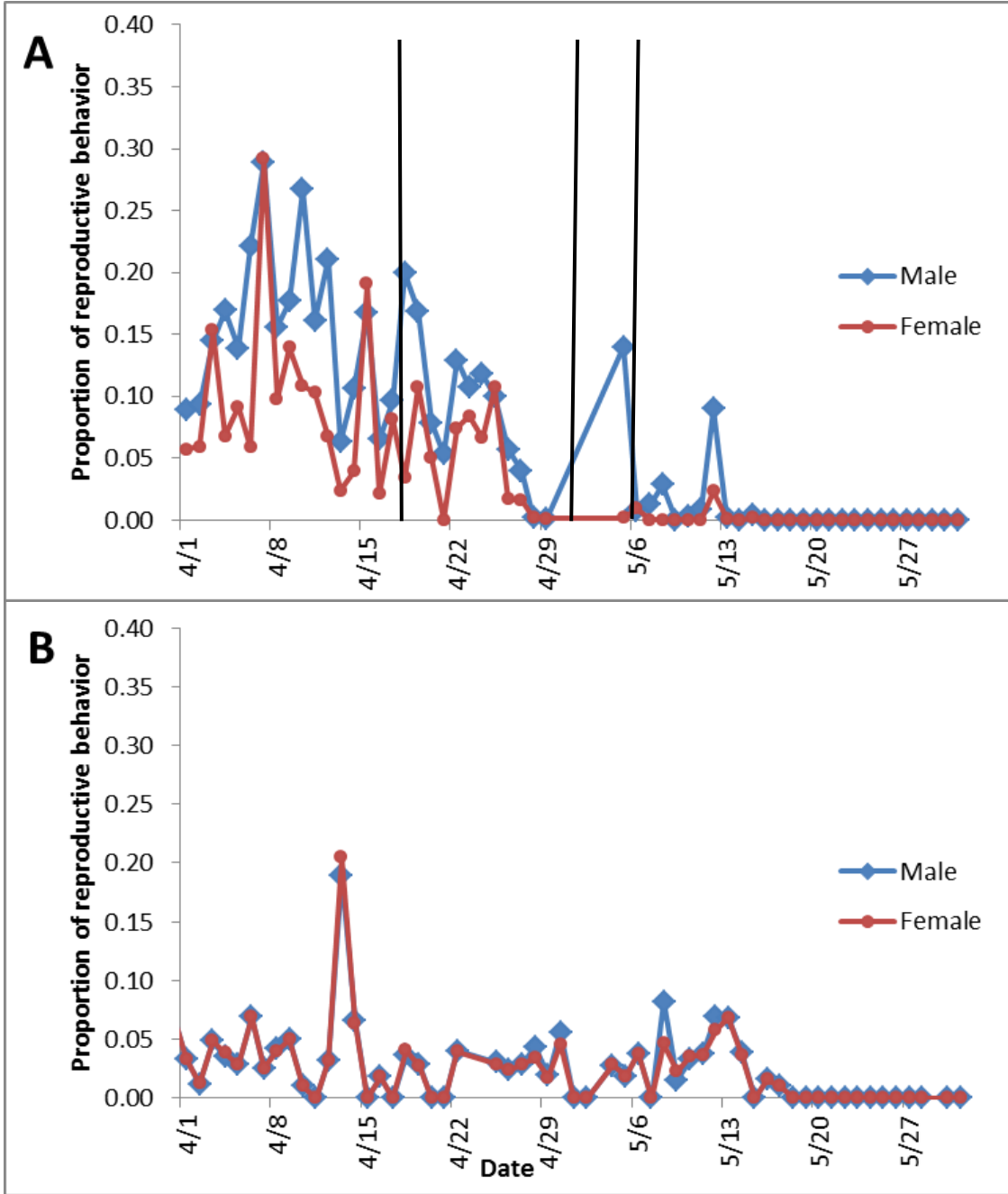
Figure 8: Increase in time spent performing reproductive behaviors approaching an egg laying event. Mean ± standard error of the mean time spent performing reproductive behaviors before and after an egg laying event (n = 5). Day 0 indicates when an egg was laid.
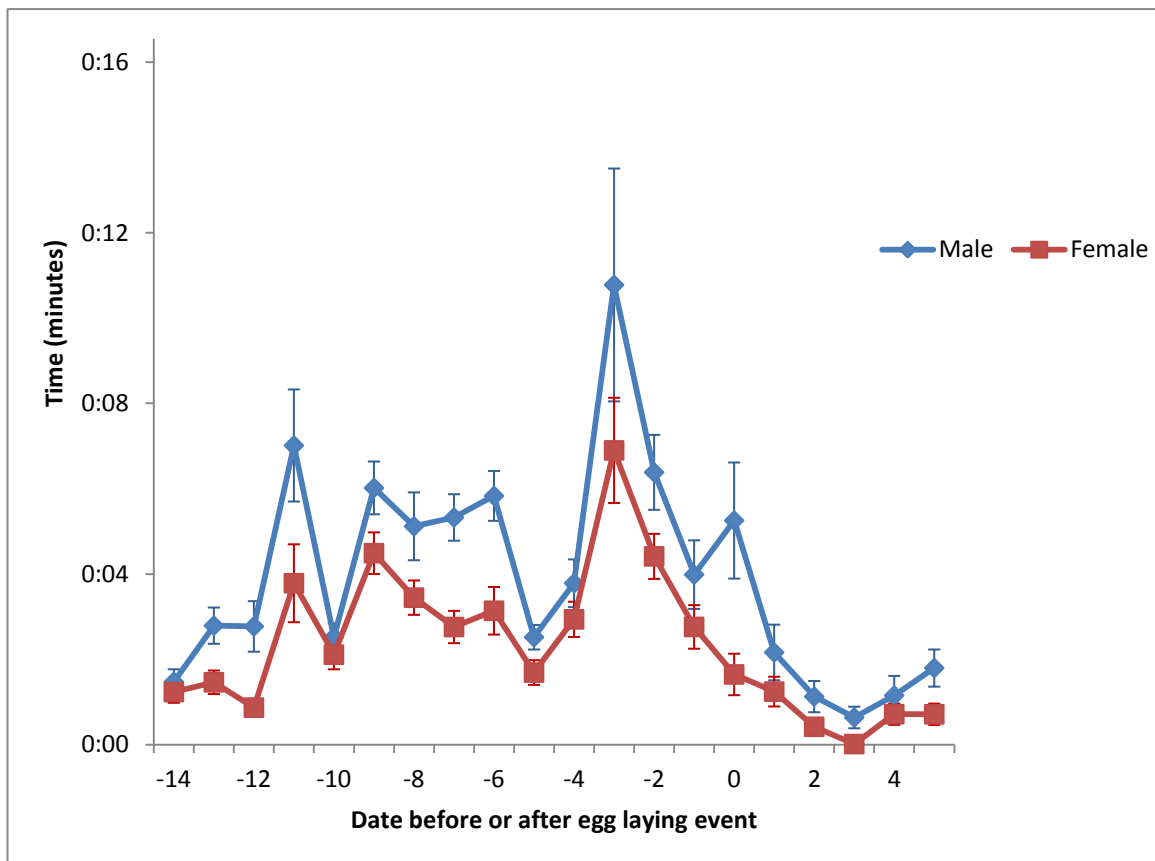
Figure 9: Behavior and hormone concentrations show same temporal pattern. Mean ± standard error of the mean of (A) estradiol concentrations and female reproductive behavior (*n* = 5) and (B) testosterone concentrations and male reproductive behaviors (n =5). Day 0 indicates when an egg was laid.
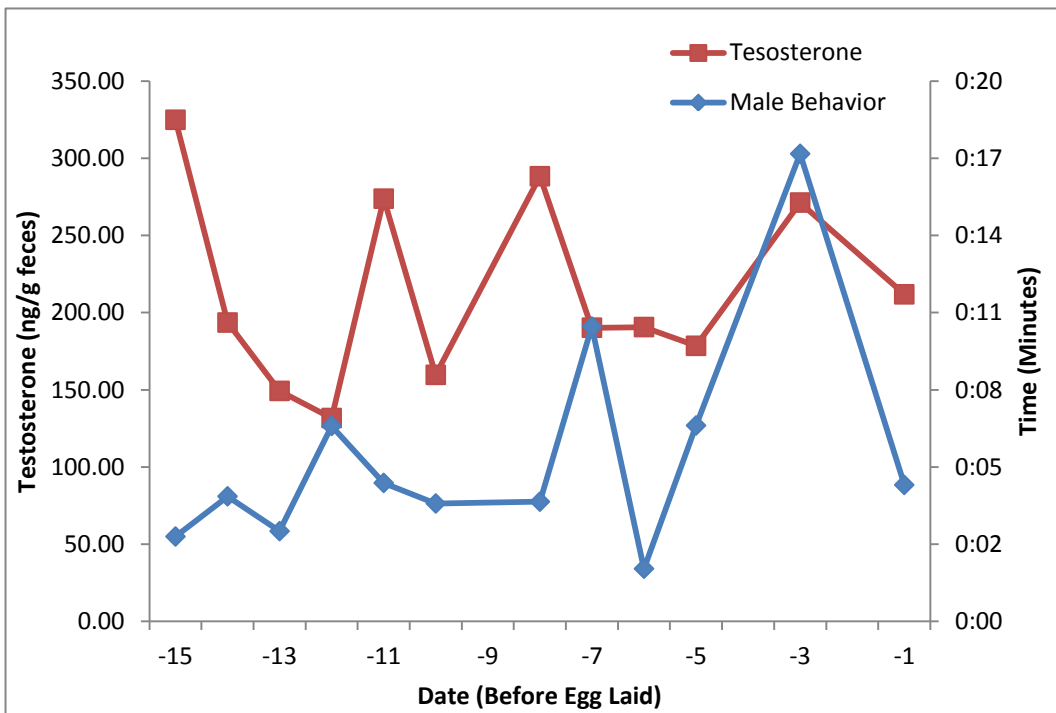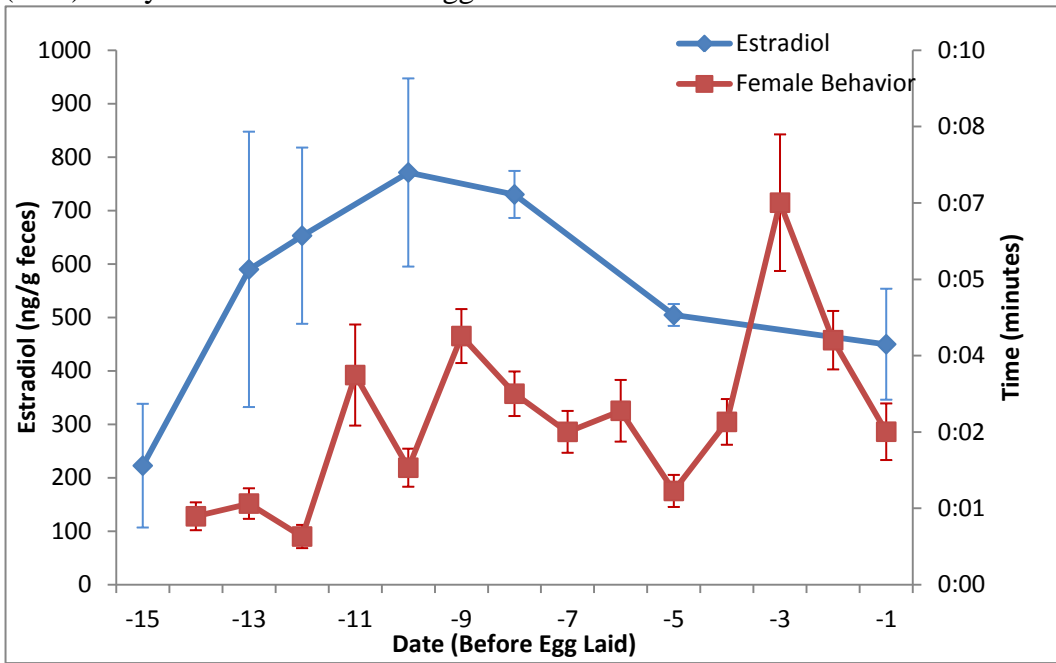
same relationship is observed to a lesser extent when male behaviors are graphed against testosterone concentrations.

There was a significant relationship between mean time spent performing reproductive behaviors in males and females (Table 4, p = 0.0022, r = 0.79). There also was a significant relationship between behaviors and corticosterone levels (Table 4, p = 0.0945, r = 0.50). A significant relationship between behaviors and other hormones was not observed, although there is evidence which supports the possibility of a strong link between the two.

When the effect of temperature on egg laying was examined, a significant relationship was observed between number of eggs per year and the difference in temperature between the months of January and February (p = 0.0420). When the difference in
temperature between the months was greater, more eggs were laid by this population, for example in 2011 and 2007, when the difference in temperature was 8 and 9.6 degrees respectively, more eggs were produced (54 and 57 eggs) than in 2002 and 2012 when the difference in temperature was only 0.2 and 3.9 degrees respectively (43 and 29 eggs).

Discussion

Non-invasive assessment of hormone metabolites in fecal samples has been widely used to track gonadal and adrenal activity and to investigate the relationship between endocrine hormones and behaviors in many wildlife species (Brown et al. 2001, Wielebnowski et al. 2002, Carlstead and Brown 2005, Brown 2006, Dorsey et al. 2010). The present study utilized this technology to longitudinally evaluate

Table 4: Correlations between behaviors and hormone concentrations. Summary of all behavioral and hormonal mean correlations with given p-values. Significant relationships are shown with an asterisk. Negative correlations are indicated with a negative sign after the p value.

	E2	Pg	FCort	Female Behavior	T	MCort	Male Behavior
Estradiol (E2)	1.0000	0.0424*	0.6467	0.6102	0.0504*	0.0771*(-)	0.9774
Progesterone (Pg)	0.0424*	1.0000	0.8258	0.5493	0.2941	0.3819	0.7926
Female Corticosterone (FCort)	0.6467	0.8258	1.0000	0.5493	0.9816	0.0699*	0.1286
Female Behavior	0.6102	0.5493	0.5493	1.0000	0.9940	0.5252	0.0022*
Testosterone (T)	0.0504*	0.2941	0.9816	0.9940	1.0000	0.4722	0.9702
Male Corticosterone (MCort)	0.0771*(-)	0.3819	0.0699*	0.5252	0.4722	1.0000	0.0945
Male Behavior	0.9774	0.7926	0.1286	0.0022*	0.9702	0.0945*	1.0000

metabolites of gonadal and adrenal hormones in captive whooping cranes and examine the relationship between endocrine and reproductive behaviors. We have established, for the first time, hormonal profiles of male and female whooping cranes, and demonstrated divergence in the patterns of gonadal steroids between females that laid eggs and individuals that failed to reproduce. In the former, gonadal hormones exhibited cyclical waves associated with an egg laying event, whereas steroid levels remain constant and sometimes abnormally low in the latter. Furthermore, we showed that abnormal patterns of gonadal hormone were not the result of chronic stress as there were no differences in patterns and concentrations of adrenal hormone between reproductively successful and unsuccessful birds. We also found no differences between gonadal and adrenal hormones among males, regardless of reproductive class and outcome. Finally, successful pairs showed higher proportion of reproductive behaviors and specific reproductive behaviors were more prevalent compared to unsuccessful pairs.

Female Endocrinology

The present study determines that there was a rise and fall in fecal estradiol and progestagen metabolite concentrations associated with egg laying events. Specifically, estradiol metabolites reached peak concentrations just prior to egg production, and declined shortly after the eggs were laid. However, progestagen metabolites remained elevated a few days after egg laying before declining to the baseline level. This hormone pattern is similar to patterns reported in other bird species, including canaries (*Serinus canaria*), great hornbills (*Buceros bicornis*), and domestic chickens (*Gallus domesticus*). It has been suggested that the decline in

estradiol concentration is regulated by factors associated with egg incubation that in turn causes the decrease in the steroid excretion from small follicles (Sockman and Schwabl, 1999). Elevated estradiol concentration has been 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, Norris 2006). Unlike estradiol, the decline of progesterone has been shown to be tightly linked with the time since ovulation of the first egg (Sockman and Schwabl, 1999). In our study, progesterone concentration declined slowly during the first 6 days post-egg laying compared to the sharp decline after 4 days in canaries. Progesterone has been revealed as a primary factor influencing timing of ovulation (Lague et al. 1975), whereby levels increase as the ovum grows, signaling LH secretion (Yang et al. 1997). The increase in LH up-regulates the conversion of androgens produced by the theca interna layer into estrogen, and signals ovulation (Moudgal and Razdan 1985). After ovulation, progesterone levels rapidly decrease until the growth of a subsequent follicle (Liu et al. 2001). The difference in the duration during which progesterone remains elevated above the baseline between the whooping crane and canary (demonstrated by the previous study) may be due to variation in lag time for steroid hormones to clear the body between different species, especially when the size difference between the species is so great (Schwarzenberger et al. 1996).

Our findings of gonadal hormone metabolites are consistent with those reported in the great hornbill (Crofoot et al. 2003), where patterns differed between laying and non-laying seasons. In laying seasons, birds displayed hormonal profiles

in which estrogen was produced and continued to rise until just before ovulation. Once a follicle is ovulated, it no longer produces estradiol and progesterone, which in turn results in the rapid decrease in the hormone concentrations. If follicles remain present on the ovary they will continue to produce steady levels of hormones (Liu et al. 2001, Liu and Bacon 2004).

Non-laying females that had significantly higher progesterone baselines even without elevated estradiol may be exhibiting polycystic ovarian follicle (PCOF) syndrome, which would prevent egg production. PCOF syndrome in birds is characterized by high basal progesterone concentrations and is observed when large follicles remain attached to the ovary and become cystic. Large F1 follicles form, but without sufficient estradiol concentrations they are not able to ovulate, and so continue to produce progesterone and block further ovarian activity (Liu and Bacon 2004).

In the present study, I chose to compare not only means and baseline corticosterone concentrations among crane pairs, but also standard deviations and number of days above baseline as it has been shown that standard deviation may be a more revealing measure of adrenal activity. For example, Carlstead and Brown (2005) showed that although there were no variations in mean corticoid concentration between stressed and non-stressed black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*), a large standard deviation which indicated increased adrenal activity was observed in the latter. In the present study, non-laying females showed more days of elevated corticosterone levels compared to laying females, although no difference between any other values was observed. Thus, it is

likely that the abnormal pattern in gonadal hormone profiles observed in non-reproductive pairs was not associated with increased adrenal activity. Our finding is similar to that observed in Manx shearwaters (*Puffinus puffinus*) (Roiu et al. (2010). Thus, other factors influencing reproduction must be considered.

High levels of variation observed within individuals may be explained by our collection method. In Sockman and Schwabl (1999), samples collected earlier in the day exhibited higher levels of both estradiol and progesterone. While we collected the newest and freshest samples that could be found, in the future it would be ideal to collect samples from earlier in the morning whenever possible to decrease potential variations in fecal sample age. In a study by Lague et al. (1975), chickens typically displayed estradiol and progesterone peaks within hours of ovulation; however, ovulations did occur without an estradiol peak and other estradiol peaks were observed in the absence of ovulation events. Seemingly random hormonal events as described in their study may explain the variation observed in our study, especially progesterone peaks observed outside of breeding season.

Male Endocrinology

There were no differences in testosterone and corticosterone concentrations and patterns between reproductive and non-reproductive seasons. 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 and frequently experiencing territory challenges had higher circulating testosterone and corticosterone concentration than those living in a low

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population density habitat. Similar findings have also been reported in brown-headed cowbirds (Wingfield et al. 1987).

Behaviors

We observed significant differences in reproductive behaviors between laying and non- laying pairs, such that successful breeders showed a higher proportion of reproductive behaviors when compared to unsuccessful birds. Behavioral observations showed that successful pairs performed more copulation attempts, unison calls, and precopulatory behavior. Frequent copulations are necessary to ensure enough sperm is available for fertilization (Jones and Nicolich 2001), and pairs are known to begin performing reproductive behaviors up to 5 weeks before an egg laying event (Swengel et al. 1996). Sheldon and Burke (1994) showed that chaffinches (*Fringilla coelebs*) increased their frequency of copulation to a peak of 4.4 attempts per hour roughly 3 days before the onset of egg laying.

We also demonstrated that male corticosterone and reproductive behaviors are highly correlated to female adrenal steroid excretion and reproductive displays, respectively. This indicates that cranes do respond to the behaviors of their respective mates. Because reproduction has been indicated as one factor stimulating adrenal response (Romero 2002, Landys et al. 2006), it is not surprising that male reproductive behaviors were highly associated with corticosterone excretion. However, we did not observe the same significant relationship between behavior and corticosterone levels in the females. We believe this occurred as successful males displayed reproductive behaviors more frequently during the breeding season than

their female counterparts (Fig. 6A, 7A, and 8), and thus, had more energy expended performing these behaviors, which in turn increased glucocorticoid levels.

Environment has a strong regulatory control on avian reproduction (Gee and Russman 1996, Norris 2006). As temperature is a key determinant in egg production in northern breeding species, perhaps a large rise in temperature shortly before the breeding season acts as a signal stimulating egg production.

In summary, our findings indicate that poor reproductive success in whooping crane pairs is associated with perturbation of gonadal function in female birds that in turn compromises follicle development and ovulation. Our research also demonstrates that the deviation of gonadal function is not associated with increased adrenal activity. Future research should focus on identifying factors influencing female gonadal functions. Potential factors include improper captive environmental and husbandry conditions which could prevent the release of critical gonadotropins in the hypothalamic-pituitary-gonadal axis. Finally, knowledge gained from this research has improved understanding of reproductive endocrinology in 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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Chapter 4: Overall Conclusions

The research performed in completion of this thesis has furthered the overall knowledge of reproductive biology of the whooping crane. My studies are the first to systemically assess seminal characteristics of whooping cranes and to longitudinally evaluate metabolites of gonadal and adrenal hormones and examine the relationship between endocrine and reproductive behaviors in captive birds.

In the first study, I was unable to detect a relationship between inbreeding coefficient and seminal quality of the whooping crane housed at the USGS Patuxent Wildlife Research Center. However, we demonstrated that stage of breeding season sperm output and characteristics. Specifically, ln(total sperm) increased as the breeding season progressed, while percent normal morphology was the highest in the early breeding season and progressively became lower across the breeding season. This study is crucial for improving genetic management of the endangered whooping crane and establishes normal range values for seminal characteristics to use in future research studies. Current values for pair fertilities at PWRC are based on how many fertile eggs have been laid in the past; however, this is not a true representation of a male's potential as it is confounded by female reproductive variables and potential problems. Thus, it would be beneficial to separate male fertility problems from overall pair fertility. This could be achieved by examining fertilizing potential of sperm obtained from each male using a spermatozoa-egg interaction assay that utilizes perivitelline membranes from recently laid, unfertilized eggs

In the second study, I established hormonal profiles of male and female whooping cranes. I demonstrated different patterns of gonadal steroids between

females that laid eggs and individuals that failed to reproduce, but observed no difference in males of similar reproductive outputs. I showed that contrary to my hypothesis, abnormal patterns of gonadal hormones were not the result of chronic stress as there were no differences in patterns and concentrations of adrenal hormone between reproductively successful and unproductive birds. Also, although reproductive behaviors occurred less frequently in non-reproductive pairs compared with reproductive birds, there was little evidence that this difference was influenced by gonadal or adrenal hormone concentrations.

Advances in the basic reproductive knowledge of a species held in captivity have enhanced the management of *ex situ* and *in situ* populations in many wildlife species. Reproductive management has already played important roles in the recovery of the whooping crane. Findings obtained from this research lay a foundation for future research focusing on improving genetic management of captive whooping cranes, through cryopreservation, which would allow for transport of genetics between captive populations and enable an individual's genetics to remain represented decades after they are no longer reproductively active. Finally, knowledge generated from this research improves our understanding about reproductive endocrinology of the whooping crane, information that is crucial for identifying potential factors impacting reproductive success of the PWRC population. In this respect, future research priorities include captive environment suitability and its influence on reproductive performance, as well as genetic influence on mate choice and pair dynamics.

Appendices

Appendix A

All models examined using multiple model selection theory. Selected models are indicated with an *

A) Inbreeding

Model Name	log(Likelihood)	AIC	Model Weight (%)
Log Total Sperm			
Inb + 1|Ind + Exp + House + AI	-99.5	213.0	3.1
Inb + 1|Ind + Exp + House	-99.48	211.0	8.5
Inb + 1|Ind + Exp + AI	-99.54	211.1	8.1
Inb + 1|Ind + House + AI	-100.3	212.5	4.0
Inb + 1|Ind + Exp	-99.5	209.0	23.1
Inb + 1|Ind + AI	-100.2	210.4	11.5
Inb + 1|Ind + House	-100.3	210.6	10.4
Inb + 1|Ind *	-100.2	208.4	31.2
Percent Normal			
Inb + 1|Ind + Exp + House + AI	-101.8	217.6	1.9
Inb + 1|Ind + Exp + House	-101.6	215.1	6.7
Inb + 1|Ind + Exp + AI	-101.4	214.9	7.4
Inb + 1|Ind + House + AI	-102.1	216.2	3.8
Inb + 1|Ind + Exp	-101.2	212.4	25.7
Inb + 1|Ind + AI	-102.2	214.3	9.9
Inb + 1|Ind + House	-102.2	214.3	9.9
Inb + 1|Ind *	-101.9	211.8	34.7
Motility			
Inb + 1|Ind + Exp + House + AI	-113.5	241.1	2.6
Inb + 1|Ind + Exp + House	-114.2	240.3	3.9
Inb + 1|Ind + Exp + AI	-113.6	239.1	7.2
Inb + 1|Ind + House + AI	-113.2	238.4	10.2
Inb + 1|Ind + Exp	-114.1	238.2	11.2
Inb + 1|Ind + AI	-114.2	238.5	9.7
Inb + 1|Ind + House	-113.2	236.5	26.2
Inb + 1|Ind *	-114.1	236.3	29.0
Total Motile Sperm			
Inb + 1|Ind + Exp + House + AI	-94.88	203.8	3.0
Inb + 1|Ind + Exp + House	-95.1	202.2	6.6
Inb + 1|Ind + Exp + AI	-94.98	202.0	7.3
Inb + 1|Ind + House + AI	-94.9	201.8	8.1
Inb + 1|Ind + Exp	-95.12	200.2	18.1
Inb + 1|Ind + AI	-95.68	201.4	9.9
Inb + 1|Ind + House	-95.02	200.0	20.0
Inb + 1|Ind *	-95.68	199.4	27.0
Total Normal Sperm			
Inb + 1|Ind + Exp + House + AI	-84.52	183.0	2.5
Inb + 1|Ind + Exp + House	-84.56	181.1	6.6
Inb + 1|Ind + Exp + AI	-84.51	181.0	6.9
Inb + 1|Ind + House + AI	-84.89	181.8	4.6
Inb + 1|Ind + Exp	-84.53	179.1	17.8
Inb + 1|Ind + AI	-84.89	179.8	12.5
Inb + 1|Ind + House	-84.87	179.7	13.2
Inb + 1|Ind *	-84.83	177.7	35.9

B) Age

Model Name	log(Likelihood)	AIC	Model Weight (%)
Log Total Sperm			
Inb + 1|Ind + Exp + House + AI	-100.9	215.8	2.63
Inb + 1|Ind + Exp + House	-100.9	213.8	7.16
Inb + 1|Ind + Exp + AI	-100.9	213.9	6.81
Inb + 1|Ind + House + AI	-101.4	214.8	4.34
Inb + 1|Ind + Exp	-100.9	211.9	18.51
Inb + 1|Ind + AI	-101.2	212.4	14.41
Inb + 1|Ind + AI	-101.5	213	10.68
Inb + 1|Ind *	-101.3	210.6	35.45
Percent Normal			
Inb + 1|Ind + Exp + House + AI	-103.5	221.1	1.62
Inb + 1|Ind + Exp + House	-103.3	218.5	5.94
Inb + 1|Ind + Exp + AI	-103.1	218.2	6.90
Inb + 1|Ind + House + AI	-103.8	219.5	3.60
Inb + 1|Ind + Exp	-102.8	215.7	24.08
Inb + 1|Ind + AI	-103.7	217.4	10.29
Inb + 1|Ind + AI	-103.8	217.5	9.79
Inb + 1|Ind *	-103.4	214.8	37.77
Motility			
Inb + 1|Ind + Exp + House + AI	-115.6	245.1	3.21
Inb + 1|Ind + Exp + House	-116	243.9	5.85
Inb + 1|Ind + Exp + AI	-116	244.1	5.29
Inb + 1|Ind + House + AI	-115.2	242.4	12.38
Inb + 1|Ind + Exp	-116.3	242.6	11.20
Inb + 1|Ind + AI	-116	242	15.12
Inb + 1|Ind + AI	-115.7	241.5	19.41
Inb + 1|Ind *	-116.4	240.8	27.55
Total Motile Sperm			
Inb + 1|Ind + Exp + House + AI	-95.75	205.5	2.80
Inb + 1|Ind + Exp + House	-95.83	203.7	6.89
Inb + 1|Ind + Exp + AI	-96.17	204.3	5.11
Inb + 1|Ind + House + AI	-95.88	203.2	8.85
Inb + 1|Ind + Exp	-96.14	202.3	13.88
Inb + 1|Ind + AI	-95.95	201.9	16.96
Inb + 1|Ind + AI	-96.11	202.2	14.60
Inb + 1|Ind *	-96.36	200.7	30.90
Total Normal Sperm			
Inb + 1|Ind + Exp + House + AI	-85.89	185.8	2.03
Inb + 1|Ind + Exp + House	-85.94	183.9	5.26
Inb + 1|Ind + Exp + AI	-85.84	183.7	5.81
Inb + 1|Ind + House + AI	-86.13	184.3	4.31
Inb + 1|Ind + Exp	-85.87	181.7	15.80
Inb + 1|Ind + AI	-86	182	13.60
Inb + 1|Ind + AI	-86.09	182.2	12.31
Inb + 1|Ind *	-85.92	179.8	40.87

C) Date

Model Name	log(Likelihood)	AIC	Model Weight (%)
Log Total Sperm			
Inb + 1|Ind + Exp + House + AI	-96.6	209.2	3.1
Inb + 1|Ind + Exp + House	-96.5	207.1	9.0
Inb + 1|Ind + Exp + AI	-96.7	207.4	7.7
Inb + 1|Ind + House + AI	-97.3	208.5	4.5
Inb + 1|Ind + Exp	-96.6	205.2	23.2
Inb + 1|Ind + AI	-97.3	206.5	12.1
Inb + 1|Ind + House	-97.4	206.8	10.4
Inb + 1|Ind *	-97.4	204.7	29.8
Percent Normal			
Inb + 1|Ind + Exp + House + AI	-97.2	210.4	2.2
Inb + 1|Ind + Exp + House	-97.0	208.0	7.3
Inb + 1|Ind + Exp + AI	-96.7	207.4	9.9
Inb + 1|Ind + House + AI	-96.7	209.3	3.8
Inb + 1|Ind + Exp *	-96.5	205.0	32.7
Inb + 1|Ind + AI	-98.2	208.3	6.3
Inb + 1|Ind + House	-97.3	206.6	14.7
Inb + 1|Ind	-97.8	205.7	23.1
Motility			
Inb + 1|Ind + Exp + House + AI	-112.7	241.4	4.4
Inb + 1|Ind + Exp + House	-113.6	241.1	5.2
Inb + 1|Ind + Exp + AI	-113.1	240.2	8.1
Inb + 1|Ind + House + AI	-112.4	238.8	16.3
Inb + 1|Ind + Exp	-113.7	239.5	11.5
Inb + 1|Ind + AI	-114.1	240.2	8.1
Inb + 1|Ind + House*	-112.9	237.7	28.3
Inb + 1|Ind	-114.3	238.6	18.0
Total Motile Sperm			
Inb + 1|Ind + Exp + House + AI	-91.3	198.7	4.7
Inb + 1|Ind + Exp + House	-91.8	197.6	8.2
Inb + 1|Ind + Exp + AI	-91.8	197.7	7.8
Inb + 1|Ind + House + AI	-91.3	196.6	13.5
Inb + 1|Ind + Exp	-92.1	196.3	15.7
Inb + 1|Ind + AI	-92.6	197.1	10.5
Inb + 1|Ind + House*	-91.9	195.8	20.2
Inb + 1|Ind	-92.0	195.9	19.2
Total Normal Sperm			
Inb + 1|Ind + Exp + House + AI	-83.4	182.7	2.4
Inb + 1|Ind + Exp + House	-83.4	180.7	6.5
Inb + 1|Ind + Exp + AI	-83.3	180.5	7.2
Inb + 1|Ind + House + AI	-83.8	181.5	4.4
Inb + 1|Ind + Exp	-83.3	178.5	19.6
Inb + 1|Ind + AI	-83.8	179.5	11.9
Inb + 1|Ind + House	-83.7	179.4	12.5
Inb + 1|Ind*	-83.6	177.3	35.6

Seminal characteristics of 29 whooping crane males housed at USGS Patuxent Wildlife Research Center

Individual's Studbook Number	Age (in 2010)	House with a Female	Used for AI	Semen Collection Experience	Normalized Inbreeding Coefficient	Volume (µl)	pH	Osmolarity (mOsm)	Concentration (x 10⁶/ml)	Total Sperm (x 10⁶)	% Motility	PMS	% Normal Morphology
1114	27	Yes	No	Yes	0.226	25	7.1	261	16	1.1	40	1.7	.
1127	26	Yes	Yes	Yes	-0.2093	30	8.5	306	71	2.1	40	2	58
1130	26	Yes	No	Yes	-0.114	58.5	6.8	150	169.5	18.1	50	2	22
1133	26	Yes	No	No	0.226	76.7	7.5	320	137.1	13.6	36.7	1.8	68
1144	24	Yes	Yes	Yes	0.0374	3	.	.	45	0.1	30	1.5	.
1147	23	Yes	Yes	Yes	0.1756	56.3	8.7	303	428.7	34.2	63.3	2	60.4
1161	22	No	No	No	0.226	196	7.8	307.8	110.1	15.8	46	1.7	71.4
1162	22	Yes	Yes	Yes	-0.1409	75	7.9	294	79.9	8.7	35	1.5	54.5
1165	22	Yes	Yes	Yes	0.1311	25	7.7	353	113	2.8	65	2.5	92.5
1187	20	No	No	Yes	0.226	47	8.4	306.3	180.8	9.8	69	2.3	70.1
1235	18	Yes	No	No	-0.1125	22	7.6	309	946.2	69.8	38.7	2.2	66.3
1244	18	Yes	Yes	Yes	0.2189	28.3	8.4	295	85.4	3.1	56.7	2.3	54.1
1340	15	Yes	No	No	-0.1125	82	8.1	196	30.9	3	44	2	48.5
1357	15	No	Yes	Yes	-0.1704	29	7.3	335.5	63	2.9	37.5	2	50.9
1420	14	Yes	Yes	Yes	0.0733	10	8	.	117.3	1.2	56.7	2.2	40.3
1422	14	Yes	Yes	Yes	0.2535	25	8.5	.	.	.	30	1.5	89.5
1423	14	Yes	Yes	Yes	-0.3595	55.8	7.9	381.5	174.2	15.5	45	2.3	82.8
1560	11	Yes	No	Yes	0.0387	91.2	7.6	288.5	76.6	4.2	33	1.5	85.2
1581	10	No	Yes	Yes	-0.354	58.1	8.6	327	57.5	0	45	1.8	75
1597	10	Yes	No	Yes	-0.38	105	8	307.8	416.3	43.4	20	1	73
1615	10	Yes	No	No	-0.1764	14	8.4	368	48	0.8	37.5	1.9	75.2
1691	8	Yes	No	No	0.0608	14.5	9	273.5	5.8	0.1	30	2	65
1717	7	No	Yes	Yes	-0.1704	43.3	8.3	284	150.3	6	66.7	2.5	68.3
1737	7	Yes	No	No	-0.4738	71.7	8.3	369.5	520.3	75.6	56.7	2.2	82.8
1775	6	Yes	No	No	-0.4939	12.3	8	346	34	0.3	43.3	1.7	66.3
1798	5	Yes	No	No	-0.4939	50	7.7	318	227.3	13.3	33.3	1.8	71.5
1840	4	Yes	Yes	Yes	-0.2686	137.5	7.7	322	27	2.9	30	1.5	57.2
1847	4	Yes	No	No	0.0535	5	7.9	.	.	.	.	.	.
1901	3	No	No	No	-0.1704	66.3	7.2	359.7	3.7	0.2	33.3	1.5	72.5

Chromic and Iron Oxides as a Fecal Marker to Identify Individual Whooping Cranes

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The whooping crane (*Grus americana*) is listed as endangered under the IUCN Red List, the United States' Endangered Species Act, and the Canadian Species at Risk Act (IUCN 2011, CWS and USFWS 2005). A major focus of recovery efforts for this endangered species is reintroduction to establish new populations (CWS and USFWS 2005). Captive populations are critical as a source of individuals for reintroduction efforts, and also serve as insurance populations. Currently, there are a total of 157 whooping cranes held in captive breeding centers across North America, with the largest at the USGS Patuxent Wildlife Research Center (PWRC) in Laurel, MD. Birds produced in this facility are currently being

released as part of efforts to establish the Eastern Migratory Population (EMP; Urbanek et al., 2005) and a non-migratory population in Louisiana. In the past decade, PWRC has produced and released annually an average of 18 birds into the wild; however, reproductive performance of birds at this facility is lower than desired. PWRC had a 60% fertility rate for eggs laid from 2000 through 2010 (J N Chandler pers. communication, 2011). Furthermore, reproductive onset in this captive population appears to be delayed compared to wild populations. In wild populations, reproductive onset (production of sperm and eggs) normally occurs ~5 years of age in both males and females, ~2 years after initial pair formation occurs (Ellis et al. 1996), while some females in the EMP have been known to lay eggs even earlier than 5 years of age (Converse et al., 2011). However, PWRC females, in some cases, do not start to lay eggs until 7 years of age (Ellis et al., 1996). Currently, the PWRC population consists of a total of 74 whooping cranes, including 22 pairs. Six of these pairs (27%) are consistently infertile (i.e., no production of fertile egg) and three other pairs (14%) have low fertility (30-45% fertility in eggs laid), which is variable from year to year. Six pairs (27%) are recently formed and have not produced eggs, and so have unknown fertility. This leaves only seven pairs (33%) which contribute maximally to PWRC's chick production (J N Chandler, pers. communication, 2011). Because of the challenges occurring within this captive colony, PWRC and Smithsonian National Zoo have initiated a joint research project to identify potential underlying causes of poor reproduction in captive whooping cranes.

One method critical to this research is non-invasive hormone monitoring, which has been used in a variety of studies focused on examining basic animal

biology, health, and reproduction, as well as physiological responses of animals to captive management. Hormone metabolite concentrations can be sampled in a variety of materials including feces, urine, hair, feathers, and saliva (Brown, 2008; Brown et al., 2001; Holt et al., 2003; Lobato et al., 2010; Moore et al., 1984; Wielebnowski et al., 2002). In the giant panda (*Ailuropoda melanoleuca*) hormone metabolites have been monitored in urine samples in order to understand the timing of estrus and ovulation, which aids in planning animal introductions and artificial inseminations (Moore et al., 1984). In the clouded leopard (*Neofelis nebulosa*), fecal hormone sampling has helped researchers understand relationships between aspects of enclosure design and location, and stress responses (Wielebnowski et al., 2002).

Already used in a variety of wild mammal species in both *ex situ* and *in situ* studies, non-invasive hormone monitoring also is gradually being adapted to birds. Most avian hormone studies to date have utilized blood sampling (Angelier and Chastel, 2009; Angelier et al., 2009; Angelier et al., 2006; Bluhm et al., 1983), a process which has been shown to cause stress (Gratto-Trevor et al., 1991). Studies have validated the effectiveness and feasibility of non-invasive hormone monitoring in some bird species. Ludders *et al* 2001 showed that serum corticosterone patterns were similar to those in fecal samples collected from the same bird in Florida sandhill cranes. Stanley *et al* 2007 validated reproductive steroid hormone assays for both golden eagles and peregrine falcons housed in a captive setting. To date, non-invasive hormone monitoring has not been used to assess gonadal activity and little work has been done assessing adrenal activity and function in whooping cranes. Ongoing data collection at PWRC is one of the first efforts to use non-invasive

hormone monitoring in an attempt to understand whooping crane reproductive biology.

The first critical step in this work was to establish a method to identify fecal samples from an individual bird within a breeding pair. Trials with different types of food dyes in varying amounts were unsuccessful. In the present study, we determined the feasibility of using chromic oxide (Cr_2O_3) and iron oxide (Fe_2O_3) as fecal markers. Both chromic oxide and iron oxide were obtained from Prince Agri Products (Prince Agri Products Inc, Quincy IL). These dyes have been used in nutritional studies in a variety of species, including chickens, ducks, cows, horses, and humans, especially in studies that involve more than one feeding trial or those aiming to assess the digestibility of a food item (Schurch et al., 1950). Both are non-biological, insoluble compounds which, when ingested, are not absorbed by the digestive system (Dansky and Hill, 1952; Schurch et al., 1950). Instead, they pass directly through the digestive tract and subsequently color the animal's feces.

In our first trial, cranes housed individually in outdoor pens were given smelt (*Osmerus mordax mordax)* containing a capsule filled with 450 mg green chromic oxide ($n = 5$ birds), or yellow ($n = 5$), red ($n = 4$), orange ($n = 3$), or black ($n = 3$) iron oxide. The appearance of color in the feces was visually determined 8 h later, with color intensity judged on a scale of 0 to 3, with 3 indicating intense color and 0 indicating no visible color. Visibility was determined in the field, where subsequent endocrine studies will take place, because it is important to know which color would be easiest to find where vegetation and other factors obscure sample visibility.

Chromic oxide in green, and iron oxide in orange, red, and black (but not yellow) were visible in feces (green = 3; red = 2; black = 1.5; orange = 1; and yellow = 0).
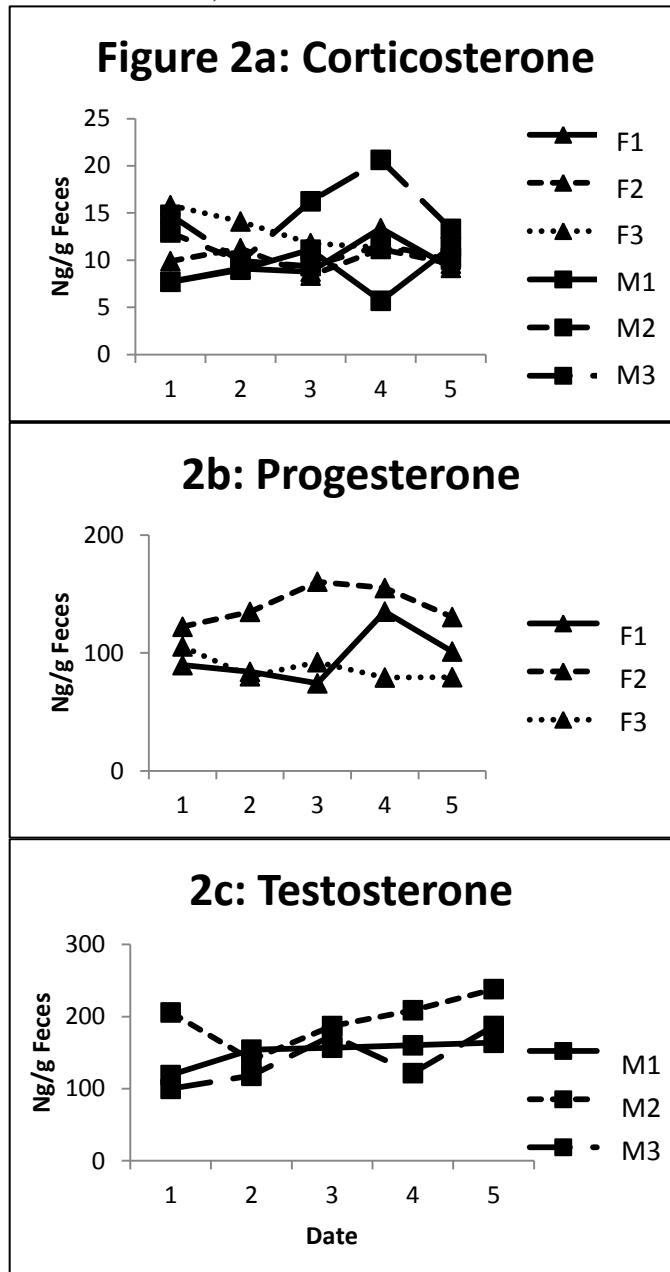
In a second trial, we assessed the time required until chromic oxide could be observed post-feeding. Four whooping cranes were housed individually in indoor pens (Fig. 1) and fed smelt containing 230 mg of green chromic oxide. The pens were checked every 30 min until first appearance of the dye in the feces, and then every hour until the end of the day (8 h post feeding). At the beginning of day 2 (24 h post-feeding), the pens were cleared of all feces to ensure that any subsequent samples which showed a presence of chromic oxide were fresh samples. The marker first appeared on average ($\pm$ SE) 1.5 ± 0.2 h after feeding and remained detectable until 27.7 ± 0.2 h for a total duration of 26.2 ± 0.2 h. Therefore, use of chromic oxide allows for a flexible collection interval and increased chance of finding an individual's fecal samples. We observed no adverse consequences of feeding either substance, as fecal production (size, consistency, and overall number of fecals) appeared normal. Finally, it was necessary to verify that chromic oxide and iron oxide would not interfere with hormone assay performance. Feces were collected daily at 07:30 A.M. for 5 days from 3 male and 3 female whooping crane adults, housed individually. On the afternoon of the second day (Day 2) each crane was given smelt containing a capsule filled with 230 mg of either green chromic oxide (females) or red iron oxide (males) so that the fecal samples collected on the morning of Day 3 were dyed. Samples were extracted with a modified dry shaking extraction using 70% ethanol (Brown, 2008). Once extracted, all samples were assessed for

Figure 1: Indoor pens where cranes were housed for trial 2. Small pens with wood shavings used as bedding allowed easy detection and identification of dyed samples.

corticosterone using a RIA kit (MP Biomedicals, Solon, OH; Fig. 2a). Female

samples were also evaluated for progestagen metabolites using an enzyme

immunoassay (EIA, monoclonal pregnane CL425; Fig. 2b), and male samples were

also examined for testosterone using an EIA (polyclonal R156/7; Fig. 2c). Antibodies

for protestagen and testosterone EIAs were obtained from Coralie Munro (University

of California, Davis, CA, USA). Hormone metabolite concentrations remained

constant over the collection period (Fig. 2), providing no evidence that either colorant

interfered with the evaluation of excreted hormones. The only individual that showed

a significant difference between the Day 3 sample and the other collected samples,

using a standard z score, was the corticosterone value for female crane number F2.

In summary, our findings indicate that both chromic oxide and iron oxide can be used

as fecal markers for non-invasive hormone monitoring. This method will aid ongoing

studies aimed at advancing the understanding of reproductive endocrinology and

underlying causes of poor reproduction in captive whooping crane. Studies are in

progress to evaluate hormone metabolite concentrations and patterns in male and

female whooping cranes during the breeding season. The method will be easily

transferrable to a host of other avian species aiding in their conservation and captive

management.

Figure 2: Corticosterone (a), progesterone (b), and testosterone (c).
metabolite concentrations assessed during a 5 day period in six adult whooping
cranes. M indicates males (*n* = 3) and F designates females (*n* = 3). Sample collected
on Day 3 contained fecal marker, iron oxide for males and chromic oxide for females.

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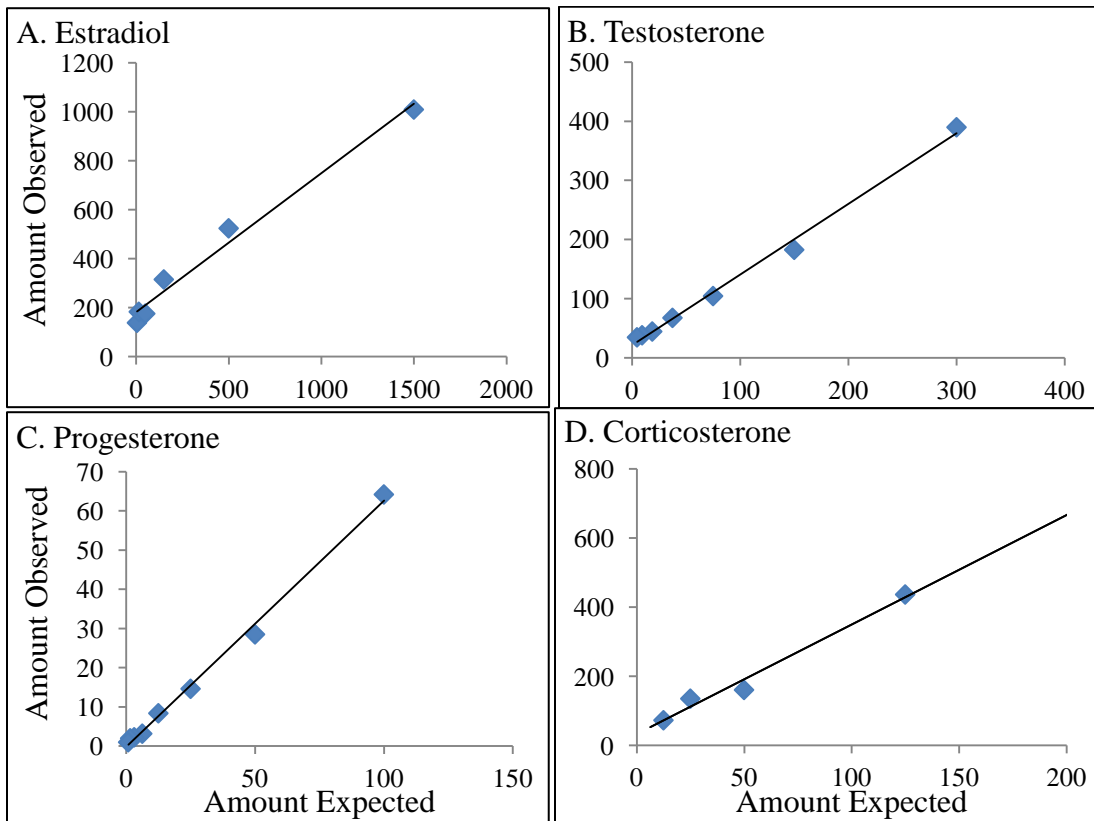
Hormone Assay Validations

Percent recovery for hormonal assays. Equation of the line and R^2 values are reported as follows: A. Estradiol: $y = 0.5678x + 180.94$ $R^2 = 0.9826$
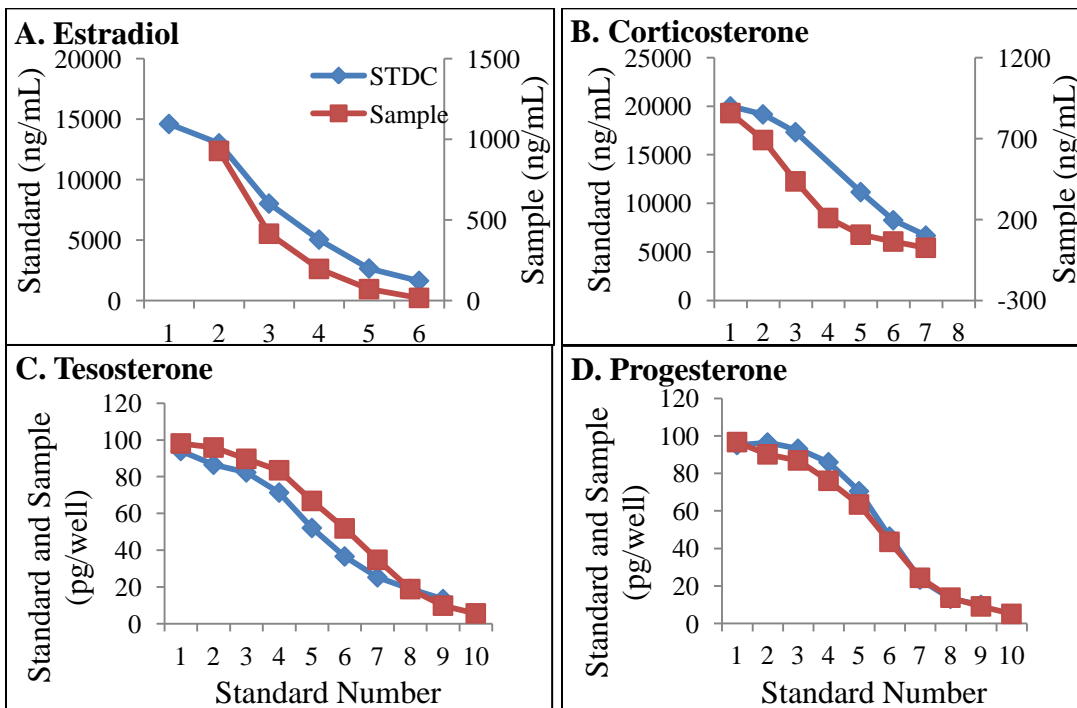B. Testosterone: $y = 1.1936x + 21.403$ $R^2 = 0.9944$
C. Progesterone: $y = 0.6277y + 0.1204$ $R^2 = 0.9959$
D. Corticosterone: $y = 3.1691x + 33.228$ $R^2 = 0.9802$

Parallelism performed for hormone assays. STDC = Standard Curve

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