

ABSTRACT

Title of Dissertation: **BIOLOGY AND MANAGEMENT OF
REPRODUCTION IN CAPTIVE CRANES**

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There are 15 species of cranes found throughout the world, 11 of which are listed as vulnerable or endangered. All 15 species are currently managed in captivity; however, with increased threats to wild crane habitats and populations, *ex situ* management becomes increasingly critical as a hedge against extinction. Reproduction and the production of offspring is required to ensure self-sustaining populations managed in *ex situ* conservation breeding programs. However, current reproductive success of the endangered whooping crane (*Grus americana*), as well as other species, maintained *ex situ* is suboptimal and hinders population sustainability and reintroduction goals. The objectives of this dissertation were to 1) develop a cryopreservation protocol for crane semen to improve genetic management in endangered cranes, 2) investigated seasonal hormone patterns and measured the impact of captive environment on hormone production and reproductive behaviors, and 3) retrospectively examine the effect of bird as well as management variables on egg fertility in whooping cranes. The findings demonstrated that 1) sperm of both whooping and white-naped crane performed better following cryopreservation when dimethyl-sulfoxide is utilized as a cryoprotectant, 2) seasonal fluctuations occur in hormone production in both sexes, while addition of a water feature to captive enclosures stimulated reproduction in females, and 3) female specific variables had the greatest influence on probability of egg fertility. Overall findings will help whooping crane management Continued research into the mechanisms controlling sperm sensitivity to cryo-damage, egg production, and fertilization are necessary to mitigate reproductive problems in captive crane species.

BIOLOGY AND MANAGEMENT OF REPRODUCTION IN CAPTIVE CRANES

by

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Dedication
For the cranes.

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Chapter 1: Literature Review

Herein, I present a review of literature focused on topics relevant to the management of captive cranes and their impact on reproduction. These topics include conservation status of the whooping and white-naped cranes, whooping crane's natural habitat, reproductive endocrinology and stress physiology in birds, avian sperm physiology, background knowledge and application of cryopreservation strategies to avian sperm, and current captive management strategies for the whooping crane.

Whooping crane (Grus americana)

The whooping crane is listed as endangered by the International Union for Conservation of Nature (IUCN 2017). The main threats to wild whooping crane populations include habitat loss and modification (Archibald and Mirande 1985; Ellis *et al.* 1992). In 1941, only 16 individuals remained in a single migratory population, which traveled between Aransas National Wildlife Refuge (ANWR), in Texas and Wood Buffalo National Park (WBNP), located in Alberta and Northwest Territories, on its yearly migration (Ellis *et al.* 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 483 individuals (IUCN 2017), which includes individuals in the Wood Buffalo migratory population, as well as reintroduced non-migratory populations in Florida and Louisiana and a migratory population that breeds in Wisconsin.

Currently, there are 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 from this captive flock are being released into the reintroduced migratory and Louisiana non-migratory populations as of 2017.

White-naped Crane (Grus vipio)

The white-naped crane is endemic to eastern Asia, summering across Northeastern Mongolia and China and wintering in the Yangtze River valley, the Korean Demilitarized Zone, and Kyushu Island of Japan (Harris and Mirande 2013). There are fewer than 4000 white-naped cranes left in the wild and the species is currently listed as vulnerable with a declining population (IUCN 2016). Because of declining *in situ* populations, *ex situ* conservation in zoos is more critical as a hedge against the extinction of this species. Currently 66 individuals (33 males, 32 female, one unknown sex) are housed in 23 institutions across North America (Database 2015). However, this captive population is not self-sustaining; it requires production of at least seven successful hatches per year in order to maintain the current population levels, a goal which is currently not met.

Whooping Crane's Habitat

Whooping cranes are a wetland species that requires aquatic habitat throughout their range, in both migratory and non-migratory populations (Ellis *et al.* 1996). The Wood Buffalo population of whooping cranes nests in the WBNP from late April through mid-May. The nesting habitat is characterized by poorly drained areas interspersed with numerous potholes and shallow, seasonally or semi-

permanently flooded marshy wetlands (Ellis *et al.* 1996; Hughes 2008) with near abundant vegetation, most often including mixed marshes of bulrush (*Scirpus validus*), cattail (*Typha latifolia*), and water sedge (*Carex aquatilis*; Timoney 1999).. This population spends its winters along the Gulf coast of Texas at ANWR, which consists of brackish to freshwater marshlands (Hughes 2008). Similar habitat is utilized by reintroduced whooping cranes at Necedah National Wildlife Refuge in Wisconsin (Fig. 1A) and the White Lake Wetlands Conservation Area in Louisiana (Fig. 1B). Spalding *et al.* (2009) correlated environmental factors with egg laying success and found that the best predictors of success were winter precipitation and marsh water depth. Specifically, deeper water resulted in earlier nests with more eggs laid, while greater precipitation levels resulted in higher fertility and hatching rates. It is hypothesized that this increased reproduction is due to deeper water increasing food availability and providing opportunities to place nests further from shore and in less hazardous areas.

In addition to work on the whooping crane habitat, there have been investigations of environmental effects on a closely related crane species which also demonstrates a strong relationship between wetland habitat quality and reproduction. Wild greater sandhill cranes (*Grus canadensis tabida*) nesting at Malheur National Wildlife Refuge in Oregon produced more chicks when nests were established early in the spring (Ivey 2007) when the water level was high. High water levels increase crane food availability, including both plants and macroinvertebrates, and helps protect nests from predators. It has been observed that sandhill cranes will desert nests when water levels drop too low (Ivey, 2007). High water levels early in the

Figure 1: Images of wild whooping cranes habitats at Necedah National Wildlife Refuge, Necedah, WI (A) and White Lake Conservation Area, LA (B).



(Photo credit: Megan Brown).

season also influenced the time at which the sandhill cranes built their nests. These results indicate the importance of proper habitat, especially the presence of water, on reproductive success in another crane species (see also Drewien et al. 1995). This increase in water depth increases the abundance of both plants and macroinvertebrates, and helps protect nests from predators. It has been observed that sandhill cranes will desert nests when water levels drop too low (Ivey, 2007). High water level early in the season also influenced the time at which the sandhill cranes built their nests. These results indicate the importance of proper habitat, especially the presence of water, on reproductive success in another crane species (Drewien et al. 1995; Ivey 2007). Similar results have been observed in greater flamingos (*Phoenicopterus ruber roseus*), another wetland dependent species, breeding in Southern France. Here, low water levels decreased the amount of prey available and reduced fledgling survival or prevented breeding altogether (Cezilly et al. 1995).

Reproduction

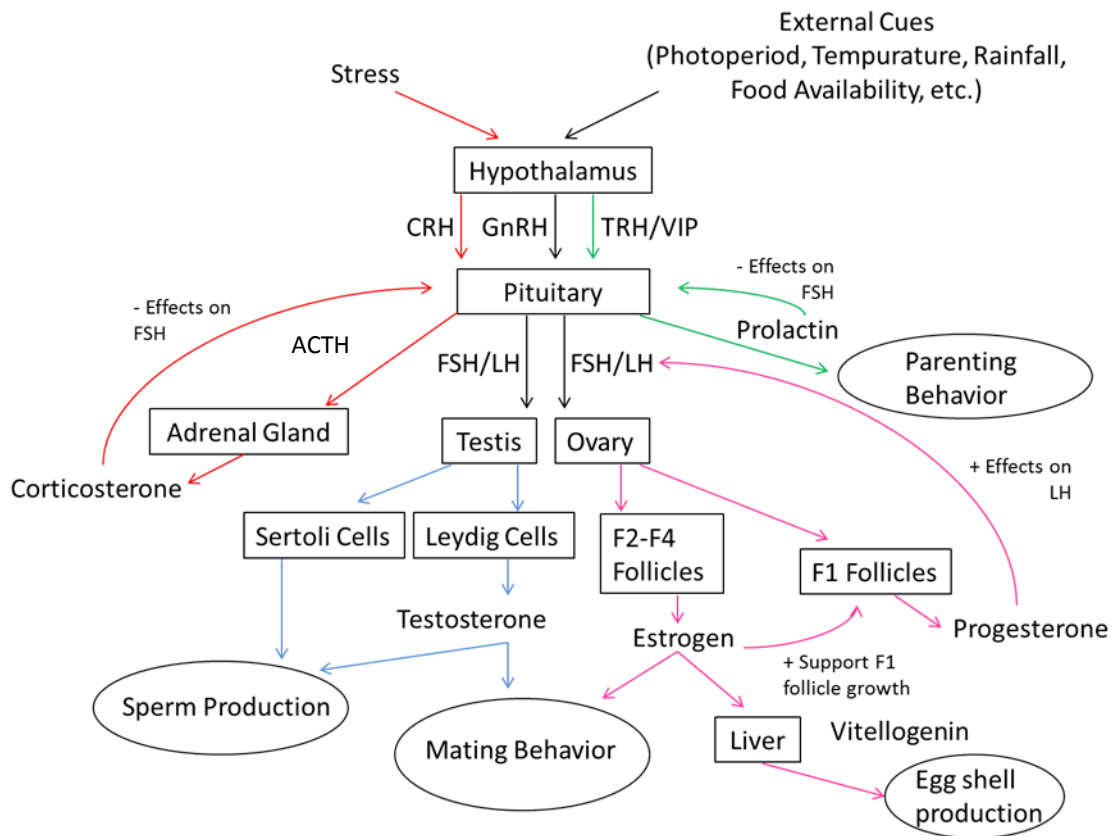
Reproductive endocrinology

The external environment influences the hypothalamo-pituitary-gonadal (HPG) axis in avian species. The primary environmental stimulus for reproduction is photoperiod (day length), while other cues are considered secondary or supplementary (Ball and Ketterson 2008). These external cues are translated through neuronal receptors in the brain to the hypothalamus, which triggers the secretion of gonadatropin releasing hormone (GnRH; Leska and Dusza 2007). GnRH acts on the anterior pituitary to mediate the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones in turn regulate gonad functions in both

males and females and the subsequent production of testosterone and estradiol (Ellis *et al.* 1996). If the appropriate signals are not sent to the hypothalamus, GnRH will not be released and the successive steps in the HPG axis will not be stimulated (Leska and Dusza 2007). There are many different isoforms of GnRH, including GnRH-1 and GnRh-2 and their functions in birds are not entirely understood. It has been suggested that GnRH-2 controls release of LH (Scanes 2014), while GnRH-1 regulates courtship behavior in birds (Ottinger and Baskt 1995; Norris 2006).

In males, FSH induces testicular growth and initiates the production of sperm by signaling the Sertoli cells (Fig. 2; Joyner 1990; Norris 2006). LH in males directs the secretion of androgens by the Leydig cells and mediates spermiation, a process by which mature sperm are released into the lumen of the seminiferous tubule (Ottinger and Baskt 1995, Norris 2006). Testosterone plays an important role in sperm production by influencing meiosis, maintaining spermatogenesis, and stimulating secondary male sex characteristics and male reproductive behaviors (Joyner 1990, Norris 2006). Without the production of testosterone, sperm production is compromised males are less likely to be successful breeders, as (Joyner 1990, and Bacon 2005). As the ovum grows, progesterone levels continue to increase and signal LH secretion (Norris 2006), which in turn up-regulates the conversion of androgen produced by the theca interna layer into estrogen and signals ovulation.

Figure 2: Hormonal Control of Avian Reproduction. External cues stimulate the hypothalamus, triggering a hormone cascade in the pituitary and gonads that regulates 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. Black arrows indicate the initial stimulus to the hypothalamic-pituitary-gonadal axis, then the blue and pink arrows indicate the differing roles of the HPG 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
- ACTH: Adrenocorticotropin Releasing Hormone
- FSH: Follicle Stimulating Hormone
- LH: Luteinizing Hormone

In females, FSH stimulates follicle growth and estrogen production (Ottinger and Baskt 1995, Norris 2006; Fig. 2). In birds, estrogen secreted from the follicle signals the liver to produce the yolk precursors, vitellogenin and very low density lipoproteins, and factors critical for egg shell production (Norris 2006). Progesterone is produced by granulosa cells of the largest preovulatory follicles on the ovary (Liu and Bacon 2005). As the ovum grows, progesterone levels continue to increase and signal LH secretion (Norris 2006), which in turn up-regulates the conversion of androgen produced by the theca interna layer into estrogen and signals ovulation. Luteinizing hormone secretion initiates when females nest and the concentration reaches peak levels at the onset of egg production and immediately declines after eggs are laid (Joyner 1990, Ottinger and Baskt 1995). After ovulation, estrogen and progesterone levels decrease until the growth of a new follicle (Ottinger and Baskt 1995).

The reproductive endocrine profile has been well characterized in other avian species (Lague *et al.* 1975; Bluhm *et al.* 1983; Sockman and Schwabl 1999; Crofoot *et al.* 2003). A previous study has found that female whooping cranes exhibit a similar reproductive profile to other birds. Specifically, estradiol concentrations are elevated for a period of weeks preceding an egg laying event, which is accompanied by a rise in progesterone concentrations just prior to ovulation (Brown *et al.* 2016). In this study, some whooping crane females displayed overall low estradiol production and very static values of progesterone which resulted in no egg production (Brown *et al.* 2016). Endocrine patterns immediately following an oviposition are currently unknown in the whooping crane. However, other avians, including white-naped

cranes, show a decreased concentration of both hormone in the days immediately following an ovulation (Lague *et al.* 1975; Proudman and Opel 1981). It is believed this is under the influence incubation behavior and increased prolactin production (Proudman and Opel 1981). The cause of these abnormal hormone profiles is unknown. Nevertheless, it is hypothesized that an inappropriate captive environment, e.g., the lack of wetland features in the enclosure, may be the cause of the perturbed steroid production (see below).

Stress physiology

Stress is a naturally occurring process in which the body responds to external stimuli. When a situation is perceived as stressful the hypothalamus releases corticotropin releasing hormone (CRH) which signals the anterior pituitary to produce adrenocorticotropin hormone (ACTH). ACTH then acts on the adrenal cortex and stimulate the release of glucocorticoids (Norris, 2006; Fig. 2). The primary glucocorticoid produced in birds is corticosterone. This molecule has the potential to act on all tissues within the body, with a variety of resulting actions, including increased gluconeogenesis, metabolism, and blood calcium (Siegel 1980). These are all classic actions seen in the fight or flight response, which help an individual cope and survive in short term stressful situations (Siegel, 1980; Norris, 2006). Although the secretion of corticosterone helps individuals cope with stressful situation, persistent elevation of this hormone (chronic stress) suppresses reproduction in an attempt to preserve body condition and maintain homeostasis (Angelier *et al.* 2009).

Stress has been shown to have a negative impact on avian reproduction. Wingfield *et al.* (1982) showed that when wild caught white-crowned sparrows

(*Zonotrichia leucophrys*) were first moved into captivity they produced higher plasma corticosterone and lower LH and dihydrotestosterone (DHT) concentrations when compared to the initial levels observed at capture. After an acclimation period, hormone production returned to normal and breeding resumed. In another study, Ouyang et al. (2011) showed that house sparrows (*Passer domesticus*) 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 with low corticosterone levels. Corticosterone also has a large influence on both reproductive and parenting behaviors. Studies of king penguins (*Aptenodytes patagonicus*; Groscolas et al. 2008) and black legged kittiwakes (*Rissa tridactyla*; Angelier et al. 2009) showed individuals with high corticoid levels are more likely to abandon nests and eggs.

While the majority of evidence confirms the inhibitory role of glucocorticoids, these “stress hormones” also serve a role in energy metabolism. In this instance glucocorticoid production prepares the body for normal physiological stressors such as migration (Koch et al. 2002; Landys et al. 2004), the energetic demands of courtship displays and copulation (Scanes 2014), and fasting during nesting and incubation (Wingfield et al. 1982).

Reproductive behavior and reproductive success

Reproductive behavior in the whooping crane has been well characterized (Ellis et al. 1996; Kuyt 1996; White 2000; Dellinger et al. 2013). Whooping cranes typically are monogamous and mate for life (Ellis et al. 1996; Cech et al. 2009). Reproductive behaviors include pre-copulatory behavior (marching), dancing, and

wing solicitation, usually performed by both members of the pair before attempting to copulate (Ellis *et al.* 1996; White 2000). This progression of behaviors synchronizes individuals in preparation for copulation and ensures that both individuals are fit and capable of the physical demands associated with mating (Ellis *et al.* 1996).

If they are successful during the breeding season, the crane pair will build a nest a few days before the first egg is laid. Wild nests are built in shallow water from vegetation and form a low but wide platform surrounded by a moat (Ellis *et al.* 1996; Cech *et al.* 2009). Whooping cranes typically lay two eggs per clutch within 2 days of each other. Once the eggs are laid, both parents incubate the clutch, taking turns and unison calling whenever they switch (Ellis *et al.* 1996).

Age and individual experience effects on reproduction

Experiences gained throughout an animal's lifetime can have a strong influence on survival and reproduction in later life (Prado-Oviedo *et al.* 2016). Reproductive success may improve as animals' age, possibly due an increase of experience (Curio, 1983). While young birds may have the skills necessary to breed and produce eggs, they do not know how to budget energy or use those skills appropriately. As birds age, they gain experience relevant to both reproduction and survival. Minton (1968) found that in general mute swans (*Cygnus olor*) must reach four years of age in order to breed successfully even if their mate has previously paired and nested. In conjunction with this, a previous successful pairing does not guarantee successful breeding when an individual is paired with a new mate. New pairs of mute swans typically spent one year as non-breeders prior to successful nesting. It has been suggested that parenting experience also increases reproductive

success in cranes (Archibald 1974; Ellis *et al.* 1996). For this reason, captive whooping crane pairs are occasionally allowed to raise a chick, to help stimulating future reproductive output (Jane Chandler, PWRC, personal communication). Others have shown that breeding/chick rearing experience is critically important in avian species. In wandering albatross (*Diomedea exulans*) breeding experience, rather than age, is a better predictor of breeding success (Angelier *et al.* 2006). Likewise, Davis (1976) showed that reproductive success in wild pairs of Arctic skua (*Stercorarius parasiticus*) was greatest if one member of the pair, regardless of sex, had previously raised a chick.

Avian sperm

Avian sperm consist of four sections, an acrosome, head, mid-piece, and flagella, each of which play critical roles during fertilization (Fig. 3; Hammerstedt and Graham 1992). The acrosome is essential for recognition of the female gamete during fertilization. The acrosome consists of an acrosomal cap and the sub-acrosomal rod (Etches 1996). In the presence of the inner perivitelline layer of the female follicle, the acrosomal reaction is initiated (Ashizawa *et al.* 2006). Upon this initiation, proteolytic enzymes are released which remove the acrosomal cap and exposes the acrosomal rod (Ahammad *et al.* 2013). It is the subacrosomal rod which binds to the female gamete and induces penetration and fertilization. The head region is filled with densely compacted chromosomal material and carries the male chromosomes (Blesbois 2012). The mid-piece and flagellar portions consist of the mitochondria and cytoskeleton of the cell and perform the necessary function of movement (Etches 1996).

Figure 3: Morphologically normal whooping crane sperm with each of the four regions, acrosome, head, mid-piece, and flagella (tail) portions labeled.

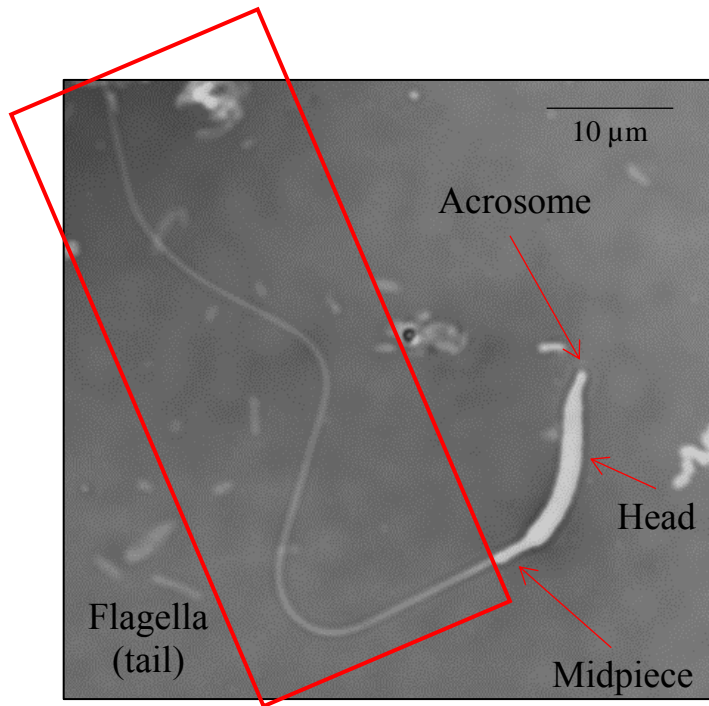


Photo credit: Megan Brown

Each region of the sperm is surrounded by a loosely fitting plasma membrane (Etches 1996). Sperm plasma membranes have three main components (Hammerstedt and Graham 1992), the most common being phospholipids, which contain a hydrophilic head and a pair of hydrophobic fatty acid tails. These phospholipids form a bilayer, with the hydrophobic heads facing the extra- and intra-cellular environments, with the hydrophobic tails protected in the space formed between the two hydrophilic layers. Avian sperm have high levels of polyunsaturated fatty acids, containing numerous double bonds resulting in kinked tails (Surai *et al.* 1998). These kinked tails prevent dense packing of phospholipids within the membrane. mitochondria and cytoskeleton of the cell and perform the necessary function of motility (Etches 1996).

The types and proportion of fatty acids differed by species; however, the most abundant found in five fowl species (chicken [*Gallus gallus domesticus*], turkey [*Meleagris gallopavo*], guinea fowl [*Numida meleagris*], duck [*Anas platyrhynchos domesticus*], and goose [*Anser anser domesticus*]) was 22:4 (n-6), or adrenic acid.

The next most common elements are membrane proteins, which act as transport channels or in cellular recognition during fertilization (Hammerstedt and Graham 1992). Finally, membranes contain cholesterol molecules, which reside within the membrane and help maintaining structure. The ratio of cholesterol: phospholipids in the membrane dictates its fluidity (Parks and Graham 1992). Higher levels of cholesterol, while making membranes more rigid at physiological temperatures, maintain membrane fluidity at low temperatures. Avian sperm have a low cholesterol:phospholipid ratio (Chicken: 0.30; Parks and Lynch 1992).

Prior to ejaculation avian sperm already possess full motility and majority of their fertilizing capability (Etches 1996; Blesbois 2012). Unlike mammals there is no long term storage and maturation in the epididymis, instead sperm are stored in the vas deferens for one to three days until ejaculation (Blesbois 2012). The approximate volume for four ejaculations is stored in the vas deferens of chickens, although the majority of sperm are expelled in the first ejaculation following any prolonged period of non-mating (Etches 1996). The concentration of sperm in commercially produced domestic poultry is typically high, (chicken: 5×10^9 cells/mL, turkey: 9×10^9 cells/mL, Japanese quail [*Coturnix japonica*]: 5×10^9 cells/mL; Etches 1996) while non-domesticated avian species have more variable sperm concentration (Budgerigar [*Melopsittacus undulates*]: 2.5×10^7 cells/mL; sandhill crane: 2×10^8 cells/mL; whooping crane: 1.8×10^8 cells/mL; ostrich [*Struthio camelus*]: 4×10^9 cells/mL; Gee 1995; Brown *et al.* 2015; Rybnik *et al.* 2007).

Upon successful copulation and ejaculation, sperm are transferred from the male's cloaca to the female's cloaca through inversion of both (Gee *et al.* 2004). Sperm are stored in the female reproductive tract in specialized invaginations called sperm storage tubules (SST). Sperm survival in these tubules varies by species (2 weeks in chickens and 9 weeks in turkey; Scanes 2014). While there is no definitive proof of SSTs occurring in cranes, paternity tests have confirmed successful fertilization by sperm inseminated up to nine days prior to oviposition in the whooping crane (Jones and Nicolich, 2001) and 16 days in the wattled crane (Swengel and Tuite, 1997). Only morphologically normal and motile sperm are able to enter the SSTs and high numbers of sperm located in the tubules are required for

successful egg fertilization (Brillard and Bakst 1990). To be accepted into the SSTs, certain sperm surface recognition glycoproteins are necessary (Wishart and Steele, 1990). These recognition proteins are typically lacking on immature sperm harvested directly from the testes, and these proteins may be damaged during cryopreservation.

Semen cryopreservation

Sperm are small cells with very little intracellular space and high surface area to cytoplasm ratio (Blesbois, 2007; Blesbois, 2012). During cryopreservation sperm are required to survive many processes and environments which can cause irreversible damage (Watson 2000). The process of cryopreservation can be divided into five stages (Hammerstedt *et al.* 1990). The first stage involves the addition of extenders and preliminary cooling to around 5°C. This stage slows metabolism, decreasing sperm motility and halting metabolic processes. The second stage exposes sperm to cryoprotectants and the packaging method specific to the freezing protocol being practiced. The exposure to a cryoprotectants initiate rapid volume changes and altered solute concentration. In this stage, if a permeating cryoprotectant is used, intracellular water diffuses out the cell as the cryoprotectant diffuses in. The length of time allotted for this stage is possibly the most important factor in cryopreservation as it is beneficial to remove as much intracellular water as possible to prevent intracellular ice formation during freezing. In this third stage (freezing) samples are cooled to sub-zero temperatures and cells are rapidly decreased in temperature from 5° to near -100°C before being plunged into liquid nitrogen and reaching a final storage temperature of -196°C. During freezing cells are again exposed to differing solute concentrations and volume changes. The use of a cryoprotectant prevents

dehydration, because the cryoprotectant has a lower freezing temperature than the extracellular water and of the cell itself. In the fourth stage, or long term storage, cells can survive for decades. All physiological processes are halted and sperm exist in a state of dormancy. The cells remain in this state until thawing in the fifth stage. Upon thawing, cells are rehydrated, membranes recover, and any damage incurred during the freezing process is manifested. In order for cells to survive these processes, a certain level of membrane plasticity is required.

Factors influencing semen cryopreservation

The most common forms of damage sperm experience is caused by volume changes and osmotic stress, which damages cellular membranes. Avian sperm are especially susceptible to damage compared to mammalian sperm because of their cylindrical head shape (Etches 1996; Blesbois 2012). This unique shape means that volume and osmotic changes, which are common during cryopreservation, become even more hazardous to avian sperm. To protect cells during freezing, protocols may stipulate use of various cryoprotectants (Watson 2000). There are two types of cryoprotectants: small molecules that cross the cell membranes known as permeating cryoprotectants (glycerol, dimethyl-sulfoxide [DMSO] and dimethyl-acetamide [DMA]), and large molecules, that cannot cross the cell membrane known as non-permeating cryoprotectants (salts and sugars). Permeating cryoprotectants enter the cell and replace the intracellular water and prevent cell dehydration and shrinkage (Blanco *et al.*, 2000, Watson, 2000).

Intracellular ice formation happens when cells are frozen too quickly and water is unable to diffuse out fully (Blanco *et al.*, 2000; Holt, 2000; Blesbois, 2011).

However, if the cells are frozen too slowly, the extended time in high solute concentrations can cause damage (Watson, 2000; Blesbois, 2011). Water outside of the cellular environment freezes before water within the cellular environment, which changes the osmotic nature of the solution (Watson, 2000). As more water outside the cell freezes, more water leaves the cell to alleviate this osmotic disparity. As water leaves, if there is nothing to replace it, the cell dehydrates and shrinks. Upon thawing, water enter into the cell too quickly, damaging the cellular membrane components and possibly rupturing the cell (Hammerstedt *et al.* 1990; Watson 2000). The low cholesterol:phosphate ratio in avian sperm causes the membrane to be less flexible and respond unfavorably to these volume changes during freezing and thawing (Parks and Lynch 1992).

Another common cryoinjury involves the phospholipid membrane of the sperm cell, and its susceptibility to the phenomenon called cold shock (Karow and Crister, 1997; Holt, 2000; Watson, 2000). During freezing, the different lipids within the membrane are able to longitudinally shift and reconfigure, not only changing the composition but also the functionality of the membrane (Karow and Crister, 1997). Specifically, classes of phospholipids have different melting points and freeze at different temperatures. As some sections of the membrane freezes, the non-frozen lipids will shift and aggregate together forming rafts within the membrane. This shift and functionality change becomes apparent when cells are thawed and are detrimental to sperm survival and fertilizing ability (Watson, 2000). When membranes become damaged they also become leaky, cellular components and ATP are lost, and calcium regulation is hampered (Karow and Crister, 1997, Blesbois, 2011).

During cryopreservation the polyunsaturated lipids in the head section of phospholipid bilayer are particularly at risk to damage from lipid peroxidation (LPO) (Surai *et al.*, 1998; Douard *et al.*, 2000; Watson, 2000). Lipid peroxidation occurs when a ROS steals a hydrogen atom from the methylene group adjacent to the double bond present in the unsaturated fatty acid (Kohen and Nyska, 2002). A ROS includes any oxygen radical with at least one unpaired electron (Bayr, 2005). The removal of this hydrogen destabilizes this double bond and ultimately leads to the rearrangement of the fatty acid and transformation into a peroxy radical. This radical will further decompose and lose all lipid properties (Kohen and Nyska, 2002). This membrane damage is likely from the buildup of ROS and the lack of natural antioxidants upon removal from the natural sperm environment (Breque *et al.*, 2003; Bansal and Bilaspuri, 2010). ROS are produced naturally during enzymatic cellular processes and have been shown to have detrimental effects on sperm in a variety of species including bull (*Bos taurus*; Chatterjee and Gagnon, 2001), ram (*Ovis aries*; Peris *et al.*, 2007), human (*Homo sapiens*; Mazilli *et al.*, 1994), and numerous species of domestic fowl (Partyka *et al.*, 2001). Fat soluble vitamins, such as vitamin E, A, and K, are highly effective in mitigating the damage of LPO (Donoghue and Donoghue 1997). These antioxidants have a higher affinity for ROS, and through binding, neutralize the threat to cellular membranes (Kohen and Nyska 2002).

Cryopreservation of avian sperm

There are unique challenges when attempting to cryopreserve avian sperm (Parks and Lynch, 1992; Blesbois, 2011). Avian sperm are more filamentous than mammalian sperm, with a large membrane surface, small cytoplasmic space, and long

tails (Blesbois, 2007; 2011). Within this small space the intracellular water content is lower compared to that of mammalian sperm (Blesbois, 2011). Avian sperm membranes are composed of different glycolipid arrangements than mammal sperm (Parks and Lynch, 1992). This difference in lipid composition, combined with their lower intracellular water content, makes avian sperm less sensitive to cold shock and temperature fluctuations, but more susceptible to membrane damage (Karow and Crister, 1997). Avian sperm are also distinctive as they must survive not only the freeze-thaw procedures, but also long periods of storage *in vivo* within the sperm storage tubules (SSTs) of the female reproductive tract (Bakst, 1998).

Since cryopreservation research was first initiated in the 1940s (Polge *et al.* 1949), most sperm cryopreservation studies in avian species have been conducted in the domestic fowl (Blesbois, 2007; 2011). Despite extensive research, sperm cryopreservation has not been routinely utilized in poultry industries. This is likely due to low fertility levels (25-35% in domestic turkeys) using cryopreserved sperm, even when the samples are from highly fertile males (Blesbois and Grasseau, 2001). This is concerning for use of the technique in endangered species which have small populations where often the males already have poor quality ejaculates (Fitzpatrick and Evans, 2009).

Historically, glycerol has been used as the main cryoprotectants for poultry semen, (Polge *et al.* 1949; Etches 1996). However, this cell permeable cryoprotectant also has sterilizing properties, especially in birds if present during artificial insemination (Hammerstedt and Graham 1992). Glycerol can be removed through gradient washing; however, avian sperm are highly sensitive to mechanical

manipulation (repeated pipetting and centrifugation) and too much handling often negates the benefit of glycerol. Due to the limitation of glycerol, studies have been conducted to examine the effectiveness of other cryoprotectants, including DMSO (Gee and Sexton 1979; Gee *et al.* 1985; Gee and Sexton 1990; O'Brien *et al.* 1999; Penfold *et al.* 2001) or DMA (Brock and Bird 1991; Tselutin *et al.* 1999; Blanco *et al.* 2000; Saint Jalme *et al.* 2003; Blanco *et al.* 2008) in protecting avian sperm against freezing injuries in numerous species, including chickens, turkeys, ducks, penguins (Spheniscidae spp.), pheasants (Phasianinae spp.), and sandhill cranes.

To alleviate damage caused through LPO, anti-oxidants may be added to freezing media. Previous studies have shown that fat soluble vitamins, such as vitamin E, A, and K, are highly effective in mitigating the damage of LPO to avian sperm, as compared to the water soluble vitamins, vitamin B and C (Mangiagalli *et al.*, 2007; Suari *et al.*, 2001; Donoghue and Donoghue, 1997). Partyka *et al.* (2012) found that after freezing, activity of the antioxidant enzyme catalase in chicken sperm significantly decreased in the seminal plasma while malondialdehyde, the main product of lipid peroxidation, increased significantly. Vitamin E, a natural antioxidant, has been found in high concentrations in semen of domesticated chicken (Surai *et al.*, 1999). When used as an added supplement to freezing media, vitamin E has also increased post thaw viability in species with naturally high concentrations of vitamin E (chickens) and those without (ducks; Surai *et al.*, 1999).

The focus of semen cryopreservation in cranes has primarily been on the greater sandhill crane (Gee *et al.* 1985). Sperm cryopreservation methods developed in the greater sandhill crane have been adapted for an endangered subspecies,

Mississippi sandhill crane (*Grus canadensis pulla*) and “Cryo-chicks” were produced at Audubon Center for Reproduction in Endangered Species (ACRES) as recently as 2008 (Meg Zuercher, ACRES, pers. communication). Research previously conducted at PWRC resulted in development of cryopreservation protocols for sandhill cranes, using DMA, which allowed maintenance of high sperm viability (77%) after freezing (Blanco *et al.*, 2011). However, low motility values, around 25%, also resulted; obtaining a high post thaw motility is paramount because motility is the most critical factor for fertilization (Froman *et al.*, 1999).

While PWRC has been at the forefront of crane semen and cryopreservation work, there has been little work utilizing semen cryopreservation in management of other endangered crane species. Successful cryopreservation of whooping and white-naped crane sperm will allow production of offspring from behaviorally incompatible or geographically separated, but genetically desirable, pairs. Furthermore, the capacity to cryopreserve semen samples from the current population allows for preservation of current genetic diversity, and the ability to restore diversity in the future, which was accomplished for black-footed ferrets (*Mustela nigripes*; Howard *et al.* 2016).

Captive Management of Whooping Cranes

Whooping cranes at Patuxent Wildlife Research Center

PWRC is the largest among the six captive breeding facilities for whooping cranes. Currently, PWRC maintains 78 whooping cranes with 24 breeding pairs. On average, the facility produces ~19 chicks annually (and more than 130 birds total since 2001; Sarah J. Converse, PWRC, personal communication). However,

whooping cranes in this captive population experience poor reproduction. Between 2007 and 2011, only 54% of eggs produced were fertile, as compared to 95% fertility seen in the Wood Buffalo population (Kuyt 1995) 71% in the reintroduced migratory population (Whitear and Lacy 2016). The reintroduced Florida Non-Migratory Flock is an exception (52%; Folk *et al.* 2005). In 2011, eight captive breeding pairs did not produce and four pairs laid only a few eggs. Furthermore, reproductive onset in captive female cranes is delayed compared to wild counterparts (eight vs. five years old, respectively; Ellis *et al.* 1996).

Captive habitat

PWRC currently houses cranes (whooping and sandhill) in dry pens, roughly between 3,000 and 5,000 ft² (Fig. 4; USGS-PWRC 2010). However, there is a great deal of anecdotal evidence that reproduction increases the more naturalistic the *ex situ* environment is (Ellis *et al.* 1996; Hughes 2008). Other crane species, such as brolgas (*Grus rubicunda*) housed at the International Crane Foundation (ICF) began breeding after sprinkler systems were used to simulate the rainy breeding season (Ellis *et al.*, 1996). At captive breeding centers and in the wild, rainfall correlated positively with breeding success of sarus cranes (Balzano 1989) and for whooping cranes at PWRC (N. Songsasen, unpublished data). Wattled cranes (at the Wildlife Survival Center, Midway, GA), whooping cranes and Siberian cranes (at ICF, Baraboo, WI) increased their reproductive output when pens were flooded seasonally (Ellis *et al.*, 1996). Finally, captive, non-reproductive sandhill cranes at ICF began laying eggs when moved from a dry pen into a pen with a full sized pond, although this was not examined experimentally (Kelly Maguire, pers. comm.).

Figure 4: Whooping cranes housed in traditional captive enclosures at Patuxent Wildlife Research Center.



Photo credit: Megan Brown

These observations of crane species serve only as anecdotal evidence in support of a more naturalistic environment facilitating crane reproduction.

As few studies have investigated captive habitat effects on cranes, it is relevant to consider research performed on other wetland species housed in captivity. Breeding in captive Caribbean flamingo flock (*Phoenicopterus ruber ruber*), was positively influenced by increasing levels of precipitation and heavy rainfall (Stevens 1991; Michael and Pichner 1989) or when nesting grounds were artificially flooded during breeding season (Brown *et al.* 1983). This flooding mimics the natural environment of the Caribbean flamingo (Michael and Pichner 1989). This again indicates a link between natural environment, specifically the presence and depth of water, and reproduction in wetland birds.

Behavior in captivity

Appropriate reproductive behaviors are critical for birds to reproduce normally. The majority of avian reproductive behaviors are visual rather than olfactory cues (Cech *et al.* 2009). When natural behaviors are restricted, reproduction could be limited or halted altogether, because behaviors are often critical to trigger necessary hormonal changes (Bohmke 1995). The simplest way that captive environments can limit reproduction involves enclosure design and size. Whooping cranes housed in open top exhibits must have their wings clipped to prevent them from escaping from the enclosure. However, this also prevents them from reproducing normally, as the species requires full use of wings for courtship displays and to copulate successfully (Ellis *et al.* 1996).

Animals are more likely to breed successfully if kept in appropriate housing situations, including social situations as well as an appropriate physical environment. For example, Caribbean flamingos at the National Zoological Park displayed more reproductive behaviors, both as individuals and as a group, when the flock's social grouping was more balanced (Stevens 1991). Wild whooping crane pairs maintain large territories during the breeding season (Ellis *et al.* 1996). It is possible that captive centers maintaining pairs in too close proximities or at high densities within one location could negatively impact breeding success.

Captive stress

Presently, few studies have examined the influence of captive environment on stress in birds. Previous studies in wild birds have shown that the stress response is modified depending on the time of year, with less corticosterone being produced during the breeding season in response to the same stimuli as compared to the non-breeding season (Wingfield *et al.* 1982). Ouyang *et al.* (2011) showed that captive birds with high levels of corticosterone prior to the breeding season have less reproductive success than those with low glucocorticoid levels. Specifically, the former exhibits a delay in egg laying, produces fewer eggs, and has fewer eggs hatch and survive to fledgling stage compared to the latter. If the captive environment is causing high stress, reproduction would be diminished and may explain why some species, including whooping cranes, have trouble breeding in captivity.

Stress associated with captive management and its link to reproductive performance has been demonstrated in several mammal species (Carlstead and Shepherdson 1994). For example, clouded leopards (*Neofelis nebulosa*) housed in zoo

enclosures with more vertical climbing space had lower stress hormone levels than their counterparts having no opportunity to climb (Wielebnowski *et al.* 2002). Male brown tree snakes (*Boiga irregularis*) housed in Guam showed decreased spermatogenesis and shrunken sexual organs, which the authors believe was caused by increased glucocorticoid concentrations due to stress from inadequate housing conditions (Aldridge and Arackal 2005).

Dissertation Objectives

The three studies discussed within this dissertation are designed to examine how captive management strategies can impact reproduction or how management protocols can be used more effectively to improve reproductive success. Specifically the goals were to 1) examined the influence of cryoprotectant type and antioxidant supplementation on post-thaw survival, in order to develop a cryopreservation protocol for whooping crane semen, 2) investigated seasonal hormone patterns and measured the impact of captive environment on hormone production and reproductive behaviors, and 3) examined factors, both management related and otherwise, and their effects on egg fertility.

Chapter 2: Cryopreservation effects on sperm function and fertility in two threatened crane species

Introduction

Currently, there are 15 extant crane species in the world, 11 of which are listed as vulnerable or endangered by the International Union for the Conservation of Nature (IUCN 2016). All 15 species are currently managed in captivity; however, with increasing threats to wild crane habitats and populations, *ex situ* management is critical as a hedge against extinction (Harris and Mirande 2013; Leito *et al.* 2015; IUCN 2016; Namgay and Wangchuk 2016).

One of the main goals of *ex situ* management is to sustain genetic diversity of populations (Holt *et al.* 2003). However, cranes are monogamous birds that mate for life, and this presents a challenge for maintaining genetic diversity in an *ex situ* setting. In many crane species, such as the whooping crane (*Grus americana*; Jones and Nicolich 2001) and white-naped crane (*Grus vipio*; Mace *et al.* 2013), artificial insemination (AI) is a crucial tool for overcoming this specific challenge. This technology allows managers to inseminate females with semen donated from a male outside her established pair. However, this breeding technique is currently only possible at institutions that house multiple males. The development of successful semen cryopreservation procedures would allow the movement of genetic material between breeding centers, which is much easier than moving live birds. Furthermore, semen cryopreservation would permit banking of genetic material for future use even long after the death of individual males. This benefit has already been demonstrated

in the black-footed ferret (*Mustela nigripes*) in which live offspring have been produced from frozen spermatozoa stored for over 20 years (Howard *et al.* 2016).

Work in semen cryopreservation has been ongoing since the 1940's, when the first chicken eggs were successfully produced with frozen chicken sperm (Shaffner *et al.* 1941) and glycerol was identified as an effective cryoprotectant (Polge *et al.* 1949). However, current protocols are still unreliable for the majority of both domesticated and exotic birds (Blesbois 2007). One of the many factors limiting the success of semen cryopreservation is that spermatozoa are small cells with very little intracellular space and are highly sensitive to damage during the freeze-thaw process (Long 2006). Avian sperm are especially intolerant to volume and osmotic changes, as compared to mammalian sperm, because of their cylindrical head shape and low surface area to volume ratio, with little cytoplasmic space and densely packed chromatic material (Etches 1996; Long 2006; Blesbois 2012), thus, increasing susceptibility to cryoinjuries.

Historically, glycerol was used as the main cryoprotectants for poultry sperm, (Polge *et al.* 1949; Etches 1996). However, this cell permeable cryoprotectant also has sterilizing properties, especially in birds if present during artificial insemination (Hammerstedt and Graham 1992). Therefore, other cryoprotectants, including dimethyl sulfoxide (DMSO; Gee and Sexton 1979; Gee *et al.* 1985; Gee and Sexton 1990; O'Brien *et al.* 1999; Penfold *et al.* 2001) and dimethyl acedamide (DMA; Brock and Bird 1991; Tselutin *et al.* 1999; Blanco *et al.* 2000; Saint Jalme *et al.* 2003; Blanco *et al.* 2008) have been examined for their effectiveness in protecting

sperm against freezing injuries in numerous species, including chickens, turkeys, ducks, penguins, pheasants, and sandhill cranes.

One of the main causes of cryoinjuries involves the poly unsaturated phospholipids of the bilayer of the head section. Unsaturated phospholipids contain one or more double bonds between adjacent carbon molecules within their lipid tails. These lipid tails are particularly at risk to damage from lipid peroxidation (LPO; Surai *et al.*, 1998; Douard *et al.*, 2000), likely caused by the buildup of reactive oxygen species (ROS) and the lack of natural antioxidants upon removal from the seminal plasma (Breque *et al.*, 2003; Bansal and Bilaspuri, 2010). ROS includes any oxygen radical with at least one unpaired electron (Bayr 2005). Lipid peroxidation occurs when these ROS remove a hydrogen atom from a methylene group that is adjacent to the carbon-carbon double bond. This results in the destabilization of the double bond and transformation of the lipid tail into a peroxy radical (Kohen and Nyska 2002). Previous studies have shown that fat soluble vitamins, such as vitamin E, A, and K, are highly effective in mitigating the damage of LPO (Donoghue and Donoghue 1997). These antioxidants have a higher affinity for ROS, and through binding, neutralize the threat to cellular membranes (Kohen and Nyska 2002).

Previous work utilizing cryopreservation in crane species has focused primarily on sandhill cranes (*Grus canadensis*; Gee and Sexton 1979; Blanco *et al.* 2012). While current cryopreservation protocols for sandhill cranes have resulted in high cell survival and produced fertile eggs (Gee *et al.* 1985; Blanco *et al.* 2012), these protocols have yet to be adapted to other crane species, and are not being routinely utilized in breeding management. The goal of this study was to develop

effective cryopreservation protocols for two endangered crane species, the whooping and white-naped crane, which can be incorporated in breeding management. Research described was designed to assess 1) the effectiveness of two permeating (DMSO and DMA) and one non-permeating (sucrose) cryoprotectant in preserving sperm function during cryopreservation in both species and 2) the influence of vitamin E on post-thaw survival of whooping crane sperm. The hypothesis was that the combination of both a permeating and non-permeating cryoprotectant would improve motility and viability of sperm post-thaw and that the addition of vitamin E to freezing media improves sperm survival and function post thaw.

Materials and Methods

Sampling design and semen collection

Whooping cranes ($n = 8$) and white-naped crane ($n = 3$) housed at Patuxent Wildlife Research Center (Laurel, MD; PWRC) and Smithsonian Conservation Biology Institute (Front Royal, VA; SCBI) respectively, were included in the study. Birds were selected based on historic semen production metrics (Brown *et al.* 2015; Panyaboriban *et al.* 2016). Males producing samples of high volume (≥ 50 uL) and above average motility ($\geq 40\%$) were considered. Males were selected by respective flock managers based on the crane's response to semen collections and on minimizing conflicts with needs of the AI program. All animal procedures associated with this study were performed with approval of Animal Care and Use Committees at USGS-PWRC (2013-04), Smithsonian Conservation Biology Institute (10-11), and University of Maryland (883522-1).

Cranes at PWRC were housed in breeding pens, all of which are outdoors and approximately 13.7 m wide and 19.8 m long. Birds were housed with conspecific females ($n = 5$) or alone ($n = 3$). All cranes were fed a pelleted breeder diet (produced by Republic Mills, Okolona, OH; recipe provided by Swengel and Carpenter 1996) in a gravity feeder and provided water *ad libitum*. All semen collections at PWRC were carried out by a team of three PWRC staff, each with over 10 years of experience using the massage collection method previously described (Gee and Temple 1978; Brown *et al.* 2015).

Cranes at SCBI are housed in breeding pens, all of which are outdoors and with a conspecific female. Pens are approximately 22.9 m wide and 45.7 m long. Subjects were each housed with conspecific females. Cranes were fed Zeigler Crane Breeder Diet (Zeigler Feed, Gardners, PA) in a gravity feeder and provided water *ad libitum*. Semen samples were obtained from males using the massage collection technique as cited above, by two SCBI staff members, each with more than 13 years of experience in crane management

At PWRC, semen samples were collected weekly from each whooping crane male during peak breeding season (April). For the white-naped cranes at SCBI, semen samples were collected opportunistically throughout the breeding season (March through May) as part of routine seminal assessment for the AI program. Males were randomly assigned to a collection day. Samples were collected in the enclosure and transported to the lab within 20 mins in a Styrofoam cooler with ice packs. Study 1 included whooping and white-naped cranes, while Study 2 only involved whooping cranes (Study 1 and 2 described below).

Semen processing and assessment

Upon arrival at the laboratory, all samples were assessed for basic seminal variables including volume and motility. Only samples that were not contaminated with feces or urates and those with >40% motility were utilized. The samples were diluted 1:2 with crane semen extender (Blanco *et al.* 2012; composition in Appendix A) then assessed for concentration (Neubauer haemocytometer), motion variables (Computer Assisted Semen Assessment [CASA], see below) and membrane integrity (SYBR-14/propidium iodine staining, see below).

Sperm cryopreservation and thawing

The diluted samples were further diluted with crane semen extender containing a combination of permeating cryoprotectants and non-permeating cryoprotectant (Study 1 DMSO/DMA or sucrose; Study 2 DMSO). Following exposure to the cryoprotectant, samples were allowed to equilibrate at 4°C for up to 30 min, loaded into 0.25 ml French straws (AgTech Inc., Manhattan, KS, USA) labeled with individual's ID, date, and treatment group, then heat sealed (Uline, Pleasant Prairie, WI). Straws were frozen using a two-step freezing method previously described (Panyaboriban *et al.* 2016). Briefly, straws were cooled in liquid nitrogen (LN₂) vapor on a Styrofoam board (2.5 cm thickness) that was placed 2.5 cm above LN₂; thus, the total distance between the straws and LN₂ was 5 cm. After 6 min, the straws were lowered to 2.5 cm above LN₂ for 14 min before being plunged into LN₂. The average cooling rate during the first step was 7 °C/min (from 4°C to -30°C) and that of the second step was 9 °C/min (from -30°C to -110°C). Samples

were kept frozen (between 6 and 8 months), then thawed in a 37°C water bath for 30 sec and assessed immediately for post-thaw survival (see below).

Motility and motion variable analysis

Sperm motility and motion variables were assessed using CASA system (Hamilton Thorne Research, Beverly, MA, USA) and eight randomly selected microscopic fields per sample. CASA measurements were recorded at 60 Hz, with sperm head size (min/ max) = 6/17 μm^2 ; low VSL cut off = 6 $\mu\text{m/s}$; low VAP cut off = 5 $\mu\text{m/sec}$; and threshold straightness = 80% as determined and reported (Panyaboriban *et al.* 2016). Variables evaluated included overall motility (%), straight-line velocity ($\mu\text{m/s}$, velocity over the straight-line distance between the beginning and end of the sperm's path), curvilinear velocity ($\mu\text{m/s}$, velocity over the actual sperm's path, including all deviations of sperm head movement), average path velocity ($\mu\text{m/s}$, velocity over a calculated, smoothed path), and straight line distance (μm , distance covered in the straight-line path).

Viability assay

The percentages of viable (i.e. membrane intact) spermatozoa were evaluated using SYBR-14/propidium iodide (PI) fluorescence staining (LIVE/DEAD Sperm Viability Kit; Invitrogen Molecular Probes, Eugene, OR, USA). SYBR-14 working solution (2 μL) at a concentration of 0.1 mg/ml and PI (5 μL) at a concentration of 2 mg/ml were added to 5 μL of semen sample. Following 15 mins of incubation, 2 μL of stained sample was placed on a glass slide, covered with a coverslip, and examined immediately using a fluorescence microscope (Olympus BX40, Olympus Optical Co., Ltd., Japan). For each sample 100 spermatozoa were assessed. Spermatozoa

fluorescing green (SYBR14 positive) were classified as being viable (having an intact membrane), while spermatozoa fluorescing red (PI positive) were considered non-viable (damaged membrane).

In vitro sperm-egg interaction assay

Inner perivitelline (IPVL) membranes from chicken eggs were utilized to examine binding ability using a method modified from that reported by Kido and Doi (1988) and Robertson *et al.* (1997). Specifically, fresh egg (within 2 days of lay) from chickens were sourced from a local organic farm (Eagle Crest Farm, Strasburg, VA). Egg yolks were isolated by hand and rinsed with 5% NaCl. Yolks were then placed in 0.01 M HCl and incubated at 37°C for 1 hour. The HCl was removed and then yolks were punctured, allowing the internal yolk contents to flow out. The membranes were then washed twice in 5% NaCl and gently teased apart where layers had begun to separate, isolating the inner (IPVL) and outer PVLs. The IPVL was cut into small sections and placed in 500 µL of minimal essential media (MEM) buffered with 10 µM HEPES and 10 mM CaCl₂ to facilitate acrosomal reaction (Brown *et al.* 2017). Approximately 1×10^6 spermatozoa (as assessed by semen concentration per sample) were added to a vial containing a section of IPVL and incubated at 37°C for one hour. Following incubation membranes were removed with tweezers, washed in 5% NaCl, stretched onto a glass slide, and covered with a cover slip. Slides were viewed under dark-field illumination at 100x magnification. Three fields of view per membrane were randomly selected and assessed for presence of spermatozoa binding determined circular holes in the membrane (Fig. 1).

Morphology Assessment

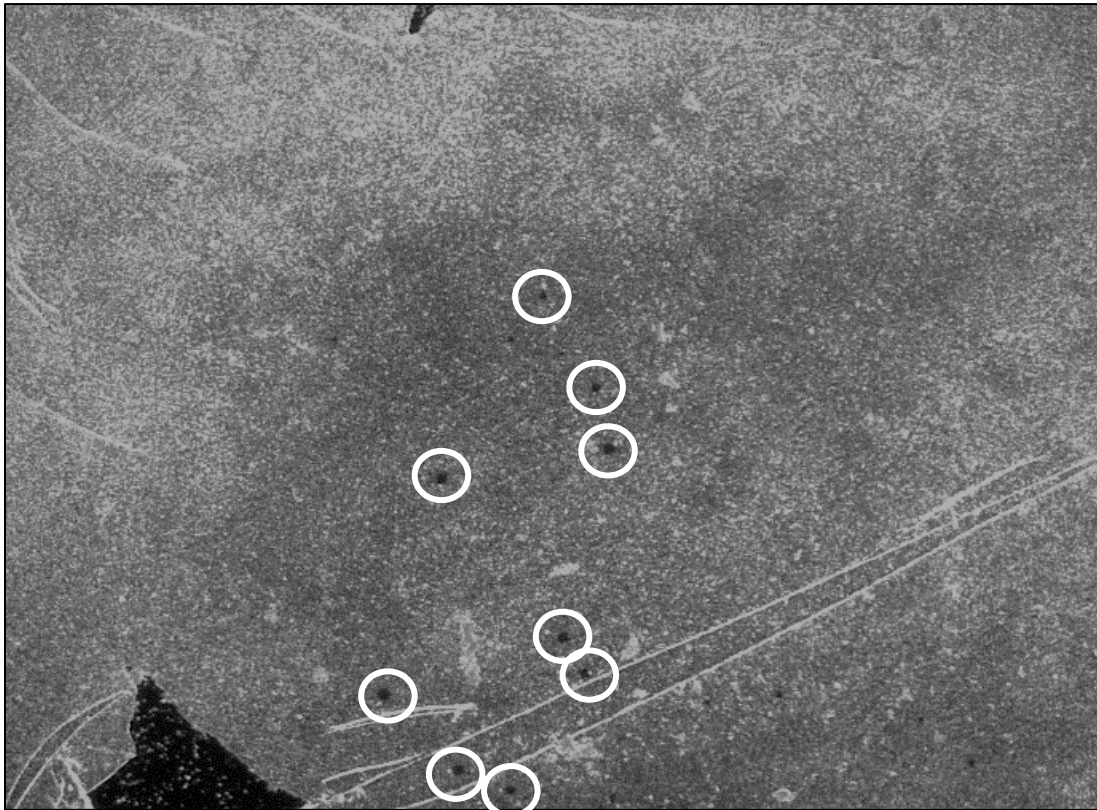
Sperm pleomorphisms were assessed as previously described for the whooping crane (Brown et al. 2015). Briefly 5 μ l of either fresh diluted or post-thaw semen sample was fixed in 0.3% glutaraldehyde and stored in a cryo-vial at 4°C. Later a subsample was stained with eosin-nigrosin (Fisher Scientific, Pittsburgh, PA), smeared on two slides, and 200 individual spermatozoa evaluated per slide using a bright field microscope (1000x in oil immersed magnification). Each spermatozoon was classified as structurally normal or abnormal.

Experimental Treatments

Study 1: Effect of cryoprotectant on sperm survival following cryopreservation

Samples were collected from both whooping cranes at PWRC ($n = 4$) and white-naped cranes at SCBI ($n = 3$). In this study we utilized combinations of two different permeating cryoprotectants: DMSO (8% for whooping crane, 10% for white-naped crane) or DMA (8% for whooping crane, 10% for white-naped crane), with one non-permeating cryoprotectant, sucrose. A total of six treatments were included: 1) DMA, 2) DMA + 0.1M sucrose, 3) DMSO, 4) DMSO + 0.1M sucrose, 5) 0.1M sucrose, and 6) 0.2M sucrose. Each of these cryoprotectants was selected based on past success cryopreserving sandhill crane sperm (Gee and Sexton 1979; Blanco *et al.* 2012). When possible samples were split equally between all six treatment groups with the requirement that $> 25 \mu$ L of diluted sample was frozen per treatment; if the sample was too small, it was randomly assigned to treatments until exhausted. The samples were frozen and thawed as described above and then assessed for overall motility and four sperm motion variables (measured by CASA), percent viability, and

Figure 1: Representative images from sperm-egg binding assay. White circles indicate holes in the membrane where crane sperm have bound and penetrated the membrane.



ability to bind in sperm-egg interaction assay. All treatments within one samples were thawed on the same day and assessed in all assays that day.

Study 2: Effect of vitamin E supplementation prior to cryopreservation

Samples were collected from whooping cranes at PWRC ($n = 8$) to assess the effect of vitamin E on post thaw survival and function of whooping crane spermatozoa. Three levels of vitamin E treatment were tested: a control (DMSO alone), 5 $\mu\text{g}/\text{mL}$, and 10 $\mu\text{g}/\text{mL}$. Vitamin E (prepared at a 3X concentration of the final tested dosage) was included in the freezing media (containing 24% DMSO) before being added to extended semen. When possible samples were split equally between all treatment groups, and $> 25 \mu\text{L}$ of diluted sample was frozen per straw; if the sample was too small it was randomly assigned to treatments until exhausted. Frozen-thawed samples were assessed for overall motility and four sperm motion variables (measured by CASA), percent viability, and percent normal morphology.

Statistical Analysis

In Study 1, seminal variables post-thaw (motility, membrane integrity, motion characteristics, fertility as measured through egg-membrane binding) were compared between treatment groups, and with a fresh control when possible. An interaction between treatment and species was included to determine if there were differences between whooping and white-naped cranes. Analysis was conducted using linear mixed models for each response variable and with individual serving as random effect in the lme4 package (Bates *et al.* 2012) in the statistical program R (R Core Team 2014). In each case, estimates of the mean effect of treatment, standard error of this effect, and 95% confidence intervals for each mean effect were calculated from the

model taking the random effect into account. Statistical significance was determined between two treatments if confidence intervals did not overlap each other (Johnson 1999).

Analysis for Study 2 was conducted similarly as above. Each seminal variable of interest (motility, membrane integrity, motion characteristics, and normal morphology) were compared between treatment groups and against a fresh control when possible. Again, estimates of the mean effect of treatment, standard error of this effect, and 95% confidence intervals for each mean effect were calculated. Statistical significance was determined between two groups if confidence intervals did not overlap each other.

Results

Initial fresh ejaculates from whooping cranes showed significantly lower percent motility compared to white-naped cranes (Table 1). However, other semen characteristics, including viability (Table 1), normal morphology (whooping crane: $73.05 \pm 1.03\%$ vs. white-naped crane: $73.83 \pm 1.97\%$), and the four CASA motion variables were comparable between the two species (included in Appendix A). Cryopreservation reduced sperm motility and plasma membrane integrity compared to the fresh ejaculates of both species (Table 1).

Effect of cryoprotectants on sperm survival following cryopreservation

Whooping Crane

Whooping crane sperm overall performed best when frozen in the DMSO treatment group. Post-thaw motility (overall %), viability, and number of sperm binding to IPVL were highest in the DMSO treatment group (Table 1). In each

variable examined the DMSO + 0.1 M sucrose treatment group was most similar to DMSO alone, but slightly lower. The DMA and DMA+ 0.1 M sucrose treatment groups displayed intermediate overall motility levels and number of sperm bindings, but were equal in percent viable sperm, compared to DMSO. Post-thaw viability (%) was similar among the four permeating cryoprotectant treatment groups. The sucrose alone treatments each had the lowest values for motility, viability, and number of sperm binding.

Of the CASA motion variables evaluated, significant differences were observed only in Straight Line Distance (μm). The highest observed values post-thaw were in the DMSO and DMA + 0.1 M sucrose treatments, which were also most similar to raw ejaculates. There were no significant differences for the remaining CASA motion variables (Straight Line Velocity, Curve Linear Velocity, and Average Path Velocity; Appendix A).

White-naped Crane

Again, the DMSO treatment group displayed the highest values for overall motility, viability, and number of sperm binding. The DMSO + 0.1 M sucrose treatment group was most similar to DMSO, although lower in each case. The two DMA treatment groups displayed intermediate motility, and viability values. The sucrose only groups had the lowest motility, motility, and sperm binding values.

As in the whooping crane, Straight Line Distance (μm) was the only CASA motion variable with significant differences among the treatment groups (Table 1). The highest observed values were in the DMSO treatment and the DMSO + 0.1 M sucrose treatments, both being higher than fresh values. All other treatments were

Table 1: Sperm characteristics (mean estimate \pm standard error of estimate (95% confidence interval)) of fresh and post-thaw samples in the whooping crane and white-naped crane.

Species	Cryoprotectant	Overall Motility (%)	Viability (%)	Sperm Binding (Number)	Straight Line Distance (μm)
Whooping Crane	Fresh	47.9 \pm 3.8 (40.4, 55.5) ^a	89.5 \pm 2.1 (85.3, 93.6) ^a	--	10.1 \pm 2.7 (9.6, 10.6) ^a
	DMA	3.1 \pm 0.6 (1.9, 4.3) ^{bc}	46.0 \pm 3.3 (39.6, 52.5) ^b	4.9 \pm 1.4 (2.2, 7.5) ^{ab}	6.7 \pm 0.8 (5.1, 8.4) ^b
	DMA + 0.1M Sucrose	1.7 \pm 0.3 (1.1, 2.4) ^c	49.8 \pm 2.9 (44.0, 55.5) ^b	2.3 \pm 1.4 (-0.4, 4.9) ^{ab}	7.9 \pm 1.0 (6.0, 9.8) ^{ab}
	DMSO	6.4 \pm 1.1 (4.2, 8.6) ^b	51.0 \pm 3.1 (45.0, 57.1) ^b	6.9 \pm 1.1 (4.7, 9.1) ^a	8.9 \pm 0.7 (7.6, 10.3) ^{ab}
	DMSO + 0.1M Sucrose	4.0 \pm 0.7 (2.6, 5.4) ^b	46.3 \pm 2.8 (40.8, 51.7) ^b	3.2 \pm 1.2 (0.9, 5.6) ^{ab}	7.9 \pm 0.7 (6.6, 9.3) ^b
	0.1M Sucrose	1.0 \pm 0.2 (0.7, 1.4) ^c	22.4 \pm 2.9 (16.8, 28.0) ^c	0.9 \pm 1.2 (-1.2, 3.2) ^b	6.3 \pm 0.8 (4.7, 7.8) ^b
	0.2M Sucrose	1.0 \pm 0.2 (0.6, 1.4) ^c	26.7 \pm 3.5 (19.9, 33.5) ^c	1.0 \pm 1.5 (-1.9, 3.9) ^b	6.4 \pm 0.9 (4.6, 8.2) ^b
White-naped Crane	Fresh	66.7 \pm 4.9 (57.0, 76.4) ^a	89.3 \pm 2.0 (85.4, 93.2) ^a	--	6.5 \pm 0.3 (5.9, 7.1) ^a
	DMA	3.4 \pm 0.7 (2.1, 4.7) ^c	47.8 \pm 3.5 (40.9, 54.6) ^{bc}	0.8 \pm 1.3 (-1.6, 3.2) ^b	7.6 \pm 0.9 (5.9, 9.3) ^{ab}
	DMA + 0.1M Sucrose	3.0 \pm 0.6 (1.8, 4.2) ^c	49.5 \pm 3.7 (42.2, 56.9) ^{bc}	3.2 \pm 1.3 (-0.8, 5.5) ^{ab}	7.9 \pm 1.0 (6.0, 9.8) ^{ab}
	DMSO	15.5 \pm 2.9 (9.8, 21.3) ^b	55.4 \pm 3.5 (48.5, 62.3) ^b	6.3 \pm 1.3 (3.0, 8.1) ^a	9.9 \pm 0.9 (8.1, 11.7) ^b
	DMSO + 0.1M Sucrose	8.7 \pm 1.7 (5.3, 12.2) ^b	52.2 \pm 3.7 (44.9, 59.6) ^{bc}	2.3 \pm 1.3 (-1.0, 4.1) ^{ab}	11.5 \pm 1.0 (9.6, 13.4) ^b
	0.1M Sucrose	1.9 \pm 0.4 (1.2, 2.7) ^{cd}	40.6 \pm 4.0 (32.8, 48.4) ^{cd}	0.2 \pm 1.8 (-3.0, 2.1) ^b	5.8 \pm 0.9 (3.9, 7.5) ^a
	0.2M Sucrose	1.1 \pm 0.2 (0.7, 1.6) ^d	30.4 \pm 4.3 (21.9, 38.9) ^d	0.2 \pm 1.2 (-3.5, 1.9) ^b	6.0 \pm 1.0 (4.1, 7.9) ^a

Different superscripts denote significant differences between treatment groups.

similar to values from fresh ejaculates. Values for the remaining CASA motion variables (Straight Line Velocity, Curve Linear Velocity, and Average Path Velocity) are included in Appendix A.

Effect of vitamin E supplementation following cryopreservation

Cryopreservation of whooping crane sperm with vitamin E supplementation reduced sperm motility and plasma membrane integrity but not sperm morphology compared to the fresh ejaculates (Table 2). Varying levels of vitamin E supplementation did not affect sperm motility, membrane integrity, or normal morphology post thaw. However, motion variables assessed by CASA displayed the highest values in the 5 µg/mL treatment compared with 0 and 10 µg/mL in straight line velocity and straight line distance although this relationship was not significant.

Discussion

Semen cryopreservation has proven useful for enhancing genetic management of several endangered species, including black-footed ferrets (Howard *et al.* 2016), pallas cats (*Otocolobus manul*; Swanson *et al.* 2007), and scimitar horned oryx (*Oryx dammah*; Morrow *et al.* 2000), among others. This technology facilitates the movement of genetic materials between captive breeding centers and allows reintroduction of genetic variability into the population using the sperm of dead individuals (Holt *et al.* 2003). For the whooping crane and white-naped crane, semen cryopreservation has not been routinely integrated into *ex situ* management, despite the extensive use of AI. The main goal of the present study was to establish a cryopreservation protocol that can preserve sperm motility and fertility post-thaw.

Table 2: Mean \pm standard error of mean percent viability, normal morphology and motility as well as sperm motion characteristics of whooping crane sperm post-thaw.

	% Viable Sperm	% Normal Morphology	% Motile Sperm	Straight Line Velocity ($\mu\text{m/s}$)	Curve Linear Velocity ($\mu\text{m/s}$)	Average Path Velocity ($\mu\text{m/s}$)	Straight Line Distance (μm)
Fresh	87.7 \pm 2.5 ^a	72.7 \pm 12 ^a	52.1 \pm 1.5 ^a	-	-	-	-
DMSO	44.1 \pm 3.0 ^b	64.1 \pm 17.1 ^a	7.9 \pm 1.3 ^b	26.3 \pm 1.9 ^a	58.6 \pm 4.7	33.6 \pm 2.4	15.3 \pm 0.9 ^a
5 μg	40.5 \pm 2.8 ^b	65.6 \pm 17.5 ^a	10.2 \pm 1.1 ^b	28.0 \pm 1.9 ^b	59.8 \pm 4.8	35.5 \pm 2.5	16.1 \pm 0.9 ^b
10 μg	40.7 \pm 2.8 ^b	65.6 \pm 17.5 ^a	9.1 \pm 1.2 ^b	26.0 \pm 1.9 ^a	59.6 \pm 4.9	34.1 \pm 2.5	14.8 \pm 1.0 ^a

Different superscripts denote significant differences between groups.

To accomplish this goal, this study investigated the influence of different cryoprotectants and a natural antioxidant, vitamin E, on post-thaw survival of whooping and white-naped crane spermatozoa. The findings demonstrated that both crane species are sensitive to damage incurred during cryopreservation, although there appear to be species-specific differences in damage tolerance. Despite these differences, DMSO proved to be the most effective cryoprotectant for both species. Supplementation with vitamin E had a modest effect on sperm motion characteristics, but did not affect overall sperm survival or motility following cryopreservation.

Overall, DMSO performed best as a cryoprotectant for sperm of both whooping and white-naped cranes, based on post-thaw motility, viability, and sperm binding assay. This finding is consistent to that reported previously in the sandhill crane (Gee *et al.* 1985). However, while sperm of both species performed best in DMSO treatments, white-naped crane sperm was generally of higher quality both pre- and post-thaw than whooping crane sperm. White-naped crane sperm displayed higher motility in raw semen, as well as better post-thaw motility (roughly 50% higher in both DMSO treatments) and viability (in 5 of 6 treatments) than the latter. Additionally, white-naped crane sperm appeared to tolerate the sucrose only treatments better than whooping crane sperm. A previous study in the sandhill crane demonstrated that sucrose, in combination with DMA, improved post-thaw cell survival compared to DMA alone (Blanco *et al.* 2011).

The differences in the responses of sperm to cryoprotectants and to cryopreservation across three crane species - white-naped crane and whooping crane in the present study and the sandhill crane in the study reported previously - may be

due to differences in plasma membrane composition. Plasma membrane composition can be species-specific, especially with respect to cholesterol content of spermatozoa in the genus galliformes (Blesbois *et al.* 2005), and this may influence sperm cryosurvival. Cholesterol is a major component in the plasma membrane which supports membrane structure (Krause and Regen 2014). High levels of cholesterol within the plasma membrane correspond to low membrane fluidity, which can decrease cell survival during cryopreservation (Blesbois *et al.* 2005). It is possible that there are species differences in cholesterol content among the crane species that result in varying sensitivity to freezing and thawing as well as varying responses to cryoprotectants. Future studies should investigate plasma membrane composition of crane sperm across the family, and if species-specific differences do exist determine how best to adapt cryopreservation protocols to differing membrane compositions.

The use of sucrose as a non-permeating cryoprotectant did not protect sperm during cryopreservation. Treatments containing sucrose alone had the lowest values for each post-thaw variable measured. White-naped crane sperm fared better in sucrose treatments, especially when paired with one of the permeating cryoprotectants; however, higher levels of motility and IPVL penetration were observed in the presence of permeating agents. Whooping crane sperm did very poorly when sucrose was used alone, especially in terms of viability and IPVL binding assays. Non-permeating cryoprotectants alter the osmotic levels of extracellular water, thus forcing out intra-cellular water dehydrating the cell (Watson 2000). Avian sperm are very sensitive to volume changes (Blesbois 2012); therefore,

it is possible that cells undergo irreversible damage when dehydrated prior to freezing.

While our results support the early work performed by Gee et al. (1985), it is contrary to the study by Blanco et al. (2012), which found that sandhill crane sperm survived better and produced more fertile eggs when using DMA as a cryoprotectant, compared to DMSO. This may be another example of species-specific differences due to composition of the sperm plasma membranes. DMA and DMSO, are similar in their cryoprotective mechanism of action (both contain two hydrophobic methyl groups). These cryoprotectants create three hydrogen bonds with water (de Ménorval *et al.* 2012), and induce membrane thinning. This thinning increases the fluidity of the membrane's hydrophobic core lipid membranes, allowing the cryoprotectant and water molecules to move more freely across the membrane. Although both cryoprotectants perform similar actions, they differ in molecular weight (DMA: 87.12 and DMSO: 78.13 gmol⁻¹) and structural components (DMA bears amide groups, whereas DMSO has a hydrophilic sulfoxide group; Iaffaldano *et al.* 2012). These differences in structure alter the affinity of each cryoprotectant to specific phospholipids and proteins of the membrane bilayer (Fuller 2004). The three species of cranes studied show varying preference to different cryoprotectants, which further indicates the need to determine membrane composition of individual species.

Because the ultimate goal of cryopreservation and genome resource banking is to thaw and use sperm to produce offspring, spermatozoa must maintain function and the ability to fertilize following freezing. In order to assess functionality, an *in vitro* assay was utilized that could be used in the absence of an egg-producing female

crane. Stewart *et al.* (2004) determined that the sperm-egg binding reaction is less species-specific in birds than in mammals. In the present study, an egg-sperm binding assay for domestic fowl was adapted (Kido and Doi 1987; Robertson *et al.* 1997) for use in cranes. Sperm of both species retain the ability to bind following cryopreservation, albeit at relatively low numbers. A similar low proportion of sperm binding following cryopreservation as compared to fresh samples was reported by Robertson *et al.* These results in the sperm-egg interaction assay corresponded to overall percent motility post-thaw, where treatments with the highest motility values (i.e., DMSO) had the highest numbers of sperm binding to the inner perivitelline layer. However, the low number of competent sperm would be unlikely to successfully fertilize an egg (Long 2006). Therefore, an improved cryopreservation protocol is still needed for this technology to be routinely incorporated into captive crane management

Vitamin E is more lipophilic than other natural antioxidants (Packer *et al.* 1979) and has improved sperm post thaw survival in other avian species, including domestic turkeys and ducks (Donoghue and Donoghue 1997; Surai *et al.* 2001). Our data showed that a moderate level of vitamin E added to semen extender enhanced some motion characteristics of whooping crane spermatozoa, but did not increase overall sperm survival. It is possible that vitamin E does not have the same affinity for crane sperm as seen in other avian species (Surai *et al.* 2001) or that a different antioxidant, such as vitamin C, may have a stronger effect. It is also possible that oxidative stress is not a major cause of poor post-thaw survival observed in cranes. Other factors, such as the inability of the cell to cope with volume changes during

freezing and thawing, may be responsible for poor survival. Therefore, future studies should explore other cryopreservation strategies. For example, altering the sperm plasma membrane components, specifically cholesterol concentrations, to allow for more fluid cell membranes which has been shown to improve cell cryosurvival (Ansah and Buckland 1982). The addition of cholesterol has been shown to improve sperm cell survival in a number of mammal species (Moore *et al.* 2005, Moce *et al.* 2010, Salmon *et al.* 2014) while the reduction in membrane cholesterol concentration has improved cell survival in domestic chickens (Partyka *et al.* 2016).

Conclusions

In summary, the findings generated from this study serve as an important foundation for future development of an improved protocol for sperm cryopreservation in cranes. The findings of species differences in the responses of sperm to cryopreservation underscore the need for studying biology of each individual species. Finally, additional work should investigate other additives to alleviate the damage caused during cryopreservation and increase post-thaw survival.

Chapter 3: Reproduction of whooping cranes maintained *ex situ* is improved in naturalized enclosures

Introduction

Whooping cranes (*Grus americana*) are a wetland species that requires aquatic habitat for roosting, foraging, and nesting (Ellis *et al.* 1996; Harris and Mirande 2013). In a previous study, the best predictors for egg laying success in wild whooping crane in central Florida were winter precipitation and marsh water depth (Spalding *et al.* 2009). Specifically, deeper water resulted in earlier nests and more eggs laid, while higher precipitation levels resulted in higher fertility and hatching rates. This relationship holds true for the greater sandhill crane (*Grus canadensis tabida*) at Malheur National Wildlife Refuge in Oregon where high water levels in early spring were critical for nesting success (Ivey 2007). In the wild the effect of water level on crane's reproductive performance may be explained by an increase in food availability in deeper wetlands (both plants and macro-invertebrates). It has been observed that sandhill cranes desert nests when water levels drop too low (Ivey 2007). These results indicate the importance of proper habitat, especially the presence of water, on reproductive success in cranes (Drewien *et al.* 1995; Ivey 2007).

The effect of water level on crane breeding success may extend to the captive environment. Anecdotal observations have suggested that more naturalistic *ex situ* environments, such as exhibits containing a pond or mimicking natural rain patterns, enhanced reproductive success (Ellis *et al.* 1996; Hughes 2008). Brolgas (*Grus rubicunda*) housed at the International Crane Foundation (ICF, Baraboo, WI) began breeding after sprinkler systems were used to simulate the rainy breeding season

(Ellis *et al.*, 1996). Breeding success of sarus cranes (*Grus antigone*) in breeding centers and in the wild is closely linked to the amount of rainfall during the laying season (Balzano 1989). Furthermore, wattled cranes at the Wildlife Survival Center, Midway, GA, as well as whooping and Siberian cranes at ICF displayed increased reproduction when pens were flooded seasonally (Ellis *et al.* 1996). Finally, captive, non-reproductive sandhill cranes at ICF began laying eggs when moved from a dry pen into a pen with a full sized pond (Kelly Maguire, pers. comm.).

Endangered whooping cranes have been housed in captivity since the late 1960s (Ellis *et al.* 1996). Captive whooping cranes play important roles in the species' recovery program as they serve as a source for reintroduction (Ellis and Gee 2001) and as a repository for retaining genetic diversity (Jones *et al.* 2002). Greater reproductive rates in captivity can better support reintroduction programs. In addition, relatively equal reproductive contribution across individuals is a recognized method for retaining genetic diversity *ex situ* (Holt *et al.* 2003). However, reproductive success in the captive flock has been poor compared to wild populations, with low egg production from captive females and low natural fertility. Reproductive contribution is also uneven. On average, 23 reproductive pairs at Patuxent Wildlife Research Center (PWRC) each produced, on average, 2.6 eggs annually (Range 1-9 eggs per pair) with an average fertility of 37% (Brown, 2017; Chapter 4). Production from individual captive pairs, subjected to egg or whole clutch removal, could be as high as to 8 or 9 eggs per season (Derrickson and Carpenter 1987).

In a recent study, successful egg production in the whooping crane was linked to the production of female gonadal hormones. Successful females produced higher

fecal estrogen metabolites but lower fecal progestagen metabolites (Brown *et al.* 2016). Additionally reproductive success was related to the amount of time spent performing reproductive behaviors, especially in females. Overall, results indicate that successful egg production may be limited by inadequate stimulation of the female hypothalamic-pituitary-gonadal (HPG) axis. However, it is not yet understood what constitutes adequate stimulation, nor how sensitive the HPG in whooping cranes is to external cues.

The captive breeding center with the largest number of whooping cranes is the PWRC, in Laurel, Maryland. PWRC, like a number of other centers, currently houses cranes in pens without standing water sources. It is possible that this captive environment could result in inadequate stimulation of the HPG axis in females of breeding pairs.

The objectives of this study were to, 1) characterize seasonal patterns of hormone production in captive whooping cranes, and 2) explore the sensitivity of whooping crane gonadal and adrenal hormone production and reproductive behavior to environmental stimuli. It was hypothesized that hormonal production in this species would show seasonal patterns, with higher concentrations of reproductive hormones (females: estrogens males: androgens) produced during the spring breeding season. Because wild whooping cranes depend on wetland ecosystems for nesting, it was also hypothesized that a captive environment containing a pond which would more closely mimic wild habitat would stimulate the HPG axis, and induce the production of hormone profiles associated with reproductive success, resulting in increased reproduction.

Methods

Pair Selection

Eight breeding pairs were selected from the 17 total pairs within the captive flock at PWRC. Pairs were considered for the study based on temperament and historically poor reproduction (2008-2012 breeding seasons). Pairs were removed from consideration if they were too nervous or too aggressive during the breeding season based on the flock manager's assessment. Poor reproduction was defined as < four eggs per breeding season in three of the previous five years. After selection based on these two criteria, eight pairs were available for study. All pairs were fed a specialized pelleted crane diet (produced by Republic Mills, Okolona, OH; recipe provided by Swengel and Carpenter, 1996) in a gravity feeder and provided water *ad libitum*. All work associated with this study was performed with approval of Animal Care and Use Committees at USGS-PWRC (2013-04), Smithsonian Conservation Biology Institute (10-11), and University of Maryland (883522-1).

For the first study year, all pairs remained in their original breeding pens, ~13.7 m wide by 19.8 m long (Baseline Pens). Pairs were housed with or without flight netting based on wing condition, and with or without photo period lights, as summarized in Table 1. In January of Year 2, pairs were randomly assigned and moved to study pens, in either the control or wetland treatment (Table 1). Control pens were similar to the original pens, ~12.2 m wide by 30.5 m long and containing no pond or photoperiod lights. Treatment pens were ~15.2 wide by 45.7m long, with no photoperiod lights and contained a large pond with a surface area equal to just under half of the total area of the pen. These treatment pens were also in full view of

Table 1: Summary of pairs and egg production during wetland study.

Study Group	Baseline Pen	Study Pen	Flight Netting (Control and Study Pens)	Year 1 (Pre-move)		Year 2 (Study Pen)		Year 3 (Study Pen)	
				Number of Eggs	Number of Clutches	Number of Eggs	Number of Clutches	Number of Eggs	Number of Clutches
Wetland Treatment	B 8	T 5	No	0	0	0	0	2*	2*
	S 26	T 7	No	0	0	0	0	3	2
	S 30	T 9	Yes	0	0	2	1	4	2
	S 32	T 11	Yes	0	0	0	0	0	0
Control Treatment	O 4	LFP 2	No	0	0	0	0	0	0
	S 36	LFP 4	No	1	1	0	0	1	1
	B 24	LFP 6	Yes	0	0	0	0	0	0
	B 12	LFP 8	Yes	1	1	1	1	1	1

*Pair is suspected of laying eggs and subsequently breaking them based on endocrine profiles and observed behaviors

a natural wetland area. Pairs were housed in their study pens during Years 2 and 3 of the study.

Sample Collection

Fecal samples (void of urate when possible; Hayward et al., 2010) were collected from all birds once per week from July 1st through January 31st (Wednesday) and three times per week from February 1st through June 30th (Monday, Wednesday, and Friday).

One sample per week was judged to be adequate to determine overall seasonal trends in hormone metabolite excretion (Akesson and Raveling 1981), while samples were collected three times per week during the breeding season to capture the increasingly dynamic nature of hormone production associated with egg laying (Brown *et al.* 2016). Chromic Oxide (Cr₂O₃) and Iron Oxide (Fe₂O₃) in capsules was delivered in lake smelt (*Osmerus mordax mordax*) the day prior to collection to allow determination of male versus female samples based on color (Brown *et al.* 2014). The samples were stored 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. Laying females were not sampled during June-August while they were incubating eggs and raising chicks. Sampling resumed during September once chicks were removed as per management protocols. Sampling of non-laying females was performed through the entire year.

Hormone Extraction and Analysis

All samples were evaluated for gonadal (males: androgen; females: estrogen and progesterone) and adrenal (males and females: glucocorticoid) hormone metabolites using immunoassays previously validated for the whooping crane (Brown *et al.* 2016). Concentrations of progesterone and androgen metabolites were quantified using enzyme immunoassays (EIA). EIA antibodies for monoclonal progesterone antibody (CL425) and polyclonal testosterone antibody (R156/7) were obtained from University of California, Davis, CA, USA. Estrogen metabolites were analyzed using radio immunoassay (RIA) for 17 β estradiol, and glucocorticoid metabolites were assessed using a corticosterone RIA kit, both obtained from MP Biomedicals (Santa Ana, CA). For both EIAs and RIAs, inter- assay variation were <15% and intra-assay variations were <10%. All hormone concentrations are expressed as mass units of hormone per gram of dry feces.

Behavioral Analysis

Crane pairs were monitored two days per week through video recordings (Vivotek, Portland, OR and Super Circuits, Austin, TX) to assess reproductive behaviors during the breeding season. Videos of individual pairs were recorded for a total of 75 min beginning 15 min before sunrise and continuing for an hour after sunrise. Behaviors were assessed based on an ethogram previously developed for this species, using continuous focal group sampling (Brown *et al.* 2016). Periods during which birds were out of camera view were omitted. For the wetland pens, time spent in the water versus on dry land was also recorded.

Health Evaluations

Health metrics, including weight, body condition, hematocrit, and fecal parasite load, were compiled from annual health exams from each year of the study. Values were compared before and after birds were moved from their original enclosure to the study pens. Parasites commonly found in crane fecal samples include *Capillaria sp.*, *Eucoleus sp.*, ascarids, acanthocephalans (*Macracanthorhynchus sp.*), and gapeworms (*Syngamus sp.*, *Cyathostoma sp.*; Ellis *et al.* 1996).

Statistical Analysis

Study 1: Influence of season on hormone production

Monthly averages for each hormone were calculated from one sample collection per week (Wednesdays) throughout the entire year. Prior to analysis, data (pair-years) from control (dry pens) were divided between laying and non-laying pairs, creating two different groups (non-laying [$n = 11$] and laying [$n = 5$]). Analyses were designed to detect whether groups varied across months, and whether there were differences across groups within a month using the lme4 package (Bates *et al.* 2012). Differences were considered significant if their 95% confidence intervals did not overlap.

Study 2: Influence of enclosure environment on hormones behaviors, and egg production

Individual models were built to determine the influence of enclosure type on each of the five hormone concentrations, reproductive behaviors (individually for males and females), and overall reproductive output (numbers of eggs laid), for a total of eight separate models. Breeding season means (March-May) were compared

within treatment and between the control (dry) and treatment (wetland) study groups. Individual models were built in the lme4 package (Bates *et al.* 2012) in R (R Core Team 2014) to determine the effect of environment on hormone concentration, proportion of time spent performing reproductive behaviors, or total number of eggs laid. In each case these variables served as the response variable, an interaction between year and enclosure type served as fixed effects, and individual birds were included as a random effect to account for repeated measures. Models which analyzed hormone concentrations and behaviors were fit with a Guassian (normal) distribution, while the models analyzing the total number of eggs laid was fit with a Poisson distribution. Significant difference was determined when 95% confidence intervals did not overlap.

Results

Study 1: Influence of season on hormone production

Female Hormones

There were significant differences in seasonal patterns of estrogen production between laying and non-laying females. Specifically, laying females excreted more estrogen and progesterone metabolites in May compared to non-laying females (Fig. 1A and B). Non-laying females showed a less dynamic progestagen patterns and produced consistent concentrations of progestagen throughout the year. Glucocorticoid metabolite production was higher during the non-breeding season (September-January; Fig. 2A). Hormone production decreased in the months prior to the breeding season (January-March) and increased again during the breeding season (April-May). Laying females also produced the highest concentrations of

Figure 1: Average monthly concentrations of hormone metabolites measured for egg laying and non-laying females (A: Estrogens, B: Progestagens). Superscripts denote significant differences within a group across months while asterisks denote significant differences within a month between groups, both based on a 95% confidence interval.

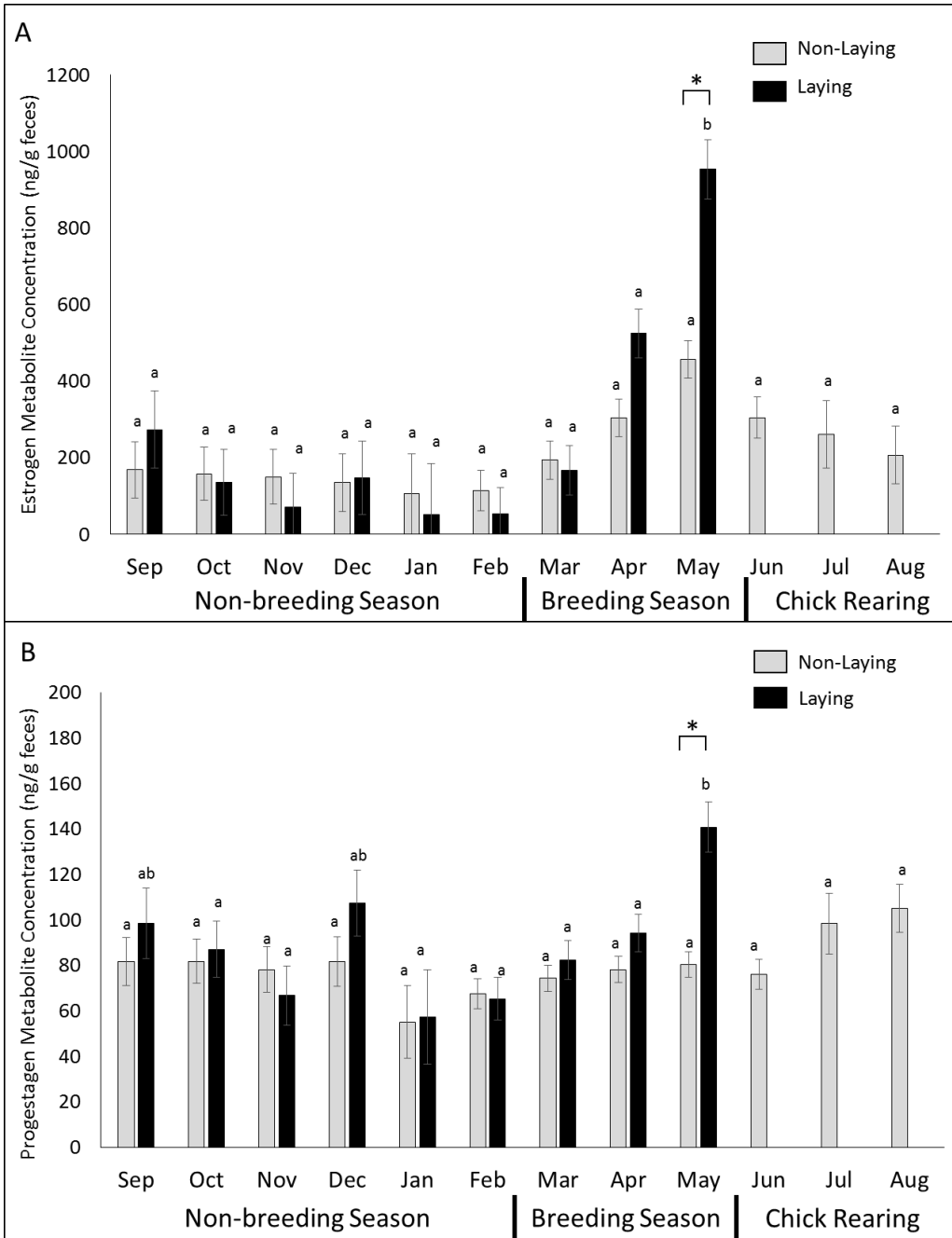
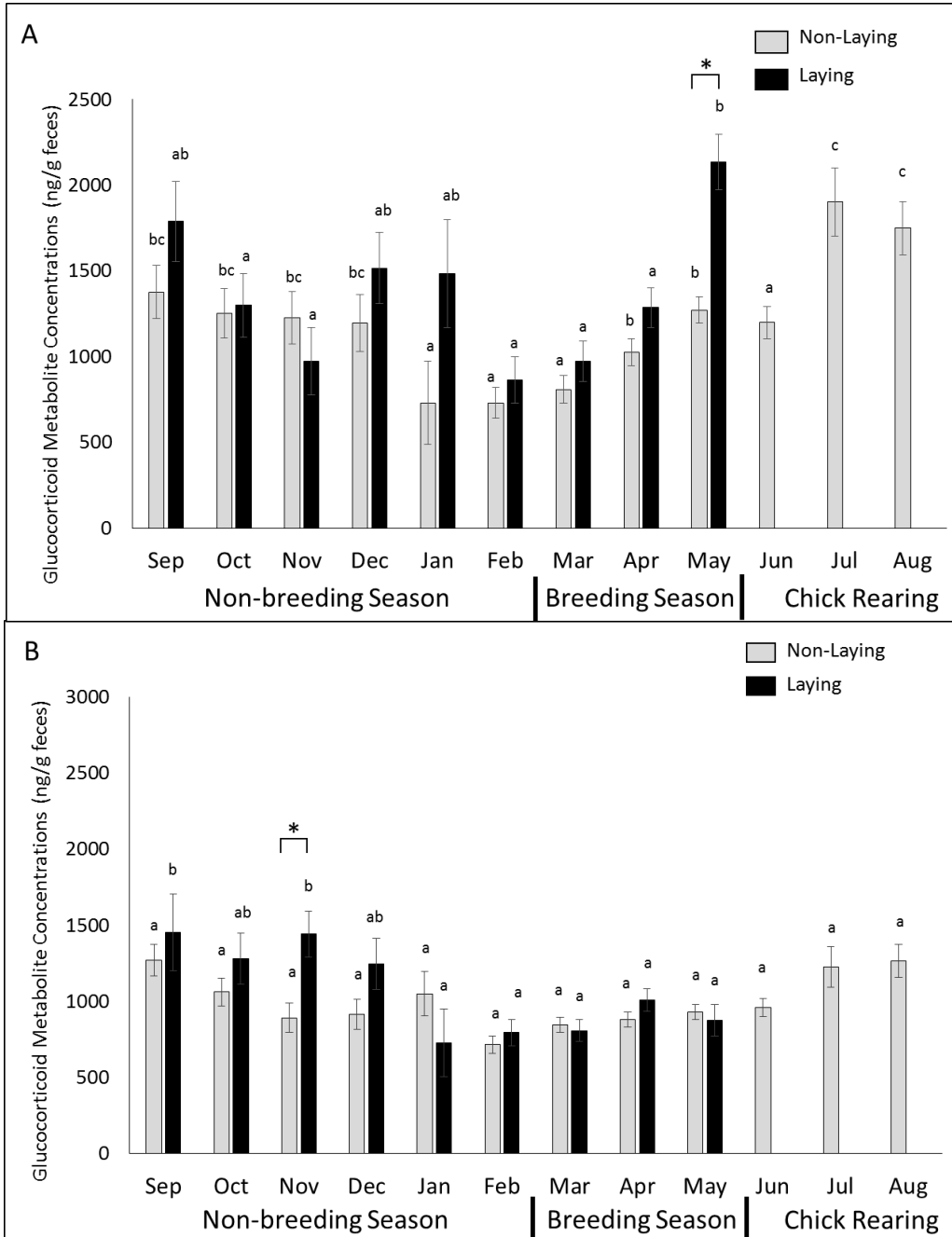


Figure 2: Average monthly concentrations of hormone metabolites measured for egg laying and non-laying pairs (A: Female Glucocorticoids, B: Male Glucocorticoids). Superscripts denote significant differences within a group across months; asterisks denote significant differences within a month between groups, both based on a 95% confidence intervals.



glucocorticoids in May, similar to patterns seen in gonadal hormone production. This was significantly higher than any other month within laying females and compared to non-laying females in the month of May.

Male Hormones

Androgen concentrations produced by males of non-laying pairs were higher in the breeding season (March-May), remained at intermediate levels from June until December, and the lowest in January and February (Fig. 3). However, there were no statistical differences in androgen metabolites concentration among months in males from laying pairs. Within a giving month, there were no differences in androgen metabolite concentration between males from laying and non-laying groups.

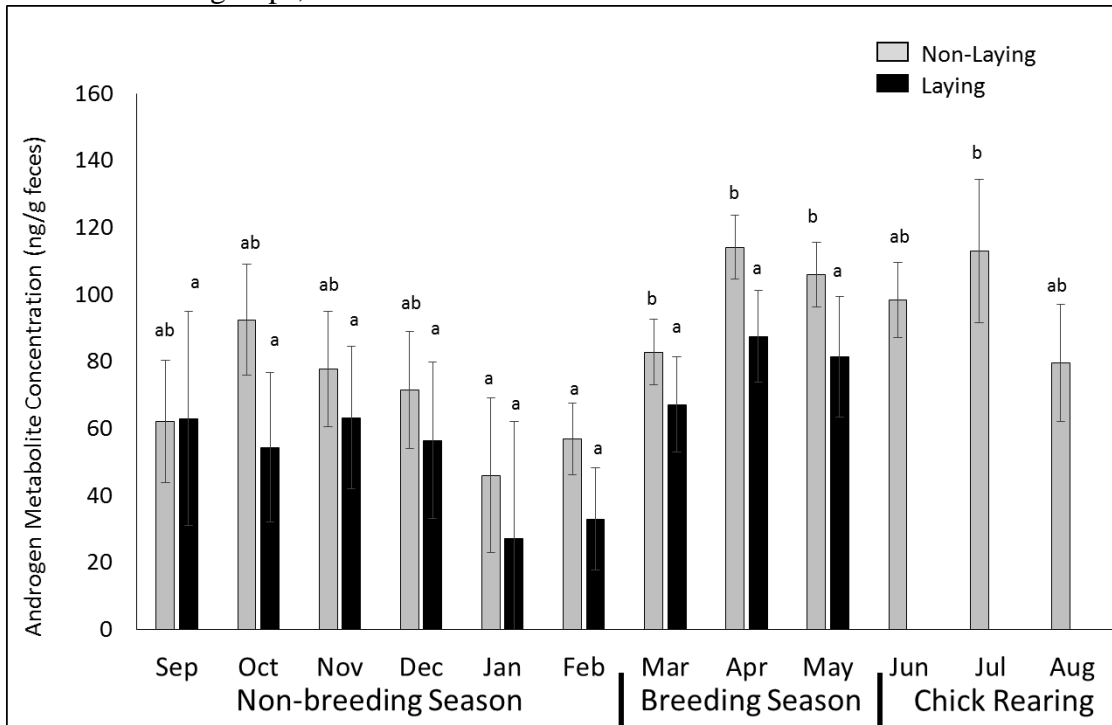
Glucocorticoid metabolite production was highest during the non-breeding season (September-December; Fig. 2B) especially in males from laying pairs. Hormone production declined in the months prior to the breeding season (February-March). Glucocorticoid metabolite levels in November were higher in males of laying pairs than in non-laying ones. Overall, there was little difference in hormone concentration patterns produced by male whooping cranes throughout the year.

Study 2: Influence of enclosure environment on hormones, behaviors, and egg production

Health Evaluations

Enclosure type had no effect on overall bird health. Weight (Males: 6.52 ± 0.11 kg; Females: 5.16 ± 0.07 kg) and body condition score (Males: 3.12 ± 0.13 ; Females: 2.6 ± 0.12) of all birds remained constant throughout the study period. The level of parasite load did not increase when birds were moved to the wetland pens.

Figure 3: Average monthly concentrations of androgen metabolites measured for males of egg laying and non-laying pairs. Superscripts denote significant differences within a group across months while asterisks denote significant differences within a month between groups, both based on a 95% confidence interval.



Endocrine Response

Enclosure environment influenced female gonadal hormone production. Specifically females in the wetland treatment produced higher estrogen than those in control pens in Year 3 (Fig. 4A; 95% CI: 22.76, 318.70). Mean progesterone concentrations were significantly higher in females housed in the control pens in Year 3, (Fig. 4B; 95% CI: 1.68, 33.18). Females in the wetland pen also produced significantly lower glucocorticoid metabolites in Year 2 (Fig. 5A; 95% CI: -571.93, -1.19), although in Year 3 corticoid excretion returned to similar levels as observed in Year 1. Androgen metabolite (Fig. 4C) and glucocorticoid metabolite (Fig. 5B) excretions in males were similar between the two groups throughout the study period.

Behavioral Response

In Year 1, there were no differences in proportion of time males or females spent performing reproductive behaviors in either study group. (Females, Control: $1.13 \pm 0.49\%$; Treatment: $1.25 \pm 0.46\%$; Fig. 6A; Males, Control: $1.09 \pm 0.66\%$; Treatment: $2.53 \pm 0.66\%$; Fig. 6B). Pairs utilized the wetland side of their enclosure for nesting and foraging behaviors. During the time observed females spent $45.5 \pm 3.7\%$ of time in the pond side of the pen, while males spent $49.3 \pm 5.1\%$ of time in the pond side of the pen.

Figure 4: Mean values of gonadal hormone metabolites produced by females (Estrogens: A; Progestagens: B) and males (Androgens: C) during wetland study. Values indicate model calculated mean averages for each treatment group in a given breeding season. Females in the wetland treatment group displayed significantly higher estrogen metabolite production in Year 3. Control pen females exhibited significantly higher progesterone metabolite production in Year 3. There was a trend toward decreased progesterone metabolite production in Year 2 and 3 for wetland pen females, but was not significant. Males exhibited no differences between groups or years in androgen metabolite production.

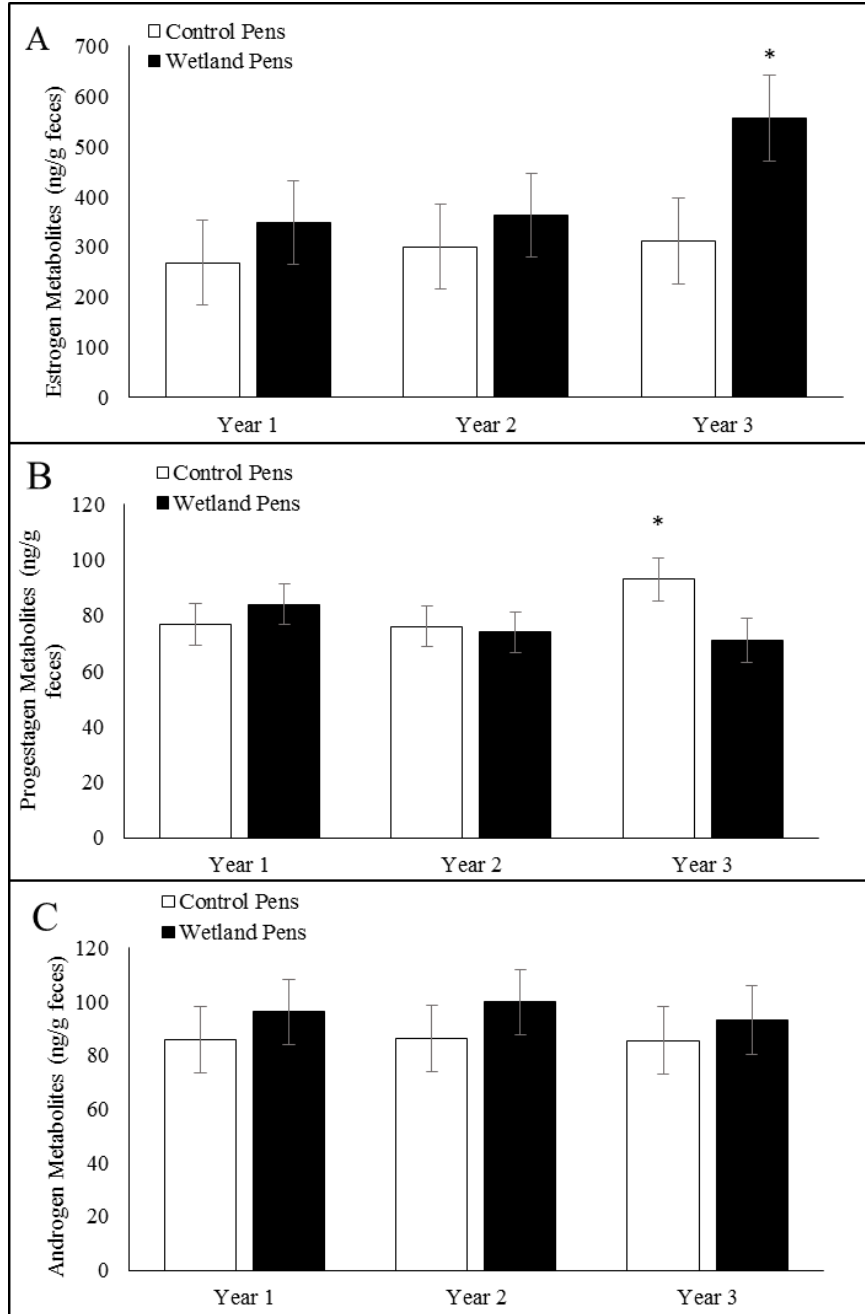


Figure 5: Mean values of glucocorticoid metabolites produced by females (A) and males (B) during wetland study. Values indicate model calculated mean averages for each treatment group in a given year. Females in the wetland treatment group had significantly lower glucocorticoids in Year 2; however Year 3 production was comparable to production in year 1 and all years in control females. For male, study year or treatment did not affect glucocorticoid production.

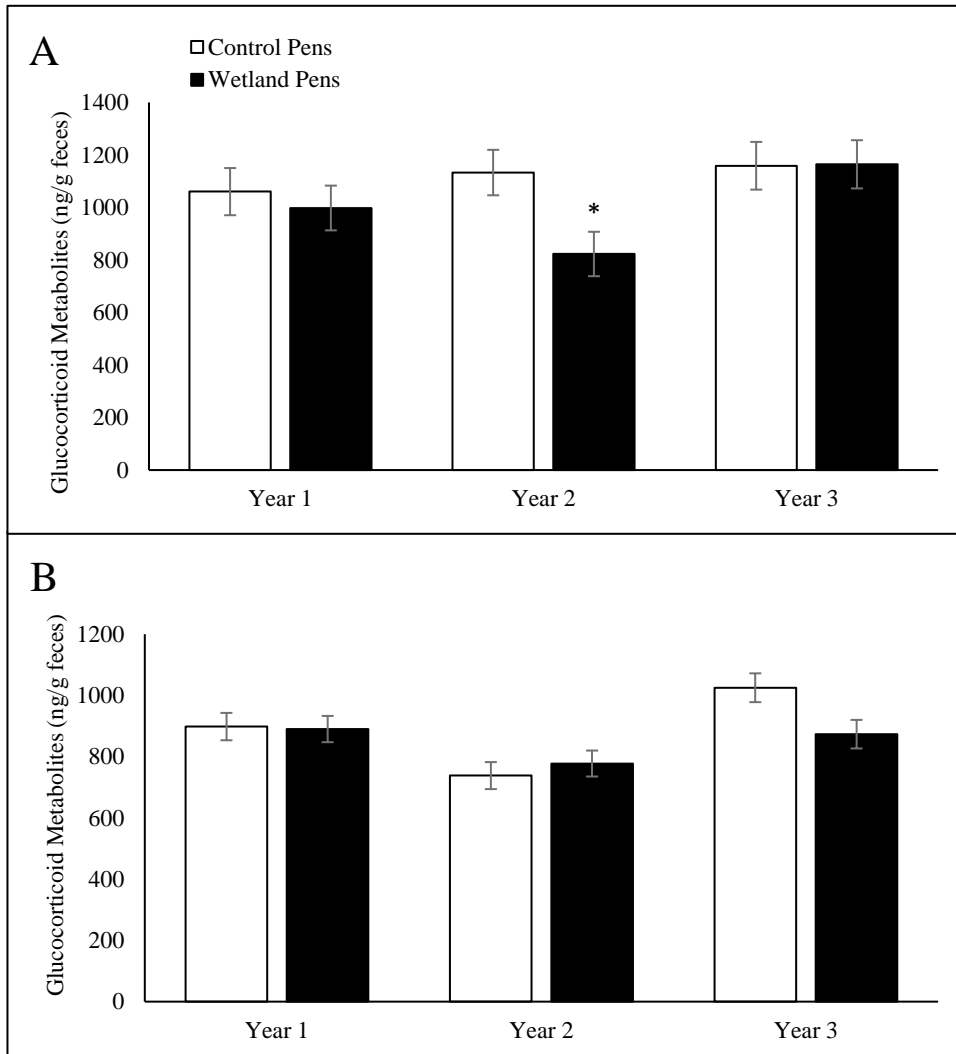
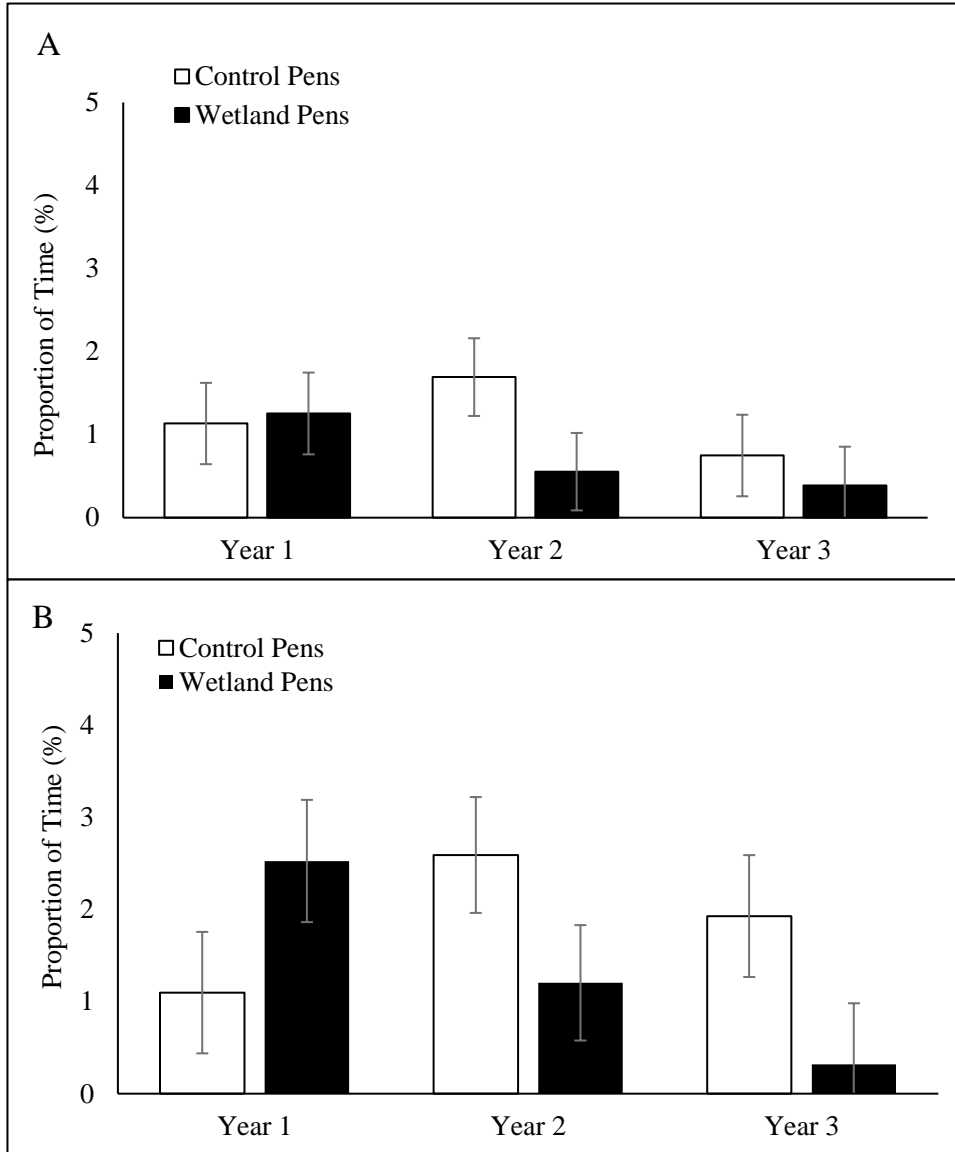


Figure 6: Behavioral response of females (A) and males (B) during wetland study. Overall there were no significant differences between control or treatment groups in either sex based on 95% confidence interval.



Egg production

In Year 1 (Table 1), there were low levels of egg laying from all pairs. Pairs in the control pens consistently laid few or no eggs in each study year. None of the pairs in the wetland treatment group laid in year 1. Once the treatment pairs were moved to the wetland pens, egg laying increased in two of the four pairs. The number of eggs laid was significantly higher in year 3 for the wetland treatment group (7 versus 2 eggs; 95% CI: 0.94, 2.67) compared to the control group.

Discussion

The present study utilized non-invasive hormone monitoring to evaluate changes in gonadal and adrenal hormone production across the annual cycles and in response to different captive environment in whooping cranes. Seasonal hormone patterns changed in both gonadal and adrenal hormones with the most striking differences observed in female estrogen and progesterone production. The findings in this study also showed that moving birds to pens containing a large pond stimulated gonadal hormone secretions and egg production in female whooping cranes.

Seasonal hormone production patterns of both female and male whooping cranes followed patterns previously described for this species (Brown *et al.* 2016). Laying females produced higher estrogen concentrations during the breeding season compared to non-laying females. Progesterone production was also highest in laying females during the month of May when all eggs were produced in this study.

Progesterone metabolite secretion was observed at moderate levels in all females throughout the year. This is unusual as progesterone is typically produced by granulosa cells of the largest preovulatory follicles prior to ovulation (Etches and

Duke 1984; Liu *et al.* 2001). In the absence of high estrogen production (as observed in non-laying females) during the breeding season and year round in laying females, it can be assumed there are no follicles present on the ovary (Etches 1996). This then begs the question what other cells could be producing progesterone. There is evidence that the adrenal gland produces progesterone, as progesterone has been measured in prepubertal and ovariectomized mammals (Asher *et al.* 1989) and in chicken embryos as early as 9 days of development (Kalliecharan and Hall 1974). In each case these animals lack follicular development and the granulosa cells which produce progesterone. In addition to its role in reproduction, progesterone is also a glucocorticoid precursor in the steroidogenic pathway (Norris 2006). The adrenal gland could be the source of progesterone observed in females throughout the year and, in particular, non-laying females during the breeding season. Corticosterone is also known to be a strong ovulatory inducer triggering the ovulatory luteinizing hormone surge similarly to progesterone (Etches 1996). The mechanism in which the adrenal gland produces progesterone and its interaction with the ovary requires further examination.

Males produced higher androgen concentrations during the breeding season (April and May) than during non-breeding period. Males from non-laying pairs produced higher levels of androgens than males from laying pairs especially in May. Typically in male birds, testosterone levels rise early in the breeding season to stimulate sperm production (Penfold *et al.* 2000) and aid in mate and territory defense (Gee 1995). In other bird species, once a pair has formed and established a breeding territory, testosterone levels decrease during nesting and incubation (Wingfield *et al.*

1987). Within this study, males from non-laying pairs never enter into incubation and lack the stimuli halting testosterone production. This may explain the elevated testosterone concentration in late summer (July-August) in non-laying males.

Females in two of the four pairs moved to the wetland treatment pens laid eggs during the study period and all pairs performed reproductive and nest guarding behaviors. Females in the wetland treatment pens, both laying and not, displayed hormone patterns most similar to those associated with reproductive success (Brown *et al.* 2016). In Year 2, females moved to the wetland pens displayed decreased glucocorticoid production while glucocorticoid production increased in females moved to control pens. However, the difference in glucocorticoid concentration was not observed in Year 3. The difference between females could be due simply to yearly variation in female glucocorticoid production. Often cranes will delay reproduction the first season once moved to a new pen (USGS-PWRC 2010). Therefore, the rise in glucocorticoid concentration in the control birds during Year 2 may be due to stress associated with relocation from the baseline pen to study pen. Such an effect was not observed in the wetland group, suggesting that the natural environment can alleviate such a stress response. However the overall results from the wetland enclosure experiment showed that being housed in a naturalized environment improved reproduction (increased egg laying and increased hormonal response) in captive whooping crane females. A similar response has been described in other species housed in captivity such as the clouded leopard (Wielebnowski *et al.* 2002) and black rhinoceros (Carlstead *et al.* 2005).

Increased photoperiod is the primary environmental stimulus for reproduction in both male and female birds (Dawson *et al.* 2001). While photoperiod is adequate as the sole stimulus for breeding in male birds, females play a greater role in regulating reproductive effort and are more sensitive to secondary environmental cues which can vary from year to year (Ball and Ketterson 2008). As seen in wild cranes (Ivey 2007; Spalding *et al.* 2009), reproduction was improved when water was present during the breeding season. Most often this increase in reproduction is believed to result from increased food availability (Spalding *et al.* 2009) While food is provided *ad libitum* and captive diets were formulated based on nutrient profiles consumed by wild cranes, the wetland pens did provide novel food/protein sources. Birds were often seen foraging and catching prey such as frogs and crayfish in the ponds. Chicks raised in these ponded pens seem to be fed exclusively from foraged food items, rather than the formulated chick diet which is provided during chick rearing. By giving these birds the ability to perform more naturalized behaviors may have stimulated neuroendocrine pathways. However the true role that the presence of water plays in the overall function of reproductive mechanisms is still unknown. As female cranes showed a hormonal response to the wetland enclosure while males did not, the addition of water to the captive pens likely served as a secondary stimulus necessary for initiating reproduction in female whooping cranes.

Conclusions

This study was the first to monitor annual hormonal cycles in captive whooping cranes. The chief findings include 1) female hormones, both gonadal and adrenal, showed fluctuations seasonally, especially in laying females, and 2) housing

birds in an enclosure containing a large pond influenced hormone concentrations and stimulated reproduction in pairs. Based on the results presented here, it is recommended that managers interested in increasing egg production consider options for adding standing water to captive environments. By ensuring that the captive environment mimics wild habitats as closely as possible, captive managers can increase the reproductive potential and the welfare of animals.

Chapter 4: Factors Affecting Egg Fertility in Captive Whooping Cranes: A Retrospective Analysis

Introduction

Egg fertility is an important component of reproductive performance in avian populations. The whooping crane (*Grus americana*) is the only crane species endemic to North America, and has been managed in captivity since the 1960s following a substantial genetic bottleneck (Kuyt 1996; Glenn *et al.* 1999; Boyce *et al.* 2005). Following major *ex situ* conservation efforts, there are 158 whooping cranes managed in 11 institutions in North America, consisting of five breeding centers and six holding and exhibit facilities (Zimorski and Jones 2015). However, the current effective population size of captive whooping cranes is only 52 birds (Zimorski and Jones 2015), meaning that only 33% of the total population is contributing to the sustainability of the captive flock and the reintroduction efforts.

The largest breeding center, the US Geological Survey's Patuxent Wildlife Research Center (PWRC; Laurel, MD), currently maintains 24 behavioral pairs, but only 16 of which consistently produce fertile eggs, and only with intensive human assistance, e.g., artificial insemination (Brain Clauss, PWRC, pers. comm.). Historically whooping crane reintroduction programs have relied on captive pairs to produce chicks for release (Ellis and Gee 2001), however, current reintroduction goals for the whooping crane are impeded by poor reproduction within the captive flock (Harrell and Bidwell 2016).

For the whooping crane, the problem of low fertility seems to be isolated to captivity (0.37 fertility rate from 2005-2015, $n = 438$; discussed here), and fertility is highly variable between individual pairs (Nicolich *et al.* 2001). High levels of egg

fertility have been reported in wild whooping crane populations, both within the remnant Aransas-Wood Buffalo Population (~73%; Ellis *et al.* 1992; Kuyt 1995) and the reintroduced Eastern Migratory (~71%; Whitear and Lacy 2016). The reintroduced Florida Non-Migratory Flock is an exception (52%; Folk *et al.* 2005). Low natural fertility in captivity has been partially addressed with the implementation of an artificial insemination program (Gee 1983). This program also allows for greater control in genetic management, overcoming a lack of flexibility in genetic pairing that is inherent when breeding a long-lived monogamous bird that mates for life (Jones and Nicolich 2001). However, the artificial insemination program is labor intensive, requiring a great deal of human investment in order to produce the fertile eggs necessary for sustaining captive populations and reintroduction programs.

Development of improved management techniques to address poor egg fertility will require a better understanding of the factors influencing fertility. To advance this understanding, in the present study a variety of potential predictors of egg fertility specific to management practices and life history events of paired and egg producing whooping cranes housed at PWRC were evaluated. The aim of this analysis was to determine how management strategies influence egg fertility in this species and to identify future research needs.

Methods

Data set development

The data set was built from breeding and husbandry records maintained at PWRC. The years 2005-2014 were selected for study because management strategies were generally standardized over this time, thus allowing the evaluation of fertility

within the context of contemporary management approaches. Data were collected for each egg produced during the study period. Information was collected relative to each male (sire) and female (dam) producer individually and related to each pair. All predictor variables are described in Table 1.

Egg fertility was visually assessed by crane staff at PWRC. Eggs which did not hatch were opened and the yolk was examined for evidence of embryo development. Eggs with obvious embryo development, whether early or late dead embryos, were included as fertile eggs, while eggs with no visible evidence of an embryo were included as infertile eggs. Any egg recorded as having unknown fertility were excluded. Because the egg fertility evaluation was done through macroscopic visual assessment, it is possible that some early dead embryos were misclassified as infertile eggs.

A statistical model set was developed focused on the following research questions: 1) How do egg-specific variables impact fertility? (*Effect of Egg*), 2) How do life history factors of individual breeders impact fertility? (*Effect of Sire and Dam* with separate models for each sex), 3) How do pair-specific characteristics affect egg fertility? (*Effect of Pair*), and 4) How do management decisions impact egg fertility? (*Effect of Captive Management*). A total of five model sets were constructed to address these questions.

Table 1: Summary of variables considered, grouped by research question, for their effect on fertility of Whooping Crane eggs produced at Patuxent Wildlife Research Center, between 2005 and 2015. *n* = numbers of egg in a given level of categorical variable.

Predictor Variables	Abbreviation	Description
Effect of Egg		
Month laid	MLD	Categorical; four levels: March (<i>n</i> = 20), April (<i>n</i> = 230), May (<i>n</i> = 182), June (<i>n</i> = 6)
Number egg of season	NES	Continuous; Range: 1-9 eggs
Number egg of clutch	NEC	Continuous; Range: 1-3 eggs
Effect of Sire and Dam: Sire		
Male age	MAGE	Continuous; Range: 5-31 years
Male number of pairings	MNP	Continuous; Range: 1-3 pairings
Wing status of male	MWS	Categorical; two levels: full winged (Full; <i>n</i> = 352) or not (injured or clipped; Not; <i>n</i> = 86)
Male rearing method	MRM	Categorical; two levels: naturally (Crane; <i>n</i> = 115) or artificially (human or costume; Human; <i>n</i> = 323)
Male age at first pairing	MAFP	Continuous; Range: 1-9 years
Male age at current pairing	MACP	Continuous; Range: 2-15 years
Effect of Sire and Dam: Dam		
Female age	FAGE	Continuous; Range: 5-41 years
Female number of pairings	FNP	Continuous; Range: 1-5 pairings
Age of female at first laying	FAFL	Continuous; Range: 5-16 years
Wing status of female	FWS	Categorical; two levels: full winged (Full; <i>n</i> = 387) or not (injured or clipped; Not; <i>n</i> = 51)
Female rearing method	FRM	Categorical; two levels: naturally (Crane; <i>n</i> = 107) or artificially (human or costume; Human; <i>n</i> = 331)
Female age at first pairing	FAFP	Continuous; Range: 3-9 years
Female age at current pairing	FACP	Continuous; Range: 3-32 years
Effect of Pair		
Years paired	YPD	Continuous; Range: 2-26 years
Pair chick rearing experience	CRE	Categorical; Yes (<i>n</i> = 344) / No (<i>n</i> = 94)
Chick reared previous year	CRPY	Categorical; Yes (<i>n</i> = 65) / No (<i>n</i> = 373)
Previously paired	PP	Categorical; four levels: Neither previously paired (<i>n</i> = 176), Male previously paired (<i>n</i> = 32), Female previously paired (<i>n</i> = 59), Both previously paired (<i>n</i> = 171)
Kinship	KIN	Continuous; Range: 0-0.125
Effect of Captive Management		
AI strategy	AI	Categorical; 3 levels, AI received in year in question from social mate (<i>n</i> = 100), from other male (<i>n</i> = 114), or no AI received (<i>n</i> = 224).
Copulation	COP	Categorical; 3 levels: Able to copulate (Able; <i>n</i> = 314); Able but copulation is prevented (AbleNot; <i>n</i> = 64); Physically not able to copulate (NotAble; <i>n</i> = 60)
Reared a chick	CHK	Categorical; Yes (<i>n</i> = 344) / No (<i>n</i> = 94)

Histograms of continuous effects are included in Appendix B.

Model set development and model selection

Models were built using variables determined to be of interest, specific to each research question (see below). Generalized linear mixed models, with a Bernoulli-distributed response variable (fertile/infertile) were built in the lme4 (Bates *et al.* 2012) package in R (R Core Team 2014). In each model, pair and year served as random effects. All combinations of the associated variables (corresponding to the four research questions) were considered in a given model set and an information-theoretic approach was taken for model selection and inference.

As per information-theoretic model selection and inference methods, Akaike's Information Criterion (AIC) was calculated for each model (Akaike 1973; Burnham and Anderson 2004). AIC uses the maximized log likelihood of a model and a penalization term for the number of parameters in the model to determine which model is the most parsimonious fit for the data. Within each model set, the model with the lowest AIC value is the best fit. AIC weights were calculated as probabilities that each model was the best fit for the data using the following equation:

$$w_i = \frac{\exp^{-0.5\Delta_i}}{\sum_{r=1}^R \exp^{-0.5\Delta_r}}$$

where w_i is the AIC weight for model i , Δ_i is the AIC value for model i minus the minimum AIC value of all models in the set, and R is the total number of models in the set. Additionally, the evidence ratio was calculated. This ratio was calculated for model i , as the AIC weight of the top model divided by the AIC weight of model i and can be interpreted as the relative evidence in favor of the top model as compared to model i (Summarized in Table 2).

Table 2: Model selection results for each of four categories of variables corresponding to research questions and their effect on egg fertility in captive Whooping Cranes at Patuxent Wildlife Research, between 2005 and 2015.

Candidate models	AIC	Δ AIC	w_i	Evidence Ratio
<i>Effect of Egg</i>				
(Pair + Year)	526.030		0.254	
MLD + (Pair + Year)	527.747	1.717	0.108	2.359
ENS + (Pair + Year)	528.104	2.074	0.090	2.820
ENC + (Pair + Year)	528.255	2.225	0.083	3.043
MLD + ENS + ENC + (Pair + Year)	532.315	6.285	0.011	23.158
<i>Effect of Sire and Dam: Sire</i>				
MAGE + (Pair + Year)	511.062		0.192	
MAGE + MWS + (Pair + Year)	511.387	0.325	0.087	2.214
MAGE + MRM + (Pair + Year)	512.652	1.590	0.085	2.261
MAGE + MNP + (Pair + Year)	512.694	1.632	0.073	2.620
MAGE + MACP + (Pair + Year)	512.988	1.632	0.071	2.718
MAGE + MRM + MWS + (Pair + Year)	513.038	1.926	0.001	131.907
<i>Effect of Sire and Dam: Dam</i>				
FAGE+ FRM + FACP + FWS + (Pair + Year)	511.538		0.076	
FAGE+ FACP + FWS + (Pair + Year)	511.854	0.317	0.065	1.171
FAGE+ FRM + FACP + (Pair + Year)	511.989	0.451	0.061	1.253
FAGE+ FACP + (Pair + Year)	512.034	0.496	0.059	1.281
FAGE+ FRM + FACP + FWS + FNP + (Pair + Year)	512.112	0.574	0.057	1.333
FAGE+ FAFP + FRM + FACP + FAFL + FWS + FNP + (Pair + Year)	515.086	3.549	0.013	5.896
<i>Effect of Pair</i>				
KSHP + PP + CRE + (Pair + Year)	507.011		0.268	
KSHP + PP + CRE + CRPY + (Pair + Year)	508.121	1.110	0.154	1.741
KSHP + CRE + NYP + (Pair + Year)	508.265	1.254	0.143	1.872
KSHP + PP + CRE + NYP + (Pair + Year)	508.764	1.753	0.112	2.402
KSHP + CRE + (Pair + Year)	508.908	1.896	0.104	2.581
KSHP + CRE + CRPY + NYP + (Pair + Year)	509.510	2.498	0.077	3.488
<i>Effect of Captive Management</i>				
COP + AI + CRE + (Pair + Year)	467.616		0.688	
COP + AI + (Pair + Year)	469.284	1.668	0.299	2.303
AI + CRE + (Pair + Year)	476.010	8.394	0.010	6.649
AI + (Pair + Year)	478.287	10.671	0.003	207.577
CRE + (Pair + Year)	518.629	51.013	0.000	1.194 x 10 ¹¹
COP + CRE + (Pair + Year)	521.561	53.945	0.000	5.177 x 10 ¹¹

For each question the top five models and the global model are presented. Akaike's Information Criterion (AIC) was used to rank models, and the model with the lowest AIC value has the best fit for the analyzed data. Also listed are the relative AIC for each model (Δ AIC), the model weight (w_i), and the evidence ratio. Variables are described in Table 1. In addition, every model contained random effects of breeding pair and year (Pair + Year).

The sum of weights for the set of models in which a variable appeared were calculated and served as a measure of the importance of that variable (Table 3). Predicted probability of egg fertility are reported based on the most supported statistical model for each research question. When more than one variable remained in the model, the prediction for the variable in question was calculated with all other covariates held at the highest value or level within the model.

Models

Effect of Egg

Three predictors for the question ‘how do egg-specific variables impact fertility?’ were considered.

Predictors included the month in which the egg was laid (categorical: March, April, May, or June; MLD). This variable was of interest to determine whether and how the timing of egg production during the season influenced probability of egg fertility. It was hypothesized that eggs laid in peak season (April) would have the highest probability of fertility.

Also included was egg sequence in the breeding season (treated as continuous variable, the first, second, third, etc., egg of the season for the pair; ENS). It is a common practice to pull eggs from pairs, allowing for increased egg production opportunity, both in captivity (Ellis et al, 1996) and in the reintroduced Eastern Migratory Population (Harrell and Bidwell 2013). The hypothesis was that eggs produced late in a pair’s seasonal sequence would have differential (lower) probability of fertility compared to eggs laid earlier.

Table 3: Summary of variable weights, regression coefficient (effect) estimates, and 95% confidence intervals, for all predictor variables of interest corresponding to research questions and their effect on egg fertility in captive Whooping Cranes at Patuxent Wildlife Research, between 2005 and 2015.

Model	Parameter	Relative Importance Value	Parameter Level	Effect Estimate	95% Confidence Interval
<i>Effect of Egg</i>	MLD	0.266	March	-0.057	(-1.108 , 0.995)
			May	0.124	(-0.344 , 0.591)
			June	-13.193	(-1.992 , 0.464)
	ENS	0.286		0.030	(-0.116 , 0.175)
	ENC	0.334		0.019	(-0.411 , 0.449)
<i>Effect of Sire and Dam: Sire</i>	MAGE	0.999		-0.008	(-0.016 , -0.001)
	MAFP	0.271		-0.003	(-0.286 , 0.253)
	MRM	0.308	Human	0.442	(-1.014 , 2.035)
	MACP	0.275		-0.020	(-0.189 , 0.128)
	MWS	0.24	Not	-0.156	(-0.928 , 0.604)
	MNP	0.298		-0.269	(-1.296 , 0.675)
<i>Effect of Sire and Dam: Dam</i>	FAGE	0.998		-0.013	(-0.021 , -0.005)
	FAFP	0.349		-0.115	(-0.440 , 0.211)
	FRM	0.556	Human	1.044	(0.318 , 2.405)
	FACP	0.811		-0.125	(-0.222 , -0.029)
	FAFL	0.305		-0.121	(-0.362 , 0.120)
	FWS	0.559	Not	-1.463	(-2.891 , -0.034)
	FNP	0.363		0.426	(-0.265 , 1.118)
<i>Effect of Pair</i>	KSHP	0.988		-43.813	(-78.117 , -9.510)
	PP	0.606	Female	-1.176	(-2.195 , -0.157)
			Male	0.446	(-0.657 , 1.548)
			None	0.223	(-0.416 , 0.862)
	CRE	0.989	Yes	1.687	(0.932 , 2.510)
	CRPY	0.359	Yes	0.333	(-0.299 , 0.965)
NYP	0.433		0.042	(-0.012 , 0.956)	
<i>Effect of Captive Management</i>	COP	0.996	AbleNot	-1.691	(-2.686 , -0.696)
			NotAble	-0.647	(-1.716 , 0.422)
	AI	0.999	Social	-0.867	(-1.578 , -0.156)
			No AI	-2.690	(-3.464 , -1.916)
CRE	0.661	Yes	0.997	(0.065 , 1.929)	

Relative Importance Values are calculated by summing the Akaike weights for all models in which the given variable appeared. Bolded variables indicate those that appeared in the top ranked model for each research question. Also included are the effect estimates and the confidence interval for each variable calculated from the highest ranked model in which the variable appeared. See Table 1 for description of variables and reference level compared for each parameter.

The final variable considered for this research question was the order the egg was laid within a clutch (first or second; ENC). A clutch was defined by laying date: if eggs were laid within 4 days of each other, they were grouped into the same clutch (Ellis *et al.* 1996). Female whooping cranes likely can store sperm in specialized sperm storage tubules (Jones and Nicolich 2001); however, it is unknown how many stored sperm are adequate to fertilize a complete clutch and if sperm stored are depleted after the first egg is laid. It was hypothesized that there would be no difference in probability of egg fertility between eggs of the same clutch.

Effect of Sire and Dam

Individual life history factors of breeders could potentially influence egg fertility. In many mammalian and avian species (Minton 1968; Angelier *et al.* 2006; Williams 2012; Prado-Oviedo *et al.* 2016), previous life events can affect an individual's ability to successfully reproduce. Separate models for the sire and dam were run to determine if specific life history factors are different between the sexes. The same six variables were considered for each sex, plus one additional variable for females.

Age of the individual (male: MAGE and female: FAGE) when the egg was laid was included in models for both sexes. In each case this variable was fit as a quadratic function to determine if there is a peak age for fertile egg production within this captive population. Captivity has increased the maximum productive age of other crane species (Ellis *et al.* 1996) and age of the individual influences nest success and chick production of reintroduced whooping crane chicks (Anne Lacy, International Crane Foundation; pers. comm.).

In addition, age of the individual when it was first paired (male: MAFP, female: FAFP) and age of the individual when it was paired with its current mate (age was treated as a continuous variable; male: MACP, female: FACP) were examined. Two variables were included to determine if the time of pairing in a bird's life, either for the first time or for subsequent pairings, had an influence on egg fertility. It was hypothesized that pairing later in life would have a negative effect on probability of egg fertility.

Rearing method was divided between cranes that were reared by crane parents (either whooping or sandhill cranes) or reared through artificial means by human caregivers (including humans without costume, humans using a puppet head, or humans in full crane costume with a puppet head; male: MRM, Female: FRM). This variable was included to investigate if rearing methods could have an effect on captive production. It was hypothesized that eggs from cranes reared by cranes would have a higher probability of fertility.

Having full use of wings has been linked to production of fertile eggs in crane species. Specifically in a captive flock of red-crowned cranes (*Grus japonensis*) fully winged pairs displayed a 76.3% egg fertility rate while pinioned birds reached only 27.0% egg fertility rate (Belterman and King 1993). The first naturally fertile eggs were produced at PWRC in 1986 by a young pair of sandhill cranes which had been allowed to remain fully-flighted (Nicolich *et al.* 2001). Thus, wing status of the individual during the season in which a given egg was laid (categorical variable, full wing or not full wing; male: MWS, female: FWS) was included to assess the impact on whooping cranes within this population. Individuals without full wings included

those who were injured and could not fully extend their wings, birds with clipped wings, and birds that had mechanical wing restraints to restrict copulation (for purposes of preventing particular genetic pairings). It was hypothesized that eggs from individuals without full wings would have a lower probability of fertility.

The number of pairs the individual had been in (including its current pairing, treated as a continuous variable; male: MNP, female: FNP) was considered. This variable was included to determine if multiple pairing attempts are detrimental to egg fertility. It was hypothesized that number of pairings would have an effect on egg fertility.

The analysis for females also included the age at which the female began laying eggs (treated as a continuous variable; FAFL). In addition to the other age-related variables, this was of interest to determine if the delayed onset of egg laying seen in captive females (Ellis *et al.* 1996) also limits fertility in these eggs. It was hypothesized that if females were older when they begin laying, probability of egg fertility would be decreased.

Effect of Pair

Crane pair bonds are critical for the success of the pair and it is possible that pair characteristics could influence the fertility of laid eggs. Four predictors were considered for this question.

First, whether either member of the pair had been previously paired with another crane (categorical variable, four levels: neither individual previously paired, male paired previously, female paired previously, both individuals paired previously, PP) was considered. It is known that individuals can successfully pair again in the

wild if a pair splits or one member dies (Ellis *et al.* 1996). It is possible that this is also the case in captivity and could impact future fertility of subsequent pairs. It was hypothesized that either individual being previously paired would not have an effect on egg fertility.

Second, the number of years the pair had been together (treated as a continuous variable, NYP) was included. The length of time a pair had been together was defined as starting in the year in which the members of the pair were determined to be behaviorally compatible to a degree that they could be safely co-housed (this definition of pairing is used consistently throughout my analyses). Typically the initial eggs laid by recently formed pairs are infertile (often the first two breeding seasons; Ellis *et al.* 1996). Therefore, it was hypothesized that pairs that had been together longer would have higher egg fertility.

Third, whether the pair had chick rearing experience together (categorical variable, yes/no, CRE) was considered. As stated previously, a pair's ability to produce and raise chicks is thought to be a critical factor in pair bond stability. It was hypothesized that chick rearing would have a positive effect on egg fertility.

Fourth, whether the pair had raised a chick in the previous breeding season (categorical variable, yes/no, CRPY) was included. This variable was used to determine if raising a chick every year was necessary to increase egg fertility. It was hypothesized that if a pair raised a chick the previous season, its eggs would have a higher probability of fertility.

Finally, the kinship of the pair (treated as a continuous variable, KNSP) was examined. Kinship of the pair was calculated using PMx software (Lacy *et al.* 2012)

using the known pedigree information and kinships previously developed through genetic analysis (Jones *et al.* 2002). It was hypothesized that kinship of the pair would have a negative effect on egg fertility.

Effect of Captive Management

It is possible that decisions made by captive flock managers through captive management could impact egg fertility. Three different predictors were considered in this model set.

The first predictor was a pair's chick-rearing experience (categorical variable, yes/no; CRE). A pair's ability to produce and raise chicks is thought to be a critical factor in pair bond stability, and often wild pairs that fail to raise a chick over successive seasons will "divorce." However, in captivity it is up to captive managers to allow a pair to raise a chick and suitability of the pair to do so is up to manager's discretion. Thus, the same variable (CRE) was included in a second model set to determine whether allowing a pair to raise a chick together had an impact on probability of egg fertility.

The second predictor described whether a pair was allowed and/or was able to copulate in the year in which the egg was produced (categorical variable, three levels, COP). Regular copulations are an integral part of pair bonding, crane reproductive behavior, and breeding success. Inhibiting copulation, for genetic or other management goals (Ellis *et al.* 1996), or the physical inability to copulate, due to injuries, likely impacts the probability of egg fertility. The predictor was categorical with three levels: 1) able and allowed to copulate (natural fertility), 2) able but not allowed to copulate (i.e., copulation was prevented to meet genetic management

goals), and 3) not physically able to copulate. Note that all pairs included in the study would have received AI if they were not able to copulate or were prevented from copulating (with sperm from either the social mate or a male other than the social mate, if the pair were not able to copulate, or a male other than the social mate, if the pair was prevented from copulating). Pairs which were able and allowed to copulate may have received supplemental AI within the pair (social mate was the semen donor) or from outside the pair.

The third predictor concerned the artificial insemination strategy for the pair in the year in which the egg was produced (AI; categorical variable, 3 levels). The AI strategy variable was split into three categories: 1) female did not receive AI (natural fertility), 2) female was inseminated with samples from her social mate (either supplemental to natural fertility or in cases where pair could not naturally copulate due to injury), and 3) female was inseminated with samples from a non-social mate (AI only as natural copulation prevented or physically impossible).

Results

In total, 438 individual eggs were included in the analysis, of which 162 were fertile. These eggs were produced by 23 unique pairs (some individuals occurred in more than one pair). Total egg production per pair during the 2005-2014 time period ranged from 1 to 46 eggs. The average number of eggs produced per pair in a given season was 2.6 ± 0.07 eggs (mean \pm SE) with a range of 1 to 9 eggs.

Effect of Egg

The highest ranked model for the research question on the effect of egg characteristics was the null model, containing no predictors (Table 2). All relative importance values were below 0.34.

Effect of Sire and Dam

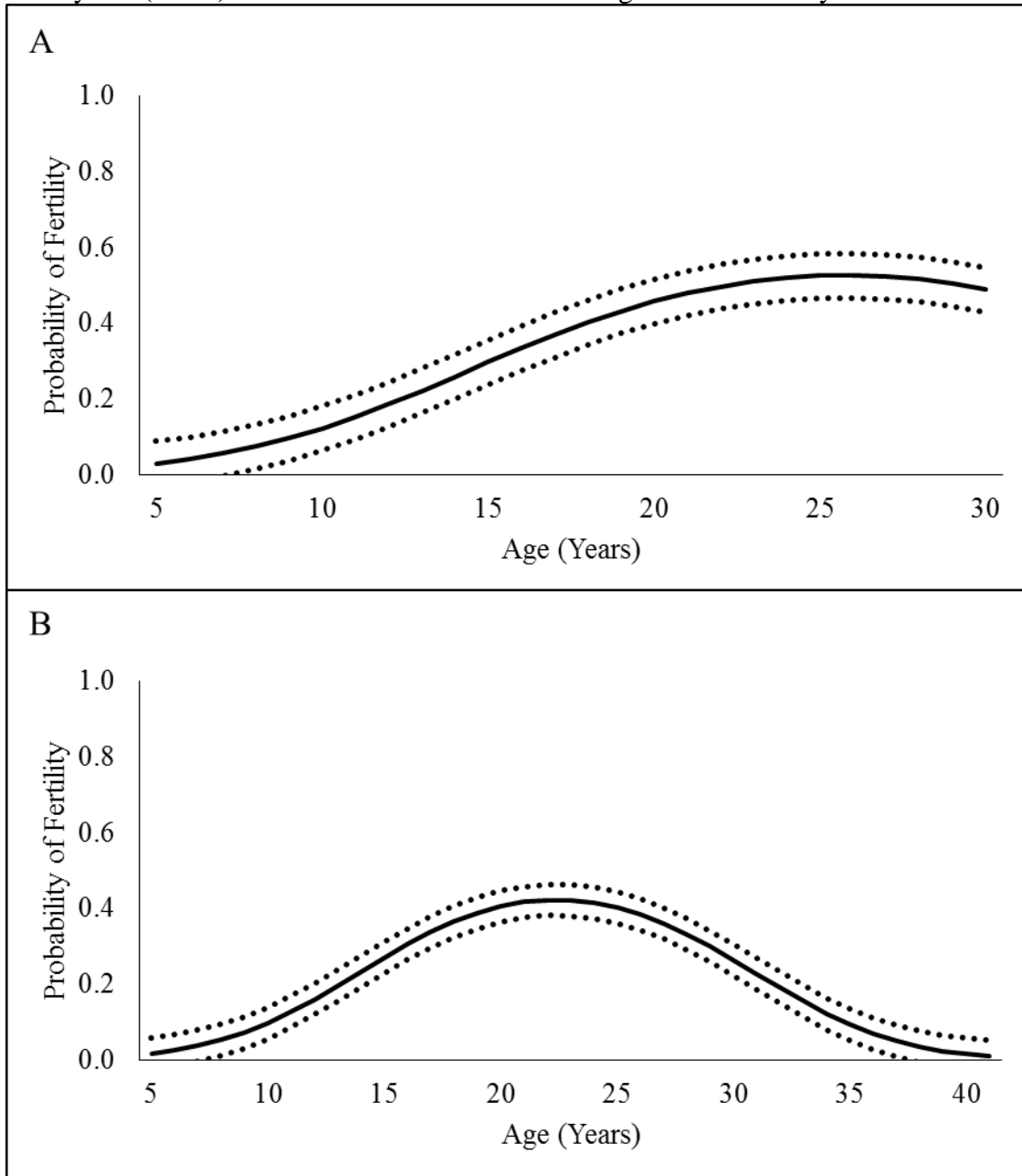
Sire

The top model in the set including predictors involving male life history contained only the age variable (MAGE; Table 2). A quadratic function of male age had a strong influence on egg fertility (relative importance value = 0.999; Fig. 1A). Probability of egg fertility for males was highest at 27 years of age (0.526; 95% CI: 0.477, 0.585) and was above 0.50 between the ages of 23 and 30 years of age. Variables that did not remain in the model included age at first pairing (MAFP), rearing method (MRM), age at current pairing (MACP), wing status (MWS), and number of pairs (MNP).

Dam

The top model in the model set including female life history effects contained four variables (Table 2). These variables were: age (FAGE; relative importance value = 0.998), rearing method (FRM: 0.556), age at current pairing (FACP: 0.811), and wing status (FWS: 0.559). Variables which did not remain in the model included age at first pairing (FAFP), age at first laying (FALF), and number of pairs (FNP).

Figure 1: Effect of individual age on probability of egg fertility (predicted probability, solid lines; 95% confidence intervals, dotted lines). The age of the male (A) and female (B) both had an effect on the probability of an egg being fertile, in both cases age was fit as a quadratic function. Probability of egg fertility was highest at a male age of 27 years (probability of egg fertility = 0.526) and was above 0.5 between the ages of 23 and 30 years of age. Probability of egg fertility was highest at a female age of 22 years (0.422) and was above 0.4 between the ages of 20 and 25 years.



Age of the female was fit as a quadratic function (Fig. 1B; Table 2). In all fertility probability predictions presented here, covariates for other variables in the top model are held at the levels with the highest fertility. Probability of egg fertility was highest at female age = 22 years old (0.422; 95% CI: 0.381, 0.463; given FRM = Human, FWS = Full, FACP = 2) and was above 0.40 between the ages of 20 and 25 years.

Female age at current pairing was fit as a linear effect (Fig. 2). The younger the female was when she was paired with her current social mate, the higher the probability that her eggs were fertile. Females paired at 2 years of age had fertility of 0.383 (95% CI: 0.279, 0.487; given FRM = Human, FWS = Full, FAGE = 22), while females paired at 12 years of age had fertility of 0.186 (95% CI: 0.082, 0.289).

For the variable FWS, females with full use of wings produced eggs with higher probability of fertility than females without (Table 2). For the variable FRM, females who were raised by human caregivers had higher probable fertility compared to those reared by crane parents. Predicted probability of egg fertility was calculated for each combination of categorical variables (FWS and FRM) under the best model set with FAGE and FACP set at values with the highest predicted fertility (22 and 2 respectively). Eggs produced by females that had full use of their wings and were raised by humans had a 0.398 probability of fertility (95% CI: 0.382, 0.414) as compared to eggs produced by females with full wings raised by cranes at 0.206 (95% CI: 0.195, 0.218), females without full wings raised by humans at 0.150 (95% CI: 0.141, 0.159), and females without full wings raised by cranes at 0.062 (95% CI: 0.057, 0.066).

Figure 2: Effect of female age at current pairing on probability of egg fertility (predicted probability, solid lines; 95% confidence intervals, dotted lines). Overall the younger the female was when entering her current pair, the higher probability of egg fertility. Females paired at 2 years of age had a probability of egg fertility of 0.383 (95% CI: 0.279, 0.487), while females entering a new pair at 12 years of age had a probable fertility of 0.186 (95% CI: 0.082, 0.289).



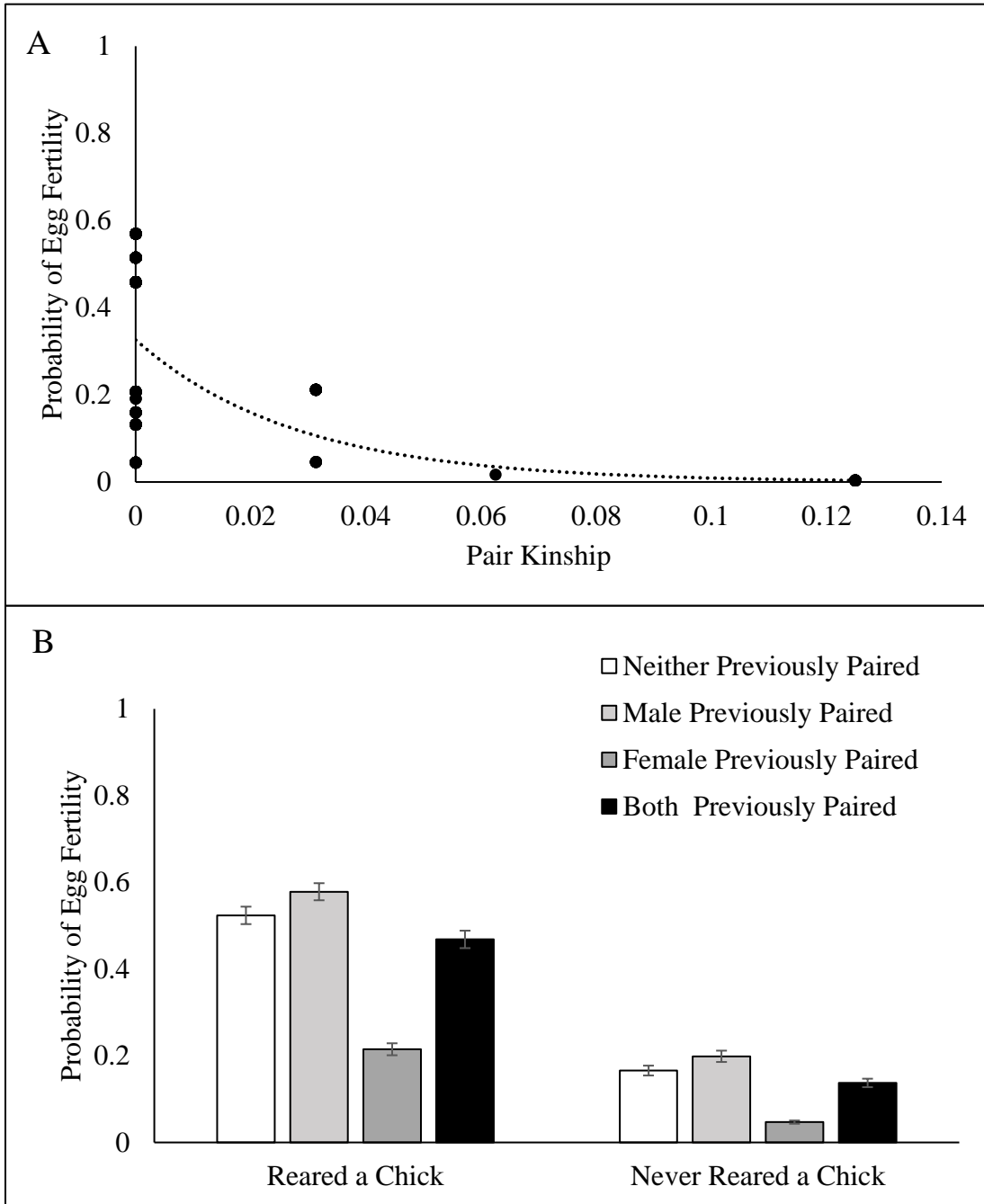
Effect of Pair

The top model in the model set focused on pair-specific factors contained pair kinship (KSHP), previously pairing of birds in the pair (PP), and if the pair had chick rearing experience (CRE; Table 2). Chick rearing experience had the highest relative importance (0.989), followed by kinship (0.988), and previous pairing (0.606).

Probability of egg fertility was calculated based on pair kinship values with the other variables held at the values with the greatest positive impact (CRE = Yes, PP = Male; Fig 3A). With a mean kinship of 0, pairs had a predicted probability of egg fertility of 0.389 (95% CI: 0.372, 0.406). As kinship increased, probability of egg fertility decreased, such that birds that were second cousins (kinship = 0.031) had probability of fertility = 0.170 (95% CI: 0.139, 0.202), and birds that were first cousins (kinship = 0.125) had probability of fertility = 0.004 (95% CI: 0.003, 0.005).

Kinship of the pair was held at the population minimum (KNSP = 0) and the probability of egg fertility for the predictors CRE and PP were estimated (Fig. 3B). Across the two predictors, probability of egg fertility was highest (0.578; 95% CI: 0.574, 0.582) for pairs which had raised a chick and in which the male had been previously paired to another female. The lowest probability of egg fertility was in pairs that had not raised a chick and the female had been previously paired to another male (0.047; 95% CI: 0.046, 0.048).

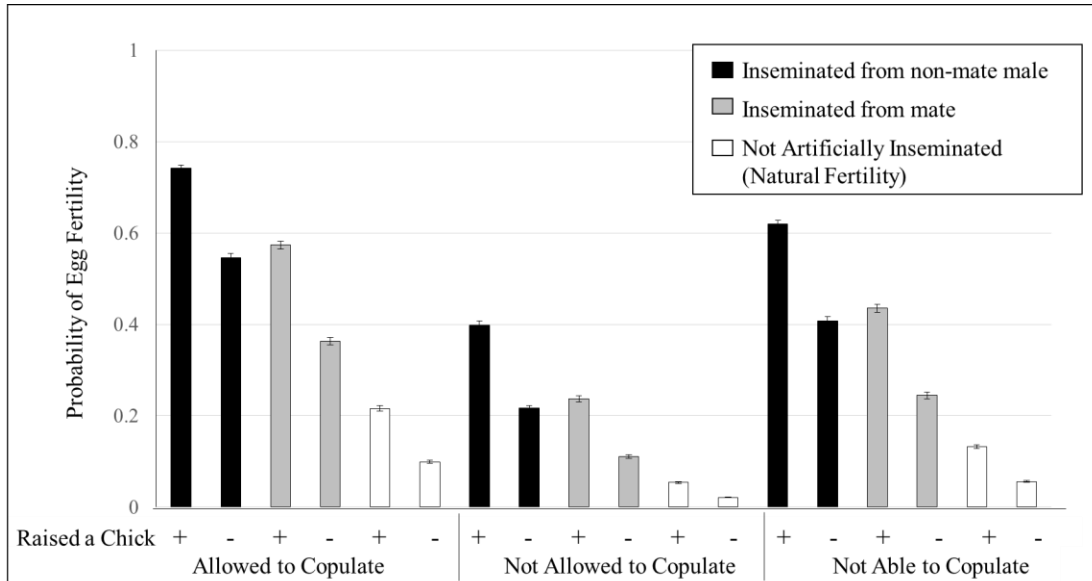
Figure 3: Effect of pair on probability of egg fertility. Probability of egg fertility based on pair kinship (KNSP) values (A) with the chick rearing experience (CRE) and if either individual was previously paired (PP) variables held at mean values and probable fertilities for the predictors of CRE and PP were estimated with KSHP of the pair was held at the population minimum (kinship of zero, assumed no relation; B). When the pair was unrelated the probability of egg was 0.389 (95% CI: 0.373, 0.406). A kinship value higher than zero resulted in lower probability of egg fertility. Chick rearing experience had a positive influence on probability of egg fertility, while a female being previously paired had a negative influence.



Effect of Captive Management

The top model for the model set focused on effects of management contained the predictors copulation (COP), artificial insemination strategy (AI), and chick rearing experience (CRE; Table 2) with relative importance values of 0.986, 1.000, and 0.661 respectively. Predictions for probability of egg fertility were calculated for each level of the three parameters in the model (Fig. 4). Eggs from pairs that were allowed to copulate, have previously raised a chick, and in which the female was inseminated by a male other than her social mate had the highest probability of egg fertility at 0.742 (95% CI: 0.729, 0.756). The next highest probability was produced by pairs which have raised a chick and were allowed to copulate, but the females received supplemental AI with only her social mate as the donor (0.573; 95% CI: 0.557, 0.598). The lowest probability of egg fertility was obtained if a pair had not reared a chick, were prevented from copulating naturally (although were physically able to) and were not inseminated (0.021; 95% CI: 0.019, 0.022).

Figure 4: Effect of captive management on probability of egg fertility. The top ranked model for the Management Question included the variables AI Strategy (AI), if the pair was able/allowed to copulate (COP), and chick rearing experience (CRE). Overall patterns indicate that probability of egg fertility is highest if the pair 1) has ever raised a chick together, 2) were allowed to copulate, and 3) females were inseminated by a male other than her social mate.



Discussion

The results presented here constitute an examination of factors that could have affected fertility of eggs produced by the whooping crane flock at PWRC between 2005 and 2014. Over time, production in this flock has decreased and the flock has required increasing levels of human assistance to produce target chick numbers to sustain the captive population and contribute to reintroduction programs (Brain Clauss, PWRC, pers. comm.). The aim of this retrospective analysis was to understand how management strategies and birds' life history events are associated with egg fertility. This information may be useful for revising management strategies.

Effect of Egg

No effects of egg specific variables was found, including seasonal timing, sequence within season, or sequence within clutch, on fertility. This is similar to results from research on domestic poultry, where the first eggs of the season do not have a higher probability of egg fertility compared to subsequent eggs (Robinson *et al.* 1991; Fasnko *et al.* 1992). Research on ostriches (*Struthio camelus*) also indicated that fertility does not vary during the reproductive season (Malecki and Martin 2003).

Effect of Sire and Dam

Sire

The only variable concerning males that influenced egg fertility was male age (MAGE). This is somewhat contrary to previous research in which age of male did not affect production or quality of semen obtained from whooping cranes maintained at the same breeding facility (Brown *et al.* 2015). Probability of egg fertility was

highest in males between the ages of 23 and 30 years of age. However, male age is likely to be closely correlated with female age, which may be the causative variable. Indeed, the correlation between male age and female age in a pair is 0.52 in this dataset. The oldest male was 31 years old, and at this age, fertility was nearly as high as at peak age. It is notable that captive males in other crane species have produced fertile eggs at 60 years of age (Ellis *et al.* 1996); better information on reproductive senescence in captive cranes will require larger datasets including older males and perhaps models including more complex polynomial functions.

Dam

This analysis showed that multiple female life history factors influence the probability of egg fertility. In the present study, highest probability of egg fertility was observed in females between the ages of 20 and 25 years. This age range was older than expected and could be related to the delay in onset of egg laying seen in captive birds (Ellis *et al.* 1992; Ellis *et al.* 1996) as females who begin laying later in life would reach peak productivity later than those who began laying earlier. Age at current pairing (FACP) was included in the model and showed that pairing females early improves lifetime fertility. It is common that the first few years of production in new crane pairs result in infertile eggs (Archibald 1974; Ellis *et al.* 1996) and that as female age increases, egg fertility increases until senescence. This pattern occurs in other wild avian species (Møller and De Lope 1999; Angelier *et al.* 2006; Rebke *et al.* 2010) and domestic poultry (Adams *et al.* 1978; Mather and Laughlin 1979; Bramwell *et al.* 1996).

Wing condition is known to impact a male's ability to fertilize eggs (Gee 1983; Stevens 1991; Chen *et al.* 2001); however, little information is available regarding the effect of wing condition on female fertility. Flight restriction has been shown to negatively impact overall welfare of captive birds (Peng *et al.* 2013), which may affect and/or stress the mechanisms controlling egg production.

Results indicated that females raised through artificial methods (by humans) had a higher probability of egg fertility. These results, while interesting, were not expected and also differ from a previous report that rearing method did not affect fertility within the whooping crane pair (Nicolich *et al.* 2001). The results from the current analysis and the previous work by Nicolich *et al.* both suggest that cranes reared by humans do not automatically decrease in reproductive potential. Females raised by humans may be better acclimated to life in captivity. This acclimation could allow for more positive response to frequent human disturbances and handling. This is important for the application of artificial reproductive techniques such as artificial insemination.

Effect of Pair

One of the most striking results from this analysis is the confirmation that parental relatedness (kinship; KNSP) negatively affects egg fertility. Previous studies in whooping cranes have failed to identify a relationship between inbreeding and semen quality (Brown *et al.* 2015), hatching success (Smith *et al.* 2011) and only a weak relationship to post-release survival of offspring (Converse *et al.* 2012). Prospective whooping crane pairs are recommended based on genetic compatibility and low levels of kinship (Ellis *et al.* 1996; Jones and Nicolich 2001). However, if no

appropriate genetic match is available, a pair will be formed between individuals who are behaviorally compatible until a genetic match is available (Ellis *et al.* 1996).

Thus, while the majority of pairs within our dataset have a kinship value of 0, there are pairs with kinship values which range from 0.3125 (second cousins) to 0.125 (first cousins). It has been shown in three long lived species, the fin whale (*Balaenoptera physalus*), the wandering albatross (*Diomedea exulans*), and the grey seal (*Halichoerus grypus*) that offspring from unrelated parents grow up to be more successful adults (Amos *et al.* 2001) and that parental genetic similarity reduces egg fertility in songbirds species (Cordero *et al.* 2004).

Chick rearing experience is influential in reproductive success of whooping crane pairs both *in situ* and *ex situ* (Archibald 1974; Kuyt and Goossen 1987; Ellis *et al.* 1996; Olsen and Converse 2017). The inclusion of this variable in multiple models, with high relative importance, confirms the recommendation that captive pairs should be given the opportunity to raise a chick, early in their reproductive life and as often as possible. These results show that rearing a chick in the previous season (CRPY) did not increase egg fertility in the subsequent year, meaning there is no immediate carry-over effect from chick rearing, but rather this experience of raising a chick may play a role in overall pair bond strength and success.

Effect of Captive Management

All of the predictors related to effects of management were found to have an influence on egg fertility. Each of these three predictors, ability to copulate (COP), AI strategy (AI), and chick rearing experience (CRE) are related to breeding success and genetic management.

Two of these predictors, ability to copulate (COP) and AI strategy (AI), are related to sperm availability during egg production. Being prevented from copulating, when a bird was physically able to, had a strong negative effect, even more so than pairs that cannot naturally copulate. Pairs are prevented from copulating through wing restraints as part of genetic management (Ellis *et al.* 1996). This allows for managers to ensure that eggs produced are sired by a new genetic match rather than the social mate. However, wing condition is thought to impact a male crane's ability to fertilize eggs (Gee 1983; Stevens 1991; Chen *et al.* 2001; Nicolich *et al.* 2001) and in the present study females with full use of their wings had higher probability of egg fertility. Courtship behavior increases production of sex steroids and stimulates egg and sperm production in other avian species (reviewed in Silver and Cooper 1983). Altered wing function (through injury or mechanically binding wings) decreases physical ability to perform courtship behaviors and may negatively impact mechanisms controlling egg production and successful fertilization.

It is generally understood that high numbers of sperm are required for fertilization to be successful (Lake 1983; Gee *et al.* 2004). Studies in other avian species have shown that not all behaviorally successful copulations transfer semen (Birkhead 1991) and multiple successful copulations are required for fertilization of an egg (Wishart 1985). The number of sperm available during natural fertilization in captive whooping crane pairs is unknown. Wild whooping cranes have been observed copulating multiple times per day (Folk *et al.* 2005; Dellinger *et al.* 2013). However, little information is known about frequency of copulation in captive cranes or the relative success of semen transfer during natural copulations. Domestic chickens have

been observed copulating up to 30 times per day while only half of these copulations result in a transfer of sperm (Etches 1996). It is most likely that the artificial insemination program increases the number of sperm present in the female tract during egg laying events beyond what would be present through natural copulation, ensuring higher levels of egg fertility. However, even if this is the case, my results indicate that female inseminated from donor males other than their social mates had higher probability of egg fertility than those given supplemental inseminations using sperm from the social mate. It is possible that natural fertility is compromised by reproductive behavior incompatibility or mis-timing of gamete production within the pair (Malecki and Martin 2003), a problem which is resolved by using artificial insemination.

Conclusions

This study would not have been possible without the extensive records kept for each individual within this population at PWRC. There is high value in examining historical records to understand the needs of captive animals.

Overall the findings confirm the value of providing opportunities for captive birds to engage in natural breeding behaviors. This includes allowing pairs to raise chicks and allowing individuals to retain full use of their wings whenever possible. Additionally, management should continue to genetically manage the population to form pairs from unrelated individuals and introduce pairs as soon as animals have reached sexual maturity to increase the number of years a pair has to be productive.

This data set represents a laying population with a skewed age structure, as the mean ages for males and females were 17 and 19 respectively. Although this is a

long-lived species, this is still troubling given the fact that in nature this species begins reproducing as early as 4 years old (Ellis *et al.* 1996). While some birds within this study are younger, the majority of eggs produced by the captive population are laid by older females. A delay in onset to egg laying has already been identified in this species (Ellis *et al.* 1996) and could indicate the captive environment has a negative impacting reproduction in this species, specifically the age in which an individual reproduces.

Of note, the results presented here propose the need to further examine reproduction in female cranes. Overall the field lacks information on female reproductive mechanisms in exotic birds (Ball and Ketterson 2008, Williams 2012). Based on the results presented here, it seems that females are more influenced by captive management and life events than males. More research on female reproduction is needed to better understand the mechanisms controlling egg laying and egg quality in the whooping crane and cranes in general. Further scrutiny and extensive documentation of semen collections and quality of inseminations should be a high priority to better understand how infertility may be attributed to female contributions rather than male variation.

This study lays the basic foundation that will help improve egg fertility in captive birds and will begin to improve captive management and reproduction. These results continue to highlight the problem of low fertility within this flock, but leave additional questions unanswered. Future research should focus on 1) if enclosure design impacts the ability of pairs to copulate successfully and inadvertently prevents natural fertilization of eggs; 2) if size of enclosure effects performance of courtship

displays and 3) if some pairs, while behaviorally compatible and able to cohabitate, reproduce poorly because of incompatibilities at the cellular or molecular level that may affect fertilization. These are all questions that require more extensive ethological studies. Additional work may differentiate effects of individual from effects of captivity and determine if any of the results presented here are attributed to generations in captivity or the overall captive setting.

Chapter 5: Overall Conclusions

The research performed in completion of this dissertation has furthered the overall knowledge of reproductive biology in two endangered crane species, the whooping crane and the white-naped crane. My studies focused on factors associated with captive management and their impacts on reproduction.

In the first study, I sought to develop a protocol for successful semen cryopreservation in two endangered crane species. I compared the effect of two different permeating cryoprotectants (DMSO and DMA) alone and combined with a non-permeating cryoprotectant (sucrose) sperm motility, viability, and ability to fertilize and egg post-thaw. Results indicated, that while there were species specific differences, overall sperm of both species performed better in the DMSO only treatment. While previous work failed to determine an effect of inbreeding on basic seminal parameters (volume, concentration, and motility), however it is possible that inbreeding becomes detrimental at more mechanistic levels and decreases the ability of sperm to survive cryopreservation. This could explain the differences observed in our work with two threatened and inbred populations compared to the more outbred sandhill crane. The effect of vitamin E was examined as an anti-oxidant to prevent damage caused by lipid peroxidation. Vitamin E displayed no significant benefit to any variable measured post-thaw. It is possible that crane sperm do not suffer from lipid peroxidation or that another anti-oxidant, such as vitamin C, would prove to be more beneficial.

In the second study, I utilized non-invasive hormone monitoring to evaluate the sensitivity of gonadal and adrenal hormone production to changes in annual

cycles and captive environment in whooping cranes. Seasonal patterns were displayed in all hormones, gonadal and adrenal, with the most striking differences observed in female estrogen and progesterone production. The findings in this study also showed that moving birds to a naturalistic exhibit stimulated gonadal hormone secretions and egg production in female whooping cranes. Overall, the study demonstrated that there are seasonal changes in hormone production in captive whooping cranes which are further stimulated by the beneficial effects of natural environment on reproductive performance. The implementation of a more naturalized captive environment indicates that water levels may act as an essential supplemental stimulus to reproduction in female cranes. However, while these results indicate the benefits of including a standing water feature in crane enclosures, there are other variables which likely influenced our results. Primarily the wetland study pens are larger than the traditional crane pens. However birds readily breed in the smaller enclosures so it is unlikely that larger space alone would stimulate breeding. The number of pairs housed in proximity to each other in the wetland pens are also fewer than the number housed in the traditional breeding series. This decreased pair density could have a positive effect by reducing intra-pair competition. Wild pairs maintain large territories and do not associate with each other during the breeding season. Being in close auditory range, even with visual barriers, may inhibit reproduction. Additionally, the increase of novel food availability and novel protein sources may have contributed to the observed increase in reproduction.

In the third study, I examined numerous variables associated with the management of captive whooping cranes and their effect on probability of egg

fertility. I found that female whooping cranes are more sensitive to the variables examined compared to males. Major results of interest are the negative effect of pair kinship, and the positive influence of chick rearing experience, full use of wings, and being allowed and/or able to copulate. My results also show the reliance on the artificial insemination program to produce fertile eggs. This research lays the basic foundation that will help improve egg fertility in captive birds and direct management decisions.

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. Management of this species has already played an important role in recovery. Findings obtained from the research presented here continue to advance this recovery effort and lay foundations for future research. Further work in cryopreservation is necessary to continue improving genetic management of captive whooping cranes. The evidence of species specific differences directs the need for classification of cellular membrane components and the development of species specific freezing protocols. Additional examinations of the HPG axis and its sensitivity to exogenous cues is necessary to understand causes of egg laying failure and mediating therapies. The final avenue of future research needs are more extensive ethological studies and further investigations of mechanisms controlling pair bond formation and the influence of captive environment on reproduction. Knowledge generated from this research improves our understanding about reproduction in whooping cranes, information which is crucial for identifying and mitigating potential factors impacting reproductive success of the PWRC population.

Appendix A

Crane semen extender recipe (Blanco *et al.* 2012).

Ingredients dissolved in 100 mL distilled H₂O and adjusted to an osmolarity of 310 mosm and a pH of 7

Ingredient	Molar Concentration	Amount
D-fructose	63.8 mM	1.15 g
Sodium glutamate	112.1 mM	2.1 g
Polyvinyl Pirolidone	8.3 μ M	0.3 g
Glycine	13.0 mM	0.2 g
Potassium acetate	50.9 mM	0.5 g

Table 1: Sperm motion characteristics (mean \pm standard error of mean) post thaw in the whooping crane and white-naped crane.

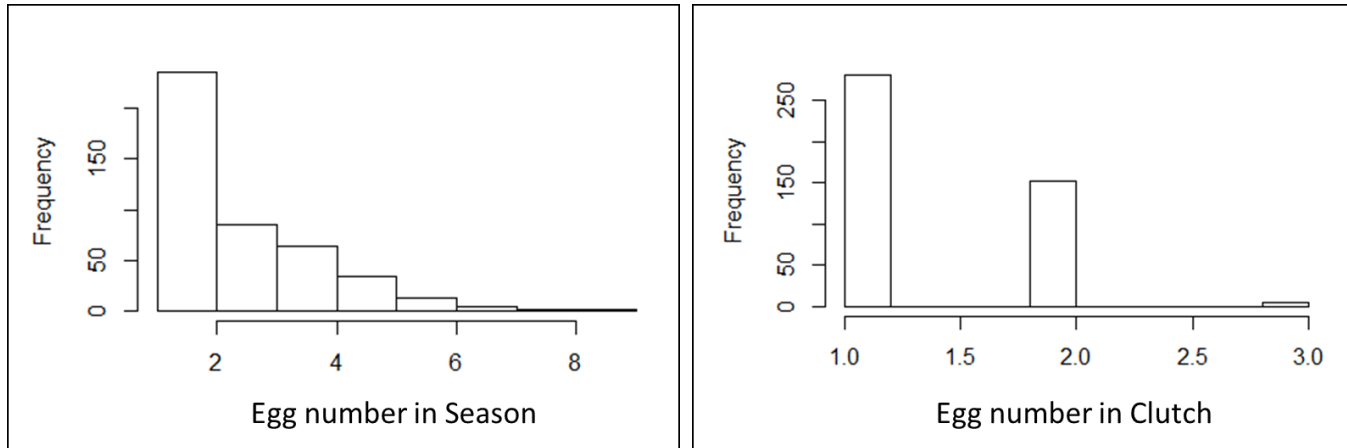
Species	Cryoprotectant	Straight Line Velocity ($\mu\text{m/s}$)	Curve Linear Velocity ($\mu\text{m/s}$)	Average Path Velocity ($\mu\text{m/s}$)	Straight Line Distance (μm)
Whooping Crane	Fresh	18.56 \pm 3.52	40.54 \pm 6.71	26.05 \pm 4.01	10.06 \pm 2.53 ^c
	DMA	18.08 \pm 3.46	45.80 \pm 16.98	27.05 \pm 6.38	7.59 \pm 0.86 ^{ab}
	DMA + 0.1M Sucrose	25.38 \pm 3.18	74.37 \pm 15.60	36.84 \pm 5.82	7.87 \pm 0.97 ^{abc}
	DMSO	19.11 \pm 2.76	48.14 \pm 13.61	26.91 \pm 5.04	9.88 \pm 0.91 ^{bc}
	DMSO + 0.1M Sucrose	20.24 \pm 2.76	57.92 \pm 13.61	31.53 \pm 5.04	11.47 \pm 0.97 ^c
	0.1M Sucrose	17.51 \pm 3.11	66.31 \pm 15.28	33.27 \pm 5.82	5.75 \pm 0.91 ^a
	0.2M Sucrose	18.77 \pm 3.78	74.42 \pm 18.47	32.91 \pm 7.13	6.02 \pm 0.97 ^a
White-naped Crane	Fresh	27.69 \pm 2.47	55.96 \pm 3.38	36.29 \pm 2.45	16.54 \pm 1.62 ^c
	DMA	19.61 \pm 6.84	45.06 \pm 9.20	28.79 \pm 6.83	6.73 \pm 0.84 ^{ab}
	DMA + 0.1M Sucrose	36.73 \pm 7.20	55.49 \pm 13.82	50.50 \pm 7.74	8.50 \pm 0.79 ^{abc}
	DMSO	24.73 \pm 7.01	45.57 \pm 9.94	31.62 \pm 7.25	8.95 \pm 0.70 ^{bc}
	DMSO + 0.1M Sucrose	27.94 \pm 7.20	59.65 \pm 10.88	35.87 \pm 7.74	7.93 \pm 0.70 ^{ab}
	0.1M Sucrose	24.02 \pm 6.94	48.80 \pm 13.82	45.93 \pm 7.13	6.25 \pm 0.77 ^a
	0.2M Sucrose	26.80 \pm 7.14	38.13 \pm 11.99	32.87 \pm 7.63	6.39 \pm 0.90 ^a

Different superscripts denote significant differences between groups.

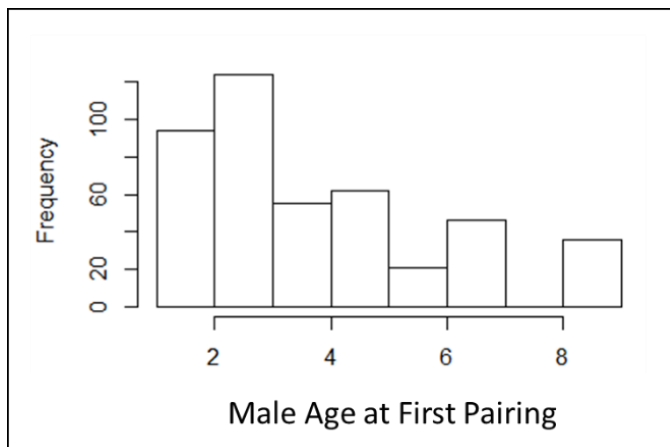
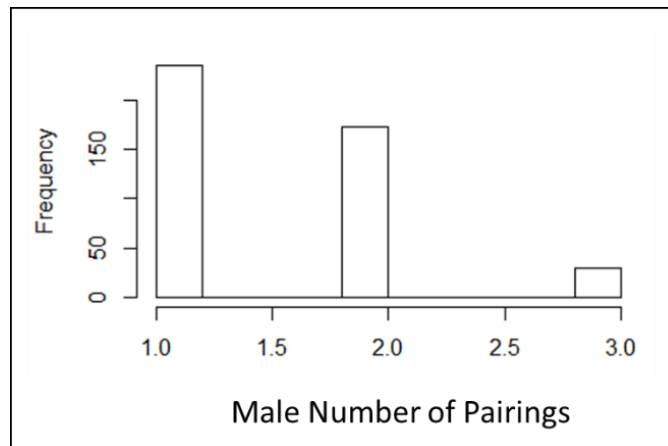
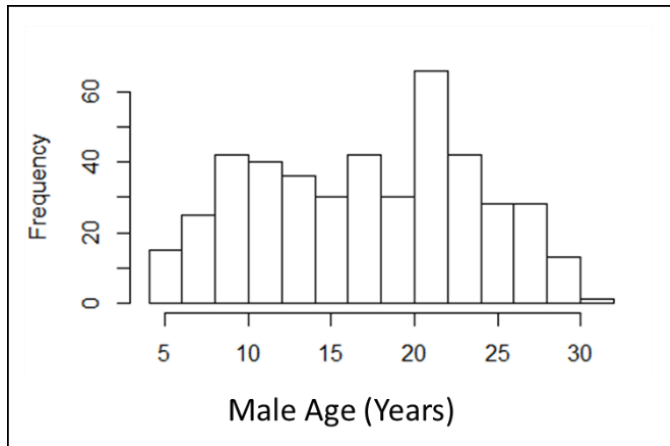
Appendix B

Histograms for continuous effects for each of five model sets

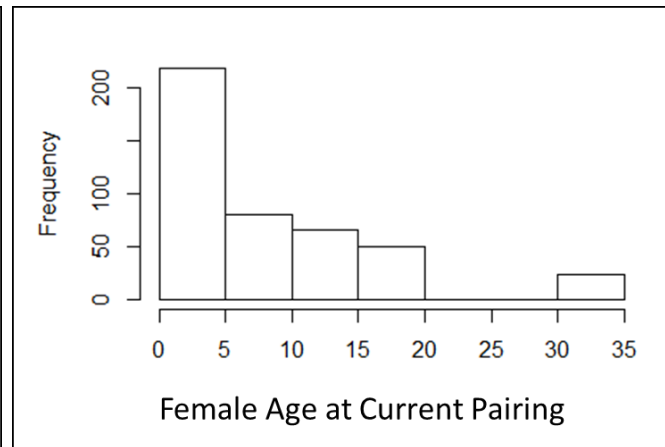
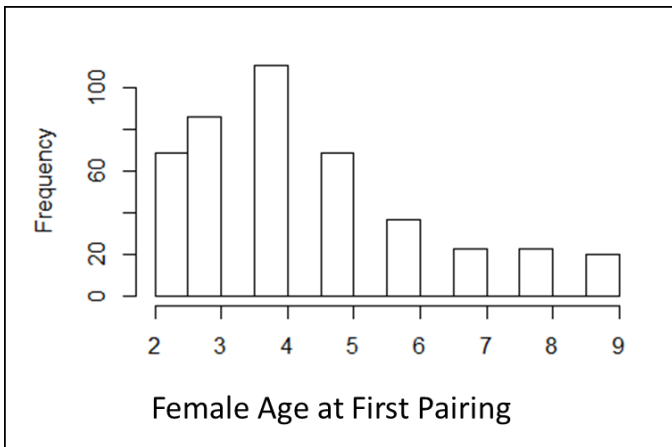
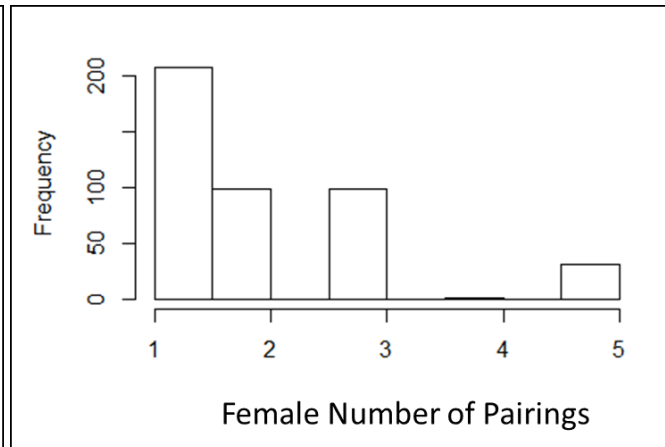
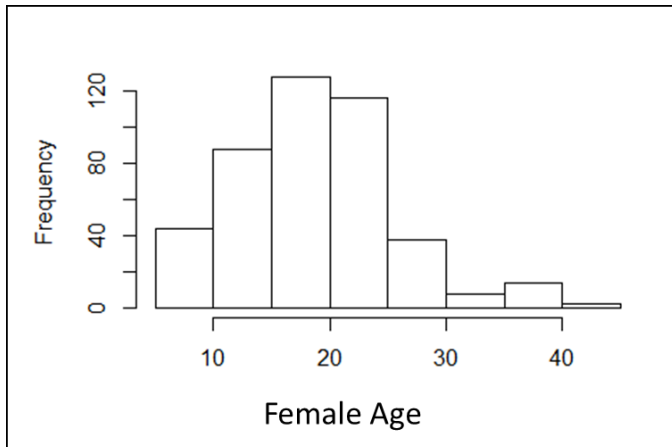
Effect of Egg:



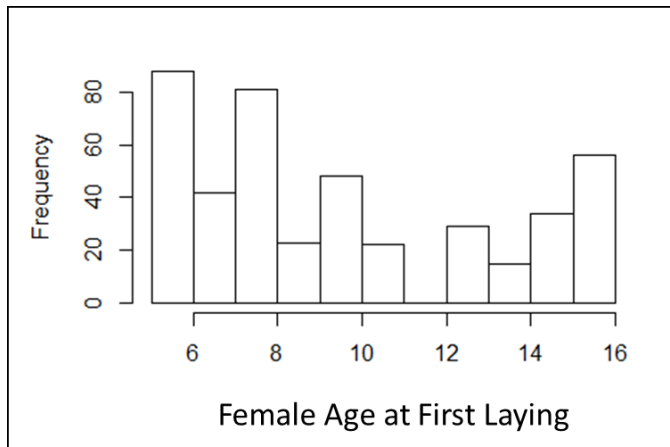
Effect of Sire:



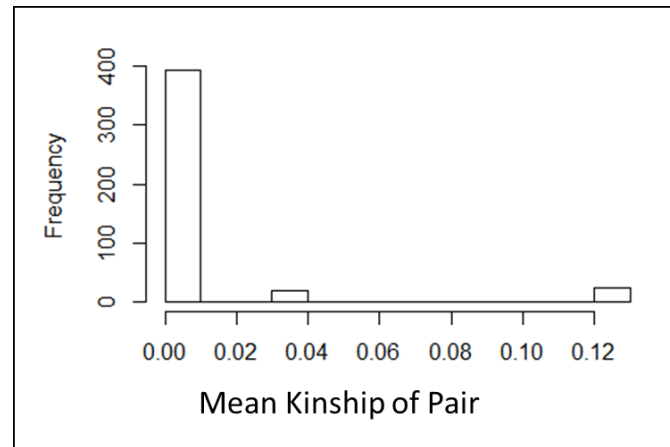
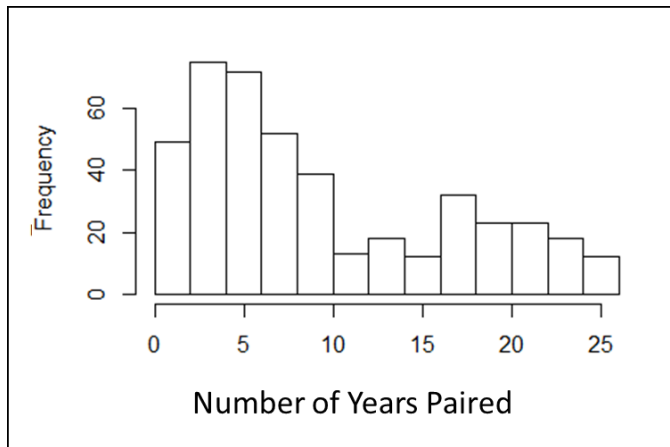
Effect of Dam:



Effect of Dam (Continued):



Effect of Pair:



Bibliography

- Adams, A., Craig, J., and Bhagwat, A. (1978) Effects of flock size, age at housing, and mating experience on two strains of egg-type chickens in colony cages. *Poultry Science* **57**(1), 48-53
- Ahammad, M., Nishino, C., Tatemoto, H., Okura, N., Okamoto, S., Kawamoto, Y., and Nakada, T. (2013) Acrosome reaction of fowl sperm: evidence for shedding of the acrosomal cap in intact form to release acrosomal enzyme. *Poultry science* **92**(3), 798-803
- Akaike, H. (1973) Maximum Likelihood Identification of Gaussian Autoregressive Moving Average Models. *Biometrika* **60**(2), 255-265
- Akesson, T.R., and Raveling, D.G. (1981) Endocrine and body weight changes of nesting and non-nesting Canada geese. *Biology of Reproduction* **25**, 792-804
- Aldridge, R.D., and Arackal, A.A. (2005) Reproductive biology and stress of captivity in male brown treesnakes (*Boiga irregularis*) of Guam. *Australian Journal of Zoology* **53**(4), 249-256
- Amos, W., Wilmer, J.W., Fullard, K., Burg, T., Croxall, J., Bloch, D., and Coulson, T. (2001) The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society of London B: Biological Sciences* **268**(1480), 2021-2027
- Angelier, F., Clement-Chastel, C., Weicker, J., Gabrielsen, G.W., and Chastel, O. (2009) How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in black-legged kittiwakes. *Functional Ecology* **23**, 784-793
- Angelier, F., Shaffer, S.A., Weimerskirch, H., and Chastel, O. (2006) Effect of age, breeding experience and senescence on corticosterone and prolactin levels in a long-lived seabird: The wandering albatross. *General and Comparative Endocrinology* **149**, 1-9
- Ansah, G. A., and Buckland, R. B. (1982). Genetic variation in fowl semen cholesterol and phospholipid levels and the relationships of these lipids with fertility of frozen-thawed and fresh semen. *Poultry Science*, **61**(4), 623-637.
- Archibald, G.W. (1974) Methods for breeding and rearing cranes in captivity. *International Zoo Yearbook* **14**, 147-155
- Asher, G., Peterson, A., and Duganzich, D. (1989) Adrenal and ovarian sources of progesterone secretion in young female fallow deer, *Dama dama*. *Journal of reproduction and fertility* **85**(2), 667-675

Ashizawa, K., Wishart, G., Katayama, S., Takano, D., Ranasinghe, A., Narumi, K., and Tsuzuki, Y. (2006) Regulation of acrosome reaction of fowl spermatozoa: evidence for the involvement of protein kinase C and protein phosphatase-type 1 and/or-type 2A. *Reproduction* **131**(6), 1017-1024

AZA Sustainability Database, (2015) White-naped Crane *Grus vipio*.

Ball, G.F., and Ketterson, E.D. (2008) Sex differences in the response to environmental cues regulating seasonal reproduction in birds. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**(1490), 231-246

Balzano, S.A. (1989) Unpubl. Climatic influences on crane production. In 'North American Crane Workshop'

Bates, D., Maechler, M., and Bolker, B. (2012) lme4: Linear mixed-effects models using S4 classes. (R package version 0.999999-0: <http://CRAN.R-project.org/package=lme4>)

Bayr, H. (2005) Reactive oxygen species. *Critical care medicine* **33**(12), S498-S501

Belterman, R., & King, C. E. (1993). Captive management and husbandry of Red-crowned Cranes in Europe. *International Zoo News* **40**(1), 6-20.

Birkhead, T.R. (1991) Sperm depletion in the Bengalese finch, *Lonchura striata*. *Behavioral Ecology* **2**(4), 267-275

Biswal, G. (1954) Additional histological findings in the chicken reproductive tract. *Poultry science* **33**(4), 843-851

Blanco, J.M., Gee, G., Wildt, D.E., and Donoghue, A.M. (2000) Species Variation in Osmotic, Cryoprotectant, and Cooling Rate Tolerance in Poultry, Eagle, and Peregrine Falcon Spermatozoa. *Biology of Reproduction* **63**(4), 1164-1171

Blanco, J.M., Long, J.A., Gee, G., Donoghue, A.M., and Wildt, D.E. (2008) Osmotic tolerance of avian spermatozoa: Influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. *Cryobiology* **56**(1), 8-14

Blanco, J.M., Long, J.A., Gee, G., Wildt, D.E., and Donoghue, A.M. (2011) Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. *Animal reproduction science* **123**(3), 242-248

Blanco, J.M., Long, L.A., Gee, G., Wildt, D.E., and Donoghue, A.M. (2012) Cryopreservation of avian spermatozoa: effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. *Anim Reprod Sci* **131**, 1-8

- Blesbois, E. (2007) Current status in avian semen cryopreservation. *World's Poultry Science Journal* **63**(02), 213-222
- Blesbois, E. (2012) Biological features of the avian male gamete and their application to biotechnology of conservation. *The Journal of Poultry Science* **49**(3), 141-149
- Blesbois, E., Grasseau, I., and Seigneurin, F. (2005) Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. *Reproduction* **129**(3), 371-378
- Bluhm, C.K., Phillips, R.E., and Burke, W.H. (1983) Serum levels of luteinizing hormone, prolactin, estradiol and progesterone in laying and nonlaying mallards (*Anas platyrhynchos*). *Biol Reprod* **28**(2), 295-305
- Bohmke, B.W. (1995) Captive design for endangered birds. In 'Conservation of Endangered Species in Captivity: An Interdisciplinary Approach.' (Eds. EF Gibbons, BS Durrant and J Demarest) pp. 291-302. (State University of New York Press: Albany, NY)
- Boyce, M.S., Subhash, R.L., and Johns, B.W. (2005) Whooping Crane Recruitment Enhanced by Egg Removal. *Biological Conservation* **126**, 395-400
- Bramwell, R., McDaniel, C., Wilson, J., and Howarth, B. (1996) Age effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. *Poultry science* **75**(6), 755-762
- Brillard, J.P., and Bakst, M.R. (1990) Quantification of spermatozoa in the perm-storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. *Biology of Reproduction* **43**, 271-275
- Brock, M.K., and Bird, D.M. (1991) Prefreeze and postthaw effects of glycerol and dimethylacetamide on motility and fertilizing ability of American kestrel (*Falco sparverius*) spermatozoa. *Journal of Zoo and Wildlife Medicine*, 453-459
- Brown, M.E., Clauss, B., Pukazhenthil, B., and Songsasen, N. In vitro methods for examining male fertility in crane species. In '14th North American Crane Workshop', January 11-15 2017, Chattanooga, TN,
- Brown, M.E., Converse, S.J., Chandler, J.N., Crosier, A.L., Lynch, W., Wildt, D.E., Keefer, C.L. and Songsasen, N., (2017). Time within reproductive season, but not age or inbreeding coefficient, affects seminal and sperm quality in the whooping crane (*Grus americana*). *Reproduction, Fertility and Development*, **29**(2), 294-306.
- Brown, M.E., Converse, S.J., Chandler, J.N., Shafer, C., Brown, J.L., Keefer, C.L., and Songsasen, N. (2016) Female gonadal hormones and reproductive behaviors as

key determinants of successful reproductive output of breeding whooping cranes (*Grus americana*). *General and comparative endocrinology* **230**, 158-165

Brown, M.E., Doyle, R.C., Chandler, J.N., Olsen, G.H., French Jr., J.B., Wildt, D.E., Converse, S.J., Keefer, C.L., and Songsasen, S. (2014) Chromic and Iron oxides as fecal markers to identify individual whooping cranes. *Proceedings of the North American Crane Workshop* **12**, 68-72

Brown, S.G., Shannon, P., and Farnell, G. (1983) Renesting and Brooding in the Caribbean flamingo. *Zoo Biology* **2**, 137-141

Burnham, K.P., and Anderson, D.R. (2004) Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods Research* **33**, 261-304

Carlstead, K., and Shepherdson, D. (1994) Effects of environmental enrichment on reproduction. *Zoo Biology* **13**, 447-458

Cech, R., Dunning, J.B.J., and Elphick, C. (2009) Behavior. In 'Sibley's Guide to Bird Life and Behavior.' (Eds. C Elphick, JBJ Dunning and DA Sibley) pp. 608. (Knopf: New York, NY)

Cezilly, F., Boy, V., Green, R.E., Hiron, G.J., and Johnson, A.R. (1995) Interannual variation in greater flamingo breeding success in relation to water levels. *Ecology* **76**(1), 20-26

Chen, G., Gee, G., Nicolich, J., and Taylor, J. (2001) The effects of semen collection on fertility in captive, naturally fertile, sandhill cranes. *North American Crane Workshop* **8**, 185-194

Converse, S.J., Royal, J.A., and Urbanek, R.P. (2012) Bayesian analysis of multi-state data with individual covariates for estimating genetic effects on demography. *Journal of Ornithology* **152**(2), 561-572

Cordero, P., Aparicio, J., and Veiga, J. (2004) Parental genetic characteristics and hatching success in the spotless starling, *Sturnus unicolor*. *Animal Behaviour* **67**(4), 637-642

Crofoot, M., Mace, M., Azua, J., MacDonald, E., and Czekala, N.M. (2003) Reproductive assessment of the greater hornbill (*Buceros bicornis*) by fecal hormone analysis. *Zoo Biology* **22**, 135-145

Davis, J. (1976) Breeding success and experience in the Arctic skua, *Stercorarius parasiticus* (L.). *The Journal of Animal Ecology*, 531-535

Dawson, A., King, V.M., Bentley, G.E., and Ball, G.F. (2001) Photoperiodic control of seasonality in birds. *J Biol Rhythms* **16**(4), 365-80

- de Ménorval, M.-A., Mir, L.M., Fernández, M.L., and Reigada, R. (2012) Effects of Dimethyl Sulfoxide in Cholesterol-Containing Lipid Membranes: A Comparative Study of Experiments In Silico and with Cells. *PLOS ONE* **7**(7), e41733
- Dellinger, T., Folk, M.T., and Spalding, M.G. (2013) Copulation behavior of non-migratory whooping cranes in florida. *The Wilson Journal of Ornithology* **125**(1), 128-133
- Derrickson, S., and Carpenter, J. (1987) Behavioral management of captive cranes--factors influencing propagation and reintroduction.
- Donoghue, A., and Donoghue, D. (1997) Effects of water-and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poultry Science* **76**(10), 1440-1445
- Drewien, R.C., Brown, W.M., and Kendall, W.L. (1995) Recruitment in Rocky Mountain greater sandhill cranes and comparison with other crane populations. *The Journal of wildlife management*, 339-356
- Dzoma, B. (2010) Some factors affecting fertility and hatchability in the farmed ostrich: a review. *Journal of Animal and Veterinary Advances* **9**(2), 229-239
- Ellis, D., Lewis, J., Gee, G., and Smith, D. (1992) Population recovery of the whooping crane with emphasis on reintroduction efforts: past and future. *Proceedings North American Crane Workshop* **6**, 142-150
- Ellis, D.H., and Gee, G.F. (2001) Whooping crane egg management: options and consequences. *Proceedings North American Crane Workshop* **8**, 17-23
- Ellis, D.H., Gee, G.F., and Mirande, C.M. (1996) 'Cranes: Their Biology, Husbandry, and Conservation.' (Department of the Interior, National Biological Service: Washington D.C. and Baraboo, WI) 308
- Etches, R., and Duke, C. (1984) Progesterone, androstenedione and oestradiol content of theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology* **103**(1), 71-76
- Etches, R.J. (1996) 'Reproduction in Poultry.' (CAB International: Wallingford, UK) 318
- Fasenko, G., Hardin, R., Robinson, F., and Wilson, J. (1992) Relationship of hen age and egg sequence position with fertility, hatchability, viability, and preincubation embryonic development in broiler breeders. *Poultry science* **71**(8), 1374-1383

Folk, M.J., Nesbitt, S.A., Schwikert, S.T., Schmidt, J.A., Sullivan, K.A., Miller, T.J., Baynes, S.B., and Parker, J.M. (2005) Breeding biology of re-introduced non-migratory whooping cranes in Florida. *Proceedings of the North American Crane Workshop* **9**, 105-109

Fuller, B.J. (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* **25**(6), 375-388

Gee, G. (1995) Avian reproductive physiology. In 'Conservation of Endangered Species in Captivity An Interdisciplinary Approach.' (Eds. EF Gibbons Jr., BS Durrant and J Demarest) pp. 241-262. (State University of New York: Albany, NY)

Gee, G., and Sexton, T. Artificial insemination of cranes with frozen semen. In 'Proceedings 1978 Crane Workshop', 1979, Rockport, TX. (Ed. J Lewis), pp. 89-94

Gee, G.F. (1983) Crane Reproductive Physiology and Conservation. *Zoo Biology* **2**, 199-214

Gee, G.F., Baskt, M.R., and Sexton, S.F. (1985) Cryogenic Preservation of Semen from the Greater Sandhill Crane. *Journal of Wildlife Management* **49**, 480-484

Gee, G.F., Bertschinger, H., Donoghue, A.M., Blanco, J., and Soley, J. (2004) Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. *Avian and Poultry Biology Reviews* **15**(2), 47-101

Gee, G.F., and Sexton, T.J. (1990) Cryogenic preservation of semen from the Aleutian Canada goose (*Branta canadensis leucopareia*). *Zoo biology* **9**(5), 361-371

Gee, G.F., and Temple, S.A. (1978) Artificial insemination for breeding non-domestic birds. In 'Symposia of the Zoological Society of London. Vol. 43.' (Ed. PF Watson) pp. 376. (Academic Press: London)

Glenn, T.C., Stephan, W., and Brayn, M.J. (1999) Effects of Population Bottleneck on Whooping Crane Mitochondria DNA Variation. *Conservation Biology* **13**, 1097-1107

Groscolas, R., Lacroix, A., and Robin, J. (2008) Spontaneous egg or chick abandonment in energy-depleted king penguins: A role for corticosterone and prolactin? *Hormones and Behavior* **53**, 51-60

Hammerstedt, R., Graham, J.K., and Nolan, J.P. (1990) Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* **11**(1), 73-88

Hammerstedt, R.H., and Graham, J.K. (1992) Cryopreservation of poultry sperm: The enigma of glycerol. *Cryobiology* **29**(1), 26-38

Harrell, W., and Bidwell, M. (2013) Report on Whooping Crane Recovery Activities (2012 breeding season-2013 spring migration). *United States Fish and Wildlife Service and Canadian Wildlife Service*

Harrell, W., and Bidwell, M. (2016) Report on Whooping Crane Recovery Activities (2015 Breeding Season - 2016 Spring Migration). <http://www.bringbackthecranes.org/technicaldatabase/recovery/pdf/WCRecoveryReportSeptToApril2016Appendices.pdf>.

Harris, J., and Mirande, C. (2013) A global overview of cranes: status, threats and conservation priorities. *Chinese Birds* **4**(3), 189-209

Holt, W., Pickard, A., Rodger, J., and Wildt, D. (Eds) (2003) 'Reproductive Science and Integrated Conservation.' (University Press: Cambridge)

Howard, J.G., Lynch, C., Santymire, R.M., Marinari, P.E., and Wildt, D.E. (2016) Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. *Animal Conservation* **19**(2), 102-111

Hughes, J.M. (2008) 'Cranes A natural History of a Bird in Crisis.' (Firefly Books: Buffalo, NY) 256

Iaffaldano, N., Di Iorio, M., and Rosato, M.P. (2012) The cryoprotectant used, its concentration, and the equilibration time are critical for the successful cryopreservation of rabbit sperm: dimethylacetamide versus dimethylsulfoxide. *Theriogenology* **78**(6), 1381-1389

IUCN (2016) Red List of Threatened Species.

Ivey, G.L. (2007) Factors influencing nest success of greater sandhill cranes at Malheur National Wildlife Refuge, Oregon. University of Oregon,

Johnson, D. H. (1999). The insignificance of statistical significance testing. *The journal of wildlife management* **63**(3), 763-772.

Jones, K.L., Glenn, T.C., Lacy, R.C., Pierce, J.R., Unruh, N., Mirande, C.M., and Chavez-Ramirez, F. (2002) Refining the Whooping Crane Studbook by Incorporating Microsatellite DNA and Leg-banding Analysis. *Conservation Biology* **16**(3), 789-799

Jones, K.L., and Nicolich, J.M. (2001) Artificial Insemination in Captive Whooping Cranes: Results From Genetic Analyses. *Zoo Biology* **20**(4), 331-342

Joyner, K.L. (1990) Avian Theriogenology. In 'Avian Medicine: Principles and Application.' (Eds. BW Ritchie, GJ Harrison and LR Harrison) pp. 53. (H B D International Inc.: Brentwood, TN)

- Kalliecharan, R., and Hall, B.K. (1974) A developmental study of the levels of progesterone, corticosterone, cortisol, and cortisone circulating in plasma of chick embryos. *General and comparative endocrinology* **24**(4), 364-372
- Kido, S., and Doi, Y. (1988) Separation and properties of the inner and outer layers of the vitelline membrane of hen's eggs. *Poultry Science* **67**(3), 476-486
- Koch, K.A., Wingfield, J.C., and Buntin, J.D. (2002) Glucocorticoids and parental hyperphagia in ring doves (*Streptopelia risoria*). *Hormones and Behavior* **41**(1), 9-21
- Kohen, R., and Nyska, A. (2002) Invited review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic pathology* **30**(6), 620-650
- Krause, M.R., and Regen, S.L. (2014) The structural role of cholesterol in cell membranes: from condensed bilayers to lipid rafts. *Accounts of chemical research* **47**(12), 3512-3521
- Kuyt, E., and Goossen, J. P. (1987). Survival, age composition, sex ratio, and age at first breeding of whooping cranes in Wood Buffalo National Park, Canada. In *Proceedings 1985 Crane Workshop* (pp. 230-244). Washington, DC: Platte River Whooping Crane Habitat Maintenance Trust, Grand Island, Nebraska, and US Fish and Wildlife Service.
- Kuyt, E. (1995) The nests and eggs of the whooping crane, *Grus americana*. *The Canadian Field-Naturalist* **109**, 1-5
- Kuyt, E. (1996) Reproductive manipulation in the whooping crane *Grus americana*. *Bird Conservation International* **6**, 3-10
- Lacy, R., Ballou, J., and Pollack, J. (2012) PMx: software package for demographic and genetic analysis and management of pedigreed populations. *Methods in Ecology and Evolution* **3**(2), 433-437
- Lague, P.C., Tienhoven, A.v., and Cunningham, F.J. (1975) Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chicken (*Gallus domesticus*). *Biology of Reproduction* **12**, 590-598
- Lake, P. (1983) Factors affecting the fertility level in poultry, with special reference to artificial insemination. *World's Poultry Science Journal* **39**(02), 106-117
- Landys, M.M., Piersma, T., Ramenofsky, M., and Wingfield, J.C. (2004) Role of the low-affinity glucocorticoid receptor in the regulation of behavior and energy metabolism in the migratory red knot *Calidris canutus islandica*. *Physiological and Biochemical Zoology* **77**(4), 658-668

- Leito, A., Bunce, R.G.H., Kllvik, M., Ojaste, I., Raet, J., Villoslada, M., Leivits, M., Kull, A., Kuusemets, V., and Kull, T. (2015) The potential impacts of changes in ecological networks, land use and climate on the Eurasian crane population in Estonia. *Landscape Ecology* **30**(5), 887-904
- Leska, A., and Dusza, L. (2007) Seasonal Changes in Hypothalamic-Pituitary-Gonadal Axis in Birds. *Reproductive Biology* **9**(2), 99-126
- Liu, H.K., and Bacon, W.L. (2005) Changes in egg production rate induced by progesterone injection in broiler breeder hens. *Poultry Science* **84**, 321-327
- Liu, H.K., Long, D.W., and Bacon, W.L. (2001) Concentration change patterns of luteinizing hormone and progesterone and distribution of hierarchal follicles in normal and arrested laying hens. *Poultry Science* **80**, 1509-1518
- Long, J. (2006) Avian semen cryopreservation: what are the biological challenges? *Poultry science* **85**(2), 232-236
- Mace, M., Lynch, W., Mirande, C.M., Songsasen, N., Durrant, B., Beall, F., Azua, J., and Schuman, A. (2013) Innovative Approach to Sustainable Management for Cranes The Role of Regional Breeding Centers. In 'AZA Connect. Vol. August.' pp. 12-15)
- Malecki, I., and Martin, G. (2003) Sperm supply and egg fertilization in the ostrich (*Struthio camelus*). *Reproduction in domestic animals* **38**(6), 429-435
- Mather, C.M., and Laughlin, K. (1979) Storage of hatching eggs: The interaction between parental age and early embryonic development. *British Poultry Science* **20**(6), 595-604
- Michael, G., and Pichner, J. (1989) The pink pheonix. *Minnesota Zoo Magazine* **Jan/Feb**, 6-7
- Minton, C.D.T. (1968) Pairing and breeding of mute swans. *Wildfowl* **19**, 41-60
- Morrow, C., Wolfe, B., Roth, T., Wildt, D., Bush, M., Blumer, E., Atkinson, M., and Monfort, S. (2000) Comparing ovulation synchronization protocols for artificial insemination in the scimitar-horned oryx (*Oryx dammah*). *Animal reproduction science* **59**(1), 71-86
- Mller, A., and De Lope, F. (1999) Senescence in a short-lived migratory bird: age-dependent morphology, migration, reproduction and parasitism. *Journal of Animal Ecology* **68**(1), 163-171
- Namgay, R., and Wangchuk, S. (2016) Habitat preferences and conservation threats to Black-necked Cranes wintering in Bhutan. *SpringerPlus* **5**(1), 1

- Nicolich, J.M., Gee, G.F., Ellis, D.H., and Hereford, S.G. (2001) Natural fertility in whooping cranes and Mississippi sandhill cranes at Patuxent Wildlife Research Center.
- Norris, D.O. (2006) 'Vertebrate Endocrinology.' 4 edn. (Elsevier Science: Oxford, United Kingdom)
- O'Brien, J.K., Oehler, D.A., Malowski, S.P., and Roth, T.L. (1999) Semen collection, characterization, and cryopreservation in a Magellanic penguin (*Spheniscus magellanicus*). *Zoo biology* **18**(3), 199-214
- Olsen, G.H., and Converse, S.J. (2017) Releasing Parent-reared Whooping Cranes in Wisconsin: A Pilot Study 2013-2015. North American Crane Workshop. (Ed. D Aborn): Chattanooga, TN) **14**
- Ottinger, M.A., and Baskett, M.R. (1995) Endocrinology of the Avian Reproductive System. *Journal of Avian Medicine and Surgery* **9**(4), 242-250
- Ouyang, J.Q., Sharp, P.J., Dawson, A., Quetting, M., and Hau, M. (2011) Hormone levels predict individual differences in reproductive success in a passerine bird. *Proceedings of Royal Society B* **278**, 2537-2545
- Packer, J.E., Slater, T., and Willson, R. (1979) Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* **278**, 737-738
- Panyaboriban, S., Pukazhenthii, B., Brown, M.E., Crowe, C., Lynch, W., Singh, R.P., Techakumphu, M., and Songsasen, N. (2016) Influence of cooling and thawing conditions and cryoprotectant concentration on frozen-thawed survival of white-naped crane (*Antigone vipio*) spermatozoa. *Cryobiology* **73**(2), 209-215
- Parks, J.E., and Graham, J.K. (1992) Effects of cryopreservation procedures on sperm membranes. *Theriogenology* **38**(2), 209-222
- Parks, J.E., and Lynch, D.V. (1992) Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology* **29**(2), 255-266
- Partyka, A., Bonarska-Kujawa, D., Sporniak, M., Strojceki, M., and Nizański, W. (2016) Modification of membrane cholesterol and its impact on frozen-thawed chicken sperm characteristics. *Zygote (Cambridge, England)*, 1-10
- Penfold, L.M., Harnal, V., Lynch, W., Bird, D., R, D.S., and E, W.D. (2001) Characterization of Northern pintail (*Anas acuta*) ejaculate and the effect of sperm preservation on fertility. *Journals of Reproduction and Fertility* **121**, 267-275

Penfold, L.M., Wildt, D.E., Herzog, T.L., Lynch, W., Ware, L., Derrickson, S.E., and Monfort, S.L. (2000) Seasonal patterns of LH, testosterone, and Semen quality in the Northern Pintail Duck (*Anas Acuta*). *Reproduction, Fertility, Development* **12**, 229-235

Peng, S.J.-L., Chang, F.-C., Sheng-Ting, J.I., and Fei, A.C.-Y. (2013) Welfare assessment of flight-restrained captive birds: effects of inhibition of locomotion. *The Thai Journal of Veterinary Medicine* **43**(2), 235

Polge, C., Smith, A.U., and Parkes, A. (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164**(4172), 666

Prado-Oviedo, N.A., Bonaparte-Saller, M.K., Malloy, E.J., Meehan, C.L., Mench, J.A., Carlstead, K., and Brown, J.L. (2016) Evaluation of demographics and social life events of Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*) in North American zoos. *PloS One* **11**(7), e0154750

Proudman, J., and Opel, H. (1981) Turkey Prolactin: Validation of a Radioimmunoassay and Measurement of Changes Associated with Broodiness. *Biology of Reproduction* **25**, 573-580

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013.

Rebke, M., Coulson, T., Becker, P.H., and Vaupel, J.W. (2010) Reproductive improvement and senescence in a long-lived bird. *Proceedings of the National Academy of Sciences* **107**(17), 7841-7846

Robertson, L., Brown, H.L., Staines, H.J., and Wishart, G.J. (1997) Characterization and application of an avian *in vitro* spermatozoa-egg interaction assay utilizing the inner perivitelline layer from laid chicken eggs. *Journal of Reproduction and Fertility* **110**, 205-211

Robinson, F., Hardin, R., Robinson, N., and Williams, B. (1991) The influence of egg sequence position on fertility, embryo viability, and embryo weight in broiler breeders. *Poultry Science* **70**(4), 760-765

Rybnik, P., Horbanczuk, J., Naranowicz, H., Lukaszewicz, E., and Malecki, I. (2007) Semen collection in the ostrich (*Struthio camelus*) using a dummy or a teaser female. *British poultry science* **48**(5), 635-643

Saint Jalme, M., Lecoq, R., Seigneurin, F., Blesbois, E., and Plouzeau, E. (2003) Cryopreservation of semen from endangered pheasants: the first step towards a cryobank for endangered avian species. *Theriogenology* **59**(3-4), 875-888

Scanes, C.G. (2014) 'Sturkie's avian physiology.' (Elsevier)

- Shaffner, C.S., Henderson, E., and Card, C. (1941) Viability of spermatozoa of the chicken under various environmental conditions. *Poultry Science* **20**(3), 259-265
- Siegel, H. (1980) Physiological stress in birds. *Bioscience* **30**(8), 529-534
- Silver, R., and Cooper, M. (1983) Avian behavioral endocrinology. *Bioscience* **33**(9), 567-572
- Smith, D.H., Converse, S.J., Gibson, K.W., Moehrensclager, A., Link, W.A., Olsen, G.H., and Maguire, K. (2011) Decision analysis for conservation breeding: maximizing production for reintroduction of whooping cranes. *The Journal of Wildlife Management* **75**(3), 501-508
- Sockman, K., and Schwabl, H. (1999) Daily estradiol and progesterone levels relative to laying and onset of incubation in canaries. *General and Comparative Endocrinology* **114**, 257-268
- Spalding, M.G., Folk, M.J., Nesbitt, S.A., Folk, M.L., and Kiltie, R. (2009) Environmental correlates of reproductive success for introduced resident whooping cranes in Florida. *Waterbirds* **32**(4), 538-547
- Stevens, E.F. (1991) Flamingo breeding: The role of group displays. *Zoo Biology* **10**, 53-63
- Stewart, S.G., Bausek, N., Wohlrab, F., Schneider, W.J., Horrocks, A.J., and Wishart, G.J. (2004) Species specificity in avian sperm: perivitelline interaction. *Comparative Biochemistry and Physiology Part A* **137**, 657-663
- Surai, P., Blesbois, E., Grasseau, I., Chalah, T., Brillard, J.-P., Wishart, G., Cerolini, S., and Sparks, N. (1998) Fatty acid composition, glutathione peroxidase and superoxide dismutase activity and total antioxidant activity of avian semen. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **120**(3), 527-533
- Surai, P., Fujihara, N., Speake, B., Brillard, J., Wishart, G., and Sparks, N. (2001) Polyunsaturated fatty acids, lipid peroxidation and antioxidant protection in avian semen. *ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES* **14**(7), 1024-1050
- Swanson, W., Stoops, M., Magarey, G., and Herrick, J. (2007) Sperm cryopreservation in endangered felids: developing linkage of in situ-ex situ populations. *Society of Reproduction and Fertility supplement* **65**, 417
- Timoney, K. (1999) The Habitat of Nesting Whooping Cranes. *Biological Conservation* **89**, 189-197

Tselutin, K., Seigneurin, F., and Blesbois, E. (1999) Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. *Poultry Science* **78**(4), 586-590

USGS-PWRC (2010) General crane care protocol index: 2010-2013. USGS Patuxent Wildlife Research Center Crane Program.

Watson, P.F. (2000) The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science* **60**, 481-492

White, J. (2000) Management of Captive Whooping Crane (*Grus americana*) to Improve Breeding Behavior and Success. University of Calgary, Calgary, Canada

Whitear, A., and Lacy, A. (2016). Egg fertility rate of the reintroduced eastern migratory whooping crane population 2005-2012. *Proceedings of the North American Crane Working Group* **13**, 138

Wielebnowski, N.C., Fletchall, N., Carlstead, K., Busso, J.M., and Brown, J.L. (2002) Noninvasive assesment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. *Zoo Biology* **21**, 77-98

Williams, T.D. (2012) 'Physiological adaptations for breeding in birds.' (Princeton University Press)

Wingfield, J.C., Ball, G.F., Dufty, A.M., Hegner, R.E., and Ramenofsky, M. (1987) Testosterone and aggression in birds. *Animal Science* **75**(602-608)

Wingfield, J.C., Smith, J.P., and Farner, D.S. (1982) Endocrine responses of white-crowned sparrows to environmental stress. *Condor* **84**, 399-409

Wishart, G. (1985) Quantitation of the fertilising ability of fresh compared with frozen and thawed fowl spermatozoa. *British poultry science* **26**(3), 375-380

Zimorski, S.E., and Jones, K.L. (2015) Whooping Crane Master Plan for 2015. Ed. WCR Team.