INFECTIOUS BURSAL DISEASE VIRUS IN WILD TURKEYS AND SANDHILL CRANES OF FLORIDA

By

KRISTEN L. CANDELORA

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INFECTIOUS BURSAL DISEASE VIRUS IN WILD TURKEYS AND SANDHILL CRANES OF FLORIDA

By

Kristen L. Candelora

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Captive-reared whooping cranes (*Grus americana)* released into Florida for the resident reintroduction project experienced unusually high mortality and morbidity during the 1997/98 and 2001/02 release seasons. Infectious bursal disease virus (IBDV) serotype 2 is currently under investigation as the factor that precipitated the mortality events. A small percentage of whooping cranes have been exposed to IBDV in the captive setting. However, many more are being exposed post-release, and prevalence of exposure increases with age or length of time the birds are in the wild in Florida. No studies have been published on the prevalence of IBDV in wild birds of North America. The goal of this study is to provide baseline data that can be used to create effective protocols and take measures to ensure that this virus does not impact the recovery of the endangered whooping crane.

To determine if wild exposure to IBDV serotype 2 is possible, captive sentinel chickens were monitored for exposure to the virus on whooping crane release sites in central Florida during the 2003/04 and 2004/05 release seasons. Wild exposure is possible as chickens on both sites became exposed to IBDV serotype 2. To examine the potential for exposure of whooping cranes from other wildlife reservoirs, blood samples were collected from wild turkeys (*Meleagris gallopavo*) and sandhill cranes (*Grus canadensis*) in 21 counties throughout Florida and 2

counties in southern Georgia. There is potential for whooping cranes, both resident and migratory, to become exposed to this virus through contact with wild birds as wild turkeys and sandhill cranes in 8 counties in Florida and 1 county in southern Georgia have been exposed to the virus. In addition, there is a significant age effect on seroprevalence in sandhill cranes. These findings are consistent with chicks having a shorter exposure time and immature immune system. The presence of higher seroprevalence and higher titers in older birds suggests that there is constant re-exposure or that birds remain carriers of the virus.

To investigate the original source and history of the virus in wild birds of Florida, archived serum samples collected from sandhill cranes were tested for antibodies to IBDV serotype 2. Although I was unable to demonstrate that sandhill cranes in Florida were exposed to IBDV prior to the introduction of captive-reared cranes, the high prevalence and wide distribution of the virus in both sandhill cranes and wild turkeys suggest that the virus has been in Florida for quite some time.

Many of the sites where blood was collected from wild turkeys and sandhill cranes overlap with the current distribution of whooping cranes in Florida. The presence of this virus in wild birds in these areas is especially concerning for the resident flock of whooping cranes because they nest and raise their chicks in Florida. The effect of exposure on whooping crane chicks is unknown at this time. However, the impact to young chickens suggests that if whooping crane chicks hatched in the wild are exposed to the virus at an early age, chick survival could be greatly reduced. Conversely, the relatively high titers maintained by adults may be passed on to the chicks and protect them at this otherwise vulnerable period in their lives.

CHAPTER 1
INTRODUCTION

The range of the whooping crane (*Grus americana*) once extended from central Canada south to Mexico, and from Utah to the Atlantic coast. In 1865, the estimated population size was 700-1,400 birds (U.S. Fish and Wildlife Service 1994). Habitat loss, unregulated hunting, and specimen collection had severe negative impacts, and by 1937 the population was reduced to a single non-migratory flock in southwestern Louisiana and a single migratory flock that wintered on the Gulf coast of Texas and nested in an unknown location (later discovered to be Wood Buffalo National Park in Canada). By 1950, the non-migratory flock had been extirpated and just 34 birds remained in the migratory flock (U.S. Fish and Wildlife Service 1994).

Congress passed the Endangered Species Preservation Act in 1966, and the whooping crane was listed as threatened with extinction in 1967. In that same year the Canadian Wildlife Service and the U.S. Fish and Wildlife Service began collecting eggs from birds in the Wood Buffalo flock, to generate a captive breeding flock of whooping cranes. The goal was to propagate whooping cranes, and reintroduce their offspring into the areas from which they were extirpated. Reintroduction of captive-reared whooping cranes began in 1993 when the Florida Fish and Wildlife Conservation Commission, in cooperation with several government and private agencies, released birds on the Kissimmee Prairie in central Florida with the goal of establishing a resident, non-migratory flock (U.S. Fish and Wildlife Service 1994).

Captive-reared whooping cranes released into Florida for the resident reintroduction project experienced unusually high mortality and morbidity during the 1997/98 and 2001/02 release seasons. Exposure to infectious bursal disease virus (IBDV) was documented, and may have been the precipitating factor for these mortality events (Spalding et al. 2006). The purpose of this study is to provide baseline data on the potential for exposure of whooping cranes to

IBDV from other wildlife reservoirs, so that effective protocols can be created and measures taken to ensure that this virus does not impact the recovery of the endangered whooping crane.

Mortality Events

In 1997/98, 14 of 22 captive-reared whooping cranes died within 6 months of their release. Another mortality event occurred during the 2001/02 release season. Seventy percent of birds released were affected. Of the 27 birds released 14 died, 5 others became sick and two of these had to be taken into permanent captivity. Birds from 4 different captive-rearing facilities in the United States and Canada were involved (Spalding et al 2006). A number of potential causes were considered (food, water, environmental toxins, herbicides, West Nile virus, coccidiosis, St. Louis and eastern equine encephalitis) and were ruled out (M. G. Spalding, University of Florida, unpublished data). Finally, because there was evidence of immunosuppression and the pattern of illness seemed to be restricted to young of the year, blood samples were tested for exposure to IBDV.

The pattern of birds testing positive matched well with those that were sick or died, suggesting that IBDV may have been the factor precipitating the mortality events (Spalding et al. 2006). As a result, an epidemiological study was initiated. This included testing archived blood samples from whooping cranes for exposure to IBDV, the initiation of a monitoring program for all captive-reared whooping cranes released into Florida and their offspring, and testing of whooping cranes in the captive flock. Results showed that some birds had been exposed to IBDV in the captive setting (Hartup and Sellers 2006). However, many more were exposed after their release into Florida and prevalence of exposure increased with age or length of time a bird was in the wild (Spalding et al. 2006).

Infectious bursal disease poses a significant threat to the successful recovery of the endangered whooping crane, and captive-reared whooping cranes released in Florida are being

exposed to the virus post-release. Very little is known about the incidence of IBDV outside of poultry operations, especially in North America. Therefore prevalence of the virus in wild birds, the etiology of the virus, and its transmission mechanism are unknown. The goals of this study are to investigate whether wild exposure is possible, how wild exposure may occur, and the origin of the virus in wild birds of Florida.

Epidemiology of Infectious Bursal Disease

Infectious bursal disease is an acute, highly contagious, viral disease that is common on poultry farms. The pathogenicity, resistance to disinfectants, and persistence in the environment make it one of the most important viruses of domestic poultry throughout the world (Lukert and Saif 2003). Infected chickens shed the virus in their feces, and can transmit the virus for at least 14 days (Ahad 2002). Water, feed, and droppings taken from infected pens in poultry houses were found to be infectious for 52 days (Benton et al. 1967).

There are two forms of this disease, clinical and subclinical. For chickens, the period of greatest susceptibility to clinical disease is between 3 and 6 weeks of age. The incubation period is very short, and clinical signs are seen within 2–3 days of exposure. Symptoms such as loss of appetite, dehydration, and diarrhea rapidly appear and the mortality rate can be as high as 30% (Lukert and Saif 2003). In chickens infected before 3 weeks of age, a severe, prolonged immunosuppression occurs (Lukert and Saif 2003). The suppression of their immune system is similar to the effect of HIV on humans, leaving the birds vulnerable to a variety of ailments that normally would not be life threatening (Allan et al. 1972). In this weakened state, the birds are also more susceptible to predation.

There are two serotypes of IBDV. Type 1 occurs in all major poultry producing areas worldwide, and can cause acute mortality/immunosuppression in chickens. Type 2 is widespread in poultry flocks in the United States. It is found in both chickens and turkeys, but is non-

pathogenic to these species (Lukert and Saif 2003). It is Type 2 that appears to have been involved in the whooping crane mortality event of 2001/02 (Spalding et al. 2006).

Anecdotal evidence suggests IBDV may be pathogenic to other avian species, but no study has directly linked exposure to clinical illness in any species other than chickens. Evidence of exposure to IBDV (serotype not identified) was found in Adelie penguin (*Pgyoscelis adeliae*) chicks following a mortality event where an infectious agent was suspected to be the cause. Clinical disease was not apparent at that time, but authors suggested that further investigation was warranted (Gardner et al. 1997). Populations of common eider (*Somateria mollissima*) in the Baltic Sea have declined significantly in some areas and juvenile mortality was reported to be the primary cause for this decline (Hollmen et al. 2000). Although authors reported no evidence of illness, exposure to IBDV serotype 1 was confirmed and exposure prevalence was highest for eiders nesting in areas where duckling survival was low (Hollmen et al. 2000). In addition, following a mortality event of African black-footed penguins (*Spheniscus demersus*) and macaroni penguins (*Eudyptes chrysolophus*) in a zoo in the United Kingdom, Jackwood et al. (2005) were able to isolate IBDV serotype 2 from birds involved. The role of this virus in the mortality event was unknown, and authors suggested that further study should be conducted to determine pathogenicity of IBDV in these species.

Impact of Disease on Population Recovery

Interactions between disease and population density play an important role in regulating animal populations, generally without threat of eliminating the population completely (Lafferty and Gerber 2002). This is especially true for large populations that occupy large geographic areas (Woodroffe 1999). As a population expands, animals are forced to live in closer proximity to one another and share resources with an increasing number of conspecifics. In some situations these circumstances lead to an increase in stress and a decrease in general condition, leaving

individuals more susceptible to disease while bringing them closer to animals from which they may contract it (Smith 2001). A decline in population density is often the result. At lower densities transmission of disease is usually reduced and diseases are spread more slowly, if at all (Lafferty and Gerber 2002). The disease's role in population regulation then becomes negligible.

The impact of disease on small populations can be very different, posing a serious threat to the persistence of such populations. If hosts are killed more quickly than they can breed, population growth rates decline (Woodruff 1999). This perpetuates the small population's vulnerability to extinction by stochastic events. One example comes from the long-term monitoring of bighorn sheep (*Ovis canadensis*). Berger (1990) found that small populations of 50 animals or less were more prone to extinction than populations numbering 100 animals or more. This is a concern for the Florida resident population of whooping cranes which now number approximately 60 individuals (Folk et al. 2006).

The threat of extinction caused by disease is also greater in small populations because they are more likely to have experienced inbreeding, and limited genetic diversity can lead to a decrease in disease resistance (Glenn et al. 1999). These populations could therefore experience greater mortality than a genetically diverse population, and disease has proven to be an issue in some species involved in reintroduction programs when there is a decline or reduction of genetic diversity in captive-bred animals (Glenn et al. 1999). In 1982 and 1983, 18 cheetahs (*Acinonyx jubatus*) in a captive breeding facility died from feline infectious peritonitis. It is unusual for this disease to cause such high mortality. O'Brien et al. (1985) suggested that mortality was so significant because the cheetahs lacked an effective immune response as a result of inbreeding. This concern also applies to whooping cranes because the population experienced a severe genetic bottleneck in the early 1940's when the migratory flock declined to just 15 or 16 birds

(U.S. Fish and Wildlife Service 1994). It is estimated that these remaining birds were highly interrelated, having an effective population size of only 1.2 birds (Jones et al. 2002). Because this was the founder population, all whooping cranes that exist today suffer reduced genetic diversity (Glenn et al. 1999).

In the conservation of small, already compromised populations such as the whooping crane, immunocompromising diseases such as IBDV could be especially problematic (Thorne and Williams 1988, Lafferty and Gerber 2002). Once infected with such a disease, host organisms become susceptible to secondary infection by common but normally benign disease organisms (Hollmen et al. 2000).

The potential for transmission of IBDV between domestic poultry and wild birds is an additional concern for the resident flock of whooping cranes in Florida as disease can be an issue in the recovery of species when the potential for transmission between domestic animals and endangered species exists. The African wild dog (*Lycaon pictus*) population has declined considerably in recent decades, and in 2002 was estimated at less than 5,500 individuals. Domestic canine diseases such as rabies and canine distemper, possibly transmitted by dogs from local villages, were some of the suggested causes of this decline (van de Bildt et al. 2002). According to Thorne and Williams (1988) canine distemper was also common in domestic dogs in Wyoming. In 1985, a drastic decline was noted in the last known wild population of black-footed ferrets (*Mustela nigripes*). To investigate the cause of the decline, ferrets were captured from widely separated locations. The fact that all died in captivity of canine distemper, indicated that the disease was widespread. The 18 known survivors of the disease outbreak were captured and placed in a captive breeding program. The free-ranging colony was essentially extirpated.

Objectives

I could find no published study confirming that wild birds in the United States have become exposed to IBDV. However, results from the epidemiological study by Spalding et al. (2006) indicated that some captive-reared whooping cranes had been exposed to IBDV following their release into Florida. Therefore, the first objective was to test the hypothesis that wild exposure to IBDV is possible without direct contact with potentially infected captive-reared whooping cranes.

To investigate how wild exposure may occur I tested the hypothesis that contact with wild birds is a potential exposure mechanism. For contact with wild birds to be a valid exposure mechanism, wild birds that whooping cranes share habitat with post-release must be positive for IBDV exposure. Therefore, the second objective was to determine if such species had been exposed to the virus and to assess the prevalence of exposure among those species.

The original source of IBDV in wild bird of Florida is unknown. There are many possibilities related to domestic poultry operations such as: transmission by people carrying the virus on contaminated footwear, inappropriate disposal of poultry products, and use of poultry litter as fertilizer in the agricultural industry. Another option is that captive-reared cranes were exposed to the virus in captivity and they introduced the virus to wild birds of Florida post-release. However, this possibility might be eliminated if it could be shown that wild birds in Florida had been exposed to the virus prior to the release of captive-reared cranes. Therefore, the third objective was to test the hypothesis that wild sandhill cranes (*Grus Canadensis*) in Florida were exposed to the virus prior to the release of captive-reared cranes, or in areas where contact with captive-reared cranes was highly unlikely.

CHAPTER 2
SENTIENEL CHICKENS

Introduction

Infectious bursal disease virus (IBDV) is a common poultry virus. It is well documented that birds in domestic operations are being exposed worldwide, and transmission mechanisms are fairly well understood in this setting (Lukert and Saif 2003). Although there are no published studies confirming that wild exposure to IBDV has occurred in North America, or demonstrating that transmission mechanisms exist, Spalding et al. (2006) found that captive-reared whooping cranes (*Grus americana*) had been exposed to IBDV following their release into Florida. To test the hypothesis that wild exposure in Florida is possible without direct contact with potentially infected whooping cranes, sentinel chickens confirmed to be free of disease were monitored for exposure to IBDV.

Materials and Methods

Six-week-old specific pathogen free (SPF) leghorn chickens were purchased from Charles River Laboratories, Inc. (251 Ballardvale Street Wilmington, MA 01887-1000). Blood samples were collected upon arrival to confirm the sentinel chickens had not previously been exposed to IBDV. Chickens were housed in 32x10x12 Tomahawk Raccoon/Feral Cat Live Traps (PO Box 323, Tomahawk, WI, 54487) and provided with fresh food and water daily.

Lake County Release Site

The first cohort of captive-reared whooping cranes for the 2003/04 release season arrived at the release site on December 8, 2003. The birds were brailed and placed in a portable pre-release pen. Brails were removed on December 21, 2003 and the birds were free to leave the pen (Figure 2-1). Three of these birds were positive for exposure to IBDV serotype 2 upon arrival at the release site. No birds seroconverted while in the pen. The second cohort arrived on

February 5, 2004 and brails were removed on February 18, 2004 (Figure 2-1). None of these cranes were positive for exposure to IBDV serotype 2 upon arrival. One bird seroconverted while in the pen (M. G. Spalding, University of Florida, unpublished data).

Eight SPF chickens were placed in cages when the first cohort arrived for release (December 8, 2003) and remained on the site through April 2004 (Figure 2-1). To increase the chance for viral exposure for another investigation, the chickens were separated into two groups of four and placed 1.07 km apart. One group of chickens was placed 1.47 km from the release site, along the edge of a small pine forest (Figure 2-2). This group of chickens was exposed to crane feces collected from the vicinity of the release pen. The second group of chickens was placed 0.51 km from the release site, along the edge of an oak hammock (Figure 2-2). This group was not exposed to crane feces. To minimize cross contamination when checking the sentinel chickens the protocol was to always visit the non-exposure group first.

Feces were collected from areas surrounding the feeders used by cranes after leaving the release pen. All were used by the current year's release birds, past years release birds, and a few wild sandhill cranes. For each collection attempt, I monitored the feeder being used by the most current year's captive-reared whooping cranes. I watched the birds, noting when and where one defecated. Feces were collected when cranes left the feeder area. When there were multiple feces in the vicinity, all were collected in an attempt to ensure collection of the target feces. Feces were placed in the cages of the exposure group when the chickens had been on the site for 43 days, 50 days, and 56 days (Figure 2-1). No feces were collected after the arrival of the second cohort of captive-reared whooping cranes.

To determine whether the likelihood of becoming exposed to IBDV was independent of exposure to crane feces, a likelihood ratio (G) test for independence was employed using the

statistical software JMP 7 (SAS Institute 2007). The test was 2-tailed and considered significant at $P \leq 0.05$.

Polk County Release Site

Five captive-reared whooping cranes arrived for release on December 8, 2004 and were debrailed on December 22, 2004 (Figure 2-3). None of the captive-reared cranes were positive for IBDV exposure upon arrival. One bird seroconverted while in the pen (M. G. Spalding, University of Florida, unpublished data).

Eight SPF chickens were placed in cages on December 11, 2004 and remained on the release site through May 2005 (Figure 2-3). All 8 chickens were placed in the same area and none were exposed to crane feces. The cages were placed in a small oak hammock surrounded by improved pasture.

Blood Collection and Analysis

Blood collection and analysis methods were the same for SPF chickens on both release sites. A blood sample was collected from each chicken approximately every 2 weeks. One to 2 mL of blood was collected from the medial metatarsal vein (Figure 2-4). A 27-gauge needle was used on young chickens, and a 25-gauge needle was used when they were full grown. After collection, blood was transferred into a lithium heparinized vacutainer. All samples were kept cold until they were spun down and the serum collected. The serum was frozen until it was sent to the Poultry Diagnostic & Research Center (College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, GA 30602) for evaluation.

All serum samples were tested for IBDV serotype 2 antibodies. Infectious bursal disease serotype 2 virus neutralizations, using the beta procedure (constant virus/diluted serum), were performed in primary chicken embryo fibroblast (CEF) cultures prepared from 9-11 day old SPF

chicken embryos. Sera were heat inactivated at 56˚C for 45 minutes, followed by centrifugation at 1200 x g for 10 minutes. Fifty microliters of serum was added to the first well of each row (rows A, B, C, D, E, F, G, and H) in a tissue culture-treated 96 well plate. Serotype 2 IBDV antigen was diluted to contain 100-500 Tissue Culture Infectious $Dose_{50}$/50µl.

Subsequently, 50 µL of diluted antigen was added to all wells (columns 1-11), except wells in column 12 (this served as a cell control). Serial dilutions were prepared by mixing the serum and antigen in column 1 and transferring 50 µL to column 2. Pipette tips were changed and contents in column 2 were mixed and 50 µL was transferred to column 3. This was repeated through column 10 where contents were mixed and 50 µL were discarded. Column 11 contained antigen only and was the virus control. Positive serotype 2 IBDV sera and negative sera were included in assay as controls. One hundred and ninety µL of CEFs were added to all wells. Cells were incubated at 37˚C for 5 days, cell culture media decanted, cells fixed with methanol for 1 minute and stained with crystal violet for 1 minute. Virus neutralizing titer was recorded as the reciprocal dilution of the last well exhibiting no cytopathic effect.

It is unknown what titer level indicates true exposure to IBDV. Previous studies of wild birds have considered titer levels ranging from 1:16 to 1:80 as evidence of exposure (Wilcox et al. 1983, Gardner et al. 1997, Ogawa et al. 1998, Hollmen et al. 2000). For the purposes of this study I assumed a titer level of 1:8 or less indicated no exposure to the virus. Birds with a titer level of 1:16 were considered possibly exposed. A titer level of 1:32 or greater was assumed to be indicative of exposure.

Bursal Fluid Aspiration

Seventeen bursal fluid samples were collected from SPF chickens on the Polk County release site in an attempt to isolate the IBDV serotype 2. The bursa of fabricius is a sac-like extension of the hindgut, located on the dorsal side of the cloaca. The chicken was placed on its

back, and a Kendall Monoject 16G x 1-1/2 aluminum hub blunt needle (tyco/Healthcare, Two Ludlow Park Dr., Chicopee, MA, 01022) was inserted into the vaginal opening of the vent, at a slightly downward angle. The needle was inserted until it dropped down into the bursa of fabricius (Figure 2-5). Fluid was aspirated and then injected into a sterile solution of phosphate buffer saline. The solution was frozen and sent to the lab for evaluation.

Results

Lake County: December 2003 through April 2004

Seroprevalence did not differ significantly between groups exposed and not exposed to crane feces ($P = 0.148$). Of the 4 chickens in the non-exposure group, three seroconverted and one was possibly exposed. Of the three chickens with titer levels high enough to indicate exposure, two had a titer level of 1:64 and one had a titer level of 1:256 indicating recent exposure. Two of these chickens seroconverted after being on the release site for 101 days. One chicken seroconverted after being on the release site for 133 days (Figure2-1).

Of the four chickens in the fecal exposure group, one became exposed to IBDV serotype 2, two were possibly exposed, and one was not exposed. The chicken with a titer level high enough to indicate viral exposure seroconverted after being on the release site for 101 days (Figure 2-1). The chicken that did not seroconvert is the bird that spent the least amount of time on the release site. It was found dead in its cage after having been on the release site for 79 days (Figure 2-1). Necropsy did not reveal cause of death, and no virus was isolated from its tissues.

Polk County: December 2004 through May 2005

Eight sentinel chickens were placed on the release site, but a raccoon killed one within the first 2 weeks. Data were analyzed on the 7 remaining chickens. Two chickens became exposed to the virus, one was possibly exposed, and four were not exposed. Of the two chickens

that became exposed, one seroconverted after being on the release site for 29 days, the other seroconverted after 66 days (Figure 2-1). One had a titer level of 1:256, indicating recent exposure.

The IBDV serotype 2 was not isolated from any bursal fluid samples. Comparison of titer level from blood collected on the same day as bursal fluid revealed that only one of the seventeen samples was collected from a bird having a titer level high enough to indicate exposure.

Discussion

Some sentinel chickens on both release sites seroconverted to IBDV serotype 2, supporting the hypothesis that wild transmission of IBDV in Florida is possible. The hypothesis is strengthened by the failure of exposure to potentially infected feces from whooping cranes to increase the seroconversion rate of the chickens. It is possible, but unlikely, that the chickens became infected directly from the captive-reared whooping cranes. Cranes may have visited the chicken cages, although this was never observed. The virus may have been carried by people when feeding or handling the chickens, although protocol dictated that chickens were visited before entering the release area to minimize this possibility.

The hypothesis is further supported by the fact that chickens on the Polk County release site became exposed to IBDV, yet none of the captive-reared whooping cranes released were positive for IBDV serotype 2 antibodies upon arrival at the release site. However, one of the captive-reared whooping cranes seroconverted while in the pen. Seroconversion could be the result of wild exposure or it is possible that the crane was exposed in captivity, but did not seroconvert until after it was put in the pen.

The finding that likelihood of exposure was not significantly affected by contact with potentially infected feces must be interpreted with caution for 2 reasons. First, the sample size

was small (n = 8). Second, it is possible that I did not collect any infected feces. Previous research indicates that chickens shed the virus in feces for 14 days (Ahad 2002). It is unknown if or how long whooping cranes may shed the virus in feces, but it is possible that exposed cranes were not shedding the virus at time of collection.

Regardless of whether or not chickens in the fecal exposure group were exposed to infected crane feces, some chickens in the non-exposure group did seroconvert. This suggests that the virus is present in the environment and available to infect susceptible hosts. Therefore, further research into wild exposure mechanisms with insects acting as the vector is warranted. A strain of IBDV (serotype not identified) was isolated from mosquitoes (*Aedes vexans*) trapped in southwestern Ontario in 1976 (Howie and Thorsen 1981). This species is found throughout the United States and utilizes a wide range of habitat types (O'Malley 1990). Whooping cranes are known to have contracted other mosquito born viruses such as Eastern Equine Encephalitis and West Nile virus (M. G. Spalding, University of Florida, unpublished data). Dung beetles may be another insect of interest as whooping cranes in Florida have become infected with a nematode that utilizes dung beetles as an intermediate host (Varela et al. 2001).

Another potential exposure mechanism is the use of poultry litter containing feces as fertilizer, a practice used regularly on the property adjoining the Lake County release site. Although the virus can be transmitted in chickens through contact with infected feces, research indicates that IBDV does not survive the Maryland Method of dead bird composting, otherwise known as two stage composting (Murphy 1990). Therefore the material itself if properly composted is probably not the source of infection. But the virus has been isolated from adult lesser mealworms (*Alphitobius diaperinus Panzer*) which commonly inhabit poultry houses, living in poultry droppings and litter (McAllister et al. 1995, Dunford and Kaufman 2006).

Mealworms could be transferred from the poultry house to the wild if litter infested with mealworms is spread on the fields, and presence of the lesser mealworm has been confirmed in numerous counties throughout Florida (Dunford and Kaufman 2006).

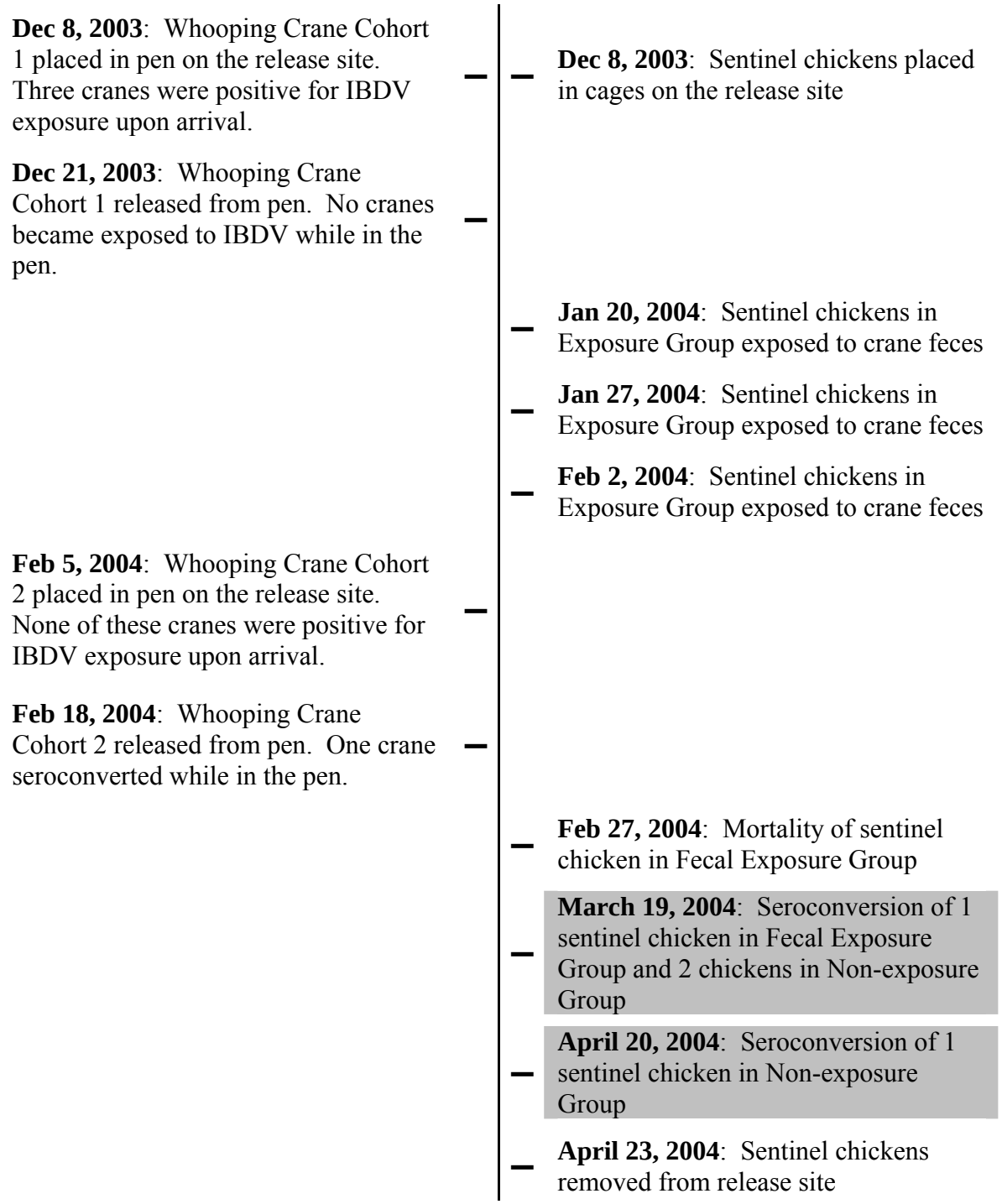

Figure 2-1. Timeline for Lake County release site.

Figure 2-2. Chicken trap locations on Lake County release site.

Dec 8, 2004: Whooping cranes placed in pen on release site. No cranes were positive for IBDV exposure upon arrival.

Dec 11, 2004: Sentinel chickens placed in cages on the release site

Dec 22, 2004: Whooping cranes released from pen. One crane seroconverted while in the pen.

Jan 9, 2005: Seroconversion of 1 sentinel chicken

Feb 15, 2005: Seroconversion of 1 sentinal chicken

May 3, 2005: Sentinel chickens removed from release site

Figure 2-3. Timeline for Polk County release site.

Figure 2-4. Collecting blood from a chicken via the medial metatarsal vein.

Figure 2-5. Aspirating bursal fluid from the bursa of fabricius.

CHAPTER 3
INFECTIOUS BURSAL DISEASE IN WILD BIRDS OF FLORIDA

Introduction

Infectious bursal disease virus has been well studied in commercial poultry operations, but very little is known about the prevalence or exposure mechanisms in wild birds (Lukert and Saif 2003). Although I could find no published literature on the incidence of IBDV in wild birds of North America, studies done in Antarctica, Australia, Crozet Archipelago in the Indian Ocean, Finland, Ireland, Japan, Nigeria, and Spain indicate that wild birds worldwide are being exposed to the virus (Nawathe et al. 1978, Wilcox et al. 1983, Gardner et al. 1997, Ogawa et al. 1998, Hollmen et al. 2000, Campbell 2001, Hofle et al. 2001, Gauthier-Clerc et al. 2002). Anecdotal evidence from these studies suggests that exposure to IBDV in wild birds of North America may be the result of spill-over, the transmission of contagious agents from reservoir animal populations (often domesticated species) to wildlife occupying the same area (Daszak and Cunningham 2000). Potential transmission mechanisms are the use of poultry farms, human activity, disposal of poultry products, the use of poultry litter as fertilizer, and contact with infected wild birds.

Use of Poultry Farms

The farm environment provides valuable habitat for wildlife, and wild birds in North America may become exposed to IBDV through their use of poultry farms. This includes both large commercial operations where wild birds may use drainage ponds and small farms with free-range poultry where direct contact with wild birds is possible. At the poultry farm of the National Veterinary Research Institute in Nigeria, evidence of exposure to IBDV (serotype not identified) was found in six of 50 wild birds captured on the farm. Chickens on the poultry farm housed in the commercial type setting and those kept as free ranging “back-yard” birds had been

exposed as well (serotype not identified). The authors did not investigate the source of exposure in these wild birds, but suggested that it could have been the domestic poultry (Nawathe et al. 1978).

When blood samples were collected from 11 species of wild water birds in Western Australia, evidence of exposure to IBDV (serotype not identified) was found in 7 species (Wilcox et al. 1983). Antibodies to the virus were most commonly detected in black ducks (*Anas superciliosa*) from the Perth area. The authors reported that farm ponds used to collect drainage from poultry sheds are common on commercial poultry farms in Perth, and that black ducks had been observed using these fresh water ponds.

Sera from king penguins (*Aptenodytes patagonicus*) on Possession Island of the Crozet Archipelago in the South Indian Ocean were examined for antibodies to IBDV serotypes 1 and 2. Chicks and adults had been exposed to both serotypes of the virus. For many years there was a poultry yard with domestic chickens and ducks in the scientific station on Possession Island. Although the authors did not know whether any of the domestic poultry had been exposed to IBDV, these domestic birds did have daily contact with wild birds (Gauthier-Cleric et al. 2002).

Human Activity and Disposal of Poultry Products

It has also been suggested that human activity and disposal of poultry products could be sources of exposure for wild birds. Gauthier-Cleric et al. (2002) reported that since the 1960s the beach of the king penguin colony where birds were found to have been exposed to IBDV, was the main landing point for people, equipment, and food destined for the research station. Although the authors did not investigate potential exposure mechanisms, the virus may have been introduced by human activity on the island or by sewage from the poultry yard that was discharged untreated into a field.

Disposal of poultry products is suspected as the source of exposure in common eiders (*Somateria mollissima*) and herring gulls (*Larus argentatus*) in two mixed species breeding colonies along the Finnish coast. The colony that was close to human development had significantly greater prevalence of exposure to IBDV serotype 1. Herring gulls from this colony were observed foraging at a nearby landfill. The authors proposed that gulls foraging at the landfill may have come in contact with the virus through waste from poultry farms, and then transmitted the virus when they returned to feed their young (Hollmen et al. 2000).

Human activity is suspected in exposure of two species of Antarctic penguins (*Aptenodytes forsteri* and *Pygoscelis adeliae*) to IBDV (serotype not identified) as evidence of exposure was found in colonies near centers of human activity, but none of the samples collected from penguins in a remote and rarely visited site had antibodies to the virus. Authors suggested that the virus may be spread by people on their footwear, clothing, equipment, or vehicles as they move around Antarctica. They also proposed that inappropriate disposal of imported poultry products may have been involved as wild birds could have become exposed when scavenging on waste and then transmitted the virus to other birds in the area (Gardner et al. 1997).

Use of Poultry Litter as Fertilizer

Poultry litter containing feces is used as fertilizer in the agricultural industry, and this practice may be involved in transmission of IBDV from domestic operations to the wild. The virus can be transmitted in chickens through contact with infected feces, but IBDV does not survive the Maryland Method of dead bird composting, otherwise known as two stage composting (Murphy 1990). Therefore, the material itself if properly composted is probably not the source of infection. But lesser mealworms (*Alphitobius diaperinus Panzer*) commonly inhabit poultry houses where they live in poultry droppings and litter, and the IBDV (serotype

not identified) has been isolated from adult lesser mealworms up to 14 days after exposure (McAllister et al. 1995, Dunford and Kaufman 2006). Mealworms could be transferred from the poultry house to the wild if litter and feces infested with mealworms is spread on fields as fertilizer (Dunford and Kaufman 2006). Presence of the lesser mealworm has been confirmed in Alachua, Broward, Charlotte, Clay, Dade, Hillsborough, Indian River, Manatee, Marion, Orange, Pasco, Pinellas, Polk, Putnam, and Volusia counties and probably occurs throughout the state of Florida (Dunford and Kaufman 2006).

Contact with Wild Birds

Wild birds for whom the virus is not pathogenic may act as reservoirs of the virus (Nawathe et al. 1978, Wilcox et al. 1983). To address this question, van den Berg et al. (2001) performed experimental infections of 3–6 week old commercial pheasants (*Phasianus colchicus*), grey partridges (*Perdix perdix*), Japanese quail (*Coturnix coturnix japonica*), and guinea fowl (*Numida meleagris*) using the very virulent strain of IBDV (serotype 1). These species were chosen because they are closely related evolutionarily to domestic fowl, and because they are commonly released for hunting or ornamental purposes.

None of these species exhibited clinical signs of illness. Guinea fowl were found to be fully refractory to infection. Some pheasants and partridges seroconverted, but none excreted the virus. Quail were susceptible to infection and shed the virus in feces for up to 7 days. Therefore, Japanese quail have the potential to act as a reservoir of the very virulent strain serotype 1 virus. However, results from this study do not support the findings of Weisman and Hitchner (1978) who found *Coturnix* quail (species not specified) to be refractory to IBDV, although they used the Classical Strain serotype 1 virus. Therefore *Coturnix* quail appear capable of transmitting and being a reservoir of at least one strain of IBDV.

Van den Berg et al. (2001) concluded that persistence of IBDV in wild bird populations is unlikely to occur and that the source of infection has to be found in poultry farms or the environment. Captive-reared whooping cranes released in Florida spend the majority of their time on farms and ranches. These range from large ranches that specialize in cattle and crops, to smaller farms where owners keep "back-yard" chickens. In addition, the use of litter from poultry operations as fertilizer is common on some farms. Therefore it is possible that whooping crane exposure may be a result of their use of the farm environment, or exposure to wild birds that act as reservoirs of the virus.

For contact with wild birds to be a potential exposure mechanism for whooping cranes, it first needs to be shown that wild birds utilizing the same habitat have been exposed to the virus. I tested the hypothesis that wild turkeys (*Meleagris gallopavo*), Florida sandhill cranes (*Grus canadensis pratensis*), and Florida bobwhite quail (*Colinus virginianus floridana*) have been exposed to IBDV. These 3 species were chosen based on likelihood of interaction (based on personal observations made during the 4 years I monitored whooping cranes), and previous research regarding susceptibility to IBDV in closely related domestic species

I found no previous research on susceptibility of sandhill cranes to IBDV. They were included because they are closely related to whooping cranes (both in genus *Grus*), and are the species I observed captive-reared whooping cranes interacting with most regularly after release. Wild turkeys were chosen because previous research indicates that domestic turkeys can be carriers of IBDV. When domestic turkeys are exposed to IBDV they respond serologically and are capable of transmitting the virus, but do not develop clinical disease (Giambrone et al. 1978, Weisman and Hitchner 1978, Jackwood et al. 1981, Barnes et al. 1982). If wild turkeys are also carriers, then they are likely candidates to be natural reservoirs of the virus. In addition, IBDV

serotype 2 has been detected in domestic turkeys in the United States (Jackwood et al. 1982, Chin et al. 1984). Although I observed minimal interaction between wild turkeys and whooping cranes, wild turkeys were observed foraging in the same pastures used by whooping cranes.

Florida bobwhite quail also share habitat with whooping cranes. Although I never observed direct interaction between these two species, both were observed using edge habitat in agricultural areas. No studies have been published on prevalence of IBDV in this species of quail. However, previous research indicates that *Coturnix* quail are capable of transmitting at least 1 strain of the virus, serotype 1 (van den Berg 2001). Although *Coturnix* quail are in a different family (Old World quail of the family Phasianidae) than bobwhite quail (New World Quail of the Family Odontophoridae), it is possible that they are capable of transmitting the virus as well. In addition, there is another mode of transmission of the virus from a domestic source into the wild in Florida as pen-raised bobwhite quail are often released for dog training and hunting (Wiley 2005).

Materials and Methods

Wild Turkey

To determine if populations of wild turkey had been exposed to IBDV, 596 blood samples were collected from wild turkeys at 24 locations in 21 counties throughout Florida (DeSoto, Gadsden, Glades, Hernando, Highlands, Holmes, Jefferson, Lake, Leon, Levy, Wakulla, Madison, Martin, Orange, Osceola, Palm Beach, Pasco, Polk, Putnam, Sumter, Wakulla) and 3 adjoining plantations in Thomas and Grady counties in southern Georgia (Figure 3-1).

Two methods were used to collect these samples. During the 2004, 2005, and 2006 spring turkey-hunting seasons, 434 blood samples were collected from hunter-harvested wild turkeys (Table 3-1). When hunters brought a bird to the check station, it was hung upside down

by the feet. An incision, 2.5–5.1 cm in length, was made in the right side of the neck. This would cut the jugular vein, releasing blood. A lithium heparinized vacutainer was held beneath the incision and as much blood as possible was collected. From December 2003 through January 2007, 162 blood samples were collected from live birds captured with rocket nets (Table 3-2). Between 1 and 2 mL of blood was collected from the medial metatarsal vein using a 25-gauge needle. After collection, blood was transferred into a lithium heparinized vacutainer.

All blood samples were kept cold until they were spun down and the serum collected. The serum was frozen until it was sent to the Poultry Diagnostic & Research Center (College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, GA 30602) for evaluation. All serum samples were tested for IBDV serotype 2 antibodies using the beta procedure (constant virus/diluted serum) described in Chapter 2.

It is unknown what titer level indicates true exposure to IBDV. Previous studies of wild birds have considered titer levels ranging from 1:16 to 1:80 as evidence of exposure (Wilcox et al. 1983, Gardner et al. 1997, Ogawa et al. 1998, Hollmen et al. 2000, van den Berg 2001). For the purposes of this study I assumed a titer level of 1:16 or less was not indicative of exposure to the virus. Birds with a titer level of 1:32 or greater were considered exposed to the virus.

The Kruskal-Wallis nonparametric ANOVA and Wilcoxon rank sum tests were used to determine if average titer level differed by location and year respectively. Post-hoc comparisons were performed using Wilcoxon rank sum tests. To determine if likelihood of exposure (# birds with titer level $\geq$ 1:32) was independent of location and year, a likelihood ratio (G) test for independence was used. Post-hoc comparisons were performed using the likelihood ratio test as well. All tests were 2-tailed and considered significant at $P \leq 0.05$. Analysis was performed using the statistical software JMP 7 (SAS Institute 2007).

Florida Bobwhite Quail

I hoped to obtain blood samples from harvested Florida bobwhite quail in a manner similar to that of harvested wild turkeys. However, due to the smaller size of bobwhite quail I was unable to get a sufficient serum sample by collecting blood in a heparinized vacutainer post mortem, spinning it down, and separating the serum. Therefore, I conducted a pilot study to test the feasibility of collecting blood with filter paper strips. This method allows for the determination of antibody titer level with a much smaller amount of blood. First, I investigated whether enough blood could be collected from dead quail to perform the analysis. Then I examined whether this method provided an accurate measure of antibody titer level for IBDV serotype 2.

To examine whether enough blood could be collected from harvested Florida bobwhite quail to perform the analysis, 11 samples were collected from birds harvested in November of 2004 on Babcock-Webb WMA in Charlotte County. Collectors were instructed to cut the jugular or brachial vein and saturate 100 diameter Nebuto blood filter strips (Advantec MFS, Inc., 6691 Owens Dr., Pleasanton, CA, 94588) with as much blood as possible. The goal was to completely saturate the filter paper strip with blood. Then the filter paper strip was placed in a small plastic bottle with a desiccant pack (Schleicher & Schuell BioScience, 10 Optical Ave., Keene, NH, 03431). Bottles with filter paper strips and desiccant were then sent to the lab for analysis.

To test the accuracy of the antibody titer level resulting from samples collected with filter paper strips, serum and filter paper samples were compared for 4 harvested turkeys and 12 live chickens. Blood was collected in a heparinized vacutainer. Then a filter paper strip was placed in the vial and saturated with blood. The remaining blood in the vial was spun down, and the serum separated. Each sample was analyzed for antibodies to IBDV serotype 2.

Florida Sandhill Crane

To determine if antibodies to IBDV serotype 2 were present in Florida sandhill cranes, 53 blood samples were collected in seven counties in central Florida: Hernando, Highlands, Lake, Orange, Osceola, Polk, and Sumter (Figure 3-2). Seven samples were opportunistically collected from adult sandhill cranes. Four samples were obtained from dead or injured birds (3 from Osceola County and 1 from Orange County), 2 were collected from nuisance birds (1 from Highlands County and 1 from Lake County), and 1 was acquired from an extremely tame bird that we were able to hand grab while capturing chicks at Moss Park in Orange County.

Forty-six samples were collected from pre-fledgling sandhill crane chicks captured during the 2004, 2005, and 2006 breeding seasons (3 from Hernando County, 7 from Polk County, 2 from Sumter County, 30 from Osceola County, and 4 from Orange County). Chicks ranged in age from approximately 25 to 65 days old.

Capture teams of 3 to 5 wildlife biologists drove through known crane-breeding areas looking for families. Chicks selected for capture were at least 2 weeks of age, and no older than approximately 70 days so they could not fly. If the chicks could be captured safely, the team drove as close as possible to the family and ran out and captured the chicks by hand.

Between 1 and 2 mL of blood were collected from the medial metatarsal vein using a 25-gauge needle. After collection blood was transferred to a lithium heparinized vacutainer. Pictures were taken of the chicks' head, wings, and body so that age estimate could be confirmed. Chicks judged to be at least 50 days old were banded with color bands and/or aluminum FWS bands. Average handling time was 15 minutes, with a range from 6 to 29 minutes. Handling time for each chick varied based on the number of chicks (single or twins), the ease with which I was able to get a blood sample, whether the chick(s) was old enough to be banded, and the capture team's level of experience. All blood samples collected from sandhill

cranes were handled in the same manner, and evaluated using the same criteria as was used for wild turkeys.

To determine if likelihood of exposure (# birds with titer level ≥ 1:32) was independent of age, a likelihood ratio (G) test for independence was used. To determine if average titer level differed by age, a Wilcoxon rank sum test was used. All tests were 2-tailed and considered significant at $P \leq 0.05$. Analysis was performed using the statistical software JMP 7 (SAS Institute 2007).

Results

Wild Turkey

Overall, 6% of wild turkeys were exposed to IBDV serotype 2 (n = 596, mean = 1:7, SE = 1, median = 1:2, range = 1:0 to 1:256, Figure 3-3). Evidence of exposure was found in 8 counties in Florida (Hernando, Highlands, Lake, Osceola, Pasco, Polk, Putnam, Sumter) and 1 county in southern Georgia (Grady).

Exposure prevalence (% samples with titer level ≥1:32) was 13% in 2003/04, 13% in 2004/05, 1% in 2005/06, and 0% in 2006/07 (Table 3-3). To investigate whether exposure prevalence and average titer level differed significantly between years, I analyzed the five sites where 10 or more samples were collected in multiple years: Caravelle Ranch WMA-2004 vs. 2005, Half Moon WMA-2004 vs. 2006, Richloam WMA-2004 vs. 2006, Three Lakes WMA-2005 vs. 2006, and Triple N Ranch WMA-2005 vs. 2006. Exposure prevalence differed significantly between years at all sites, and average titer level differed significantly between years at all sites except Richloam WMA (Table 3-4). Exposure prevalence and titer level decreased over time at all sites except Caravelle Ranch WMA where there was an increase (Figure 3-4).

Site specific exposure prevalence ranged from 0% to 30%, with 14 sites having no detectable evidence of exposure (Figure 3-1). Exposure prevalence ($P=0.0003$) and average titer level ($P<0.0001$) differed significantly between the sample sites. When performing individual comparisons I eliminated all sites where less than 10 samples were collected (Andrews WMA, Choctawhatchee River WMA, Tosohatchee WMA, and Upper Hillsborough WMA). The two sites with the highest exposure prevalence, Triple N Ranch WMA (30%) and Half Moon WMA (24%), did not differ significantly from each other but had significantly higher exposure prevalence than most other sites. Exposure prevalence on Triple N Ranch WMA was significantly greater than exposure prevalence on all sites except Brooksville (8%) and Lake Panasoffkee WMA (8%). Exposure prevalence on Half Moon WMA was significantly greater than exposure prevalence on all sites except South Georgia (5%), Brooksville, and Lake Panasoffkee WMA.

When locations were combined into regions (area with a 10.5 mile radius) there was still a significant difference in exposure prevalence ($P=0.0020$) and average titer level ($P<0.0001$). The two regions with the highest exposure prevalence were the one that included Half Moon WMA and the one that included Triple N Ranch WMA. Exposure prevalence decreased to 19% on Half Moon WMA and 9% on Triple N Ranch WMA. This caused the exposure prevalence of Half Moon WMA to become significantly higher than that of Triple N Ranch WMA. When locations were combined the number of sites that Half Moon WMA was significantly greater than was reduced from 18 to 14, and the number of sites that Triple N Ranch WMA was significantly greater than was reduced from 19 to 10. The only site that Triple N Ranch WMA did not differ from alone but differed from when combined by region was the site that was combined with Half Moon WMA.

Florida Bobwhite Quail

None of the 11 filter paper samples collected on Babcock-Webb WMA were completely saturated with blood. All were analyzed, and each had a titer level of 1:0. However, of the 16 serum and filter paper samples compared, none resulted in matching titer levels. For 6 birds the filter paper method overestimated antibody titer level, and for 10 birds it underestimated antibody titer level (Figure 3-7).

Florida Sandhill Crane

Overall, 7.5% of sandhill cranes were exposed to IBDV serotype 2 (n = 53, mean = 1:10, SE = 3, median = 1:4, range = 1:0 to 1:128,). Exposure prevalence (p=0.0025) and average titer level (p=0.0155) were significantly higher in adults than juveniles (Figure 3-6). Forty-three percent of adults were exposed to the virus (mean = 1:37, SE = 17, median = 1:16, range = 1:0 to 1:128). Two percent of juveniles were exposed to the virus (mean = 1:6, SE = 1, median = 1:3, range = 1:0 to 1:32).

Discussion

The hypothesis that wild turkeys and sandhill cranes in Florida have been exposed to IBDV serotype 2 was supported. Overall prevalence of exposure in wild turkeys remained constant during the 2003/04 and 2004/05 sampling years and then decreased thereafter, suggesting that viral occurrence may be cyclic in nature. This was supported by exposure prevalence decreasing significantly between years on all sites tested except Caravelle Ranch WMA where exposure prevalence increased. But this is the only site where both years used for comparison were during the sampling years (2003/04 and 2004/05) when overall exposure prevalence remained constant.

I was unable to assess site specific variation in exposure prevalence of sandhill cranes due to small samples sizes, but prevalence of exposure in wild turkeys did vary among sites. However, this result must be interpreted with caution as 12 of the 14 sites with no detectable evidence of exposure were sites sampled only in 2006, a year with extremely low exposure prevalence statewide. If viral occurrence is cyclic in nature then differences in site specific exposure may be the result of populations being in different phases of this cycle.

Adult sandhill cranes captured for this study had significantly higher seroprevalence and average titer level than juveniles. Although the adult sample size was small, this is consistent with findings of Spalding et al. (2006) that exposure prevalence in whooping cranes increases with age.

I was unable to assess the prevalence of exposure to IBDV serotype 2 in Florida bobwhite quail. Collecting a sufficient amount of blood for analysis from harvested birds proved difficult, and results from the filter paper comparative study demonstrated that the resulting titer levels were not accurate. Therefore the use of filter paper strips was not a reliable method to determine the antibody titer level for IBDV serotype 2 in Florida bobwhite quail.

Hunters involved in the pilot study on Babcock-Webb WMA were cooperative and exhibited interest in being involved in the investigation of disease prevalence in Florida bobwhite quail. Large numbers of quail are harvested each year and present an opportunity to investigate disease issues in this species. Alternative methods for collecting blood from harvested quail that could be used for disease studies should be explored. But it appears that future research investigating prevalence of IBDV would have to involve sacrificing quail specifically for the disease study or live trapping to collect blood samples.

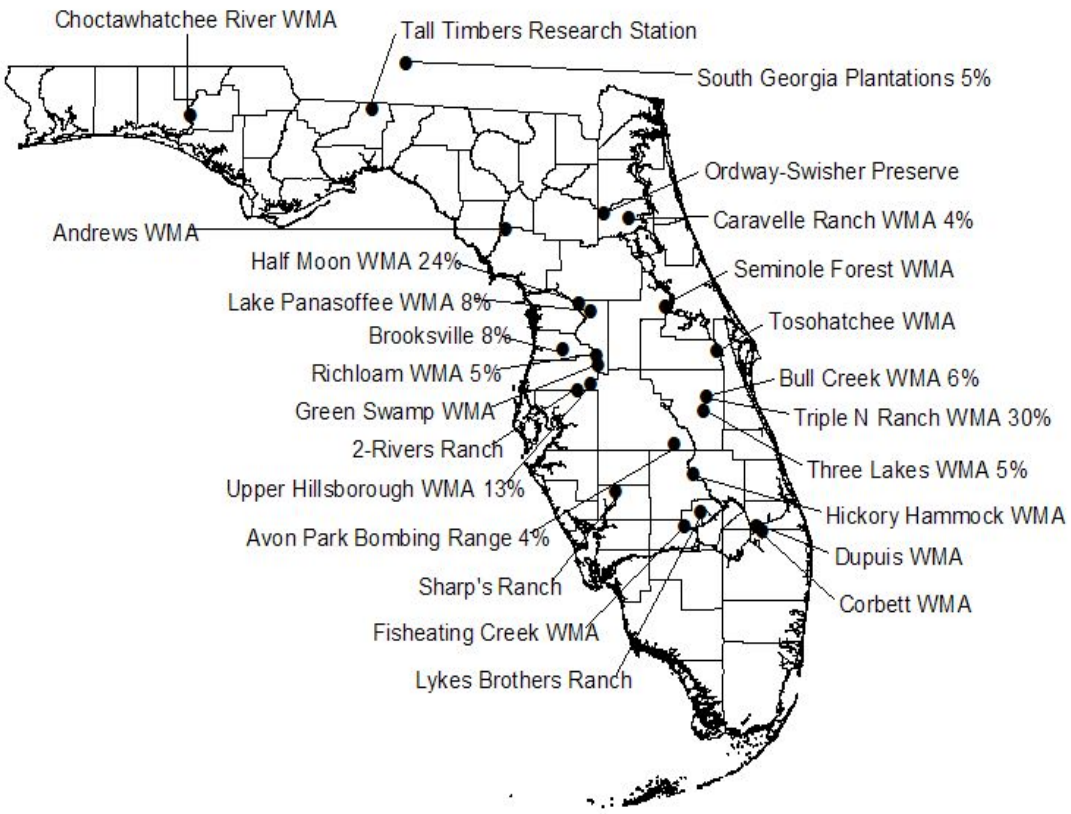

Figure 3-1. Blood collection sites for wild turkey samples collected from December 2003 through January 2007 (n=596). Exposure prevalence is listed beside each location. Locations with no percent listed had 0% exposure prevalence.

Table 3-1. Number of blood samples collected from harvested wild turkeys during the 2004, 2005, and 2006 spring turkey seasons.

Location	County	n
Andrews WMA	Levy	5
Avon Park Bombing Range	Polk, Highlands	25
Private properties near Brooksville	Hernando	13
Bull Creek WMA	Osceola	34
Choctawhatchee River WMA and private property	Holmes	8
Corbett WMA	Palm Beach	17
Dupuis WMA	Martin, Palm Beach	14
Fisheating Creek WMA	Glades	16
Green Swamp WMA	Polk, Sumter, Lake, Pasco	53
Half Moon WMA	Sumter	41

Table 3-1. Continued

Location	County	n
Hickory Hammock WMA and private property	Highlands	11
Lake Panasoffkee WMA	Sumter	13
Richloam WMA	Hernando, Pasco, Sumter, Lake	44
Seminole Forest WMA	Lake	16
Tall Timbers Research Station and private property	Madison, Wakulla, Leon, Gadsen, Jefferson	11
Three Lakes WMA	Osceola	74
Tosohatchee WMA	Orange	8
Triple N Ranch WMA	Osceola	23
Upper Hillsborough WMA	Polk, Pasco	8

Table 3-2. Number of blood samples collected from wild turkeys using rocket nets from December 2003 through January 2007.

Location	County	n
Caravelle Ranch WMA	Putnam	71
Ordway/Swisher Preserve	Putnam	21
Lykes Brothers Ranch	Glades	18
Sharp's Ranch	DeSoto	13
2-Rivers Ranch	Hillsborough	13
Three Lakes WMA	Osceola	7
Private Plantations, South Georgia	Thomas, Grady	19

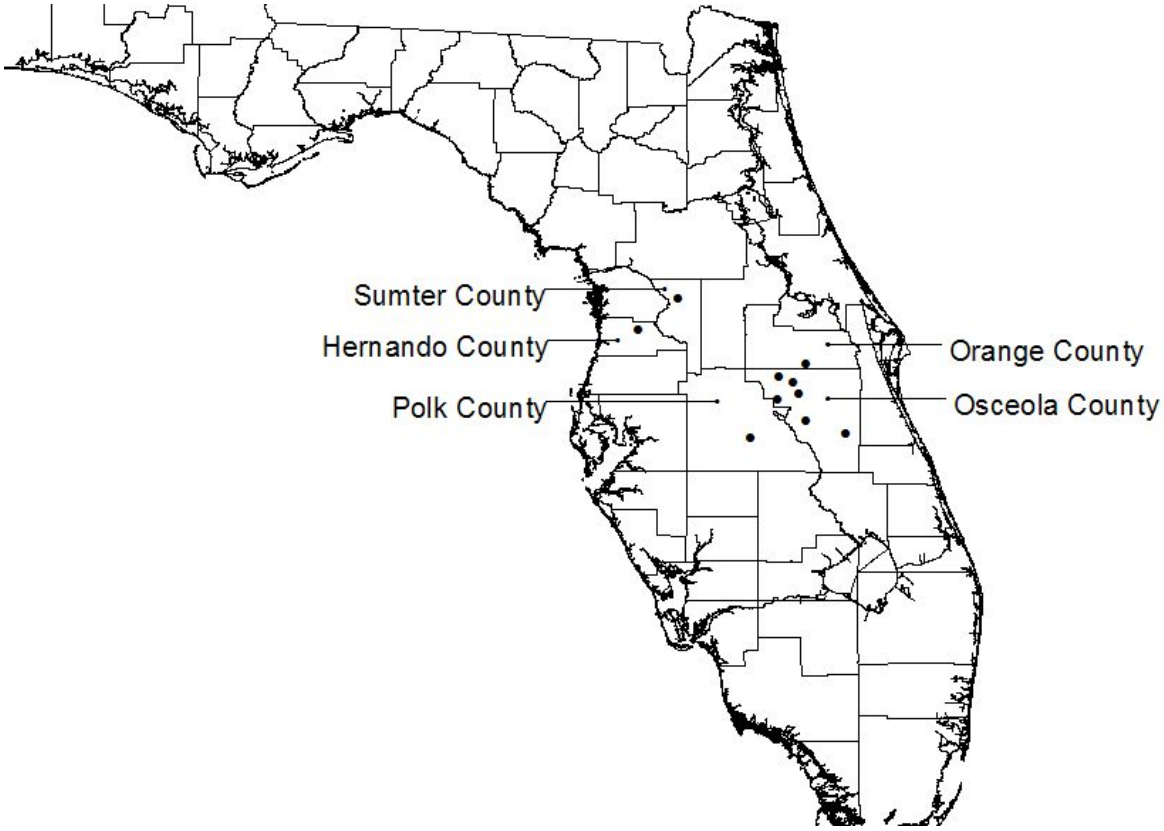

Figure 3-2. Blood collection sites for Florida sandhill crane samples collected during 2004, 2005, and 2006 (n=53).

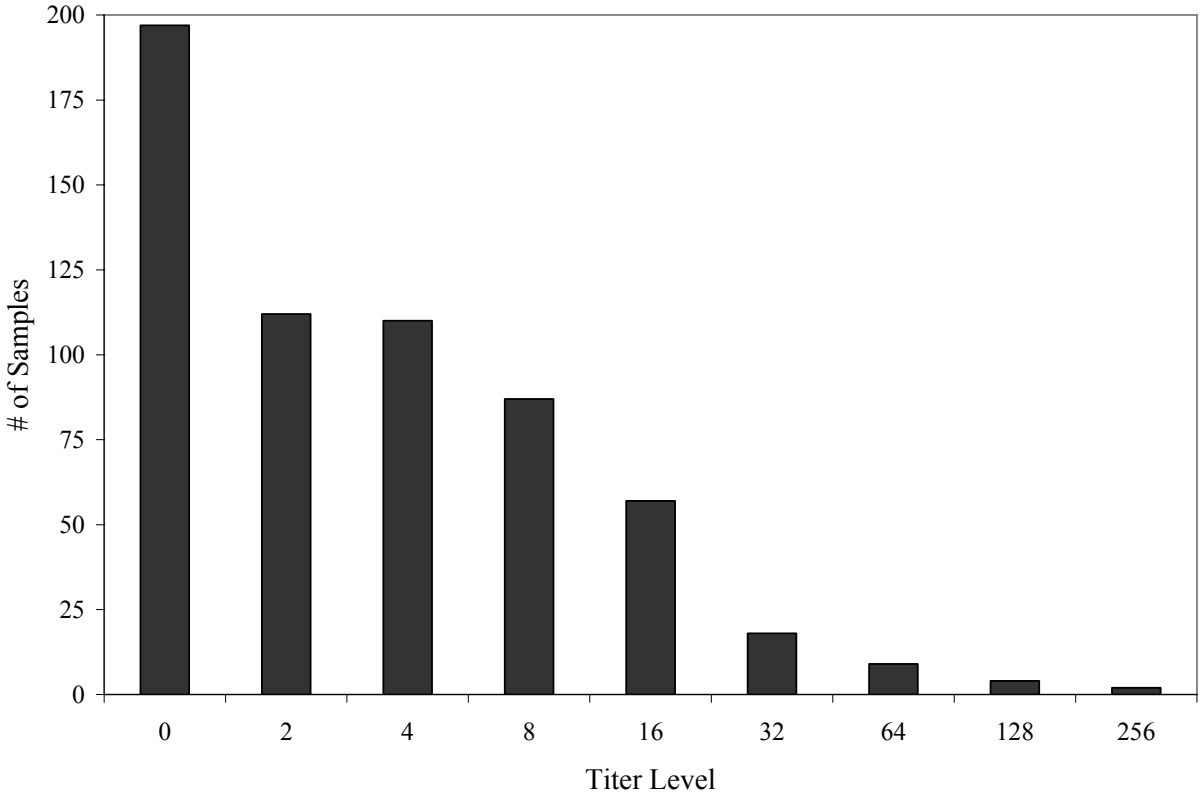

Figure 3-3. Frequency of titer levels for all wild turkey samples collected from December 2003 through January 2007 (n=596).

Table 3-3. Statistical results for yearly exposure prevalence in wild turkeys.

Year	n	Mean	SE	Median	Range
2003/04	95	1:14	4	1:02	1:0 to 1:256
2004/05	128	1:12	2	1:04	1:0 to 1:128
2005/06	353	1:05	0.3	1:02	1:0 to 1:32
2006/07	20	1:00	0.1	1:00	1:0 to 1:2

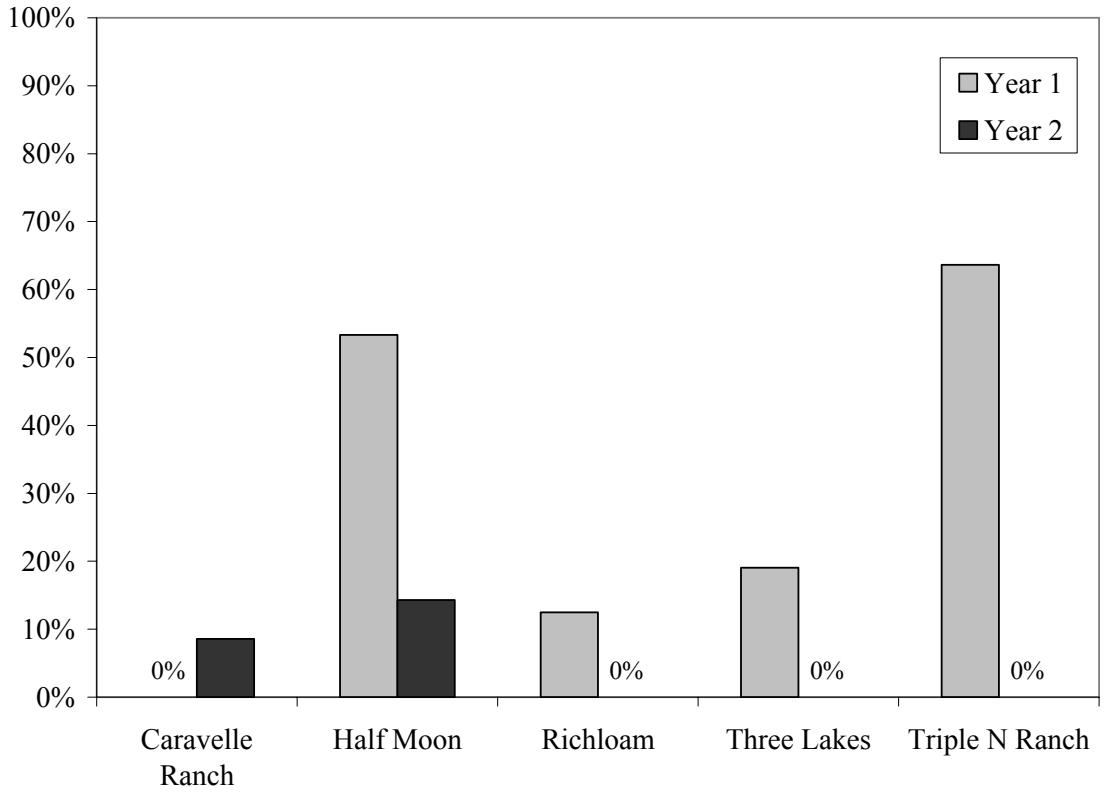

Figure 3-4. Wild turkey exposure prevalence (% birds with titer level ≥1:32) at five sample sites where 10 or more samples were collected in multiple years.

Table 3-4. P-values for difference in yearly exposure prevalence and average titer level in wild turkeys at sites where more than 10 samples were collected.

Location	n_1	n_2	Average Titer Level	Exposure Prevalence
Caravelle Ranch WMA	36 in 2004	35 in 2005	P<0.0001	P=0.0364
Half Moon WMA	15 in 2004	19 in 2005	P=0.0002	P=0.0011
Richloam WMA	16 in 2004	28 in 2006	NSD	P=0.0401
Three Lakes WMA	21 in 2005	53 in 2006	P=0.0362	P=0.0012
Triple N Ranch WMA	11 in 2005	12 in 2006	P=0.0080	P=0.0002

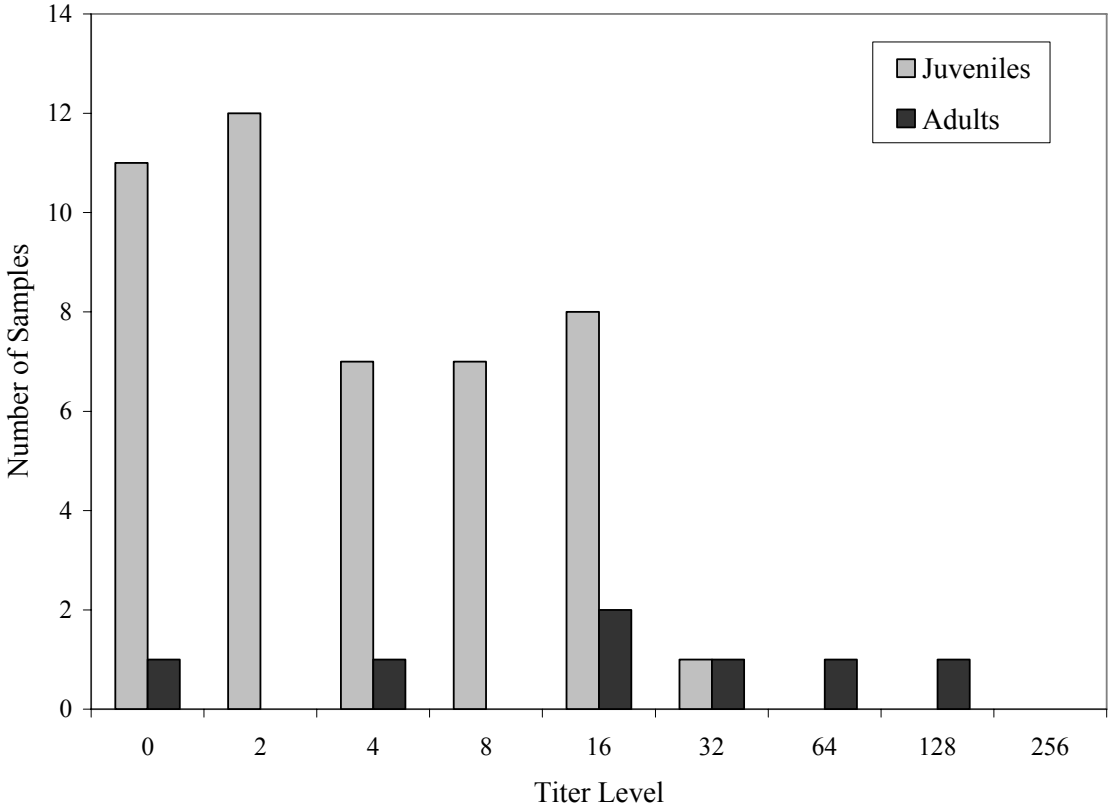

Figure 3-5. Frequency of titer levels for Florida sandhill cranes captured in 2004, 2005, and 2006 (n=53).

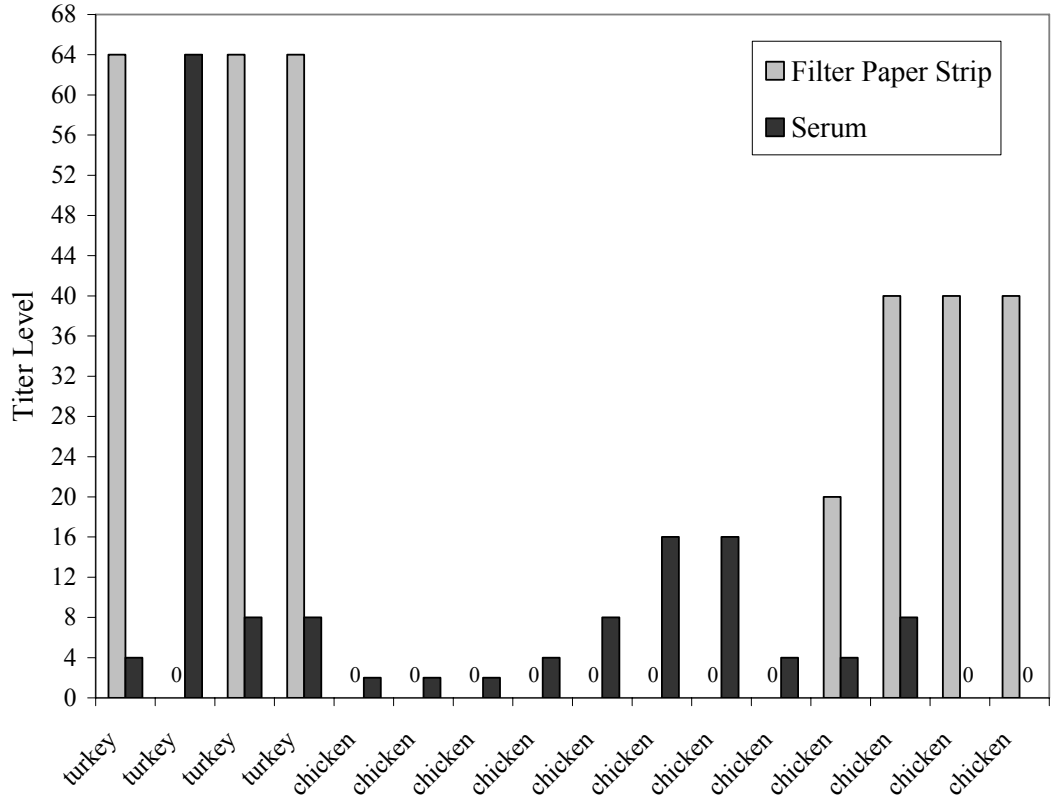

Figure 3-6. Titer level results for serum/filter paper comparisons.

CHAPTER 4
ARCHIVED SAMPLES

Introduction

The original source of IBDV serotype 2 in wild bird of Florida is unknown. There are many possibilities related to domestic poultry operations such as transmission of the virus by poultry workers on contaminated footwear, inappropriate disposal of poultry products, and the use of litter containing feces as fertilizer in agricultural operations. But once it was determined that some cranes in captive-rearing facilities had been exposed to IBDV, there was concern that captive-reared cranes may be responsible for introducing the virus to wild birds of Florida (Hartup and Sellers 2006). There have been instances when captive-bred animals exposed to a pathogen in the captive facility, exposed wild animals in and around the release site to those pathogens (Spalding et al.1996, Snyder et al.1996, Woodford and Rossiter 1994). This possibility could be eliminated if it was determined that wild birds in Florida had been exposed to the virus prior to the release of captive-reared cranes, or in areas where contact with captive-reared cranes was highly unlikely.

Sandhill cranes reared at Patuxent Wildlife Research Center in Laurel, Maryland were sporadically released into Florida from 1971 - 1991 as preliminary trials to develop release techniques for captive-reared whooping cranes. Fourteen 5-month-old captive-reared sandhill cranes were released in 1971 near Palmdale in Glades County (Figure 4-1). None of these birds were observed associating with wild sandhill cranes and all died within 3 months without leaving the immediate area (Nesbitt 1978). From 1974 - 1976, 4 captive-reared sandhill cranes ranging from 6 months to 4 years-of-age were released on Paynes Prairie in Alachua County (Figure 4-1). Three died and 1 paired with a wild sandhill crane and set up a territory on Paynes Prairie (Nesbitt 1978). Additional releases took place on Paynes Prairie from 1986 - 1987. Twenty-

seven 9 to 10-month-old captive-reared sandhill cranes were released. Survivors dispersed to Gilchrist, Levy, Marion, Putnam, and Sumter counties (Nesbitt and Carpenter 1993) (Figure 4-1). In 1991, 15 captive-reared sandhill cranes ranging from 1 to 2 years-of-age were placed in a holding pen on Kanapaha Prairie in Alachua County (Figure 4-1). The 11 surviving birds were then moved to a release pen on the Prairie Unit of Three Lakes WMA in Osceola County (Figure 4-1). Some experimental birds interacted with wild sandhill cranes, and two formed pairs with wild sandhill cranes (Nesbitt and Folk 1992). In 1993, the first captive-reared whooping cranes were released at Three Lakes WMA.

Materials and Methods

I was unable to locate any blood samples collected prior to 1971. As a result I could not test the hypothesis that wild birds in Florida were exposed to IBDV serotype 2 prior to the release of captive-reared cranes. Instead, I analyzed samples collected from wild birds in an area where contact with captive-reared cranes was highly unlikely, and hypothesized that these birds had been exposed to the virus.

From 1991 - 2000, Dr. Marilyn Spalding archived 477 wild sandhill crane serum samples collected in 7 counties (Alachua, Citrus, Lake, Levy, Marion, Osceola, Sumter) in Florida. Samples were eliminated from consideration if they were collected in a county after captive-reared cranes had been released there or were known to have dispersed there. Unfortunately, this eliminated from consideration all but 3 samples collected in Lake County in 1993 (Figure 4-1). These samples were tested for antibodies to IBDV serotype 2. The archived samples also included serum collected from captive-reared sandhill cranes prior to their release on Three Lakes WMA in 1991. Samples for 10 of the 11 birds released were tested for antibodies to IBDV serotype 2.

To gain further insight into prevalence of exposure and length of time the virus has been present in wild sandhill cranes of Florida, 108 archived serum samples collected in Alachua County (northern Florida) and Osceola County (central Florida) from May 1992 - March 1998 were tested for antibodies to IBDV serotype 2. These samples came from 98 individuals.

All serum samples were sent to the Poultry Diagnostic & Research Center (College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, GA 30602) for evaluation. They were tested for IBDV serotype 2 antibodies using the beta procedure (constant virus/diluted serum) described in Chapter 2.

It is unknown what titer level indicates true exposure to IBDV. Previous studies of wild birds have considered titer levels ranging from 1:16 to 1:80 as evidence of exposure (van den Berg 2001, Hollmen et al. 2000, Ogawa et al. 1998, Gardner et al., 1997, Wilcox et al. 1983). For the purposes of this study I assumed that a titer level of 1:16 or less was not indicative of exposure to the virus. Birds with a titer level of 1:32 or greater were considered exposed to the virus.

I detected an age effect on seroprevalence in the sandhill crane samples collected for this study (reported in Chapter 3); however, the adult sample size was small (n = 7). Analysis of the archived samples separated by age allowed for further investigation of this finding. Samples were separated into age categories. Juveniles ranged in age from 55 days to 10 months. Subadults ranged from 12 months to 2.7 years. Adults ranged from 3 to 10+ years. To determine if likelihood of exposure (# samples with titer level $\geq$ 1:32) was independent of age, the likelihood ratio (G) test for independence was used. Post-hoc analysis on each pair of treatments was done using the same test. To determine if average titer level differed by age, a

Kruskal-Wallis nonparametric ANOVA was used. Post-hoc analysis on each pair of treatments was done using the Wilcoxon rank sum test.

Exposure prevalence and average titer level of wild turkeys sampled in this study (reported in Chapter 3) differed significantly among locations. Analysis of archived samples allowed for further investigation of this result. To determine if mean titer level differed between sandhill cranes captured in Alachua and Osceola counties, a Wilcoxon rank sum test was used. To determine if likelihood of exposure (# samples with titer level $\geq$ 1:32) was independent of location, a likelihood ratio (G) test for independence was used. All tests were 2-tailed and considered significant at $P \leq 0.05$. Analysis was performed using the statistical software JMP 7 (SAS 2007).

Results

Of the three samples collected from wild sandhill cranes in Lake County in 1993, all birds were exposed to IBDV serotype 2. Titer levels ranged from 1:32 to 1:128. None of the samples collected from captive-reared sandhill cranes released on Three Lakes WMA in 1991 had titer levels high enough to indicate exposure. Titer levels ranged from 1:0 to 1:4.

Forty-six percent of samples collected from wild sandhill cranes in Alachua and Osceola counties had titer levels high enough to indicate exposure to IBDV serotype 2 (n = 108, median = 1:16, range = 1:0 to 1:1024, mean = 1:52, SE = 11). Sixty-three percent of adults, 56% of subadults, and 13% of juveniles had been exposed to the virus (Table 4-1, Figure 4-2). Juveniles had significantly lower exposure prevalence than both adults ($P<0.0001$) and subadults ($P<0.0002$), but there was not a significant difference between adults and subadults. Average titer level differed among all age groups ($P<0.0001$) with adults having significantly higher average titer levels than subadults ($P=0.0043$) and juveniles ($P<0.0001$), and subadults having significantly higher average titer levels than juveniles ($p=0.0001$).

Samples collected in Alachua County had significantly higher average titer level (P=0.0291) and exposure prevalence (P=0.0307) than those collected in Osceola County. In Alachua County, 54% of samples had titer levels high enough to indicate exposure, and earliest evidence of exposure came from samples collected on May 7, 1992. (Table 4-2, Figure 4-3). In Osceola County, 38% of birds had titer levels high enough to indicate exposure, and earliest evidence of exposure came from samples collected on October 1, 1992 (Table 4-2, Figure 4-3).

Discussion

All 3 sandhill crane samples collected in Lake County prior to the release/dispersal of captive-reared cranes to that area, had titer levels high enough to indicate exposure to IBDV serotype 2. Although this may be evidence that the release of captive-reared cranes was not the original source of the virus, I cannot discount the possibility that wild birds having contact with captive-reared cranes dispersed to the area. Therefore, I am unable to rule out the possibility that captive-reared cranes were the original source of the virus in wild birds of Florida. However, because 10 of the 11 captive-reared sandhill cranes released on Three Lakes WMA in 1991 did not have titer levels high enough to indicate exposure (1 crane was not tested), we can be fairly confident that this particular release was not the source of the virus in wild birds of Florida.

Significantly higher exposure prevalence and average titer levels were found in adult sandhill cranes when compared to juvenile cranes. Subadults were intermediate between adults and juveniles as there was no statistical difference in prevalence of exposure between adults and subadults, but there was a significant difference in mean titer level. This is because the majority of subadult samples indicating exposure (75%) had a titer level of 1:32, the lowest possible titer level to indicate exposure. There were very few samples at the higher end of the spectrum, with

no subadult birds having a titer level higher than 1:128. In contrast, 31% of adults had titer levels greater than 1:128 and only 19% had a titer level of 1:32.

The significant trend toward higher titer levels as the birds age suggests that there is constant re-exposure or that birds remain carriers of the virus. If sandhill cranes are re-exposed to the virus throughout their lifetime they could mount a more effective immune response with each subsequent exposure thus leading to higher titer levels as the birds age. Although unknown for IBDV in cranes, birds that are carriers of a virus could have a latent infection that results in intermittent shedding of the virus throughout their life. This could result in higher titer levels as the birds age as well.

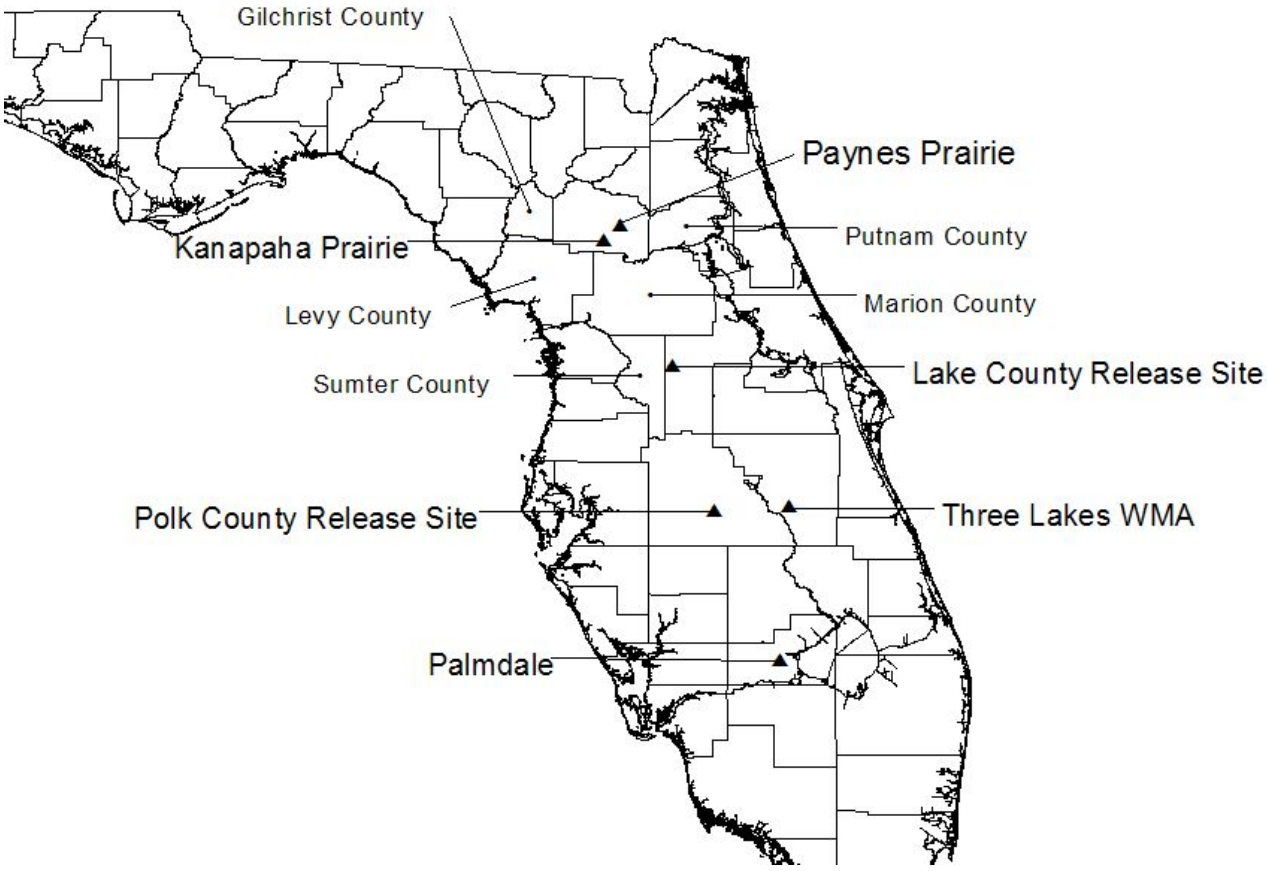

Figure 4-1. Captive-reared sandhill crane and whooping crane release sites (depicted with triangles) and dispersal areas (depicted with dots). Polk and Lake County release sites are locations where sentinel chickens were placed (Chapter 2).

Table 4-1. Statistical results for exposure prevalence by age of archived sandhill crane samples collected in Alachua and Osceola counties from May 1992 to March 1998.

Age	n	Mean	SE	Median	Range
Adult	41	1:104	27	1:64	1:4 to 1:1024
Subadult	36	1:28	4	1:32	1:2 to 1:128
Juvenile	31	1:12	3	1:04	1:0 to 1:64

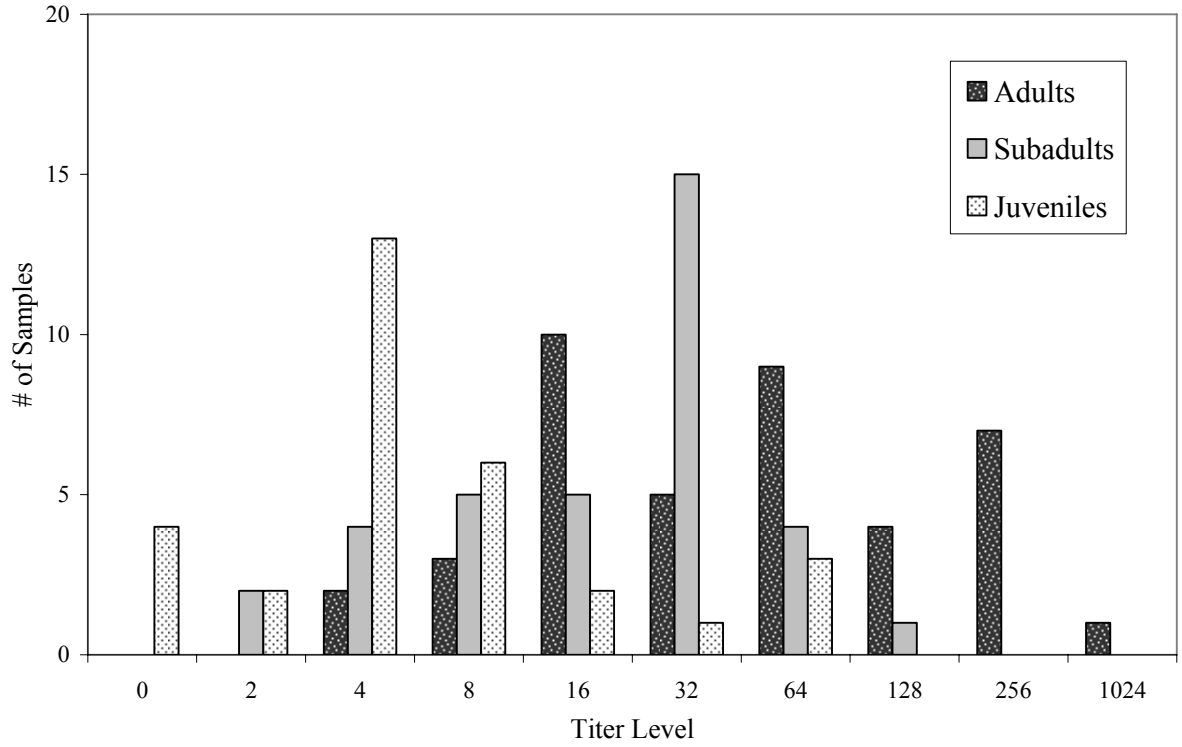

Figure 4-2. Frequency of titer levels for juvenile, subadult, and adult archived sandhill crane samples collected in Alachua and Osceola counties from May 1992 to March 1998 (n=108).

Table 4-2. Statistical results for exposure prevalence by county of archived sandhill crane samples collected in Alachua and Osceola counties from May 1992 to March 1998.

County	n	Mean	SD	Median	Range
Alachua	55	1:70	149	1:32	1:2 to 1:1024
Osceola	53	1:33	53	1:16	1:0 to 1:256

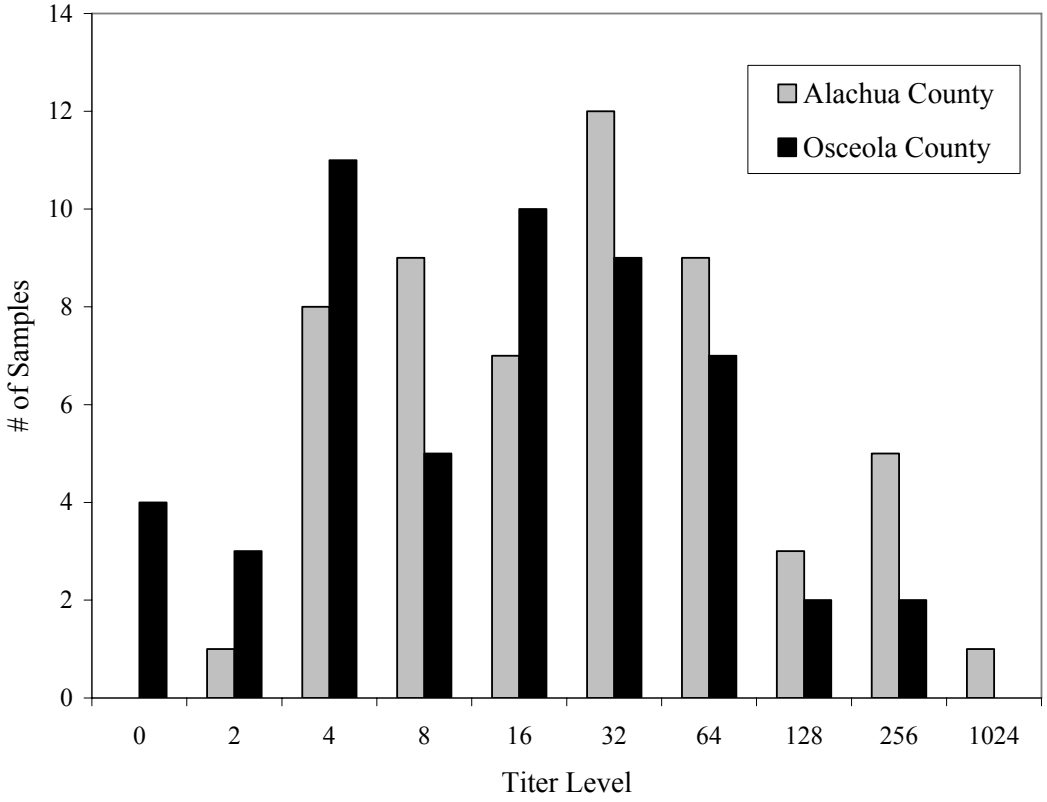

Figure 4-3. Frequency of titer levels for archived sandhill crane samples collected in Alachua and Osceola counties from May 1992 to March 1998 (n=108).

CHAPTER 5
SYNTHESIS AND SIGNIFICANCE

Wild turkeys and sandhill cranes throughout Florida have been exposed to IBDV serotype 2. The virus has been present in Florida for at least 15 years and is available to infect susceptible hosts. Because we know so little about the distribution of this virus in the environment and its mode of transmission, it is imperative that we conduct further research in order to learn what, if any, steps can be taken to minimize the effects of this virus on the survival of endangered whooping cranes. The presence of the virus in Florida could not be linked with certainty to the reintroduction project, but the evidence is consistent with the virus being present in the environment for a long time.

Implications for the Whooping Crane Reintroduction Project

Many of the wild turkey and sandhill crane blood collection sites overlap with areas where whooping cranes are currently found or have been found in the past. Therefore whooping cranes, both resident and migratory, could come in contact with wild turkeys and sandhill cranes that have been exposed to IBDV serotype 2. In addition, although these findings do not rule out other potential exposure mechanisms, they do suggest that post-release interaction with wild birds of Florida is one potential exposure mechanism for whooping cranes involved in the 1997/98 and 2001/02 mortality events.

The presence of this virus in wild turkeys and sandhill cranes of Florida is especially concerning for the resident flock of whooping cranes because they nest and raise their chicks in Florida. When chickens are exposed to IBDV between 3 and 6 weeks of age, symptoms rapidly appear and mortality rates can approach 30%. When chickens are exposed before 3 weeks of age a severe, prolonged immunosuppression results, leaving the birds vulnerable to normally benign disease agents (Lukert and Saif 2003). The effect of exposure to IBDV on prefledgling

whooping crane chicks is unknown at this time. However, the impact to young chickens suggests that if whooping crane chicks hatched in the wild are exposed to the virus at an early age, this could greatly reduce chick survival potential. Conversely, the relatively high titers maintained by adults may be passed on to the chicks and protect them at this otherwise vulnerable period in their lives.

Age Effect on Seroprevalence

Significantly higher exposure prevalence and average titer levels were found in adult sandhill cranes captured for this study and in archived samples, when compared to juvenile cranes. These findings are consistent with the findings of Spalding et al. (2006) that exposure prevalence in whooping cranes increased with age. The lower seroprevalence and titer levels in juveniles could be explained by juvenile cranes having a shorter exposure time and immature immune system. The higher seroprevalence and titer levels in older birds suggest that there is constant re-exposure or that birds remain carriers of the virus. However, there is also the possibility that this age effect on seroprevalence reflects decreased survival of sandhill cranes infected at a young age (Schettler et al. 2001, Garvin et al. 2004). The effect of exposure on sandhill crane chicks is unknown at this time. However, the impact to young chickens suggests that if sandhill crane chicks hatched in the wild are exposed to the virus at an early age, chick survival could be greatly reduced. If exposed chicks are less likely to survive then they are consequently less likely to be sampled, biasing the chick samples toward birds that have not been exposed to the virus. Therefore, investigation into the pathogenicity of IBDV in sandhill cranes is warranted.

Variation in Exposure Prevalence among Sites

Prevalence of exposure in wild turkeys and archived sandhill crane samples varied among sites. Future research should investigate exposure prevalence in relation to potential

sources of infection such as domestic poultry facilities and small family farms with free-ranging back yard poultry. However, it is important that the research project control for effect of year as viral occurrence may be cyclic in nature.

Transmission Mechanisms

There are 2 subspecies of the sandhill crane found in Florida. The Florida sandhill crane (*Grus canadensis pratensis*) is resident, and the greater sandhill crane (*Grus canadensis tabida*) is migratory. These 2 subspecies interact when greater sandhill cranes are in Florida during the winter. The role that greater sandhill cranes play in the epidemiology of IBDV in wild birds of Florida is unknown, and certainly warrants further investigation. Out of the 108 archived samples analyzed, 6 came from greater sandhill cranes. One of these birds had been exposed to the virus. Therefore, greater sandhill cranes may become exposed to the virus on their wintering grounds in Florida and carry the virus north with them. Or it is possible that greater sandhill cranes were exposed first, and are the original source of the virus in wild birds of Florida. Either way, this is a potential transmission mechanism for IBDV throughout the flyway.

The possibility that wild birds were exposed to the virus as a result of domestic poultry operations or disposal of poultry products has not been addressed in this study but certainly remains a potential source of exposure. The use of poultry litter containing feces as fertilizer in Florida's agricultural industry remains one potential mechanism for transmission of the virus from domestic operations to the wild. Farmers should be educated on the importance of properly composting chicken litter to eliminate the possibility of disease transfer. In addition, investigation into methods that minimize the transfer of mealworms within the litter could greatly reduce the potential for transmission from domestic operations to the wild. Research into potential insect vectors is needed as well, and should focus on mealworms, dung beetles, and mosquitoes.

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BIOGRAPHICAL SKETCH

Kristen Lee Candelora was born in Gainesville, FL in 1974. She grew up in Tampa, graduating from Chamberlain High School in 1992. Kristen earned a B.A. in psychology from the University of North Carolina at Wilmington in 1997. Following graduation, she returned to Tampa to work as a Crisis Counselor on a Baker Act Unit.

Kristen earned a B.S. in wildlife ecology and conservation from the University of Florida in 2002. Upon graduation, she began work as a Whooping Crane Biologist for the Florida Fish and Wildlife Conservation Commission (FWC). After working for FWC for two years, Kristen entered the Wildlife Ecology and Conservation M.S. program at the University of Florida. She continued working for FWC while completing her master's project. She received the Best Student Paper award at the 10th North American Crane Workshop in Zacatecas, Mexico and the Florida Chapter of The Wildlife Society spring meeting in Cocoa Beach, Florida.

Upon completion of her M.S. program, Kristen began work as the Private Lands Coordinator for the Upland Ecosystem Restoration Project.