DISEASE RISKS TO WHOOPING CRANES (*Grus americana*) DETERMINED BY NON-INVASIVE SAMPLING AND USE OF THE SANDHILL CRANE (*Grus canadensis*) AS A SURROGATE

A Dissertation

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ABSTRACT

The only self-sustaining wild population of endangered whooping cranes (*Grus americana*) has grown to approximately 308 individuals. However, the population growth is not consistent with species recovery goals, and the impact of parasite infection on whooping crane populations is largely unknown. Our goal was to quantify the prevalence of fecal parasites and hemoparasites in whooping cranes and to compare the prevalence of infection between whooping and sandhill cranes (*Grus canadensis*). We assessed the prevalence and phenology of *Eimeria* oocysts in whooping crane fecal samples collected across two winter seasons (November 2012 – April 2014) at the Aransas National Wildlife Refuge along the Texas Gulf coast. Across both years, 26.5% (n=328) of fecal samples were positive for *Eimeria* based on microscopy. We noted nematode eggs in 30% (n=327) and 2.7% (n=75) of whooping and sandhill crane fecal samples, respectively. However, sequences from these samples aligned with soil-dwelling nematodes, indicating environmental contamination. We noted trematode eggs in 11.1% (n=63) and 50% (n=20) of whooping and sandhill crane samples, respectively. We identified three species of trematode, one cestode, one acanthocephalan, and one nematode in sandhill cranes on necropsy. *Orchipedum jolliei* was the most common trematode and was noted in 42% (n=108) of sandhill cranes. The prevalence of *O. jolliei* was significantly higher in sandhill cranes wintering along the Texas Gulf Coast than in the Texas panhandle or New Mexico. We used three different PCR assays to screen samples for Haemosporida and detected an infection prevalence of 59.5% (n=163) across all birds. Infection prevalence was high in whooping cranes and sympatric sandhill cranes, but significantly lower in allopatric sandhill cranes. *Haemoproteus antigonis* was present in 46% of samples from both crane species and was phylogenetically distinct from other avian Haemosporida. We demonstrate that non-invasive fecal collections combined with PCR and DNA sequencing techniques provides a useful tool for monitoring coccidia and helminth infection in cranes. We also document a high prevalence of *H. antigonis* in whooping cranes and sympatric sandhill

cranes, supporting the use of sandhill cranes as a surrogate species for understanding health threats to the endangered whooping crane.

DEDICATION

For my parents, who have always encouraged and supported me; and for Pete, whose smiling eyes and wagging tail say I can do anything.

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CHAPTER I
INTRODUCTION

The whooping crane (*Grus americana*) and the sandhill crane (*Grus canadensis*) are the only crane species in North America, and wild populations occur exclusively in North America. Though some subpopulations of the sandhill crane were historically persecuted and suffered great population declines, the populations today in general are strong and not of conservation concern. In contrast, the whooping crane experienced a severe population decline in the early 20th century and has been listed as endangered since 1967. The species has rebounded from a low of 15 individuals in 1941 to a total of 451 wild and reintroduced birds, and 157 captive birds in 2013[1]. The Aransas-Wood Buffalo population (AWBP), which nests in Wood Buffalo National Park, Alberta and Northwest Territories, Canada and winters among coastal marshes at the Aransas National Wildlife Refuge in Texas, USA, is the only self-sustaining wild population of whooping cranes. Population projections, however, indicate that whooping cranes may not achieve the down-listing criterion of a reaching a population size of 1000 individuals[2] until the mid-2060s[3], and the species therefore remains endangered and is highly susceptible to stochastic events that could decimate the population.

Surprisingly little is known about the diversity of diseases that affect the wild whooping cranes, and almost nothing is known about their epidemiology[2]. This is due in part to the difficulty of establishing surveillance and monitoring programs given that these birds are sensitive to disturbance, and invasive sampling is not desirable. A variety of infectious diseases have been reported in captive and reintroduced whooping cranes[4,5], however a 1978 publication documenting coccidia infection in one-third of sampled cranes is the only published report concerning parasites infecting the AWBP whooping cranes[6]. Coccidian parasites in the genus *Eimeria* are the most commonly reported infection in cranes. Unlike the common poultry-associated *Eimeria* species that cause localized infections and variable enteric disease, *E. gruis* and *E. reichenowi* can spread systemically in cranes, causing disseminated visceral coccidiosis (DVC)[7,8], which

can be fatal, especially in young chicks[7]. Clinical signs of DVC depend on the tissues affected and the severity of infection, and can include enteritis, hepatitis, bronchopneumonia, myocarditis, and splenitis. Oocysts develop in the intestine or respiratory tract and are shed in the feces[9]. DVC is an important cause of crane chick mortality in captivity[8,10-12], and has also been described in captive adult cranes[13]. Whooping crane chicks have high mortality (27% - 68%) during the first 20 days after hatching[14-16], and the role of DVC as a cause of wild chick mortality is poorly understood[7]. Additionally, infection with these *Eimeria* species may make surviving birds more susceptible to other disease or predation[8].

In addition to coccidia, a variety of helminths have been reported in sandhill cranes and captive whooping cranes[17,18], however no studies have been published concerning helminth parasites of the AWBP whooping cranes. While helminths may not directly cause clinical disease, they can impact the immune function of their host rendering it more susceptible to other infections. In particular, there is a polarized immune response to extracellular (helminth) versus intracellular (viruses and bacteria) parasites in which the up-regulation of the immune response to an extracellular parasite results in the downregulation of the immune response to subsequent microparasite challenges, leaving the animal more susceptible to attack by the latter[19]. The prevalence of helminth infection and the role of helminths as risk factors for other diseases in whooping cranes warrant further evaluation.

There is also increasing evidence that biting dipteran flies are a nuisance to whooping cranes on their breeding grounds. Swarms of black flies are contributing to nest abandonment and chick mortality in the reintroduced population of whooping cranes in Wisconsin (bringbackthecranes.org/whatwedo/PDF/wcep13.pdf). Although no studies have been done to determine if these vectors also pose a risk to the AWBP whooping cranes in Canada, it is logical to suspect that they do, given the known distributions of black fly and mosquito species[20]. Whooping cranes are likely exposed to the parasites transmitted by these flies, which include at least four genera of hemoparasites (blood parasites). The only published information about hemoparasites in

whooping cranes is a citation for unpublished data in which *Haemoproteus antigonis* was detected in a small number of whooping cranes in Florida[21]. No studies have been conducted to describe the hemoparasites in the AWBP whooping cranes. Two species of *Haemoproteus,* one species of *Plasmodium* and one species of *Leucocytozoon* have been reported in sandhill cranes[21].

Hemoparasites can have a broad range of negative effects on host birds. For example, *Plasmodium* infection in naïve populations has played a key role in severe declines of populations of Hawaiian honeycreepers[22]. At the other end of the spectrum, *Plasmodium* infection causes no detectable detrimental effects in some endemic populations of Dutch house martins[23], but has been associated with decreased reproductive success in other populations[24]. The majority of hemoparasite infections reported in sandhill cranes do not cause significant disease, however severe anemia has been associated with *Haemoproteus balearicae* infection in two sandhill crane chicks[21]. The significance of hemoparasite infections in terms of host health is likely to vary based on physiological and immunological factors that operate at the individual host animal level, as well as ecological factors. Prior to individual level studies, baseline epidemiological investigations must be conducted to learn if these parasites are relevant at the population level.

Knowledge of the parasites carried by whooping cranes, and the ways in which they may be hindering the growth of the population, is prerequisite for achieving the management goals for this species. For example, the use of managed freshwater ponds and supplemental feeding sites on the wintering grounds has the potential to encourage the spread of parasites and pathogens due to aggregation of birds around a common source. The purpose of this study was to explore the disease threats to whooping cranes using a two-fold approach. First, a direct approach through non-invasive sampling of feces, and secondly, an indirect approach through the use of sandhill cranes as a surrogate. The surrogate species approach is useful when sample sizes are limited in an endangered species. A closely related abundant species can be used a model for the endangered species. For example, the bobwhite quail (*Colinus virginianus*) has been

used as a surrogate for the Attwater's prairie chicken (*Tympanuchus cupido attwateri*)[25]. The sandhill crane is an appropriate surrogate for the whooping crane because it is the closest relative to the whooping crane in North America and some populations are sympatric with whooping cranes. Additionally, sandhill cranes are actively hunted throughout the wintering range, providing access to large sample sizes. The objectives of this project were to characterize the species richness, infection prevalence, and genetic diversity of enteric parasites and hemoparasites in whooping cranes and three wintering populations of sandhill cranes and to determine the associations between parasite infection and gross and histologic lesions in three populations of sandhill cranes as a model for understanding the pathology in whooping cranes.

CHAPTER II

COCCIDIAN PARASITES AND CONSERVATION IMPLICATIONS FOR THE ENDANGERED WHOOPING CRANE (*Grus americana*)*

Overview

While the population of endangered whooping cranes (*Grus americana*) has grown from 15 individuals in 1941 to an estimated 304 birds today, the population growth is not sufficient to support a down-listing of the species to threatened status. The degree to which disease may be limiting the population growth of whooping cranes is unknown. One disease of potential concern is caused by two crane-associated *Eimeria* species: *Eimeria gruis* and *E. reichenowi*. Unlike most species of *Eimeria*, which are localized to the intestinal tract, these crane-associated species may multiply systemically and cause a potentially fatal disease. Using a non-invasive sampling approach, we assessed the prevalence and phenology of *Eimeria* oocysts in whooping crane fecal samples collected across two winter seasons (November 2012 – April 2014) at the Aransas National Wildlife Refuge along the Texas Gulf coast. We also compared the ability of microscopy and PCR to detect *Eimeria* in fecal samples. Across both years, 26.5% (n=328) of fecal samples were positive for *Eimeria* based on microscopy. Although the sensitivity of PCR for detecting *Eimeria* infections seemed to be less than that of microscopy in the first year of the study (8.9% vs. 29.3%, respectively), an improved DNA extraction protocol resulted in increased sensitivity of PCR relative to microscopy in the second year of the study (27.6% and 20.8%, respectively). The proportion of positive samples did not vary significantly between years or among sampling sites. The proportion of *Eimeria* positive fecal samples varied with date of collection, but there was no consistent pattern of parasite shedding between the two

*Reprinted with permission from "Coccidian parasites and conservation implications for the endangered whooping crane (*Grus americana*)" by Bertram, M., Hamer, G. L., Snowden, K. F., Hartup, B. K., and Hamer, S. A.. 2015. *PLoS ONE,* 10(6). doi: 10.1371/journal.pone.0127679

years. We demonstrate that non-invasive fecal collections combined with PCR and DNA sequencing techniques provides a useful tool for monitoring *Eimeria* infection in cranes. Understanding the epidemiology of coccidiosis is important for management efforts to increase population growth of the endangered whooping crane.

Introduction

The whooping crane (*Grus americana*) experienced a severe population decline in the first part of the 20th century and has been listed as endangered since 1967. The species has rebounded from a low of 15 individuals in 1941 to a total of 451 wild birds, including reintroduced populations, and 157 captive birds in 2013[1]. The Aransas-Wood Buffalo population (AWBP), which nests in Wood Buffalo National Park, Alberta and Northwest Territories, Canada and winters among coastal marshes in and around the Aransas National Wildlife Refuge in Texas, USA, is the only self-sustaining wild population of whooping cranes. During the 2013-2014 winter, the population was estimated at 304 individuals (95% CI = 260–354; CV = 0.08)[26]. The International Recovery Plan[2] sets a goal of down-listing the species to threatened by 2035. One criterion for down-listing the species requires the AWBP to maintain a population of at least 1000 individuals[2]. A second criterion relaxes this requirement to at least 400 individuals in the AWBP if a second self-sustaining flock is established[2]. Population projections indicate the probability of the AWBP reaching 400 individuals by 2040 is greater than 80%[27], however the probability of this population reaching 1000 individuals by 2040 is essentially zero[27], and the AWBP is not likely to reach 1000 individuals until at least the mid-2060s[3]. The species therefore remains endangered and is highly susceptible to stochastic events that could decimate the population.

Disease is cited as one of the factors for listing the whooping crane as endangered[2], however little is known about diseases affecting these birds. A variety of infectious diseases have been reported in captive and reintroduced whooping cranes[5,28], however similar studies for wild cranes are lacking. To our knowledge, a 1978 publication is the only published report concerning bacterial, viral, or parasitic pathogens affecting the AWBP whooping cranes, and that study analyzed a single fecal sample

each from 19 individuals. In that publication, nearly one third of cranes sampled were shedding coccidia[6].

Coccidia are obligate intracellular protozoan parasites in the phylum Apicomplexa. Coccidian parasites in the genus *Eimeria* infect a wide range of vertebrate and invertebrate hosts[29]. *Eimeria* have a direct fecal-oral life cycle. Noninfective oocysts are passed in the feces and undergo sporulation in the environment to become infective. Oocysts are hardy and can survive a wide range of environmental conditions. The sporulated oocyst (sporocyst) is ingested in food or water and undergoes asexual and sexual reproduction in host epithelial cells. Oocysts are the product of sexual reproduction and are excreted in the feces, and detecting oocysts within voided fecal samples is the most common method of diagnosing coccidian infection of a host[29]. The majority of *Eimeria* species infect intestinal epithelial cells, and the remainder infect renal epithelial cells, with few exceptions[30]. The *Eimeria* species infecting cranes (*E. gruis* and *E. reichenowi*) are two such exceptions. Unlike the common poultry-associated *Eimeria* species that cause localized infections and variable enteric disease, *E. gruis* and *E. reichenowi* can spread systemically in cranes, causing disseminated visceral coccidiosis (DVC)[7,8]. Clinical signs of DVC depend on the tissues affected and the severity of infection, and can include enteritis, hepatitis, bronchopneumonia, myocarditis, and splenitis. Oocysts develop in the intestine or respiratory tract and are shed in the feces[9]. Chronic infections are characterized by granulomas disseminated throughout many organs[7]. DVC is an important cause of crane chick mortality in captivity[8,10-12], and has also been described in captive adult cranes[13]. In one study, experimentally infected sandhill crane (*Grus canadensis*) chicks all developed granulomas, and 23.8% of wild sandhill cranes had granulomas at necropsy[31]. A separate study found 84% of wild sandhill cranes that had granulomas were also shedding oocysts in the feces[32]. Wild whooping crane chicks are associated with high mortality (27% - 68%) during the first 20 days after hatching[14-16], and the role of DVC as a cause of wild chick mortality is poorly understood[7]. Additionally, infection with these *Eimeria* species may make surviving birds more susceptible to other disease or predation[8].

Eimeria species have been described in at least eight species of cranes[7] worldwide, and probably infect all crane species, however, only *Eimeria gruis* and *Eimeria reichenowi* are diagnosed commonly[10]. *E. gruis* and *E. reichenowi* have been described in wild and captive whooping, sandhill, white-naped (*Grus vipio*), and red-crowned cranes (*Grus japonensis*), and additionally in captive demoiselle (*Anthropoides virgo*), sarus (*Antigone antigone*), and Eurasian cranes (*Grus grus*)[7,12]. Phylogenetically, the *E. gruis* and *E. reichenowi* that were isolated from hooded, white-naped, and red-crowned cranes cluster in a clade separate from the *Eimeria* species infecting other birds and mammals[33,34], but the genetics of *Eimeria* infecting whooping cranes has not previously been explored. Here, our objectives were to (*i*) determine the prevalence and phenology of coccidia shedding in the wintering AWBP population of whooping cranes; (*ii*) compare microscopic and molecular detections of coccidia species; and (*iii*) determine the phylogenetic relationships among the *E. gruis* and *E. reichenowi* isolated from whooping cranes to those from other crane species, and other *Eimeria* species.

Methods

Fecal sample collection

Whooping crane fecal samples were collected every three weeks during two winter seasons, Nov 2012-March 2013 and Nov 2013-April 2014, at the Aransas National Wildlife Refuge in Aransas, Refugio, and Calhoun counties on the Texas Gulf Coast (28.313449,-96.804022)(Figure 2.1). Because invasive sampling for health surveillance of these endangered birds is not desired due to their conservation status, our sampling approach is based on analyses of voided fecal samples. Fecal samples were collected from a series of ten artificial freshwater ponds on the refuge. Whooping cranes utilize freshwater sources for drinking when salinity levels in the marsh are high[35], as during the ongoing drought conditions in Texas which persisted through the study period. Although cranes are distributed across the refuge on territories, we prioritized fecal searching and collection at the ponds for the following reasons: (i) multiple family

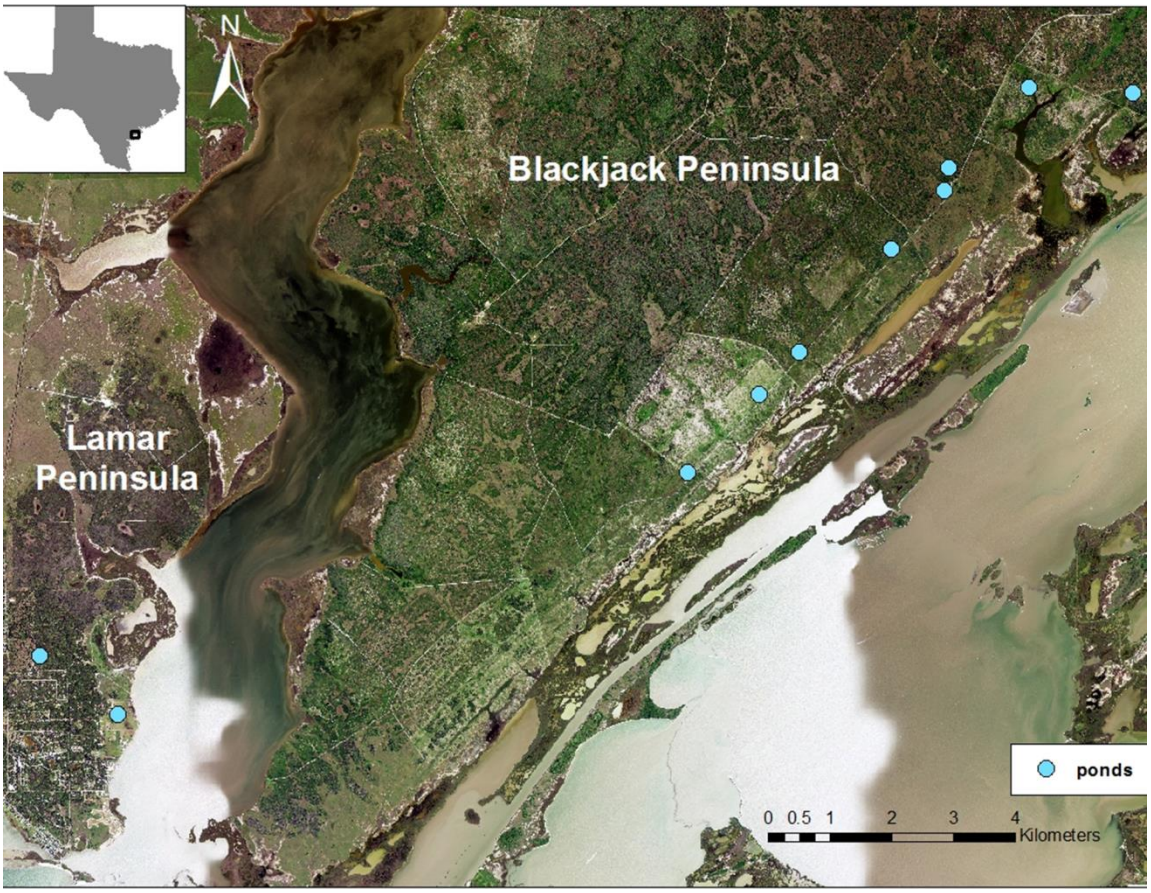

Figure 2.1. Pond sites on the Blackjack and Lamar peninsulas. The Aransas National Wildlife Refuge is located along the Texas Gulf Coast and encompasses the Blackjack Peninsula and Matagorda Island. Eight pond sites in this study were located on the Blackjack Peninsula. The two pond sites on the Lamar Peninsula were included during 2012-2013. The map image was created by the USDA National Agriculture Imagery Program (NAIP) and downloaded as a GIS file, and the figure was produced using ArcMAP 10 (Esri, Redlands, CA). Reprinted with permission from Bertram et al. 2015.

groups use the same ponds such that many individuals may be sampled from the same focal area; (ii) ponds are accessible from land and did not require boats to access; and (iii) disturbance to the birds due to the presence of our research team was limited because all ponds were located along the main service road and birds are habituated to occasional traffic along this road. Additionally, aggregation of cranes at the ponds may facilitate parasite transmission, resulting in higher prevalence of infection. We deployed infrared game cameras (Trophy Cam HD, Bushnell, Overland Park, KS) to determine patterns of whooping crane use of the ponds, and planned our collection excursions to occur after cranes departed. We collected fresh (estimated to be <24 hrs old) feces from 8 pond sites on the Blackjack peninsula during the study, and from an additional 2 pond sites on the Lamar peninsula during the 2012-2013 season (Figure 2.1). The two sites on the Lamar peninsula yielded very few samples and were eliminated from the sampling sites in the 2013-2014 season. We searched each pond site for fresh feces twice during each two-day collection trip. Feces were collected into Whirl-pak bags, after which air was removed manually, and samples were stored on ice for transportation and storage at 4^{o}C in the lab. Feces were selected for collection when they met the appearance of whooping crane scat (Figure 2.2) based on food contents (blue crab and wolfberry[36,37]) and in combination with evidence of recent whooping crane presence at the pond (tracks, game camera pictures). Sandhill cranes cohabitate with whooping cranes in the study area, and we also collected several sandhill crane fecal samples for comparison.

Figure 2.2. Whooping crane scat collection. Game camera photo of two adult whooping cranes and one juvenile (far left) at a freshwater pond on Aransas National Wildlife Refuge (A). Scat produced by a whooping crane feeding primarily on blue crab and other invertebrates (B) and wolfberry (C). Scat was collected after the cranes naturally left the pond. Reprinted with permission from Bertram et al. 2015.

Host species confirmation

To confirm host species, the *Grus* genus-wide primers Grus16SF and Grus16SR[38] were used to amplify a 470-bp fragment of the mitochondrial 16S rRNA gene for a systematic random sample of 10% of fecal samples that we suspected were from whooping cranes based on field observations. Additionally, for proof-of-principle, we also analyzed eight fecal samples that we suspected were from sandhill cranes. PCR was performed in 15 µl reactions consisting of 1X FailSafe PCR Premix A, 0.15 µl FailSafe Enzyme, 0.25 µM each primer, 0.1 µg/µl BSA, and 1 µl fecal DNA. Cycling parameters were as described previously[38]. Positive samples were purified using

ExoSAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Purified samples were submitted for bi-directional sequencing to Eton Bioscience Inc. (San Diego, CA). Sequences were compared to known crane sequences using the BLAST tool in GenBank, and a representative sequence of a whooping and a sandhill crane were deposited in GenBank (Accession #KP966312 and KP966313).

Fecal flotation

All samples were subjected to fecal flotation within 5 days of collection following standard veterinary protocol[39]; our own trials suggested the ability to detect coccidia microscopically and molecularly was not altered within this period (unpublished data). Briefly, one gram of feces was suspended in 10 ml zinc sulfate solution (s.g. 1.18), strained through a double layer of gauze, and transferred to a 15 ml centrifuge tube. Zinc sulfate solution was added to a final volume of 15 ml and a coverslip was placed over the top of the tube. Samples were centrifuged in a swinging-bucket centrifuge at 2000xg for 5 minutes. The coverslip was immediately placed on a slide and examined for the presence of coccidia oocysts using a compound light microscope. The entire coverslip was examined at 125X magnification, and suspected oocysts were further examined and measured at 500X magnification. Oocyst shape (pyriform or round/oval), presence and number of sporocysts in the oocysts, burden of infection, and single vs. mixed species infection were noted. Burden of infection was defined qualitatively as low (<2 oocysts per high power field), medium (2-10 oocysts/hpf), or high (>10 oocysts/hpf).

Molecular detection of coccidia

All samples (regardless of fecal flotation result) were subjected to a second fecal flotation to generate a template with concentrated oocysts for DNA extraction. We modified the flotation procedure described above by spinning tubes without a coverslip. Immediately after centrifugation, 100 µl of liquid at the surface (which would contain concentrated oocysts in positive samples) was transferred from the surface of the tube into a microcentrifuge tube. During the 2012-2013 season, the resulting samples were immediately stored at -20ºC until DNA extraction. During the 2013-2014 season, the

resulting samples were washed twice with 300 µl water to remove residual zinc sulfate and then stored at -20ºC until DNA extraction. The DNA was extracted using the QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) following manufacturer's instruction during the 2012-2013 season. During the 2013-2014 season, DNA was extracted using the E.Z.N.A. Stool DNA Extraction Kit (Omega Biotek, Norcross, GA), and samples were processed in a cell disruptor (Mini-beadbeater 96, BioSpec Products, Inc., Bartlesville, OK) for 90 seconds to break open the oocysts, then incubated at 55ºC overnight. We then proceeded with DNA extraction following the manufacturer's instructions. All samples were eluted in two rounds of 25µl (50 µl total) into the same tube.

Coccidia were detected using PCR to amplify a portion of the internal transcribed spacer regions (ITS) using two previously published assays. A 466-bp region of the first internal transcribed spacer (16S – 5.8S rRNA region) was amplified using the primers BSEF and BSER[40] at a concentration of 0.5 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 1 µl of sample template. Cycling parameters were as described by Gerhold et al.[41], except annealing temperature was changed to 55ºC because this temperature was determined to be optimal after our pilot trials. Alternatively, a nested reaction was used to amplify a 400-bp region of the second internal transcribed spacer (5.8S – 28S rRNA region). The initial PCR used the primers EITSF2 and EITSR2[38] at a concentration of 0.25 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix A, 0.15 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 1 µl sample template. The second PCR used the primers WW2 and WW4r[42] at 0.25 µM in a 15 µl reaction. The first PCR product was diluted 1:50 and 1 µl of the diluted product was used in the second PCR. All other reaction components were identical to the first PCR. Cycling parameters were run as previously described[38].

We used an independent PCR for a different *Eimeria* gene on a random subset of positive samples and negative samples for confirmatory purposes. The primers 1FE and 4RB[43] were used to amplify a 358-bp region of the 18S rRNA gene at a concentration of

1 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix E, 0.15 µl FailSafe Enzyme, and 1.5 µl fecal DNA. Cycling parameters were run as previously described[33].

To complement the morphological differences we noted between crane-associated *Eimeria* species, we confirmed the identity of coccidia species using DNA sequencing for all three amplified regions (ITS-1, ITS-2, and 18S rRNA). All positive sequences were purified and sequenced as described above. Forward and reverse sequences were aligned and a consensus sequence was determined using Clustal W within Mega 6.0[44]. Sequences were compared to known *Eimeria* sequences using the BLAST tool in GenBank. Consensus sequences were then aligned along with publicly available *Eimeria* species sequences and analyzed in Mega 6.0 using a neighbor-joining tree using the bootstrap method with 1000 replicates. Samples with poor quality sequences or multiple peaks were excluded from phylogenetic analysis. All sequences produced during this project and utilized in the phylogenetic analysis were deposited in GenBank (Accession #KP966299 – KP966311).

Statistical analysis

Statistical analysis was performed using SAS software, version 9.4 (Cary, NC). Proportion of samples positive and confidence intervals were calculated accounting for clustering at the pond level. The chi-squared test was used to compare proportion of positive samples between ponds and between years. Fisher's exact test was used to compare proportion of positive samples between dates of collection within each year due to small samples sizes during 2013-2014. Proportion of samples positive based on microscopy was compared to that based on PCR using the chi-squared test.

Results

Sample collection and host confirmation

We collected a total of 339 fecal samples, with 227 collected during 2012-2013 and 112 collected during 2013-2014. Of these, 11 were suspected to come from sandhill cranes, whereas the remainder was attributed to whooping cranes based on visual characteristics. A total of 79 samples, including 9 that were suspected to come from

sandhill cranes, were subjected to a molecular confirmation of host species. All 9 that were suspected to come from sandhill cranes based on appearance were confirmed to contain sandhill crane DNA based on DNA sequence analysis; sandhill crane samples were excluded from further analysis. Of the remaining 70 samples, 37 were confirmed to contain whooping crane DNA based on sequence analysis and 5 sequences were poor quality and could not be matched to species. The remainder of samples did not amplify using PCR, but nonetheless are included in the analysis as originating from whooping crane due to field identification and presence of whooping cranes immediately preceding collection based on camera trap and observational data. The lack of amplification could be attributed to a lack of host DNA in the floated fraction of fecal material that was subjected to DNA extraction, or degradation of host DNA in the feces while in the field.

Microscopic examination

We identified two types of oocysts based on morphology (Figure 2.3). The first type was pyriform and measured 18 µm x 12 µm (range 16-20 µm x 10-14 µm), and matched size descriptions of *Eimeria gruis*[45,46]. The second type was round to oval and measured 20 µm x 16 µm (range 12-22 µm x 12-20 µm), and matched size descriptions of *Eimeria reichenowi*[45,46].

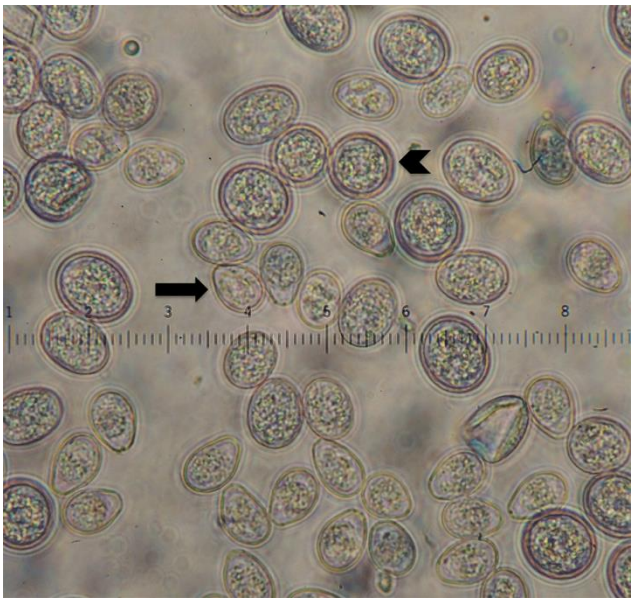

Figure 2.3. Coccidia observed during fecal flotation. Fecal flotation under 500X magnification showing a mixed infection with two species of *Eimeria*. The smaller, pear-shaped oocysts are consistent with *Eimeria gruis* (arrow) and the larger, round to oval oocysts are consistent with *Eimeria reichenowi* (arrowhead). Reprinted with permission from Bertram et al. 2015.

In total, 87 of 328 (26.5%; 95% CI: 20.3% – 32.8%) samples were positive for *Eimeria* on microscopy. The majority of samples (63.2%, n=55) had a low burden of infection, 20 (23%) had a medium burden of infection, and 12 (13.8%) had a high burden of infection. The burden of infection did not differ significantly across the study ($p=0.22$, $\chi^2=4.45$, $df=3$). Fifty-seven samples (65.5% of positive samples) were single infections with *E. gruis*, 17 (19.5%) were single infections with *E. reichenowi*, and 12 (13.8%) were mixed infections with both *Eimeria* species. Data for the two species were combined for further analysis due to low sample numbers for *E. reichenowi*. There was no significant difference in overall proportion of positive samples between 2012-2013 and 2013-2014 (29.3% and 20.8%, respectively; $p=0.08$, $\chi^2=3.17$, $df=1$). The proportion of *Eimeria* positive samples varied significantly across the season during 2012-2013 (Fisher's exact test, $p<0.001$), but not during 2013-2014 (Fisher's exact test, $p=0.361$). Across the November-April winter collection season, prevalence peaked in December and again in April during 2012-2013, whereas there was a single peak in January during

2013-2014 (Table 2.1). The proportion of *Eimeria* positive samples did not vary significantly among ponds (p=0.43, χ^2=9.10, df=9).

Table 2.1. Phenology of *Eimeria* shedding in winter based on microscopy in whooping crane feces. Reprinted with permission from Bertram et al. 2015.

	2012-2013		2013-2014	
	Positive Samples (%)	Total Samples	Positive Samples (%)	Total Samples
November	8 (2.0)	25	5 (18.5)	27
December	27 (58.7)	46	5(25.0)	20
January	11 (23.4)	47	2 (50.0)	4
February	8 (12.7)	63	6 (18.2)	33
March	4 (14.8)	27	3 (33.3)	9
April	7 (50.0)	14	1 (7.7)	13

Molecular examination

Samples that were very dry or very small (n=66) were excluded from molecular analysis. In total, 43 of 262 (16.4%; 95% CI: 10.5% - 22.4%) samples were positive for *Eimeria* using PCR for either ITS-1 or ITS-2 regions. The proportion of samples that tested positive during the 2012-2013 season was 8.9% (95% CI: 1.7% - 16.1%; n=157), and was significantly less than during the 2013-2014 season (27.6%; 95% CI: 13.2% - 42.0%; n=105; p<0.0001, χ^2=14.66, df=1). The large difference between years was attributed to the improved sample preparation and DNA extraction protocol we used in the 2013-2014 season.

A total of 41 samples was subjected to PCR for *Eimeria* 18S rRNA for an independent assessment. Of the 38 that were positive based on PCR for either ITS region of *Eimeria*, 28 (73.7%) were also positive in the 18S rRNA PCR. Of the 3 that were negative based on PCR for the ITS region, 2 (66.7%) were also negative in the 18S rRNA PCR.

The proportion of samples that tested positive as determined by PCR for ITS-1 or ITS-2 was significantly lower than that which was determined by microscopy during 2012-2013 ($p<0.0001$, $\chi^2=22.13$, df=1), but during 2013-2014, the proportion of positive samples as determined by PCR for ITS-2 was significantly greater than that determined by microscopy ($p<0.0001$, $\chi^2=46.23$, df=1). During 2013-2014, PCR had a sensitivity of 86.4% and a specificity of 88.0% compared to microscopy for detection of coccidia. Of the 105 samples collected during 2013-2014, 19 samples were positive and 73 samples were negative based on both microscopy and PCR, whereas 3 samples were positive on microscopy but negative on PCR, and 10 samples were negative on microscopy but positive on PCR.

Phylogenetic analysis

We obtained forward and reverse DNA sequences from either the ITS-1 or ITS-2 regions from 21 samples to include in the phylogenetic analysis. The five samples for which we determined ITS-1 sequences were identical to each other and matched closely with a previously published *E. gruis* sequence from a hooded crane in Japan[38], and the crane-associated clade is more closely related to poultry *Eimeria* species than to cattle *Eimeria* species (Figure 2.4). The 15 ITS-2 sequences produced similar results, with the crane *Eimeria* species forming a separate clade that was more closely related to poultry *Eimeria* species than to cattle *Eimeria*. However, our ITS-2 sequences showed three distinct lineages. One lineage, comprised of nine nearly identical sequences, grouped with previously published *E. gruis* sequences. Another lineage was comprised of four sequences and grouped with previously published *E. reichenowi* sequences. The third lineage, comprised of two identical sequences, formed a unique group within the crane *Eimeria* clade.

We obtained forward and reverse DNA sequences from the 18S rRNA gene for 28 samples. Upon manual examination of the chromatograph traces, six samples had double nucleotide peaks at two polymorphic sites within the alignment that were among those that differentiated *E. gruis* and *E. reichenowi* and were excluded from phylogenetic analysis. All analyzed sequences were within the clades that contained the

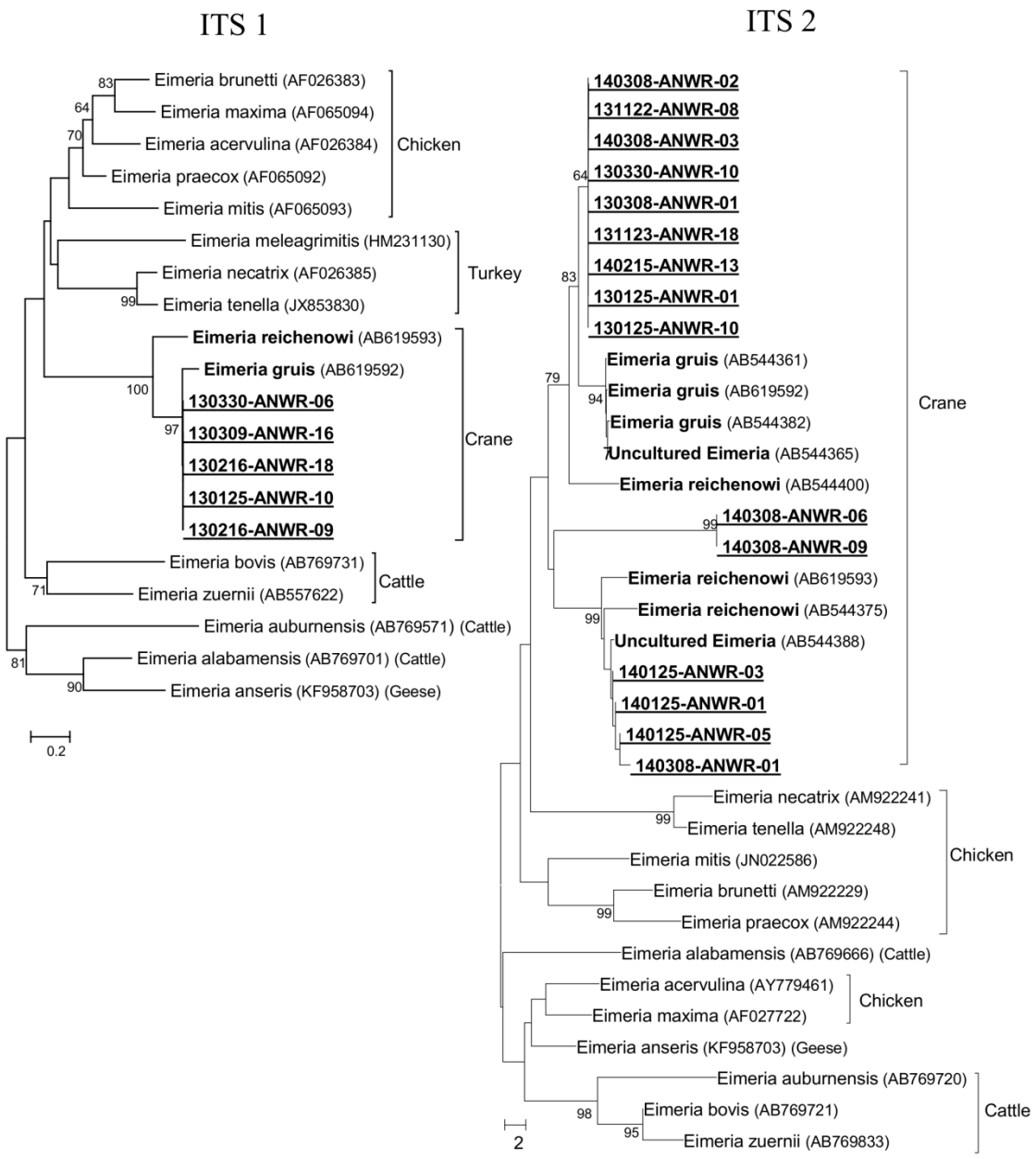

Figure 2.4. Phylogenetic tree using *Eimeria* ITS-1 and ITS-2 sequences. Phylogenetic trees using the neighbor-joining method on ITS-1 (466 bp) or ITS-2 (400 bp) sequences from *Eimeria* species. Bootstrap values are based on 1000 replicates and shown where greater than 60. Bold species indicate isolates from cranes, and underlined species indicate sequences generated in this study. The GenBank accession number of each isolate is shown in parentheses, and the known vertebrate host is also shown. Reprinted with permission from Bertram et al. 2015.

previously published *E. gruis* and *E. reichenowi* sequences. The crane *Eimeria* species formed a clade with *E. anseris* from domestic geese that was separate from all other *Eimeria* species investigated. Using 18S rRNA sequences, *Eimeria* species from poultry, cattle, and rodents are more closely related to each other than to crane *Eimeria* (Figure 2.5). Two of the three *E. reichenowi* published sequences (from a crane in Japan) and the sequence we generated from a whooping crane formed a unique clade. The third *E. reichenowi* published sequence formed a separate branch in the crane *Eimeria* clade. Among samples (n=10) for which we generated both ITS and 18S rRNA sequences, the *Eimeria* species assignment was congruent based on analysis of both loci for all but two samples (140125-ANWR-01; 140125-ANWR-03), which grouped with *E. reichenowi* at the ITS locus and grouped with *E. gruis* at the 18S rRNA locus. Microscopic assessment of the oocysts in these two samples revealed both round and pear-shaped oocysts, indicative of mixed species infections.

Overall, DNA sequence analysis supported morphologic analysis for species-level identification. Samples containing the pear-shaped oocysts on microscopy aligned with *E. gruis* on the DNA sequence analysis. Several samples that contained both types of oocysts, but had many more pear-shaped than round oocysts, also aligned with *E. gruis* on DNA sequence analysis. One of the samples containing only the round oocysts produced a good quality sequence and aligned with *E. reichenowi* on the DNA sequence analysis.

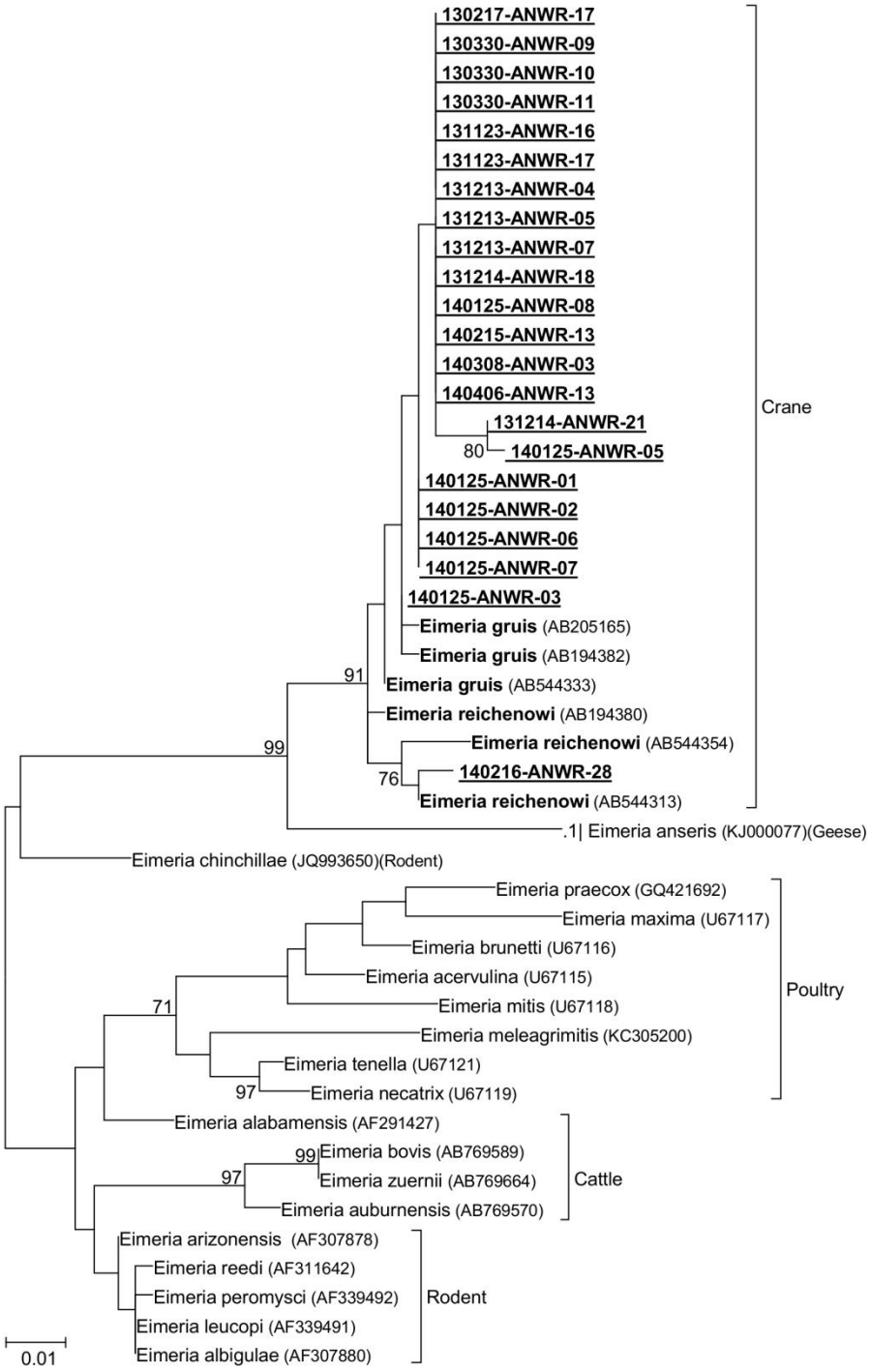

Figure 2.5. Phylogenetic tree using *Eimeria* 18S rRNA sequences. Phylogenetic tree using the neighbor-joining method on 18S rRNA sequences (358 bp) from *Eimeria* species. Bootstrap values are based on 1000 replicates and shown where greater than 60. Bold species indicate isolates from cranes, and underlined species indicate sequences generated in this study. The GenBank accession number of each isolate is shown in parentheses, and the known vertebrate host is also shown. Reprinted with permission from Bertram et al. 2015.

Discussion

We document that nearly one-third of fecal samples collected from the only wild migratory population of whooping cranes on their wintering grounds harbor *Eimeria* species coccidian parasites, based on visualization of oocysts and PCR analysis of voided fecal samples. These data underscore the importance of understanding how coccidian parasites may impact population health. Our findings are similar to those reported in the only previous published assessment of coccidia in this population of whooping cranes (31.5%)[6]. Although these two datasets suggest that coccidia infection may have remained stable while this population increased in size over the past 35 years, the study from the 1970s was based on a single fecal sample from only 19 individuals. Although the exact number of individual cranes represented in our analysis is not known, the ponds from which we collected samples are utilized by birds of multiple family groups. During December and January 2012-2013, approximately 17 individuals were documented in the marshes adjacent to our study sites, increasing to 45 individuals in late February (Elizabeth Smith, personal communication); we therefore expect our study samples represent a subset of this number of birds. In contrast to the AWBP whooping cranes, only 13% (n=54) of reintroduced whooping cranes in Florida were found to be shedding coccidia[47]. The reintroduced cranes had access to feed containing a coccidiostat, which likely explains the lower prevalence of *Eimeria* among this population[47]. In other avian host species, oocyst shedding can vary with the time since infection and the time of day when the feces is voided[48,49]. Numerous studies of *Eimeria* and *Isospora* species in other avian hosts have shown that oocyst shedding is lowest in the morning and increases through the day[50-52]. Although we collected samples in the mid- to late-afternoon each day, the samples were voided by cranes throughout the day. If *Eimeria* in cranes follow the same diurnal shedding pattern, oocysts may not be present in fecal samples deposited in the morning even if the crane is infected. Furthermore, infected birds may not shed oocysts across the full time frame of infection. Novilla et al.[53] found oocysts in fecal samples from three of four captive sandhill cranes with DVC, and a study of hunter-harvested wild sandhill cranes found oocysts in fecal

samples of 84% (n=64) of cranes with DVC[32]. Accordingly, if we assume the fecal samples we studied are representative of the crane population on the refuge, our results suggest the true coccidia infection prevalence in the whooping cranes is likely higher than the results of our fecal analysis indicate.

Through a longitudinal assessment, we found that prevalence of coccidia shedding varied across the season, but the variation was not consistent across the two years of the study. During the first year, prevalence peaked in December and again in April, however there was only a single nonsignificant peak in January during the second year. The lack of a significant trend in the proportion of positive fecal samples during 2013-2014 may be due to small sample sizes during January and March. Our results suggest that birds arrive infected and maintain a low level of shedding throughout the winter season. Interestingly, previous studies in wild red-crowned cranes (*Grus japonensis*) in Japan found a similar level of *Eimeria* infection in fecal samples (26%) and a higher percentage of infection in samples collected in December compared to January through April[54]. The authors of that study suggest two possible explanations for the decrease in *Eimeria* infection over the winter: 1) temperatures are too cold for sporulation to occur, therefore new infections do not occur; and 2) coccidiosis is a self-limiting disease, and recovered cranes no longer shed oocysts[54]. We suggest other factors must be involved in the phenology of oocyst shedding in our study, since winter temperatures along the Texas Gulf Coast remain mild enough for sporulation to occur[55], and *E. gruis* and *E. reichenowi* spread systemically, unlike other *Eimeria* species. Viable schizonts have been seen in granulomas in multiple tissues, which potentially prolong the infection[31]. Hartman et al.[56] documented temporal peaks in shedding of *E. gruis* and *E. reichenowi* in fecal samples collected from communally roosting sandhill cranes in Wisconsin during the summer. Temporal shedding and communal roosting likely increase transmission, and communal roosting is common among whooping cranes at the ANWR. We are currently investigating the degree to which physiological stress may contribute to *Eimeria* shedding.

The discrepancy we observed in prevalence estimates based on microscopic vs. molecular examination during 2012-2013, in which significantly more matched samples were positive using microscopy, is likely attributed to an inefficient DNA extraction protocol used in the 2012-2013 season. Specifically, we did not include a mechanical breaking step, as we were following the protocol of Honma et al.[38] and assumed there may be enough free DNA released from opened or degraded oocysts. We refined our extraction method during 2013-2014 to include a mechanical breaking step in addition to rinsing oocysts to remove excess flotation solution since high salt concentrations are detrimental to PCR reactions. With these modifications, we found that the proportion of positive samples was greater than we detected in the previous field season. Furthermore, more samples were determined to be positive based on PCR than on microscopy. Honma et al.[38] concluded that PCR is less sensitive than microscopy, however we showed that with proper sample preparation, PCR can detect more positive samples than microscopy, and may be used as a conservation tool to monitor the prevalence of *Eimeria* in the whooping crane population.

The ITS regions can be used to determine the species of *Eimeria*, however there are multiple copies of these regions in the *Eimeria* genome, and sequence length can vary within a single oocyst, limiting the utility of these regions for investigating phylogeny[29,57]. The 18S rRNA gene is more conserved, making it more suitable as a marker for both species identification and phylogenetic analysis. Previous studies have shown that the *E. gruis* and *E. reichenowi* that infect cranes in Japan are phylogenetically distinct from other *Eimeria* species[33,34], and our results show this is true for the *E. gruis* and *E. reichenowi* that infect cranes in North America. Furthermore, many 18S rRNA sequences from our study are identical to each other, but distinct from previously published *E. gruis* and *E. reichenowi* sequences, suggesting there may be different lineages of these parasites infecting cranes in North America and in Japan. Although recent studies on *Eimeria* species that infect poultry indicate that the 18S rRNA gene is not suitable by itself for identification and phylogenetic analysis at the species level, and propose using the cytochrome *c* oxidase subunit I (COI) gene

instead[58,59], we elected to use the ITS regions and 18S gene in this study because they have been characterized for crane-associated *Eimeria* species, whereas COI has not.

We found that searching for freshly-voided fecal samples around freshwater ponds at ANWR is an efficient means of collecting a large number of fecal samples. One drawback to this method, however, is that it is difficult to match samples to individual birds at the time of collection because these ponds are used communally by a large number of birds. An alternative means of collection is to monitor family groups on territories in the marshes and search for feces after the birds have vacated an area. This method is time-consuming and yields low numbers of samples[60], but may be necessary to represent a large number of individual birds during wet years when pond use is diminished.

We have detected a high and persistent prevalence of coccidian parasites in whooping cranes, and the degree to which parasites regulate the whooping crane population remains unknown. Previous studies provide a framework for understanding the potential for parasites to regulate wild vertebrate host populations. For example, long-term experimental reductions in the burden of a parasitic nematode resulted in increased fecundity and a prevention of population crashes of free-living red grouse (*Lagopus lagopus scoticus*) in England[61]. A meta-analysis investigating the effect of parasites on wild vertebrates revealed a significant negative effect of parasites at the population-level which resulted from reduced clutch size, hatching success, young produced, and survival[62]. Current evidence indicates coccidia infecting cranes frequently spread systemically to cause DVC[7,31,32], although mortality is low in adult birds[47]. Mortality from DVC is likely much higher in chicks, and the disease may exert a population-level effect by reducing survivorship of this life stage. However, the cause of death of chicks is exceedingly difficult to ascertain due to the remote location of the breeding grounds in Wood Buffalo National Park in northern Canada. The prevalence of coccidian parasites within whooping crane fecal samples at the key refuge used for overwintering of the species underscores the importance for considering DVC as a disease that may be regulating the population growth of this species. Understanding the

times and locations important in *Eimeria* transmission will aid conservation efforts and inform management decisions aimed at the recovery of the AWBP whooping cranes.

CHAPTER III

MORPHOLOGIC AND MOLECULAR CHARACTERIZATION OF HELMINTHS IN ENDANGERED WHOOPING CRANES (*Grus americana*) AND CONGENERIC SANDHILL CRANES (*Grus canadensis*)

Overview

The single migratory wild population of whooping cranes (WHCR, *Grus americana*) is endangered, and the extent to which parasites may be impacting the population is largely unknown. The goal of this study was to characterize the helminth community in whooping cranes using classical and molecular parasitological approaches with direct sampling from WHCR as well as sampling sandhill cranes (SACR, *Grus canadensis*) as a surrogate species. Classical parasitological approaches revealed 11 different nematode egg morphotypes in 14.8% (n=327) and 4.0% (n=75) of fecal samples from WHCR and SACR, respectively. The majority of nematode DNA sequences recovered from fecal samples aligned with soil-dwelling nematodes, suggesting their presence was due to consumption by the cranes or an artifact of sample collection of voided feces. We noted three different trematode egg morphotypes in 18.3% and 13.3% of WHCR and SACR samples, respectively. We identified adults of three species of trematode (*Orchipedum jolliei*, *Prohyptiasmus grusi*, *Paratanaisia bragai*), one cestode (*Gruitaenia gruis*), one acanthocephalan (*Polymorphus minutus*), and one nematode (*Tetrameres grusi*) in SACR on necropsy. Whooping cranes and sandhill cranes are parasitized by diverse helminth taxa, and further studies are needed to determine the health consequences of helminth infections in cranes.

Introduction

Despite habitat acquisition and reintroduction efforts, whooping cranes (WHCR, *Grus americana*) remain endangered, and the Aransas-Wood Buffalo Population (AWBP) is the only self-sustaining migratory population of WHCR. During winter 2015-2016, this population was estimated at 329 individuals (95% CI = 293–371; CV = 0.073)[63] in and around the Aransas National Wildlife Refuge on the Texas Gulf Coast;

however, population growth is insufficient to meet species recovery goals[2,3]. The degree to which parasitic infection may be one factor limiting population growth of the wild whooping cranes is largely unknown, as reports of parasites in whooping cranes come from studies of reintroduced populations[17,64,65], and no studies have been published concerning helminth parasites of the AWBP whooping cranes.

Helminths have been shown to regulate host populations. For example, the nematode *Trichostrongylus tenuis* has been shown to regulate population cycles in red grouse (*Lagopus lagopus scoticus*)[61]. Further, a recent meta-analysis showed that parasites, including helminths, have a significant negative effect on clutch size, hatching success, and young produced[62]. In addition to population effects, helminths may directly cause clinical disease in host individuals and impact the immune function of their host rendering it more susceptible to other infections. In particular, there is a polarized immune response to extracellular (helminth) versus intracellular (viruses and bacteria) parasites in which the up-regulation of the immune response to an extracellular parasite results in the downregulation of the immune response to subsequent microparasite challenges, leaving the animal more vulnerable to attack by the latter[19]. The effects of helminths on crane populations have not been explored.

Helminth identification in feces via classical parasitology methods, such as fecal flotation or sedimentation, is based on morphology of eggs or larval stages. Often identification can be made only to the family or genus level due to similarities between eggs at the species level. Recently, molecular techniques have been used to complement classical parasitology methods for detection of medically important helminth parasites in feces[66-69]. When DNA preparation methods sufficiently lyse eggs, PCR may be more sensitive than traditional microscopy, and DNA sequencing facilitates the identification of parasites to a lower taxonomic unit, dependent on the depth of reference sequences. For example, PCR has been used to identify helminth species[70-72] and to match larval and adult stages of helminths[73-75].

Parasitological studies of wildlife have mostly relied on lethal sampling, limiting the host species that can be studied. Because our efforts to characterize the helminth

parasites in WHCR are largely limited to methods that are non-invasive to the target population, we included assessments of sandhill cranes (SACR, *Grus canadensis*) as a surrogate species. The surrogate species approach is useful in studies of endangered species where sample sizes are limited. For example, this approach has been successful in studies of the Attwater's prairie chicken (*Tympanuchus cupido attwateri*) using the northern bobwhite (*Colinus virginianus*) as a surrogate[25]. The SACR is a useful surrogate as the closest North American relative to the WHCR, its abundance, and overlapping range with WHCR. A variety of helminths have been reported in the Mid-Continent population of sandhill cranes[18,76-79], and in the eastern population of greater sandhill cranes (*G. c. tabida*)[56,80,81], and non-migratory Florida sandhill cranes (*G. c. pratensis*)[80,82,83], which are sympatric in Florida. The purpose of this study was to characterize the fecal helminth community in WHCR and SACR using traditional parasitological and molecular methods, and to characterize and compare the adult helminth community in three different SACR populations at necropsy.

Methods

Fecal collection

Whooping crane fecal samples were collected as previously described[84]. Briefly, we collected fresh (estimated to be <24 hrs old) feces at the Aransas National Wildlife Refuge (ANWR) in Aransas, Refugio, and Calhoun counties on the Texas Gulf Coast approximately every three weeks during two winter seasons from November to April of 2012 - 2014. Feces were collected under Special Use Permit #21531-13-003 and #21530-14-03-DI. Feces were selected for collection when they met the appearance of WHCR scat based on food contents (blue crab and wolfberry[36,37]) and in combination with evidence of recent WHCR presence at the pond (tracks or from game camera photos). SACR are sympatric with WHCR in the study area, and we also collected several SACR fecal samples for comparison. We used PCR and DNA sequencing to confirm host species for a subset of fecal samples as we previously described[84].

Historic whooping crane necropsy records

The National Wildlife Health Center in Madison, WI, has served as the central laboratory for necropsies of captive and wild whooping cranes. We received necropsy records from all WHCR necropsies performed on AWBP birds that died between 1993 – 2014 for comparison with data obtained from wild WHCR and SACR during this study.

Sandhill crane necropsy

We sampled hunter-harvested SACR collected in winter months between November 2012 and January 2014 through relationships with the Texas Parks and Wildlife Department, New Mexico Department of Game and Fish, and private hunting clubs and outfitters. Hunter-harvested sandhill cranes were salvaged under Federal Fish and Wildlife Permit #MB89164A-0 and Texas Parks and Wildlife Department Permit #SPR-0512-917. Sandhill cranes from the following three populations were sampled: 1. Mid-continent population wintering on the Texas Gulf Coast (harvested in Jackson County, TX). 2. Mid-continent population wintering in the Texas panhandle (harvested in Armstrong and Carson Counties, TX). 3. Rocky Mountain population wintering in New Mexico (harvested in Socorro County, NM). Some birds harvested in New Mexico may also have been part of the Mid-continent population. The Mid-continent population is comprised of Lesser (*G. c. canadensis*) and Greater (*G. c. tabida*) subspecies, whereas the Rocky Mountain population is comprised of the Greater subspecies only[85,86]. The Rocky Mountain population serves as an out-group for comparison because their breeding, migration, and wintering ranges do not overlap with WHCR. All birds were either subjected to necropsy in the field immediately post-harvest or frozen at -20ºC immediately post-harvest and subjected to necropsy in the laboratory at a later date. Each carcass was subjected to a full gross necropsy, at which time all apparent helminths were collected in 70% ethanol and intestinal contents were collected from the lower intestine. All tissues were flushed with 0.9% saline and examined using a dissecting microscope to facilitate recovery of helminths.

Adult trematodes, cestodes, and acanthocephalans were stained in Semichon's carmine[87], cleared in xylene, mounted in Canada balsam (Alpha Aesar, Haverhill, MA)

and examined under 125X and 500X magnification. Wet mounts of unstained nematodes were also examined under 125X and 500X magnification. Morphologic identification was made using previously published keys and species descriptions[77,78,83,88-90] in combination with molecular results.

Microscopic examination of feces

All fecal samples were subjected to a centrifugal fecal flotation test using $ZnSO_4$ flotation solution[39] to concentrate parasite eggs, cysts, and oocysts, followed by microscopic examination at 125X and 500X magnification[84]. Images including a calibrated eyepiece grid were captured for measurements and morphologic comparisons among the various observed eggs.

Additionally, fecal samples of sufficient volume were subjected to a fecal sedimentation test[39] for identification of heavy eggs that could not be recovered on flotation. For sedimentation, 2 g of feces was suspended in 10 ml 0.9% saline, strained through a double layer of gauze, then decanted into a 15 ml centrifuge tube, and 0.9% saline was added to fill the tube. Samples were allowed to sit one hour at room temperature, the supernatant was removed, and the sediment was re-suspended in 15 ml 0.9% saline. This step was repeated until the supernatant was clear, after which the supernatant was removed. Ten drops of sediment were placed on slides with coverslips and examined at 125X magnification. Images including a calibrated eyepiece grid were captured for measurements and morphologic comparisons among the various observed eggs. Images were taken under 500X magnification. The remaining sediment was transferred to a microcentrifuge tube and stored at -20ºC until DNA extraction.

Molecular detection of helminths

The DNA extraction of fecal flotation samples was performed as previously described[84]. Sedimentation samples were extracted using the E.Z.N.A. Stool DNA Extraction Kit (Omega Biotek, Norcross, GA). All sedimentation samples were processed in a cell disruptor (Mini-beadbeater 96, BioSpec Products, Inc., Bartlesville, OK) for 90 seconds, then incubated at 55ºC overnight. We then proceeded with DNA extraction following the manufacturer's instructions.

Nematodes. The DNA extracted from fecal flotation samples was subjected to PCR to amplify a 900 bp region of the 18S ribosomal RNA gene of nematodes. The primers Nem18SF and Nem18SR[91] were used at a concentration of 0.5 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 2 µl of sample template. Cycling conditions were as described by Floyd et al.[91]. DNA extracted from *Porrocaecum ensicaudatum* and a *Synhimantus* sp., each removed from an American robin (*Turdus migratorius*) and stored in glycerin[92], were used as positive reference controls. Samples were considered positive if the closest match to the resulting sequence in GenBank was a nematode species.

Trematodes. The DNA extracted from fecal sedimentation samples was subjected to PCR to amplify an 800 bp region of the 18S ribosomal RNA gene of Platyhelminthes. The primers TremCF and TremAR[73] were used at a concentration of 0.5 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 2 µl of sample template. Cycling conditions were as described by Routtu et al.[73]. The DNA extracted from adult trematodes (*Orchipedum jolliei, Prohyptiasmus grusi, Paratanaisia bragai*) removed from SACR at necropsy in this study was used as a positive control. Samples were considered positive if the closest match to the resulting sequence in GenBank was a trematode species.

Acanthocephalans. The DNA extracted from fecal sedimentation samples was subjected to PCR to amplify a 1000 bp region of the 18S ribosomal RNA gene of acanthocephalans. The primers Acanth18SF and Acanth18SR[93] were used at a concentration of 0.5 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 2 µl of sample template. Cycling conditions were as described by Herlyn et al.[93]. DNA extracted from an adult acanthocephalan (*Polymorphus minutus*) removed from a SACR at necropsy in this study was used as a positive control. Samples

were considered positive if the closest match to the resulting sequence in GenBank was an acanthocephalan species.

DNA Sequencing

Amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Purified samples were submitted for bi-directional sequencing to Eton Bioscience Inc. (San Diego, CA). Forward and reverse sequences were aligned and a consensus sequence was determined using Clustal W within Mega 6.0[44]. Sequences were compared to helminth sequences using the BLAST tool in GenBank. Consensus sequences were aligned along with publicly available helminth species sequences and phylogenetic relationships were analyzed in Mega 6.0 using the maximum likelihood method based on a Kimura 2-parameter with gamma distribution and evolutionarily invariable sites (K2+G+I) model of evolution using the bootstrap method with 1000 replicates[94]. The model was selected based on fit estimated by the Aikake information criterion (AICc) and Bayesian information criterion (BIC). Samples with poor quality sequences or double nucleotide peaks were excluded from phylogenetic analysis. Representative unique sequences produced during this project and utilized in the phylogenetic analysis were deposited in GenBank (Accession #KX172098 - KX172129).

Statistical analysis

Statistical analysis was performed using R software (www.R-project.org). The chi-squared test was used to compare fecal helminth prevalence between crane species, and to compare the prevalence of each helminth species found at necropsy among the three SACR populations. Logistic regression was used to investigate the relationship between the presence of *Orchipedum jolliei* and SACR population, age, and sex.

Results

Fecal analysis

We collected 327 WHCR fecal samples from the ANWR and 75 SACR fecal samples, of which we collected 11 from the ANWR, 28 from the Texas Panhandle population, 21 from the Gulf Coast population, and 15 from the New Mexico population.

On fecal flotation, 14.8% (95% CI 11.3%, 19.2%) of WHCR samples and 4.0% (95% CI 1.0%, 12.0%) of SACR samples were positive for nematode eggs overall (Table 3.1). Although nematodes were not noted in samples from SACR harvested in New Mexico, sample sizes were small and there were no significant differences in the proportion of positive samples among the SACR populations (Fisher's exact p=0.556). The proportion of positive samples was significantly higher for WHCR than for SACR (χ^2=6.39, df=1, p=0.01).

We noted 11 different nematode egg morphotypes, identified to six different superfamilies[95], and two cestode egg types in WHCR and SACR fecal samples. Two egg morphotypes were consistent with nematodes in the Ascaridoidea superfamily (Figure 3.1A, B). One was found in 2 (0.6%) WHCR samples and was oval, with a smooth thick shell, and measured 120-124 μm x 68-80 μm. The other was found in one (0.3%) WHCR sample, and was round to oval, with a rough thick shell, and measured 34 μm x 32 μm. One egg morphotype was consistent with nematodes in the superfamily Trichinelloidea, or capillarid-type eggs, and was found in 3 (0.9%) WHCR samples and one (1.3%) SACR sample (Figure 3.1C). This egg was yellow, elongated, slightly asymmetric, with bipolar plugs, and measured 52-58 μm x 24-28 μm. One egg morphotype consistent with nematodes in the superfamily Dioctophymatoidea was found in 5 (1.5%) WHCR samples and was oval, with a rough shell, and measured 118-134 μm x 56-80 μm (Figure 3.1D). One egg morphotype consistent with nematodes in the superfamily Habronematoidea was found in 2 (0.6%) WHCR and one (1.3%) SACR samples and was oval, with a thin shell, and measured 44-52 μm x 26-32 μm (Figure 3.1E). One egg morphotype consistent with nematodes in the superfamily Rhabditoidea was found in 4 (1.2%) WHCR samples and was oval, larvated, with a thin shell, and measured 50-76 μm x 24-36 μm (Figure 3.1F). Five egg morphotypes were consistent with nematodes in the order Strongylida, but could not be identified to superfamily. Eggs were oval to elongated, with smooth thin walls, and were embryonated, with some beginning to differentiate into larva (Figure 3.1G-K). Egg sizes and prevalences are listed in Table 3.1. Two WHCR samples were infected with two different nematode egg

Table 3.1. Types and prevalence of helminth eggs in whooping crane and sandhill crane fecal samples. Nematode, cestode, and small trematode eggs were observed on fecal flotation; large trematode eggs were observed on fecal sedimentation. N = number of fecal samples examined.

	Whooping Crane			Sandhill Crane		
		Prevalence			Prevalence	
	N	(%)	95% CI	N	(%)	95% CI
Nematode eggs	337			75		
Ascaridoidea						
120-124 µm x 68-80 µm		0.6	0.1, 2.4		0	na
34 µm x 32 µm		0.3	0.01, 1.9		0	na
Trichinelloidea						
52-58 µm x 24-28 µm		0.9	0.2, 2.8		1.3	0.07, 8.2
Dioctophymatoidea		1.5	0.5, 3.6		0	na
Habronematoidea		0.6	0.1, 2.4		1.3	0.07, 8.2
Rhabditoidea		1.2	0.4, 3.2		0	na
Strongylida						
48-60 µm x 22-30 µm		3.6	1.9, 6.3		0	na
50-64 µm x 18-20 µm		2.1	0.9, 4.4		0	na
74-82 µm x 28-36 µm		1.8	0.7, 4.0		1.3	0.1, 8.2
94-98 µm x 44-56 µm		1.2	0.4, 3.2		0	na
112-129 µm x 68-70 µm		0.9	0.2, 2.8		0	na
Total		14.8	11.3, 19.2		4.0	1.0, 12.0
Cestode eggs	337			75		
rough shell		0.3	0.01, 1.9		0	na
smooth shell		0	na		1.3	0.1, 8.2
Total		0.3	0.01, 1.9		1.3	0.1, 8.2
Trematode eggs						
30-38 µm x 14-20 µm	337	16.9	13.2, 21.4	75	0	na
70-80 µm x 46-50 µm	63	1.6	0.1, 9.7	20	40	20.0, 63.6
96-124 µm x 56-78 µm	63	9.5	3.9, 20.2	20	10	1.7, 33.1
Total	337	18.3	14.5, 23.0	75	13.3	6.9, 23.6

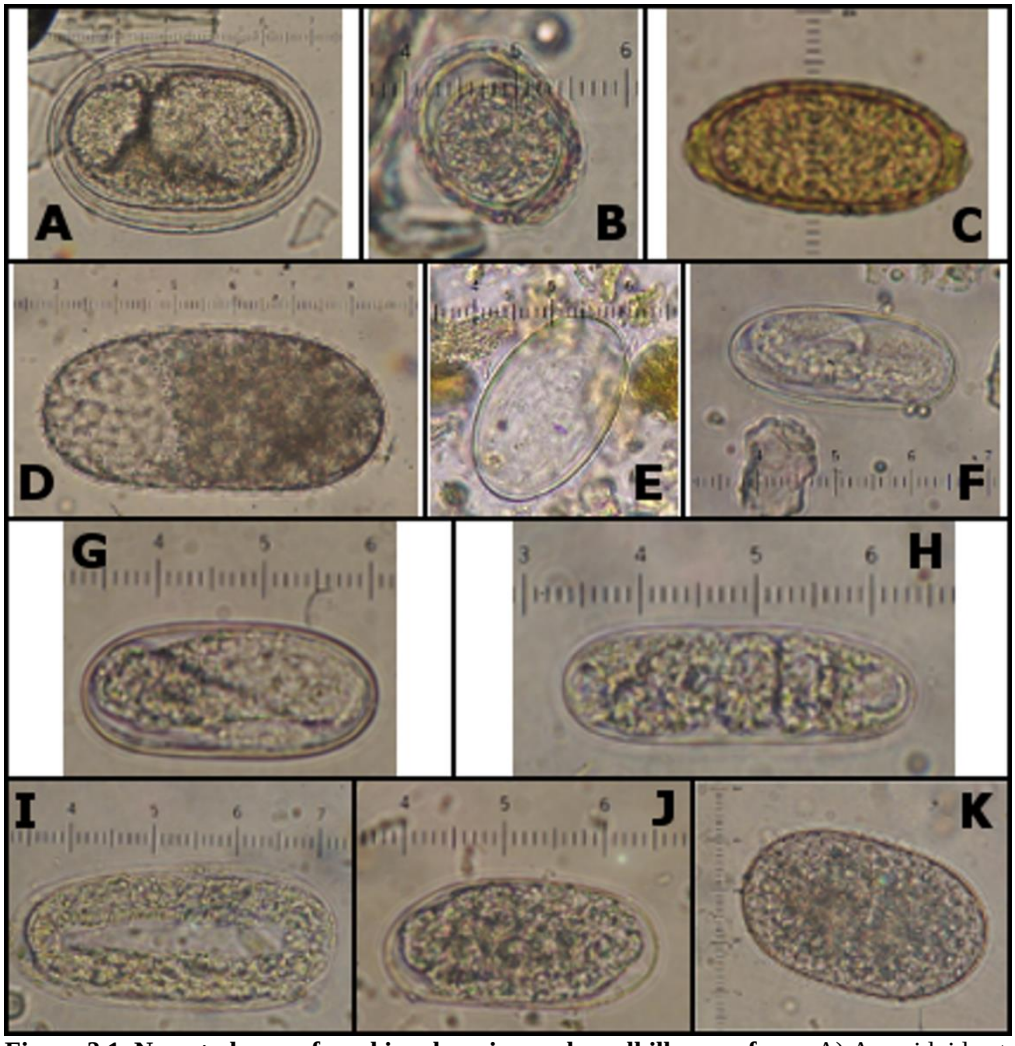

Figure 3.1. Nematode eggs found in whooping and sandhill crane feces. A) Ascaridoidea type egg from sandhill crane feces. B) Ascaridoidea type egg from whooping crane feces. C) Trichinelloidea type egg. D) Dioctyphymoidea type egg. E) Habronematoidea type egg. F) Rhabditoidea type egg. G-K) Strongylida type eggs. Scale: each major demarcation is 20 µm.

morphotypes. One was infected with egg morphotypes consistent with Dioctophymatoidea and Strongylida, and one was infected with two different morphotypes of eggs consistent with Strongylida. Additionally, nematode larvae or free-living adult nematodes were noted in 31 WHCR samples and one SACR sample. Cestode eggs of two different morphologies (Figure 3.2) were noted in one (0.6%) WHCR and one (1.3%) SACR sample.

We performed fecal sedimentation on a subset of 63 WHCR and 20 SACR fecal samples. Overall, 18.3% (95% CI 14.5%, 23.0%) of WHCR samples and 13.3% (6.9%, 23.6%) of SACR samples overall were positive for trematode eggs (Table 3.1). Trematode eggs were noted in at least one sample in each SACR population, and there were no significant differences in the proportion of positive samples among SACR populations (Fisher's exact p=0.79). There was also no significant difference in the overall proportion of positive samples between whooping and SACR (χ^2=1.07, df=1, p=0.30). We identified three types of trematode eggs based on morphology. One egg morphotype was found on fecal flotation in 57 (16.9%) WHCR samples and was oval, slightly asymmetric, tan, with an operculum on one end, and measured 30-38 µm x 14-20 µm (Figure 3.3A). This egg morphotype is consistent with *Tanasia*[90], *Brachylaima*[90], *Stomylotrema*[96], or *Amphimerus*[97], and was found in significantly more WHCR than SACR samples (χ^2=14.67, df=1, p=0.0001). The other two trematode egg morphotypes were found on fecal sedimentation. One was oval, yellow, with an operculum on one end, and measured 70-80 µm x 46-50 µm (Figure 3.3B). This egg morphotype is consistent with *Orchipedum jolliei* and was found in one (1.6%) WHCR sample and 8 (40%) SACR samples. The proportion of positive samples was significantly higher for sandhill than for WHCR for this egg type (χ^2=23.17, df=1, p<0.0001). The second egg morphotype found on sedimentation was oval, yellow, with an operculum on one end, and measured 96-124 µm x 56-78 µm (Figure 3C). This egg morphotype is consistent with *Prohyptiasmus grusi*[78], *Echinostoma revolutum*[98], *Strigea gruis*[92], or *Philophthalmus gralli*[99], and was found in 6 (9.5%) WHCR samples and 2 (10%) SACR samples. There was no significant difference in the proportion of positive samples

between WHCR and SACR for this egg morphotype (χ^2=0.003, df=1, p=0.95). Acanthocephalan eggs were not found in any samples.

Historic whooping crane necropsy records

We received parasitological reports from eight AWBP whooping cranes necropsied at the National Wildlife Health Center. Nematodes belonging to three orders, trematodes belonging to three orders, cestodes, and acanthocephalans were noted in AWBP whooping cranes (Table 3.2). Seven (87.5%) whooping cranes were infected with at least one species of helminth, and one bird was parasitized by seven species of helminth.

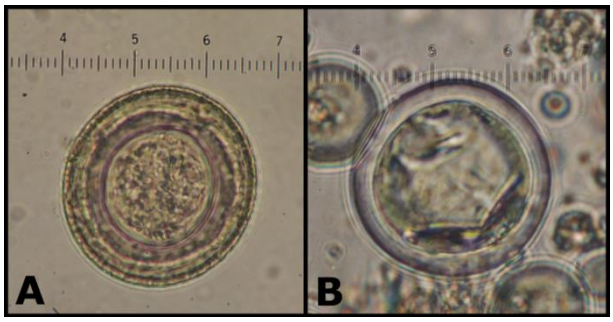

Figure 3. 2. Cestode eggs found in whooping and sandhill crane feces. A) Cestode egg from whooping crane feces. B) Cestode egg from sandhill crane feces. Scale: each major demarcation is 20 µm.

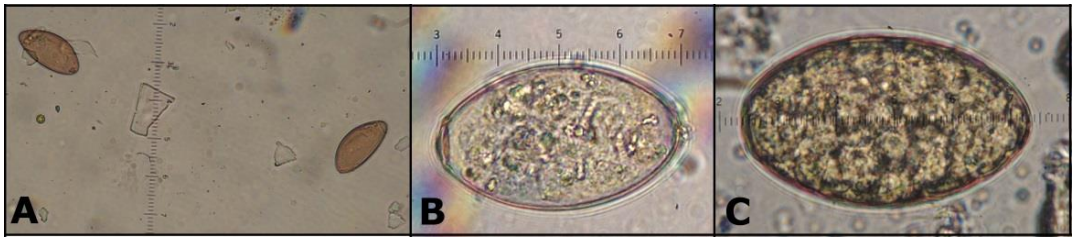

Figure 3.3. Trematode eggs found in whooping and sandhill crane feces. A) smallest egg type, consistent with *Brachylaima*, *Stomylotrema*, or *Amphimerus*.B) Egg type consistent with *Orchipedum jolliei*. C) Largest egg type, consistent with *Prohyptiasmus grusi*, *Echinostoma revolutum*, *Strigea gruis*, and *Philophthalmus gralli*. Scale: each large demarcation is 20 µm.

Table 3.2. Helminths noted in whooping and sandhill cranes at necropsy. Whooping crane data provided by the National Wildlife Health Center, Madison, WI. Whooping cranes were necropsied between December 1993 – January 2014.Sandhill crane data generated in this study. Sandhill cranes were necropsied between November 2012 – January 2014. Prov = proventriculus.

	Whooping Crane		Sandhill Crane		
Helminth	No. Examined	No. Infected	No. Examined	No. Infected	Organ Infected
Nematode					
Trichinelloidea					
Capillaria sp.	8	2			Intestine
Eucoleus sp.	4	1			Esophagus
Spirurida					
Pectinospirura sp.	6	1			Proventriculus
Schistorophus sp.	8	1			Gizzard, Intestine
Habronematoidea					
Tetrameres grusi			26	13	Proventriculus
Strongylida					
Epomidiostomum sp.	6	1			Proventriculus
Unidentified	7	2			Gizzard
Unidentified Larvae	8	2			Prov. Intestine
Unidentified Eggs	4	1			Feces
Trematode					
Echinostomida					
Paratanaisia bragai			108	1	Kidney
Echinostomatidae	8	1			Intestine
Opisthorchiida					
Amphimerus sp.	7	1			Liver
Heterophyidae	4	1			Crop
Ascocotyle filippei	8	1			Intestine
Ascocotyle gemina	8	1			Intestine
Leighia sp.	8	1			Gizzard, Intestine
Plagiorchiida					
Orchipedum jolliei			108	45	Trachea
Prohyptiasmus grusi			108	6	Body Cavity
Renicola sp.	7	1			Kidney
Unidentified Larva	8	1			Intestine
Cestode					
Gruitaenia gruis			99	9	Intestine
Unidentified Cestode	8	2			Intestine
Acanthocephala					Intestine
Unidentified	8	1	99	2	Intestine

Sandhill crane necropsy

We necropsied 108 sandhill cranes, including 44 from the Texas Panhandle, 24 from the Gulf Coast, and 40 from New Mexico. Nine birds from New Mexico had intestines removed prior to necropsy and could not be examined for cestodes or acanthocephalans. The proventriculus from a subset of birds (17 from the Gulf Coast, 9 from New Mexico) was examined specifically for *Tetrameres* sp. We identified three species of trematode (*Orchipedum jolliei*, *Prohyptiasmus grusi*, *Paratanaisia bragai*), one cestode (*Gruitaenia gruis*), one acanthocephalan (*Polymorphus minutus*), and one nematode (*Tetrameres grusi*) on necropsy (Figure 3.4). Overall, 54 (50%) birds were infected with one to four species of helminths, and single-species infections were most common. Fifteen birds (13.9%) were infected with two helminth species; two with *O. jolliei* and *P. grusi*, three with *O. jolliei* and *G. gruis*, and 10 with *O. jolliei* and*T. grusi*. Two birds (1.9%) were infected with three helminth species; one with *O. jolliei*, *T. grusi*, and *P. grusi*, and one with *O. jolliei*, *T. grusi*, and *G. gruis*. One bird (0.9%) was infected with four helminth species; *O. jolliei*, *P. grusi*, *P. bragai*, and *G. gruis*. *O. jolliei* was the most common helminth and was found in 45 (42%) birds overall, with a significantly higher prevalence in the birds from the Gulf Coast (χ^2=27.8, df=2, p<0.0001)(Table 3.3). Bird age and sex were not significant predictors of infection. The odds of infection with *O. jolliei* in birds from the Gulf Coast population was 12.5 (95% CI 3.6, 62.4) times higher than the odds in the Texas Panhandle population and 21.8 (95% CI 5.9, 113.7) times higher than the odds in the New Mexico population. Among infected individuals the average worm burden was 9.2, with a range from 1 – 93 (Figure 3.5). The other helminth species were each found in <10 individuals, with no significant differences between populations.

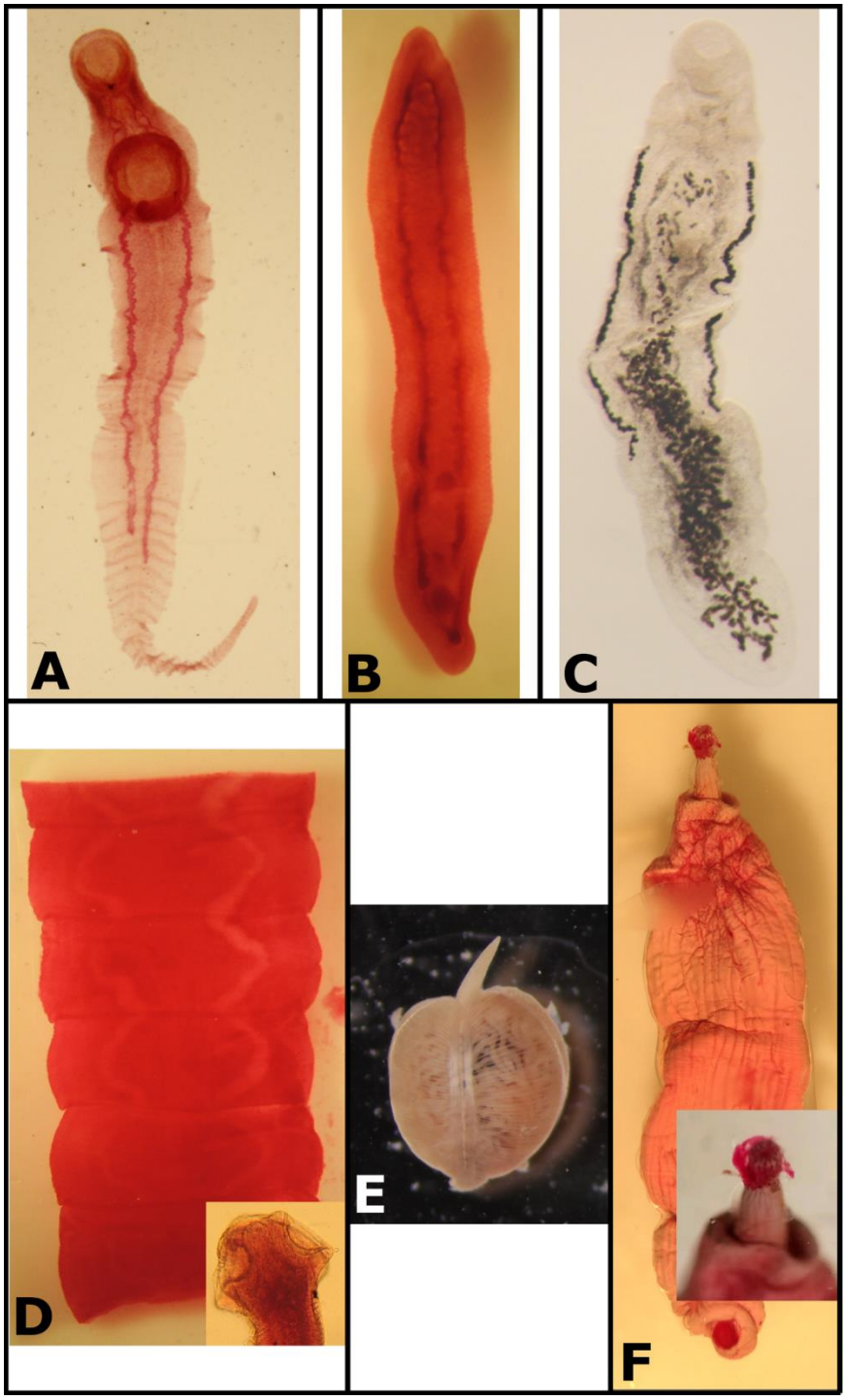

Figure 3.4. Helminths recovered from sandhill cranes at necropsy. A-C) Trematodes. A) *Orchipedum jolliei*. B) *Prohyptiasmus grusi*. C) *Paratanaisia bragai*. D) Cestode *Gruitaenia gruis*. Inset: scolex. E) Nematode *Tetrameres grusi* (female). F) Acanthocephalan *Polymorphus minutus*. Inset: proboscis.

Table 3.3. Prevalence of six helminth species in three wintering populations of sandhill cranes detected during necropsy.

	Texas Panhandle	Texas Gulf Coast	New Mexico	Total
N	44	24	40	108
Orchipedum jolliei	34.1%	87.5%	22.5%	41.7%
95% CI	21.0, 49.2	69.2, 96.5	11.8, 38.0	32.3, 51.4
Prohyptiasmus grusi	9.1%	8.3%	0	5.6%
95% CI	3.2, 21.0	1.5, 26.2	na	2.5, 11.7
Paratanaisia bragai	2.3%	0	0	0.9%
95% CI	0.1, 11.7	na	na	0.1, 4.8
*Tetrameres grusi**	na	70.6%	11.1%	50%
95% CI	na	45.6, 87.6	0.6, 44.3	30.0, 70.0
Gruitaenia gruis^	4.5%	8.3%	16.1%	9.1%
95% CI	0.8, 15.2	1.5, 26.2	6.6, 33.4	4.6, 16.4
Polymorphus minutus^	4.5%	0	0	2%
95% CI	0.8, 15.2	na	na	0.4, 6.7

* Gulf Coast N=17, New Mexico N=9
^ New Mexico N=31.

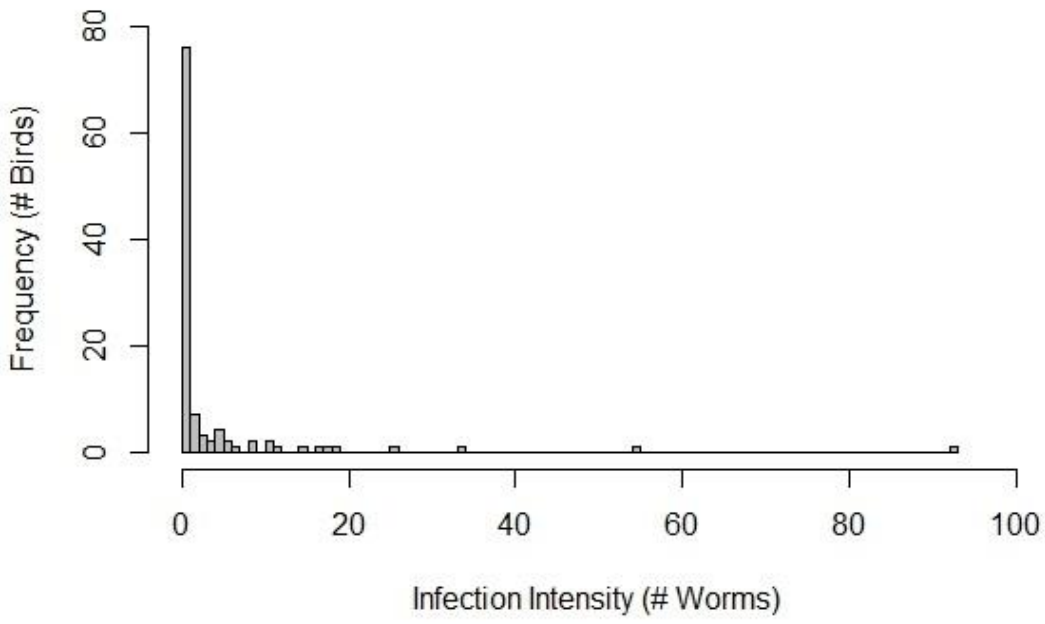

Figure 3.5. Distribution of *O. jolliei* worm burden in sandhill cranes (n=108).

Molecular analysis

We used PCR and DNA sequencing to further characterize the nematode eggs observed in fecal samples. Many sequence attempts failed or resulted in non-target amplification (e.g. blue crab DNA). Nematode sequences were recovered from 16 WHCR and one SACR samples. Three WHCR and the single SACR samples produced identical sequences which aligned most closely with *Strongyloides* sp. (Figure 3.6). The remaining 13 sequences aligned with soil-dwelling nematodes; including *Panagrolaimus* sp. (one sequence), *Acrobeloides* sp. (three sequences), *Aphelenchoides* sp. (two sequences), *Plectus* sp. (one sequence), *Diplolaimelloides* sp. (one sequence), *Monhystrella* sp. (one sequence), *Amblydorylaimus* sp. (two sequences), and *Ethmolaimus* sp. (two sequences).

We also used PCR and DNA sequencing to further characterize the trematode eggs observed in fecal samples and the adult Platyhelminthes (trematodes and cestodes) recovered from SACR at necropsy. We produced sequences from 16 WHCR and one SACR fecal samples, as well as the three trematodes and one cestode recovered from SACR at necropsy. *Gruitaenia gruis* formed a clade with three other cestode species, which served as the out-group for the analysis (Figure 3.7). Twelve WHCR samples were identical to each other and a previously published *Tanaisia fedtschenkoi* sequence[100]. *Paratanaisia bragai* also aligned in this clade, but the sequence was not identical to the previously published *P. bragai* sequence[101]. *Prohyptiasmus grusi* aligned with *Cyclocoelum* sp. Two WHCR sequence aligned with *Dicrocoelium* sp., one aligned with *Phagicola* sp., and one aligned with *Clinostomum* sp. The SACR fecal sample sequence was identical to *Orchipedum jolliei*.

The sequence of the acanthocephalan recovered from SACR was identical to a previously published *Polymorphus minutus* sequence over 882 bp[102]. We did not detect any positive samples using this acanthocephalan PCR on DNA extracted from fecal sedimentation samples (n=63 and n=20 for WHCR and SACR, respectively).

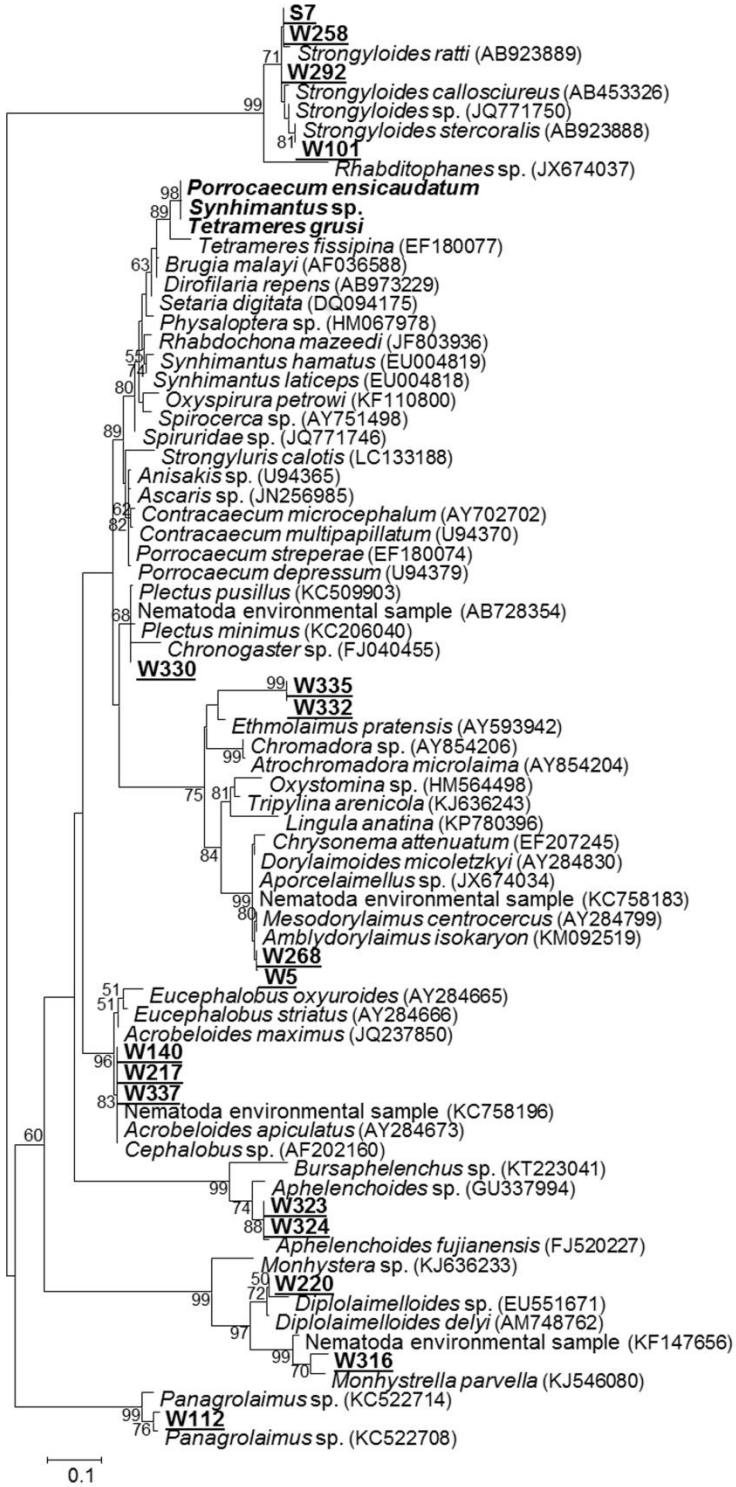

Figure 3.6. Phylogenetic analysis of nematode 18S rRNA sequences (453 bp) recovered from fecal samples. Sequences in bold were generated in this study; the single sample beginning with 'S' is from a sandhill crane; all those beginning with 'W' are from whooping cranes.

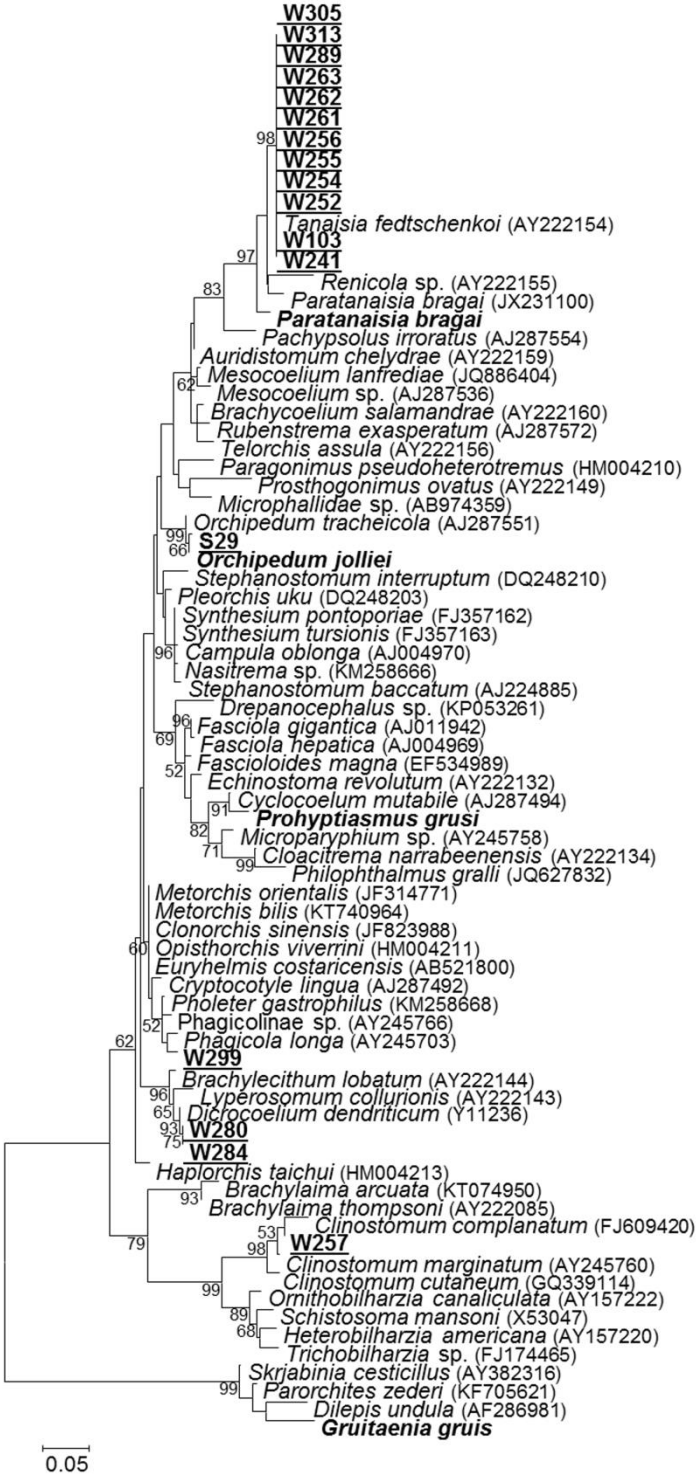

Figure 3.7. Phylogenetic analysis of trematode 18S rRNA sequences (491 bp) from whooping and sandhill crane fecal samples and adult trematodes recovered from sandhill cranes at necropsy. Sequences in bold were generated in this study; the samples beginning with 'S' are from sandhill cranes; all those beginning with 'W' are from whooping cranes. Fecal samples are underlined.

Discussion

A critical first step toward an understanding of the health impact of parasites on a target host population is a descriptive analysis of the parasite community in the target host species. We employed non-invasive sampling in combination with a surrogate species approach with a priority interest in characterizing the helminth parasites that may impact endangered whooping cranes while also gathering information on the specific parasites of SACR. We noted a higher prevalence and greater variety of nematode eggs in WHCR feces compared to SACR feces, with no difference in the prevalence of nematode eggs among the three SACR populations. We detected 11 different morphotypes of nematode eggs in WHCR fecal samples, but only three in SACR fecal samples. Although WHCR and SACR are sympatric at ANWR, they utilize different food resources (whooping cranes feed primarily on wolfberry, blue crab, clams, and aquatic snails in salt marshes during the winter; sandhill cranes feed primarily on waste grain, wolfberry, acorn, and insects in upland sites[60]), which may result in different exposures to nematodes. Further, differences in nematode communities between the two crane species may be due to differences in the microenvironment to which the cranes are exposed. Whooping cranes inhabit coastal salt marshes during the winter, whereas sandhill cranes prefer more upland habitat[85,103,104].

Historic data we present here revealed that nematodes in the order Strongylida were noted in AWBP whooping cranes at necropsy, specifically the genus *Epomidiostomum*, and we noted one egg type consistent with this genus in WHCR fecal samples (Strongylida-type egg, 94-98 µm x 44-56 µm)[95,105]. Nematodes in the superfamily Trichinelloidea were also historically noted in AWBP whooping cranes at necropsy, and we noted one egg type consistent with this superfamily in whooping and SACR fecal samples[106,107]. However, we cannot determine nematode species from egg morphology, and the two crane species may be parasitized by different nematode species within the superfamily Trichinelloidea. We also noted eggs consistent with the superfamilies Ascaridoidea, Dioctophymatoidea, Habronematoidea, and Rhabditoidea, which were not previously reported in the AWBP whooping cranes, but have been

reported in WHCR in Florida[17]. We recovered eggs consistent with the superfamily Habronematoidea from one SACR infected with *Tetrameres grusi*, lending further support to this egg type identification. Additionally, one sample with an egg consistent with the Rhabditoidea superfamily aligned with previously published Rhabditoidea sequences in the molecular analysis. However, the majority of nematode sequences matched most closely with soil-dwelling nematodes. Soil-dwelling nematodes may have been inadvertently consumed by the cranes during normal feeding behavior or may represent environmental contamination of the fecal samples. Fecal samples collected at ANWR were voided samples exposed to the environment for up to 24 hours prior to collection, which may have resulted in contamination with soil-dwelling nematodes. Samples collected from SACR at necropsy were collected directly from the large intestine, which prevented environmental contamination and may have contributed to the difference in the proportion of positive samples between WHCR and SACR. While the majority of nematodes in our study may be non-pathogenic or otherwise represent pass-through or contamination by soil-dwelling nematodes, we recovered *Strongyloides* sp. DNA sequences from one SACR and three WHCR samples, all voided feces from ANWR. These sequences may represent true infections, since *Stongyloides* sp. have been reported in WHCR and SACR[17,80,82], but environmental contamination with the free-living stage of *Strongyloides* sp. cannot be ruled out. Although representatives of each nematode order or superfamily reported in cranes were included in our phylogenetic analysis when available, few superfamilies are represented in GenBank. Accordingly, while we report many soil-dwelling nematode species as the closest available genetic matches to the nematodes in crane samples, these samples may in fact be other species not yet represented with genetic information in the public database.

The proportion of fecal samples positive for trematode eggs was not significantly different between WHCR and SACR samples, however we noted different trematode egg types in the two crane species. The smallest egg type was found only in WHCR samples and was morphologically and molecularly consistent with *Tanasia fedschenkoi*. *T. fedschenkoi* has been previously reported in Florida SACR, along with trematodes

that have morphologically similar eggs including *Brachylaima* sp, and *Stomylotrema* sp.[17,80,82]. The second egg type was found predominantly in SACR samples and was morphologically and molecularly consistent with *Orchipedum jolliei*[88], and our observations include an individual sandhill in which *O. jolliei* adults were found during necropsy and eggs were in the feces. The largest egg type was found mostly in WHCR samples and was consistent with *Prohyptiasmus grusi*[78], *Echinostoma revolutum*[98], *Strigea gruis*[92], or *Philophthalmus gralli*[99], which have been previously reported in North American cranes[17,76,82], however we cannot further identify this egg type. *O. jolliei* was the most common trematode in SACR fecal samples, but was not noted in WHCR samples, whereas *T. fedtschenkoi* was the most common trematode in WHCR fecal samples, but was not noted in SACR fecal samples. Differences in trematode communities are likely due to differences in diet between the two crane species leading to different exposures to the invertebrate intermediate hosts for these species. However, the life-cycles of these trematode species have not been elucidated, and the intermediate hosts are unknown. Differences in trematode communities may also be due to differential host-compatibility between WHCR and SACR. However, *T. fedtschenkoi* has a wide host range, including SACR[82,90], and *O. jolliei* has been reported in mallard ducks (*Anas platyrhynchos*) in Iraq[108] and in a flamingo (*Phoenicopterus ruber*) in Canada[109] in addition to reports in SACR.

O. jolliei was also the most common trematode noted at necropsy and adults were present in 42% of SACR overall, however the prevalence was significantly higher in birds harvested in the Gulf Coast compared to the Texas Panhandle and New Mexico. The birds harvested in New Mexico are part of the Rocky Mountain population, whereas birds harvested in the Panhandle and along the coast are part of the Mid-continent population. This difference could be related to a differential distribution of the intermediate host for *O. jolliei*, which is currently not identified. Although they are both part of the Mid-continent population, birds wintering along the Gulf Coast may breed further east than birds wintering in the Texas Panhandle[86], therefore these two subpopulations may be exposed to different habitats and intermediate hosts on the

breeding grounds. Because SACR in this study were harvested shortly after their arrival on the winter grounds, and any parasites acquired on the winter grounds would not have developed to adults prior to harvest, and therefore we conclude that *O. jolliei* transmission likely occurs on the breeding grounds or during migration.

DNA sequencing and morphologic characteristics suggest the identity of the acanthocephalan recovered from SACR in this study is *Polymorphus minutus,* which has not previously been reported in cranes. The acanthocephalan genera *Centrorhynchus kuntzi* and a *Southwellina* sp. have been reported in reintroduced WHCR in Florida[17], and an unidentified acanthocephalan has been reported in one SACR harvested in west Texas[110] and in one historical AWBP whooping crane. Acanthocephalans appear to be rare parasites in sandhill cranes and whooping cranes.

The majority of infected SACR harbored a single helminth species at necropsy, and apparent adult worm burden was low (<5 worms) in the majority of infections. Similarly, based on historic data, the majority of infected WHCR also harbored a single helminth species at necropsy. Interestingly, we did not note any gross pathology associated with infection in SACR, and all birds were in good body condition at necropsy. The apparent helminth community presented here is likely a conservative estimation due to incomplete parasite recovery from birds during necropsy, and small sample sizes of birds from some populations. Further, infected birds may shed eggs intermittently. To minimize the effect of intermittent egg shedding on parasite detection, we increased the likelihood of finding infections by repeated sampling at ANWR.

Whooping cranes and sandhill cranes are exposed to different helminth communities, likely due to differences in diet and habitat preference. Nonetheless, SACR may provide some insight into the potential clinical effects, or lack thereof, of helminths on WHCR, and therefore SACR may be useful as a surrogate species in this context. A recent study found comparable patterns of nematode communities using non-invasive and invasive sampling of African buffalo[111], and we show that non-invasive fecal samples can be used to monitor the helminth community in AWBP whooping cranes. Future studies are needed to investigate the clinical and histologic implications of

helminth infection on individuals, but also population-level impacts on survival and fecundity in the context of species conservation.

CHAPTER IV

A NOVEL HAEMOSPORIDA CLADE AT THE RANK OF GENUS IN NORTH AMERICAN CRANES (AVES: GRUIFORMES)

Overview

The unicellular blood parasites in the order Haemosporida are highly diverse, infecting many vertebrates, are responsible for a large disease burden among humans and animals, and have reemerged as an important model system to understand the evolutionary and ecological dynamics of host-parasite interactions. The phylogenetics and systematics of Haemosporida are limited by poor sampling of different vertebrate host taxa. We surveyed the Haemosporida of wild whooping cranes (*Grus americana*) and sandhill cranes (*Grus canadensis*) (Aves: Gruiformes) using a combination of morphological and molecular approaches. We identified *Haemoproteus antigonis* in blood smears based on published morphological descriptions. Phylogenetic analysis based on partial cytochrome *b* (cyt*b*) and cytochrome oxidase (coI) sequences placed *H. antigonis* parasites in a novel clade, distinct from all avian Haemosporida genera for which cyt*b* and/or coI sequences are available. Molecular clock and divergence estimates suggest this crane clade may represent a new genus. This is the first molecular description of *H. antigonis* and the first report of *H. antigonis* in wild whooping cranes, an endangered bird in North America. Further sampling of Haemosporida, especially from hosts of the Gruiformes and other poorly sampled orders, will help to resolve the relationship of the *H. antigonis* clade to other avian Haemosporida genera. Our study highlights the potential of sampling neglected host species to discover novel lineages of diverse parasite groups.

Introduction

Haemosporida are protozoan parasites that infect diverse vertebrate host tissues and are vectored by various dipteran biting flies. The order contains the agents of human malaria and related parasites. Avian Haemosporida have been described from a wide range of host species and geographic localities. Over 200 species of avian Haemosporida

have been described, with most species descriptions based on morphologic characteristics of blood stages and host range[112]. Recently, many researchers have used molecular techniques to detect Haemosporida, although the depth of screening across host taxa remains very heterogeneous. The MalAvi database has been established as a publicly available repository for Haemosporida sequences of the 5' end of the mitochondrial cytochrome *b* (cyt *b*) gene[113]. Molecular studies have shown some Haemosporida species can infect a broader range of host species than previously thought, including host species in different families[114-116]. While the advent of these molecular techniques have uncovered an unexpected diversity in Haemosporida and their interactions with avian hosts, the increasing use of molecular techniques to identify Haemosporida infections has led to numerous sequences in the GenBank (http://www.ncbi.nlm.nih.gov) and MalAvi databases that are identified only to genus. This underscores the importance of combining molecular and morphologic descriptions to accurately identify species.

Phylogenies based on molecular data can vary widely depending on the species included and the method of analysis[117]. There are wide discrepancies in the literature as to the phylogenetic relationship of Haemosporida genera, depending on the gene sequence(s) analyzed, the method of analysis, and the Haemosporida species included in the analysis. Analysis of parasite cyt *b* sequences from a variety of bird, lizard, and mammal hosts show *Plasmodium* is paraphyletic, forming one clade with *Hepatocystis* and a second clade with *Haemoproteus*[118]. A recent study by Lutz et al.[119] also suggests that *Plasmodium* is paraphyletic. In contrast, analysis of four genes showed avian *Haemoproteus* fall into two clades which are sister to *Plasmodium*[120]. Alternatively, phylogenetic analyses of only avian parasites show the subgenus *Haemoproteus* (*Haemoproteus*) as a sister clade to *Plasmodium* and *Haemoproteus* (*Parahaemproteus*)[121,122]. Additionally, a group of parasites from raptors formed a unique clade not closely related to *Plasmodium* or *Parahaemoproteus*[123]. All of these phylogenetic hypotheses, developed with maximum-likelihood or Bayesian techniques, place *Leucocytozoon* as an outgroup to *Plasmodium* and *Haemoproteus*. However, an

analysis using relaxed molecular clock methods showed *Plasmodium* as paraphyletic with two major subgroups: mammalian *Plasmodium* and *Hepatocystis*, and avian *Plasmodium, Leucocytozoon, Haemoproteus*, and *Parahaemoproteus*[124]. In contrast, Borner et al.[125] analyzed a set of 21 nuclear genes, and the resulting phylogeny showed *Plasmodium* as monophyletic and *Leucocytozoon* in a basal position to the rest of the Haemosporida. Further complicating the picture, the majority of researchers in North America use PCR assays targeting the 3' end of the cyt *b* gene[126,127], whereas the majority of researchers in Europe use PCR assays targeting the 5' end of the cyt *b* gene[113,116], with inadequate overlap to compare sequences generated with different assays. While both portions of the gene are represented in GenBank, the MalAvi database consists of only sequences generated with assays targeting the 5' end[113].

Phylogenetic relationships are greatly influenced by the taxa included in the analysis, and the majority of published avian Haemosporida sequences were recovered from passerine and columbiform hosts, while studies of hosts in other orders are severely lacking. Two crane species (Gruidae, order: Gruiformes) occur in North America, the endangered whooping crane (*Grus americana*) and the abundant sandhill crane (*Grus canadensis*). Prior studies of Haemosporida in cranes of North America are based on examination of blood smears and include descriptions of *Haemoproteus antigonis, Haemoproteus balearicae*, *Plasmodium-polare*-like, and *Leucocytozoon grusi* in sandhill cranes[128-131], and *Haemoproteus antigonis* in a small number of non-migratory whooping cranes in Florida[21]. Hemoparasites have not been previously studied in the only self-sustaining migratory population of whooping cranes (the Aransas-Wood Buffalo population; AWBP). Associated with our broad surveys of parasites infecting AWBP whooping cranes[84], we identified *Haemoproteus antigonis* on several blood films based on morphology. We also present a phylogenetic analysis of the Haemosporida that challenges the placement of *H. antigonis* in the genus *Haemoproteus*. Instead, our analysis suggests these parasites represent a novel evolutionary lineage of parasites identified in North American cranes, and highlight the importance of sampling neglected

vertebrate taxa to resolve the evolutionary relationships of malaria parasites and related Haemosporida.

Methods

Sample collection

Whooping crane blood samples were collected by one of the authors (BKH) as part of an ongoing telemetry and health monitoring study of the AWBP whooping cranes[132]. Birds were captured manually (pre-fledging juveniles) or using a remote triggered snare and manually restrained under valid federal, state and provincial permits. Blood was drawn from the jugular vein. A blood smear was made immediately after sample collection, air dried and fixed with methanol within 8 hours in the field. An aliquot of whole blood was preserved in Longmire's buffer (0.1M Tris, 0.1M EDTA, 0.01M NaCL, 0.5% SDS, pH 8.0). All field techniques were approved by a University of Wisconsin Animal Care and Use Committee (protocol no. V01506-0-10-11). Samples included in this study were collected during the summer at Wood Buffalo National Park (WBNP) and during the winter at Aransas National Wildlife Refuge (ANWR) between December 2009 and February 2014.

We collected blood samples from hunter-harvested sandhill cranes at necropsy between November 2012 and January 2014 through relationships with the Texas Parks and Wildlife Department, New Mexico Department of Game and Fish, and private hunting clubs and outfitters. All birds were either subjected to necropsy in the field immediately post-harvest or frozen at -20ºC immediately post-harvest and subjected to necropsy in the laboratory at a later date. Each carcass was subjected to a full gross necropsy, at which time we collected a blood sample, either whole blood or blood clot which had pooled in the coelomic cavity. Blood samples were frozen at -20ºC until DNA extraction.

Morphologic detection of Haemosporida

Approximately 2 cm^2 of the red blood cell monolayer on each blood smear was examined at low magnification (X500), and at least 100 fields were examined at high magnification (X1250), as recommended by Valkiunas[112]. Each blood smear was

examined for 15-20 minutes, and any parasites noted were examined and measured at 1250X. Morphologic identification of parasites was determined using a published taxonomic key[112].

Molecular detection of Haemosporida

DNA extraction. DNA was extracted from 100 µl of whole blood using the E.Z.N.A Tissue Extraction kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions for tissue extraction with modifications including an overnight lysis step at 55ºC and elution into 100 µl of elution buffer.

Haemosporida screening. First, *Plasmodium* and *Haemoproteus* infections were detected using a nested PCR reaction targeting an approximately 500 bp region of the 3' end of the cyt *b* gene. The first PCR reaction used the primers 3932F[133] and DW4[118] at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix E (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 1 µl of sample template. The second PCR reaction used the primers 413F and 926R[126] at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR was used as the template. In both rounds of PCR, cycling parameters were as described by Fecchio et al.[133]. A sample collected from a northern cardinal (*Cardinalis cardinalis*) and known to be infected with *Plasmodium* was used as a positive control[134].

To generate a longer portion of the gene, we also used a nested PCR reaction targeting an approximately 700 bp region of the 5' end of the cyt *b* gene. The first PCR reaction used the primers DW2 and DW4[118] at a concentration of 0.4 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, and 1 µl of sample template. The second PCR used the primers LeucoF and LeucoR[135] at a concentration of 0.4 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, and 2 µl of the product from the first PCR, diluted 1:20. In both rounds of PCR, cycling parameters were as described by Sehgal et al.[135].

Finally, we used a nested PCR reaction targeting an approximately 900 bp region of the mitochondrial cytochrome oxidase subunit I (*coI*) gene. The first PCR reaction used the primers *coI*/outerF and *coI*/outerR[120] at a concentration of 0.3 µM in a 15 µl reaction with remaining reaction components as outlined above. The second PCR used the primers *coI*/nestedF and *coI*/nestedR[120] at a concentration of 0.3 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR, diluted 1:20, was used as the template. In both rounds of PCR, cycling parameters were as described by Martinsen et al.[120]. The same positive control used for cyt *b* PCR reactions was also used for *coI* PCR reactions.

Sequencing and phylogenetic analyses

Amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Purified samples were submitted for bi-directional sequencing to Eton Bioscience Inc. (San Diego, CA). Forward and reverse sequences were aligned and a consensus sequence was determined using Clustal W within Mega 6.0[44]. Samples were considered positive if a DNA sequence was obtained for which the identity matched most closely to a Haemosporida species in GenBank. For samples which produced a sequence for both cyt *b* assays, sequences were aligned for each sample and a consensus sequence was generated. Sequences for the cyt *b* amplicons overlapped by approximately 400 bp, resulting in an approximately 800 bp consensus sequence. Chromatographs were examined manually, and sequences with double nucleotide peaks were separated using phasing. For otherwise clean samples with the same double nucleotide peaks in both the forward and reverse sequences, sequences containing all possible combinations of nucleotides at the base pairs with double nucleotide peaks were created and compared to clean sequences generated in this study and in GenBank. The two sequences which were identical to clean sequences were used in the phylogenetic analysis. All sequences were compared to known Haemosporida sequences using the BLAST tool in GenBank and were aligned with the closest matches and additional publicly available avian Haemosporida species sequences representative of unique clades in previous studies[115,118,120,136-139] (Table 4.1). We also compared cyt *b*

sequences to previously published avian Haemosporida sequences using the BLAST tool in MalAvi, however the longer sequences available in GenBank were used for phylogenetic analysis. After alignment, sequences were cropped to the same length at the first conserved base-pair closest to each end of the sequence. Samples with poor quality sequences in one or both directions were excluded from phylogenetic analysis. Representatives of all unique sequences produced during this project and utilized in the phylogenetic analysis were deposited in GenBank (Accession #KX223839 – KX223846) (Table 4.2).

Phylogenetic relationships were first explored in Mega 6.0 using the maximum likelihood method based on a general time reversible with gamma distribution (GTR+G) model of evolution using the bootstrap method with 1000 replicates[94]. Using BEAST (v.1.7; [140], we reconstructed phylogenetic trees (GTR+I+**Γ**, Yule process, 10,000,000 generations sampling every 1,000 trees) under two sets of priors: one with a strict molecular clock and one with a relaxed (uncorrelated lognormal) molecular clock. After determining that model parameter values were stable (ESS > 200, Tracer v1.6[141], we calculated BayesFactors (in Tracer v1.6) to determine which clock model provided a better estimate of the data. Using TreeAnnotator[140], we reconstructed the maximum clade credibility tree and then visualized the tree using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Divergence estimates

Using uncorrected distances, we calculated dissimilarity matrices within and between all major groups from the phylogenetic analyses. The groups we compared were: crane versus non-crane parasites, crane versus *Leucocytozoon* parasites, crane versus *Haemoproteus* parasites, crane versus *Parahaemoproteus* parasites, and crane versus *Plasmodium*/*Polychromophilus* parasites.

Table 4. 1. Species and accession numbers of GenBank sequences used in phylogenetic analyses.

Species Name (cyt *b*)	GenBank Accession #	Species Name (coI)	GenBank Accession #
Plasmodium berghei	DQ414646	*Plasmodium circumflexum*	KM434214
Plasmodium circumflexum	JN164734	*Plasmodium Haemamoeba* sp.	EF011227
Plasmodium elongatum	AF069611	*Plasmodium relictum*	EU254579
Plasmodium falciparum	JF923761	*Haemoproteus turtur*	EU254592
Plasmodium gallinaceum	AY099029	*Haemoproteus passeris*	EU254599
Plasmodium gemini	EU834708	*Parahaemoproteus vireonis*	NC012447
Plasmodium giganteum	AY099053	*Haemoproteus belopolskyi*	EU254603
Plasmodium knowlesi	EU880496	*Haemoproteus fringillae*	EU254604
Plasmodium lutzi	KC138226	*Haemoproteus columbae*	FJ168562
Plasmodium relictum	JN164731	*Leucocytozoon fringillinarum*	FJ168564
Plasmodium vivax	KF591834	*Leucocytozoon majoris*	FJ168563
Plasmodium yoelii	XM721750	*Parahaemoproteus* sp.	GU251987
Plasmodium sp.	EU627831	*Leucocytozoon* sp.	EU254565
Plasmodium sp.	EU627835	*Haemoproteus* sp.	EU254602
Plasmodium sp.	GQ141560	*Plasmodium* sp.	EU254590
Plasmodium sp.	GQ141588	*Plasmodium* sp.	EU254593
Plasmodium sp.	GQ141594	*Plasmodium* sp.	EU254588
Plasmodium sp.	GQ141604	*Plasmodium* sp.	EU254589
Plasmodium sp.	HQ724295	*Parahaemoproteus* sp.	GU251985
Plasmodium sp.	JN164732	*Parahaemoproteus* sp.	GU251979
Haemoproteus anatolicum	KM068154	*Haemoproteus* sp.	EU254605
Haemoproteus balmorali	DQ630014	*Haemoproteus* sp.	KJ499987
Haemoproteus belopolskyi	DQ630006	*Leucocytozoon* sp.	EU254564
Haemoproteus columbae	FJ168562	*Leucocytozoon* sp.	KM610046
Haemoproteus iwa	KC754966	*Haemoproteus lanii*	KM434211
Haemoproteus lanii	DQ630012	*Plasmodium relictum*	HQ228562
Haemoproteus majoris	JN164727	*Plasmodium Bennettinia* sp.	EF011230
Haemoproteus mesnili	KF049514	*Haemoproteus minutus*	KM434207
Haemoproteus minutus	DQ630013	*Haemoproteus belopolskyi*	KM434209
Haemoproteus pallidus	DQ630005	*Haemoproteus pallidus*	KM434210
Haemoproteus payevskyi	DQ630009	*Haemoproteus tartakovskyi*	KM434212
Haemoproteus sp.	GQ395668	*Haemoproteus parabelopolskyi*	KM434216
Haemoproteus sp.	EU627834	*Plasmodium ashfordi*	KM434215
Haemoproteus sp.	EU627829	*Plasmodium relictum*	KM434213
Haemoproteus sp.	GQ141564	*Plasmodium Giovannolaia* sp.	EF011228
Haemoproteus sp.	GQ141567	*Plasmodium relictum*	EF011226
Parahaemoproteus vireonis	FJ168561	*Plasmodium Novyella* sp.	EF011224
Parahaemoproteus sp.	GQ141557	*Plasmodium chabaudi*	EF011200
Parahaemoproteus sp.	GQ141584	*Plasmodium Huffia* sp.	EF011211

Table 4.1 continued

Species Name (cyt *b*)	GenBank Accession #	Species Name (coI)	GenBank Accession #
Parahaemoproteus sp.	GQ141586	*Plasmodium berghei*	EF011199
Parahaemoproteus sp.	GQ141589	*Plasmodium gallinaceum*	KP025674
Parahaemoproteus sp.	GQ141621		
Parahaemoproteus sp.	GU251998		
Polychromophilus sp.	KF159714		
Leucocytozoon fringillinarum	FJ168564		
Leucocytozoon majoris	FJ168563		
Leucocytozoon sp.	KF479480		
Leucocytozoon sp.	EU627797		

Table 4.2. Results of blood film examination and three PCR assays targeting avian Haemosporida for 27 AWBP whooping cranes.

Crane ID	Blood Film	cyt *b* (724 bp)	coI (370 bp)
W01	None	0	*Plasmodium*
W02	None	*H. antigonis*	*Plasmodium*
W03	None	0	0
W04	None	*Plasmodium*	0
W05	None	0	*Plasmodium*
W06	*H. antigonis*	*H. antigonis* (KX223839)	0
W07	None	0	0
W11	*H. antigonis*	*H. antigonis*	*H. antigonis* (KX223845)
W12	None	0	*Plasmodium*
W14	None	*H. antigonis*	*Plasmodium*
W15	None	0	0
W18	*H. antigonis*	*H. antigonis* (KX223840)	*H. antigonis*
W19	*H. antigonis*	*H. antigonis*	0
W20	None	0	0
W21	None	0	*H. antigonis*
W22	None	0	*H. antigonis*
W23	*H. antigonis*	*H. antigonis* (KX223841)	*H. antigonis* (KX223846)
W24	*H. antigonis*	0	*H. antigonis*
W26	None	0	*H. antigonis*
W27	None	*H. antigonis*	0
W28	None	0	0
W30	*H. antigonis*	0	0
W31	None	0	0
W32	*H. antigonis*	0	0
W33	*H. antigonis*	0	*Plasmodium*
W51	None	*H. antigonis* (KX223844)	*H. antigonis*
W55	None	*Plasmodium*	*Plasmodium*

Results

Morphological screening

We examined blood smears from 27 whooping cranes (Table 4.2). We noted Haemosporida infection on nine (33.3%) blood smears on microscopy, none of which had morphologic evidence of mixed infection. All infections showed low parasitemia (<1 parasite per 1000 red blood cells). Macrogametocyte description and dimensions noted in this study were compatible with *Haemproteus antigonis* noted on paratype and voucher blood films and with previously published descriptions (Table 4.3)[112,131].

Macrogametocyte (Figure 4.1A and B). Broadly sausage-shaped, with the ends sometimes slightly enclosing the host cell nucleus, but not completely encircling it. The average parasite length and width were 13.3 µm and 5.0 µm, respectively. The host cell nucleus was markedly displaced laterally, and the average nucleus displacement ratio (NDR) was 0.3. The cytoplasm was granular and stained deep blue, with medium-sized pigment granules (average 21) scattered randomly throughout. The parasite nucleus stained pale to deep pink and was located in the middle 1/3 of the parasite. The average length and width of the parasite nucleus were 3.0 µm and 1.8 µm, respectively.

Microgametocyte (Figure 4.1C). Similar in configuration to the macrogametocyte. The microgametocyte cytoplasm stained pale blue, and the nucleus was diffuse and difficult to identify.

Table 4.3. Morphometric parameters of gametocytes and host erythrocytes of *Haemoproteus antigonis*. Parasites noted on WHCR blood films in this study and *H. antigonis* noted on paratype and voucher blood films are listed along with previously published *H. antigonis* parameters. Length and width are given in micrometers. NDR is the nucleus displacement ratio, calculated as described in Valkiunas[112].

	This study			*H. antigonis* voucher			Bennett et al. 1975		
Parameter	n	mean	sd	n	mean	sd	n	mean	sd
Uninfected erythrocyte	30			30			50		
Length		14	1.1		13.3	1.2		13.7	1
Width		7.6	0.5		7.3	0.7		7.5	0.5
Nucleus length		6.2	0.8		6.2	0.6		6.1	0.6
Nucleus width		2.7	0.4		2.8	0.5		2.8	0.3
Erythrocyte parasitized by macrogametocyte	30			30			50		
Length		14.9	1.1		13.4	1.0		13.6	1.2
Width		8.6	0.7		8.3	0.6		8.5	1.1
Nucleus length		5.8	0.8		6.0	0.5		5.9	0.8
Nucleus width		2.5	0.3		2.6	0.4		2.4	0.4
Erythrocyte parasitized by microgametocyte	9			15					
Length		14.1	1.2		13.6	1.1			
Width		8.2	0.5		8.6	0.8			
Nucleus length		5.2	0.7		5.9	0.5			
Nucleus width		2.7	0.3		2.7	0.3			
Macrogametocyte	30			30			50		
Length		14.1	1.4		13.8	1.3		13	2
Width		4.7	0.5		4.6	0.9		4.8	1.1
Nucleus length		2.5	0.3		3.0	0.4		3.6	0.9
Nucleus width		2.1	0.3		1.9	0.4		3.4	1.1
No. pigment granules		20.5	2.8		18.7	3.7		19.3	4
NDR		0.4	0.2		0.4	0.1		0.4	
Microgametocyte	9			15					
Length		14.5	2.1		14.1	1. 6			
Width		4.6	0.7		4.3	0.6			
Nucleus length				11	6.4	1.5			
Nucleus width				11	2.9	0.6			
No. pigment granules		17.1	2.9		15.0	2.5			
NDR		0.4	0.2		0.5	0.2			

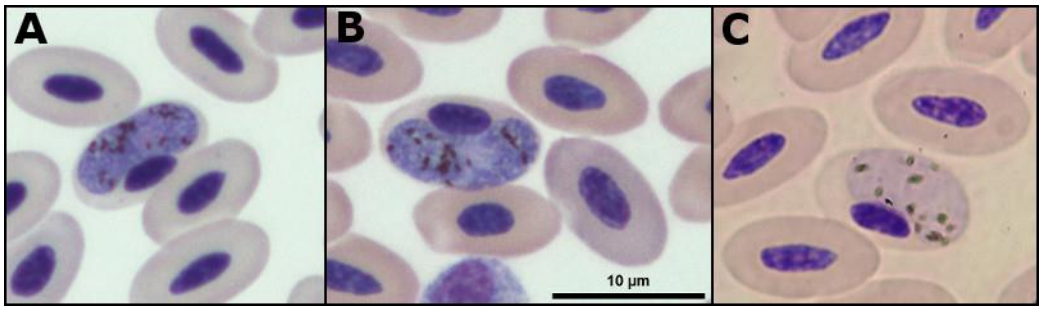

Figure 4.1. Crane Haemosporida identified as *Haemoproteus antigonis*. A,B) Mature macrogametocyte. C) Mature microgametocyte.

Molecular analysis

We screened 61 whooping crane and 102 sandhill crane samples, and obtained 724 bp consensus DNA sequences from the cyt *b* gene of *Haemoproteus antigonis* for 14 samples, and 370 bp consensus sequences from the *coI* gene of *H. antigonis* for 22 samples from both crane species, which were included in the phylogenetic analysis. Twelve sequences from the cyt *b* gene, represented in GenBank by # KX223839, KX223840, KX223841, KX223844, were identical to each other. One sequence (KX223843) differed at one base pair, which resulted in a change in the encoded amino acid from histidine to arginine. One sequence (KX223842) differed from the twelve identical sequences at six base pairs, however only two base pair changes resulted in changes to the encoded amino acid (histidine to leucine, and isoleucine to threonine). Eight sequences from the *coI* gene, represented in GenBank by #KX223845, KX223846, were identical to each other, and the remaining 14 sequences, all isolated from sandhill cranes and represented in GenBank by #KX223854, KX223855 (see Chapter V), were identical to each other, but differed from the eight at one base pair. The single base pair change results in a change in the encoded amino acid from phenylalanine to serine. Table 1 shows the results of the two PCR assays and GenBank accession numbers for the 27 whooping cranes for which we also had blood smears.

Phylogenetic analyses. The data were best described with a relaxed molecular clock (BayesFactors $\Delta = 37.64$). Phylogenetic relationships between major clades were not

well supported, but generic clades were very well supported with posterior probabilities of 1 in most cases, including the crane parasites (Figure 4.2). *Plasmodium*, *Haemoproteus, Parahaemoproteus*, and *Leucocytozoon* were each monophyletic, with *Leucocytozoon* and *Haemoproteus* forming a clade sister to the clade formed by *Plasmodium* and *Parahaemoproteus*. *Haemoproteus antigonis* sequences recovered from sandhill crane samples were identical to *H. antigonis* sequences recovered from whooping crane samples, and *H. antigonis* formed a novel clade at the level of genus, sister to *Plasmodium* and *Parahaemoproteus*.

Divergence estimates. Uncorrected distances within crane parasites ranged from 0.008 to 0.0014 and that between crane parasites and other genera ranged from 0.075 to 0.198 (Table 4.4).

Table 4. 4. Uncorrected (p) distances between crane parasites and other clades (i.e., genera).

Comparison	Uncorrected distances
Cranes-*Leucocytozoon*	0.158-0.197
Cranes-*Haemoproteus*	0.087-0.135
Cranes-*Parahaemoproteus*	0.080-0.113
Cranes-*Plasmodium*/*Polychromophillus*	0.075-0.198

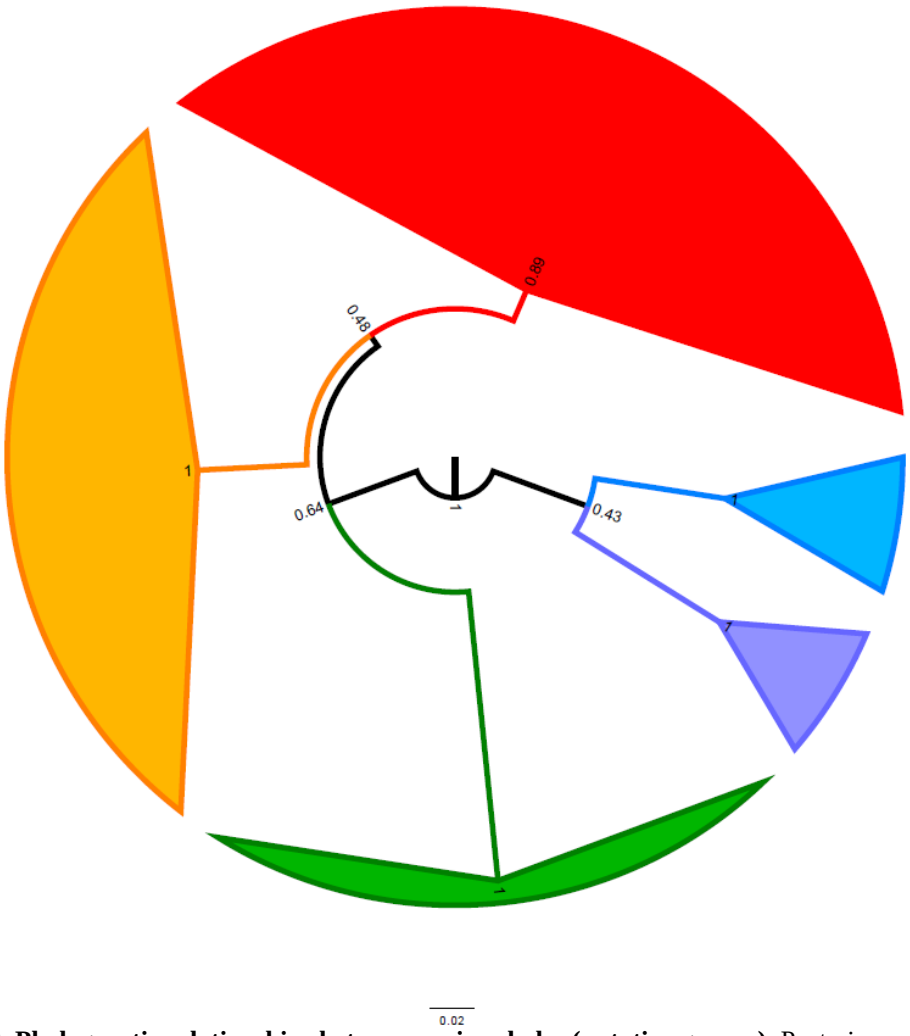

Figure 4.2. Phylogenetic relationships between major clades (putative genera). Posterior probability values are indicated on branches. Colors correspond to genera: *Haemoproteus* (blue), *Leucocytozoon* (purple), *Parahaemoproteus* (orange), *Plasmodium* (and close relatives, red), Crane parasites (green).

Discussion

Our results are similar to previous studies showing monophyly of the common haemosporidian genera[120-122,125]. However, as in these previous studies, we found poor support for relationships between the major clades. The difficulty in resolving the deep phylogenetic relationships among Haemosporida is due in part to poor taxonomic

sampling[117], and our analyses included a haemosporidian from Gruiformes, a poorly sampled avian taxon.

Using a combination of morphologic and molecular methods, we detected for the first time Haemosporida infection in endangered whooping cranes. Parasites observed on blood smears from AWBP whooping cranes were identified as *H. antigonis*, and we provide the first molecular characterization of the species. Sequences recovered from sandhill crane samples were identical to *H. antigonis* sequences recovered from whooping crane samples for both cyt *b* and *coI* sequences. *Haemoproteus antigonis* was not previously represented in either the GenBank or MalAvi database, and our *H. antigonis* sequences formed a novel clade when analyzed with previously published avian Haemosporida sequences. However, our molecular data cannot support inclusion of the novel clade in the genus *Haemoproteus.* The novel clade forms a polytomy with all Haemosporida. Many of the avian Haemosporida species described molecularly to date were isolated from passerines and doves, and our novel clade may reflect evolutionary differences between the parasites of these divergent groups. Although the vectors for many avian Haemosporida, including *H. antigonis*, are unknown, the clade might reflect differences in the vector communities encountered by cranes and passerines.

The discovery of new species and even genera is becoming commonplace in haemosporidian research as we continue to increase the host-taxonomic and geographic breadth of our sampling. The phylogenetic uniqueness of the crane parasites compares with other recent discoveries in raptorial birds[123,142] and white-tailed deer[143], to name a few, and is most likely a new genus. However, as in these prior studies, the placement of the putative genus of crane parasites is unclear. This is likely the result of "undiscovered" diversity in neglected host taxa. The phylogenetic tree of Haemosporida parasites is incomplete, and will likely change as we continue to sample more hosts. Further sampling of Haemosporida, especially from poorly sampled host taxa, will help to resolve the relationship of *H. antigonis* to other avian Haemosporida genera, and will help to resolve the deep phylogenetic relationships among haemosporidians.

CHAPTER V

MOLECULAR EPIZOOTIOLOGY OF HAEMOSPORIDA IN WILD WHOOPING CRANES (*Grus americana*) AND SANDHILL CRANES (*Grus canadensis*)

Overview

The only self-sustaining wild population of endangered whooping cranes (*Grus americana*) has grown from a low of 15 to approximately 329 individuals. However, the population growth is not consistent with species recovery goals, and the impact of parasite infection on whooping crane populations is largely unknown. Our goal was to quantify the prevalence of Haemosporida in cranes and to compare the prevalence of infection between whooping cranes and sandhill cranes (*Grus canadensis*). We used three different PCR assays, interpreted in parallel, to screen samples for Haemosporida, and detected an overall infection prevalence of 83.6% (n=61) in whooping cranes and 45.1% (n=102) in sandhill cranes. While whooping cranes and sympatric sandhill cranes captured in Texas shared a high infection prevalence, that of allopatric sandhill cranes captured in New Mexico was significantly lower. *Haemoproteus antigonis* was the most abundant hemoparasite in cranes, present in 57.4% of whooping crane samples and 39.2% of sandhill crane samples, and was phylogenetically distinct from other avian Haemosporida. *Plasmodium* was present in 29.5% of whooping crane and 6.9% of sandhill crane samples and *Leucocytozoon* was present in 4.9% of whooping crane and 3.9% of sandhill crane samples. This study documents a high prevalence of Haemosporida, with shared parasite lineages between the two species, supporting the use of sandhill cranes as a surrogate species for understanding health threats to the endangered whooping cranes.

Introduction

The Aransas-Wood Buffalo population (AWBP) is the only self-sustaining wild population of endangered whooping cranes (*Grus americana*). This population nests in Wood Buffalo National Park (WBNP), Northwest Territories, Canada, and winters among coastal marshes at the Aransas National Wildlife Refuge (ANWR) in Texas,

USA. During winter 2015-2016, this population was estimated at 329 individuals (95% CI = 293–371; CV = 0.073)[63], an encouraging increase from the low of 15 individuals in 1941[1]. Population projections, however, indicate that whooping cranes may not achieve the down-listing criterion of a population size of 1000 individuals[2] until the mid-2060s[3]. Parasitic infection may be one factor limiting population growth, and a variety of parasites have been reported in captive and reintroduced whooping cranes[4,5]. However, a 1978 publication[6] and our recent study[84], both documenting coccidia infection in approximately one-third of sampled cranes, are the only published reports concerning parasites infecting the AWBP whooping cranes, and the impact of parasites on the population has not been assessed.

There is increasing evidence that biting dipteran flies are a nuisance to whooping cranes on their breeding grounds. Swarms of black flies (Simuliidae) contribute to nest abandonment and chick mortality in the reintroduced population of whooping cranes on their breeding grounds in Wisconsin[144,145]. Black flies have also been shown to cause nest abandonment in common gulls (*Larus canus*)[146] and contribute to nestling mortality in great horned owls (*Bubo virginianus*)[20] and red-tailed hawks (*Buteo jamaicensis*)[147]. In addition to being a nuisance, these flies and other hematophagous Diptera vector a variety of Haemosporida[112], including the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in North America. Acute Haemosporida infection has been shown to cause mortality in birds, and has been implicated in the decline and extinction of native Hawaiian bird species[148]. For example, *Plasmodium* infection has played a key role in severe declines of populations of Hawaiian honeycreepers including ‘I’iwi (*Vestiaria coccinea*)[149] and ‘apapane (*Himatione sanguinea*)[22]. Additionally, *Leucocytozoon* infection is associated with increased chick mortality in great horned owls (*Bubo virginianus*)[20] and yellow-eyed penguins (*Megadyptes antipodes*)[150].

Due to the short, transient nature of the acute stage of infection, most studies of Haemosporida infections in wild populations have investigated the chronic phase of infection, which is characterized by low levels of parasitemia and few or mild clinical signs. Chronic Haemosporida infections have often been assumed to be non-pathogenic,

however recent studies have documented a broad range of direct and indirect effects of chronic Haemosporida infections on wild bird populations. For example, *Plasmodium* infection does not appear to have any detrimental effects in some endemic populations of Hawaii amakihi (*Hemignathus virens*)[151] or house martins (*Delichon urbicum*)[23], but has been associated with decreased reproductive success in other populations of house martins[24] and great reed warblers (*Acrocephalus arundinaceus*)[152]. Another recent study of great reed warblers showed individuals with chronic avian malaria (*Plasmodium* or *Haemoproteus*) infection had increased rates of telomere shortening and shortened life spans compared to uninfected individuals[153]. Although overt clinical signs are rare, it is becoming clear that chronic Haemosporida infections can have detrimental effects on infected individuals.

Most studies to detect avian haemosporidians rely on either morphologic identification of parasites on blood smears or a PCR test targeting fragments of the mitochondrial cytochrome *b* (cyt *b*) gene. Morphologic studies may underestimate infection prevalence, especially in chronic infections with low parasitemia[154], and prevalence estimates in morphologic studies are dependent on the quality of the blood smear and the search effort[155]. Additionally, many haemosporidians are difficult to identify to species based on morphology, especially when infection intensity is low and not all stages of the parasite are represented in the sample. Molecular studies, on the other hand, can detect infections with low parasitemia, and may even detect infections in which parasitemia is too low for the infection to be transmitted to another host[122]. Molecular methods in the absence of morphologic identification, however, typically cannot allow determination of whether the parasites are. PCR assays may also fail to detect coinfections if the more abundant parasite is preferentially amplified[156-158]. Additionally, use of a single gene target limits the diversity of parasites that can be detected in a study, and prevalence estimates are affected by the gene target and PCR assay used in the study[154]. For example, Zehtindjiev et al.[159] described a *Plasmodium* species that failed amplification with five different PCR assays. Prior studies of Haemosporida in cranes of North America are based on examination of blood smears

and include descriptions of *Haemoproteus antigonis, Haemoproteus balearicae, Plasmodium-polare*-like, and *Leucocytozoon grusi* in sandhill cranes (*Grus canadensis*)[128-131], and *Haemoproteus antigonis* in a small number of non-migratory whooping cranes in Florida[21]. The majority of chronic Haemosporida infections reported in sandhill cranes have not been reported to cause disease, however severe anemia has been associated with acute *Haemoproteus balearicae* infection in two sandhill crane chicks[129]. We previously reported *Haemoproteus antigonis* infections in AWBP whooping cranes and sympatric sandhill cranes, and described a novel molecular clade formed by the parasite species (see Chapter IV). Our objective was to determine the prevalence of infection with Haemosporida (*Plasmodium, Haemoproteus, Leucocytozoon*) in AWBP whooping cranes and three different wintering populations of sandhill cranes with differing levels of sympatry to the whooping cranes.

Methods

Sample collection

Whooping crane blood samples were collected as previously described (see Chapter IV). Briefly, we collected blood samples from hunter-harvested sandhill cranes at necropsy between November 2012 and January 2014 through relationships with the Texas Parks and Wildlife Department, New Mexico Department of Game and Fish, and private hunting clubs and outfitters. Sandhill cranes from the following three populations were sampled: 1. Mid-continent population wintering on the Texas Gulf Coast (harvested in Jackson County, TX). 2. Mid-continent population wintering in the Texas panhandle (harvested in Armstrong and Carson Counties, TX). 3. Rocky Mountain population wintering in New Mexico (harvested in Socorro County, NM). Some birds harvested in New Mexico may also have been part of the Mid-continent population. The Mid-continent population is comprised of Lesser (*G. c. canadensis*) and Greater (*G. c. tabida*) subspecies, whereas the Rocky Mountain population is comprised of the Greater subspecies only[85,86]. The Rocky Mountain population serves as an out-group for comparison because their breeding, migration, and wintering ranges do not overlap with whooping cranes (Figure 5.1). All birds were either subjected to necropsy in the field

within 6 hours post-harvest or frozen at -20ºC immediately post-harvest and subjected to necropsy in the laboratory at a later date. Each carcass was subjected to a full gross necropsy, at which time we collected either whole blood or blood clot which had pooled in the coelomic cavity. Blood samples were frozen at -20ºC until DNA extraction.

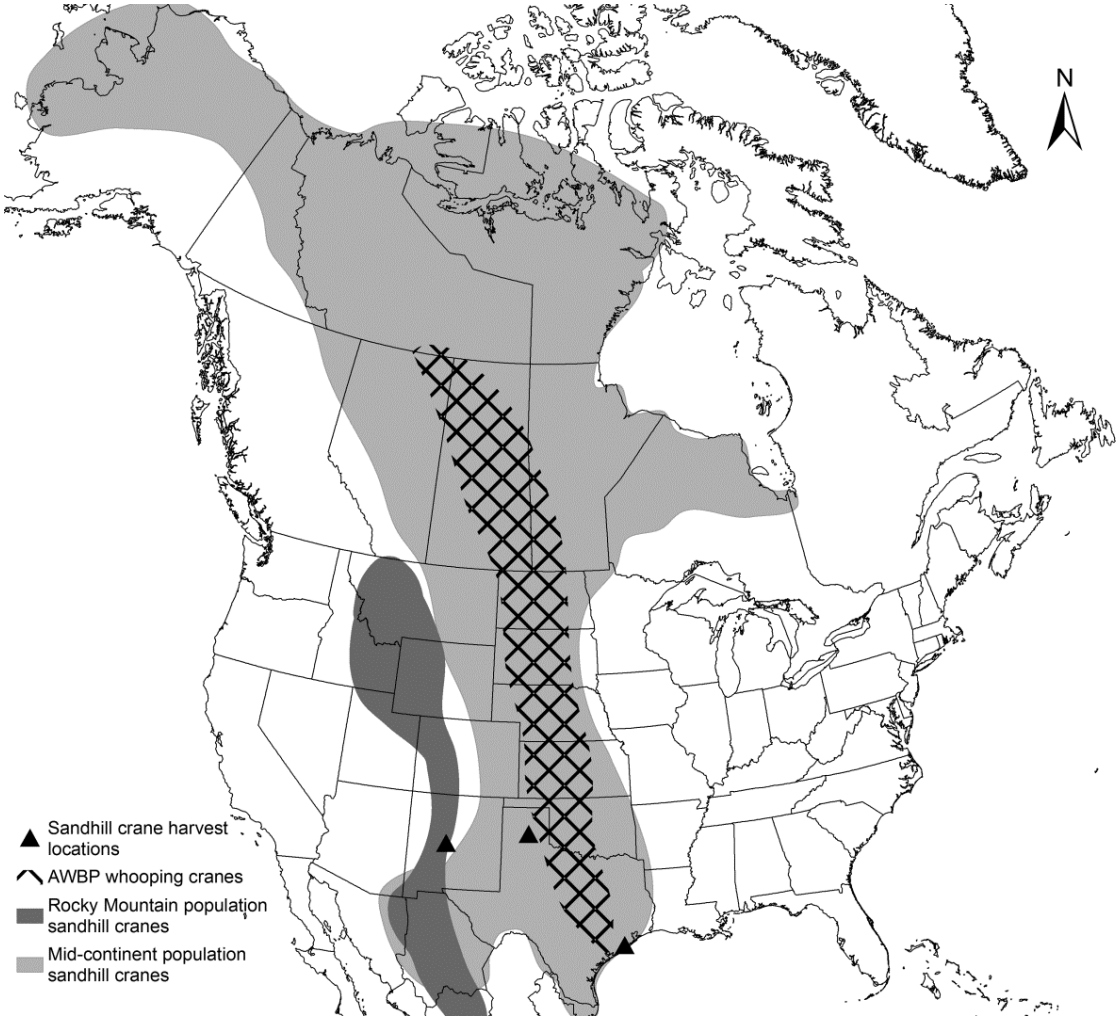

Figure 5.1. Ranges of the Mid-continent and Rocky Mountain populations of sandhill cranes in North America, and AWBP whooping cranes. Ranges shown include breeding, winter, and migration routes [3,85,86]. Locations where sandhill cranes included in this study were harvested are indicated.

Molecular detection of Haemosporida

DNA extraction. DNA was extracted from 100 µl of whole blood or blood clot using the E.Z.N.A Tissue Extraction kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions for tissue extraction with modifications including an overnight lysis step at 55ºC and elution into 100µl of elution buffer.

Haemosporida screening. All DNA samples were screened for Haemosporida using three separate PCRs to target different genes and results were interpreted in parallel. A sample was considered positive for overall prevalence estimates if it met the criteria for positivity on at least one assay.

First, Haemosporida infections were detected using a nested PCR reaction targeting an approximately 500 bp region of the 3' end of the mitochondrial cytochrome *b* (cyt *b*) gene. The first PCR reaction used the primers 3932F[133] and DW4[118] at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix E (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 1 µl of sample template. The second PCR reaction used the primers 413F and 926R[126] at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR was used as the template. In both rounds of PCR, cycling parameters were as described by Fecchio et al.[133]. A sample collected from a northern cardinal (*Cardinalis cardinalis*) and known to be infected with *Plasmodium* was used as a positive control[134].

Second, we used a nested PCR reaction targeting an approximately 900 bp region of the mitochondrial cytochrome oxidase subunit I (*coI*) gene. The first PCR reaction used the primers *coI*/outerF and *coI*/outerR[120] at a concentration of 0.3 µM in a 15 µl reaction with remaining reaction components as outlined above. The second PCR used the primers *coI*/nestedF and *coI*/nestedR[120] at a concentration of 0.3 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR, diluted 1:20, was used as the template. In both rounds of PCR, cycling parameters were as described by Martinsen et al.[120]. The same positive control used for cyt *b* PCR reactions was also used for *coI* PCR reactions.

Third, DNA samples were screened specifically for *Leucocytozoon* infections using a nested PCR reaction targeting an approximately 700 bp region of the mitochondrial cyt *b* gene. The first PCR reaction used the primers DW2 and DW4[118] at a concentration of 0.4 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, and 1 µl of sample template. The second PCR used the primers LeucoF and LeucoR[135] at a concentration of 0.4 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, and 2 µl of the product from the first PCR, diluted 1:20. In both rounds of PCR, cycling parameters were as described by Sehgal et al.[135]. DNA extracted from bird blood known to be positive for *Leucocytozoon* was obtained from Ravinder Sehgal at San Francisco State University, San Francisco, CA, and used as a positive control.

Sequencing and phylogenetic analyses

Positive PCR amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Purified samples were submitted for bi-directional sequencing to Eton Bioscience Inc. (San Diego, CA). Forward and reverse sequences were aligned and a consensus sequence was determined using Clustal W within Mega 6.0[44]. Samples were considered positive for prevalence estimates if a DNA sequence was obtained for which the identity matched most closely to a *Plasmodium* or *Haemoproteus* species in Genbank. Due to the sequence homology in the *cytb* gene across *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* species, the PCR assay used to detect *Leucocytozoon* has been shown to produce false positive results for *Leucocytozoon* due to the presence of *Plasmodium* or *Haemoproteus*[160]. Accordingly, we considered a sample positive for *Leucocytozoon* infection only if a DNA sequence was obtained for which the identity matched most closely to a *Leucocytozoon* species in Genbank. Chromatographs were examined manually, and sequences with double nucleotide peaks, indicating mixed infections, were separated using phasing. For otherwise clean samples with the same double nucleotide peaks in both the forward and reverse sequences, sequences containing all possible combinations of nucleotides at the

base pairs with double nucleotide peaks were created and compared to clean sequences generated in this study and in GenBank. The two sequences which were identical to previously generated clean sequences were used in the phylogenetic analysis. All sequences were compared to known Haemosporida sequences using the BLAST tool in GenBank and were aligned with the closest matches and additional publicly available avian Haemosporida species sequences representative of unique clades in previous studies[78,115,118,120,137-139]. Phylogenetic relationships were analyzed in Mega 6.0 using the maximum likelihood method based on a general time reversible with gamma distribution (GTR+G) model of evolution using the bootstrap method with 1000 replicates[94]. The model was selected based on fit estimated by the Akaike information criterion (AICc) and Bayesian information criterion (BIC). Samples with poor quality sequences or double nucleotide peaks were excluded from phylogenetic analysis. All unique sequences produced during this project and utilized in the phylogenetic analysis were deposited in GenBank (Accession #KX223847- KX223877).

Statistical analysis

Statistical analysis was performed using SAS software version 9.4 (Cary, NC). Overall prevalence and confidence intervals were calculated accounting for clustering at the population level. The chi-squared test and logistic regression were used to investigate the relationships between hemoparasite infection and population, age, and sex. Fisher's exact test was used to compare *Leucocytozoon* prevalence among populations due to small numbers of positive samples.

Results

Molecular screening

We collected blood samples from 163 individual cranes, including 61 AWBP whooping cranes, 47 sandhill cranes captured in the Texas Panhandle, 22 sandhill cranes captured along the Texas Gulf Coast, and 33 sandhill cranes captured in New Mexico (Table 5.1). When results from all three Haemosporida assays were interpreted in parallel, we detected Haemosporida in 83.6% (95% CI 74.0, 93.2) of whooping cranes and 45.1% (95% CI 35.3, 54.9) of sandhill cranes, including *Haemoproteus antigonis* in

57.4% (95% CI 44.6, 70.2) of whooping cranes and 39.2% (95% CI 29.6, 48.9) of sandhill cranes, *Plasmodium* spp. in 29.5% (17.7, 41.3) of whooping cranes and 6.9% (1.9, 11.9) of sandhill cranes, and *Leucocytozoon* spp. in 4.9% (0.0, 10.5) of whooping cranes and 3.9% (0.1, 45.1) of sandhill cranes.

Haemoproteus antigonis. Among the four crane populations, we detected significantly fewer infections with *H. antigonis* in the New Mexico population of sandhill cranes than in the other three populations (χ^2=26.99, df=3, p<0.0001) (Table 5.1). Age and sex were not significant predictors of infection (χ^2=0.12, df=1, p=0.743; and χ^2=0.27, df=1, p=0.604 respectively). However, age and sex were retained in the regression model because previous studies in other avian species have found differences in infection prevalence between juveniles and adults[22,150,161,162], and sex was not distributed equally across populations in our data. When controlling for age and sex of the bird, the odds of infection were 22.9 (95% CI 4.8, 109.1), 27.4 (95% CI 5.1, 148.2), and 17.5 (95% CI 3. 3, 92.6) times higher in AWBP whooping cranes, sandhill cranes captured in the Panhandle, and along the Gulf Coast than in sandhill cranes captured in New Mexico, respectively. *H. antigonis* infection was present in hatch-year birds, including 3 of 21 hatch-year whooping cranes captured at Wood Buffalo National Park, 4 of 5 hatch-year sandhill cranes captured along the Gulf Coast, and 21 of 32 hatch-year sandhill cranes captured in the Panhandle.

Table 5.1. Prevalence of Haemosporida in AWBP whooping cranes (WHCR) and three populations of sandhill cranes (SACR). Overall prevalences (%) are given for each species, and prevalences for age (juvenile or adult) and sex (male, female, or unknown) are given for each population. The 95% confidence intervals are given in parentheses.

Population	N	*Haemoproteus antigonis*	*Plasmodium*	*Leucocytozoon*	All Haemosporida
WHCR	61	57.4 (44.6, 70.2)	29.5 (17.7, 41.3)	4.9 (0.0, 10.5)	83.6 (74.0, 93.2)
Female	24	66.7	33.3	8.3	95.8
Male	26	46	64.6	0	76.9
Unknown	11	63.6	9.1	9.1	72.7
Juvenile	22	13.6	68.2	9.1	81.8
Adult	39	82.1	7.7	2.6	84.6
SACR	102	39.2 (29.6, 48.9)	6.9 (1.9, 11.9)	3.9 (0.1, 7.8)	45.1 (35.3, 54.9)
Panhandle	47	57.5 (42.8, 72.1)	4.3 (0.0, 10.3)	2.1 (0.0, 6.4)	59.6 (45.0, 74.1)
Female	6	33.3	0	16.7	33.3
Male	29	51.7	3.5	0	51.7
Unknown	12	83.3	8.3	0	91.7
Juvenile	32	65.6	4.3	3.1	68.8
Adult	15	40	0	0	40
Gulf Coast	22	50 (27.3, 72.7)	18.2 (0.7, 35.7)	9.1 (0.0, 22.1)	63.6 (41.8, 85.5)
Female	8	62.5	37.5	0	75
Male	14	42.9	7.1	14.3	57.1
Juvenile	5	80	20	0	80
Adult	17	41.2	17.7	11.8	58.8
New Mexico	33	6.1 (0.0, 14.7)	3 (0.0, 9.2)	3 (0.0, 9.2)	12.1 (0.4, 23.9)
Female	14	0	7.1	0	7.1
Male	19	10.5	0	5.3	15.8
Juvenile	3	0	0	0	0
Adult	30	6.7	3.3	3.3	13.3

Plasmodium. Among the four crane populations, we detected significantly more infections with *Plasmodium* in the AWBP whooping cranes than in the three sandhill crane populations (χ^2=17.87, df=3, p=0.0005), and hatch-year birds were significantly more likely to be infected than adults (χ^2=10.73, df=1, p=0.0011). When controlling for population and sex of the bird, the odds of infection were 6.62 (95% CI 2.16, 20.32) times higher in hatch-year than in adult birds, and when controlling for age and sex the odds of infection were 34.4 (95% CI 4.0, 333), 1.35 (95% CI 0.3, 5.6), and 8.26 (95% CI 0.9, 71.4) times higher in the AWBP whooping cranes than in the Panhandle, Gulf Coast, and New Mexico populations, respectively. *Plasmodium* infection was present in hatch-year birds, including 14 of 21 hatch-year whooping cranes captured at Wood Buffalo National Park, 1 of 5 hatch-year sandhill cranes captured along the Gulf Coast, and 2 of 32 hatch-year sandhill cranes captured in the Panhandle.

Leucocytozoon. We found an overall prevalence of 4.29% (95% CI 0.75, 7.84) of *Leucocytozoon* infection in cranes, and we did not detect a significant difference in prevalence among the four crane populations (Fisher's exact test, p=0.601). *Leucocytozoon* infection was present in hatch-year birds, including 2 of 21 hatch-year whooping cranes captured at Wood Buffalo National Park, and 1 of 32 hatch-year sandhill cranes captured in the Panhandle.

Comparison of PCR assays and phylogenetic analysis

The three primer sets produced different results for some samples with respect to infection status (Table 5.2). Results agreed between the Haemosporida cyt *b* and *coI* assays for 117 (72%) samples, between the Haemosporida cyt *b* and *Leucocytozoon cytb* assays for 116 (71%) samples, and between the Haemosporida *coI* and *Leucocytozoon* cyt *b* assays for 121 (74%) samples.

*Haemosporida cyt b.*We obtained consensus DNA sequences from the cyt *b* gene for 41 samples. Upon manual examination of the chromatograph traces, four samples had double nucleotide peaks. Sequences for these samples were separated using phasing, and a total of 45 sequences were included in the phylogenetic analysis.

Phylogenetic analysis revealed one sequence from a whooping crane sample aligned in a clade with a previously published *Plasmodium* sequence (Figure 5.2). Thirty-seven sequences, identified as *H. antigonis* were identical to each other and formed a novel clade, along with an additional six sequences which each differed from the sequence of the majority clade at the same single base pair. *H. antigonis* was detected in all four crane populations. The four samples with mixed infections each consisted of the two strains in the *H. antigonis* clade.

Table 5.2. Prevalence of Haemosporida detected by each PCR assay in 163 whooping crane and sandhill crane blood samples.

	Haemoproteus antigonis		*Plasmodium*		*Leucocytozoon*	
Assay	Prevalence (%)	95% CI	Prevalence (%)	95% CI	Prevalence (%)	95% CI
Haemosporida cyt *b*	30.1	6.4, 53.7	1.2	0.0, 4.5	0.0	na
Haemosporida *coI*	21.5	1.1, 41.9	12.3	0.0, 31.8	0.0	na
Leucocytozoon cyt *b*	38	3.9, 72.2	10.43	0.0, 27.9	4.3	0.8, 7.8

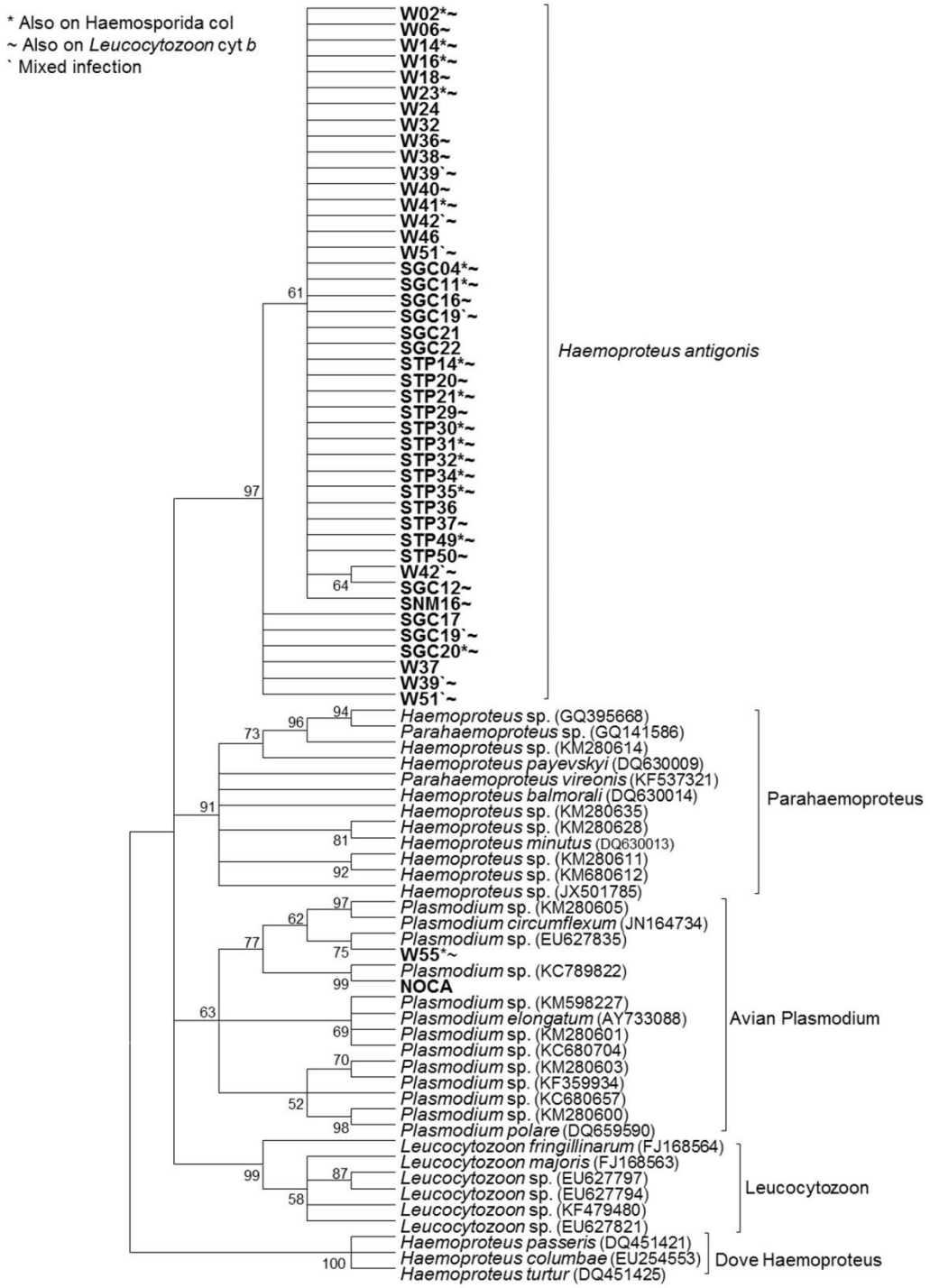

Figure 5.2. Phylogenetic tree using avian Haemosporida cyt *b* sequences (399 bp). The tree was created using the maximum likelihood method with a GTR+G model of evolution. Bootstrap values are based on 1000 replicates, and nodes with <50% support are collapsed. Sequences in bold were generated in this study. NOCA – positive control sample from a northern cardinal. W - AWBP whooping crane, STP – sandhill crane harvested in the Texas, SGC – sandhill crane harvested on the Texas Gulf Coast, SNM – sandhill crane harvested in New Mexico.

Haemosporida coI. We obtained consensus sequences from the *coI* gene for 32 samples. One sample had double nucleotide peaks, indicating mixed infection, and a total of 33 sequences were included in phylogenetic analysis. One sequence aligned in a clade with a previously published *Plasmodium relictum*[120] sequence, two identical sequences aligned with a previously published *Plasmodium circumflexum* sequence, and an additional eight identical sequences formed a separate, closely related clade (Figure 5.3). One sample in this clade (W55) also aligned with *Plasmodium* in the cyt *b* and *Leucocytozoon* analyses, two (W02, SGC04) aligned with *Plasmodium* in the *Leucocytozoon* analysis, but aligned with *H. antigonis* in the cyt *b* analysis, and two (W14, SGC20) aligned with *H. antigonis* in the cyt *b* and *Leucocytozoon* analyses. Twenty-two *H. antigonis* sequences, including 12 samples that were also represented in the cyt *b* analysis, formed a novel clade with two lineages, as was seen on the cyt *b* phylogenetic analysis. The sample with a mixed infection consisted of the two *H. antigonis* strains. AWBP whooping cranes and sandhill cranes capture in the Panhandle and along the Gulf Coast were represented in each of the unique clades, whereas sandhill cranes captured in New Mexico were not represented in the *coI* phylogenetic analysis.

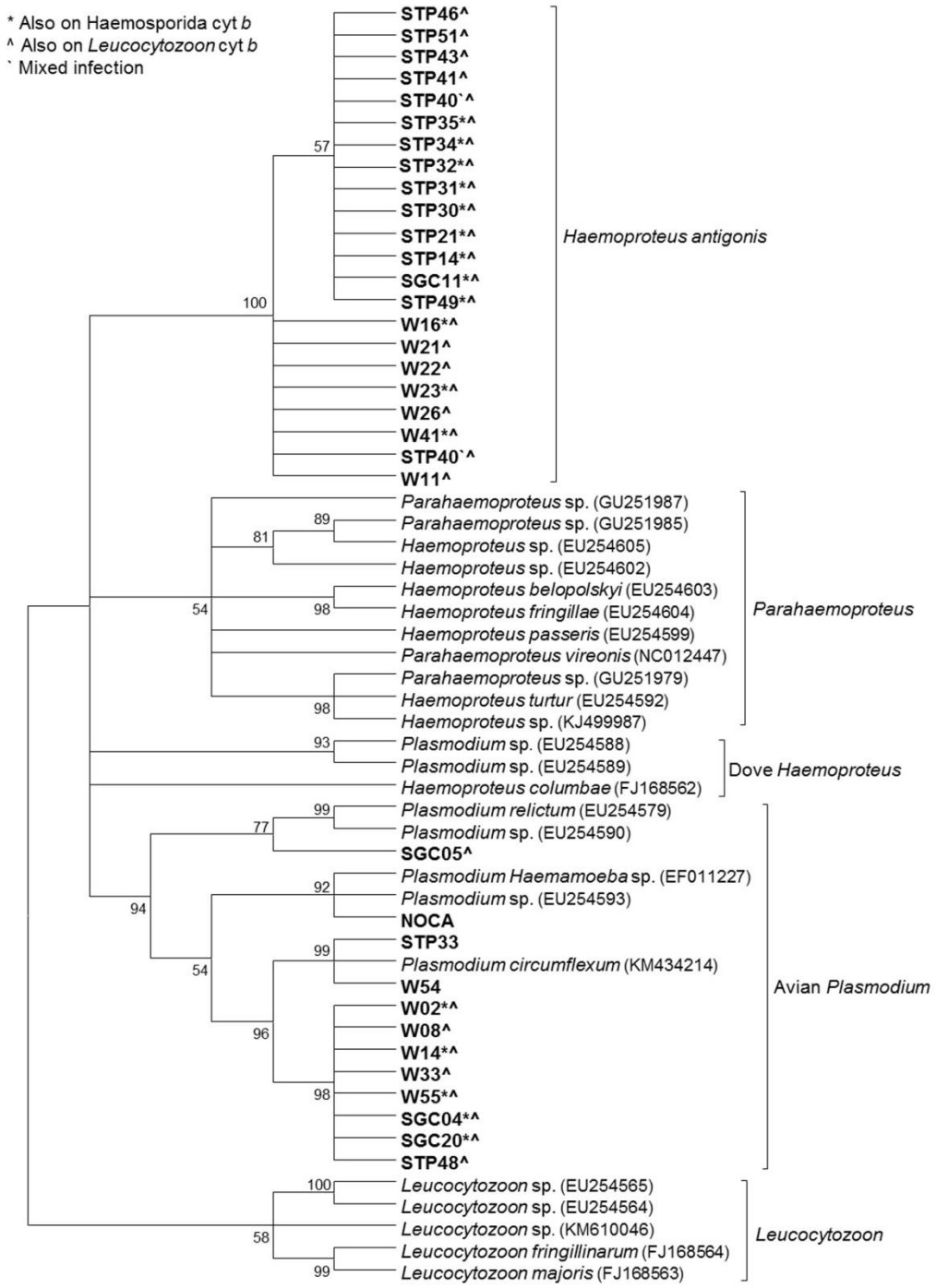

Figure 5.3. Phylogenetic tree using avian Haemosporida *coI* sequences (370 bp). The tree was created using the maximum likelihood method with a GTR+G model of evolution. Bootstrap values are based on 1000 replicates, and nodes with <50% support are collapsed. Sequences in bold were generated in this study. NOCA – positive control sample from a northern cardinal. W - AWBP whooping crane, STP – sandhill crane harvested in the Texas, SGC – sandhill crane harvested on the Texas Gulf Coast, SNM – sandhill crane harvested in New Mexico.

Leucocytozoon cyt b. Although 76 of 163 samples produced a sequence from the *Leucocytozoon* PCR assay, a majority of the sequences (94%) revealed the presence of other Hemosporida species, which was not unexpected given the sequence homology across taxa[160]. We obtained consensus DNA sequences from the cyt *b* gene for 76 samples, including 15 samples with mixed infections as revealed by double nucleotide peaks in the chromatographs. Sequences for these 15 samples were separated using phasing, and a total of 91 sequences were included in the phylogenetic analysis. Six sequences aligned with a clade containing previously published *Leucocytozoon* sequences, however the crane sequences formed a distinct group within this clade (Figure 5.4). All four crane populations were represented in this group. One sample (STP14) had a mixed infection with two different *Leucocytozoon* strains and was also represented in the *H. antigonis* clade in the Haemosporida cyt *b* and *coI* analyses. One additional sample (SGC05) was also represented in the *coI* analysis, in which it grouped with *Plasmodium* sequences.

Of the 85 non-*Leucocytozoon* Haemosporida sequences obtained from this assay, eight were identical sequences and grouped with a previously published *Plasmodium polare* sequence. An additional six sequences grouped with *Plasmodium circumflexum*. Four of these sequences and one sequence from the *P. polare* group were also represented in the *coI* analysis, in which they also grouped with *Plasmodium circumflexum*. The *Plasmodium polare* group consisted of whooping crane samples exclusively, whereas all four crane populations were represented in the other *Plasmodium* group. The remaining 71 sequences, representing all four crane populations, were *H. antigonis* and formed a novel clade, consistent with results from our analyses of Haemosporida cyt *b* and *coI*. One sample (W02) had a mixed infection with *Plasmodium* and *H. antigonis*. The remaining 12 mixed infections consisted of the two *H. antigonis* strains. Sixteen samples were represented in the novel *H. antigonis* clade in all three analyses, 12 were represented in the *H. antigonis* clade in the *Leucocytozoon* and *coI* analyses, and 15 were represented in the *H. antigonis* clade in the *Leucocytozoon* and Haemosporida cyt *b* analyses.

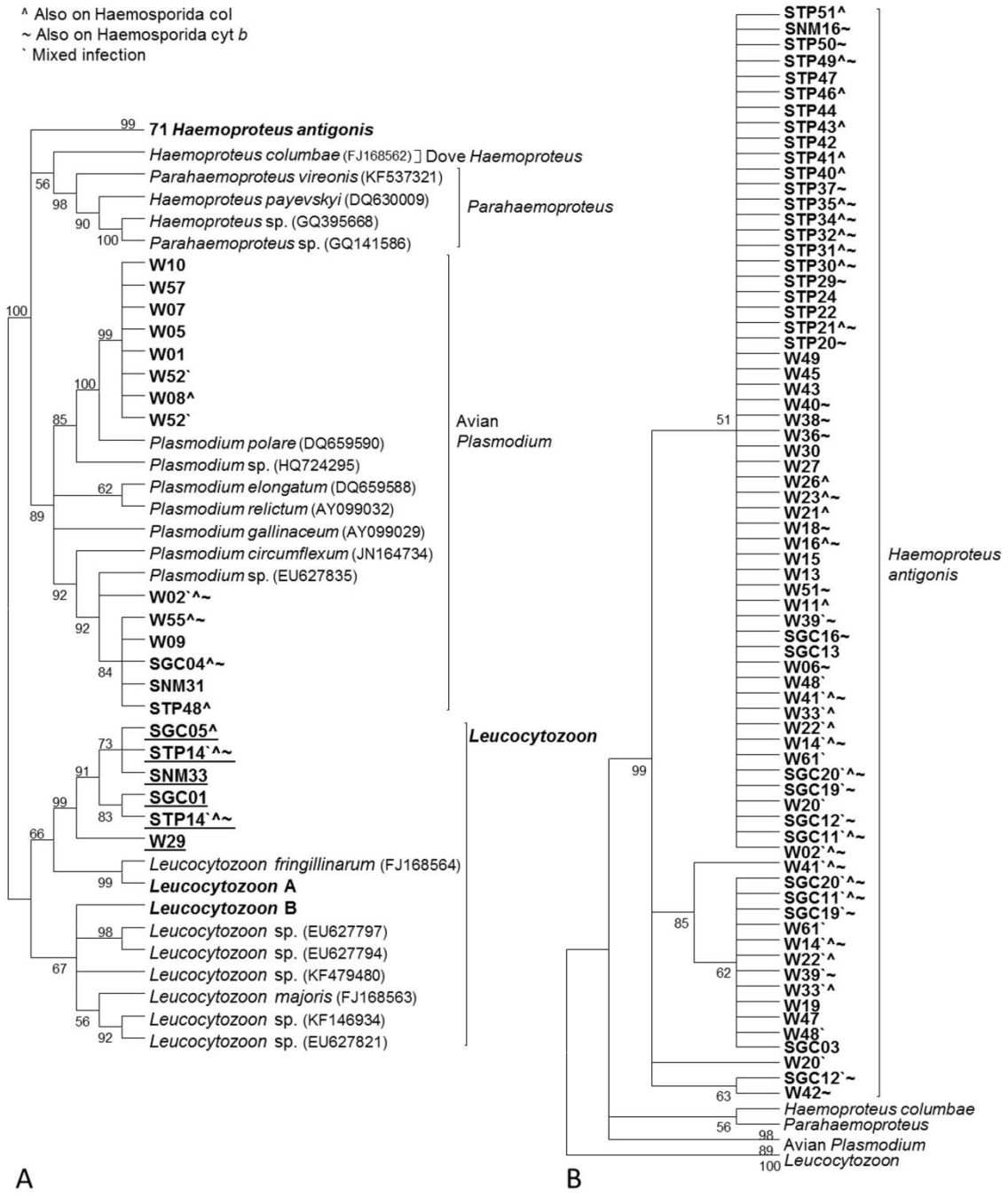

Figure 5.4. Phylogenetic tree using *Leucocytozoon* cyt *b* sequences (617 bp). A) Tree showing samples that do not fall in the novel crane Haemosporida clade. B) Tree showing samples that are included in the novel clade. The tree was created using the maximum likelihood method with a GTR+G model of evolution. Bootstrap values are based on 1000 replicates, and nodes with <50% support are collapsed. Leucocytozoon A and B are positive control samples. Sequences in bold were generated in this study, and *Leucocytozoon* sequences are underlined. W - AWBP whooping crane, STP – sandhill crane harvested in the Texas, SGC – sandhill crane harvested on the Texas Gulf Coast, SNM – sandhill crane harvested in New Mexico.

Discussion

We noted infections with Haemosporida in the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* in AWBP whooping cranes and each of three wintering populations of sandhill cranes. We found a high prevalence (50% - 57%) of *Haemoproteus antigonis* in AWBP whooping cranes and the two sympatric sandhill crane populations that winter in Texas using a combination of three different PCR assays. In contrast, the sandhill crane population wintering in New Mexico, which has no overlap with whooping cranes, had a significantly lower prevalence (6%) of Haemosporida. Additionally, we noted infection in 3 juvenile whooping cranes sampled on their breeding grounds at Wood Buffalo National Park, indicating transmission is occurring on the breeding grounds. We also noted infection in 21 juvenile sandhill cranes harvested on the wintering grounds. The prepatent period for most Haemosporida is 11 days to three weeks[112], and the sandhill cranes were harvested shortly after arrival to the wintering grounds, which suggests transmission occurred prior to the arrival of the birds to the wintering grounds. This supports previous studies, including one on sandhill cranes, which indicate birds usually acquire hemoparasite infections on their breeding grounds[128,163].

Environmental differences, including differences in vector insect species, contribute to differences in prevalence among different bird populations[162], and likely help to explain differences in prevalence among the four crane populations in this study. The lower prevalence in sandhill cranes harvested in New Mexico may reflect that the majority of sandhill cranes wintering in New Mexico are part of the Rocky Mountain population which breeds in and around southeastern Idaho, whereas the two Texas wintering sandhill crane populations are part of the Mid-continent population which breeds in northern Canada. The specific vector species is unknown for most avian haemosporidians, including those infecting cranes. However, previous studies have suggested *Culicoides* midges[161] and Hippoboscid flies[128,164] are the primary vectors for *Parahaemoproteus* and *Haemoproteus*, respectively, and mosquitoes are the primary vectors for *Plasmodium* (reviewed by Valkiunas[112]). Interestingly, the most common

haemosporidian parasite (*H. antigonis*) in this study infected all four crane populations, suggesting its vector has a broad geographic range.

This is the first study to use molecular techniques to detect hemoparasite infection in sandhill cranes, and the prevalence reported here is higher than previous studies that relied on microscopy for parasite detection because we have likely detected birds with chronic infections that have low-levels of parasite in the blood[154]. Previous studies have reported *Haemoproteus* prevalence of 8% - 14% in mixed-age sandhill cranes from Florida and from western North America[80,82,128], and Dusek et al.[129] reported a higher prevalence (36%) among sandhill crane chicks in Florida.

Due to differences in gene targets, sensitivity, and specificity among the three PCR assays, our results underscore the importance of using multiple assays to increase probability of detection of Haemosporida. Nonetheless, 46 of 97 positive samples were represented in at least two of the three phylogenetic analyses, and all assays provided support for the novel clade formed by *H. antigonis* sequences. Different primers may have differing affinities for different haemosporidian lineages, which can result in bias, especially when mixed infections are present. Additionally, when there are large differences in parasitemia between co-infecting lineages, amplification will be biased toward the lineage with the higher concentration of DNA in the sample. In our study, the *coI* and *Leucocytozoon* cyt *b* primers detected a higher prevalence of *Plasmodium* than the Haemosporida cyt *b* primers. Additionally, through the use of multiple primer sets we were able to detect mixed species infections in seven samples because these samples produced a *Plasmodium* sequence in one assay and a *H. antigonis* sequence in another assay.

We previously reported for the first time the DNA sequences of *H. antigonis* and the parasite's placement in a novel, well-supported clade at the level of genus within the Haemosporida phylogeny (see Chapter IV). *H. antigonis* was previously reported based on morphology only in sandhill cranes[131]. In the current study, *H. antigonis* was the most abundant parasite, and all three phylogenies support that this parasite forms a highly supported Haemosporida clade that does not include any other publically-available

sequences. Many of the avian Haemosporida species described molecularly to date were isolated from passerines, and our novel clade may reflect evolutionary differences between the parasites of gruiformes and passerines. Additionally, the clade reflects differences in the vector communities encountered by cranes and passerines. Further sampling of Haemosporida, especially from gruiform hosts, will help to resolve the relationship of the novel crane Haemosporida clade to other avian Haemosporida genera.

We detected a low prevalence of *Leucocytozoon* infection (2% - 9%) in the four crane populations, which constitutes the first report of *Leucytozoon* infection in whooping cranes and in sandhill cranes wintering in Texas and New Mexico. This infection prevalence is conservative because we required sequence confirmation of *Leucocytozoon* to consider a sample positive. Although we screened for coinfections as evidenced by double nucleotide peaks in the sequence chromatographs, we may have failed to detect coinfections, especially if another haemosporidian was present at higher levels than *Leucocytozoon*. A single species of *Leucocytozoon*, *L. grusi*, has been reported in sandhill cranes[130]. Previous studies, based on microscopy, have reported a prevalence of 8% - 18% in the non-migratory Florida sandhill cranes[122,130]. In contrast, infection prevalence was 50% in one study examining Florida sandhill crane chicks only[129]. Forrester et al.[122] did not detect *L. grusi* in any of 51 Greater (migratory) sandhill cranes wintering in Florida, and *Leucocytozoon* infection has not previously been reported in whooping cranes[21]. The Florida sandhill cranes were likely infected during the summer, when Simuliid flies, the vector for *Leucocytozoon*, were abundant[21,164]. Our observations of infections of *Leucocytozoon* in two juvenile whooping cranes sampled on the breeding grounds in Canada and one sandhill crane harvested in the Texas Panhandle provides further evidence that exposure is occurring during the summer on breeding grounds for both species.

We found many identical parasite sequences in samples from whooping and sandhill cranes, suggesting these parasites are shared between the two crane species. This is likely the result of generalist hematophagous arthropod vectors that feed opportunistically on either crane species as well as the parasite's propensity to infect

closely related hosts[134,165]. This highlights the utility of the sandhill crane as a surrogate for the whooping crane. The surrogate species approach is useful in studies of endangered species, where sample sizes are limited. For example, this approach has been successful in studies of the Attwater's prairie chicken using the northern bobwhite as a surrogate[25]. The sandhill crane is a useful surrogate for the whooping crane because it is the closest North American relative to the whooping crane, it is abundant, and the Mid-continent population of sandhill cranes has an overlapping range with whooping cranes. The impact of avian malaria infection on crane populations is unknown, and studies in other wild bird populations show conflicting results. Future studies investigating the effect of avian malaria on fitness in sandhill cranes and whooping cranes will aid in the management of these species.

CHAPTER VI
CONCLUSION

The whooping crane is a flagship species for conservation efforts in the US, serving as a highly visible indicator of ecosystem health and drawing tourism income to local economies. Local communities near wintering grounds and migration stop-over sites receive an estimated $20 million annually from tourism due to whooping crane-related activities[2]. Annual whooping crane recovery costs are estimated at $6 million, over two-thirds of which go to captive breeding programs and efforts to establish reintroduced populations[2]. Despite tremendous investments, efforts to establish self-sustaining reintroduced populations have not been successful to date (bringbackthecranes.org/conservationinaction), highlighting the importance of the AWBP. This project contributes to knowledge about disease threats to the AWBP whooping cranes, which can inform conservation and management consultants and decision-makers for this critically important population.

To our knowledge, this is the first study to evaluate the prevalence of fecal parasites in the AWBP whooping cranes using both traditional parasitologic and molecular techniques. Identification of the coccidia and helminth species associated with cranes is a critical step in collecting baseline data from which future changes can be monitored, and also for beginning to explore the role that coccidia and helminth infections may play in health and disease of North American cranes. Furthermore, the non-invasive approach employed, relying on analysis of voided fecal samples, can serve as an important conservation tool that can be used for years to come to monitor the parasite burden in these birds with minimal disruption to the population.

This is also the first study to describe hemoparasite infections in the AWBP whooping cranes. Additionally, it provides much-needed information on hemoparasite prevalence in sandhill crane populations, which could have implications for management and harvesting regulations of this species. With the exception of one publication describing hemoparasite infections in sandhill cranes from Canada and Texas[128], all

other studies have been limited to sandhill cranes in Florida[21], where vector species and exposure to hemoparasites are likely different. Comparisons of hemoparasites among whooping and sandhill cranes indicates that transmission is occurring on the breeding grounds. Furthermore, *Haemoproteus antigonis* is likely endemic in these two crane species and has been parasitizing cranes for a long time, as indicated by its position in a novel clade on phylogenetic analysis. This study can inform management decisions for the critically important AWBP and lays the groundwork to assess the degree to which these parasites may be impacting crane health.

Although the sandhill crane is not endangered, management of this species can be challenging due to its growing popularity as a game species. This study provides data about parasite infections in sandhill cranes, which will aid management of this species.

Finally, this study adds to the body of research supporting the use of a surrogate species for studying species of conservation concern. Experimental study designs and more invasive procedures yield valuable information that shapes species management plans, however, these types of studies are not feasible with endangered species. Use of a surrogate species yields similarly valuable information without compromising populations of endangered species[25]. This study supports the use of the sandhill crane as a surrogate for the whooping crane, and future studies investigating the impact of parasites on whooping cranes can employ a surrogate approach using sandhill cranes.

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