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PROTOZOOLOGY - SHORT COMMUNICATION

Haemosporidia of grey crowned cranes in Rwanda

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Abstract
Grey crowned cranes (*Balearica regulorum*) have been facing significant and long-term population declines in East Africa. Studies of Haemosporidian infections are essential to gain insight into pathogenic threats and help infer vector-host relationships, resolve parasite relationships, and support conservation efforts. As part of a program to reintroduce captive cranes in Rwanda back to their natural habitats, through health checks and initial microscopic examination, 120 grey crowned cranes were selected under suspicion of harboring Haemosporidian infections following initial peripheral blood smear examinations. Of these, 104 were infected with *Haemoproteus* and 3 were coinfected with *Leucocytozoon* as detected by PCR and microscopy. Sequencing allowed us to identify 2 distinct unreported lineages of *Haemoproteus antigonis* and one lineage of *Leucocytozoon* in the subspecies of Grey Crowned Cranes endemic to East Africa, *B. r. gibbericeps*. Molecularly, our two lineages of *Haemoproteus antigonis* differ by 32 base pairs and matched with about 95 percent identity to previously reported sequences of *H. antigonis* found in other species of cranes. No visible morphologic differences were found when compared to images of *H. antigonis* from previous studies. Our work demonstrates not only a need for increased testing within the family Gruidae, but also to investigate the possibility of cryptic speciation within the morphospecies *Haemoproteus antigonis*.

Keywords Avian blood parasites · Haemosporida · *Leucocytozoon* · *Haemoproteus* · Crane · *H. antigonis* · Rwanda

Introduction

With 11 of the 15 described crane species threatened worldwide, the need to bolster and support conservation efforts has become critical (Bertram et al., 2017a, b). Cranes, family Gruidae, represent an understudied taxon of birds in ongoing investigations of avian Haemosporidian infections (Clark et al., 2014). Capturing and handling larger birds poses more of a challenge than with passerines, but now, as conservation programs increase capacity to protect these sensitive bird communities, so do opportunities to test for potential pathogens and gain insight into disease dynamics (Václav et al., 2016). Avian Haemosporida of the genera *Plasmodium*, *Leucocytozoon*, and *Haemoproteus* are transmitted by dipteran insects and have the potential to decimate wild populations (Valkiūnas 2005, Ortiz-Catedral et al., 2019**.** High virulence and mortality have mainly been attributed to *Plasmodium* spp. when introduced to native populations either by migration or an introduced invasive species, but climate change and fragmented habitats can also contribute to spillover events, both of which affect the cranes within this study (Fecchio et al., 2011). As taxonomic sampling of Haemosporida within crane species broadens, we can better understand host-vector relationships and ecological forces that may alter transmission. Here, we provide information of Haemosporidian parasites in a recovering population of cranes in Rwanda.

The East African grey crowned crane (*Balearica regulorum gibbericeps*) is one of two subspecies of grey crowned crane mainly distributed in Eastern Africa. The East African Grey Crowned Crane residing in Rwanda is non-migratory and isolated from other crane species. This species has been

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on the IUCN red list as endangered since 2012 and has suffered long-term population declines with the main causes being loss of habitat and illegal domestic and international trade (Morrison 2015). Utilizing a multi-pronged approach, the Rwanda Wildlife Conservation Association (RWCA) partnered with the Rwandan government since 2014 to combat illegal poaching and capture of wild cranes by implementing community-wide education programs, registering captive cranes, and reintroducing a subset of healthy captive cranes to a protected habitat within Akagera National Park (ANP).

Following an initial examination of peripheral blood smears, 120 samples were chosen which were suspect to harbor blood parasites. Applying polymerase chain reaction (PCR) and microscopy, we tested for the three genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* with the goal of identifying infections and sequence lineages. We expected the results to reflect a biodiverse habitat abundant with both migratory and endemic birds host to a variety of ectoparasites. Though studies on crane Haemosporidia are limited, there are a few molecular descriptions of specialist parasites affecting the Gruidae including *Haemoproteus antigonis*, *Leucocytozoon grusi*, and *Haemoproteus balearicae*. Neither *L. grusi* or *H. balearicae* have been identified to the molecular level in GenBank. *H. antigonis* has been identified to species level but the molecular studies have questioned its placement within the subgenus *Haemoproteus*, instead suggesting it belongs in a novel clade sister to *Plasmodium* (Bertram et al., 2017a, b). The phylogenetic placement differs when utilizing outgroup rooting versus outgroup-free relaxed molecular clock methods (Outlaw and Ricklefs 2011; Galen et al., 2018). Since the phylogeny of blood parasites based on cytochrome *b* gene testing generally groups according to insect vectors, this may imply that *H. antigonis* is spread by a different insect than the Hippoboscidae which are known to transmit *Haemoproteus* (*Haemoproteus*) (Yabsley et al., 2018). As taxonomic sampling increases we can better resolve these phylogenetic relationships and strengthen our knowledge of the complex host–pathogen ecology that shapes Haemsoporidian infections.

Methods

Sampling

Upon intake into the quarantine facility by the RWCA, each crane was hooded to minimize stress and physically restrained for examination. At this time, multiple samples were taken for various tests including whole blood obtained from the right jugular vein and placed into a lithium heparinized tube, a fecal sample, cloacal swab, and tracheal swab. Blood smears were immediately made and air-dried. An aliquot (0.15 ml) of the whole blood was also placed into a cryotube containing lysis buffer solution for PCR testing. Blood smears were then fixed with methanol and stained with either Diff-quik (Siemens, Malvern, PA) or Giemsa (Azer Scientific, Morgantown, PA) according to manufacturer's instructions. The cryotubes were frozen at -80 °C and shipped with the stained slides to San Francisco State University (SFSU) for Haemosporidian parasitic screening.

Extraction and amplification

Extraction of DNA was performed at SFSU with Promega Wizard SV Genomic DNA Purification System (Promega, Madison, WI) using the centrifugation methods according to the manufacturer's instructions. The volume of nuclease-free water added to the microcolumn was kept at 125 µl to obtain a more concentrated elution. After extraction, brain-derived neurotrophic factor (BDNF) was amplified from each sample to ensure the presence of DNA (Sehgal and Lovette 2003). PCR was performed to detect the Haemosporidians *Haemoproteus, Plasmodium,* and *Leucocytozoon.* The nested protocol for *Haemoproteus* and *Plasmodium* targeted a region of the parasitic mitochondrial cytochrome *b* as described in Waldenström et al. (2004) with the first round utilizing primers NF/NR2 and the second round F/R2 to target the 479 bp fragment for amplification (Waldenström et al., 2004). The nested *Leucocytozoon* protocol followed that in Hellgren et al. (2007) utilizing first round primers NFI/NR3 and second round primers FL/R2L. PCR reactions were conducted using Promega GoTaq Polymerase with a final volume of 25 µl. Master mix for PCR included 10.875 µl purified H20, 5.0 µl MgCl2, 0.5 µl dNTP's, 1.0 µl forward and reverse primer, 0.125 µl Taq polymerase, and 4 µl of extracted sample to account for lower yield of parasite to host DNA. Included in each reaction plate was a negative control of purified water and a positive control of, in most reaction plates, a previously positive sequenced woodpecker sample (Groff et al, 2019), otherwise a sequenced and positive crane sample was used. Only gels with a successful amplification of positive and negative controls were included in the study. Samples were run in triplicates to check consistency of results and positive PCR results were compared to available prepared blood slides. Gel electrophoresis was performed with 5 µl of product on an 1.8% gel stained with ethidium bromide and visualized under a UV transmitter. Positive samples were then purified either, in house with ExoSAP-IT (Affymetrix Inc. Santa Clara, Ca) cleanup reagent per manufacturer's guidelines, or at Elim Biopharm (Hayward, Ca) when samples were sent for sequencing. Bi-directional sequences of contigs were assembled using Geneious (v.11.0.4) (https://www.geneious.com). Sequences were then deposited into GenBank (accession numbers:

#MT497526, #MT497527, #MT497528) and compared with sequences in the database using NCBI nucleotide BLAST (Basic Local Alignment Search Tool).

Evaluation of blood slides

Each blood smear was inspected by one individual (JS) under the oil immersion objective (×1000) with a Nikon e200. Morphologic analysis was performed on two slides per sample, each examined for at least 20–30 min or 100+fields of view on low power (400×) and 100+fields of view on high power (1000×). Scanning was kept to the monolayer and feathered edge if parasites where identified; otherwise, the search was expanded to detect infections with low parasitemia (Godfrey et al., 1987). Morphologic identifications were compared to the parameters in the published taxonomic key by Valkiūnas (2005).

Results and discussion

We tested 120 grey crowned crane samples, 89 of those samples had both blood for PCR and a blood smear, 25 samples had only blood for PCR, and 6 samples had only a blood smear to evaluate. 104 (86%) of the 120 samples total tested positive for Haemosporidia by either PCR or microscopy (Table 1). Of the 89 samples that had both blood for PCR and a blood smear there were 9 samples that would not show positive on PCR, but we did find parasites upon the blood smear evaluation (see 68/77 under Positive Haemoproteus in Table 1).

From the blood samples available, 90 samples of grey crowned cranes tested positive by PCR for Haemosporidian parasites of the genera *Haemoproteus* with 3 carrying a coinfection of *Leucocytozoon*. Of those that tested positive for *Haemoproteus*, 48 samples returned high quality sequences which could be aligned and trimmed. All 3 positive for *Leucocytozoon* returned a high-quality sequence. We found two distinct lineages of *H antigonis* and one lineage of *Leucocytozoon*. The sequence of *Leucocytozoon* most closely matched, with 96 percent identity, a sequence found in a Grey plantain-eater (*Crinifer piscator*) (Accession #LC271258). Our two lineages of *H. antigonis* differed in 32 base pair positions. One sample was found to have double peaks at each of those base pair positions indicating a co-infection of each lineage (Van Rooyen et al., 2013). Our *H. antigonis* lineage 1 matched with a percent identity of 95.34 to a *H. antigonis* spp. found in a Sandhill crane and lineage 2 matched with percent identity of 95.07 to a *H. antigonis* spp. found in a Whooping crane (Accession #KX223875 and #KX223844 respectively). Of the three samples that tested positive for a *Leucocytozoon* coinfection, we were able to identify that two were coinfected with *H. antigonis* lineage 1 but could not return a sequence to distinguish the third.

Thirty-two samples which tested positive for *H. antigonis* lineage 1 and four samples of *H. antigonis* lineage 2 also had slides for evaluation (Table 2). All of those confirmed the presence of *H. antigonis* infection with no visible morphologic differences between the lineages. Most showed a low parasitemia (<1/1,000 RBC). Morphologic features matched those described in the *H. antigonis* species (Valkiūnas 2005) with young gametocytes showing a wavy cleft next to the nucleus without touching it (Fig. 1A, B). Macrogametocytes and microgametocytes caused marked displacement of the nucleus (Fig. 1D arrow 1) often with a cleft separation (Fig. 1D arrow 2) while closing around the nucleus but never encircling or touching ends fully. Few vacuoles were found and pigment granules (Fig. 1D arrow 3) were sporadic through the parasite cytoplasm and limited in number. Note the similarities between morphological presentation of our *H. antigonis* and previously described species, even with marked differences in speciation molecularly (Bertram et al., 2017a, b). The two samples that tested positive for

Table 2 Summary of high-quality sequences obtained showing the number of samples infected with each lineage of *H. antigonis*

	Lineage 1	Lineage 2	Coinfection of Lineage 1 and 2
48 Sequences aligned of *H. antigonis*	39	7	1
Blood smears available to compare	32	4	0

Table 1 Results from PCR and Blood smear evaluation of 120 Grey crowned crane samples

	Number Tested	Positive *Haemoproteus*	Positive *Leucocytozoon* coinfection
PCR/Blood smear evaluation (Samples with both available)	89	68/77	2/0
PCR only (No smear available)	25	22	1
Blood smear only (No PCR available)	6	5	0
Total	120	104	3

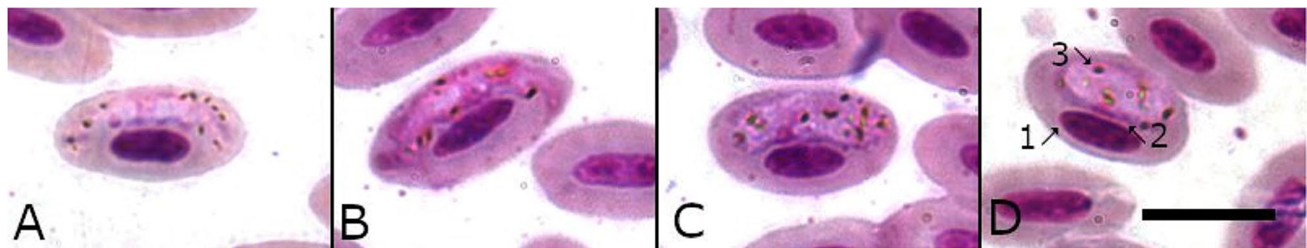

Fig. 1 *Haemoproteus antigonis* identified from the blood of Grey Crowned Cranes (*Balearica regulorum*) sampled in Rwanda. A, B Growing gametocytes with a wavy cleft next to the nucleus. C, D Mature gametocytes displacing the nucleus laterally 1. Marked displacement of the nucleus in a mature gametocyte 2. Cleft between the parasite and nucleus 3. Few pigment granules sporadic through cytoplasm of parasite. Bar = 10 microns

Leucocytozoon by PCR also had corresponding slides but there were no *Leucocytozoon* cells found. We thus surmise that *Leucocytozoon* parasitemia is low since it is only detectable using PCR.

To date, this is the largest investigation of Haemosporidia infections in Grey Crowned Cranes as previous studies have been limited to captive ex situ zoo collections holding a small number of birds to sample. A recent study performed at the Beijing zoo sampled 10 Grey Crowned Cranes on exhibit but reported no Haemosporidian infections (Jia et al., 2018). The in situ RWCA program is under the constraints of only testing birds that come into the quarantine facility from captivity. Because of this we were unable to survey the population as a whole or ascertain the exact location of the acquired infections. This leaves open the question whether they were exposed in the wild before capture, or on the grounds they were kept. Even small-scale habitat changes can affect parasite prevalence including proximity to water, altitude, or temperature (Sehgal 2015). Establishing the true prevalence of these parasites would be difficult as it would require constructing a stress free and ethically sound plan to capture and test the whole flock at random. This will need to be addressed in the future as to whether testing will rely solely on sick birds who are easily caught or a larger effort to assess flock health.

Reporting the specialist parasite *H. antigonis* in these cranes is not surprising, as it has been commonly found in other Gruidae though mainly by morphological identification. With advancements in molecular testing, it has become clear that the taxonomic groupings of Haemosporidia may be broader than the accepted 3 genera *Haemoproteus*, *Leucozytozoon*, and *Plasmodium*, and methods such as multi-locus testing should be investigated to better infer evolutionary relationships (Barrow et al., 2019). This parasite has been placed in a novel sister clade to *Plasmodium* in multiple publications, and could illustrate the need to reclassify the genera of Haemosporidia to include the cryptic diversity of parasite species found in non-passerines (Galen et al., 2018).

Our intention here was to add data to better understand *H. antigonis* diversity and evolution. Our lineages had 23 (#MT497527) and 24 (#MT497526) different base pairs compared to the lineage reported in Whooping cranes (Bertram et al., 2017a, b). *H. antigonis* lineages reported in Sandhill, Whooping, and a White-naped crane differ by less than 1% among each other. Variation in geographic location and isolation in our crane population's distribution could account for the greater than 4% divergence of our reported lineages. The relatedness and phylogeny among the crane species should also be considered as this specialist parasite may have diverged early and evolved within our crane population (Krajewski et al., 1994). Future studies should include taxonomically close relatives such as the Southern African Grey Crowned Crane or Black Crowned Crane while in their endemic habitats. Though we reported a sequence of *Leucocytozoon*, we were unable to find any of these parasites upon microscopic examination, which prevented us from identifying the parasite to the species level. The closest match in GenBank came from a captive Grey plantain-eater (*Crinifer piscator*) in a study which was also unable to identify gametocytes upon morphologic examination (Kakogawa et al., 2019). Additional sampling and morphologic review will be necessary to identify this *Leucocytozoon* to the species level.

Rwanda is an incredibly biodiverse and species rich location (Brown 1984). Aside from the parasites we identified, we expected to find more pathogen species than we did. Rwanda contains multiple habitats including wetlands, swamp, savannah, and montane that the cranes traverse with a variety of vectors being recorded at various aquaculture sites (Deutsch 1992). Studies in Rwanda also show a presence of *Plasmodium* species in passerines suggesting the possibility for host-crossover events to occur, but along with their absence in our study there have been no reports of crane mortalities which could signal virulent parasite crossover events were taking place (Lauron et al., 2015). Low population density of the cranes could be influencing parasite prevalence (Ellis et al., 2017). Increased land fragmentation has also been shown to alter vector ecology and Rwanda has

undergone large-scale anthropogenic land manipulation to sustain the needs of a dense human population (Laurance et al., 2013). Vector competence and host relationships are complex and work is greatly needed in this area to understand how it may affect or threaten a recovering avian community such as this (Faust et al., 2017). We recommend both increasing Haemosporidia testing in the threatened crane populations for improved conservation success and investigations into pathogen-related ecological factors.

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Declarations

Ethics approval All procedures were conducted under valid Rwanda Development Board Conservation Department permits, and international shipment was conducted under valid CITES export and USDA import permits.

Conflict of interest The authors declare no competing interests.

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