THE CONSEQUENCE OF CO-CIRCULATING PARASITES FOR VECTOR-BORNE PATHOGEN TRANSMISSION

A Dissertation

by

ANDREW JOHN GOLNAR

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Chair of Committee,	Gabriel Lee Hamer
Committee Members,	Michel Slotman
	Micky Eubanks
	Jay Walton
Head of Department,	Pete Teel

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ABSTRACT

The unprecedented rates of vector-borne pathogen emergence and re-emergence highlights the necessity to identify ecological drivers of transmission heterogeneity. Mosquitoes ingest a variety of viruses, protozoan, and macroparasites that circulate among avian and mammalian hosts. The consequence of these co-circulating parasites for vector-borne pathogen population dynamics remains largely unknown. In this dissertation, field research, controlled laboratory transmission experiments, and mathematical modeling techniques are combined to assess how polyparasitism may influence vector-borne pathogen transmission dynamics. Field techniques used in this dissertation highlight a community of blood-parasites in the Great-tailed grackle (*Quiscalus mexicanus*), results that document the propensity of co-circulating parasites to influence zoonotic vector-borne pathogen systems. Additionally, two compartmental models that describe the dynamics of vector-borne pathogen transmission structured by host and vector co-infection status were constructed, analyzed, and parameterized in an effort to define a quantitative framework of use for evaluating the impact of polyparasitism on vector-borne pathogen population dynamics. Importantly, results quantitatively illustrate that avian malaria parasites may simultaneously increase and decrease the reproductive number of WNV through multiple mechanisms of interaction. Finally, numerous experimental transmission assays were completed to evaluate how *Plasmodium* influence rates of midgut infection and dissemination in mosquitoes simultaneously infected with West Nile and *Plasmodium* parasites and sequentially infected with *Plasmodium* and then West Nile virus during a subsequent bloodmeal. Results suggest there are no patterns driving *Plasmodium*-induced changes in rates of infection and dissemination, suggesting *Plasmodium* parasites may not be a key driver of natural WNV transmission heterogeneity. Overall, results of this dissertation stress a need to empirically and

analytically evaluate the impact of polyparasitism on transmission dynamics as a compounding consequence of multiple ecological and biological mechanisms. Although complex, enhancing our understanding of how patterns of polyparasitism impact zoonotic pathogens can help elucidate novel mechanisms of disease control and surveillance.

DEDICATION

This dissertation is dedicated to all my friends and family that showed patience as I adapted to the lifestyle of a scientist. Additionally, this is dedicated to all the canaries and great-tailed grackles that lost their lives in the pursuit of science.

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NOMENCLATURE

BCS	Bryan/College Station, Texas
CDC	Center for Disease Control and Prevention
C.I.	Confidence Interval
DFE	Disease-free Equilibrium
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
FBS	Fetal Bovine Serum
GTGR	Great-tailed Grackle
HIV-1	Human Immunodeficiency virus
NCBI	National Center for Biotechnology Information
ODE	Ordinary Differential Equation
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PRCC	Partial Rank Correlation Coefficient
rcf	relative centrifugal force
R_0	Basic Reproductive Number
RVFV	Rift Valley fever virus
TB	Tuberculosis
USA	United States of America
WNV	West Nile virus

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

1.1 Synopsis

Vector-borne pathogens are a persistent challenge for public health agencies as globalization continues to enhance rates of disease emergence and spread. The unprecedented rates of vector-borne pathogen emergence and re-emergence highlights the necessity to identify ecological drivers of transmission heterogeneity. Mosquitoes ingest a variety of viruses, protozoan, and macroparasites that circulate among avian and mammalian hosts. The consequence of these co-circulating parasites for vector-borne pathogen population dynamics remains largely unknown. In this dissertation work, field research, controlled laboratory transmission experiments, and mathematical modeling techniques are combined to assess how polyparasitism may influence vector-borne pathogen transmission dynamics. Chapter II uses field and lab techniques to elucidate the assemblage of vector-borne blood-parasites found in Great-tailed grackles (*Quiscalus mexicanus*). In Chapter III, I construct, analyze, and parameterize two compartmental models that describe the dynamics of vector-borne pathogen transmission structured by host and vector co-infection status in an effort to define a quantitative framework of use for evaluating the impact of polyparasitism on vector-borne pathogen population dynamics. In Chapter IV, controlled laboratory transmission assays were completed to study how *Plasmodium* parasites influence rates of midgut infection and dissemination in mosquitoes that are simultaneously exposed to West Nile virus and *Plasmodium* parasites and sequentially infected with *Plasmodium* parasites and then West Nile virus during a subsequent bloodmeal.

1.2 Introduction

Historical records and ongoing surveillance efforts make it clear that the rate at which infectious diseases are emerging and reemerging globally is unprecedented (Jones et al. 2008; Joly et al. 2016). Throughout the last century, integrated disease management strategies have significantly reduced the impact of vector-borne diseases through drug development, vector abatement programs, and repellent. Even amid these technological advances, globalization, shifting socio-economic behaviors, and environmental changes continue to alter our global epidemiological landscape. These effects facilitate the dispersal and emergence of pathogens worldwide at the cost of millions of lives and billions of dollars (Mayer et al. 2017, Hassell et al. 2017). In just two decades, we have witnessed the global emergence of multiple arboviruses in the western hemisphere that have infected millions of individuals and claimed thousands of lives, for diseases such as West Nile virus (WNV) in 1999, chikungunya virus in 2013, and Zika virus in 2015. Moreover, hemorrhagic arboviruses, such as Yellow Fever virus, Rift Valley fever virus (RVFV) and Japanese Encephalitis, continue to re-emerge despite advancements in vaccine technologies**.** As global commerce continues to grow, and ecological communities continue to change, there is a desperate need to identify ecological and environmental drivers of vector-borne pathogen spillover. These would allow us to effectively focus disease prediction, prevention, and detection efforts on ecological communities and landscapes prone to disease emergence (Kading et al. 2018, Kilpatrick et al. 2012).

Field and laboratory studies strongly demonstrate that arboviruses persist in a transmission cycle between blood-feeding vectors and vertebrate amplification hosts and are often studied from a single-pathogen framework (Collinge and Ray 2006, Vogels et al. 2019).

This simplified perspective has been monumental in identifying key factors that affect parasite transmission, disease incidence, and the dynamics of host-parasite systems, which in turn have significantly advanced disease control strategies (Anderson and May 1978). However, vector-borne pathogens do not exist in isolation, but persist among a variety of co-circulating organisms including viral, protozoan, fungal and bacterial agents (Woolhouse et al. 2005, Petney and Andrews 1998, Keusch and Migasena 1982). Mounting evidence continues to demonstrate that within-host community heterogeneity can modulate infectious disease heterogeneity (Petney and Andrews 1998, Jolles et al. 2008, Cattador et al. 2008, Ezenwa et al. 2016, Fenton et al. 2008, Wobeser 2008, Cox et al. 2001, Woolhouse et al. 2015). However, the consequence of co-circulating parasites on vector-borne pathogen population dynamics remains poorly explored (Vogels et al. 2019, Vazquez-Prokopec et al. 2016). This is surprising considering advancements on this topic may facilitate novel control strategies to identify and target biological communities more prone to zoonotic amplification and pathogen spillover (Belden and Harris 2007, Pederson and Fenton 2007, Moore 2008).

Zoonotic parasites are being increasingly studied because of their direct ties to human health and food security, however the role of non-zoonotic pathogens that co-circulate with zoonotic pathogens at individual, population, and community scales is also fragmentary (Wobeser 2008). A growing body of knowledge continues to demonstrate that co-infection can shape the distribution of disease at the individual and population level (Cox 2001). For example, Lloyd-Smith et al. [2008] used clinical and epidemiological evidence to simulate how co-infections alter individual level disease dynamics to influence population level disease dynamics. Specifically, they showed that HIV-1 increases the invasion threshold for many parasitic diseases, such as Leishmania and schistosoma infections. Further, Pathak et al. [2012]

demonstrated that the respiratory bacterium, *Bordetella bronchiseptica* can facilitate gastrointestinal helminth infection by *Graphidium strigosum* at the population level in European rabbits (*Oryctolagus cuniculus*). Finally, a long-term study of African Buffalo populations has been instrumental in connecting the effects of parasites on individual hosts to disease outcomes at the population level. Ezenwa et al. [2010] has shown that nematode infections make the host more vulnerable to microparasite infections by depressing the Th1 response of the host's immune system. This group went on to demonstrate with a mathematical model that nematode infections are affecting bovine tuberculosis (TB) dynamics in buffalo (Ezenwa and Jolles 2011). However, this study made it clear that nematode infection impacts TB transmission through multiple contradicting interactions. In a more recent study, TB prevalence in one herd of buffalo was shown to make individual buffalos two times more likely to become infected with Rift Valley fever virus (RVFV), a mosquito-borne virus endemic to the region of study, compared to a neighboring herd [Beechler et al. 2015]. Through mathematical models it was further concluded that larger RVFV outbreaks were expected in herds exposed to higher TB burdens, leading to higher rates of fetal abortion and mortality. Ultimately, these examples make it increasingly clear that we need to investigate infectious diseases in the context of multi-host, multi-parasite communities.

In the context of arboviral disease, West Nile virus (WNV) has become one of the most important causative agents of viral encephalitis in the world (Chancey et al. 2015). After its introduced to New York in 1999, it quickly spread throughout the contiguous United States (US) leading to large-scale declines in bird populations and the largest neuroinvasive disease outbreaks in US history, which in total has resulted in more than 48,000 human cases of disease and over 2,000 deaths (5,674 cases and 286 deaths were reported in 2012 alone) (CDC, Kramer

et al. 2008, LeDeau et al. 2007, Peterson et al. 2013). While much progress has been made understanding WNV transmission in nature, many patterns of when and where WNV occurs remains unresolved and difficult to predict or explain (Nasci 2013). For example, WNV exhibits incredible plasticity in the ability to thrive in some regions for some years causing large epidemics (i.e Chicago, Illinois), while in other regions or other years the virus remains at endemic levels or absent all together (Bertolotti et al. 2008, Peterson et al. 2013). Much of temperate North America appears suitable for fueling rapid amplification events of WNV in the late summer whereas many subtropical, tropical, and temperate regions elsewhere in the Americas has evidence of sporadic WNV transmission but not large amplification events resulting in epidemics (Chancey et al. 2015). Classically, WNV is studied in the one-host, one-pathogen framework, yet wild birds are known to host a suite of parasites that can influence individual fitness and drive population dynamics (Hudson et al. 2002).

The aim of this dissertation is to assess the impact of co-circulating parasites on vector-borne disease ecology using West Nile virus as a case study. Mosquito and bird hosts involved in the transmission of WNV are exposed to a number of different parasites, such as Culex flavivirus, haemospridians, Trypanosomatids and filarioid nematodes (Booth et al. 2015, Newman et al 2011, Hamer et al 2013, Hamer et al 2013b). Within these hosts several lineages of *Plasmodium* spp. (i.e. avian malaria) and *Haemoproteus* spp. (Medeiros et al 2013). have been documented to range from zero to 62%. Overall *Plasmodium* prevalence rates have been shown to be 51% in American robins, with peak prevalence rates in juvenile birds (75%) being synchronized with the increase in *Culex* mosquito WNV infection rates in August. Even more, it has been shown that mosquitoes are not only exposed to *Plasmodium*, *Haemoproteus*, and *Trypanosoma* parasites, but can be simultaneously co-infected with WNV (Boothe et al. 2015)

Given these findings, it is clear that WNV is co-circulating in mosquito and birds hosts with a variety of parasites, yet their role in WNV epidemic dynamics remains unknown.

In this dissertation, field research, controlled laboratory transmission experiments, and mathematical modeling techniques are combined to assess how polyparasitism may influence vector-borne pathogen transmission dynamics. In Chapter II, field and laboratory methods are used to characterize the infection prevalence and lineages of blood parasites impacting Great-tailed Grackles in College Station, Texas. In Chapter III, a mathematical framework is presented to quantify the impact of co-circulating parasites on vector-borne pathogen systems through multiple mechanisms of indirect interaction in vectors and hosts. In Chapter IV, classic methods of experimental transmission are utilized to evaluate the impact of *Plasmodium* parasites on metrics of vector competence. Understanding how within-host interactions of organisms scale-up to impact population-level dynamics of zoonotic arboviruses will help to develop disease management strategies for allocating resources for research and surveillance.

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CHAPTER II

VECTOR-BORNE BLOOD-PARASITES OF THE GREAT-TAILED GRACKLE (*QUISCALUS MEXICANUS*) IN EAST-CENTRAL, TEXAS

2.1 Synopsis

Great-tailed grackles (GTGR; *Quiscalus mexicanus*) have dramatically expanded into North America over the past century. As carriers of *Salmonella*, West Nile virus, and Saint Louis Encephalitis, GTGR are important zoonotic pathogen reservoirs, however little is known about the non-zoonotic community of parasites they support. Here, we use field and laboratory methods to characterize vector-borne blood-parasite prevalence in College Station, Texas, an urban area with a large abundance of GTGR. In 2015, 61 GTGR were captured using mist nets and blood samples were taken. Field microscopy and molecular diagnostics demonstrate that 87% of GTGR have at least one blood-parasite. Multiple parasite species were found in 50% of GTGR, including: *Haemoproteus* (*Paraheamoproteus*) species (63%), *Plasmodium cathemerium* (2%), avian trypanosomes (24%), and filarioid nematodes (52%). Parasite community structure was not determined to be dictated by any interspecific interactions among parasites. This is the first study to document trypanosome, Haemosporida, and filarial nematode parasite density distributions in GTGR.

2.2 Introduction

Haemosporida, filarioid nematodes, and trypanosomes are vector-borne blood parasites that persist in a wide range of avian hosts (Greiner et al., 1975). Although these parasites often manifest as chronic infections associated with limited anemia in birds, certain lineages can impact the long-term demographics of host populations and cause severe symptoms depending

on the specific host-parasite combination (Atkinson et al., 2009; Bennett et al., 1993; van Riper et al., 1986). The role of introduced *Plasmodium relictum* in decimating Hawaiian avifauna is a classic example demonstrating the extent to which blood parasites can influence population and community dynamics (van Riper et al., 1986). Because of their widespread presence, genetic diversity, and relative ease of sampling, haemosporida and other blood-parasites are frequently used to study ecological, evolutionary, and behavioral questions in wildlife systems (Merino et al., 2000; Valkiunas, 2004).

Populations of GTGR have dramatically expanded into North America over the past century (DaCosta et al., 2008; Wehtje, 2003). Prior to 1865, populations of GTGR were documented in Central America, Mexico, and the southernmost tip of Texas. Between 1880 and 2000, GTGR expanded their breeding range into the United States from an estimated 64,000 km^2 to more than 3,561,000 km^2, an annual expansion rate of 3.4% (Wehtje, 2003). The simultaneous expansion of GTGR subspecies *Q. m. nelson*, *Q. m. monsoni*, and *Q. m. prospodicola* into North America indicates GTGR have pre-adapted characteristics conducive to range expansion (Grabucker and Grabucker, 2010, Selander and Giller 1961). The notable rapidity of GTGR expansion is likely facilitated by their ability to adapt to food resources and safe habitats provided by human-modified environments. For example, adaptive behaviors observed in GTGR include eating dead insects off license plates (Grabrucker and Grabrucker, 2010), shadowing farm machinery to collect uncovered invertebrates (Rappole et al. 1989), or roosting in well-lit urban parking lots (Wehtje, 2003). The explosive expansion of GTGR is certainly consequential to ecosystem function and evolutionary trajectories in non-native territories (Mooney and Cleland, 2001).

Birds host a suite of parasites that impact individual fitness and population dynamics (Atkinson et al., 2009; Hudson et al., 2002). For example, the invasive house sparrow (*Passer domesticus*) is a well-known amplification host for arthropod-borne viruses like St. Louis Encephalitis and West Nile virus (Hamer et al., 2011; Reisen et al., 2005). However, these birds also support a community of lesser-studied non-zoonotic parasites, such as trematodes, cestodes, nematodes, trypanosomes, and Haemosporida, that are known to influence individual health and population dynamics (Hamer et al., 2013; Hamer and Muzzall, 2013; Hatcher et al., 2014; Martinez-Padilla et al., 2014; Medeiros et al., 2014b). As known carriers of West Nile virus, Saint Louis Encephalitis, and *Salmonella*, the expansion of GTGR populations throughout North America may have epidemiological importance, however little is known about the non-zoonotic community of parasites circulating in these hosts (Grigar et al., 2016; Komar et al., 2013; Morales-Betoulle et al., 2013) that may impact the health of GTGR or other avian species.

The objective of this study is to document the prevalence of vector-borne blood-parasites circulating in GTGR, a poorly studied host that continues to expand its range into North America. A null model of parasite colonization was used to test if parasite community structure in GTGR is dictated by any interspecific interactions among vector-borne blood-parasites. Additionally, genetic bar coding and phylogenetic analyses were utilized to assess genetic variability among parasite isolates. To our knowledge, this is the largest study documenting the trypanosome, haemosporida, and microfilariae blood-parasite assemblage in GTGR. Results provide a comparative foundation GTGR blood-parasite community structure that is surprisingly underrepresented in the published literature.

2.3 Methods

Bird Collection: GTGRs were collected from five communal roost locations in urban parking lots with artificial lighting of College Station, Texas outlined previously (Grigar et al., 2016). Sampling events occurred on seven different nights between February and July, 2015. Communal roosts were principally composed of GTGRs and European starlings (*Sturnus vulgaris*), however house sparrows and Eurasian-collard doves (*Streptopelia decaocto*) were also present. Birds were captured using nylon mist nets (20 meter length; Association of Field Ornithologists, Portland, Maine) that were erected to a height of 6-meters so nets reached the level of the canopy of arboreal communal roost sites. To stabilize nets at the appropriate net height, poles were made up of either a rigid conduit pipe or galvanized pipes connected by a threaded couplet. Mist nets were initially composed of 31mm mesh until larger grackles were observed escaping nets and a 61 mm mesh nets were substituted. The nets were erected before dusk as the communal birds were staging, approximately 1-4 hours before sunset, and run 3-4 hours each night.

After extraction from nets, blood samples of a volume less than 1% of the bird's body weight were collected with a 1 mL tuberculin syringe by jugular or brachial venipuncture. Blood was processed for field microscopy (see below) and the rest was placed in microcentrifuge tubes for molecular diagnostics. The birds were banded with uniquely numbered leg bands issued by the Bird Banding Laboratory of the US Geological Survey and released at the site of capture. All fieldwork followed methods permitted by the Institutional Animal Use and Care Committee at Texas A&M University (2012-100), US Fish and Wildlife Service (Migratory Bird Scientific Collection Permit MB89164A-0), US Geological Survey (Federal Bird Banding Permit 23789),

Texas Parks and Wildlife Department (Scientific Research Permit SPR-0512-917), and private property owners.

Trypanosome and filarioid nematode parasite detection: To determine the infection prevalence of trypanosome and filarioid nematode parasites, approximately 65uL of whole blood was transferred to a heparinized capillary tube, centrifuged, and screened for the presence of trypanosomes and filarioid nematodes in the field using a 40X compound microscope focused on the buffy coat layer as previously described (Hamer et al., 2013). The entire region of the capillary tube containing the buffy coat and plasma were searched by microscopy. After parasite detection, the buffy coat portion from the capillary tube for positive samples was transferred to a microcentrifuge tube for genetic barcoding of parasites. Whole blood not used during field microscopy was separated into serum and clot fractions by centrifugation (14,000 rpm for 6 minutes) and stored in a -20°C freezer for molecular diagnostics.

Haemosporida parasite detection: The following methods were used to determine the lineage and infection prevalence of *Haemoproteus* and *Plasmodium* species in GTGR. Nucleic acid from avian blood clots was extracted using the Biotek E.Z.N.A tissue DNA kit (Omega Bio-tek, Inc., Norcross, Georgia) after overnight incubation in proteinase K. Initially, DNA from avian blood clots were screened by PCR targeting a 154bp fragment of the haemosporida family 16S rRNA gene. Samples with amplicons at the correct molecular weight were identified as suspect-positive for haemosporida infection (Fallon et al., 2003; Fecchio et al., 2013). These suspect positive samples were then subjected to a nested PCR that targets a ~590bp fragment of the mitochondrial cytochrome b gene (*cytB*) in *Haemoproteus* and *Plasmodium* species (Fallon et al., 2003). To determine haemosporida prevalence, samples that produced PCR amplicons of the

correct size for both PCR reactions species were considered positive. Samples that produced an amplicon in only the screening assay and not the confirmatory assay were considered negative.

Plasmodium and *Haemoproteus* parasite lineages were determined by comparing genetic sequences produced from the *cytB* PCR amplicon to publicly available nucleotide data maintained by NCBI's Nucleotide Database and MalAvi (Bensch et al., 2009; Benson et al., 2008). Briefly, *cytB* amplicons were purified with ExoSap-It (Affymetrix USB, Cleveland, Ohio) and sequenced using the forward primer of the nested *cytB* PCR (413F) (Eton Biosciences Inc., San Diego, CA). Sequence chromatographs were inspected individually using 4Peaks version 1.8 (Nucleobytes, Netherlands) to assess quality by ensuring proper base calls, identifying sequence discrepancies and double peaks. Sequences of high quality were used to assign haemosporida parasite lineages using a 0.6% sequence divergence threshold from previously described parasite lineages available on MalAvi (n=1348) and population datasets published on NCBI's nucleotide database. Lineage sequences were aligned in MAFFT (Katoh and Standley, 2013), trimmed to an even length, and sequence divergence was measured based on a distance matrix generated in Geneious version 9.1.4. Sequences that did not match known *cytB* lineages with >99.4% accuracy were considered unique avian malaria lineages (Ricklefs et al., 2005).

Trypanosome and filarioid nematode phylogenetics: To evaluate the genetic diversity of trypanosome and filarioid nematode parasites, DNA was extracted from the buffy coat of a subset of trypanosome (n=4) and filarioid nematode (n=6) samples identified as positive by field microscopy and compared to sequences available through NCBI's nucleotide database. Trypanosome DNA was amplified using a nested PCR targeting a 326bp fragment of the trypanosome SSU rRNA (Sehgal et al., 2001). Filarioid nematode sDNA was amplified using a PCR targeting a 688bp fragment of the conserved nematode mitochondrial cytochrome oxidase 1

(COI) (Hamer et al., 2013; Sehgal et al., 2001). After bi-directional sequencing as described above, sequence chromatographs were assessed for quality and when mismatches were present, IUPAC nucleotide ambiguity codes were substituted. Aligned forward and reverse consensus sequences were used for phylogenetic analysis.

Following prior methods, *Thelazia lacrymalis* (GenBank: AJ271619) and *Bodo caudatus* (GenBank: AY490218) were selected as outgroup taxa for phylogenetic analysis of filarioid nematode and trypanosome datasets, respectively (Hamer et al., 2013). Trypanosome and filarioid nematode sequence datasets were aligned using ClustalW alignment in Geneious version 9.1.4 and trimmed to equal length (Kearse et al., 2012). The aligned datasets consisted of 219 positions for the trypanosome SSU rDNA dataset and 527 positions for the filarioid nematode COI gene dataset. Phylogenetic trees were constructed using both Bayesian and Maximum likelihood methods to corroborate tree topology using RAxML version 7.2.8 and Mr. Bayes version 3.2.6 plugins in Geneious (Huelsenbeck and Ronquist, 2001). Appropriate rates of evolution were selected based on the statistical results of Jmodeltest (Darriba et al., 2012; Fungiflora and Gascuel, 2003).

Polyparasitism assembly analysis: To determine if haemoparasite co-infection in GTGR were more or less frequent than expected by chance, a null model of expected co-infection frequencies based on the apparent prevalence of parasites detected during this study was generated following methods described by Janovey et al. (1995). Blood samples from GTGR that were screened for trypanosome, filarioid nematode, and haemosporida were utilized for this analysis (n=45). Significant deviations from the null model were evaluated using the chi square statistic and may indicate interspecific interactions between parasites that influence blood-parasite assembly patterns (Poulin, 1996)**.**

2.4 Results

Bird processing: In total 61 GTGR were captured, of which, 59 were adults and two were juvenile. Of the adult birds, 49 were female and 10 were male. Sex for juvenile birds was undetermined. Blood samples were obtained from 60 GTGR.

Trypanosome and filarioid nematode parasites detection: Of 60 GTGR blood samples, 46 samples yielded sufficient blood volume to screen for trypanosome and filarioid nematodes by hematocrit centrifugation and field microscopy. Of these, 52% (n=24; CI: 37-66%) were visually infected with filarioid nematodes and 24% (n=11; CI: 12-36%) were infected with avian trypanosomes (Table 1). The motility of the parasites in the buffy coat under 40X magnification was recorded for one GTGR (Supplemental file 1).

Haemosporida parasite detection: Overall, 73% (n=44; CI: 62-85%) of GTGR individuals were determined to be infected with *Plasmodium* or *Haemoproteus* parasites (Table 1). The initial PCR targeting the 16S rRNA gene amplified DNA in 47 of the 60 avian blood clot samples. Follow up nested PCR of the 47 samples produced 44 visible PCR amplicons of correct size. Three reactions failed, which were subsequently determined to be uninfected. High quality sequences allowed for lineage determination in 38 of these 44 samples. Parasite lineages include *Haemoproteus* (*Parahaemoproteus)* lineage CHI18PA (n=31), CHI22PA (n=6), and CHI18PA/CHI22PA mixed infection (n=1). The *cytB* gene of the *Plasmodium* species detected during this study (n=1) most closely matched *Plasmodium cathemerium* in GenBank with 99% identity (GenBank Accession AY377128.1).

Trypanosome and filarioid nematode phylogenetics: The GTGR filariod nematode sequence 150622-B04 forms a distinct clade with two *Chandlerella quiscali* sequences isolated from a northern cardinal and a common grackle (*Quiscalus quiscula*) in the USA (Figure 1;

GenBank: MH379969). The GTGR isolate 150413-B16 matches an *Onchocercidae* species isolated from a common grackle in the USA with 99% identity and an *Onchocercidae* species isolated from an American robin (*Turdus migratorius*) with 91% identity (Figure 1; GenBank: MH379968). GTGR COI gene sequences 150413-B15 (GenBank: MH379967), 150622-B13 (GenBank: MH379970), 150325-B05 (GenBank: MH379965), and 150331-B15 (GenBank: MH379966) form a monophyletic clade in comparison to other filarioid nematode COI gene sequences (Figure 1).

The SSU rDNA sequence from GTGR 150218-B13 (GenBank: MH379963) is identical to avian trypanosome sequences isolated from a Eurasian sparrowhawk (*Accipiter nisus*) in the Czech Republic, a yellowhammer (*Emberiza citronella*) from the Czech Republic, a house sparrow from the USA, and a village weaver (*Ploceus cucullatus*) from Gabon (Figure 2). These six sequences form a unique clade with posterior probability support of 1. The trypanosome species isolated from GTGR 150622-B07 (GenBank: MH379964) is identical to an avian trypanosome sequence generated from a yellow-breasted chat (*Icteria virens*) captured in the USA and a wood warbler (*Phylloscopus sibilatrix*) captured in the Czech Republic. These three isolates form a clade with 0.93 posterior probability support. The SSU rDNA sequence of GTGR 150325-B12 (GenBank: MH379962) is identical to six trypanosome sequences isolated from a Latham francolin (*Francolinus lathami*) captured in Cameroon, a biting midge (*Culicoides festivipennis*) (unknown location), an Ashy robin (*Hateromyias albispecularis*) from Australia, an American robin from the USA, a house sparrow from the USA, and a collared flycatcher (*Ficedula albicollis*) from the Czech Republic. GTGR 150413-B16 (GenBank: MH379961) is identical to trypanosome genetic sequences isolated from the blood of a northern cardinal (*Cardinalis cardinalis*) in the USA and an ashy robin from Australia.

Polyparasitism assembly analysis: Complete data on haemosporida, trypanosome, and filariod nematode infection status was obtained for 45 individuals. Of these 45 individuals, 53% (n=24) of GTGR were infected with multiple haematoparasite species (Table 2). Haemosporida-filarioid nematatode co-infections were most common (n=16), followed by haemosporida-trypanosome (n=4), and three-way infections of haemosporida-trypanosome-filarioid nematode (n=4) (Table 2). There were no filarioid nematode-trypanosome co-infections without a haemosporida infection. There were 6 (13%) GTGR with no detected haematoparasites (Table 2). In comparison to a null model, rates of parasite co-infection did not vary from what was expected suggesting parasite colonization is not dictated by interactions among different parasite species (Table 2) (X^2= 3.9309, df= 7, p-value= 0.7877).

Table 1 Haematoparasite prevalence in great-tailed grackles (*Quiscalus mexicanus*), College Station, Texas, 2015

Haematoparasite	Genus	Lineage	Count	Sample size	Est. Prevalence	95% C.I.
Filarioid nematode	-	-	24	46	0.52	0.37-0.66
Avian Trypanosome	-	-	11	46	0.24	0.12-0.36
Haemosporida	-	-	44	60	0.73	0.62-0.85
	**Haemoproteus*	-	38	60	0.63	0.51-0.76
	-	*CHI18PA*	31	60	0.52	0.37-0.63
	-	*CHI22PA*	6	60	0.1	0.03-0.18
	-	*CHI18PA/CHI22PA*	1	60	0.02	0-0.05
	Plasmodium	*Unclassified*	1	60	0.02	0-0.05
	Undetermined	-	5	60	0.08	-

** Haemoproteus (Parahaemoproteus) species*
C.I. = Confidence Interval

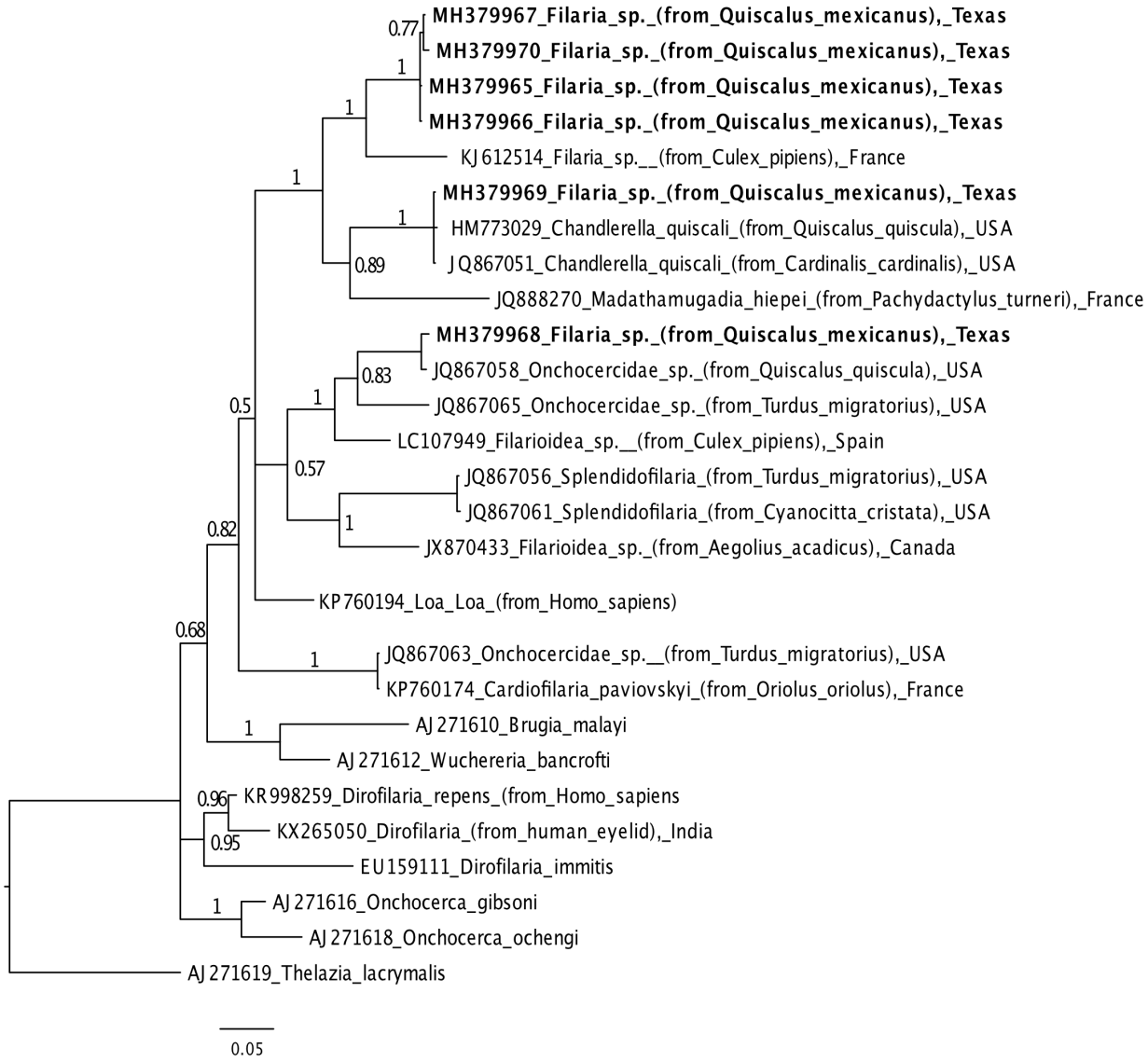

Figure 1 A phylogenetic tree constructed from a 527 base pair segment of filarial nematode DNA inferred using mitochondrial cytochrome oxidase 1 gene sequences from 27 organisms using *Thelazia lacrymalis* (GenBank: AJ271619.1) as an outgroup. Filarial nematode sequences from great-tailed grackles captured in College Station, Texas are listed in bold font.

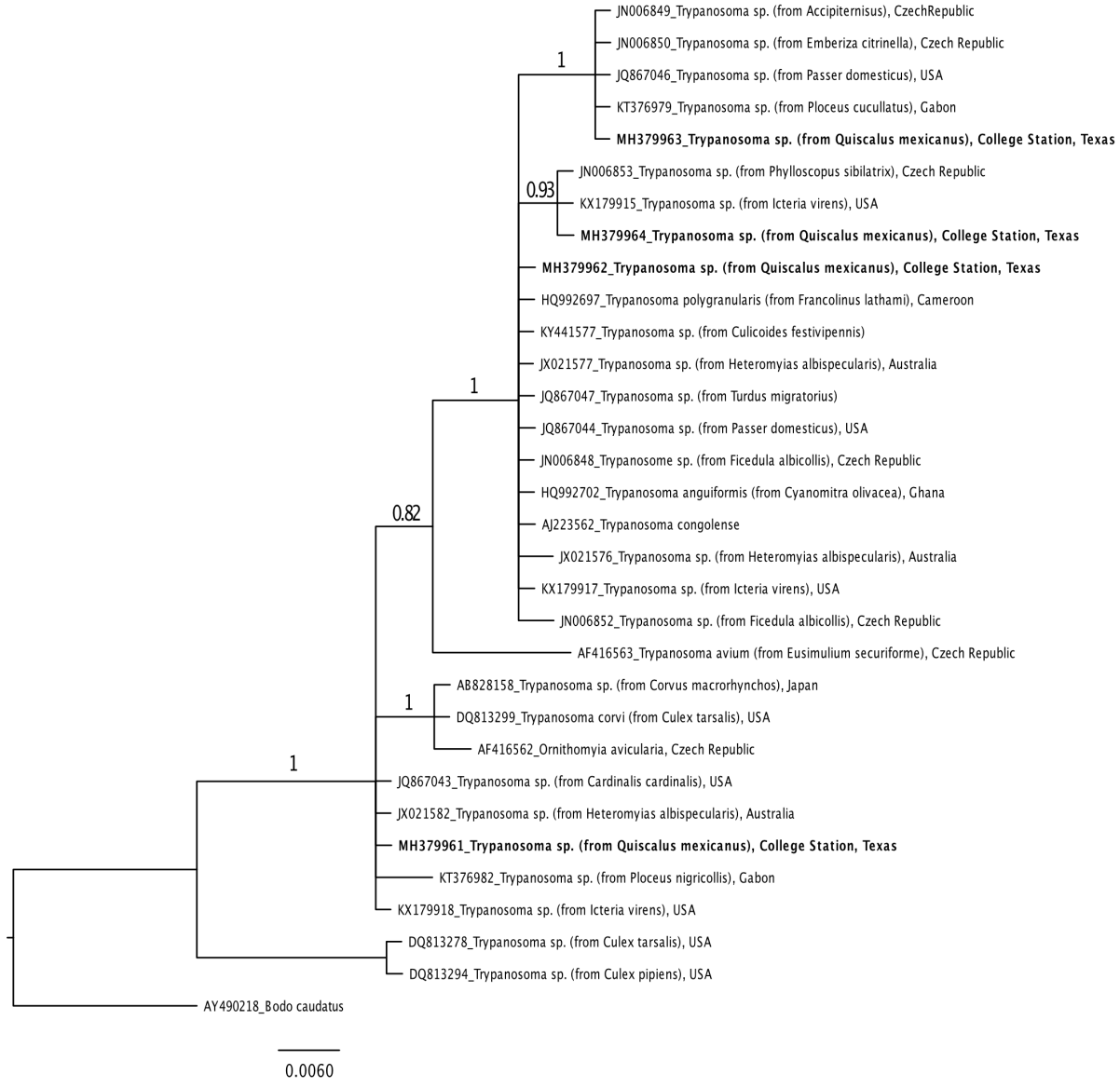

Figure 2 A phylogenetic tree constructed from a 527 base pair segment of filarial nematode DNA inferred using mitochondrial cytochrome oxidase 1 gene sequences from 27 organisms using *Thelazia lacrymalis* (GenBank: AJ271619.1) as an outgroup. Filarial nematode sequences from great-tailed grackles captured in College Station, Texas are listed in bold font.

Table 2 Co-infection of great-tailed grackles (*Quiscalus mexicanus*), College Station, Texas, 2015

Infection Status	Sample size	Expected	Observed	Estimated proportion of population	95% Confidence Interval
No infection	*45*	4	*6*	0.13	0.03-0.22
Haemosporida sp. (H)	45	12	9	0.20	0.08-0.32
Filarial Nematode sp. (F)	45	5	4	0.09	0.01-0.17
Trypanosome sp. (T)	45	1	2	0.04	0-0.10
H:F co-infection	*45*	13	*16*	0.36	0.22-0.50
H:T co-infection	*45*	4	*4*	0.09	0.01-0.17
F:T co-infection	*45*	1	*0*	0.00	0
H:F:T co-infection	*45*	4	*4*	0.09	0.01-0.17

To determine if parasite colinization in GTGR was more or less frequent than expected by chance, the frequency of co-infection between multiple parasites was compared to a null model of expected co-infection frequencies based on the apparent prevalence of parasite colonization detected during this study (Janovey et al. 1995). Significant deviations from the null model were evaluated with the chi-squared statistic. Only blood samples from GTGR that were screened for trypanosome, filarioid nematode, and haemosporida infections were utilized for this analysis (n=45). The null hypothesis (there are no interactions between parasite species dictating host colonization) is rejected if the chi-squared p-value is less than 0.05. The chi-squared for given probabilities = 3.93, df=7, p=0.79.

2.5 Discussion

GTGR in east-central Texas harbor abundant and diverse vector-borne blood-parasites (Table 1) with only 13% of birds having no detectable parasitism. Considering avian blood-parasites are geographically and taxonomically widespread, the presence of these parasites in GTGR is not unusual (Chagas et al., 2017; Greiner et al., 1975; White et al., 1978). These results suggest GTGR are subjected to a relatively high level of vector-borne blood-parasitism. In contrast, a prior study in Tempe, Arizona documented no Haemosporida, *Trypanosoma*, or filarioid nematode infections in 23 GTGR individuals screened for blood-parasites (Toomey et al., 2010). Whether differences between these studies are due to genetic (*Q. m. prosopidocola* are predominantly in Texas, while *Q. m. nelson* and *Q. m. monsoni* are in Arizona) (Selander and Giller 1961), environmental, ecological, or methodological factors, rates of vector-borne blood-parasitism in College Station, Texas GTGR appear to be elevated.

A 1970s survey of blood-parasites in 388 North American bird species documented that 19.5% of birds were infected with *Heamoproteus* species, 17.7% with *Leucocytozoan* species, 3.9% with *Trypanosoma* species, 3.8% with *Plasmodium* species, and 3.1% with filarioid nematodes (Greiner et al., 1975). The prevalence of *Haemoproteus*, *Trypanosoma*, and filarioid nematode infected detected in GTGR are markedly higher than those averages across diverse avian species. The prevalence of *Haemoproteus* (20.3%), *Trypanosoma* (2%), and filarioid nematode (3%) blood-parasitism in the closest taxonomic relative to GTGR in the US- the common grackle (*Quiscalus quiscula*) (Greiner et al., 1975)- are lower than those detected among the GTGR. In contrast, the prevalence of *Plasmodium* (2%) in GTGR was lower than both common grackles (13.3%) and the average across North American bird species (3.8%) (Greiner et al., 1975). Differences in parasitism may be associated with individual, population, or community consequences.

Filarioid nematodes, *Haemoproteus* species, *Plasmodium* species, and avian trypanosomes are vector-borne parasites widely found in avian populations across the globe (Atkinson et al. 2009; Zidkova et al., 2012). Generally, these parasites are believed to be non-pathogenic in avian hosts, but on occasion can lead to severe avian disease (Atkinson et al., 2009; Stone et al., 1971; Valkiunas, 2004). A band was recovered and reported from a GTGR individual 380 meters from the original sampling site 35 months after capture (Band Number: 1713-79042). Blood from this GTGR tested positive for *Haemoproteus* CHI18PA at the initial time of sampling. Although the cause of death is unknown, recovery of a banded individual 35 months after the detection of *Haemoproteus* CHI18PA suggests this *Haemoproteus* lineage may be a non-debilitating, chronic infection in GTGR. This may explain why CHI18PA was the most frequently documented parasite in GTGR from this study (Table 1). Conversely, if *Plasmodium*

species, such as *P. cathemerium,* are causing GTGR mortality and/or reduced mobility it could explain the relatively low rates of *Plasmodium* detection in GTGR (Table 1). Nonetheless, the clinical outcomes associated with the blood-parasites detected in GTGR remain unknown.

Documentation of *Haemoproteus* lineage CHI18PA in GTGR in the current study and northern cardinals (*Cardinalis cardinalis*) based on prior work (Medeiros et al., 2016) suggests that CHI18PA may be a generalist parasite of the superfamily Passeroidea. In contrast, *Haemoproteus* lineage CHI22PA appears to be an ictarid specialist having been documented in the common grackle (*Quiscalus quiscula*), red-winged blackbirds (*Agelaius phoeniceus*) (Medeiros et al., 2014a) and in GTGR by this current study. Genetic variability observed in a subsample of COI gene sequences suggests multiple filarioid nematode lineages may be circulating in GTGR populations (Figure 1). Similarly, genetic variability observed among *Trypanosoma* SSU rDNA gene sequences suggests multiple *Trypanosoma* lineages may be circulating in GTGR (Figure 2). Whether this genetic variability represents multiple filarioid nematode or *Trypanosome* species remains unknown.

Overall, 87% of GTGR were determined to have blood-parasites and over 50% of individuals had multiple blood-parasites. Considering filarioid nematodes, *Haemoproteus* species, *Plasmodium* species, and avian trypanosomes are vectored by a composite of lice (order Phthiraptera), hippoboscids, mites, mosquitoes, biting midges and black flies (Simuliidae), the centrality of GTGR to a variety of feeding vectors highlights their predisposition to influence vector-borne transmission networks (Atkinson et al., 2009, Votýpka and Svobodova, 2004). As a follow-up to the observations of the current study, we sampled *Culicoides* from this same urban location in College Station, TX in 2016 and documented 10 species with *C. crepuscularis* positive for *Onchocercidae* sp. and *Haemoproteus* sp. DNA (Martin et al. 2019). Whether high

blood-parasitism in GTGR is a result of elevated exposure to vectors or a consequence of GTGR susceptibility, results suggest GTGR may have a propensity to impact vector-borne parasite dynamics as amplification hosts and parasite spillback (Greiner et al., 1975; Kelly et al., 2009; Miller et al., 2017; Nelson et al., 2015).

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CHAPTER III

THE CONSEQUENCE OF CO-CIRCULATING PARASITES FOR VECTOR-BORNE PATHOGEN TRANSMISSION

3.1 Synopsis

Mosquitoes ingest a variety of viruses, protozoan, and macroparasites that circulate among avian and mammalian hosts. The consequence of these co-circulating parasites for vector-borne pathogen population dynamics remains largely unknown. Here we construct, analyze, and parameterize two compartmental models that describe the dynamics of vector-borne pathogen transmission structured by host and vector co-infection status. To illustrate the utility of this framework, vector and host co-infection models are parameterized to the West Nile virus (WNV) system. Results from global sensitivity analyses demonstrate that the WNV reproductive number (R_0) is the most sensitive to changes in avian host competence, mosquito extrinsic incubation period, mosquito feeding rates, the proportions of mosquitoes expected to become infected and the proportion of hosts expected to succumb to disease. Further, we use case studies to demonstrate that avian malaria parasites may theoretically alter estimates of R_0 by as much as 48% by modulating epidemiological parameters indirectly through changes in vector feeding behavior, host mortality, vector competence, or vector survivorship. Importantly, results quantitatively illustrate that avian malaria parasites may simultaneously increase and decrease the reproductive number of WNV through multiple mechanisms of interaction. These results stress a need to empirically and analytically evaluate the impact of polyparasitism on transmission dynamics as a compounding consequence of multiple ecological and biological mechanisms. Although the ecological and evolutionary impacts of co-circulating parasites on vector-borne disease systems

remains complex, the mathematical framework presented here provides an analytical structure to prioritize theoretical mechanisms of parasite interaction that may impact vector-borne pathogen transmission the most.

3.2 Introduction

The health and economic impact of vector-borne diseases has been significantly reduced in the last century due to strategies of integrated disease management, drug and vaccine development, and dedicated financial support. Even amid this success, the dispersal and emergence of pathogens worldwide continues to accelerate in response to pressures such as globalization, shifting socio-economic behaviors, population growth, land conversion and environmental change (Mayer et al. 2017, Hassell et al. 2017). In just two decades, we have witnessed the global emergence of multiple vector-borne viruses (arboviruses) that have infected millions of individuals and claimed thousands of lives, including West Nile virus (WNV), chikungunya virus, and Zika virus. Moreover, hemorrhagic arboviruses, such as Yellow Fever virus, Rift Valley fever virus (RVFV), Japanese Encephalitis, and dengue virus continue to re-emerge (Tatem 2006, Hatcher et al. 2012, Parham et al. 2015). The unprecedented rate at which vector-borne pathogens continue to emerge and reemerge highlights the importance of understanding vector-borne pathogen transmission ecology (Kading et al. 2018, Kilpatrick et al. 2012, Ogden et al. 2019, Jones et al. 2008; Joly et al. 2016).

Field and laboratory studies strongly demonstrate that arboviruses persist in a transmission cycle between blood-feeding vectors and vertebrate amplification hosts and are often studied from a single-pathogen framework (McKenzie et al. 2005, Vogels et al. 2019). This simplified perspective has been monumental in identifying key factors that affect parasite

transmission, disease incidence, and the dynamics of host-parasite systems, which in turn have significantly advanced disease control strategies (Anderson and May 1978, May and Anderson 1978). However, vector-borne pathogens do not exist in isolation, but persist among a variety of co-circulating organisms including viral, protozoan, fungal and bacterial agents (Woolhouse et al. 2005, Petney and Andrews 1998, Keusch and Migasena 1982). As mounting evidence continues to demonstrate within-host community heterogeneity can modulate infectious disease heterogeneity (Petney and Andrews 1998, Jolles et al. 2008, Cattador et al. 2008, Ezenwa et al. 2016, Fenton et al. 2008, Wobeser 2008, Cox et al. 2001, Woolhouse et al. 2015), the consequence of co-circulating parasites on vector-borne pathogen population dynamics remains poorly explored (Vogels et al. 2019, Vazquez-Prokopec et al. 2016). This is surprising considering advancements on this topic may facilitate novel control strategies to identify and target biological communities more prone to zoonotic amplification and pathogen spillover (Belden and Harris 2007, Pederson and Fenton 2007, Johnson et al. 2015, Moore 2008).

The aim of this paper is to present a mathematical framework that can be used to quantify how vector-borne pathogen population dynamics are impacted by interspecific interactions with co-circulating parasites. Pathogen communities are diverse globally presenting countless mechanisms of direct and indirect interaction that can modulate pathogen transmission (Fenton 2008, Cox 2001). Here we construct two compartmental models that describe the dynamics of vector-borne pathogen transmission in hosts and vector populations supporting a sympatric parasite. This model helps to evaluate how changes in host mortality, vector survivorship, host-vector contact, transmission competence, and rates of host recovery can impact arboviral transmission systems. Understanding how within-host interactions scale-up to impact population-level dynamics of zoonotic arboviruses will help to develop disease management strategies for

allocating resources for research and surveillance.

To illustrate the utility of this framework, the consequence of polyparasitism for WNV transmission is explored using avian malaria as a case study. Numerous studies clearly demonstrate that WNV overlaps with the etiological agents of avian malaria (*Plasmodium* and *Haemoproteus* species parasites) which circulate in the same mosquito and avian hosts (Medeiros et al. 2016, Booth et al. 2015, Hughes et al. 2010). Further, field evidence indicates interactions between these parasites may have population level consequences, however the consequence of these parasites for arboviral transmission remains unclear (Medeiros et al. 2014). Although mechanisms of viral-protozoan interaction in vectors and hosts are numerous, fortunately multiple empirical studies provide hypotheses on how avian malaria may indirectly influence WNV transmission by modulating behavior, susceptibility to infection, transmission competence, or survivorship in host or mosquito vectors (Cator et al. 2012, Lefevre and Thomas 2008, Busula et al 2017, Yan et al. 2018, Cornet et al. 2013a, Cornet et al. 2013b, Ferguson and Read 2004, Pigeault and Villa 2018, Vezilier et al. 2012, Lalubin et al. 2012, Vaughan et al 2012, Koella et al. 1998, Ferguson and Read 2002, Noland et al. 2007). By a) quantifying the sensitivity of WNV transmission to changes in parameter input we aim to identify key parameters through which polyparasitism may impact vector-borne transmission systems. Further, we aim to b) quantitatively examine mechanisms through which avian malaria may modulate WNV population dynamics based on various empirically-guided case studies.

3.3 Methods

Mathematical Model Description: Generally, SIR based vector-borne compartmental models use mass-action kinetics to describe cross-infection between host and vector populations

over a single outbreak season (Wonham et al. 2004). In one such model, the classical SIR model for malaria transmission was expanded into an eight-compartment model with host compartments (susceptible, infectious, recovered, dead) and vector compartments (larval, susceptible, exposed, and infectious) to capture features unique to the WNV system in a single outbreak season (Wonham et al. 2004, Anderson and May 1991). Here we expand the transmission dynamics proposed by Wonham et al. [2004] to build two compartmental models structured by co-infection status in hosts (Figure 1a) and vectors (Figure 1b). For simplicity, host populations are modeled as a closed system where the total number of hosts (Nb) is a constant equal to $Sb_i + Ib_i + Rb_i$ and defined at the beginning of the outbreak season. Considering the relatively short generation time of most vector populations, vital dynamics for vector populations are included in the model and treated as density dependent. The total number of adult vectors in the system (Nm) is equal to $Sm_i + Em_i + Im_i$ where immature vectors enter adult populations (Sm_i) from the larval class (Lm_i) at a rate dependent on the vectors larval population carrying capacity. In these models it is assumed that host-vector cross infection is the only mode of transmission, therefore mechanisms of vertical transmission among vectors and horizontal transmission between hosts are ignored. Ordinary differential equations for host and vector co-infection models are described below in Equations 1-10 and Equations 11-20 respectively.

In both host and vector co-infection models, adult vectors lay eggs at a constant daily rate of B_m and persist in a larval environment with a constant carrying capacity of km. Larvae suffer from a constant daily mortality rate of u_L and mature at a constant daily rate of m. Adult vectors suffer from a daily mortality rate of u_i**.** Transmission of the focal vector-borne pathogen from an infectious vector to a susceptible host is dependent on the daily feeding rate of vectors (a_i), the transmission rate of an infectious vector with a disseminated infection (b_i) and the proportion of

hosts that develop an infection (assumed to be 1). The proportion of avian hosts expected to die from infection with the focal pathogen is defined as x_i, while the proportion of hosts expected to recover from the focal vector-borne pathogen is 1- x_i. Hosts clear the focal vector-borne pathogen at a daily recover rate (r_i) and suffer from a daily mortality rate (x_{di}). Transmission of the focal pathogen from an infectious host to a vector is dependent on the daily feeding rate of vectors (a_i), the efficiency of pathogen transmission from an infectious host to a feeding vector (c_i), the proportion of exposed vectors expected to become infectious (ir_i), and the proportion of vectors expected to develop a disseminated infection (dr_i). The extrinsic incubation period (k_i) is the rate at which exposed vectors develop a disseminated infection at which point they enter the infected class (Im_i). The proportion of hosts and vectors with a co-circulating parasite (P) is defined in the initial conditions. Mature larvae enter susceptible vector subpopulations based on a proportion of vector co-infection (P) where $Nm = (1-P)A_{m1} + PA_{m2}$. Similarly, the proportion of hosts that carry a co-circulating parasite (P) in the Host co-infection model is defined in the initial conditions, where $Nb = (1-P)A_{b1} + PA_{b2}$. For both host and vector co-infection models, parameter values for a_i, b_i, i_{ri}, d_{ri}, c_i, x_i, x_{di}, k_i, r_i, u_i, are dependent on co-infection status, where subscript 1 indicates no co-infection while subscript indicates co-infection (Table 3).

Table 3. Theoretical parameter definitions for host and vector co-infection models.

Parameter	Epidemiological description	Single infection	Co-infection
a_i	*M daily feeding rate*	a_1	a_2
b_i	*Proportion of infectious vectors transmitting virus to hosts while feeding*	b_1	b_2
I_{ri}	*Proportion of vectors expected to become infected following exposure to infectious host*	I_{r1}	I_{r2}
d_{ri}	*Proportion of infected vectors that develop a disseminated infection and become infectious*	d_{r1}	d_{r2}
c_i	*Transmission efficiency of pathogen from infectious host to a feeding vector*	c_1	c_2
x_i	*Host disease-induced mortality fraction*	x_1	x_2
x_{di}	*Rate of mortality*	x_{d1}	x_{d2}
k_i	*Vector extrinsic incubation period*	k_1	k_2
r_i	*Rate of parasite recovery in hosts*	r_1	r_2
u_i	*Daily vector death rate*	u_1	u_2
m	*Daily larval maturity rate in vectors*	-	-
Bm	*Daily vector per capita birth rate*		
km	*Larval vector carrying capacity*	-	-
uL	*Larval vector mortality rates (daily)*		
V:H ratio	*Vector to host ratio*	-	-
P	*Prevalence of co-circulating parasite in hosts and vectors*	-	-

Model Analysis and Formulation of the Basic Reproductive Number: One common approach for exploring the dynamics of infectious disease is by solving for the basic reproductive number (R_0), a useful index for transmission intensity that establishes important threshold criteria for disease persistence (Smith et al. 2007). Frequently, transmission intensity in a system is assumed to be directly related to the magnitude of R_0. This equation also defines a critical equilibrium that aids in predicting when a pathogen may persist in a system or go extinct - when $R_0 > 1$, the system is unstable and viral invasion is expected to lead to an outbreak**,** when $R_0 < 1$,

the disease-free equilibrium (DFE) is locally stable and viral invasion is expected to go extinct. Here, we define R_0 for both host and vector co-infection models.

Disease-free equilibrium of Host co-infection model

If $A_{b1} + A_{b2} = N_b$, the disease-free equilibrium of the host co-infection model S_{b10}, I_{b10}, R_{b10}, S_{b20}, I_{b20}, R_{b10}, L_{m0}, N_{m0}, E_{m0}, I_{m0}, is Ab1, 0, 0, Ab2, 0, 0, Lm0, Nm0, 0, 0, when N_m, L_m, A_{b1}, and A_{b2} are the positive solutions of the algebraic system where:

$$0 = B_m \left(1 - \frac{Lm_0}{km}\right) N_{m0} - (m + u_l)\, Lm_0\,,$$

$$0 = m\, Lm_0 - u_1 N_{m0},$$

given by:

$$Lm_0 = \frac{B_m N_m}{\frac{B_m N_m}{km} + (m + ul)} \text{ and } N_{m0} = \frac{m L_{m0}}{u_1}$$

Disease-free equilibrium of vector co-infection model

If $A_{m1} + A_{m2} = N_{m0}$, the disease-free equilibrium of the host co-infection model S_b, I_b, R, L_{m0}, S_{m1}, E_{m1}, I_{m1}, S_{m2}, E_{m2}, I_{m2} is Ab1, 0, 0, Ab2, 0, 0, Lm0, Nm0, 0, 0, when L_{m0}, A_{m1}, and A_{m2} are the positive solutions of the algebraic system where:

$$0 = B_m \left(1 - \frac{Lm_0}{km}\right) N_m - (m + u_l)\, Lm_0\,,$$

$$0 = m\, Lm_0 (1 - P) - a_1 c_1 \frac{I_b}{N_b} A_{m1} - u_1 A_{m1},$$

$$0 = m\, Lm_0 P - a_2 c_1 {}_{i_{r2}\, d_{r2}} \frac{I_b}{N_b} A_{m2} - u_2 A_{m2},$$

given by:

$$Lm_0 = \frac{B_m N_m}{\frac{B_m N_m}{km} + (m + ul)}\ , A_{m1} = \frac{mB_m N_m (1-P)}{U_1\left(\frac{B_m N_m}{km} + (m + ul)\right)} \text{ and } A_{m2} = \frac{mB_m N_m P}{U_2\left(\frac{B_m N_m}{km} + (m + ul)\right)}$$

To solve for R_0 in each model, we followed methods described by van den Driessche & Watmough [2002]. Supplementary files provide detailed information on how next generation matrices were defined and evaluated to arrive at the expression of R_0 for each system (Supplementary file 1). Briefly, the Jacobian matrices for the linear system of ODEs described

host and vector co-infection were decomposed into two matrices defined based on the appearance of new infections in a compartment, T(x), the rate of transfer of individuals into a compartment, Σ(x), and rate of individuals exiting compartments (Diekmann et al. 2010, van den Driessche and Watmough 2002). The dominant eiegenvalue of each system was computed as the spectral radius of the matrix $-T\,\Sigma^{-1}$ using the computer algebra software wxMaxima version 5.34.1 (Maxima.sourceforge.net) (Diekmann et al. 2010). The R_0 expression for host and vector co-infection models are defined in equation 21 and 22, respectively.

Equation 1: Reproductive number (R_0) for host co-infection model:

$$R_0 = \sqrt{\left(\frac{i_{r1}\, d_{r1}\, a_2^{\,2}\, b_1\, k_1\, c_2\, N_{m0}\, A_{b2}}{u_1\,(u_1 + i_{r1}\, d_{r1}\, k_1)\,((1-x_2)\, r_2 + x_2\, x_{d2})\, N_b^{\,2}} + \frac{i_{r1}\, d_{r1}\, a_1^{\,2}\, b_1\, k_1\, c_1\, N_{m0}\, A_{b1}}{u_1\,(u_1 + i_{r1}\, d_{r1}\, k_1)\,((1-x_1)\, r_1 + x_1\, x_{d1})\, N_b^{\,2}} \right)}$$

Equation 2: Reproductive number (R_0) for vector co-infection model:

$$R_0 = \sqrt{\left(\frac{k_2\, a_2^{\,2}\, b_2\, i_{r2}\, d_{r2}\, c_1\, A_{m2}}{N_b\,(u_2 + i_{r2}\, d_{r2}\, k_2)\,(u_2)\,(x_1\, x_{d1} + (1-x_1)\, r_1)} + \frac{i_{r1}\, d_{r1}\, k_1\, a_1^{\,2}\, b_1\, i_{r1}\, d_{r1}\, c_1\, A_{m1}}{N_b\,(u_1 + i_{r1}\, d_{r1}\, k_1)\,(u_1)\,(x_1\, x_{d1} + (1-x_1)\, r_1)} \right)}$$

It is important to emphasize that the model parameters can be tailored to different vector-borne parasite systems to facilitate quantitative predictions on how co-circulating parasites may influence the transmission of a focal vector-borne pathogen. Importantly, both host and vector co-infection models include important entomological parameters known to influence vector-borne pathogen transmission (Table 3), such as proportions of viral infection, dissemination, and transmission in vectors. For simplicity, these parameters are often combined into a single term (vector competence), but remain separated in these models to aid in the mechanistic exploration of vector-borne pathogen transmission. The application of these models is illustrated using the WNV system, a vector-borne transmission system that has frequently been studied through mathematical models. Importantly, this framework can be adapted to any system where parasites persist in arthropod vectors and vertebrate hosts.

Example: Impact of co-circulating parasites on West Nile virus: Ecological and experimental infection studies strongly indicate that WNV persists in a cycle between passerine birds and bird-biting *Culex* mosquitoes (Kilpatrick et al. 2005, Hamer et al. 2009, Hamer et al. 2011), a dynamic system described by numerous mathematical models (Wonham et al 2004, Wonham et al. 2006, Simpson et al. 2011, Reiner et al. 2013). Published data available in the literature was utilized to parameterize host and vector compartmental models in the absence of co-infection (Table 4). To fully capture the range in parameter space observed in the WNV system, the minimum and maximum values observed in published studies were used to define the minimum and maximum boundaries of parameter space (Wonham et al. 2004, Vogels et al. 2017, Turrell et al. 2001, Sardelis et al. 2001, Goddard et al. 2003, Komar et al. 2003, Kilpatrick et al. 2008, Jones et al. 2012). Mean parameter estimates were estimated based on the average of minimum and maximum values observed in the literature (Table 4).

Sensitivity Analysis*:* To evaluate how R_0 changes in response to changing parameter input in the WNV system, methods of global sensitivity analysis applied (Barradas and Caja Rivera 2018, Samsuzzoha et al. 2013). Specifically, Latin Hypercube Sampling was used to sample parameter space uniformly from the parameter space defined in Table 3 under varying conditions of co-infection (Barradas and Caja Rivera 2018, Marino et al. 2008). Model sensitivity was evaluated under varying conditions of parasite co-infection in host and vectors which ranged from 0-100%. Parameters were sampled 10,000 times for 10,000 runs in program R using the following packages: “deSolve”, “lhs” and “sensitivity” (R Core Team 2018, Carnell 2018, Soetaert et al. 2010, Iooss et al. 2018). Partial rank correlation coefficients (PRCC) were calculated to rank the influence of parameters on R_0 with the sensitivity package (Iooss et al. 2018). The resulting PRCC values fall between -1 and 1 and permit quantitative comparisons

among different model inputs with the magnitude representing the relative importance. Positive values imply a positive association with R_0 while negative values imply a negative association.

Case studies: To further illustrate the utility of host and vector co-infection models, the theoretical impact of avian malaria on WNV transmission was quantified using the R_0 equations for host and vector co-infections derived above. Based on published empirical studies, quantitative hypotheses were defined that explicitly define how avian malaria may theoretically influence epidemiological parameters in the WNV system. For each case study, R_0 was calculated at varying levels of avian malaria prevalence (5%, 10%, 20%, 40%, 80%) using equations 1 and 2. The consequence of each case study for WNV transmission was calculated as the percent error difference between baseline R_0 values (R_0^N) and the adjusted R_0 values (R_0^A) $[(R_0^{Ai}-R_0^{Ni})/ R_0^{Ni}]$.

For each case study, the R_0 expression was estimated 10,000 times using parameters that were uniformly and randomly selected from parameter space. The values for the baseline calculation of R_0 are defined in Table 4. For each case study, all parameters are defined based on the values in Table 4 except for the parameters hypothetically impacted by each case study (a_1, $a_{,2}$, d_{r2}, x_2, k_2, and u_2) which are re-defined based on the quantitative hypotheses of each case scenario. The initial conditions for the host model are S_{b1}= (1-P)A_{b1}, S_{b2}= P A_{b2}, I_{b1}=0, I_{b2}=0, R_{b1}=0, R_{b2}=0, S_m=N_{m0}, E_m=0, I_m=1, L_m=HL_{m0}, where P is defined as 0.05, 0.1, 0.2, 0.4, and 0.8 depending on the level of avian malaria prevalence. The initial conditions for the vector co-infection model are S_b=200, I_b=0, R_b=0, S_{m1}=(P-1)Am1, S_{m2}=PAm2, E_{m1}=0, E_{m2}=, I_{m1}=1, I_{m2}=1, L_m=VL_{m0}, where P is defined as 0.05, 0.1, 0.2, 0.4, and 0.8 depending on the level of avian malaria prevalence.

Table 4. Epidemiological parameters of West Nile virus transmission defined from the published literature.

Notation	Epidemiological description	Minimum and maximum boundaries of Parameter space	Null mean value	Notation for avian malaria infection status (-/+)	Referenced literature
a_i	*M daily feeding rate*	*0.12-0.23*	*0.175*	*a1: (-)* *a2: (+)*	*Vogels et al. (2017)*
b_i	*M with disseminated infection that transmitted virus to B by bite; Assuming a viremia above 6 PFU*	*73-100*	*0.865*	*b1: (-)* *b2: (+)*	*Turrell et al. (2001); Sardelis et al. (2001)*
I_{ri}	*M infection rate >6PFU*	*28-100*	*0.64*	*Ir1: (-)* *Ir2: (+)*	*Sardelis et al. (2001); Goddard et al. (2003)*
d_{ri}	*M proportion of infected developing disseminated infection*	*0.14-0.32*	*0.23*	*dr1: (-)* *dr2: (+)*	*Sardelis et al. (2001)*
c_i	*WNV transmission B to M*	*0-0.68*	*0.34*	*c1: (-)* *c2: (+)*	*Komar et al. (2003)*
x_i	*B mortality fraction per infected*	*0.33-1*	*0.665*	*x1: (-)* *x2: (+)*	*Komar et al. (2003)*
x_{di}	*Rate of mortality*	*0.076-0.33*	*0.203*	*xd1: (-)* *xd2: (+)*	*Komar et al. (2003)*

Table 4. Continued.

Notation	Epidemiological description	Minimum and maximum boundaries of Parameter space	Null mean value	Notation for avian malaria infection status (-/+)	Referenced literature
k_i	*M latent period, days to dissemination*	*0.071-0.25*	*0.1605*	*k1: (-)* *k2: (+)*	*Wonham et al. (2004); Kilpatrick et al. (2008)*
r_i	*B rate of viremia clearance*	0-0.22	0.11	*r1: (-)* *r2: (+)*	*Komar et al. (2003)*
u_i	*Daily mosquito death rate*	0.072-0.12	0.096	*u1: (-)* *u2: (+)*	Jones et al. (2012)
m	*Daily larval maturity rate*	*Constant*	*0.068*	-	*Wonham et al. (2004)*
Bm	*Daily Per capita Birth Rate*	*Constant*	*2*	-	*Wonham et al. (2004)*
km	*Larval Carrying Capacity*	*Constant*	Host: 34000 Vector: 4000	-	-
uL	*Proportion of larval mortality (daily)*	*Constant*	*0.05*	-	-
M:H ratio	*Mosquito to host ratio*	Constant	Host: 32000 Vector: 2000	-	*Vogels et al. (2017)*
P	*Prevalence of avian malaria in birds and mosquitoes*	*0-1*	0.5	-	-

3.4 Results

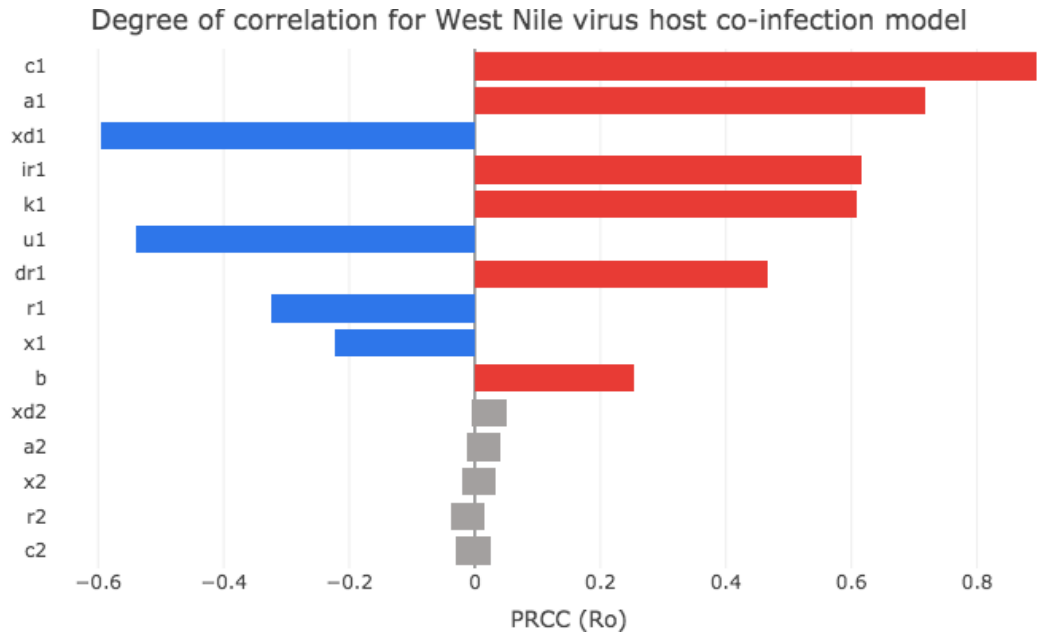
Sensitivity analysis: In the absence of co-infection, PRCC results indicate that the R_0 expression for the host co-infection model is most sensitive to host competence (c_1: 0.80), rates of mosquito feeding (a_1: 0.7), the rate of avian mortality due to WNV disease (x_{d1}: -0.61), the mosquito extrinsic incubation period (k_i: 0.59), fraction of exposed mosquitoes expected to develop (i_{r1}: 0.59), the rate of mosquito mortality (u_i: -0.54), and the fraction of infected mosquitoes expected to develop a disseminated infection (dr_1: 0.44) (Figure 3). The value of R_0 is moderately sensitive to the rate of avian recovery from WNV (r_i: -0.35), the fraction of avian hosts expected to die due to disease (x_1: -0.25), and the fraction of mosquitoes with a disseminated infection expected to transmit virus when feeding on a susceptible avian host (b_i: 0.23). The sensitivity of R_0 to parameters representing parasite co-infection (x_{d2}, a_2, x_2, r_2, and c_2) do not statistically vary from zero when the system is analyzed in the absence of a co-circulating parasite (Figure 3a).

In the absence of co-infection, PRCC results demonstrate that the R_0 expression for the vector co-infection model is the most sensitive to host competence (c_i: 0.89), rates of mosquito feeding (a_1: 0.67), the rate of WNV induced avian mortality (x_{di}: -0.59), the fraction of exposed mosquitoes expected to develop infection (i_{r1}: 0.57), the mosquito extrinsic incubation period (k_1: 0.56) and rates of mosquito mortality (u_1: -0.54) (Figure 3). The value of R_0 is also moderately influenced by the fraction of infected mosquitoes expected to develop a disseminated infection (d_{r1}: 0.42), the rate of avian recovery from WNV (r_i: -0.32), the fraction of avian hosts expected to die due to disease (x_i: -0.24), and the fraction of mosquitoes with a disseminated infection expected to transmit virus when feeding on a susceptible avian host (b_i: 0.20). The sensitivity of

R_0 to parameters representing parasite co-infection (k_2, d_{r2}, a_2, b_2, i_{r2} and u_2) are not statistically different from 0.

Tables 3 and Table 4 illustrate how PRCC values for a_i, b_i, i_{ri}, d_{ri}, c_i, x_i, x_{di}, k_i, r_i, and u_i are dependent on the prevalence of co-circulating parasites in hosts and mosquito vectors, respectively. Regardless of the amount of co-infection occurring in a system, a_i, b_i, i_{ri}, d_{ri}, c_i, and k_i are always positively associated with R_0, while x_i, x_{di}, r_i, and u_i are negatively associated with R_0 in both host and vector co-infection models. It is important to note that PRCC results for parameters describing dynamics in co-infected populations (a_2, b_2, i_{r2}, d_{r2}, c_2, x_2, x_{d2}, k_2, r_2, and u_2) are inversely related to those parameters describing the dynamics of WNV in the absence of a co-infecting organisms (a_1, b_1, i_{r1}, d_{r1}, c_1, x_1, x_{d1}, k_1, r_1, and u_1) (Tables 2 and 3). As the prevalence of co-infection grows in both host and vector co-infection systems, the sensitivity of R_0 to a_2, b_2, i_{r2}, d_{r2}, c_2, x_2, x_{d2}, k_2, r_2, and u_2 grows, while the sensitivity of R_0 to a_1, b_1, i_{r1}, d_{r1}, c_1, x_1, x_{d1}, k_1, r_1, and u_1 shrinks (Tables 3 and 4). Generally speaking, changes in the sensitivity of R_0 to a_2, b_2, i_{r2}, d_{r2}, c_2, x_2, x_{d2}, k_2, r_2, and u_2 is more pronounced at lower levels of co-infection in the system (Tables 3 and 4).

a)



b)

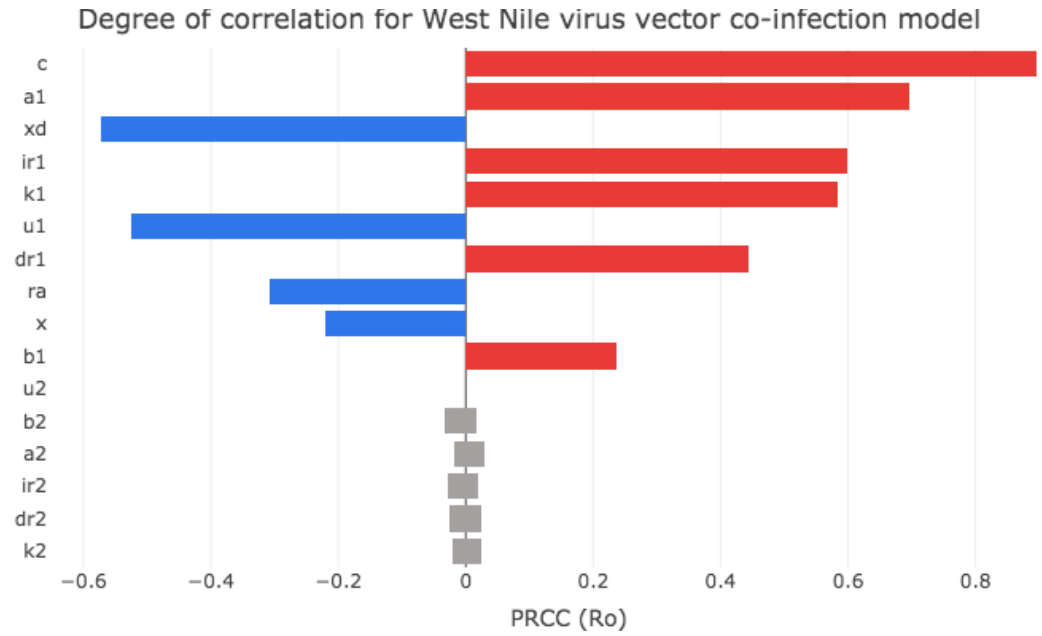


Figure 3. Tornado plot of global sensitivity analysis of R_0 with respect to all (a) host and (b) vector co-infection model parameters using partial rank correlation coefficient (PRCC).

Table 5. The sensitivity of R0 to changes in parameter input under different levels of mosquito co-infection with a co-circulating parasite. Latin Hypercube Sampling was used to sample parameter space uniformly from the parameter space defined in Table 3 under varying conditions of co-infection. Model sensitivity was evaluated under varying conditions of parasite co-infection in host and vectors which ranged from 0-100%. Parameters were sampled 10,000 times for 10,000 runs in program R using the following packages: "deSolve", "lhs" and "sensitivity". The resulting PRCC values fall between -1 and 1 and permit quantitative comparisons among different model inputs with the magnitude representing the relative importance. Positive values imply a positive association with R0 while negative values imply a negative association.

	Prevalence of WNV co-infection with co-circulating parasite in host										
Param.	**0%**	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**	**100%**
c2	-0.01	0.22	0.39	0.51	0.60	0.68	0.73	0.78	0.82	0.86	0.87
ki	0.68	0.71	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.71	0.68
a2	0.01	0.15	0.26	0.34	0.40	0.46	0.51	0.56	0.60	0.64	0.66
iri	0.44	0.47	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.47	0.44
dri	0.31	0.34	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.34	0.31
bi	0.21	0.23	0.24	0.24	0.25	0.25	0.25	0.25	0.25	0.24	0.22
r1	-0.32	-0.30	-0.27	-0.25	-0.22	-0.20	-0.17	-0.14	-0.11	-0.06	0.01
c1	0.87	0.85	0.82	0.78	0.73	0.68	0.61	0.52	0.40	0.23	0.01
xd1	-0.58	-0.55	-0.52	-0.47	-0.43	-0.39	-0.34	-0.28	-0.21	-0.12	0.01
a1	0.66	0.64	0.60	0.55	0.51	0.45	0.40	0.33	0.24	0.13	-0.01
x1	-0.23	-0.22	-0.20	-0.17	-0.15	-0.14	-0.12	-0.10	-0.07	-0.05	-0.01
x2	0.01	-0.04	-0.07	-0.09	-0.12	-0.14	-0.15	-0.17	-0.20	-0.22	-0.23
r2	-0.01	-0.08	-0.13	-0.17	-0.20	-0.22	-0.25	-0.27	-0.30	-0.32	-0.34
ui	-0.47	-0.51	-0.53	-0.53	-0.54	-0.54	-0.54	-0.54	-0.53	-0.52	-0.48
xd2	0.02	-0.11	-0.20	-0.28	-0.34	-0.39	-0.43	-0.48	-0.52	-0.56	-0.59

Table 6 The sensitivity of R_0 to changes in parameter input under different levels of mosquito co-infection with a co-circulating parasite. Latin Hypercube Sampling was used to sample parameter space uniformly from the parameter space defined in Table 3 under varying conditions of co-infection. Model sensitivity was evaluated under varying conditions of parasite co-infection in host and vectors which ranged from 0-100%. Parameters were sampled 10,000 times for 10,000 runs in program R using the following packages: "deSolve", "lhs" and "sensitivity". The resulting PRCC values fall between -1 and 1 and permit quantitative comparisons among different model inputs with the magnitude representing the relative importance. Positive values imply a positive association with R0 while negative values imply a negative association.

	Prevalence of WNV co-infection with co-circulating parasite in vector										
Param.	**0%**	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**	**100%**
ci	0.88	0.89	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.89	0.87
k2	0.00	0.11	0.20	0.29	0.37	0.44	0.50	0.55	0.59	0.63	0.66
a2	0.00	0.11	0.22	0.30	0.38	0.44	0.50	0.55	0.59	0.62	0.64
ir2	-0.01	0.05	0.11	0.16	0.20	0.25	0.29	0.33	0.37	0.40	0.42
dr2	0.00	0.04	0.08	0.11	0.14	0.17	0.20	0.23	0.25	0.28	0.30
b2	-0.01	0.02	0.05	0.07	0.10	0.12	0.14	0.15	0.17	0.18	0.19
b1	0.19	0.18	0.17	0.15	0.14	0.12	0.10	0.08	0.06	0.04	0.01
dr1	0.30	0.28	0.26	0.24	0.21	0.18	0.15	0.12	0.09	0.05	0.01
a1	0.64	0.62	0.59	0.55	0.50	0.44	0.38	0.30	0.22	0.12	0.01
ir1	0.42	0.40	0.37	0.33	0.30	0.26	0.21	0.17	0.12	0.06	0.01
u1	-0.46	-0.44	-0.41	-0.37	-0.33	-0.29	-0.24	-0.19	-0.14	-0.07	0.00
k1	0.66	0.63	0.59	0.54	0.49	0.43	0.36	0.28	0.20	0.10	0.00
xi	-0.23	-0.25	-0.26	-0.27	-0.28	-0.28	-0.28	-0.28	-0.27	-0.26	-0.24
ri	-0.31	-0.33	-0.35	-0.35	-0.36	-0.36	-0.36	-0.36	-0.35	-0.33	-0.31
u2	-0.02	-0.09	-0.15	-0.21	-0.26	-0.31	-0.35	-0.38	-0.42	-0.45	-0.47
xdi	-0.56	-0.59	-0.61	-0.62	-0.63	-0.63	-0.63	-0.63	-0.61	-0.60	-0.56

The following parameters were treated as constants and therefore were not assessed by sensitivity analysis: larval maturity rate (*m*), daily per capita birth rate (*Bm*), larval carrying capacity (km), proportion of larval morality (u_L) and vector - host ratio (*M:H*).

Case study: The theoretical impact of avian malaria parasites on WNV transmission was quantified through changes in a_1, a_2, d_{r2}, x_2, k_2, and u_2 which varied based on hypotheses presented in each case study (Table 5). In case study one, the quantitative impact of increasing rates of mosquito feeding on hosts infected with avian malaria (a_2) by 11% was quantified using the R_0 expression for both host and vector co-infection models. Results from Koella et al. [2005] demonstrate that *P. falciparum* infection can increase rates of daily bloodmeal acquisition in *Anopheles gambiae* from 10% in uninfected mosquitoes to 22% in infected mosquitoes (Koella et al. 2005). If daily vector:host contact rates increase by 11% due to Haemosporida infection (a_2), R_0 is estimated to increase between 0.4% and 9.3%. The change in R_0 depends on the R_0 expression used to evaluate this case study and prevalence of avian malaria in hosts and vectors (Table 5).

In case study two, the impact of shifting mosquito feeding behavior due to malaria infection status was quantified. When given an option between infected and uninfected avian hosts, Cornet et. al. [2013b] observed that 62% of mosquitoes fed on avian hosts carrying *P. relictum*, a departure from the null hypothesis of 50% (Cornet et al. 2013b). In a follow up choice experiment, Cornet et al. [2013a] observed a similar result where 60.3% of *Culex pipiens* mosquitoes were attracted to birds with a *P. relictum* infection. If a_2 is increased by 24% and a_1 decreased by 24% to represent a shift in mosquito feeding preferences towards avian malaria infected hosts, R_0 is estimated to decrease by 21.6% or increase by 15% depending on the prevalence of avian malaria in hosts (Table 5).

In case study three, the impact of malaria avoidance behavior in feeding vectors was quantified. In contrast to the studies by Cornet et al., a study by Lalubin et al. [2012] demonstrated that mosquitoes can be significantly more attracted to birds that are uninfected with *Plasmodium* species. By decreasing a_2 by 19% and increasing a_1 by 19% R_0 is estimated to increase by as much as 14.6% at low host avian malaria prevalence (5%) and decrease R_0 by 8.6% at high levels of avian malaria prevalence in hosts (80%) (Table 5).

In case study four, the impact of increased avian mortality due to avian malaria-WNV co-infection was quantified. A field study by Medeiros et al [2014] documented an inverse association between *Plasmodium* infection status and antibodies to WNV in a community of adult avian species in Chicago, Illinois. Although the mechanisms driving the negative association between WNV serostatus and avian malaria infection status remain unclear, a reduction in host survival due to parasite co-infection is a potential mechanism of interaction proposed by the authors. Increasing WNV-induced host mortality (x_2) by 100% in avian hosts infected with avian malaria is estimated to increase R_0 between 18.3% and 12.7% at low (5%) and high (80%) levels of avian malaria prevalence, respectively (Table 5).

In case study five, the impact of *Plasmodium* sporozoite enhancement of WNV transmission was quantified. A study by Vaughan and Turell [1996] demonstrated that malaria sporozoites can disrupt salivary gland barriers and facilitate the transmission of RVFV in *Anopheles stephensi* mosquitoes by up to 32%. If viral dissemination in mosquitoes exposed to avian malaria (d_{r2}) increases by 329%, R_0 is estimated to increase between 2.6% and 30.2%, dependent on the prevalence of avian malaria in vectors (Table 7).

Table 7. The potential consequences of co-circulating avian malaria parasites for West Nile virus transmission. The theoretical impact of avian malaria parasites for West Nile virus (WNV) transmission was quantified using compartmental models parameterized with data-driven hypotheses derived from empirical studies documenting changes in vector and host biology due to Haemosporida infection. The R0 expressions for host and vector co-infection models were used to quantify WNV transmission in 10 case studies under varying levels of avian malaria prevalence (5%, 10%, 20%, 40%, 80%). The change in outbreak potential due to avian malaria was estimated as the percent change between the baseline R_0 value(R_0^N) and the alternative R_0 value (R_0^A) estimated for each case study [(R_0^A-R_0^N)/ R_0^N]. The initial conditions used in the host co-infection model were Sb1=100, Sb2=100, Ib1=0, Ib2=0, Rb1=0, Rb2=0, Sm=Nm0, Em=0, Im=1, and Lm=HLm0. The initial conditions for the vector co-infection model were S_b=200, I_b=0, R_b=0, S_{m1}=Am1, S_{m2}=Am2, E_{m1}=0, E_{m2}=, I_{m1}=1, I_{m2}=1, L_m=VLm0. Data-driven hypotheses quantifying how avian malaria may impact epidemiological parameters in the WNV system were developed from the results of published empirical studies (Koella et al. 2002, Cornet et al. 2013a,b, Lalubin et al. 2012, Medeiros et al. 2014, Vaughen et al. 1996, Vaughen et al. 2012, Turell et al 1984, Vezilier et al. 2012, and Valkiunas et al. 2014.)

Case Study	Data-driven hypotheses	Params.	Change in parameter	Adjusted parameter space	Co-infection model	5%	10%	20%	40%	80%	Reference
1	*Host malaria infection increases vector feeding rates*	a_2	*11%*	a_1: *0.12-0.23* *a2: 0.13-0.26*	*Host* *Vector*	*0.4%* *0.9%*	*2%* *0.9%*	*2.4%* *2.8%*	*4.8%* *4.6%*	*8.7%* *9.3%*	*Koella et al. (2005)*
2	*Vectors feed preferentially on malaria infected hosts*	a_1 a_2	*a1: -24%* *a2: 24%*	a_1: *0.09-0.17* a_2: *0.15-0.29*	*Host*	*-21.6%*	*-17.4%*	*-12.1 %*	*-2.1%*	*15.3%*	*Cornet et al. (2013 a,b)*
3	*Vectors avoid hosts infected with Plasmodium*	a_1 a_2	*a1: 19%* *a2: -19%*	a_1: *0.14-0.27* a_2: *0.1-0.19*	*Host*	*14.6%*	*13.8%*	*9.6%*	*3.9%*	*-8.6%*	*Lalubin et al. (2012)*
4	*Host co-infection increases host mortality by a factor of 2*	x_2	*100%*	x_1: *0.33-1* x_2: *0.66-1*	*Host*	*18.3%*	*18.9%*	*17.1 %*	*15.9%*	*12.7%*	*Medeiros et al. (2014)*

Table 7. Continued

Case Study	Data-driven hypotheses	Params.	Change in parameter	Adjusted parameter space	Co-infection model	5%	10%	20%	40%	80%	Reference
5	*Disruption of midgut and salivary gland barriers increases viral dissemination in malaria-infected mosquitoes*	d_{r2}	*329%*	*dr_1: 0.14-0.32 dr_2: 0.46-0.64*	*Vector*	*2.6%*	*4.2%*	*9.0%*	*16.0%*	*30.2%*	*Vaughen et al. (1996)*
6	*Malaria oocyst penetration of midgut reduces viral EIP by half*	k_2	*100%*	*k_1: 0.071-0.25 k_2: 0.14-0.25*	*Vector*	*0.7%*	*0.5%*	*2.1%*	*3.5%*	*7.4%*	*Vaughen et al. (2009)*
7	*Malaria decreases the risk of mosquito mortality by 7%*	u_2	*-7%*	*u_1: 0.07-0.12 u_2: 0.08-0.13*	*Vector*	*0.7%*	*0.6%*	*2.1%*	*3.4%*	*7.1%*	*Vezilier et al. (2012)*
8	*Haemoproteus infection increases mosquito mortality*	u_2	*580%*	*u1: 0.07-0.12 u2: 0.17-0.7*	*Vector*	*-2.6%*	*-5.9%*	*-10.7%*	*-21.9%*	*-48.4%*	*Valkiunas et al. (2014)*
9	*The simultaneous impact of scenarios 1,2,3, and 4*	a_i x_2	*-*	*a_1: 0.09-0.27 a_2: 0.1-0.29 x_2: 0.66-1*	*Host*	*22.1%*	*22.9%*	*21.3%*	*20.2%*	*16.6%*	*-*

Table 7. Continued.

Case Study	Data-driven hypotheses	Params.	Change in parameter	Adjusted parameter space	Co-infection model	5%	10%	20%	40%	80%	Reference
10	*The simultaneous impact of cases 1, 5, 6 and 7*	a_i dr_2 k_2 u_2	-	a_2: *0.13-0.26* a_1: *0.12-0.23* dr_2: *0.46-64* k_2: *0.14-0.25* u_2: *0.08-0.7*	*Vector*	*-1.7%*	*-4.1%*	*-7.1%*	*-14.9%*	*-32.5%*	-

In case study six, the impact of reducing EIP in mosquito vectors was quantified. Multiple studies demonstrate that the concurrent ingestion of parasites with arboviruses can shorten the time required for a vectors to become infectious (i.e. EIP) (Turell et al 1984; Turell et al. 1987). In the case of avian malaria, oocyst penetration of the midgut may reduce EIP by disrupting barriers to dissemination. A study by Vaughan et al. [2012] suggests microfilarial parasites may reduce the EIP of Dengue virus dissemination by half. Reducing the EIP of mosquitoes infected with avian malaria (k_2) by half increased R_0 between 0.7% and 7.4%, depending on the prevalence of avian malaria in vectors (Vaughan et al. 2012) (Table 7).

In case study seven, the consequence of decreasing rates of vector mortality due to malaria infection status was quantified. Results published by Vezilier et al. [2012] suggest that *Plasmodium*-infection increases the median survival of *Culex pipiens* by 1.3 days compared to an uninfected control (Vezilier et al. 2012). The impact of increasing vector survivorship in co-infected populations (u_2) by 7% is estimated to increase R_0 between 0.6% at low levels of avian malaria and 7.1% at high levels of avian malaria prevalence in vectors (Table 7).

In case study eight, the impact of increased vector mortality due to avian malaria exposure was quantified. In contrast to Vezilier et al [2012], results from Valkiunas et al. [2014] demonstrate that the *Heamoproteus* species can reduce rates of *Ochlerotatus cantanas* survivorship by as much as 580%. The consequence of reducing vector survivorship in mosquitoes exposed to avian malaria (u_2) by 580% is estimated to reduce R_0 between 2.6% and 48.5% depending on the prevalence of avian malaria in vectors (Table 7).

In case study nine, the compounding impact of case studies 1-4 was quantified by adjusting the parameter space of a_1, a_2, and x_2 based on the minimum and maximum parameter space observed across case studies 1-4. The compounding impact of avian malaria based on the

case studies presented is estimated to increase R_0 between 16.6% and 22.1% depending on the prevalence of avian malaria in hosts (Table 7).

In case study ten, the compounding impact of case studies 1 and 5-8 R_0 expression was quantified by adjusting the parameter space of a_1, a_2, $d_{r2,}$ k_2 and u_2 based on the minimum and maximum parameter space observed across the case studies. The impact of avian malaria acting through multiple mechanisms is estimated to reduce R_0 between 1.7% and 32.5% depending on the prevalence of avian malaria in vectors (Table 7).

3.5 Discussion

The extension of individual parasite models into multi-parasite models has proven valuable for assessing the population-level consequences of polyparasitism, but remains an underexplored topic in vector-borne disease systems (Fenton et al. 2008, Vogels et al. 2019). In this study we present a theoretical framework to help quantify the impact of co-circulating parasites on vector-borne pathogen transmission that can be parameterized to match the biological components of any simple vector-borne pathogen system. Further, we derived expressions for R_0, a key index of pathogen transmission intensity, to facilitate the quantitative evaluation on how co-infection can change parameter input and alter pathogen transmission at the population level. To illustrate the utility of this quantitative framework in evaluating the impact of co-circulating parasites on vector-borne transmission, models were parameterized to the WNV system to a) explore the sensitivity WNV transmission to parameter input under varying levels of co-infection and b) quantitatively estimate how avian malaria parasites may modulate WNV transmission based on multiple mechanisms of indirect interaction empirically described in the literature. Sensitivity analyses highlight important parameters through which

sympatric parasites, including avian malaria, may impact WNV transmission; valuable information for establishing disease control policies, strategizing effective resource allocation, and prioritization future research directions (Smith et al. 2005, Baily 1975).

Sensitivity analyses provide a powerful tool for quantifying uncertainty in complex models and pinpointing critical parameters that impact model outcomes (Marino et al. 2008). Through sensitivity analysis we demonstrate that WNV outbreaks are significantly influenced by the efficiency of host transmission to feeding vectors (c_i); a result that re-iterates the importance of understanding within-host interactions between co-infecting parasites especially as rates of co-infection prevalence increase in a population (Table 5 and 6) (Kellly-Hope et al. 2006, Fenton et al. 2007, Fleming et al. 2006, Ezenwa et al. 2010, Lloyd-smith et al. 2008, Pathak et al. 2012, Ezenwa et al. 2011, and Beechler et al. 2015). How avian malaria parasites influence WNV kinetics in infected hosts remains unclear as different parasites induce different immune responses that may enhance or suppress the effects of a concomitant infection (Pederson and Fenton, 2007). For example, malaria may interact with WNV through avian immunosuppression, polarization of the Th1/th2 dichotomy, or eliciting the host production of non-specific immune effector molecules (Cox et al. 2001). Evidence even suggests parasites themselves may produce immunomodulatory factors that exert immune-modulatory effects that may impact infection dynamics (Fallon et al. 2006). Nonetheless, results of this analysis restate that changes in host infectiousness can have important consequences for WNV transmission and should remain a focus of empirical studies.

Additionally, sensitivity results demonstrate that any co-circulating parasite that increases vector feeding rates (a_i), the probability that an exposed mosquito will become infected (I_{ri}), and viral extrinsic incubation period (k_i) could significantly increase WNV transmission. On the other hand, results also suggest that any co-circulating parasite that increases rates of host disease

induced mortality could lead to a significant reduction in WNV transmission potential as PRCC results demonstrate that the rate of disease induced mortality in hosts (x_{di}) has a significant inverse proportional association with R_0. Importantly, as the level of co-infection increases in a system, the sensitivity of R_0 to these parameters also increases, quantitatively confirming that the frequency of co-infection in a population is an important factor capable of dictating vector-borne disease transmission heterogeneity (Tables 3 and 4) (Susi et al. 2015). As such, identifying and monitoring parasites that impact vector-borne disease transmission may provide a credible surveillance tool to predict which environments are more supportive of vector-borne disease transmission. Additionally, the augmentation of these parasite populations may provide a means to mitigate vector-borne pathogen transmission.

Furthermore, we demonstrate that this framework is useful for quantitatively estimating the population-level impact of empirical and theoretical hypotheses. Through multiple mechanisms of indirect interaction posited by the literature it is apparent that avian malaria parasites are capable of increasing or decreasing WNV transmission by modulation multiple epidemiological parameters. For example, avian malaria may increase WNV transmission by increasing proportions of host mortality (Case study 4), facilitating viral dissemination from the mosquito midgut (Case study 5), decreasing the mosquito EIP (Case study 6), or increasing vector mortality (Case study 8) (Table 7). Results also suggest avian malaria parasites may decrease WNV transmission through vector parasite avoidance behavior and increased vector survivorship (Case study 2 and 7, respectively). Importantly, results from case study 1 and 2 demonstrate that adjustments in WNV parameter input based on interactions between parasites may not always impact population transmission dynamics in a unidirectional manner. In case study 2, WNV transmission was estimated to decrease at low to moderate levels of malaria infection. As

mosquitos focused feeding on malaria infected hosts virus transmission is expected to increase in this subgroup of the avian population, however overall population is expected on the larger scale because infectious mosquitoes are avoiding the larger population of hosts that do not carry avian malaria. Interestingly, at high rates of infection (80%) R_0 was estimated to increase in case study 2 as mosquitoes preferentially feed on the larger proportion of hosts (Table 7). Conversely, in case study 3, vector-parasite avoidance behavior was estimated to increase R_0 at low levels of avian malaria prevalence and decrease R_0 at high levels of avian malaria prevalence as vectors would preferentially feed on the smaller sub-population of birds uninfected with avian malaria increasing transmission among that subpopulation (Table 7).

The simultaneous impact of multiple hypotheses were explored through case studies 9 and 10. The results of these scenarios illustrate that the consequence of co-infection for vector-borne pathogen transmission is dictated by multiple mechanisms that are concurrently increasing and decreasing viral transmission potential. Although the hypotheses explored through the case studies above are not a comprehensive representation of all the potential interactions that may exist between avian malaria and WNV, the simultaneous impact of hypotheses tested through the host co-infection model are predicted to increase in transmission, while the simultaneous impact of hypotheses tested through the vector co-infection model are predicted to reduce WNV transmission (Table 7). Ultimately, to fully appreciate the net impact of co-circulating parasites on vector-borne pathogen transmission future studies should aim to study the net impact of concurrent ecological interactions in both host and vectors. Extending this modeling framework into a comprehensive model that tracks co-infection in both vectors and hosts simultaneously is an intuitive next step, however analysis of the system may be prohibitive.

Vector-borne transmission systems are particularly complex as real populations of vectors and hosts are heterogenous in space in time and dynamically respond to environmental and ecological factors (Ezenwa et al. 2006, Wonham et al. 2006). It is important to note that the theoretical models presented here are a simplification of natural processes and make many biological assumptions, which influence the outcome of model predictions (Wonham et al. 2006). For example, assuming host populations are fixed during an outbreak season and do not grow or shrink due to natural migration, birth, or death likely reduce estimates of R_0. Furthermore, the assumption of density dependence is an important concept that must be considered for each arthropod species. Ultimately, the modeling structure here can be adapted to include numerous environmental, ecological or evolutionary extensions, but, amid these complications, it is important that communication between mathematicians and biologists continue to effectively track disease dynamics and produce rational control strategies (Wonham et al. 2006, Roberts and Heesterbeek, 1993). The analyses of R_0 presented here was completed utilizing computational software and techniques outlined in the published literature that facilitate model analysis. Importantly, it is explicitly assumed that the DFE for both vector and host models exists and is stable near the threshold, however, it has been shown that some models have unstable endemic equilibria near the DFE for R0 <1 suggesting that a disease may persist even when DFE is locally stable (van den Driessche and Watmough 2002). As such, quantifying scenarios of disease elimination through the models presented above should be cautious with interpretation.

Overall, this work demonstrates that the consequence of co-circulating parasites for vector-borne pathogen transmission is complex with multiple mechanisms of interaction occurring concurrently. Results reiterate the difficulty in assessing the population-level impact of co-

infection when studying hypothetical mechanisms of interaction in isolation (Fenton 2008). Importantly, parasites that co-occur in host and vector populations may impact transmission dynamics differently in each organism. As such, studies focused on identifying factors that increase or reduce the persistence of a vector-borne parasite should consider the impacts of polyparasitism in both hosts and vectors tandemly. The quantitative models presented here provide a simple framework to begin understanding how sympatric parasite interactions impact vector-borne pathogen transmission and can help prioritize future research and the development of novel vector-borne disease control strategies in the future.

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CHAPTER IV

WEST NILE VIRUS INFECTION AND DISSEMINATION RATES IN *CULEX QUINQUEFASCIATUS* MOSQUITOES SIMULTANEOUSLY AND SEQUENTIALLY EXPOSED TO *PLASMODIUM* PARASITES

4.1 Synopsis

Mosquito-borne diseases pose a persistent problem for human health and agricultural systems highlighting a need to identify novel mechanisms of control. Numerous studies have determined that midgut infection and escape barriers are important factors that influence vector transmission efficiency. As such, any mechanism that changes rates of viral infection or dissemination can significantly impact vector-borne disease transmission. Here, the consequence of *Plasmodium*-West Nile virus infection in mosquito vectors is assessed by measuring rates of midgut infection and dissemination in simultaneously infected and sequentially infected mosquitoes. Contrasting results between experiments suggest that certain mechanisms associated with *Plasmodium* infection may modulate WNV kinetics. Yet, considering the majority of results demonstrate no changes in rates of viral infection and dissemination due to *Plasmodium* infection, *Plasmodium* parasites are likely not a key driver of natural WNV transmission heterogeneity.

4.2 Introduction

Vector-borne pathogens are a persistent challenge for public health agencies as globalization continues to enhance rates of disease emergence and spread (Kading et al. 2018). The amplification and transmission of arthropod-borne pathogens depends on three factors: the presence of competent vectors, the presence of competent hosts, and the frequency of contact between these competent organisms (Komar et al. 2003, Golnar et al. 2014, Kilpatrick et al.

2012). It follows that *a priori* prediction and prevention strategies focused on mitigating the emergence and re-emergence of vector-borne pathogens in the US are contingent on identifying competent vectors and hosts capable of transmitting a particular pathogen (Golnar et al. 2014). As such, vector competence assays provide fundamental data necessary for developing effective arthropod-borne viruses (arbovirus) control strategies, as every arthropod species demonstrates variability in their ability to transmit a particular pathogen (Hardy et al. 1983).

Generally, vector competence is evaluated by exposing individual vectors to a known quantity of virus and sampling individual tissues at various time intervals (Vogels et al. 2017). For example, the mosquito midgut can be dissected and tested for virus to determine rates of viral infection and the legs can be removed and assayed for virus to determine rates of viral dissemination from the midgut into the hemocoel. Further, mosquito saliva can be collected and tested for virus as a metric of transmission potential (Hurlbut 1966). Overall, decades of published findings demonstrate that the ability of viruses to infect, disseminate, and be transmitted by mosquito vectors is regulated by a suite of extrinsic (temperature, rainfall, and competition) and intrinsic (genetics) factors (Kenney and Brault 2014, Kramer and Ebel 2003). In mosquito vectors, the midgut barrier has long been known to be the most important barrier to the infection process (Kramer et al. 1981, Hardy et al. 1983)**.** Therefore, any mechanism that modulates rates of midgut viral infection and escape can theoretically modulate arbovirus transmission intensity (Vaughan et al. 2012).

West Nile virus (WNV) was introduced to New York in 1999 and quickly spread throughout the contiguous United States (US) leading to large-scale declines in bird populations and causing the three largest neuroinvasive disease outbreaks in US history, which resulted in more than 48,000 human cases of disease and over 2,000 deaths (5,674 cases and 286 deaths

were reported in 2012 alone) (CDC, Kramer et al. 2008, LeDeau et al. 2007, Peterson et al. 2013). Since it was first described in 1937, WNV has become one of the most important causative agents of viral encephalitis in the world (Chancey et al. 2015). While much progress has been made understanding WNV transmission in nature, many patterns of when and where WNV occurs remains unresolved and difficult to predict or explain (Nasci 2013). For example, WNV exhibits incredible plasticity in the ability to thrive in some regions for some years causing large epidemics (i.e Chicago, Illinois), while in other regions or other years the virus remains at endemic levels or absent all together (Bertolotti et al. 2008, Peterson et al. 2013). Much of temperate North America appears suitable for fueling rapid amplification events of WNV in the late summer whereas many subtropical, tropical, and temperate regions elsewhere in the Americas has evidence of sporadic WNV transmission but not large amplification events resulting in epidemics (Chancey et al. 2015). Classically, WNV is studied in the one-host, one-pathogen framework, yet wild birds are known to host a suite of parasites that can influence individual fitness and drive population dynamics (Atkinson et al. 2009, Hudson et al. 2002). Numerous field studies demonstrate that avian *Plasmodium* species and WNV overlap temporally and spatially in *Culex* vectors and Passerine hosts, illustrating the theoretical propensity for avian malaria parasites to influence WNV transmission (Medeiros et al. 2016, Booth et al. 2015, Hughes et al. 2010). Considering WNV and avian *Plasmodium* species may interact while naturally infecting *Culex* mosquito species, the focus of this study is to determine if *Plasmodium* parasites can modulate rates of viral infection and dissemination in the *Culex quinquefasciatus* vector.

4.3 Methods

Mosquitos: Wild *Culex quinquefasciatus* egg rafts (BCS strain) were collected from multiple locations in College Station, Texas in 2018. Over 100 egg rafts were reared in a quarantine facility on a diet of liver powder and yeast as larvae and 10% sugar ad libitum as adults. Defibrinated sheep blood and chicken blood were offered to mosquitoes through an artificial membrane feeder to establish the colony. Adults were maintained in the BSL1 insectary with a natural light photoperiod at 26-27C and 45-50% humidity. Following these same methods, the *Culex quinquefasciatus* Sebring mosquito strain, originally colonized in Florida in 1998, was maintained in lab since 2014. The BCS strain of *Culex quinquefasciatus* mosquitoes used for vector competence studies were between 5 and 12 generations removed from wild populations during infection assays. For all vector competence studies, groups of pupae within four days of age were separated into a container for experimental infection. Adults were provided 10% sugar *ad libitum* until one day prior to experimental infection. On the day prior to infection, adult mosquitoes were chilled on ice and females were separated into three containers of equal numbers. These female mosquitoes were starved of sugar and water 18 hours before offering the first bloodmeal.

West Nile virus: Vero cells were maintained in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 Celsius. A strain of West Nile virus isolated from mosquitoes in El Paso, Texas (WNV TX AR-9-5282-P2) was obtained from the University of Texas Medical Branch (UTMB) and propagated in Vero cells by infection at an MOI of 0.01. Supernatant was harvested 3 days post infection, clarified by centrifugation at 4C and aliquoted into single use vials before freezing at -80C. The WNV 5282

strain was passaged three times on Vero cells and split into 500ul aliquots that were stored at -80C until viral infections. Viral titers of frozen aliquots were determined by plaque assay.

***Plasmodium* species:** *Plasmodium relictum* KV115, originally isolated from an infected bird in Hawaii, was obtained from a colleague with the National Instititue of Health as a cryopreserved sample in canary blood. Additionally, a lineage of *Plasmodium* was isolated from a Blue Jay (*Cyanocitta cristata*) in College Station, Texas on September, 2018. Blood was cryoprotected with glycerin and stored in liquid nitrogen. Frozen aliquots were thawed, deglycerinated, and intravenously inoculated into susceptible domestic canaries (*Serinus canaria)*. Both male and female canaries were obtained from US vendors (The Finch Farm, Co., Washington, USA; Pet Supplies Plus, Texas, USA) and held in an aviary protected from biting flies until infection with *Plasmodium* species. After seven days, blood was serially transferred between birds until parasitemia was detected (Valkiunas et al. 2004). To diagnose blood infection, blood smears were made after collecting bird blood through wing venipuncture. Blood smears were allowed to air dry, fixed in 100% methanol for 5 seconds, and stained using a 10% Giemsa solution (Valkiunas et al. 2004). After 90 minutes, blood smears were analyzed under oil immersion microscopy for infection. When gametocytes were observed, birds were immobilized with elastic bandage wrap for 30 minutes to allow mosquitoes to feed or anesthetized using Isoflurane and exsanguinated by jugular venipuncture using a heparin coated syringe to stop blood coagulation. Bird blood was either cryopreserved for future experiments or stored on ice for vector competence assays.

Sequential infection: To test how prior exposure to *Plasmodium* parasites influences the vector competence of *Cx. quinquefasciatus* mosquitos, groups of mosquitos were permitted to feed on infected and uninfected canaries immobilized using elastic bandages for no longer than

30 minutes. Following feeding, a small blood sample was collected from each donor bird through brachial venipuncture as described above to quantify parasitemia based on a total count of 10,000 cells per slide (Godfrey et al 1987). Engorged mosquitoes from experimental and control treatments were separated into containers and provided sugar *ad libitum* for 7-9 days. At day 4 a cup full of water was placed in each cage to induce oviposition. The total number of egg rafts from each treatment group was noted. After mosquitoes were permitted time to oviposit, all mosquitoes were exposed to WNV. All bloodmeals were prepared with the same concentration of pelleted chicken red blood cells, 0.1mM of adenosine triphosphate, Fetal Bovine Solution, 10% sugar, and WNV 5282 P3 with a final viremia titer of 10^7 PFU/ml. Prior to bloodmeal preparation, whole chicken blood was washed with PBS by repeatedly pelleting red blood cells at 706 relative centrifugal force (rcf). After washing, 90% of remaining supernatant was removed and cells were resuspended in the remaining 10% of PBS. Mosquitoes were permitted to feed for 2 hours. In some cases, multiple bloodmeals were offered in succession to increase the numbers that fed. A sample of blood was stored at the onset of feeding and following feeding (after 2 hours) for titration.

Concomitant infection*:* For each duel infection experiment, a canary with microscopy confirmed *Plasmodium* gametocytes was euthanized by exsanguination through jugular venipuncture and isofluorane antiesthetic. Control blood without *Plasmodium* infection was obtained from canaries uninfected with *Plasmodium* or uninfected chicken blood was substituted. Syringes were coated with heparin anticoagulant. Canary blood was stored on ice until bloodmeals were prepared in the BSL3. Frozen WNV-5282-P3 stocks were thawed to room temperature before preparing bloodmeal treatments. All treatments contained avian blood washed following the methods described above, with 0.1mM of adenosine triphosphate, FBS,

10% sucrose solution, which was mixed with 200ul of WNV stock solution for a final volume of 710ul. In each experiment, two bloodmeal treatments were created, one that contained blood with *Plasmodium* gametocytes and another with no *Plasmodium* gametocytes. Mosquitoes were permitted to feed on each bloodmeal treatment 2-hour segments by the use of a Hemotek membrane feeding apparatus warmed to 37C. A 50ul aliquot of each blood meal was stored before mosquitoes began feeding for back titration by plaque assay. After 2 hours, another blood sample was stored for back titration. When possible, multiple attempts to feed mosquitos were completed to increase sample sizes. Transmission experiments were repeated 1-3 times within a 12-hour window to increase the number of female mosquitoes that fed.

Following oral infections, mosquitoes were chilled on ice. Blood-fed mosquitoes were separated for each group into separate cups containing fully engorged and partially engorged mosquitoes. Mosquitoes were maintained with 10% sugar *ad libitum* at 27C with a 16:8 light:dark photocycle and 60% relative humidity. Mosquito mortality was recorded daily until mosquitoes were dissected on day 7 or 14. On dissection day, mosquitoes were chilled on ice and legs and wings were removed and placed in 200ul of 10% FBS cell culture media containing 1 metal BB. Mosquito midgut was removed each individual and stained with 0.05% mercurochrome to aid in oocyst burden estimation under microscopy. The head/thorax and midgut were stored in separate tubes containing 200ul of 10% FBS cell culture media and 1 metal bb. Mosquito tissues (midgut, legs/wings, head/thorax) were homogenized at in the Qiagen TissueLyser at 25hz/sec and clarified by centrifugation at 5,000rpm for 1min. Subsamples (50ul) of each tissue was combined with 350ul lysis solution, vortexed, and removed from the BSL3 for nucleic acid extraction and PCR following the rt-PCR protocol outlined by Lanciotti et al [2000] with a cutoff ct value of 32.5.

Data analysis: Rates of WNV infection and dissemination were determined based on rt-PCR results evaluating midgut tissues and wings/legs, respectively (Turell et al. 2010, Richards et al. 2012). Rates of midgut infection and dissemination in infected individuals were compared among treatments in the same experiment using the Fisher's exact test.

Viral infection and dissemination outcomes from sequential and simultaneous infection experiments were analyzed by generalized linear mixed models (GLMMs) using the glmer function in the R package *lme4* (Bates et al 2015, Bates et al 2014). We coded the midgut infection and dissemination status of every mosquito as unsuccessful or successful and modelled the probability of infection success using a binomial distribution using multiple fixed variables. Each independent experiment was treated as a random variable. Mosquito lineage, *Plasmodium* lineage, *Plasmodium* status, and the presence of Oocysts were treated as Bernoulli variables. Oocyst burden and *Plasmodium* parasitemia were coded as numeric variables. Multiple models were run testing the predictive power of different combinations of fixed variables using mosquito midgut infection and dissemination as the response variable. The most parsimonious model was selected by comparing the differential Akaike's information criterion (dAIC <2) and weight between the full model and all nested models.

4.4 Results

Sequential feeding: Six different experiments exposed Sebring and BCS strains of *Cx. quinquefasciatus* mosquitoes to domestic canaries of known *Plasmodium* infection status prior to WNV experimental infection. *Plasmodium* parasitemia were calculated to range between a 0.3% red-blood cell infection rate to 29% red-blood cell infection rate (Table 8). The number of days necessary for mosquitoes to lay eggs was generally higher in BCS mosquitoes, but ranged

between 6 and 11 days (Table 8). In four experiments infection and dissemination rates were assessed at day 7 post infection and in two experiments infection and dissemination rates were assessed at day 14. Overall, rates of midgut infection were higher than rates of viral dissemination in infected mosquitoes (Table 8). Based on the Fisher's exact test statistic, rates of midgut infection and viral dissemination were the same more frequently than they differed (Table 8). In experiment 3, Sebring mosquitoes exposed to the BCS lineage of *Plasmodium* were much more likely to have a disseminated infection compared to mosquitoes infected with *P. relictum* (p=0.02) and the control group of mosquitos (p=0.0004) (Table 3).

The most parsimonious GLMM model predicting mosquito midgut infection using sequential feeding experiments as a random factor included Oocyst infection status (p=0.99), mosquito lineage (p=0.99), and days post WNV infection (p=0.99) as predictive factors with an AIC of 38.6. Across 235 observations and 5 random experiments, no fixed effect was determined to be a significant predictor of the response variable.

The most parsimonious GLMM model predicting viral dissemination using sequential feeding experiments as a random factor included *Plasmodium* parasitemia (p=0.997), *Plasmodium* lineage (p=0.998), and Mosquito lineage (p=0.998) as predictive factors with an AIC of 26.8. Across 243 observations and 5 experiments, no fixed effect was determined to be a significant predictor of the response variable.

Concomitant infection experiments: Three experiments exposed Sebring and BCS mosquitoes to WNV and *Plasmodium* parasites simultaneously (Table 9). At day 7 post infection, infection and dissemination rates in Sebring mosquitoes exposed to the BCS *Plasmodium* lineage were statistically the same (Table 9). On day 14 post infection, dissemination rates were found to be lower in BCS mosquitoes exposed to *P. relictum* (p=0.02,

Table 9). In experiments 2 and 3, midgut infection rates were almost statistically different in BCS mosquitoes exposed to *P. relictum* at an alpha of 0.05, however the results are completely inverse (Table 9). In experiment 2, midgut infection rates decreased in BCS mosquitoes exposed to *P. relictum* (p=0.057), while midgut infection rates increased in BCS mosquitoes exposed to *P. relictum* in experiment three (p=0.054) (Table 9).

The most parsimonious GLMM model predicting mosquito midgut infection with WNV using simultaneous feeding experiments as a random factor included *Plasmodium* exposure treatment (p=0.9998), the number of Oocysts (0.0856), *Plasmodium* lineage (0.9997), and mosquito lineage (0.9996) as predictive factors with an AIC of 54.6. Across 221 observations and 2 random experiments, no fixed effect was determined to be a significant predictor of the response variable. Experiment 2 was removed from this analysis because preparation of the bloodmeal was significantly different than experiment 1 and experiment 3 (blood was not washed with PBS and serum removed prior to bloodmeal preparation).

The most parsimonious GLMM model predicting WNV dissemination using simultaneous feeding experiments as a random factor included Oocyst infection status (p=0.75), mosquito lineage (p=0.21), and days post WNV infection (p=0.059) as predictive factors with an AIC of 166.1. Across 222 observations and 2 random experiments, no fixed effect was determined to be a significant predictor of the response variable. Experiment 2 was removed from this analysis because preparation of the bloodmeal was significantly different than experiment 1 and experiment 3 (blood was not washed with PBS and serum removed prior to bloodmeal preparation).

Table 8 Infection and dissemination rates in *Culex quinquefasciatus* mosquitoes ingesting West Nile virus after exposure to Plasmodium parasites in a prior feeding event.

Exp.	Mosquito	*Plasmodium* status	Lin.	Parasit.	Incb.	DPI	I.R.	p-value	D.R.	p-value
1	Sebring	Yes	BCS	0.0014	8	7	73% (11/15)	0.69	18% (2/11)	1
1	Sebring	no	-	-	8	7	83% (25/30)		20% (5/25)	
1	Sebring	Yes	P.R.	0.003	8	7	83% (25/30)		20% (5/25)	
2	Sebring	Yes	BCS	0.128	6	7	95% (18/19)	0.7	33% (6/18)*	5E-05
2	Sebring	No	-		6	7	89% (50/56)		0% (0/50)	
2	Sebring	Yes	P.R.	0.0014	6	7	86% (25/29)		4% (1/25)	
3	BCS	Yes	P.R.	0.032	9	7	55% (11/20)	0.1	0% (0/11)	0.45
3	BCS	No	-	-	9	7	90% (9/10)		11% (1/9)	
4	BCS	Yes	P.R.	0.285	11	14	66% (4/6)	1	25% (1/4)	1
4	BCS	No	-	-	11	14	56% (5/9)		20% (1/5)	
5	BCS	Yes	BCS	0.01	9	14	56% (5/9)	0.3	0% (0/5)	1
5	BCS	No	-	-	9	14	86% (6/7)		17% (1/6)	

**Statistically different than the control group at an alpha <0.05 based on Fisher's exact test*
Exp. = Experiment
Lin. = Lineage
Parasit. = Parasitemia
Incb. = Incubation between first feeding event and exposure to WNV
I.R. = Infection Rate
D.R. = Dissemination Rate

Table 9 Infection and dissemination rates in *Culex quinquefasciatus* mosquitoes concurrently ingesting *Plasmodium* and West Nile virus

Exp.	Mosquito	Plasmodium status	Lin.	Parasit.	DPI	Infection rate	p-value	Dissemination rate	p-value
1	Sebring	Yes	BCS	0.1	7	100% (49/49)	0.47	6% (3/49)	0.7
1	Sebring	No	-	-	7	98% (42/43)		10% (4/42)	
1	Sebring	Yes	BCS	0.1	14	100% (47/47)	1	19% (9/47)	0.8
1	Sebring	No	-	-	14	100% (41/41)		15% (6/41)	
2	BCS	Yes	P.R.	0.052	14	44% (7/16)	*0.0009	0% (0/7)	0.06
2	BCS	No	-	-	14	100% (14/14)		43% (6/14)	
3	BCS	Yes	P.R.	0.019	14	84% (16/19)	*0.001	19% (3/16)	1
3	BCS	No	-		14	31% (8/26)		13%(1/8)	

**Statistically significant at an alpha <0.05 based on Fisher's exact test*
Exp. = Experiment
Lin. = Lineage
Parasit. = Parasitemia
DPI = Days post infection

4.5 Discussion

Prior transmission studies demonstrate that *Plasmodium* parasites can modulate barriers of viral infection and transmission in mosquito vectors. This current study evaluates how avian malaria may influence rates of WNV infection and dissemination in the southern house mosquito, *Culex quinquefasciatus*. Briefly, over five experiments we exposed 239 mosquitoes to WNV with *Plasmodium* as an experimental treatment in a sequential pattern: first mosquitoes fed on *Plasmodium* infected or uninfected birds and then they were exposed to WNV through artificial feeders. We also simultaneously exposed 167 mosquitos to WNV, where bloodmeals contained *Plasmodium* parasites as the experimental treatment or contained blood without *Plasmodium* parasites. Overall, we see no consistent pattern that would suggest *Plasmodium* influences WNV infection and dissemination in mosquitoes. Results and future directions are discussed below.

Prior work demonstrates that co-infecting parasites can mechanically disrupt barriers (i.e. midgut penetration) or elicit immunological response (i.e. immune priming), which can impede viral kinetics. For example, prior infection with *Plasmodium berghei* was shown to alter the salivary gland barrier in *Anopheles stephensi* mosquitoes resulting in an increase in Rift Valley fever virus transmission (Vaughen and Turell, 1996). Yet, the role of *Plasmodium* in arbovirus transmission is not clear as prior studies demonstrate that co-ingested *Plasmodium* parasites were not shown to impact vector competence in both *Culex tarsalis* and *Aedes aegypti* mosquitoes (Barnett 1956, Rozeboom et al. 1966).

Here, we demonstrate that in some cases *Plasmodium* can increase rates of dissemination in mosquito vectors and in other cases decrease rates of viral dissemination, however the mechanism remains unknown. Based on both the sequential and concomitant exposure of

mosquitoes to *Plasmodium* and WNV, *Plasmodium* infection does not appear to impact rates of WNV infection and dissemination in a predictable or applicable fashion. In sequential experiment 2, infection with the BCS lineage of *Plasmodium* during an initial bloodmeal was found to increase rates of viral dissemination by an estimated 30% in mosquitoes that were exposed to WNV in a subsequent feeding (Table 8). In contrast, duel infection with *P. relictum*-WNV was shown to decrease rates of viral dissemination in almost a significant fashion ($p=0.06$), albeit under small sample sizes (Table 9). Furthermore, *P. relictum* KV115 co-infection decreased midgut infection rates by about 56% ($p=0.0009$) in simultaneous infection experiment 2 and increased midgut infection rates by about 53% ($p=0.001$) in simultaneous infection experiment 3 (Table 9). Contrasting results between experiments may suggest that certain mechanisms associated with *Plasmodium* infection can modulate WNV kinetics, however it may also just be due to sample size.

Other uncontrolled factors may also exist that impact viral kinetics in the mosquito, such as avian immunity. Here we used a combination of canary blood and chicken to evaluate how *Plasmodium* parasites may influence WNV infection and dissemination in the mosquito vector. Immune factors in each individual bird are likely heterogenous. In this study, to control for variations in avian immunity, during artificial exposures blood was washed with PBS multiple times to remove any proteins that may inhibit viral infectiousness or impact mosquito vectors.

Interpretation of these inconsistent outcomes is difficult as these contrasting scenarios imply *Plasmodium* parasites may increase arboviral transmission or decreases arboviral transmission. Bloodmeal preparation likely played a large role in dictating the results of experiment 2, where mosquitoes simultaneously exposed to WNV and *Plasmodium* parasites were exposed to a bloodmeal that was unwashed with PBS and spiked with a denser

concentration of sugar. Regardless of these inconsistencies, statistical analyses using mixed logistic models suggest that *Plasmodium* infection has no statistical impact on WNV infection or dissemination in both simultaneous and duel infection experiments. Even when coding *Plasmodium* infection as a binary treatment, as a numerical factor based on *Plasmodium* parasitemia, as the presence of Oocysts, or as the burden of Oocysts, no significant predictors were identified. Larger sample sizes may enhance our ability to identify significant predictors should *Plasmodium* slightly increase or decrease viral kinetics. However, results of this study suggest *Plasmodium* parasites are likely not impacting WNV transmission through the modulation of midgut infection or midgut escape barriers.

Although results demonstrate no clear pattern between midgut infection and viral dissemination, results of infection assays demonstrate that BCS and Sebring mosquito lineages were susceptible to WNV with rates of detection generally being higher in midguts than legs, reiterating the importance of midgut escape barriers in regulating viral transmission (Table 8 and 9) (Kramer et al. 1981, Hardy et al. 1983). Further, this study did not test viral transmission efficiency or rates of viral dissemination into the salivary glands, which is frequently utilized as a surrogate to viral transmission. Therefore, *Plasmodium* parasites still may influence vector competence by modulating rates of viral transmission.

Additionally, interpretation of these results must keep in mind that viral infection was diagnosed by detecting the presence of RNA through rt-PCR. Although this is a sensitive method to detect infection, the presence of viral RNA does not indicate whether an infectious virion is present. Midgut infection rates were generally high, which makes sense as ingested blood always contained WNV. Whether RNA detected in midguts or legs/wings indicates infectious virus remains to be tested. One technique to infer viral infectiousness is through Plaque assay using

Vero cells. The benefit of cell culture as a diagnostic technique would be the detection of infectious virions, but it would also provide a means for quantifying infectiousness, a metric that is perhaps more important when assessing the presence of virus in the saliva. If *Plasmodium* is impacting viral evolution this may be detected through cell culture, however, comprehensive evolutionary studies should utilize a mix of cell culture and next generation sequencing to detect viral population diversity.

Avian malaria parasites are incredibly diverse globally and known to demonstrate heterogeneity in vector compatibility (Valkiunas et al. 2004, Carlson et al. 2015). Considering the spatial and ecological overlap of arboviral agents with *Plasmodium* parasites in avian and mosquito vectors, the capacity for *Plasmodium* parasites to modulate midgut infection and escape barriers remains high, however evidence from experimental transmission suggests this ecological overlap does not appear to impact viral infection or dissemination in mosquito vectors.

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CHAPTER V

SUMMARY AND FUTURE RESEARCH

Overall, this work demonstrates that the consequence of co-circulating parasites for vector-borne pathogen transmission is complex as multiple mechanisms of interaction can occur concurrently with different consequences at individual and population levels. These results reiterate the difficulty in assessing the population-level impact of co-infection when studying hypothetical mechanisms of interaction in isolation. Importantly, parasites that co-occur in host and vector populations may impact transmission dynamics differently in each organism. As such, studies focused on identifying factors that increase or reduce the persistence of a vector-borne parasite should consider the impacts of polyparasitism in both hosts and vectors tandemly. Chapter II illustrates that Great-tailed Grackles are heavily parasitized by blood-parasites. As these birds continue to expand, they provide a great model system to study how patterns of parasite communities adjust in response to invasive organisms. Chapter IV illustrates visits the impacts of one of these parasites found to circulate in GTGR individuals, *Plasmodium*. Although the results of experimental transmission studies were mixed, it appears that in some cases Plasmodium parasites may influence rates of midgut infection and dissemination suggesting these parasites may indeed influence population level transmission dynamics of mosquito-borne diseases. To evaluate the relative consequence of these changes in infection rate and rates of dissemination, two models were defined and analyzed in Chapter III. As a case study, sensitivity analysis suggests viral dissemination rates are a key factor driving WNV transmission heterogeneity implying results from Chapter IV may indeed result in population level impacts for WNV transmission. Yet, with no consistent pattern detected, it remains difficult to predict how various lineages of *Plasmodium* may influence WNV transmission. Overall, it seems unlikely that the *Plasmodium* parasite is

modulating WNV transmission through changes in infection and dissemination rates in mosquitoes. However, it is important to note that empirical studies already document changes in vector feeding behavior, vector survivorship, and host survivorship, which may have severe impacts on transmission dynamics. As pointed out in Chapter III, future studies should focus on host co-infection dynamics with the avian malaria parasite to evaluate if viremia levels change due to *Plasmodium* infection status.

In summary, the focus of this dissertation was to evaluate the role polyparasitism plays in vector-borne pathogen transmission ecology using WNV as a model system. Polyparasitism is a rule in nature and identifying patterns of parasite assembly or elucidating drivers of parasite population dynamics requires experimentation from multiple angles. In this dissertation field, lab, and theoretical techniques were utilized to understand how avian malaria may impact WNV transmission. Theoretically, there are numerous mechanisms through which avian malaria may influence WNV transmission. However, empirical studies demonstrate no predictable pattern through which WNV transmission could be predicted based on the presence of avian malaria parasites. As it stands, this dissertation did not proof any mechanisms through which WNV could be controlled by avian malaria. As a control strategy, there is theoretical merit to the idea of manipulating parasite communities to alter viral transmission or utilizing parasite communities as a surveillance tool. Yet, from a practical standpoint this strategy is still in development and requires much more study from an empirical standpoint, theoretical standpoint, and a policy standpoint.