DISSERTATION

ENHANCING THE USE OF MOSQUITOES IN DISEASE SURVEILLANCE THROUGH SPATIALLY EXPLICIT ENTOMOLOGICAL RISK INDICES AND THE VALIDATION OF XENOSURVEILLANCE

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ABSTRACT

ENHANCING THE USE OF MOSQUITOES IN DISEASE SURVEILLANCE THROUGH SPATIALLY EXPLICIT ENTOMOLOGICAL RISK INDICES AND THE VALIDATION OF XENOSURVEILLANCE

Mosquitoes transmit an array of pathogenic organisms that pose a serious risk to human and veterinary health. Due to difficulty with diagnosis, drug resistance, and lack of viable treatments and preventative vaccines, the most effective way to combat mosquito-borne diseases remains surveillance and control of vector populations. Although specific surveillance programs vary between disease systems, surveillance data is invariably used to dictate vector control policy.

Arthropod-borne viruses (arboviruses) vectored by mosquitoes are routinely surveyed for in portions of the United States. Multiple factors are measured in an attempt to determine potential risk to human populations for arbovirus infection, including variables independent of the mosquito vectors, such as temperature, precipitation, virus detection in sentinel and domestic animals, and human case monitoring. Data collected from adult mosquitoes likely give the most direct measures of arbovirus exposure to human populations. These data, which include virus detection within mosquitoes, result in 3 important measures: (1) estimates of vector population size, (2) approximation of infection rates within vector populations, and (3) Vector Index (VI), a measure that combines population size and infection rates into a single quantitative value. The VI is an estimate of the number of infected mosquitoes collected per trap night and has proven to be a useful indicator of risk for arbovirus transmission.

West Nile virus (WNV), the leading cause of viral encephalitis in the United States, is enzootic in northern Colorado with spillover transmission into humans occurring annually. Regular sampling of *Culex tarsalis* and *Culex pipiens pipiens* mosquitoes, the most abundant vector species of WNV in Fort Collins, has taken place across the city since 2006. We aimed to test the hypothesis that entomological risk for WNV infection is not homogenously distributed across the city. Accordingly, we curated historical trap data from 42 individual Centers for Disease Control and Prevention (CDC) miniature light traps and 10 gravid traps placed throughout the city from 2006-2013. For each species, we analyzed the number of trap nights, vector abundance, and the presence of WNV RNA detected from pools of mosquitoes. We retrospectively split the city into four operationally relevant zones, and with these data we calculated estimates of vector populations, infection rates, and VI for each week during the transmission season for each zone. Our results demonstrate that the city of Fort Collins is heterogeneous for all 3 entomological risk measures calculated. Further, our data indicate that increased VI in each zone is correlated with an increase in human WNV cases. Given this, a finer-scale for calculating entomological risk, which dictates vector control strategies, is appropriate. However, it remains to be determined how specific VI values should influence vector control policies.

Traditional surveillance programs provide crucial information regarding risk of vector-borne disease transmission. Nevertheless, the advancement of Next Generation Sequencing (NGS) technologies provides an opportunity to not only improve on traditional surveillance techniques, it also enables researchers to explore aspects of surveillance samples more in-depth than previous technology allowed. Researchers are now able to determine gene flow between vector species, detect insecticide resistance alleles in vector mosquitoes and drug resistance alleles in pathogens, determine population structures of both vectors and pathogens, as well as identify microbes that may interrupt disease transmission within a vector. We sought to expand on the information that can be collected through

routine vector surveillance by advancing methodology that utilizes blood-fed mosquitoes to survey human populations for non-vector-borne pathogens called Xenosurveillance.

Xenosurveillance is a technique that exploits the hematophagous behavior of some arthropods to survey humans for pathogens circulating within a population. Previous work indicates that human viruses can be detected in the blood meals of mosquitoes by multiple molecular methods, however it remains to be assessed if: (1) Xenosurveillance is suitable for the detection of bacteria and parasites as well as viruses, and (2) Xenosurveillance can be used to detect pathogens in humans at similar levels compared to traditional sampling techniques. We therefore used real-time reverse transcription quantitative polymerase chain reaction (qRT-PCR) as well as NGS on both laboratory and field derived samples to evaluate the effectiveness of Xenosurveillance.

Laboratory colonies of *Anopheles gambiae* mosquitoes were fed blood meals containing various amounts of *Trypanosoma brucei gambiense*, *Bacillus anthracis*, as well as two RNA viruses, Middle East respiratory syndrome coronavirus (MERS-CoV), and Zika virus (ZIKV). Blood fed mosquitoes were held for up to 24 hours to determine if genetic signatures of these pathogens could be detected: (1) when mosquitoes were fed blood containing clinically relevant concentrations of pathogens and (2) in a biologically relevant period. Using pathogen specific qRT-PCR, we determined that genomes (viruses) or transcripts (bacteria and parasites) could be detected below clinical pathogenemias and up to 24-hours post blood meal. Next, we sought to determine the efficacy of Xenosurveillance in a field setting. We enrolled households from two villages in northern Liberia into an Institutional Review Board (IRB) approved study. Upon enrollment individuals within the dwelling provided us capillary blood by blood finger prick which was placed on Flinders Technology Associates (FTA) cards, referred to as human dried bloodspots (H-DBS). Blood fed *An. gambiae* mosquitoes were aspirated from within homes every other day for the next two weeks. Blood meals were subsequently removed with microdissection techniques and placed onto FTA cards, referred to as mosquito dried bloodspots (M-DBS). FTA cards were shipped

to our laboratory where both M-DBS and H-DBS were subjected to RNA extraction, cDNA synthesis, library preparation, and NGS sequencing on an Illumina platform. Data obtained through NGS was sent through an in-house computational pipeline in order to taxonomically assign nucleotide sequences. These sequences aligned to multiple viruses and parasites known to infect humans. Sequences aligning to these pathogens were detected in both M-DBS and H-DBS at similar levels. Our laboratory and field studies taken together indicate that Xenosurveillance is a non-invasive method to sample human blood for genetic signatures of viruses, bacteria, and parasites circulating in a human population while making use of mosquitoes collected as part of regular vector surveillance programs.

The use of NGS to process surveillance samples also allows for the detection of commensal microbes that may disrupt mosquito-borne disease transmission. This has led to an unprecedented increase in the number of novel viruses described from insects. Insect viruses span over 15 families, including *Flaviviridae, Parvoviridae* (subfamily *Densovirinae*), and *Baculoviridae*. Within the family *Flaviviridae*, viruses that are only capable of infecting insects and/or insect cells exist in phenotypically diverse clades. Multiple studies have indicated that co-infection of mosquitoes with insect-specific flaviviruses and vector-borne viruses may affect virus-vector interactions, possibly interrupting transmission of the pathogenic virus. *Anopheles* species mosquitoes had not previously been assessed for the presence of insect-specific flaviviruses. Therefore, we queried previous RNA-sequencing datasets generated from *Anopheles* mosquitoes collected in West Africa for the presence of commensal viruses. Using an in-house pipeline, we were able to assemble 6 full genomes of novel viruses, presumed to be insect-specific. Of these, two appear to be classical insect-specific flaviviruses (cISV). Their genomes group phylogenetically with other cISVs found in *Culex* and *Aedes* mosquitoes, however they form their own distinct clade. Due to sampling techniques, virus isolation was not possible; therefore these viruses remain to be characterized *in vivo*.

Surveillance remains a critical component of programs aimed at controlling the emergence and transmission of infectious diseases. Collectively, the work described above indicates the need for a finer-scale application of traditional mosquito-borne disease surveillance data, as well as the importance of utilizing samples collected during routine mosquito-borne disease surveillance. We have demonstrated that proper evaluation of these samples provides information about pathogen circulation in a human population and has the potential to improve disease surveillance in resource poor areas, as well as provide a more thorough understanding of the basic biology of medically important arthropods.

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To paraphrase Richard Lewontin in his lecture turned book “Biology as Ideology”, science as an institution has two goals: to provide an explanation for how our world (and others) works, and to manipulate them in such a way as to improve the quality of life. The work detailed below represents a minuscule contribution to these not necessarily mutually exclusive goals, and I don’t think I could be more proud of it. Science does not exist in a vacuum, and therefore successes cannot be attributed to one person alone. Thus far on my ceaseless journey into science, life really, I am immeasurably fortunate to have been influenced by incredible people. Some motivated and inspired. Others meticulously mentored and taught. Some had to drag me along kicking and screaming, metaphorically, thankfully. Many have done a bit of it all. For better or worse, they all had a hand in shaping who I am as a scientist and a person, an identity that is now inseparable to me. This section is for those people.

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Historical perspective

Mosquitoes have been, and continue to be, a menace to humankind. These flies proved more than a nuisance when Patrick Manson demonstrated that filarial worms were transmitted to humans by the bite of an infectious mosquito (1). Manson then took a budding malarialogist under his wing and revealed his hypothesis regarding mosquitoes transmitting malaria. A British Surgeon General in the Indian Medical Service named Ronald Ross first demonstrated that mosquitoes are the vectors of *Plasmodium,* the causative agent of malaria. Building off the germ theory of disease set forth by Louis Pasteur, and the notion that arthropods can in fact spread infectious agents laid out by Smith and Kilbourne and Manson, on July 4th, 1898 Ross identified "thread like bodies" in the salivary glands of *Culex* mosquitoes that had fed on parasitemic sparrows. Subsequently, an Italian scientist named Giovanni Batista Grassi worked out the life cycle of the human protozoan in *Anopheles* mosquitoes. Almost 20 years prior to this discovery in mosquitoes, a French physician named Charles-Louis-Alphonse Laveran spotted *Plasmodium* in the blood of a malarious patient, and was the first to identify the parasite (2).

The idea of mosquitoes being vectors of human disease extended into other areas of the world, most notably with yellow fever virus in the Americas. Yellow fever is a disease of the African tropics brought to the Americas by way of the slave trade, around 300 to 400 years ago (3). Unlike *Plasmodium*, where the agent was observed before the vector was identified, modern virology had not yet advanced enough to identify the causative agent of this disease. Instead, multiple scientists demonstrated that

yellow fever is spread from human to human by the bite of an infectious mosquito and not environmental contamination. Carlos Finlay, a Cuban physician, was the first to propose that mosquitoes were responsible for this cycle of pestilence. American military physicians Walter Reed and Jesse William Lazear were stationed in Cuba as part of the Yellow Fever Board. While in Cuba, Lazear obtained *Aedes aegypti* eggs from Finlay and began conducting transmission experiments. James Carroll, another member of the Yellow Fever Board and a pronounced skeptic of the mosquito hypothesis, volunteered to let one of Lazear's mosquitoes take a blood meal from him. This mosquito had previously fed on a patient dying of yellow fever, and successfully transmitted the then unknown virus. Miraculously, Carroll and another volunteer, solider William E. Dean, both contracted yellow fever virus, developed disease, and subsequently recovered. Lazear, the man behind the experiments, would not be so fortunate. While feeding a mosquito on a patient in a yellow fever ward, he was exposed by the bite of *Ae. aegypti* himself, and would succumb to the disease a week later. His pioneering experiments motivated Walter Reed to build "Camp Lazear", where the mystery of mosquito transmission would be solved. Reed ordered construction of two buildings both sealed off from the outside environment. In one building, three volunteers would stay for 20 nights with piles of soiled linens and clothes from patients who died of yellow fever. This was to combat the prevailing notion that yellow fever was directly transmitted from human to human. The other building consisted of two rooms separated by mesh screens. In one room, a consenting volunteer (the human consent form was allegedly created during these experiments) laid in a room occupied by mosquitoes captured from the yellow fever ward. The other room was the control. The concept of an extrinsic incubation period did not yet exist, so multiple exposures to mosquitoes were necessary to produce an infection. Throughout the course of these experiments, the people in the control room as well as the room with soiled material did not develop yellow fever, while those in mosquito room did. This laid to rest the direct exposure hypothesis and established that mosquitoes were in fact the harbinger of a few of man's worst diseases (4).

Through the work of these physicians, scientists, and volunteers, the field of medical entomology was born. What would follow is over a century of work by diligent and assiduous scientists that have advanced the field in ways that Reed, Lazear, Finlay, Manson, Ross, and the other giants that stood before us could only have been proud of.

Diversity of mosquitoes and the pathogens they spread

Mosquitoes (family *Culicidae*) are an exceptionally diverse group of insects. Over 3500 species exist in greater than 40 genera comprising 2 sub-families (5) The natural history and ecology of these animals varies widely, as do their ability to transmit pathogens. These pathogens span multiple phyla, including protozoa (6), parasitic worms (7), and viruses (8). Mosquito-borne diseases are a massive cause of morbidity and mortality that disproportionately affect underserved populations throughout the world (9).

The subfamily Anophelinae is a monophyletic group composed of greater than 475 individual species, principally within the genus *Anopheles* (5). The genus Anopheles is further divided into seven subgenera based largely on morphological characteristics of male mosquito genitalia (10). Of these, only a small proportion are biologically relevant malaria vectors (11). Within the subgenus *Cellia* exist the *Anopheles gambiae* complex, the most important vectors of malaria is sub-Saharan Africa (12, 13). This complex currently consist of 8 morphologically indistinguishable subspecies (12). These species have varying geographic distributions (14), use different ecological niches (15-17), and display variation in feeding behavior (18, 19) and vector competence for malaria (20), all of which have implications for malaria control. These species can be distinguished using various molecular methods (21, 22). The identification of vector species is a fundamental aspect of mosquito surveillance and control.

Members of the sub-family Culicinae, the other major sub-family of Culicidae, are important vectors of human disease. Rao and Rai (1987) concluded that Anophelinae and Culicinae evolved from a

common ancestor, but have diverged into separate sub-families (23). Interestingly, the array of human pathogens transmitted by these genera appear to follow this evolutionary relationship, with members of Anophelinae being important vectors of parasites and members of Culicinae being important vectors of arthropod-borne viruses (arboviruses). Albeit, there are clear and notable exceptions to this general rule with examples including Rift Valley fever virus (24) and O'nyong-nyong virus (25) transmission by *Anopheles* species, and filarial worm transmission by *Culex* and *Aedes* species (26, 27). The sub-family Culicinae is split into multiple tribes, two of which, Aedini and Culicini, are demonstrably monophyletic and contain numerous disease vectors, namely species in the genus *Culex* and *Aedes* (5). Member species of these two genera are responsible for vectoring emerging and re-emerging arboviruses. The importance of mosquito taxonomy is emphasized in regard to malaria and arbovirus control.

Human malaria is a febrile illness caused by multiple species of parasitic apicomplexan protozoa in the genus *Plasmodium*. Currently, five species are known to infect humans. *P. falciparum* (28), *P. vivax* (29), *P. ovale* (30)*, P. malariae* (30), and the zoonotic *P. knowlesi* (31) are differentiated by morphology, geographical distribution, and disease manifestation, among a multitude of other factors. *P. falciparum* causes the most virulent form of the human disease (32). Falciparum malaria is endemic throughout the tropics, although over 90% of malaria cases and deaths attributed to malaria occur in sub-Saharan Africa (33). *P. vivax* is responsible for the majority of the remaining disease burden. *P. vivax* distribution occurs throughout the tropics of South and Latin America, as well as the tropics of Asia (34). *P. ovale* and *P. malariae* are less prevalent than *P. falciparum* and *P. vivax* (33), with their distributions being confined to the tropics as well (35). *P. knowlesi,* a parasite of macaques, has recently been shown to infect humans as well (36). While all human species of *Plasmodium* were zoonotic in the distant past (37), only *P. malariae* and *P. knowlesi* are known to circulate in both humans and non-human primates consistently (38). Human malaria parasites exhibit similar lifecycles in respect to their vertebrate and invertebrate host, *Anopheles* spp. mosquitoes.

As well as malaria, *Anopheles* mosquitoes contribute to the transmission of filarial nematodes, parasitic animals that cause a variety of diseases, including lymphatic filariasis. Three species of worms within the family *Onchocercidae* are etiological agents. 90% of cases worldwide are caused by *Wuchereria bancrofti,* while *Brugia malayi,* and to a much lesser extent *Brugia timori*, contribute to the remaining disease burden (27). At least 77 species of multiple genera of mosquitoes have been shown to be competent vectors in a laboratory setting, although fewer species are biologically relevant vectors (39). Lymphatic filariasis is distributed throughout the tropics and is endemic in greater than 70 countries (40). While *Anopheles* species play an important role in the transmission of filarial nematodes in sub-Saharan Africa (41), other genera of mosquitoes, including *Culex, Aedes*, and *Mansonia* are important vector species in sub-Saharan Africa (42, 43), as well as the Americas (26) and Asia (44, 45).

Viruses transmitted to vertebrates by arthropods are referred to as arboviruses (**AR**thropod-**Bo**rne **VIRUSES**). As of April 2017, the Arbovirus Catalog maintained by the Centers for Disease Control and Prevention (CDC) and the American Committee on Arthropod-Borne Viruses contains 537 unique species or genotypes of arboviruses (46). Mosquitoes vector a substantial number of these viruses that cause significant human disease. Mosquito transmitted viruses are contained within five separate viral families: Bunyaviridae, Flaviviridae, Togaviridae, Reoviridae, and Rhabdoviridae, all of which possess an RNA genome (47). While an exhaustive list of mosquito transmitted viruses is outside the scope of this work, it is worth highlighting viruses that have recently emerged/remerged. Zika virus (ZIKV, Family Flaviviridae), first isolated from a febrile rhesus macaque in the Zikka Forest of Uganda (48), has caused a pandemic in the Americas beginning in 2013 (49). This epidemic has been exceptional due to the large scale (50), unique transmission routes (51), and unexpected pathology in fetuses (52). Anthropophilic behavior (53, 54), as well as wide distribution of the primary ZIKV vectors *Aedes aegypti* and *Aedes albopictus* mosquitoes (55, 56) contribute to the scale of virus transmission. Prior to ZIKV emergence in the Americas, ZIKV was thought to cause only a mild febrile illness during sporadic outbreaks across

Africa and Asia (57). Chikungunya virus (CHIKV, Togaviridae), also transmitted by *Ae. aegypti* and *Ae. albopictus*, emerged in the Americas in 2013 and is thought to have caused hundreds of thousands of cases in greater than 20 countries (58). All told, Chikungunya virus has caused disease in more than 100 countries since it's discovery in Tanzania in 1953 (59, 60). Many of these countries experience sustained virus transmission. West Nile virus (WNV, Flaviviridae) was first isolated from the blood of a Ugandan woman in 1937 (61) and is now found on every continent save Antarctica (62-68). WNV was detected in New York City in 1999 (69), spread across the continental United States within 5 years (70), and has subsequently become endemic. WNV can be considered an ecological generalist, in part due to the high number of competent mosquito vectors. *Culex* spp. mosquitoes transmit WNV in nature. The genus *Culex* includes the *Culex pipiens* species complex. Mosquitoes making up this species complex are highly competent vectors of WNV (71, 72), and the most widely distributed group of mosquitoes in the world (73). Proper identification of relevant disease vectors and the pathogens they harbor is critical for the organization and implementation of vector control methods.

Ecology of mosquito-borne pathogens

In order for bona fide biological transmission of mosquito-borne pathogens to occur, host (vertebrate), pathogen, and vector (mosquito) must overlap in time and space. In short, a pathogen must undergo specific development within the mosquito vector, the mosquito must then seek out a vertebrate host and through the process of blood feeding, or attempted blood feeding (74), the pathogen is transmitted to the host. Propagation of the transmission cycle occurs if the vertebrate develops high enough pathogenemia to be infectious to another mosquito that feeds on that host later in time. Clearly, this general cycle does not always apply (e.g. mechanical aerosol transmission of some arboviruses) however, in order to quantify mosquito-borne disease transmission risk, it is imperative to focus on the rules and not their exceptions.

Multiple factors affect whether or not a mosquito will transmit a pathogen, and the interactions between these factors are incredibly complex. A relatively simple mathematical model, referred to as the Ross-Macdonald model (75), Macdonald's equation (76), or vectorial capacity makes basic assumptions about interactions between variables important for vector borne disease transmission. This equation is indispensable for our basic understanding of vector-borne disease transmission dynamics. Vectorial capacity is defined as the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding on a single host in 1 day (76), or the daily reproductive number (77). The formula for vectorial capacity is:

$$\mathrm{V} = \frac{m \times a^2 \times p^n \times b}{-\ln p}$$

where *m* is the density of the mosquito vector in relation to the vertebrate host, *a* is the probability a vector feeds on a host in a day, *p* is daily survival, *n* is the extrinsic incubation period (EIP), or the amount of time the pathogen takes to develop and be infectious within a mosquito (76) , *b* is vector competence, or the intrinsic permissiveness of an arthropod vector for infection, development, and transmission of a vertebrate pathogen (76), and –ln *p* refers to the number of days a mosquito survives post EIP. While the equation for vectorial capacity is general and applicable to all mosquito-borne diseases, specific conclusions cannot be made across different vector-pathogen-host systems. For the sake of clarity, I will discuss each variable in relation to WNV transmission, specifically in Northern Colorado when applicable.

m- vector density in relation to host: The most biologically relevant vectors of WNV in Northern Colorado are *Culex tarsalis* and *Culex pipiens pipiens* mosquitoes (62). Adult *Culex* population density varies widely between and within years (78), also between locations in close proximity (79). A variety of biotic and abiotic factors can influence adult populations size, including larval predation (80), vegetation (81), temperature (82), and precipitation (83), among others. These factors interact and affect *Cx. tarsalis* and

Cx. pipiens differently (84, 85), making it difficult to reliably model adult population sizes, and therefore vector to host ratios. As well, factors influencing mosquito population density are dynamic, further complicating modeling (76). Vector density is estimated during routine mosquito-borne disease surveillance and is a useful measure of risk.

a- probability a vector feeds on a host in a day: Two independent variables, vector blood meal preference and days between blood meals, make up this value. WNV exists in an enzootic cycle, primarily between birds and *Culex* mosquitoes, where birds act as a reservoir/amplifying host for the virus (86). Assuming no vertical transmission of WNV within the mosquito, which very little appears to happen in Northern Colorado (62), humans become infected with WNV when a mosquito acquires WNV from an infected bird, and subsequently feeds on a human (87). Therefore, blood meal preference of mosquito vectors influences transmission dynamics. Kent et al. empirically determined the blood meal preferences of *Cx. tarsalis* mosquitoes in Northern Colorado by collecting engorged female mosquitoes, subjecting DNA from the blood meal to traditional Sanger sequencing, and comparing amplicons to known vertebrate sequences (88). Using the forage ratio technique, essentially controlling for host abundance when calculating proportion of blood meals from any given host (89), the authors determined humans make up a small portion of *Cx. tarsalis* blood meals overall, but this proportion increases throughout the transmission season. In addition, early in the transmission season, 60% of *Cx. tarsalis* that were blood fed *and* WNV positive had obtained their blood meal from robins, indicting robins as important amplifying host for WNV. These host preference dynamics clearly influence WNV transmission in Northern Colorado. As with all variables of vectorial capacity, host preference varies substantially depending on location and time (90-93)

Female mosquitoes seek blood meals in order to supplement nutritional needs and develop a clutch of eggs, which is referred to as the gonotrophic cycle (94). The length of the gonotrophic cycle dictates how often a female mosquito will seek a blood meal. Estimations from natural populations of

Cx. tarsalis suggest a gonotrophic cycle is completed in 4-5 days (95, 96). However, *Culex* mosquitoes have demonstrated autogeny, a process by which the mosquito completes a gonotrophic cycle and lays a clutch of eggs without having taken a blood meal, which can confound field observations of parity (97). The length of a gonotrophic cycle can decrease in *Cx. tarsalis* mosquitoes at higher temperatures, thus increasing rates of host contact (98). What a vector feeds on and how often it feeds is also a dynamic process that is influenced by multiple environmental and genetic factors.

p- daily survival, n- extrinsic incubation period: This measure aims to assess the probability of a mosquito surviving the extrinsic incubation period of the pathogen. Vectorial capacity is most sensitive to small changes in daily survival, because the effects are both in the numerator and denominator of the equation and are non-linear (76). Daily survivorship is estimated using a variety of methods, including mark-release-recapture studies (99) and age grading techniques (100). Mark-release-recapture studies of *Cx. tarsalis* mosquitoes are influenced by multiple variables, including time of release and recapture methods (101). Mark-release-recapture studies on *Culex* mosquitoes in Colorado have been conducted to determine overwintering conditions (102), but specific studies on daily survival of these populations have not been conducted. The extrinsic incubation period of WNV is related to vector competence, and is influenced by virus load (103), temperature (104), virus genotype (105) and many other factors. Again, both of these values are highly variable.

b- vector competence: Vector competence is a complex phenotype influenced in part by genetic factors of the vector, as well as environmental conditions (76). In order for transmission to occur and a mosquito to be deemed competent, there are various barriers within the mosquito a virus must overcome. First, it must infect the midgut epithelium (midgut infection barrier, MIB), replicate within and escape from midgut epithelial cells (midgut escape barrier, MEB), disseminate into the hemocoel, infect salivary gland tissue (salivary gland infection barrier, SIB), replicate within the salivary glands and escape into the saliva (salivary gland escape barrier, SEB). Virus in saliva after a period of time is

indicative of a competent vector (106). The vector competence of WNV in *Culex* mosquitoes has been studied extensively, and is variable between species, populations, and location(107-111).

Vectorial capacity is useful in shaping how we understand and model mosquito-borne disease transmission and consequences of control measures, however it is an indirect and cumbersome way to measure transmission rate of a specific pathogen from a specific vector population (112). Many aspects of this equation need to be measured empirically in controlled laboratory studies (explicitly *b* and *n*), and variables that can be observed or measured in the field are subject to confounders and assumptions such as gonotrophic discordance (113) or homogenous feeding behavior within populations of vector species, respectively (114). Due to random and systemic error inherent to systems as complex mosquito-borne pathogens, Dye (1992) suggested using a comparative opposed to an absolute approach, and limiting the number of variables measured (112, 115). This idea is reflected in large-scale vector borne disease surveillance programs (116).

The necessity of mosquito surveillance

Mosquito control remains the most effective way to mitigate transmission of most mosquito-borne pathogens (117). Currently lymphatic filariasis can be effectively treated with repeated mass drug administrations (MDA) using various combinations of the relatively inexpensive drugs albendazole, ivermectin, and/or diethylcarbamazine (DEC) (118-121). The World Health Organization (WHO) identified lymphatic filariasis, the only mosquito-borne diseases contained in the list, as potentially eradicable due to lack of a zoonotic reservoir and identification of effective treatments (122). The Global Programme to Eliminate Lymphatic Filariasis (GPELF) has two main goals: 1) interrupt transmission and 2) control morbidity (123). Substantial progress has been made in this endeavor and eradication is on the horizon due in large part to the development of more successful treatment plans using combinations of all three drugs, previously thought to be infeasible due to potential adverse effects due to the use of

ivermectin in areas co-endemic with onchocerciasis (120). Vector control is used to supplement MDA and is a contributing factor to the success of GPELF (124). Interestingly, recent work has demonstrated that ivermectin has insecticidal effects on *An. gambiae* mosquitoes for up to two weeks post MDA (125). While this phenomenon is clearly useful for controlling malaria transmission due to its effect on the vector, it likely has secondary outcomes that interrupt transmission of LF parasites from an entomological standpoint.

Valuable treatments such as MDA do not exist for other mosquito-borne diseases. No drugs are currently approved for treatment of mosquito-borne viruses, and medical care is supportive (126). In addition to the lack of antiviral therapies, only two FDA approved human vaccines exists for mosquito-borne viruses, yellow fever virus (YFV, Family Flaviviridae) and Japanese encephalitis virus (JEV, Family Flaviviridae) (127). An efficacious vaccine for WNV exist for horses (128), and a human vaccine is in development (129). As well as WNV, human vaccines are being developed for other mosquito-borne viruses, including Dengue virus (DENV, Family Flaviviridae) (130), CHIKV (131), and ZIKV (132). Most of these vaccines are still in clinical trials and have substantial hurdles to overcome before approval for human use. A potent vaccine against human *Plasmodium* has eluded researchers since the 1960's (133). Dozens of malaria vaccine candidates targeting various life stages of *Plasmodium* are currently in clinical trials (134). *Plasmodium* parasites have evolved some level of resistance to every drug attempted to thwart it (135). Resistance to chloroquine (136), mefloquine (137), primaquine (138) pyrimethamine (139), and artemisinin (140) have been thoroughly documented. The current recommended treatment for falciparum malaria is artemisinin-based combination therapies (141). The potential of artemisinin resistant malaria to spread globally could undermine malaria treatment and control programs (142). Contributing to the spread of malaria resistance is the overuse of anti-malarial drugs largely stemming from misdiagnosis (143). Malaria presents clinically with non-specific symptoms, and in combination with limited infrastructure to perform necessary diagnostic test in endemic areas, malaria is often

mis/over-diagnosed (144-148). Due to an unfortunate combination of lack of preventive therapies, drug resistance, and poor diagnostic capabilities, the best way to mitigate mosquito-borne pathogens is to reduce exposure through mosquito control. The underpinning to every useful mosquito control program is surveillance.

Arbovirus surveillance in the U.S.

Nation-wide federal funding for arbovirus surveillance did not exist in the United States prior to the introduction of WNV to the United States in 1999 (149), despite transmission of arboviruses in the United States, including YFV (150), Eastern equine encephalitis virus (EEEV, Family Togaviridae) (151), La Crosse virus (LACV, Family Bunyaviridae) (152), St. Louis encephalitis virus (SLEV, Family Flaviviridae) (153), Western equine encephalitis virus (WEEV, Family Togaviridae) (154), and likely DENV (155) were recognized as early as the 19th century and throughout the 20th century. Post WNV introduction, arbovirus surveillance systems were developed at some capacity in all 50 states (149), and surveillance guidelines were expanded to include WNV (156).

According to Moore et al. in Guidelines for Arbovirus Surveillance in the United States, the purpose of arbovirus surveillance systems is to "quantify disease activity at a given time, predict the probable future course of the disease cycle, and indicate when control should be started to prevent epizootic of epidemic transmission" (116). In order to accomplish these goals for mosquito-borne viruses, four areas need to be taken into consideration; meteorological data, mosquito surveillance, vertebrate host surveillance, and human case data (116). Due to substantial investment in arbovirus surveillance following the WNV introduction in 1999, this disease system is well defined throughout the country. Measuring virus activity in mosquitoes, birds, horses, and humans are typical aspects of WNV surveillance systems (157). I will review what information has been learned through WNV surveillance

programs, and which aspects of surveillance are the most useful for the prediction of WNV disease in a human population.

As stated above, WNV was determined to be the etiological agent of surge of encephalitis cases in New York city in 1999 (158). The subsequent spread of WNV was rapid, reaching the remainder of the continental United States by the summer of 2004 (159). Within that time, WNV had been identified in 58 species of mosquitoes and 284 species of birds (160). While all of those species are not biologically relevant, the exceptionally high number of competent vectors and enzootic hosts highlights the necessity for surveillance systems to identify local species that support transmission. *Cx. pipiens* was implicated as vector species during the initial outbreak in New York (161). *Culex pipiens quinquefasiatus* mosquitoes were the primary vector during the 2012 WNV outbreak in Dallas, Texas (162, 163) as well as a 2010 outbreak in Phoenix Arizona (164). An outbreak in the western plains and Front Range of the Rocky Mountains in 2003 was largely fueled by *Cx. tarsalis* and *Cx. pipiens* (62, 165). In addition to these important vector species, *Cx. nigripalpus* (166), *Cx. Restuans* (110), *Cx. salinarius* (167), multiple species of *Aedes* (168-171), *Culiseta* (107, 167, 172), and may play some role in enzootic transmission and/or acting as a "bridge-vector", transmitting the virus from birds to humans. The concept of alternative species acting as a bridge-vector is debated. Kilpatrick et al. suggest that WNV transmission to humans is due to a shift in host preference of *Culex* mosquitoes, instead of the involvement of other, mammalophilic species of mosquitoes (173, 174). Land use practices can provide indications of important vector species (175, 176). In general, outbreaks in urban environments stem from peridomestic species (e.g. *Cx. pipiens* and *Cx. quinquefasciatus* (177, 178)), while transmission in more rural settings is driven by species associated with agriculture (e.g. *Cx. tarsalis* (171, 179, 180).

The role of specific bird species as amplifying hosts during these outbreaks is more difficult to determine through surveillance strategies, in large part because the birds that die from WNV infection, and therefore are reported to health departments, are not necessarily pertinent hosts (181-183).

Viremias in birds typically have to reach ~10^5 plaque forming units (PFU)/ml of blood to be considered infectious to feeding mosquitoes (182). Multiple species of birds are thought to be relevant amplifying host as determined by immunological surveys indicating infection, viremia profiles, and mosquito host preference (184). This list includes the Northern cardinal (*Cardinalis cardinalis*) (185), the house sparrow (*Passer domesticus*) (186), the American Robin (*Turdus migratorius*) (90), and the American crow (*Corvus brachyrhynchos*) (187), among many other species (182). American crows reach high viremia titers, ~10^8 PFU/ml, throughout the course of infection (86). Increased viremia and mortality rates in American crows has been linked to a single nucleotide polymorphism (SNP) in the NS3 helicase gene of WNV (188). WNV is pathogenic to American crows, and massive die offs occurred following the introduction of WNV (189).These birds are common in North America (190) and in urban environments (191), further underscoring their role in WNV transmission. Regardless of the American crow's role in enzootic transmission, they are often infected and suffer high mortality rates during outbreaks making them a useful indicator of enzootic WNV activity (192, 193). Other species of birds, typically domestic chickens (*Gallus gallus domesticus*), are used as sentinels for arbovirus transmission because they usually do not succumb to disease, develop measurable amounts of antibodies, and do not develop a high enough viremia to contribute to local enzootic transmission (194-198). Multiple states make use of dead bird counts and sentinel chickens for assessing enzootic WNV activity and subsequent risk to humans (199-202).

WNV causes noticeable disease in ~20% of persons exposed to the virus, typically manifesting in a mild febrile illness (203). Of people exposed to WNV through an infectious mosquito bite, ~1% experience neurologic disease and the frequency of neuroinvasive WNV increases in immunocompromised and elderly patients (204). WNV neuroinvasive disease typically manifests as meningitis, encephalitis, or acute flaccid paralysis (205). Diagnosis typically occurs based on symptoms, and is confirmed using a variety of methods to detect anti-WNV IgM and/or WNV RNA (206, 207).

However, diagnosis can be difficult due to antibody cross-reactivity with similar flaviviruses (208), low-level and transient viremia in serum (209), and the necessity of biosafety level 3 laboratory space to perform virus isolation (210). West Nile virus is defined as a notifiable condition as part of the National Notifiable Diseases Surveillance System (NNDSS). The goal of the NNDSS is to collect data from local, state, federal, and international health care systems in order to better inform public health policy (211). In addition to typical reporting scenarios, the United States blood supply is consistently screened for the presence of WNV RNA to avoid transmission by transfusion (212).

The fact remains that the location, timing, and severity of WNV outbreaks remains unpredictable (156). Mosquitoes, birds, and humans are routinely surveyed for the presence of WNV activity at some capacity in all 50 states (149), but their ability to predict WNV activity differs. Epidemiological surveillance of human WNV cases provides much needed information about disease incidence, risk factors associated with WNV disease, geographical distribution, demography, and much more (213-215) However, *human cases are poor predictors of risk*. The time from an infectious mosquito bite to the onset of symptoms to a clinical diagnosis can range from 2-4 weeks and at times longer (205). Reisen and Brault (2007) highlight the importance of a proactive opposed to reactive approach to mosquito control based off of differences in passive (human) and active (environmental, mosquito and bird) surveillance (216). Figure 1.1 demonstrates the inherent error in using passive surveillance data to mitigate WNV epidemics.

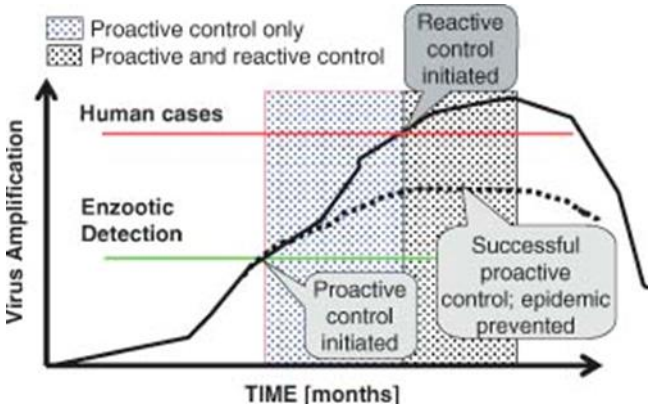


Figure 1.1 Importance of proactive versus reactive mosquito control in controlling WNV transmission to humans. From Reisen and Brault 2007 (216).

As Figure 1.1 indicates, detection of enzootic transmission occurs well before human disease is recognized (217), therefore environmental samples are more predictive of WNV transmission risk. As stated above, there are benefits of utilizing avian mortality and sentinel chickens for WNV surveillance efforts. However, avian mortality has decreased since initial WNV outbreaks (218, 219), indicating that the utility of using dead bird surveys as a predictor of risk is waning. As well, sentinel chickens take time to seroconvert and produce detectable levels of antibodies, thus minimizing their utility as a predictive measurement (220-222).

Mosquito-based surveillance is the foremost tool to quantify and predict risk of WNV transmission (116, 156). The three main entomological risk measures are estimates of vector population size, approximation of infection rates within vector populations, and the Vector Index (VI), a measure that combines population size and infection rates into a single quantitative value. High mosquito population densities are correlated with an increased risk for arbovirus transmission (79, 223, 224). However, mosquito populations can reach high densities with a low rate of enzootic transmission, so population size alone needs to be supplemented with infection rates (225). Infection rates alone have been shown to be indicative of human risk (180). Again, this number is better suited when coupled with population densities because infections rates can be comparably high, but a low mosquito population

density results in a lower risk of transmission (226). This demonstrates the importance of use the VI as an indicator of WNV transmission. This value is more predictive than either population size or infection rate alone (217), and has proven to be a useful indicator of WNV transmission risk (164, 217, 227, 228) in multiple localities. Mosquito population sizes are estimated based on adult trap numbers. There is a variety of tools used to trap adult mosquitoes (76, 229, 230). Host-seeking mosquitoes are typically trapped with a CO_2- baited CDC Miniature Light Traps. Mosquitoes caught with these traps can provide a good indication of population size, however these mosquitoes may have just emerged and have not yet taken a blood meal, so infection rates in these samples may underrepresent actual infection rates in nature (231). Gravid traps can be used for a better estimation of infection rates. As the name implies, gravid traps are designed to capture female mosquitoes that have previously taken a blood meal and are searching for an oviposition location (232). Because this population of mosquitoes has previously taken a blood meal, the likelihood of infection is higher (156). Currently, mosquitoes are pooled by date, location, and species to test for the presence of WNV. Virus isolation can be attempted, however, as stated earlier, virus isolation requires BSL-3 laboratories (210). WNV-specific qRT-PCR has proven to be a sensitive, cost and time effective method for the detection of WNV infected mosquitoes (209, 233). Pooling mosquitoes then requires an estimate of infection. A maximum likelihood estimate (MLE) for infection has been developed by the CDC and made freely available online (234). This is an improvement over the previous minimum infection rate that assumed only a single mosquito within a pool is infected (235). Mosquito population densities and infection rate estimates are determined for each species in a given area and combined to calculate the VI. The VI is summed across all biologically relevant vector species to give an estimate of the number of infected mosquitoes in an area. West Nile virus transmission is heterogeneous on a national, regional, and sometimes local scale. Because of this, mosquito-based surveillance should be conducted over a number of seasons in order to understand usual mosquito and virus activity to best understand when an increase of either, or both, of those

factors is indicative of increased risk (156). If thresholds are established, entomological based risk indices should be used to proactively determine when emergency control is necessary (Figure 1.1).

Ultra-low volume insecticides are used to control for adult populations of mosquitoes in the City of Fort Collins (236). Until 2014, the City of Fort Collins would determine risk of WNV transmission using a combination of entomological and human indices. However, this risk was determined upon a citywide basis, resulting in a substantial, and likely unnecessary, amount of insecticide being applied in the result of an adulticiding event. It remains to be assessed whether the City of Fort Collins is homogenous for these risk measures. Finer scale measures of risk would result in a more targeted and timely response in emergency spraying, as well as a smaller amount of insecticide being applied more effectively.

What other information can be obtained from mosquito surveillance samples?

Routine surveillance of vector populations is a crucial component of mosquito control throughout the world (237). As outlined above, routine vector surveillance typically consist of collecting mosquitoes to determine 1) vector population densities, and 2) infection rates of known pathogens within the population. However, more information can be obtained from these samples. For example, insecticide resistance alleles have been identified from mosquito populations that are collected in similar manners to routine vector surveillance (238, 239). As well, gene flow between mosquito populations can be determined from field collections of mosquitoes (240-243). These studies should inform which insecticide will be most effective in mosquito control efforts. Genetic variation and population structure of arboviruses can be ascertained from viruses isolated from mosquitoes that have been collected as part of routine vector surveillance programs (244-247). The microbiome of field caught and laboratory-reared mosquitoes has been the subject of much attention due to its ability to modulate mosquito physiology and susceptibility to pathogen infection (248-251). *Wolbachia* species of bacteria, first identified in field caught mosquitoes (252), have shown to modify vector competence of mosquito

populations and induce cytoplasmic incompatibility in mosquito offspring (253-259). Novel microbes are being discovered at an increasing rate in field caught mosquitoes, largely due to the advancement of NGS (260). Interestingly, there has been an explosion of literature on the diversity and richness of insect-specific viruses in the last decade (261, 262). Many descriptions of insect-specific viruses in mosquitoes were described from routine mosquito surveillance and virus identification (263). Previous literature showed that infection with two similar viruses can result in the phenomenon known as superinfection exclusion, where a previous virus infection prevents secondary infection with another virus (264). Due to the ubiquity of insect-specific viruses, researchers sought to determine the effect of co-infection in mosquitoes between pathogenic arboviruses and closely related insect-specific viruses (265, 266). Multiple studies have examined the consequences of co-infection in mosquitoes with insect-specific flaviviruses on transmission outcomes of pathogenic flaviviruses, with varying results. Using *Culex flavivirus* (CxFV, Family Flaviviridae), Bolling et al. (2012) showed co-infection of *Cx. pipiens* with CxFV significantly reduced dissemination and negatively affected vector competence of WNV (267). In a similar system, Kent et al. (2010) showed that prior infection with CxFV did not alter *Cx. quinquefasciatus* mosquitoes ability to transmit WNV (268). However, these two groups used different mosquito species and infection methods, so a direct comparison may not be appropriate. Multiple groups have also seen a negative effect on vector competence of mosquitoes for pathogenic flaviviruses when co-infected with insect-specific flaviviruses (269, 270). Field collected, WNV positive *Culex* mosquitoes were 4 times more likely to be co-infected with CxFV than their uninfected counterparts, indicating a positive association shown between CxFV and WNV in nature (271), further complicating the role of insect-specific viruses in arbovirus transmission. The vast majority of insect-specific viruses, specifically insect-specific flaviviruses, have been described from Culicinae species leaving the Anophelinae subfamily comparatively understudied. As well, their role in modulating vector competence for parasites in co-infected mosquitoes remains unknown.

Use of insects in disease identification

Using hematophagous arthropods to identify pathogens in humans is not a new concept. Xenodiagnosis is a diagnostic technique that allows a non-infected, laboratory-reared competent vector to blood feed on a human thought to be infected with a pathogen. After a specific incubation period, the vector is assessed for the proper developmental stage of the suspected pathogen (39). This technique is commonly used in the triatome/*Trypanosoma cruzi* system. Xenodiagnosis is useful when traditional diagnostic measures are not sensitive enough, for example when parasitemia is too low to be identified by traditional microscopy (272). Xenodiagnosis has been applied to multiple other disease systems, including *Borrelia burgdorferi*/*Ixodes scapularis* (273), *Leishmania* spp./*Lutzomyia* spp./*Phlebotomus* spp. (274-277), *Pediculosis humanus corporis*/*Rickettsia prowazekii* (278), and *W. bancrofti*/*Culex* spp (279). Xenodiagnosis is technically challenging, due to the necessity of keeping "clean" colonies of triatomes, ticks, mosquitoes, sandflies, or lice on hand. As well, it does not provide an immediate result as some of these pathogens can take weeks to develop within the vector (280). Molecular techniques such as PCR have proven to be more sensitive than xenodiagnosis (281). However, xenodiagnosis can be useful in determining the presence of a pathogen following drug treatment (282).

In that same vein, hematophagous arthropods have also been used in laboratory settings to perform blood draws for serial sampling. Traditional venipuncture can be difficult in some animals, and using mosquitoes to draw blood from these animals is a viable alternative (283). Mosquito blood meals can be used to measure virus titers in a variety of small animals, including birds and hamsters infected with arboviruses (284, 285). Triatomes take a substantially larger blood meal than mosquitoes, and their utility for drawing blood has been demonstrated in rabbits, seabirds, and the Iberian lynx (286-290). This technique has proven immensely helpful in the world of ornithology, specifically when measuring stress hormones in blood. Serial bleeding by venipuncture can induce the production of stress hormones (e.g. corticosterone), thereby confounding studies. Using triatomes in hollow "eggs" allows research to

collect large quantities of blood without stressing the animal (291). Taken together, this work provides precedence for the use of hematophagous arthropods to sample vertebrate blood non-invasively.

Xenomonitoring is a method to survey mosquitoes for the presence of a pathogen that they spread. This technique was pioneered in order to detect filarial worms in their mosquito vectors as a way to estimate transmission (292), and is proving to be especially useful to monitor lymphatic filariasis activity post MDA (293). Traditional xenomonitoring involved dissection of mosquitoes in search for immature larval stages of filarial worms, however this technique has benefited from advances in molecular biology (294, 295). The success of xenomonitoring demonstrates the feasibility of using field caught mosquitoes to determine the presence or absence of a pathogen in a specified area. However, this is a targeted approach and requires *a priori* knowledge about the pathogen in question.

Xenosurveillance is a technique that exploits the hematophagous behavior of some arthropods to survey vertebrates for pathogens circulating within a population (296). This technique has been applied to a variety of hematophagous arthropods, including tsetse flies, horse flies, and stable flies to identify pathogens within their blood meals (297). These flies are typically generalist feeders and take their blood meals from a range of vertebrates. In hopes of collecting human blood to survey for pathogens, we have turned to mosquitoes, the most efficient collectors of human blood on the planet. Combined with the rapid progression of NGS technologies, mosquitoes can be utilized to sample humans for genetic signatures of pathogens that may be circulating in their blood. Multiple groups have demonstrated that mosquito blood meals can be used to survey vertebrates for viral nucleic acid, including H5N1 (298), Papillomaviruses (299), and myxoma virus (300). Grubaugh et al. (2015) defined the methodology of and the term Xenosurveillance (296). This study demonstrated the feasibility of the technique to identify human viruses in a laboratory setting. For the initial proof of principle experiments, mosquitoes were fed on a water jacketed artificial membrane feeders containing serial dilutions of human immunodeficiency virus 1 (HIV, Family Retroviridae), WNV, pirital virus (PIRV, Family

Arenaviridae), and CHIKV and held for up to 24 hours post exposure. Using species specific qRT-PCR, the researchers could detect viral nucleic acid at levels well below clinical viremia and for up to 24 hours. Similar experiments were conducted in an animal model with Syrian golden hamsters to demonstrate this technique in an *in vivo* system. There was no significant difference between mosquitoes fed on an artificial membrane feeder or a live animal. Field studies were also conducted in Liberia, West Africa. The researchers' aspirated blood fed *An. gambiae* mosquitoes from the inside of homes and these were subjected to RNA sequencing with the goal of identifying human pathogen nucleic acid within the blood meal. Multiple sequencing reads aligned to Epstein-Barr virus (EBV, Family Herpesviridae) in all sequencing pools. This was confirmed using species-specific qRT-PCR. Together, Grubaugh et al demonstrated the feasibility of this novel technique to identify human viruses in mosquito blood meals in both a laboratory and field setting. However, it remains to be determined if xenosurveillance can be used to detect human pathogens other than viruses, specifically bacteria and parasites. In addition, xenosurveillance remains to be compared to more traditional human surveillance methodology like finger prick blood of venous blood draws.

Novel surveillance techniques

Emerging and re-emerging infectious diseases are a significant cause of morbidity and mortality throughout the world (301). These diseases disproportionately affect developing countries throughout the tropics, where both persistent infections (6) and intermittent outbreaks (302) are still the leading cause of disability adjusted life years in these areas (303). Many emerging and reemerging pathogens cause an acute febrile illness that presents with non-specific symptoms, complicating diagnosis (304). Furthermore, healthcare infrastructure is severely lacking in areas most commonly afflicted with these diseases, which vastly hinders healthcare professional's capacity to perform proper diagnostic tests (305). Inability to perform proper diagnostic procedures results in over-diagnosis of common illnesses (306-308), under-diagnosis of uncommon illnesses (309, 310), and can allow emerging and re-emerging

pathogens to go under the radar of healthcare workers and public health officials. This creates an environment that allows newly emerged pathogens to go undetected in an area for a period of time, often resulting in enhanced transmission. This point is highlighted by recent outbreaks of Ebola virus in West Africa (311), Zika virus in the Americas (312), and Middle Eastern Respiratory coronavirus in the Middle East (313). All of these viruses emerged into a new area and sustained transmission for months before the etiological agent was recognized. Outbreaks of these viruses emphasizes the need for vigilant surveillance of emerging and remerging pathogens.

Disease surveillance began as early as 1851 as outbreaks of cholera ravaged Europe (314). The Pan American Sanitary Bureau was created in 1902 to improve health in order to better facilitate trade across the Americas. This organization is now known as the Pan American Health Organization (315). The WHO was created in 1948 following World War II due to lack of coordination of international health groups (314). Since 1951, the International Health Regulations issued by the WHO are the premier standards for global health. These standards are multifaceted, with their main goal stated as "to prevent, protect against, control, and provide a public health response to the international spread of disease in ways that are commensurate with and restricted to public health risks, and which avoid unnecessary interference with international traffic and trade" (316). These goals cannot be accomplished without effective infectious disease surveillance. There has been a dizzying expansion of global surveillance programs since the turn of the century (317). Below, we determine how 1) traditional surveillance techniques can be improved at a local level, 2) a technique that combines field, molecular, and computational biology can create novel data-streams that inform surveillance programs, and 3) we can increase the amount of data collected from routine surveillance samples.

Chapter 2. Temporal and spatial variability of entomological risk indices for West Nile virus infection in Northern Colorado: 2006-2013

Introduction

West Nile virus (WNV, Family Flaviviridae) is a mosquito-borne virus that was first detected in North America during the summer of 1999 in New York City (69). The virus subsequently spread across the United States within 5 years (70) including to Colorado in 2002 (318) where it caused major epidemics in 2003 and 2004 (319). The virus has persisted in Colorado for more than a decade in an enzootic cycle mainly involving passerine birds (88) and peridomestic *Culex* mosquitoes, particularly *Culex tarsalis* Coquillett and *Culex pipiens* L. (62, 182). The northern Front Range of the Rocky Mountains has emerged as a high-risk area for WNV disease in humans. Larimer County alone reported >850 WNV disease cases from 2003–2013 (320). Therefore, the City of Fort Collins, Larimer County, developed an extensive WNV management program. The main priorities of this program are to provide a wide range of public education and outreach, execute routine larval control programs, extensively monitor mosquito populations, perform weekly testing for the presence of WNV in mosquito populations, and finally, to inform the need for emergency control campaigns on an as-needed basis when risk measures are elevated (321). This program was initiated in 2003 and is still in place, with annual surveillance activities spanning from June to late August or early September. Since the initiation of this program, emergency control campaigns have been executed in five trapping seasons, including three years in this study (2007, 2012, and 2013.)

Weekly data analysis included the calculation of three mosquito based risk indices: 1) *Culex* female abundance, 2) WNV infection rate, and 3) the vector index (VI). The VI is an indicator of the

abundance of WNV-infected female mosquitoes collected per trap night (322), and has proven to be a useful predictor for human risk of WNV infection (163, 164, 217, 227, 228). The City of Fort Collins traditionally based weekly risk on a city wide VI. However, based on noticeably disparate trap counts within the city and multiple studies conducted with *Cx. pipiens* and *Cx. tarsalis* (79, 323), we hypothesized that the city is spatially and temporally heterogeneous for *Culex* female abundance, WNV infection rates, and subsequently VI. These dynamics would indicate the entomological risk for WNV infection may be higher in specific portions of the city compared with others.

In this report, we present descriptive data collected from 2006– 2013 to discern yearly and seasonal trends for all three entomological risk measures. Furthermore, we divided the city into logistically relevant and equal-sized zones to ascertain whether there are specific portions of the city that are at a higher entomological risk for human WNV infection. Our goal was to retrospectively determine if the newly defined zones varied in respect to *Culex* female abundance, WNV infection rate, and VI. In parallel with entomological risk measures, we measured relative risk for each zone in the study using historical human case data. This allowed us to determine if calculating the risk indices for more precisely defined areas within the City of Fort Collins, and perhaps other cities, will better inform emergency control campaigns and result in an improved WNV management program.

Materials and methods

Study area

The study area consisted of the City of Fort Collins, CO. The city has a population of roughly 152,000 and covers 84 km2 (324). Fort Collins is located near the foothills of the Rocky Mountains in the high plains ecological zone (325). The climate is semiarid, with cold winters and hot and dry summers, low humidity, and variable precipitation (326). Fort Collins borders on extensively irrigated agricultural lands to the north, east, and south, whereas the western edge lies along the uncultivated foothills. For

analytical purposes, the city was divided into four zones using major thoroughfares: College Avenue was the East–West boundary, and Drake Road was the North–South boundary **(Figure 2.1)**.

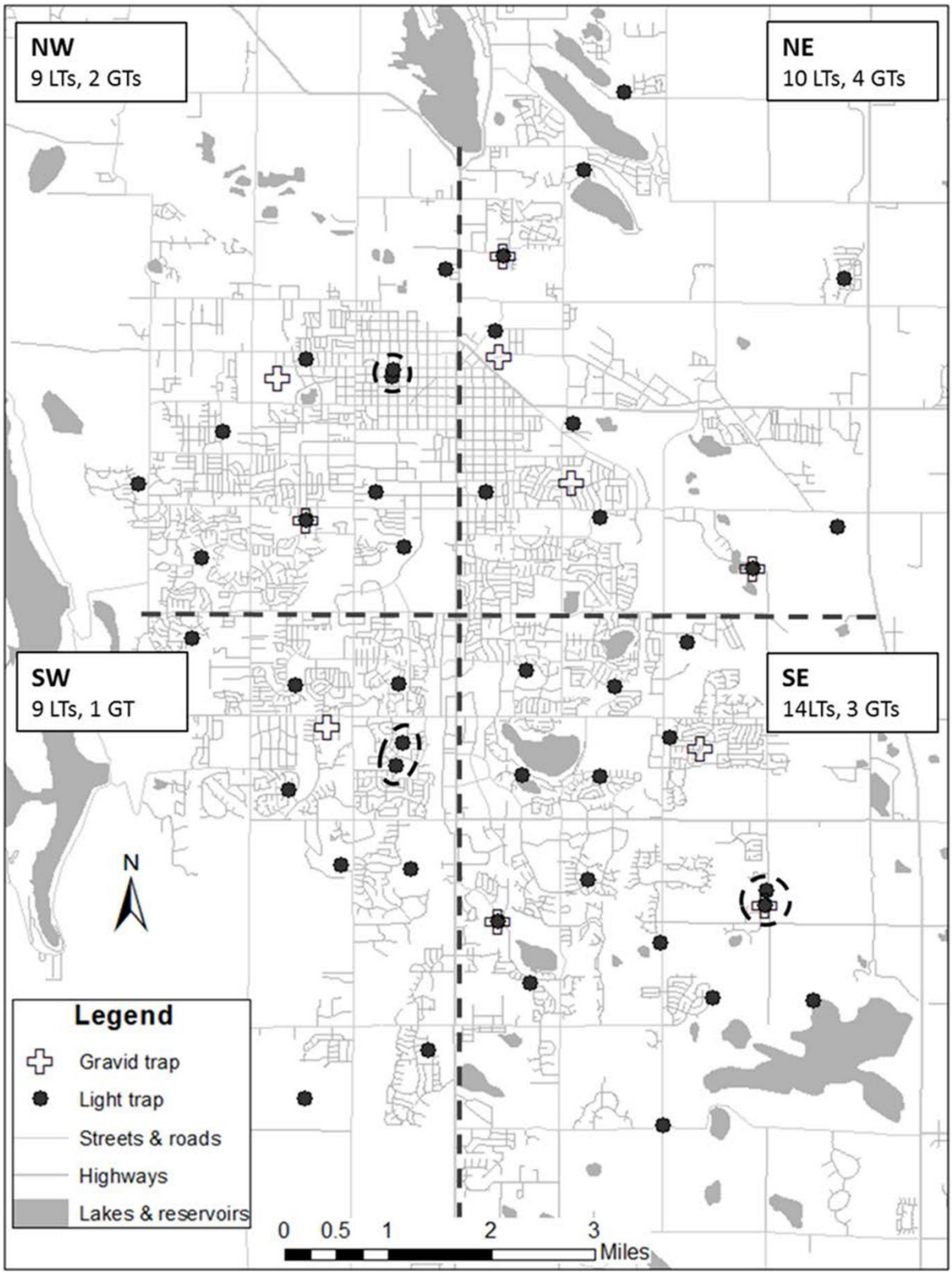


Figure 2.1 Map of the City of Fort Collins divided into four zones and showing trap placement for WNV surveillance. Black circles indicate permanent light traps. White crosses indicate gravid traps. Dashed circles

indicate relocations of traps. NW—northwest, NE—northeast, SE—southeast, SW—southwest zones.

Mosquito trapping and processing

Colorado Mosquito Control, Inc. conducted weekly trapping and identification of adult mosquitoes for the entirety of the study. Trap locations within Fort Collins were consistent over the 8-year study period. Trapping was performed weekly using 42 CO2 (dry ice)-baited Centers for Disease Control and Prevention (CDC) miniature light traps (BioQuip Products, CA) distributed in a grid-like pattern across the city, 1.3 km apart **(Figure 2.1)**. In addition to the light traps, up to 10 gravid traps were operated in any given week to attract oviparous female mosquitoes. Trapping began in Morbidity and Mortality Weekly Report epidemiological week 23 (early June) and was continued through week 35 (early September) in all years except 2011 when trapping was concluded in week 32 due to low values for entomological risk indices. Each trap was run one night per week from late afternoon until the following morning. Mosquitoes were collected and then sorted by site, date, species, and sex. Female *Culex* mosquitoes were pooled, typically in pools of no more than 50 specimens, and submitted for WNV screening. Pools were submitted as *Cx. tarsalis*, *Cx. pipiens*, or, for some specimens lacking certain body parts required for species identification, as *Culex* species (spp.). Based on the results from molecular identification assays showing that the vast majority of Culex spp. pool specimens were *Cx. pipiens* (CDC, unpublished data), *Culex* spp. mosquitoes were included as *Cx. pipiens* in calculations for mosquito abundance, infection rate, and VI.

Screening of mosquito pools for WNV

From 2006–2008, mosquito pools were processed by CDC Division of Vector-Borne Diseases personnel for the presence of WNV RNA as described previously (161, 209). From 2009–2013, mosquito pools were processed by the Colorado State University Arthropod-Borne and Infectious Diseases

Laboratory using the following methodology. Mosquito pools were homogenized in 1 ml of mosquito diluent (80% PBS, 20% FBS, supplemented with penicillin, streptomycin, gentamicin, and amphotericin B) with a single steel ball bearing using a Retsch Mixer Mill 400 (Retsch GmbH, Haan, Germany) at 24 Hz for 45 s. Homogenates were then centrifuged at 20,000 g for 5 min, and 50 ml of cleared supernatant was used for RNA extraction. RNA was extracted using either QIAamp Viral RNA Mini Kit (Qiagen, CA), or Mag-Bind Viral DNA/RNA kit (Omega, GA) with the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, MA) according to manufacturer's protocol. The extracted RNA was amplified via reverse transcriptase polymerase chain reaction (RT-PCR) using the following primers: forward 212 5' TTGTGTTGGCTCT CTTGGCGT 3', reverse 619c 5' CAGCCGACAGCACTGGACATT 3' (327). RT-PCR products were run on a 1% agarose gel stained with ethidium bromide in order to visualize the 408 base pair target sequence.

Entomological data analysis

Weekly *Culex* abundance and infection rate were calculated separately for females of 1) *Cx. tarsalis*, 2) *Cx. pipiens*, and 3) all *Culex*. Abundance was based solely on collections from light traps, whereas collections from both light traps and gravid traps were included in the WNV infection rate calculation, following the protocol established by (322). Infection rates were calculated per 1,000 females using the bias-corrected maximum likelihood estimate (MLE) in the Excel add-in, PooledInfRate (234). MLE was used in favor of the minimum infection rate (MIR) because it is more accurate at high infection rates and mosquito abundances (235). The VI is calculated by multiplying the abundance per trap night of a given mosquito species with the estimated proportion of infected females for that species (156);

$$VI = \sum \bar{N}_i \hat{P}_i$$

where *i* refers to the specific mosquito species, *N* is the abundance per trap night of the *i*th species, and *P* is the estimated infection rate per one female of the *i*th species. The VI is calculated separately for each vector species in a given area, in this case *Cx. tarsalis* and *Cx. pipiens*, and the subsequent addition of the VIs for all vector species provides an overall VI value. Abundance per trap night, which is the total *Culex* abundance divided by the number of traps operated in that zone, infection rates, and VI were calculated on a weekly basis for each year in the study for each zone.

Weather data analysis

Weather data from 2006–2013 were obtained from the Colorado Agricultural Meteorological Network. The average daily temperature and precipitation was recorded at the Fort Collins Agricultural Engineering Research Center on the CSU Foothills campus. The average weekly temperature for all years was calculated by taking the average of the daily temperatures for the week. Total precipitation was calculated by taking the sum of all precipitation for the week.

Human case data

Anonymized human case data from 2006–2013 were provided by the Larimer County Department of Health and Environment (LCDHE). Cases were assigned to zones by LCDHE using Google Fusion Tables (an open source geocoding service) based on the home address. Annual zone populations were estimated by determining the correspondence of 2000 and 2010 census blocks with a physical LCDHE map of Fort Collins (328, 329). The resulting census block lists were verified by matching them to U.S. Census Bureau TIGER/Line 2000 and 2010 census block shapefiles using ArcGIS (330, 331). The 2000 and 2010 census population data for each zone by census block were then obtained using American Fact Finder (332, 333). Annual zone populations were estimated using a compound annual growth rate calculated based on the change between the 2000 and 2010 census population falling within each zone by census block (334). These estimates were used with mapped case data to calculate annual and

cumulative zone incidence rates. Relative risks among zones were calculated using cumulative zone incidence rates. The NW zone is used as the reference group for the analysis, as it has the lowest cumulative incidence.

Statistical analysis

Because the entomological data did not follow assumptions for normality or equal variance, a Friedman's test was conducted followed by Dunn's test of multiple comparisons to determine differences between zones for Culex abundance per trap night, infection rate, and VI. The Spearman correlation was used to determine the relationship between the VI and human cases. Significance of relative risk for human cases was determined through calculation of 95% confidence intervals; a 95% confidence interval that does not include 1 (reference zone risk) is considered statistically significant at the 0.05 level. Statistics were calculated with Prism (GraphPad, CA).

Results

Seasonal trends for *Culex* abundance, infection rate, and VI

Over the 8-year study period a total of 602,420 female mosquitoes were trapped city wide, of which 131,777 were *Culex* species (21.9%). Of the *Culex*, 115,882 (87.9%) were identified as *Cx. tarsalis*, and 15,895 (12.1%) were identified either as *Cx. pipiens* or *Culex* spp. and assigned to *Cx. pipiens*. Using data from the whole city for all years, weekly 8-year averages for abundance, infection rate, and VI were calculated **(Figure 2.2)**. *Cx. tarsalis* weekly 8-year average abundance peaked in week 29, whereas *Cx. pipiens* abundance peaked in week 32 **(Figure 2.2A)**. There was no discernible difference between the two species for the seasonal peak in WNV infection rate **(Figure 2.2B)**. Infection rates did not exceed 1 per 1,000 females until week 29, and gradually increased through the end of the trapping season. Infection rates tended to peak at the end of the trapping season in week 35. The weekly 8-year average

VI rose sharply and peaked in week 29 for *Cx. tarsalis*, and rose gradually and peaked in week 32 for *Cx. pipiens* **(Figure 2.2C)**. The 8-yearr average VI was higher for *Cx. tarsalis*.

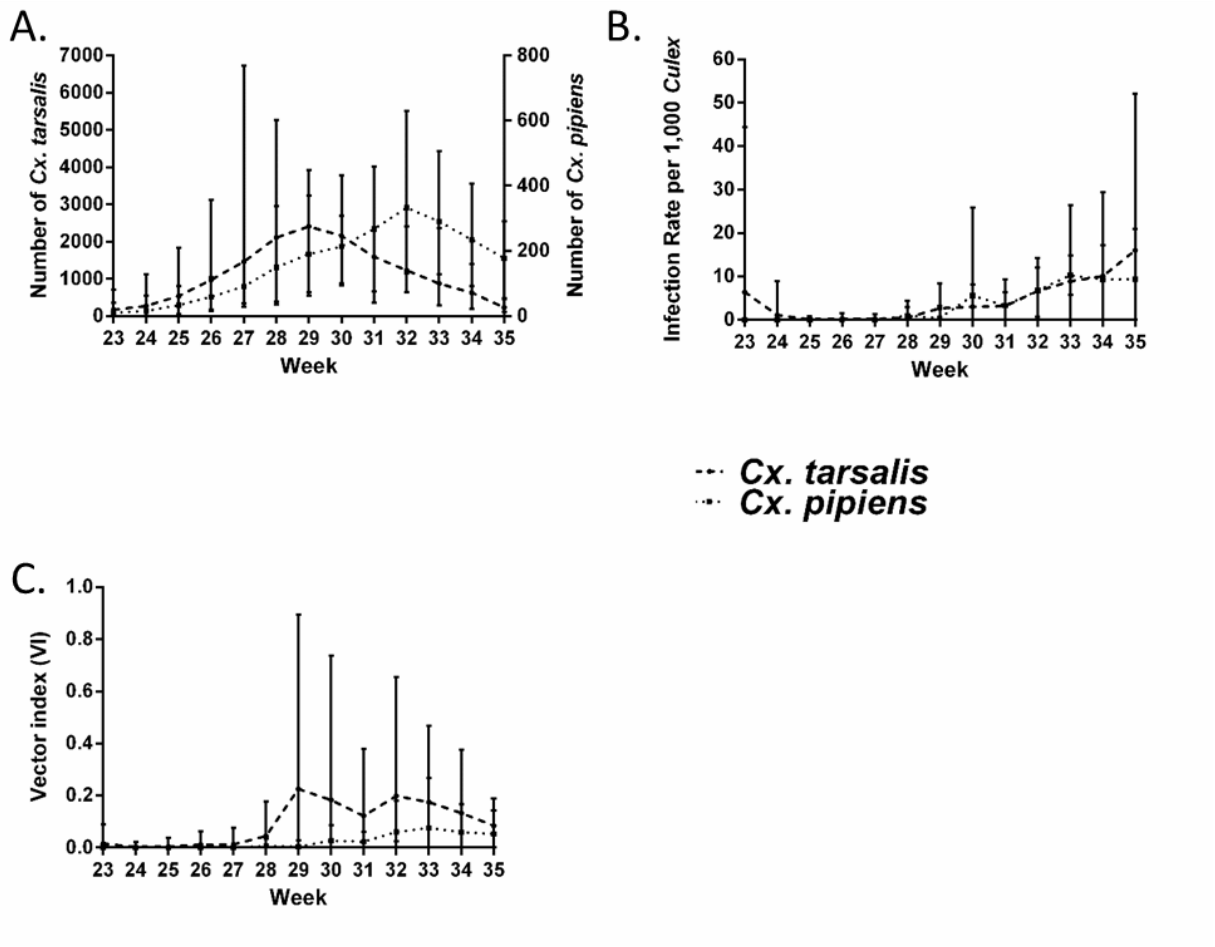
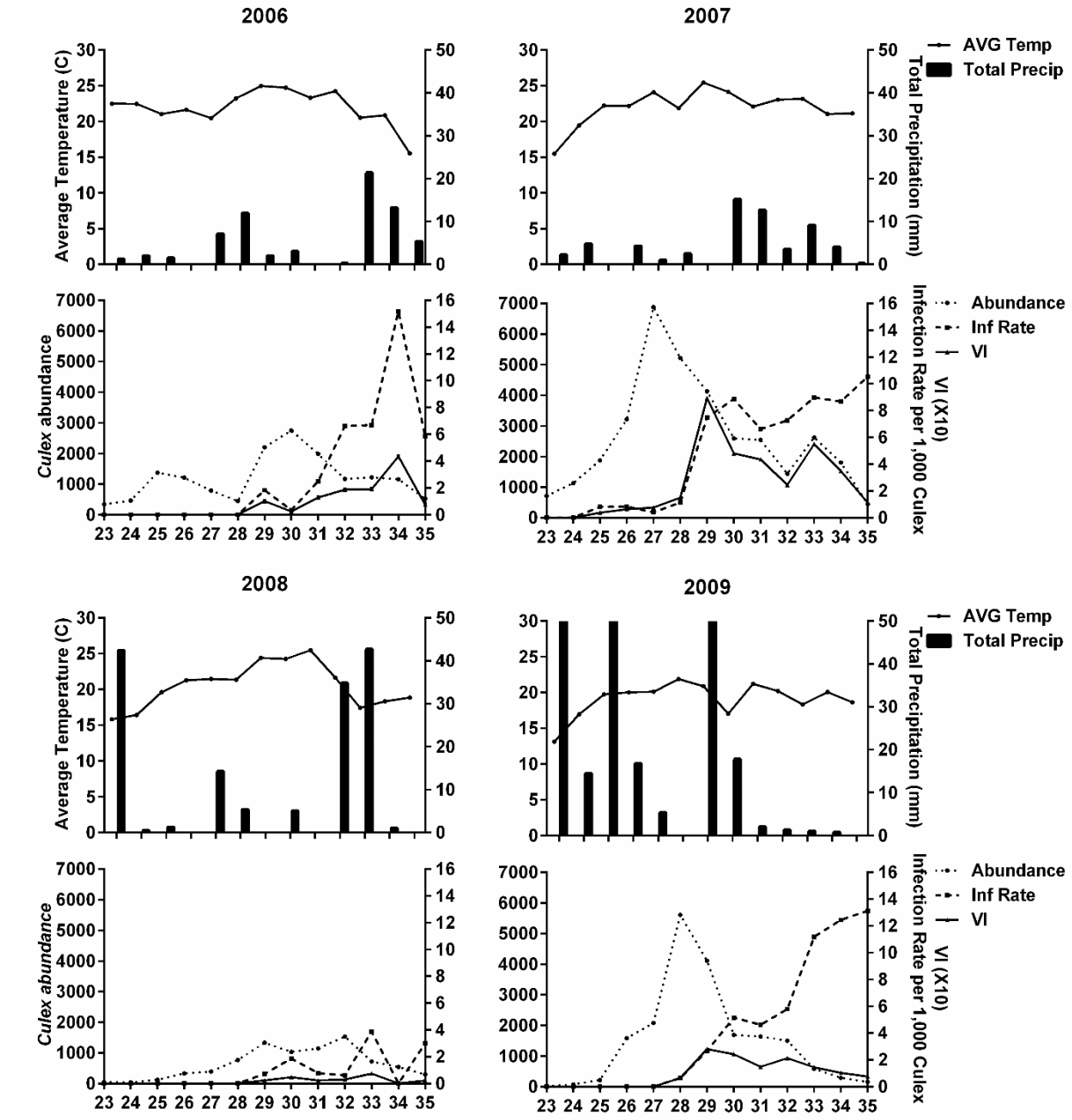


Figure 2.2 Seasonal trends for entomological risk indices, Fort Collins, CO, 2006–2013. Historical city wide data were averaged together for each week to discern seasonal trends for (A) *Culex* abundance, (B) WNV infection rate (per 1,000 mosquitoes), and (C) VI. Error bars represent the range for each week.

Yearly trends for *Culex* abundance, infection rate, and VI

The general trends for *Culex* abundance, infection rate, and VI remained constant year to year; however, the values varied substantially between years **(Figure 2.3)**. *Culex* abundance peaked prior to infection rate and VI in all years. The infection rate continued to rise throughout the trapping season, while the VI decreased with *Culex* abundance late in the season. 2007 had the highest *Culex* abundance

with a total of 34,608 females captured, which accounted for over 25% of the total abundance for the 8-year study. The majority of *Culex* captured in 2007 were *Cx. tarsalis* (32,314; 93.3%) as opposed to *Cx. pipiens* (2,294; 6.7%). 2012 had the highest average weekly infection rate with 10.3 per 1,000 females infected. 2007 and 2013 had the highest average weekly VIs of 0.40 and 0.43, respectively. Average weekly temperature fluctuated mildly within years, typically being lowest at the beginning of the season (May), rising throughout the season, and decreasing at the end (September). The temperatures between years varied more substantially. Weekly precipitation was sporadic and varied between years. In several years, there appeared to be a positive relationship between peaks in precipitation and subsequent peaks in vector abundance **(Figure 2.3— 2006, 2009, 2010)**, but in other years that pattern was missing or greatly reduced **(Figure 2.3—2007, 2008, 2012)**.


2006
2007
2008
2009
Average Temperature (C)
Total Precipitation (mm)
AVG Temp
Total Precip
Culex abundance
Infection Rate per 1,000 Culex
VI (X10)
Abundance
Inf Rate
VI
23 24 25 26 27 28 29 30 31 32 33 34 35

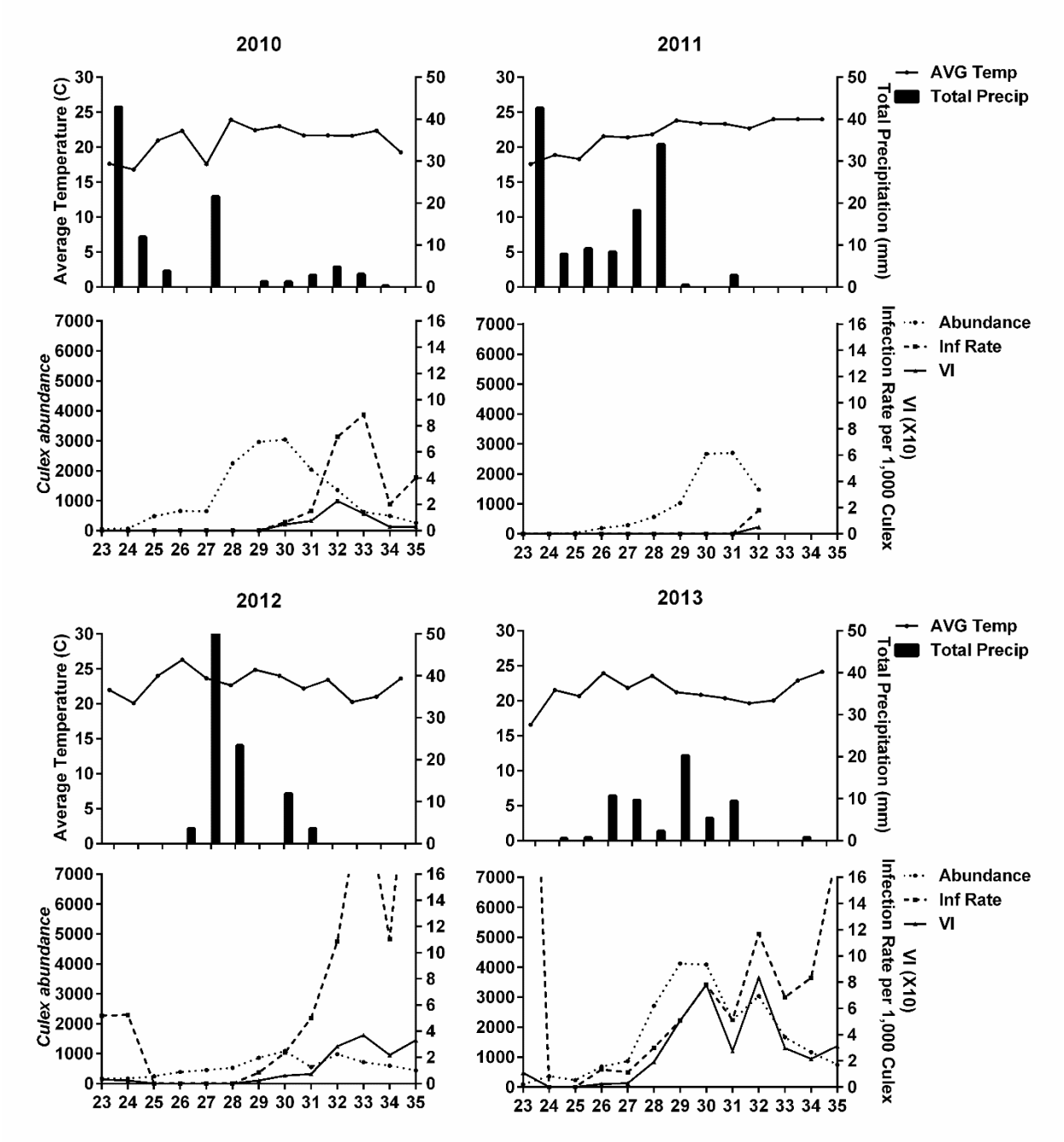


Figure 2.3 *Cx. pipiens* and *Cx. tarsalis* abundance, WNV infection rate, and VI, 2006–2013. These measures varied substantially between years. Each year in the study is represented by two graphs: The upper graph shows yearly weather data and the lower shows entomological risk indices. Yearly seasonal data show that *Culex* female abundance rises prior to WNV infection rate and VI each year, although the values for these entomological risk indices vary substantially from year to year. Average weekly temperature remains relatively consistent between years. Total weekly precipitation varies within and between years.

Zone comparisons

Fort Collins was divided into four zones in order to test the hypothesis that the city is heterogeneous for mosquito-based risk measures **(Figure 2.1)**. We found significant differences for each zone comparison for *Cx. tarsalis* and *Cx. pipiens* abundance per trap night (CO2-baited light traps), save the NE versus SE zones for *Cx. tarsalis* **(Table 2.1)**. The only significant difference in infection rate was between the NW and SE zones for *Cx. tarsalis*, with the SE zone having a higher infection rate. The SE zone had a significantly higher VI when compared with the NW and SW zones for *Cx. tarsalis* and all *Culex* VI. The differences in VI for each zone, week, and year are visually represented in **Figure 2.4**. In general, the eastern zones had higher *Culex* abundance and VI when compared with the western zones. Total human cases for each zone in each year are highly correlated with the sum of the zone VI in the same year **(Figure 2.5)**. Relative risk follows the same pattern as the VI. Inhabitants of the SE and NE zones have a significantly higher risk (P-value < 0.05) of contracting WNV as opposed to those living in the western zones **(Table 2.2)**.

Table 2.1 Statistical analysis of entomological risk indices between zones 2006-2013

	Abundance per trap night		WNV Infection rate		Vector index		
[a]Zone Comparison	*Cx. tarsalis*	*Cx. pipiens*	*Cx. tarsalis*	*Cx. pipiens*	*Cx. tarsalis*	*Cx. pipiens*	All *Culex*
NE vs. NW	**** (NE)	** (NE)	ns	ns	ns	ns	ns
NE vs. SE	ns	**** (NE)	ns	ns	ns	ns	ns
NE vs. SW	**** (NE)	****(NE)	ns	ns	ns	ns	ns
NW vs. SE	*** (SE)	** (NW)	* (SE)	ns	* (SE)	ns	* (SE)
NW vs. SW	*** (NW)	**** (NW)	ns	ns	ns	ns	ns
SE vs. SW	**** (SE)	*** (SE)	ns	ns	*** (SE)	ns	** (SE)
Friedman Stat	205.1	161.2	23.43	4.87	35.21	5.85	29.68
Treatments	4	4	4	4	4	4	4
Subjects	104	104	104	104	104	104	104
D.F.	3	3	3	3	3	3	3

Significance was determined with a Friedman test and Dunn's post test.
*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001; ns—not significant. The zone with the higher value is in parenthesis.
[a] NW, northwest; NE, northeast; SE, southeast; SW, southwest.

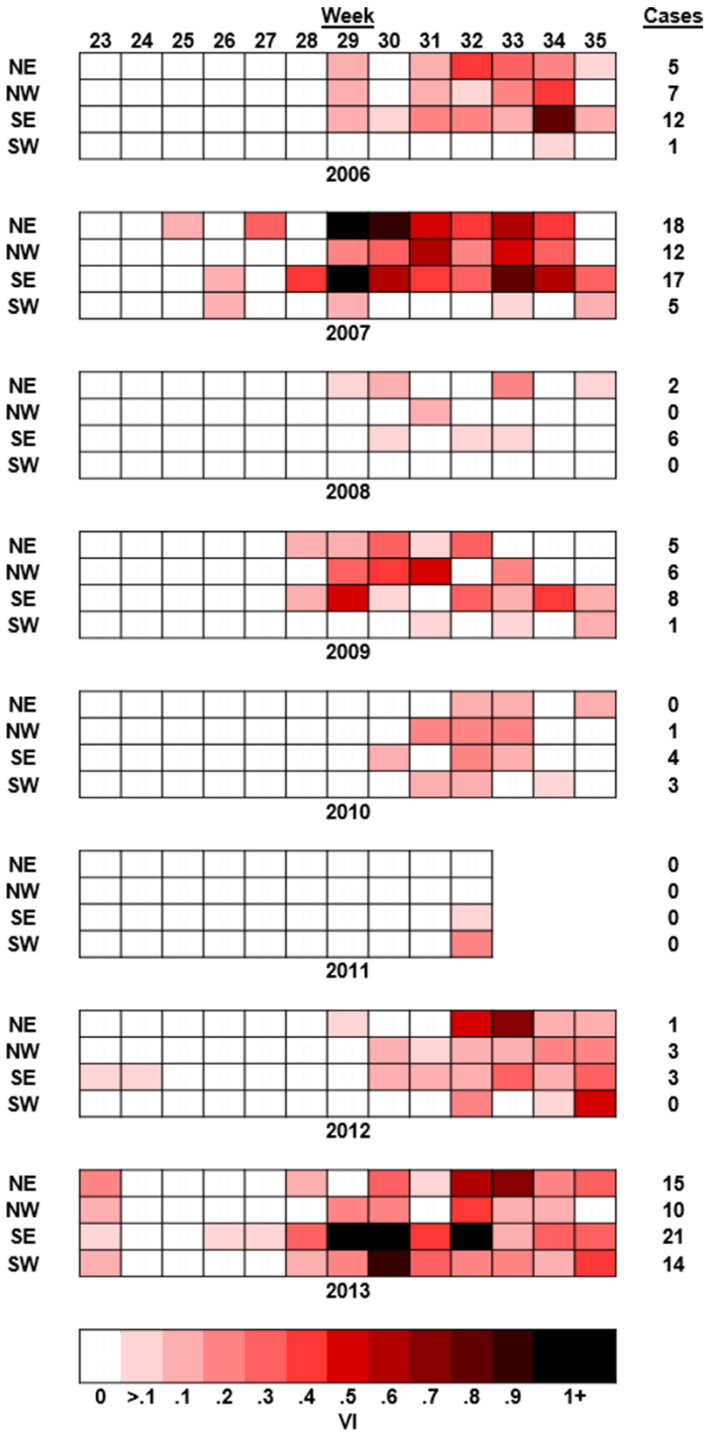


Figure 2.4 Heat map showing VI by week, zone, and year, 2006–2013, Fort Collins, CO. The VI is heterogeneous between zones. There is dramatic variation in VI within years and between years; however, the NE and SE zones consistently produce a higher VI, and therefore, reflect higher entomological risk for human exposure to WNV compared with the NW and SW zones. Total annual reported human cases are listed in the far right column.

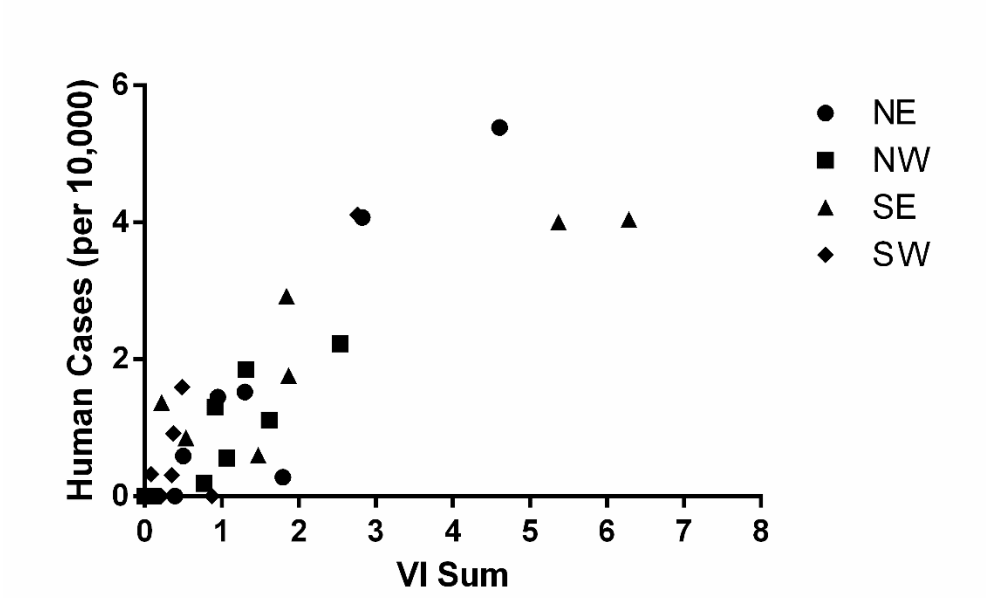


Figure 2.5 Incidence rates (cases per 10,000 population) and VI in Fort Collins, CO, 2006–2013. The VI is highly correlated with human WNV cases. The sum of the VI for each zone in each year is related to the total number of human WNV cases in the same year and zone. r = 0.8171, P-value < 0.0001.

Table 2.2 Relative risk compared between zones 2006-2013

Zone	Total Cases	Cumulative Incidence per 100,000	Relative Risk	95% CI	
NW	39	9.04	1.00		
NE	46	16.52	1.83*	1.19	2.80
SW	24	9.23	1.02	0.61	1.70
SE	71	19.22	2.13*	1.44	3.14

*Denotes statistical significance. 95% confidence intervals that do not span 1 (reference zone risk) are considered statistically significant.
NW zone= reference

Discussion

Entomological risk of WNV, summarized in the VI, can be affected by several factors. These include ecological variables such as temperature, precipitation, and land usage and cover (176, 179, 335,

336). In addition, mosquito feeding preferences (183, 337), availability of larval and adult habitats (323), and local avian species diversity (338) can also influence WNV transmission. These factors and others likely contributed to the seasonal and spatial differences in VI we observed during the study period.

Annual variation in these factors resulted in fluctuations in all mosquito-based risk measures **(Figure 2.3)**. However, our city wide data demonstrate consistent patterns for entomological risk **(Figure 2.2)**. *Culex* abundance gradually rose through the middle of the trapping season and fell at the end, as late season emerging females prepared to enter diapause and are no longer seeking blood meals. *Cx. tarsalis* tended to reach peak abundance prior to *Cx. pipiens*, which is consistent with previous local studies (79, 217) **(Figure 2.2A)**. The abundance values of these two important vector species varied substantially. However, this can partly be attributed to the sampling bias of CDC miniature light traps, which preferentially collects *Cx. tarsalis* (339, 340). The infection rate typically remained at zero until week 29 and then continued to rise steadily through the remainder of the season, even while abundance decreased **(Figure 2.2B)**. This is expected as the host-seeking (nondiapausing) population ages due to a lack of new emerging females entering the population. As the population ages, the likelihood of infection increases with multiple blood-feeding cycles and the overall increasing intensity of enzootic WNV transmission. Interestingly, the VI more closely followed the trend of *Culex* abundance than infection rate **(Figure 2.2C)**. While an infection rate above zero is necessary to have a VI above zero, the VI tended to be dictated more by *Culex* abundance than infection rate when the latter is above zero. Overall, we found that the seasonal trends remained relatively consistent despite dramatic year to year variability in values.

2007–2008 provided an example of this variation: in 2007, almost 35,000 *Culex* females were trapped in the 13 week trapping season as opposed to just 9,000 trapped the following summer in 2008 **(Figure 2.3)**. The WNV infection rate was highest in 2012, where it exceeded 20 per 1,000 females in two sampling weeks. We observed WNV infection early in the trapping seasons (week 23) in 2012 and 2013.

This may have been due to infections in newly emerged offspring of overwintering mosquitoes. WNV has been detected in pools of overwintering *Culex* mosquitoes (341, 342), and natural vertical transmission of WNV has been demonstrated on multiple occasions (343, 344). This mechanism could play an important role in maintaining WNV endemicity in northern Colorado, although Bolling et al. (2007) tested over 9,000 overwintering *Culex* females from the Front Range without detecting WNV (62). Although 2012 had the highest WNV infection rate throughout the season, 2007 and 2013, which had the highest abundances, also proved to have the highest VIs. A weak association exists within our data in regard to seasonal rainfall and mosquito population increases. As noted above, in multiple years, increased precipitation was followed by an increase in *Culex* abundance 1–3 weeks later. In other years, however, that pattern was not observed. Precipitation will increase the amount of available larval habitat for adult female *Culex* to oviposit. There is also a casual association between warmer weekly temperatures and increased *Culex* abundance. These associations do not appear to be reflected in infection rate or VI. Neither precipitation nor temperature appears to be predictive of entomological risk alone. This demonstrates the dynamic nature of WNV transmission within our study area. The impact of weather on seasonal WNV patterns is complex and requires further study.

Four zones, of approximately equal size, within the City of Fort Collins were established and retrospectively analyzed to determine if the city was homogenous for *Culex* abundance, WNV infection rate, and VI **(Figure 2.1)**. We found significant differences between all zones, save the NE versus SE, for both *Cx. tarsalis* and *Cx. pipiens* abundance per trap night, leading us to conclude that the city was heterogeneous for *Culex* abundance **(Table 2.1)**. We found fewer significant differences in infection rate between zones, with the only significant difference occurring between the NW versus SE zones for *Cx. tarsalis*. This result indicates that the city was more homogenous for WNV infection rate than for *Culex* abundance. Evaluating zone comparisons for the VI, we took into account both species of *Culex* and a combined VI. We found significant differences between the SE zone and both western zones for *Cx.*

tarsalis VI and all *Culex* VI. Our data provided no evidence of mosquitoes becoming infected at a higher rate in particular portions of the city. Rather, there was a larger abundance of mosquitoes in the eastern portion of the city as opposed to the western portion, resulting in a higher VI and higher risk for WNV infection in the east. These data are consistent with other studies done in the area. Eisen et al. (2010) found a positive association between proximity to irrigated agriculture and elevated WNV incidence (179). Schurich et al. (2014) demonstrated a negative correlation between elevation and *Cx. tarsalis* abundance within Fort Collins (323). They also showed proximity to irrigated agriculture is highly associated with increased *Cx. tarsalis* abundance. As the elevation is higher on the western side of the city and irrigated agriculture is the predominant landscape on the eastern edge, these data help to provide an explanation for the greater *Culex* abundance and VI seen in the eastern portion of the city in this study.

Our data clearly establish that Fort Collins is heterogeneous for VI **(Figure 2.4)**. The disparity in VI within the city is reflected in the relative risk for human WNV infection **(Table 2.2)**. These data suggest that the entomological risk and relative risk for human WNV infection is correlated, and is higher on the eastern portion of the city than the west **(Figure 2.5)**. This observation has implications for control policy and public outreach. Currently, the City of Fort Collins's control policy is dictated by a rise in the city wide VI over the established threshold of 0.75, although a more thorough evaluation of specific VI values in relation to human risk of WNV exposure is necessary and currently being addressed. The assumption that the city is spatially homogenous for mosquito-based risk measures and relative risk is not supported by our data. Moreover, this assumption and the use of a city wide VI prevent early recognition of increased risk in portions of the city where the inhabitants are at greatest risk for contracting WNV. With growing public concern against emergency adulticiding and pesticide use in general, a more targeted spray policy (based on zones rather than the entire city) likely would result in less overall pesticide usage by only spraying areas at elevated risk levels and avoiding treatment of areas

that present little or no risk. This also allows for more targeted public health interventions by letting citizens know which areas of the city are at the highest risk. Overall, our data demonstrate that seasonal trends can be discerned and that dividing the city into zones may better inform spray policy and help mitigate human risk for WNV infection in Fort Collins.

Chapter 3: The use of xenosurveillance to detect human bacteria, parasites, and viruses in mosquito blood meals

Introduction

Over 400 million acute febrile episodes occur in African children every year, with only a small percentage of them receiving a definitive diagnosis due to proximity of healthcare infrastructure, limitations in diagnostic capabilities, and the presumption of a malaria infection (345). However, a substantial portion of febrile episodes in sub-Saharan Africa are likely caused by pathogens other than *Plasmodium* (144). Emerging and re-emerging infectious diseases are caused by a variety of pathogenic organisms, are increasing in frequency, and often occur in areas with limited disease surveillance (346). Accordingly, novel surveillance strategies able to detect a wide array of etiological agents could have a considerable public health impact.

The term xenosurveillance refers to a technique that makes use of the hematophagous behavior of some arthropods to survey vertebrates for the presence of infectious disease agents (296). Previously, we and others have used xenosurveillance to identify genetic signatures (i.e. genomes) of viruses from *Anopheles gambiae* mosquito blood meals in laboratory- and field-based studies (296, 347). Understanding the natural history of the hematophagous arthropods, including pathogens transmitted, that are used for xenosurveillance is imperative. *An. gambiae* mosquitoes, the main malaria vector in sub-Saharan Africa (14, 348), are highly anthropophilic (349), endophilic (350), and endophagic (351) This behavior makes them important malaria vectors, but also highly efficient, non-invasive samplers of human blood that are relatively simple to collect.

The effectiveness of mosquitoes for sampling vertebrate viruses, including some non-vector borne agents, is now well documented. Vertebrate virus nucleic acids from influenza H5N1 (298), Papillomaviruses (299), and myxoma virus (300) have been detected in field-derived mosquito blood meals. Arboviruses also have been detected in the blood meal of laboratory-reared mosquitoes fed by an artificial feeder (347) and fed by various viremic animals (285). Less attention has been paid to the detection of vertebrate derived bacteria or parasites. Fernandez de Marco et al. 2016 successfully identified a cow infecting parasite, *Theileria orientalis*, in the blood meal of field-caught *Culiseta annulata* mosquitoes (352). However, the ability of this method to detect human parasites and bacteria remains unknown.

Therefore, we sought to determine whether xenosurveillance may be used to detect genetic signatures of bacteria and parasites, in addition to medically relevant viruses. Specifically, *An. gambiae* mosquitoes were fed blood meals containing *Trypanosoma brucei gambiense*, *Bacillus anthracis*, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), or Zika virus (ZIKAV). To assess the sensitivity of xenosurveillance, blood meals containing serial 10-fold dilutions of each pathogen were fed to mosquitoes. Mosquitoes were also fed and held for up to 24 hours to determine the sensitivity of xenosurveillance over time. Specific qRT-PCR assays were employed to detect RNA from each pathogen. Using this technique, we could detect RNA at or below typical clinical concentrations for up to 24 hours. These results indicate that xenosurveillance is a sensitive and effective means of detecting pathogens in blood samples collected by mosquitoes at clinically and operationally relevant concentrations and timescales.

Materials and methods

Mosquitoes and microbes

An. gambiae sensu stricto mosquitoes were used in all experiments. Mosquitoes were derived from the laboratory G3 strain (origin The Gambia) or from a recently colonized field strain from Burkina Faso (353). Larvae were reared at 28-31° C and fed fish food daily. Adults were held in 80% relative humidity on a 14:10 light:dark photoperiod and were provided with water and a 10% sucrose solution *ad libitum*. Adult mosquitoes used for experiments were 3-7 days' post emergence. *Bacillus anthracis* Sterne 34F2 strain bacteria and *Trypanosoma brucei gambiense* STIB 386 strain was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. Bacteria were propagated a day prior to feeding in tryptic soy broth at 37° C. Parasites were maintained in a T-75 tissue culture flask with HMI-9 culture media (354). Trypanosomes were passaged weekly by removing media, centrifuging at 350xg for 5 minutes, and re-suspending parasites in fresh culture media in a new flask. Stocks of MERS-CoV (obtained from Dr. Tony Schountz) and ZIKAV (strain PRVABC59) were grown on Vero cells as previously described (355).

Serial dilution blood feed

To determine limits of detection for each pathogen, various dilutions of a blood/pathogen mixture were used. 500µL of live cultures (*B. anthracis/T.b. gambiense*) or stocks of virus (MERS-CoV/ZIKAV) were diluted with 500µL of defibrinated sheep blood and successive serial dilutions were made to a final concentration of one volume pathogen to 10,000 volumes blood. Cartons of mosquitoes were exposed to the blood/pathogen mixture using a water jacketed membrane feeding apparatus and held for 12 hours post bloodfeed **(Figure 3.1)**. Mosquito blood meals were stored on CloneSaver FTA cards (GE Healthcare) and processed as previously described with slight modification (296). Briefly, blood fed mosquitoes were anesthetized with triethylamine and blood meals were removed from the

mosquito abdomen with forceps, placing the anterior end of the abdomen to the FTA card, and squeezing out the blood bolus. Forceps were used to push the blood bolus onto the FTA card and up to 20µL of RNA Later (ThermoFisher) was added to the mosquito dried blood spots (M-DBS) to enhance diffusion into the card and stabilize nucleic acid. Forceps were cleaned with 70% ethanol between processing of each sample. Each FTA card was left to dry overnight at room temperature and moved to storage in a -80° C freezer for up to 2 weeks.

The starting concentration of each pathogen was determined by qRT-PCR analysis of RNA extracted from 50µL of undiluted stock agent. In addition, RNA from 2µL of each blood/pathogen mixture was pipetted directly onto FTA cards (referred to as dried blood spots (DBS)) and tested by qRT-PCR as above. The volume of 2µL was chosen because it is the approximate volume of a mosquito blood meal. DBS samples served as a positive control, as well as a measure for how much pathogen RNA was lost during the process of bloodfeeding.

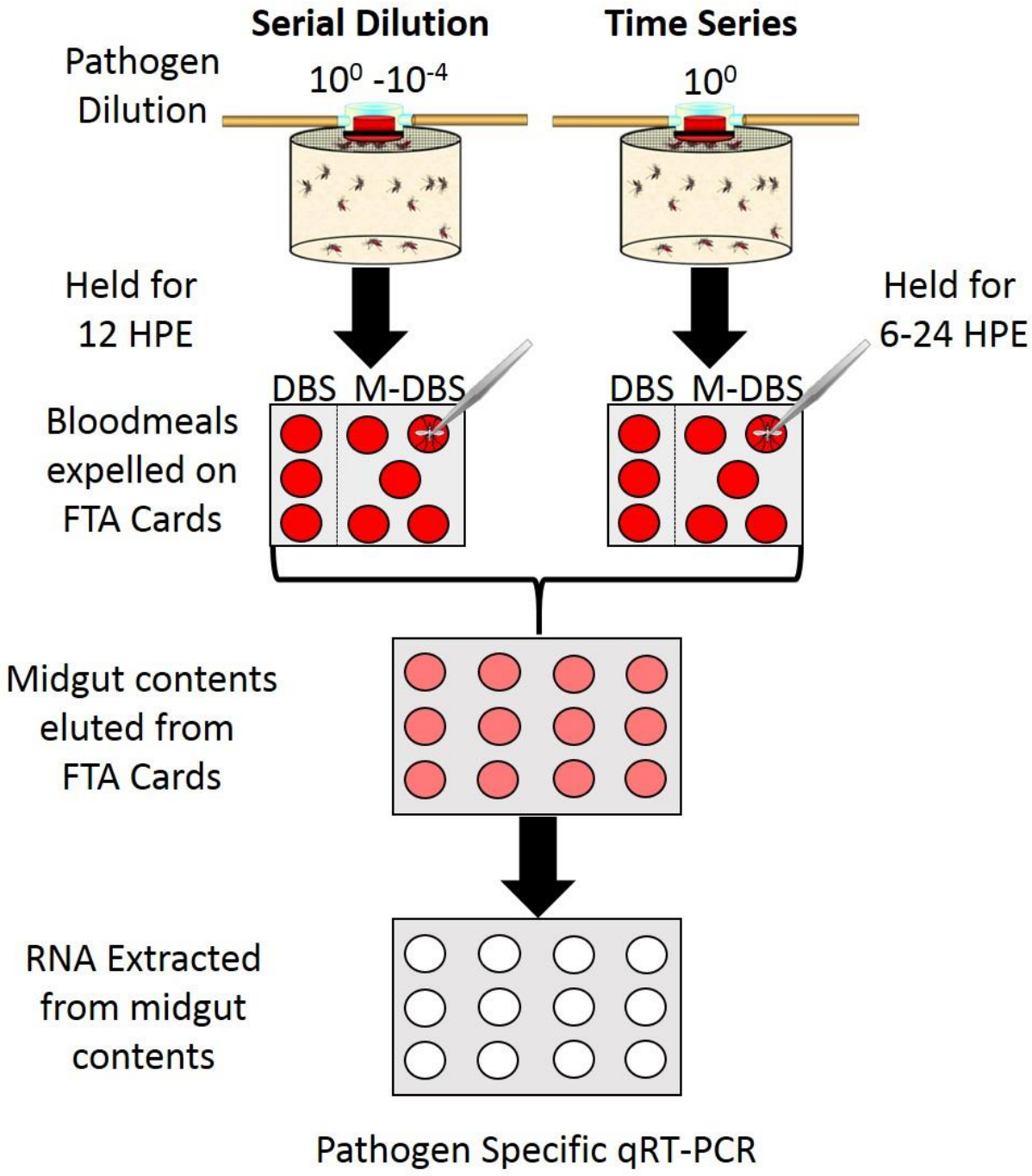


Figure 3.1 Methodology of xenosurveillance in a laboratory setting. Mosquitoes were allowed to fed on pathogenemic blood meals. Engorged mosquitoes were held for a specified period of time (hours post exposure (HPE)) and blood meals were expelled onto FTA cards (M-DBS). Dried blood spots (DBS) consisted of 2µL of the pathogenemic blood meals. FTA cards were allowed to dry at room temperature overnight, and were then transferred to a -80-degree freezer for storage. M-DBS and DBS were eluted off of FTA cards for RNA extraction. Extracted RNA was used for pathogen specific qRT-PCR. (Figure adapted from Grubaugh et al 2015 (296))

Time series blood feed

After pathogen exposure, mosquitoes were sampled at 6-hour time points up to 24 hours in order to determine how long pathogen RNA could be detected. Cartons of mosquitoes were exposed to 500µL of culture/stock mixed with 500µL of defibrinated sheep blood and held until sampling **(Figure 2.1).**

Sample processing

A single punch was removed from each DBS and M-DBS card using a Harris 3 mm micro-puncher (GE Healthcare) and placed into an 8-strip PCR tube containing 70µL of RNA Rapid Extraction Solution (ThermoFisher) supplemented with 1% 0.5 mm EDTA. A total of 5 M-DBS per pathogen were used for each dilution experiment and time point. PCR tubes were placed in a Talboys Standard Microplate Vortex Mixer (Southern Labware) at 800 rpm for up to 16 hours at 4° C to elute nucleic acids. 50µL of elution was used for DNA/RNA extraction with the Mag-Bind Viral DNA/RNA kit (Omega Bio-Tek) according to manufacturer protocol.

qRT-PCR analysis

Due to specific knowledge about pathogens spiked into mosquito blood meals, we opted to develop pathogen specific qRT-PCR approaches opposed to a more unbiased approach (e.g. Next Generation Sequencing). As well, qRT-PCR was chosen over qPCR in order to detect all pathogens assessed using the same methodology. To prepare PCR standards, extracted RNA from each pathogen culture/stock was reverse transcribed with a forward primer containing a T7 transcription site on the 5' end. Subsequent DNA was transcribed using the MEGAscript T7 Transcription kit (ThermoFisher) **(Appendix 1, Supplemental Figure 3.1)**. Transcripts were diluted to $1x10^8$ transcripts per reaction, and further log-serially diluted to $1x10^2$ transcripts per reaction. Specific primer and FAM probe sets were utilized for each individual pathogen **(Table 3.1)**. Primers and probes were designed with the Primer3 software using Geneious version 9.0.3. For *T. b. gambiense* and *B. anthracis*, primers were designed to

highly and constitutively expressed genes, Alpha tubulin (TriTrypDB# Tb927.1.2340) and the RNA polymerase beta subunit (GenBank# AF205325.1) genes, respectively (356, 357). Primer and probe sequences were blasted against the NCBI NT database to confirm specificity. For both MERS-CoV and ZIKAV, previously established primer/probe sets designed by the Centers for Disease Control and Prevention for clinical purposes were used (358, 359). Run parameters for each set of diluted standards is shown in **(Appendix 1, Supplemental Figure 3.1)**. The Reed-Muench method was employed to calculate 50% end points for each pathogen (360). 50% end points were calculated with titers of pathogen in each serial dilution blood meal, opposed to titers of pathogen recovered from M-DBS. This value reflects the amount of pathogen required in a blood meal (i.e. bacteremia, parasitemia, and viremia, referred to as "pathogenemia") to be detectable by xenosurveillance 50% of the time.

Table 3.1 Primer and probe sequences for RT-qPCR analysis

Primer	5'-3' Sequence	Product Size
T.b.g. Alpha tubulin F	AAGTCCAAGCTCGGCTACAC	182
T.b.g. Alpha tubulin R	TACGTGGGGCGCTCAATATC	
T.b.g. Alpha tubulin P	ACCGCAGGTGTCGACGGCTGTCGTGG	
B. anthracis RNA Pol Beta Subunit F	CCACCAACAGTAGAAAATGCC	175
B. anthracis RNA Pol Beta Subunit R	AAATTTCACCAGTTTCTGGATCT	
B. anthraics RNA Pol Beta Subunit P	ACTTGTGTCTCGTTTCTTCGATCCAAAGCG	
MERS-CoV Nucleocapsid F	GGCACTGAGGACCCACGTT	75(358)
MERS-CoV Nucleocapsid R	TTGCGACATACCCATAAAAGCA	
MERS-CoV Nucleocapsid P	CCCAAATTGCTGAGCTTGCTCCTACA	
Zika 3' NS1 F	CCGCTGCCCAACACAAG	77(359)
Zika 3' NS1 R	CCACTAACGTTCTTTTGCAGACAT	
Zika 3' NS1 P	AGCCTACCTTGACAAGCAGTCAGACACTCAA	

Results

Pathogens are detected in mosquito blood meals at clinically relevant levels

Xenosurveillance with *An. gambiae* mosquitoes fed blood containing serial tenfold dilutions of *B. anthracis*, *T. b. gambiense*, MERS-CoV, and ZIKAV were used to determine the limits of parasite, bacteria, and virus detection from mosquito blood meals. In all cases, genetic signatures of pathogens were detectable by xenosurveillance when mosquitoes were fed a blood meal containing pathogens at or below clinically reported levels **(Figure 3.2)** (361-364). Transcripts from *B. anthracis* were detected in all mosquitoes at all dilutions (input range: $2.21x10^5$-$2.21x10^1$), resulting in 50% end-point titer of $6.98x10^1$ transcripts/2µL **(Figure 3.2A)**. Transcripts from *T. b. gambiense* were detected in the first 3 ten-fold serial dilutions (input range: $3.86x10^6$-$3.86x10^4$), resulting in a 50% end-point titer of $1.16x10^5$ transcripts/2µL **(Figure 3.2B)**. ZIKAV RNA was detected in all mosquitoes tested in the first 3 ten-fold serial dilutions, and in 4/5 mosquitoes in the 4th (input range: $2.50x10^7$-$2.50X10^4$), resulting in a 50% end-point titer of $5.94x10^4$ GE/2µL **(Figure 3.2C)**. RNA from MERS-CoV was detected in all mosquitoes in the first 4 dilutions (input range:$1.47x10^7$- $1.47x10^4$), resulting in a 50% end-point titer of $4.65x10^4$ GE/2µL **(Figure 3.2D)**.

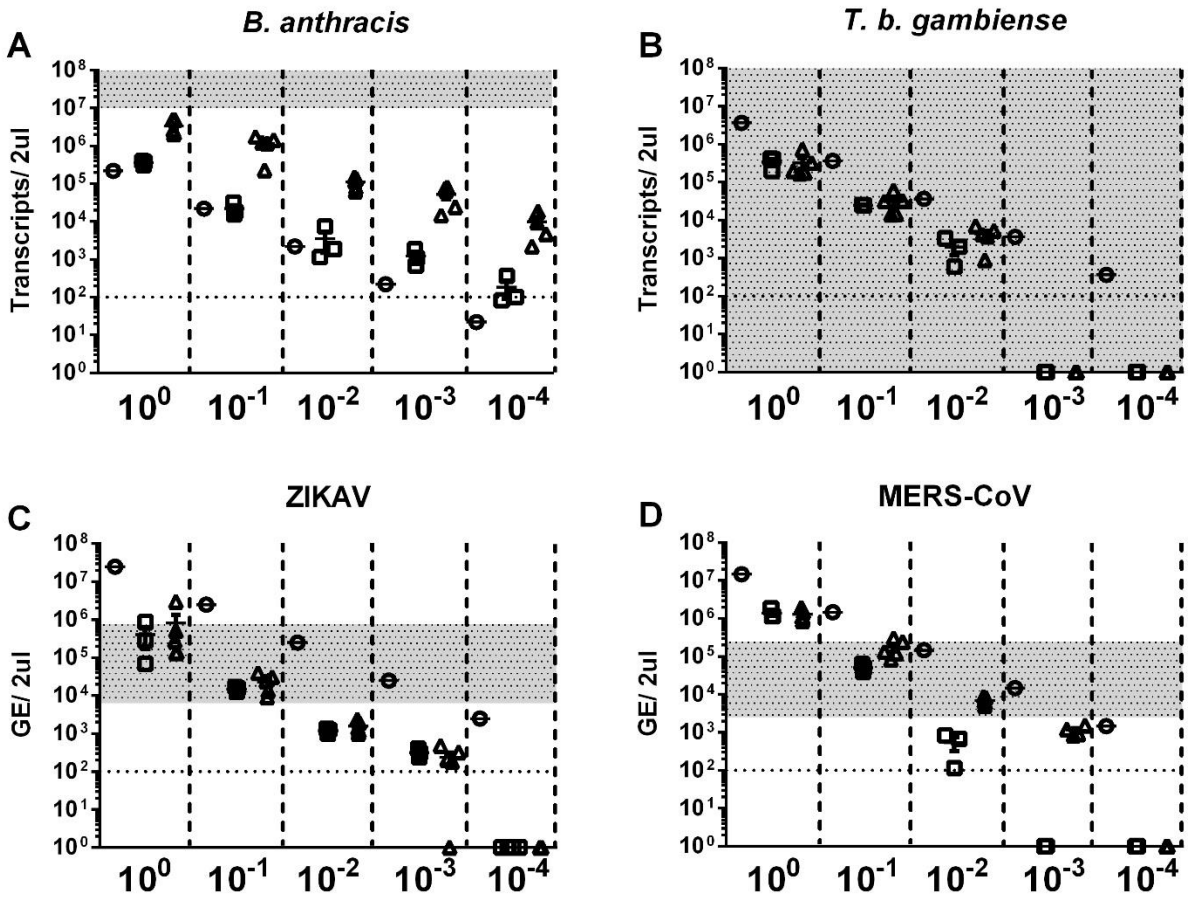


Figure 3.2 Detection of genes and genomes from human bacteria, parasites, and viruses at low levels in mosquito blood meals. Input N=1 (circle), DBS N=3 (squares), M-DBS (triangles) N=5 for each pathogen. Vertical dashed lines demarcate each pathogen:blood dilution. Horizontal dashed lines indicate the lower limit of the qRT-PCR assay. Shaded areas show reported clinical ranges of parasitemia, bacteriemia, and viremia. Data points on the y-axis at 10^0 indicate samples that were tested by qRT-PCR but were negative. Error bars represent the standard error of the mean.

Pathogens can be detected in mosquito blood meals up to 24 hours post feeding

Mosquitoes were fed a 1:1 mixture of pathogen/blood and held for up to 24 hours post blood meal. Mosquitoes were sampled at 6,12,18 and 24 hours post bloodfeed. RNA from all of the pathogens examined was stable in the mosquito and detectable by xenosurveillance for up to 24 hours post blood meal **(Figure 3.3)**. The amount of RNA detected remained similar or unchanged for both *B. anthracis* and MERS-CoV for each time point sampled **(Figure 3.3A,D)**. RNA from *T. b. gambiense* and ZIKAV dropped

compared to the input at each time point, but remains at detectable levels for up to 24 hours post bloodfeed **(Figure 3.3B,C)**.

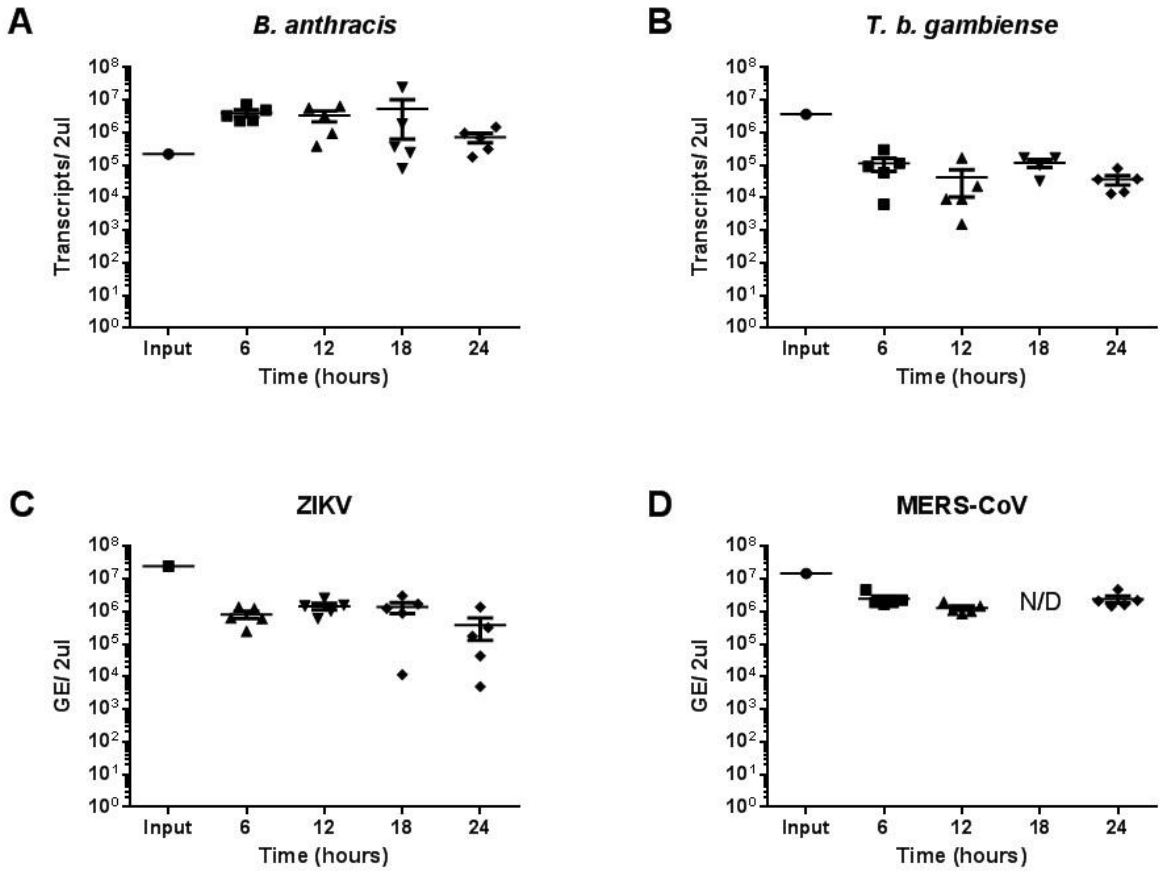


Figure 3.3 Genes and genomes can be detected from 4 major human pathogens in mosquito blood meals up to 24 hours post bloodfeed. N=5 for each pathogen. Horizontal bars represent the mean of each timepoint. Error bars represent the standard error of the mean.

Discussion

Xenosurveillance has proven to be effective at detecting viral genomes in both laboratory and field conditions (296), however, it remains to be determined how useful xenosurveillance is at detecting genetic signatures of human infecting bacteria and parasites. To address this, we performed dilution and

time-course experiments using *B. anthracis, T.b.gambiense,* MERS-CoV, and ZIKAV representing 3 different taxa of disease causing agents.

For xenosurveillance to be a useful tool, mosquitoes must feed on a pathogenemic host and transcripts or genomes from these pathogens must be detectable in mosquito blood meals. Human pathogenemia can vary widely between pathogen taxa, as well as between similar species of pathogens. We performed a serial dilution bloodfeeding experiment with four microbes representing three broad taxa of infectious agents to determine the efficacy of xenosurveillance at various levels of pathogenemia **(Figure 3.2)**. Every pathogen assessed was detectable by xenosurveillance when mosquitoes were fed blood meals at or below reported clinical values. *B. anthracis* was detected by xenosurveillance in all dilution experiments, subsequently resulting in the lowest 50% end-point of $6.98x10^1$ **(Figure 3.2A)**. More transcripts were detected in M-DBSs compared to both the input and DBS controls at each dilution. This phenomenon may be the result of *B. anthracis* actively replicating while in the mosquito midgut/blood meal. *Anopheles*, and mosquitoes in general, have diverse microbiomes containing multiple species of commensal bacteria (365). Non-exposed M-DBSs used as a control were negative by qRT-PCR, ruling out the possibility of cross-reactivity with commensal bacteria. Transcripts from *B. anthracis* could be detected by xenosurveillance below the clinical bacteremia reported for this species (361). Xenosurveillance to detect MERS-CoV and ZIKAV resulted in the next lowest 50% end-points, $4.65x10^4$ and $5.94x10^4$, respectively **(Figure 3.2C,D)**. These end-points fall within the clinically reported viremia for both viruses (362, 363). *T.b. gambiense* detection by xenosurveillance resulted in the highest 50% endpoint of $1.16x10^5$ **(Figure 3.2B)**, which falls within the reported clinical parasitemia. However, the amount of *T. b. gambiense* parasites in the blood of an infected individual at any one time can highly vary, from more than $1x10^8$ to virtually none (364). Relapsing parasitemia, the result of antigenic variation, is common in *T. b. gambiense* (366), as well as other blood-borne protozoan parasites.

Nevertheless, these data demonstrate that xenosurveillance can reliably detect genetic signatures from 3 separate taxa of pathogens at or below clinically reported pathogenemia.

The amount of time *An. gambiae* mosquitoes require to process blood meals, as well as the amount of time they rest indoors post bloodfeeding, are critical factors to the success of xenosurveillance. The rate of blood meal digestion varies between genus and species of mosquito (367, 368), as well as within species due to environmental conditions (369). A study conducted in The Gambia showed *An. gambiae* mosquitoes can have a gonotrophic cycle as often as every 2 days (370). Multiple studies show the success of blood source identification using DNA extracted from the blood meal of *Anopheles* mosquitoes significantly decreased after 30 hours (371, 372). These data, along with previous field experience (296), demonstrate the amount of time to capture a blood fed *An. gambiae* mosquito indoors that can be used for xenosurveillance is about one day. We experimentally demonstrated that RNA can be reliably detected up to 24 hours post blood meal for all 4 species **(Figure 3.3)**. Similar to the dilution experiments, *B. anthracis* transcripts were more abundant in M-DBSs than the input blood meal **(Figure 3.3A)**. For the remaining pathogens, *T. b. gambiense,* ZIKAV, and MERS-CoV, transcripts/GEs were less in M-DBSs compared to input, however transcripts/GE were detected in all mosquitoes at each time point **(Figure 3.3B,C,D)**. It is important to note that these pathogens are not vectored by mosquitoes, therefore RNA detected in M-DBS arose from the blood meal, although it does appear there is slight replication of *B. anthracis* in the mosquito midgut.

An. gambiae mosquitoes were used in this experiment to best replicate field conditions in sub-Saharan Africa, where we believe xenosurveillance could have the biggest impact. Due to their close association with humans, engorged *An. gambiae* mosquitoes are relatively easy to collect inside homes opposed to other common mosquito species. Pathogens used in this study were selected because their taxonomic diversity, representing different taxa of disease causing organisms, as well their availability, culturability, select-agent status (*B. antrhacis*), recent emergence, and epidemic potential. Currently,

we have demonstrated that xenosurveillance can be used to detect parasites and bacteria in a laboratory setting, however this remains to be assessed in the field. While Grubaugh et al. 2015 (296) demonstrated the utility of xenosurveillance to detect viruses in the field, future field based xenosurveillance studies that utilize Next Generation Sequencing techniques need to be conducted. This will determine if xenosurveillance can detect broad groups of pathogens, as well as determine the sensitivity of xenosurveillance compared to more traditional surveillance strategies.

Recent outbreaks of infectious diseases have demonstrated the need for improved surveillance and pathogen detection strategies, especially in resource limited areas where a majority of pathogens have emerged/re-emerged (346). Currently, there are numerous systems developed by local and national governments (211, 373, 374), research groups (375), and non-profit organizations (376, 377) aimed at predicting/detecting the next disease outbreak. The majority of these systems are dependent on a clinician or healthcare provider to report into a larger network. This is problematic in areas like sub-Saharan Africa where it is estimated that less than 20% of febrile episodes come to the attention of any formal healthcare system (345). The practicality of xenosurveillance allows for crucial data collection in low-tech environments. This novel data stream can potentially help inform public health officials about specific etiological agents circulating in these environments.

Chapter 4: Detection of human viruses in West Africa by xenosurveillance compared to traditional approaches

Introduction

Emerging and remerging infectious diseases pose a major public health threat throughout the world (378). Nowhere is the burden of infectious disease, both persistent and emerging, felt more strongly than in the developing countries of the tropics (379). The amount of disability-adjusted life years (DALYs) and years of life lost (YLL) due to communicable diseases has decreased globally in the last decade, however in developing areas in the tropics, specifically in sub-Saharan Africa, infectious diseases still account for the majority of DALYs and YLL (303). The consequences of malaria infections in the tropics cannot be understated. In fact, a systematic review of malaria burden by Murray and colleagues (2012) suggest that malaria causes significantly more deaths, particularly in people over the age of 5, than previously reported (380). However, these findings are contradicted on a local level. Crump et al. found that other etiological agents caused the majority of febrile illness clinically diagnosed as malaria (144). These findings are not mutually exclusive, and it is likely that we are underestimating the burden of both malaria and other pathogens that cause febrile illness in the tropics (381). Contributing to the onerous nature of this problem is the difficulty of proper disease diagnosis. Healthcare clinics in rural parts of Africa are often under-funded and lack basic infrastructure, thus severely hindering health care professional's ability to diagnosis disease (382-384). The emergence/reemergence of pathogens, as well as the introduction of pathogens in previously naïve areas, compounds this issue. This is highlighted by immense outbreaks of Ebola virus in West Africa (385), Zika virus in the Americas (386), and Middle East Respiratory Syndrome coronavirus in the Arabian Peninsula (387), all of which have occurred within 5

years of each other. Often, these outbreaks go undetected for a period of time before they are formally recognized by healthcare professionals (388-390). Taken together, these realities emphasize the importance of vigilant disease surveillance.

Global health surveillance is a key factor in an effective international response to outbreaks of infectious diseases. There has been a large expansion of global health surveillance networks since the turn of the century, consisting of programs funded by international organizations, national governments, academics, and private sector groups. These programs are thoroughly reviewed by Castillo-Salgado (2010) (317). A substantial portion of these systems are aimed at predicting and detecting the emergence/reemergence of pathogens, including the Center for Disease Control and Prevention's (CDC) Global Disease Detection (GDD) program (391), as well as the World Health Organizations (WHO) Global Outbreak Alert and Response Network (GOARN) (377). Both GDD and GOARN have made substantial investments in disease surveillance and pathogen detection, in large part due to their extensive network of international collaborators (389). Portions of these surveillance programs consist of biosurveillance, or a general expansion of disease surveillance to include more than just human case detection (392). This is crucial link in infectious disease surveillance, as it is estimated that over 60% of emerging infectious diseases are zoonotic in origin (346). Multiple groups have attempted to detect potentially zoonotic pathogens by active sampling in wildlife (393, 394). These techniques provide an interesting and novel dataset, there are many barriers to pathogen host switching and the majority of wildlife pathogens will never become zoonotic (395, 396). Taking similar active sampling approaches in humans will provide a novel data stream representing pathogens that are actively circulating in a human population. However, substantial hurdles exist in active human blood sampling, not the least of which is the need to obtain Institutional Review Board oversight and obtain informed consent.

Mosquitoes are one of the most efficient collectors of human blood on the planet. We have previously described a novel surveillance approach called xenosurveillance, a technique that makes use of the hematophagous behavior of some arthropods to survey vertebrates for the presence of pathogens (296). This study sought to improve on previous xenosurveillance methodology, as well as to compare the ability of xenosurveillance to detect human pathogens to that of a more traditional sampling method, in this case human finger prick blood. Here we show that xenosurveillance can reliably detect human viruses from as little as 2ul of blood taken from the abdomen of a mosquito. Further, detection of viruses was comparable between xenosurveillance and human finger prick blood. These data, along with previously published work, suggest that xenosurveillance is a viable, non-invasive sampling technique that can be used to detect human viruses.

Materials and methods

Ethics statement

Human subject sampling was approved by the Institutional Review Board at Colorado State University (CSU) (protocol 15-5896H) and by the National Research Ethics Board of Liberia (NREB-0017-15) in partnership with the Liberian Institute for Biomedical Research (LIBR). A local public health worker explained the process of the study and acquired signatures and/or thumb prints for consent. Informed consent was first obtained from the Heads-of-Households, followed by individual members of the household. A single finger prick of blood as well as body temperature was collected from each individual within the household at the beginning of the study. Any febrile patient was offered a SD Bioline Malaria Antigen rapid diagnostic test to determine the presence of malaria parasites (397). Patients with a positive test were offered treatment with artemisinin combination therapy by a nurse and public health worker per WHO Standards (141). No adverse events were reported.

Study location, sampling, mosquito processing and storage

Prior to the study, researchers from CSU and LIBR traveled to Northern Liberia in order to recruit villagers into the study presented here. Multiple villages in Foya County, Liberia were visited. Two villages were ultimately enrolled into the study **(Figure 4.1)**.

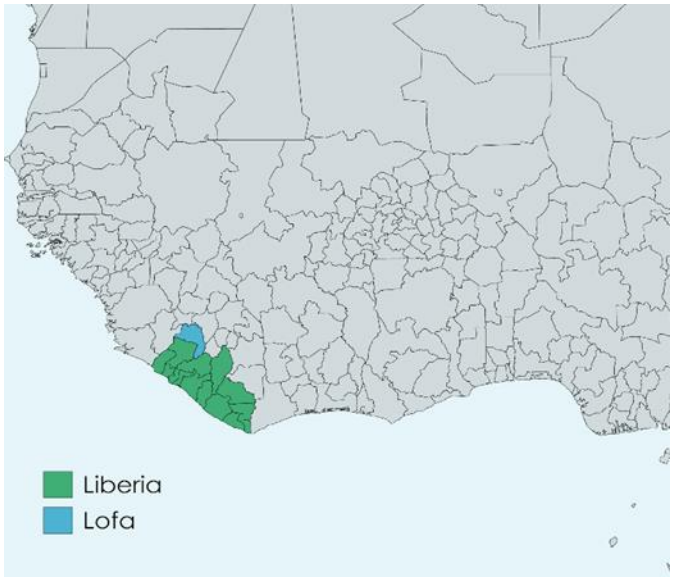


Figure 4.1 Map of West Africa showing Liberia (Green) and specifically Lofa county (Blue) where two villages were enrolled into our study

Upon enrollment, all members of the household provided blood via finger prick performed by a local nurse recruited into the study. The finger surface was swabbed with an ethanol wipe prior to blood collection. Finger prick blood was was pipetted onto CloneSaver FTA cards (GE Healthcare), hereafter referred to as human dried bloodspots (H-DBS). Each H-DBS was immediately soaked in RNA*later* (ThermoFisher Scientific) in order to facilitate diffusion of blood into the FTA card as well as stabilize the nucleic acid.

Following enrollment of households, villages were visited every other day for up to two weeks in order to collect engorged female mosquitoes as previously described with slight modification (296). Mosquitoes were aspirated prior to sunrise in order to collect mosquitoes that fed the previous night.

Researchers equipped with InsectaZookas (Bioquip, USA) surveyed the inside of homes to aspirate blood fed mosquitoes. Aspiration collections were sorted by date and location. Collections were transported to the LIBR research station in Bolahun, Liberia. Mosquitoes were identified to the lowest taxonomic level. Abdomens were dissected from blood fed mosquitoes using forceps and blood meals were applied to FTA cards as previously described (398), these are hereafter referred to as mosquito-dried bloodspots (M-DBS). FTA cards containing both H/M-DBS were placed in multi-barrier pouches (GE Healthcare) containing desiccant beads to reduce humidity and prevent microbial growth. Samples were stored at 4°C until stored on icepacks and shipped to our laboratory at Colorado State University. Pouches containing H/M-DBS were stored at -80°C until further processing.

Library preparation for next generation sequencing

Laboratory processing of samples has been described previously (398). Next Generation Sequencing (NGS) was used to assess H/M-DBS samples for the presence of pathogen-derived nucleic acid. RNA NGS was performed on M/H-DBS samples from a single home in Village A. The remaining samples from Village A were subject to DNA NGS. RNA NGS samples were separated into two pools by location and sample type. Total nucleic acid extraction on M/H-DBS was performed using the Mag-Bind Viral DNA/RNA kit (Omega) and eluted into 50µl of water. Samples were pooled by volume. H-DBS pools from each household were composed of 25µl of total RNA where M-DBS pools were composed of 10µl of total RNA. Each pool was DNAse treated using DNA-*free* DNA Removal Kit (Invitrogen). Pools were purified using a 2x solution of RNA clean XP beads (Beckman Coulter) and eluted into 30µl of water. In order to increase reads to potential pathogen nucleic acid, pools were subjected to an in-house ribosomal RNA (rRNA) depletion protocol. DNAse treated samples were incubated with custom designed oligonucleotides that aligned to the entirety of the *An. gambiae* 18s and 28s ribosomal genes, as well as dNTP. The mixture was then heated to 95 °C for 2 minutes, and the temperature was dropped to 50 °C

at a rate of 0.1C/s. The sample was then incubated at 50 °C for two hours in the presence of AMV reverse transcriptase, RNase inhibitor, and AMV reverse transcriptase buffer (New England BioLabs, USA). Following reverse transcription of rRNA, the sample was incubated with RNAse H (New England BioLabs, USA) in order to rid the sample of RNA contained in an RNA/cDNA hybrid at 37 °C for 30 minutes. The samples were then DNAse treated and purified as described to eliminate the generated cDNA. Following rRNA depletion, complementary double-stranded DNA (cDNA) was created from the remaining RNA. First-strand synthesis was performed using the SuperScript III First-Strand Synthesis System (Invitrogen) following manufactures protocol. 2^{nd} strand cDNA synthesis was conducted immediately following 1^{st} strand synthesis using a Klenow Fragment (3'-5'exo-) according to manufactures protocol. DNA NGS samples were quantified fluorometrically using the Quibit 3.0 High Sensitivity DNA assay. cDNA created from RNA NGS pools was not quantifiable using a Quibit. Both RNA and DNA NGS samples were subject to library preparation using the "tagmentation" enzyme supplied with Nextera XT following manufactures protocol with slight adjustment. Due to low concentrations from RNA NGS samples, the Amplicon Tagment Mix was diluted 10-fold in order to fragment cDNA and add adaptors (399). A dual indexing strategy was used for each NGS pool, unique Illumina indices were added to each pool using a Kapa Library Amplification Kit (Kapa BioSystems, USA). Individual libraries were quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, USA). If necessary, libraries were re-amplified using the Kapa Library Amplification Kit in order to have a proper quantity for sequencing on various platforms. Libraries were diluted to equal concentrations and pooled by volume. DNA NGS samples were sequenced on an Illumina MiSeq platform using a 600 cycle (2x300 reads) MiSeq v3 reagent kit at the CSU NGS facility. RNA NGS samples were sequenced on an Illumina NextSeq platform using a 300 cycle (1x300 reads) NextSeq Mid-Output Kit at the CSU NGS facility.

Sequencing analysis

The goal of the sequencing analysis is to search for reads aligning to human derived pathogens within the DBS. The processing pipeline is similar to that laid out in Fauver et al. 2016 (400) (found on line at https://github.com/stenglein-lab/taxonomy_pipeline) Following the taxonomic assessment pipeline, contiguous sequences (contigs) were viewed in Microsoft Excel. Multiple contigs aligning to pathogens were discovered, including two viruses, GB-virus C (GBV-C, Family Flaviviridae) (401-403) and Hepatitis B virus (HBV, Family Hepadnaviridae) (404, 405), as well as multiple species of filarial worms. These contigs were used as a "road-map" to determine which pathogen nucleic acid may be in the dataset and required further investigation. This initial dataset was produced following the removal of mosquito genomic and rRNA. Human genomic DNA was removed using Bowtie2 version 2.2.5 with parameters –sensitive-score-min C,60,0 . Reference files for GBV-C (Accession #KM670099.1) and HBV (Accession # KU736927.1) were downloaded from NCBI GenBank. Whole genome FASTA files for multiple species of filarial worms, including *Brugia malayi* (BioProject #PRJNA10729*, Dracunculus medinensis* (BioProject #PRJEB500)*, Enterobius vermicularis* (BioProject #PRJEB503)*, Onchocerca volvulus* (BioProject #PRJEB513)*, Loa loa* (BioProject # PRJNA60051), *Wuchereria bancrofti* (BioProject #PRJNA275548)*,* and *Caenorhabditis elegans* (BioProject # PRJNA13758) were downloaded from WormBase (http://parasite.wormbase.org/index.html) and concatenated into a single FASTA file. Reference FASTA files were indexed using the –build option in Bowtie2 (406). Following removal of mosquito genomic DNA, mosquito rRNA, and human genomic DNA, paired or single end reads were aligned to indexed reference files using –x and –very-sensitive options in Bowtie2 and exported as .SAM files (Sequence Alignment Map) using the –S option. Aligned .SAM files were converted to .BAM files (Binary Alignment Map) and sorted to their reference genes using the view and sort options in SAMtools, respectively (407). Individual reads that aligned were then assessed visually and with the BLASTn tool (408).

PCR confirmation

In order to validate data obtained through NGS, we designed species-specific PCR primers to 1) confirm the presence of our target of interest in individual DBS and/or sequencing pools, and 2) determine the prevalence of these viruses in our samples. Primers were designed using the Primer3 software version 2.3.4 in Geneious **(Appendix 1, Supplemental Table 4.1)** (409). The presence GBV-C was determined from individual DBS using Qiagen OneStep reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen, Germany). The sequencing reaction was run on a 1% agarose gel to visualize the amplified product. Samples that produced visible bands were sent for Sanger sequencing using the forward primer at Quintarabio labs. Chromatogram files were then aligned to the reference genome in Geneious to confirm specificity. The presence of HBV was determined from pooled sequencing samples using the iTaq Universal One-Step RT-qPCR Kit (Bio-Rad, USA) containing SYBR green on a real-time PCR platform. Positive samples sequencing and confirmation analysis were performed as stated above.

Results

Enrollment information and sample collection

Two villages in Northern Liberia were enrolled in our study **(Figure 4.1).** Village A was sampled on 6 separate days, and Village B was sampled a total of 3 times. Upon enrollment, no individuals presented as, febrile based on body temperature. Malaria is endemic throughout Liberia, and West Africa (410), so body temperature was collected during each sampling period. Throughout the course of sampling, a total of two individuals in both villages presented as febrile based on a body temperature >39°C. Both individuals were considered positive for *Plasmodium falciparum* based on the results of a SD Bioline Malaria Antigen rapid diagnostic test and were treated with artemisinin combination therapy. *Anopheles gambiae sensu lato* was the most commonly collected species of mosquito from within

homes in both villages, making up over 80% of mosquitoes collected during the study **(Table 4.1)**. Few other taxa of mosquitoes were collected from inside homes. Of the other taxa, *Aedes* and *Culex* mosquitoes were the most common, with 9 and 3 females, respectively. The vast majority of *A. gambiae* mosquitoes collected contained a full blood meal, indicating they took the blood meal the night before. Village A was slightly more populated than Village B, resulting in a higher number of people enrolled into the study. As well, a greater number of blood fed *A. gambiae* mosquitoes were collected from homes in Village A **(Table 4.1)**. Ultimately, H/M-DBS from Village A were used for the remainder of our study.

Table 4.1 Summary of enrollment and sampling data

Sample	Village A	Village B
Households	23	20
H-DBS	105	80
Mosquitoes Aspirated	198 (81%)[a]	55 (87%)
M-DBS	161	48

[a] Number in parenthesis refers to percentage of aspirated mosquitoes that were both *An. gambiae* and blood fed

Sequencing analysis

Two separate sequencing runs were performed in this study. First, a small subset of samples were subjected to RNA sequencing on an Illumina NextSeq platform **(Table 4.2)**. All H/M-DBS originated from the same home in Village A. This home was selected because it had, on average, a higher number of individuals enrolled, as well because it produced the highest number of M-DBS, resulting in 7 H-DBS and 34 M-DBS, respectively. Prior to quality control and host filtering, each DBS produced over 1.8 million reads. Following quality control and host filtering, around 50,000 reads remained for H-DBS and 11,000 for M-DBS. The greatest reduction in reads was seen after the dataset was collapsed to unique reads, removing PCR duplicates. The remaining samples were subjected to DNA sequencing on an Illumina MiSeq platform. Based on the observation that the majority of reads in the RNA NGS dataset

were lost due to PCR duplicates from library amplification, we decided to increase the number of DBS in each sequencing pool. In total, 96 H-DBS were pooled alongside 120 M-DBS. The H-DBS produced over 700,000 reads while the M-DBS pool produced almost 900,000. The volume of sample loaded onto the sequencer explains the differences in total number of reads between pools sequenced on the same platform. The greatest reduction in reads from DNA sequencing samples was observed following filtering of the human and mosquito genome.

Table 4.2 NGS Read breakdown following quality control and filtering

Sample	N.A. [a]	N	Total Reads	Reads/DBS	Reads Post QC	Reads Post Duplicate Removal	Reads Post rRNA Removal	Reads Post Mosquito Genome Removal	Reads Post Human Genome Removal	Reads Per DBS Post Filter
H-DBS	RNA	7	12,748,338	1,821,191	10,752,673 (15.6) [b]	626,190 (94.2)	600,279 (4.1)	565,846 (5.7)	340,497 (39.8)	48,642
M-DBS	RNA	34	65,750,180	1,933,828	55,231,317 (16)	3,221,542 (94.2)	3,186,837 (1.1)	948,599 (70.2)	383,672 (59.6)	11,284
H-DBS	DNA	96	713,637	7,433	554,546 (22.3)	524,159 (5.5)	524,087 (0.01)	513,406 (2.0)	984 (99.8)	10.25
M-DBS	DNA	120	898,739	7,489	707,383 (21.3)	588,146 (16.9)	582,608 (0.9)	162,485 (72.1)	59,271 (63.5)	493.925

[a] Nucleic Acid
[b] Number in parenthesis below total reads refer to percentage of reads lost at each filtering step

Taxonomic assessment

GB Virus C

GBV-C has a 9.3 kb long, single stranded, positive sense RNA genome that contains a single open reading frame that encodes for two structural and five non-structural proteins (411). Individual contigs aligning to a West African strain of GBV-C were produced from both H-DBS and M-DBS from our RNA sequencing datasets (403). This genome was then used to make a reference index and individual reads from both H-DBS and M-DBS sequencing dataset were aligned **(Figure 4.2)**. In total, 15 and 28 individual reads aligned to GBV-C from H-DBS and M-DBS, respectively. On average, these reads aligned with over 90% pairwise nucleotide similarity. Mean individual read length was 128 nucleotides, and these reads spanned ~40% of the genome **(Table 4.3)**.

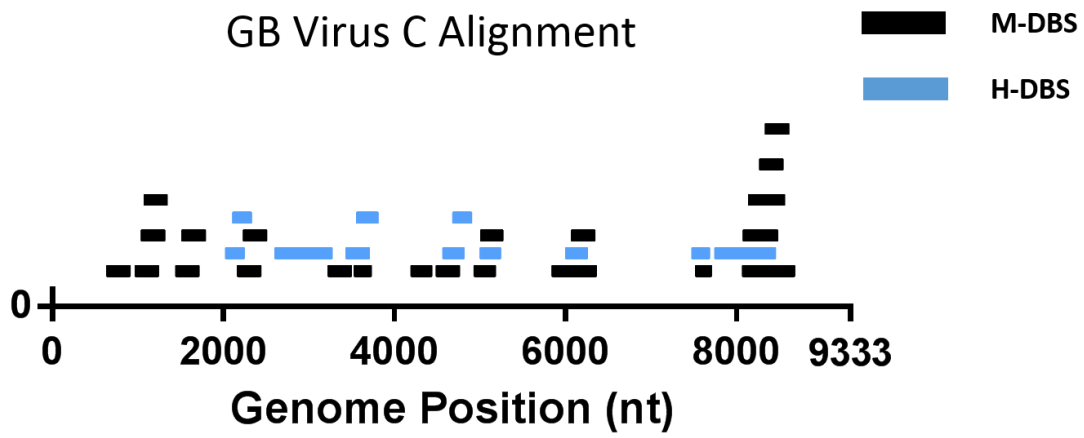


Figure 4.2 Reads from both mosquito and human dried blood spots align to and span the GB virus C genome.

Table 4.3 NGS reads aligning to GBV-C

Sample	Nucleic Acid	N	Reads aligning to GBV-C	% N.T. similarity Gbv-C strain[+]	% Genome Coverage[#]
H-DBS Pool	RNA	7	15	91.8	18.8
M- DBS Pool	RNA	34	28	93.8	26.5

+ West African Strain of GB virus C, N.T. (Nucleotide)
# 39.8% of total genome covered

Hepatitis B virus

HBV is a DNA virus with a 3.2 kb partially double stranded relaxed circular DNA genome (412). A single contig from our H-DBS DNA sequencing dataset produced an alignment to an African strain of HBV. This genome was then used to make an indexed reference, and reads from both H-DBS and M-DBS were aligned **(Figure 4.3)**. A total of 6 and 2 reads aligned from the H-DBS and M-DBS DNA sequencing dataset, respectively. Reads from both dataset aligned with up to 99% percent pairwise nucleotide identify and combined to cover ~25% of the genome **(Table 4.4)**. The mean read length was 142 nucleotides.

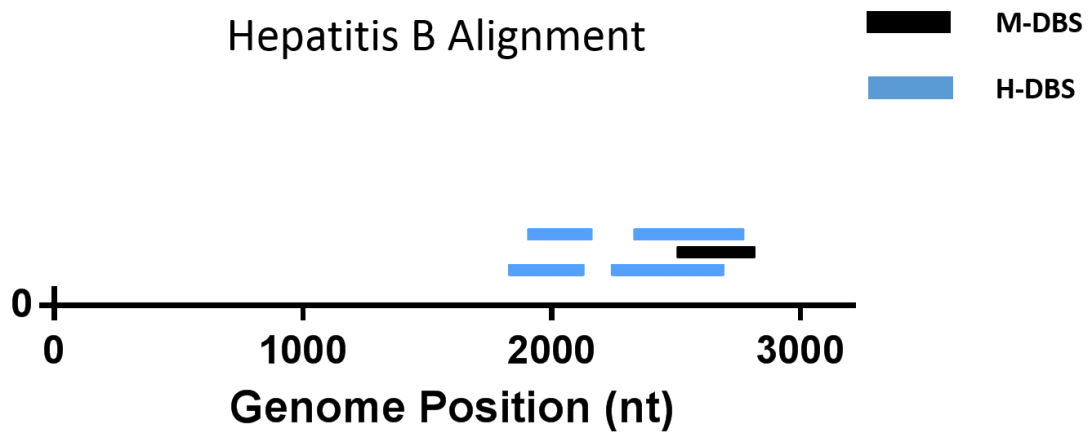


Figure 4.3 Reads from both human and mosquito dried blood spots align to an African strain of hepatitis B virus.

Table 4.4 NGS reads aligning to HBV

Sample	Nucleic Acid	N	Reads aligning to HBV	% NT similarity HBV[+]	% Genome Coverage[#]
H-DBS	DNA	96	6	98.9	24.4
M- DBS	DNA	120	2	99.3	8.5

+ African strain of HBV, N.T. (Nucleotide)
# 25.8% of total genome covered

Parasites

Our initial taxonomic assessment identified multiple contigs aligning to various species of parasitic nematodes. In order to further scrutinize these reads, a reference file consisting of *Brugia malayi, Dracunculus medinensis, Enterobius vermicularis, Onchocerca volvulus, Loa loa, Wuchereria bancrofti,* and *Caenorhabditis elegans* was indexed in order to competitively align individual reads to these genomes. Following removal of mosquito and human sequences, over 5,000 individual reads produced an alignment to a portion of these various genomes. Alignments were viewed in Geneious to examine their authenticity. The following criteria was used to remove spurious alignments: 1) Parasite did not produce top hit in Blastn search, 2) low complexity reads (e.g. ATAT repeats), 3) less than 90%

nucleotide similarity, or 4) reads aligned to ribosomal RNA sequences. Following these criteria, no nematode parasite contigs or reads were deemed legitimate.

Prevalence

Virus-specific RT-PCR and qPCR was employed to determine the prevalence of GBV-C and HBV, respectively, from individual H-DBS and M-DBS that made up the RNA NGS pools **(Table 4.5)**. We determined that a single individual from a single home assessed in Village A was GBV-C positive, resulting in a prevalence of 14.3%. Out of a total of 34 M-DBS, three were determined to have fed on that individual resulting in a prevalence of 8.8% in M-DBS. A total of 7 H-DBS from 4 separate homes were determined positive for HBV. A total of 17 M-DBS from 8 separate home also tested positive for HBV. At least one positive mosquito was collected from each home that contained at least one positive individual.

Table 4.5 Prevalence of GBV-C in H-DBS and M-DBS

	H-DBS	M-DBS
N	7	34
# Positive for GBV-C	1	3
Prevalence	14.3%	8.8%

Table 4.6 Prevalence of HBV in H-DBS and M-DBS

	H-DBS	M-DBS
N	96	120
# Positive for GBV-C	7	17
Prevalence	7.3%	14.2%

Discussion

In this study, we sought to improve on existing methodology while comparing the detection ability xenosurveillance to a more traditional sampling method, human finger prick blood spotted onto FTA cards. Over 40 homes from two villages were enrolled, resulting in a total of 185 participants **(Table 4.1)**. From these homes, a total of 253 mosquitoes were aspirated from the homes. Of these, 209 mosquitoes identified as *An. gambiae sensu lato* were blood fed, resulting in as many M-DBS. Due to a higher number of participants and a greater number of mosquitoes caught, samples from Village A were used for the remainder of the study.

A high number of reads were produced in both our RNA and DNA NGS datasets. However, we observed a remarkable reduction in reads following quality control and host filtering, greater than 99% of reads were removed in all sequencing libraries **(Table 4.2)**. This is due to multiple factors. In our RNA sequencing data, we saw a greater than 90% reduction in reads following removal of PCR duplicates. Illumina sequencing platforms require at least 1nM of prepared library to dilute and load onto the sequencer (413). The amount of RNA recovered from individual dried blood spots is undetectable by typical methods (e.g. Bioanalyzer). Thus, a substantial amount of PCR amplification is required to bring libraries to a usable quantity of nucleic acid, resulting in extensive PCR duplicates. DNA appears to be more stable on FTA filter cards than RNA, and a higher quantity of DNA is eluted off each DBS. In addition to pooling more samples together to create DNA NGS pools, the amount of reads lost to PCR duplicates was negated. However, most of the remaining reads were removed post host filtering, showing most of the sequenced nucleic acid was derived from either humans or mosquitoes.

Following quality control and host filtering, enough reads remained to detect genetic signatures of two viruses in our RNA and DNA NGS datasets, GBV-C and HBV **(Figures 4.2 and 4.3, Tables 4.3 and 4.4)**. GBV-C infects and replicates in CD4-positive T cells and is not known to be pathogenic to humans

(414). Interestingly, infection with GBV-C is associated with an increased rate of survival in patients co-infected with HIV (415). GBV-C viremia is highly variable. Patients with detectable viremia can range anywhere from $2.3x10^3$ genome equivalents per milliliter (ml) of serum/plasma to $6.5x10^8$ GE/ml as determined by qRT-PCR (416). GBV-C has an estimated prevalence between 10-28% in West African countries, which is similar to our findings, albeit ours is a small sample size **(Table 4.5)** (403). Chronic HBV infection is the leading causes of chronic hepatitis and hepatocellular carcinoma, and is responsible for up for up to 30% of cirrhosis of the liver globally (417, 418). The replicative life cycle of HBV is complex, consisting of both RNA and DNA forms, and viremias can vary substantially during subsequent stages of infection, from $1.4x10^3$ GE/ml of serum to $1.7x10^9$ GE/ml of serum (419, 420). Prevalence is also highly variable across Africa, but is estimated to be between 4-8% in Liberia as determined by HBV surface antigen (421). Due to these viruses' replicative ability and small genome size, these results demonstrate that xenosurveillance can detect comparably small amounts of pathogen nucleic acid in pools of over 100 M-DBS.

Following our initial taxonomic assessment pipeline, it appeared that a number of contigs were aligned to parasite genomes, indicating the presence of these parasites in both H-DBS and M-DBS. However, following further investigation, the vast majority of these contigs and individual reads, if not all of them, were misaligned. Misalignment appears to be a common problem in the literature, and is likely the result of spurious alignments or misannotation of host genomes (422-424). Our data emphasizes the importance of proper vetting of any NGS data.

Taken together, these data show that xenosurveillance is a viable method for detecting human viruses, and thus likely other pathogenic organisms with larger genomes (e.g. bacteria and parasites) when they are present in human blood at a similar level compared to more traditional sampling methods. Xenosurveillance can supplement current biosurveillance programs as a non-invasive way to sample large numbers of people for pathogens that may be circulating in the human population.

Chapter 5: West African *Anopheles gambiae* mosquitoes harbor a taxonomically diverse virome including new insect-specific flaviviruses, mononegaviruses, and totiviruses

Introduction

Mosquitoes (Diptera: Culicidae) are the most important vectors of human disease. *Anopheles gambiae* and other anopheline mosquitoes are the vectors of *Plasmodium* parasites in Africa, which cause nearly 200 million malaria cases annually on the continent (425). These mosquitoes are also responsible for transmitting O'nyong-nyong virus, a human pathogenic alphavirus capable of causing large disease outbreaks (25, 426). In addition, *Anopheles* spp. mosquitoes are vectors of *Wuchereria bancrofti* roundworms, the causative agent of lymphatic filariasis, which affects over 100 million people in sub-Saharan Africa (427). Current interventions for these diseases are inadequate, and future strategies need to deploy current and novel interventions to disrupt pathogen transmission.

Constituents of arthropod microbiomes are being increasingly scrutinized for their potential to alter the arthropod's ability to transmit co-infecting human pathogens (428). The microbiome of *Anopheles* species, especially in the gut, has been shown to be quite diverse and can vary depending on the mosquito's environment (296, 429). Furthermore, certain bacteria can influence *Plasmodium* development in the mosquito (430-434). During the last decade there has been increased attention to insect-specific viruses (ISVs) and their potential role in disrupting pathogen transmission. (263, 265, 435, 436). The majority of ISVs have been described in mosquitoes, although they are known to occur in several arthropod orders, including Hemiptera (i.e. true bugs) (262) and Parasitiformes (e.g. ticks) (437). ISVs belong to taxonomically diverse virus families including *Bunyaviridae* (438-442), *Flaviviridae* (436, 443-447), *Reoviridae* (448-450) *Rhabdoviridae* (451-453), and *Togaviridae* (454). In addition, ISVs in the

Birnaviridae (455-457), *Nodaviridae* (458), *Tymoviridae* (459), and *Parvoviridae* (460) families have been characterized, the latter from which an *Anopheles*-specific densovirus is being examined as a paratransgenesis candidate (460, 461). Recently discovered ISVs include those in the family *Mesoniviridae* (462-467) and a variety of positive sense ssRNA viruses including the negeviruses (468, 469). Recently, two new RNA viruses, a dicistrovirus and a cypovirus, were identified in *Anopheles* species mosquitoes (470). Mosquitoes are divided into two subfamilies: Culicinae and Anophelinae, however, a disproportionate amount of mosquito ISVs have been identified from culicine mosquitoes, leaving anopheline mosquitoes relatively understudied.

We therefore used metagenomic sequencing to identify viruses infecting wild *An. gambiae*, *An. funestus,* and *An. rufipes* mosquitoes in West Africa. We sampled adult mosquitoes from villages in rural Burkina Faso, Liberia, and Senegal, sequenced RNA, and searched datasets for virus sequences. We identified sequences from multiple new viruses. For several of these, we generated coding complete genome sequences, performed comparative and phylogenetic analyses, and determined the prevalence from our field-collected samples. Our findings indicate that anopheline mosquitoes naturally harbor multiple viruses including flaviviruses.

Materials and methods

Mosquito samples used in this study were collected from 2012-2015 on separate trips to Senegal, Liberia, and Burkina Faso (**Figure 5.1** and **Table 5.1**). Indoor resting blood fed mosquitoes were collected in Senegal (125, 471) and Liberia as (296) previously described. Mosquitoes from Burkina Faso were colonized and reared at the Colorado State University (CSU) Arthropod-Borne and Infectious Disease Laboratory prior to being sampled.

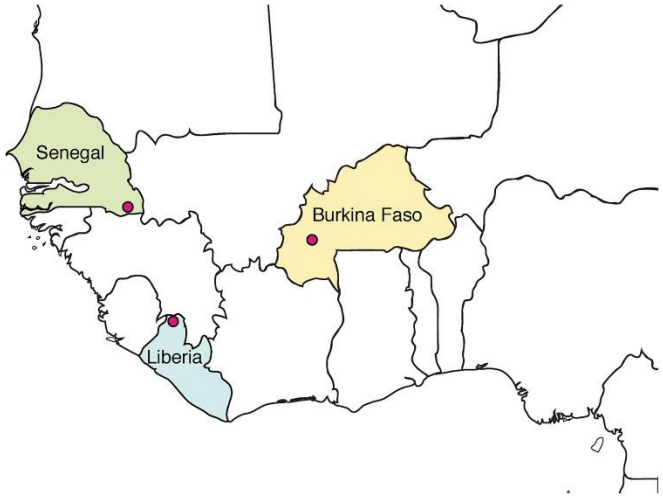


Figure 5.1 Mosquito collection sites in Liberia, Senegal, and Burkina Faso. In Liberia, mosquitos were sampled from 6 villages within an area with an approximate radius of 16 km. The collection site in Burkina Faso for mosquito eggs that were used to found a laboratory colony is indicated.

Table 5.1 Summary of mosquito samples analyzed

Sample set	Location	Date	Number mosquitos analyzed	Mosquito species[a]	Analysis method
1	Senegal	8/22/2012	41[b]	*An. funestus/ An. gambiae/ An. rufipes*	NGS (MiSeq)
2	Burkina Faso	1/5/2015[c]	17	*An. gambiae*	NGS (NextSeq)
3	"	12/30/2015[c]	3	"	"
4	Liberia (village A)	6/11&13/2013	31	*An. gambiae*	NGS (HiSeq)
5	"	6/15/2013	38	"	"
6	"	6/19/2013	24	"	"
7	"	6/21/2013	41	"	"
8	"	6/23/2013	29	"	"
9	"	6/25/2013	57	"	"
10	Liberia (village B)	6/10&14/2013	51	"	"
11	"	6/22&26/2013	57	"	"
12	Liberia (village C)	3/30/2015	15[d]	*An. gambiae*	PCR / Sanger sequencing
13	Liberia (village D)	3/31/2015	15	"	"
14	Liberia (village E)	4/1/2015	15	"	"
15	Liberia (village A)	4/2/2015	15	"	"
16	Liberia (village F)	4/3/2015	15	"	"

a) As determined by field identification and molecular analysis (see Materials and Methods)
b) Mosquitos in sample sets 1-11 were pooled (1 pool per sample set) for sequencing
c) These mosquitoes were sampled on these dates from a laboratory colony that was derived from *An. gambiae* larvae collected in Burkina Faso
d) Mosquitos in sample sets 12-16 were analyzed individually (i.e. not pooled) using PCR and Sanger sequencing to validate NGS results and measure prevalence

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Sample preparation

Burkina Faso: A laboratory colony of *An. gambiae s.s.* was established by the Institut de Recherche en Sciences de la Santé from larvae collected in Burkina Faso in 2014 and eggs from this colony were subsequently shipped to Colorado State University (CSU). Mixed sex, non-blood fed mosquitoes from the colony at CSU were homogenized in 1 ml of mosquito diluent (80% PBS, 20% FBS, supplemented with penicillin, streptomycin, gentamicin, and amphotericin B (78)) with a steel ball bearing for RNA extractions. 50 µl of cleared supernatant was used for RNA extraction with the Mag-Bind Viral DNA/RNA kit (Omega, Georgia, USA) with the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer's protocol. Libraries were prepared using the Ovation RNA-Seq System V2 (NuGEN, California, USA) and Ovation Ultralow DR Multiplex System 1-96 (NuGEN) and sequenced on an Illumina NextSeq at the CSU NGS facility.

Senegal: Field-caught blood fed mosquitoes were pooled by date and stored in RNA Later (Ambion) at -80˚C. Pools were thawed and RNA Later was removed. 1 ml of PBS was added and mosquito pools were centrifuged for 5 minutes. 140 µl of supernatant was used for RNA extraction using Qiagen Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with slight modification. Extracted RNA was subjected to DNase treatment (Thermo Fisher Scientific) and purification using Agencourt RNAclean XP beads (Bechman Coulter Genomics, Pasadena, CA). cDNA was amplified using the Ovation RNA-Seq System V2 and prepared for library construction using the Ovation

Ultralow DR Multiplex System 1-96 as described (472). Libraries were sequenced on a NextSeq instrument at the CSU NGS facility.

Liberia: Mosquitoes were collected and processed as previously described (296). Briefly, blood fed mosquitoes were knocked down with triethylamine and blood meals were expelled onto Whatman Flinders Technology Associates (FTA) clone saver cards (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Cards were stored at -20˚C and shipped to CSU for additional processing. Mosquito dried blood spots (M-DBS) were removed using a Harris 3 mm micro-puncher (GE Healthcare Life Sciences) and placed into RNA Rapid Extraction Solution (Ambion, Texas, USA) to elute nucleic acid off of the cards. RNA was extracted using the Mag-Bind Viral DNA/RNA kit with the KingFisher Flex Magnetic Particle Processor according to manufacturer's protocol. Libraries were prepared using the Ovation RNA-Seq System V2 and Nextera XT DNA Sample Preparation Kit (Illuminia, San Diego, CA) and sequenced on an Illumina HiSeq (Beckman Coulter Genomics, Danvers, MA).

Sequence analysis

Sequencing datasets were processed with the goal of taxonomically assigning non-mosquito reads. First, low quality and Illumina adapter sequences were removed using the Trimmomatic tool version 0.32 with the following settings: ILLUMINACLIP:NexteraPE:1:30:10:4:true LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:60 (473). Potential PCR duplicate sequences were collapsed using the CD-HIT-EST tool, version 4.6 with parameter –c 0.96 (474). Then, mosquito sequences were removed by aligning reads to databases of *An. gambiae* genomic sequences using Bowtie2 version 2.2.5, with parameters –sensitive –score-min C,60,0 (13). Remaining sequences were *de novo* assembled into contiguous sequences using the SPAdes genome assembler (406, 475). Resulting contigs and non-assembling reads were then taxonomically assessed, first by using the gsnapl tool, version 2014-12-28, to align to the NCBI nt nucleotide database (476). Sequences that did not produce a

nucleotide-level alignment were then searched via translated-nucleotide to protein alignments against the NCBI nr protein sequence database using the Rapsearch2 tool, version 2.23, with parameters –a t, -1 20, and –e1 e-2 (477). Draft virus genome sequences were validated by mapping individual reads to assemblies using Bowtie2 as above and in some cases using PCR and Sanger sequencing. Resulting alignments were imported into Geneious software version 9.0.4 and manually inspected (478). Sequence datasets have been deposited in the NCBI Short Read Archive (SRA) with accession PRJNA327220.

Analysis of predicted viral protein sequences

ORFs in viral genome assemblies were predicted using Geneious software. Homologs of predicted protein sequences were detected using the BLASTP tool (version 2.2.25+) to search the NCBI non-redundant protein database (nr) (479). For sequences with no detectable similarity by BLASTP, the HHpred homology detection and structure prediction tool (version 2.0) was also used (480). The transmembrane prediction tool in Geneious was used to predict transmembrane domains. Virus and virus-like contigs longer than 500 nt long were aligned to the NCBI nr protein sequence database using BLASTX to determine taxonomic classification, closest relative, and percent similarity to closest related sequences.

Phylogenetic analysis

Predicted viral protein sequences were used to query the NCBI nr protein database using the BLASTP tool (408). Database sequences that aligned with an E-value less than 10^{-3} and that were full length or nearly full-length were downloaded. These were collapsed using the CD-HIT tool, version 4.6 using parameter –c 0.9 (474). These representative sets of sequences were aligned using the MAFFT software, version 7.221, using the LINSI mode (481). Phylogenetically uninformative columns were removed from multiple alignments using the GBlocks tool, version 0.91b, with parameter –b5=n (482).

These trimmed alignments were used to create phylogenies with the MrBayes version 3.2.5 with commands preset aamodelpr=mixed and mcmc ngen=1000000 (483). Convergence was confirmed by inspecting the standard deviation of split frequencies. Phylogenies were visualized using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Validation of mosquito species collected

We used a molecular strategy to corroborate field identification of the species of collected mosquitoes**.** We collected a representative set of mosquito cytochrome c oxidase subunit 1 (CO1) gene sequences (available online: http://www.sciencedirect.com/science/article/pii/S0042682216302070) and used Bowtie2 to align sequences from our quality-filtered datasets to these sequences and tabulated the fractions of reads aligning to each mosquito species. This analysis corroborated field-based species identifications **(Table 5.1)**.

Prevalence of *Anopheles* viruses

The abundance of virus reads in sequencing datasets was calculated by using Bowtie2 to map all unique, host-filtered reads to all of the virus sequences identified in this study. The abundance was defined as the number of mapping reads per million unique reads in each dataset. Field prevalence of Anopheles Flavivirus, Bolahun Anopheles virus, and Anopheles Totivirus was determined using independent sample sets collected in 2015 **(Table 5.1)**. RNA isolated from individual Liberian M-DBS was tested for the presence of each virus by RT-PCR using the Qiagen OneStep RT-PCR kit according to manufacturer's protocol with virus specific primers **(Appendix 1, Supplemental Table 5.1)**. 15 M-DBS from 5 villages (75 total) were used to calculate field prevalence.

Results

We collected adult *Anopheles* mosquitoes from several West Africa locations and performed metagenomic sequencing of midgut RNA-derived libraries to search for viruses. In total, we analyzed 328 adult *An. gambiae* from Liberia and 41 mixed *An. gambiae/An. funestus/An. rufipes* from Senegal (~1/3 of each species). These mosquitoes were collected over the course of several years from multiple villages **(Table 5.1)**. We also analyzed 20 mosquitoes from a laboratory colony established in 2014 from Burkina Faso *An. gambiae s.s.* larvae. These colony mosquitoes were sampled in 2 batches ~12 months apart. Mosquitoes were combined into pools of between 3 and 57 mosquitoes each, and pools were sequenced on Illumina instruments to a median depth of 4.8×10^7 150 nt read pairs per pool. After filtering low quality, duplicate, and mosquito-derived reads, a median of 3.0×10^5 reads remained (0.6%). Remaining reads were de novo assembled and taxonomically assigned by comparison to sequences in Genbank, first by nucleotide-to-nucleotide alignments, then by translated-nucleotide to protein alignments. We identified a number of putative viral sequences and determined coding-complete genome segment sequences where possible (484). Sequencing depth was determined for each coding complete genome **(Appendix 1, Supplemental Figure 5.1)**. We also used sequencing data to confirm the species composition of the collected mosquito sample sets **(Table 5.1)**.

Anopheles flavivirus

We identified flavivirus sequences in datasets from mosquitoes collected in Liberia and Senegal **(Figure 5.2 and Table 5.1)**. The genus *Flavivirus* (Family Flaviviridae) includes viruses whose life cycle involves alternating replication in vertebrate and arthropod hosts and members whose life cycle is restricted to one host or the other (485). Typical flaviviruses have positive-sense ssRNA genomes that encode a single large polyprotein. We assembled two coding-complete flavivirus genomes from Liberia datasets, which shared 95% pairwise nucleotide identity. We designated these as Anopheles flavivirus

(AnFV) – variants 1 and 2. We also assembled partial sequences from at least one additional flavivirus from the Senegal datasets, which we called Anopheles flavivirus-like sequences 1 and 2 **(Table 5.1)**. Anopheles flavivirus-like sequence 1 shared ~79% pairwise nucleotide identity with the AnFV sequences. These and other genome sequences represent the consensus of sequences that shared > 99.5% pairwise identity. Single nucleotide variants were evident in the various datasets **(Appendix 1, Supplemental Figure 2)**. Given that these datasets derive from pools of mosquitoes, this variation could represent intra- or inter-host diversity, or both.

The genome organization, gene content, phylogenetic placement, and dinucleotide usage supports the classification of AnFV as a "classic" insect-specific flavivirus (cISFV) (263, 436). The AnFV genome contains an ORF of 10032 nt predicted to encode a polyprotein of 3,341 amino acids (**Figure 5.2A**). The polyprotein is predicted to be co- and post-transcriptionally cleaved to produce the typical 3 structural and 7 non-structural flavivirus proteins and we identified putative cleavage sites. The polyprotein shares 35-43% global pairwise amino acid identity with sequences from other cISFVs.

In addition to the polyprotein ORF, the genome contains an 840 nt ORF overlapping with the NS2 coding region (**Figure 5.2A**). The reading frame of this ORF is -1 relative to the polyprotein ORF, and 5 nt downstream of the predicted NS2A cleavage site, a putative "slippery" sequence (GGAUUUU) was identified as a likely site of ribosomal frameshifting. These "fifo" ORFs (fairly interesting flavivirus ORF) are a characteristic of cISFV genomes (436, 486). The predicted AnFV FIFO protein possesses no detectable sequence similarity with other cISFV FIFO proteins. However, like other cISFV FIFO proteins, the AnFV protein contains predicted transmembrane domains (486). Phylogenetic analysis corroborated the designation of AnFV as a cISFV **(Figure 5.2B,C and Appendix 1, Supplemental Figure 5.3)**. In Bayesian phylogenies based on alignments of NS5 protein sequences, the AnFV sequences occupy a well-supported branch within the cISFV clade. Analyses based on alignments of full polyprotein sequences produced phylogenies with essentially identical topologies.

Viral genomes often exhibit patterns of dinucleotide usage similar to that of their hosts. On this basis, cISFV genomes can be distinguished from those of flaviviruses whose lifecycles include replication in vertebrates (436). Based on dinucleotide usage, AnFV clusters with cISFVs **(Figure 5.2D).**

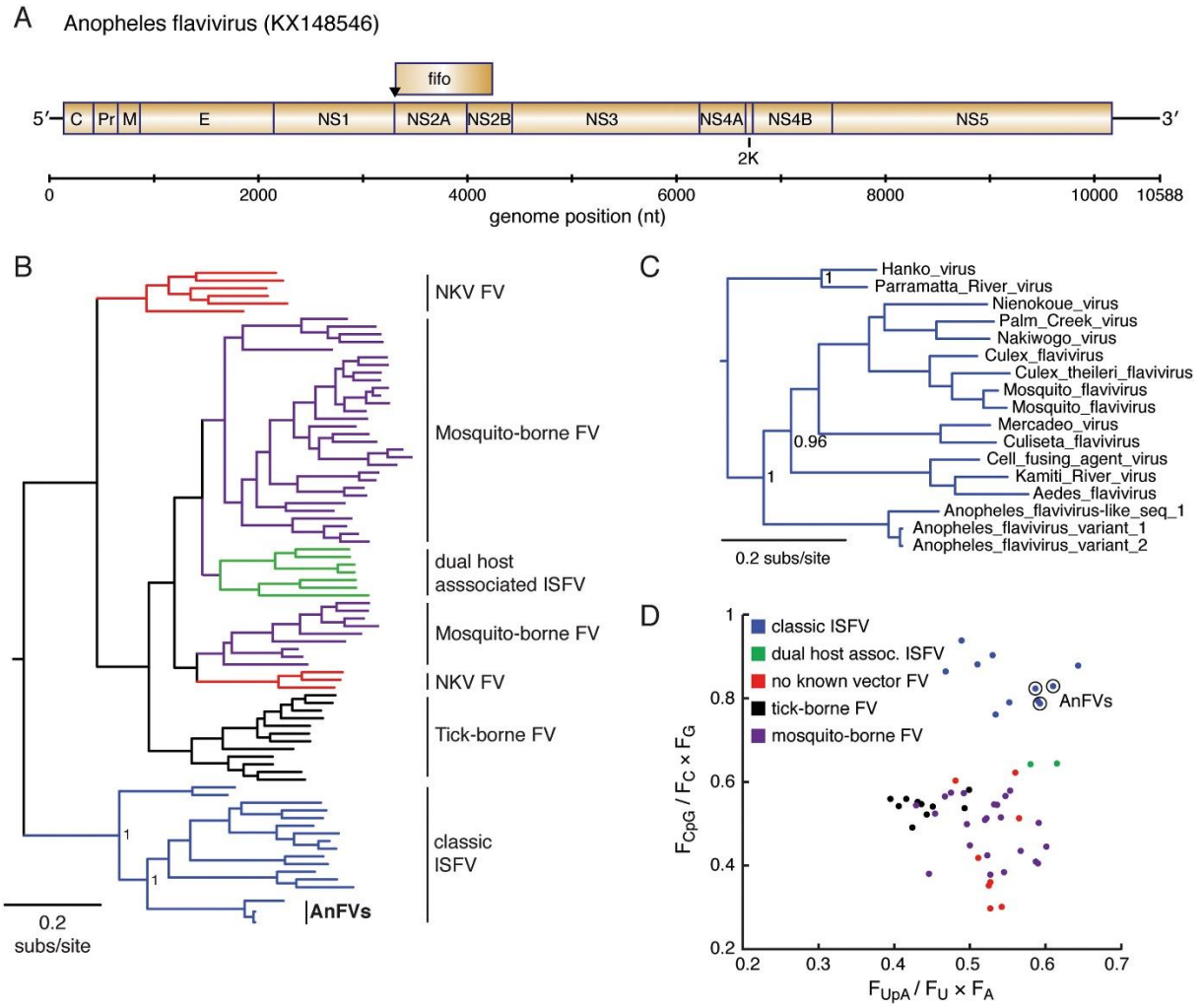

Figure 5.2 Anopheles flavivirus genome organization, dinucleotide usage, and phylogeny (A) Genomic organization of anopheles flavivirus. Predicted functional domains of the viral polyprotein are indicated as is the fifo ORF and the predicted ribosomal frameshift "slippery" sequence at its beginning is indicated by a triangle. The other AnFV and AnFV-like sequences identified in this study (KX148547-KX148549) have a similar organization. (B) AnFV clusters phylogenetically with classic ISFVs. A multiple sequence alignment of flavivirus NS5 protein sequences was used to create a Bayesian phylogeny. The phylogeny was rooted on the branch to Tamana bat virus (not shown). Posterior probabilities of select nodes are indicated. A fully-labeled version of this phylogeny including accession numbers and node posterior probabilities is available as Supplemental Figure 5.3 **(Appendix 1)**. (C) Phylogeny as in (B) but focused on the cISFV clade. (D) Dinucleotide usage in AnFV supports its categorization as a classic insect-specific flavivirus (ISFV). CpG and UpA dincucleotide frequencies for viruses in the genus *Flavivirus* in the NCBI RefSeq database are indicated. Points are color coded according to flavivirus categories as indicated and as in Blitvich and Firth (2015) (436). FV: flavivirus. Anopheles flavivirus points are indicated.

Bolahun and Gambie viruses

We identified mononegavirus sequences in mosquitoes from Liberia and Senegal and from our Burkina Faso-derived colony. *Mononegavirales* is a large and diverse order of viruses that have single-stranded negative polarity RNA genomes and evolutionarily related RNA dependent RNA polymerase (RdRp) genes (487). We assembled apparently coding-complete mononegavirus genomes from each of these three datasets **(Figure 5.3)**. The genomes from Liberia and Burkina Faso were closely related (94% pairwise nt identity), and we designated these as Bolahun virus (BOAV) – variants 1 and 2. The sequence from Senegal, which we named Gambie virus (GAMV), shared ~60% pairwise nt identity with BOAV.

The BOAV and GAMV genomes share a similar overall genome organization, with 6 non-overlapping ORFs (**Figure 5.3A**). In mononegaviruses, the ORF nearest the 3' end of the genome encodes the viral nucleoprotein. The BOAV and GAMV ORF1 is predicted to encode protein of 446 amino acids (lengths given for BOAV, accession KX148552) with no transmembrane domains and an isoelectric point of 8.7. By BLASTP search, the only identifiable homologous sequence was that encoded by Xincheng mosquito virus ORF1 (262), which shares 19% pairwise global amino acid identity. These are likely nucleoproteins for these viruses. ORF2 encodes a predicted small transmembrane domain-containing protein of 65 residues. ORF3 encodes a protein predicted to be 446 AA long with no detectable similarity to known proteins by BLASTP or HHPRED analyses w/ E-value cutoff 0.1 (480). ORF4 encodes the predicted viral glycoprotein, a 638 AA protein with 3 transmembrane domains and sequence similarity to a variety of mononegavirus glycoproteins. As with the putative nucleoprotein, the most similar sequence is from Xincheng mosquito virus, with 38% global pairwise identity. As in all mononegaviruses, the last ORF is predicted to encode the large RdRp (L) protein. BOAV and GAMV proteins share between 36% (ORF2 protein) and 72% (glycoprotein) global amino acid identity.

In phylogenies based on alignments of L protein sequences, BOAV and GAMV form a well-supported clade with Xincheng mosquito virus and Shuangao fly virus, which were identified in samples from China from an *Anopheles sinensis* mosquito and a *Psychoda alternata* fly (**Figure 5.3B and Appendix 1, Supplemental Figure 5.4**). These viruses form a sister clade with those of the *Bornaviridae* and the *Nyamiviridae*, two mononegavirus families (488-490).

In the 3 BOAV and GAMV genomes, and in the genomes of Xincheng mosquito virus and Shuangao fly virus 2, there is a small ORF upstream of the L ORF (**Figure 5.3C**). These ORFS range from 123-162 nt and encode predicted proteins of 40-53 AA with CXXC motifs characteristic of zinc ribbon type zinc finger domains (491) (**Figure 5.3D**). By HHPRED analysis, similarity to various cellular zinc finger domains was detected. In addition to their phylogenetic placement, a defining characteristic of the viruses in this clade may be the presence of these small ORFs.

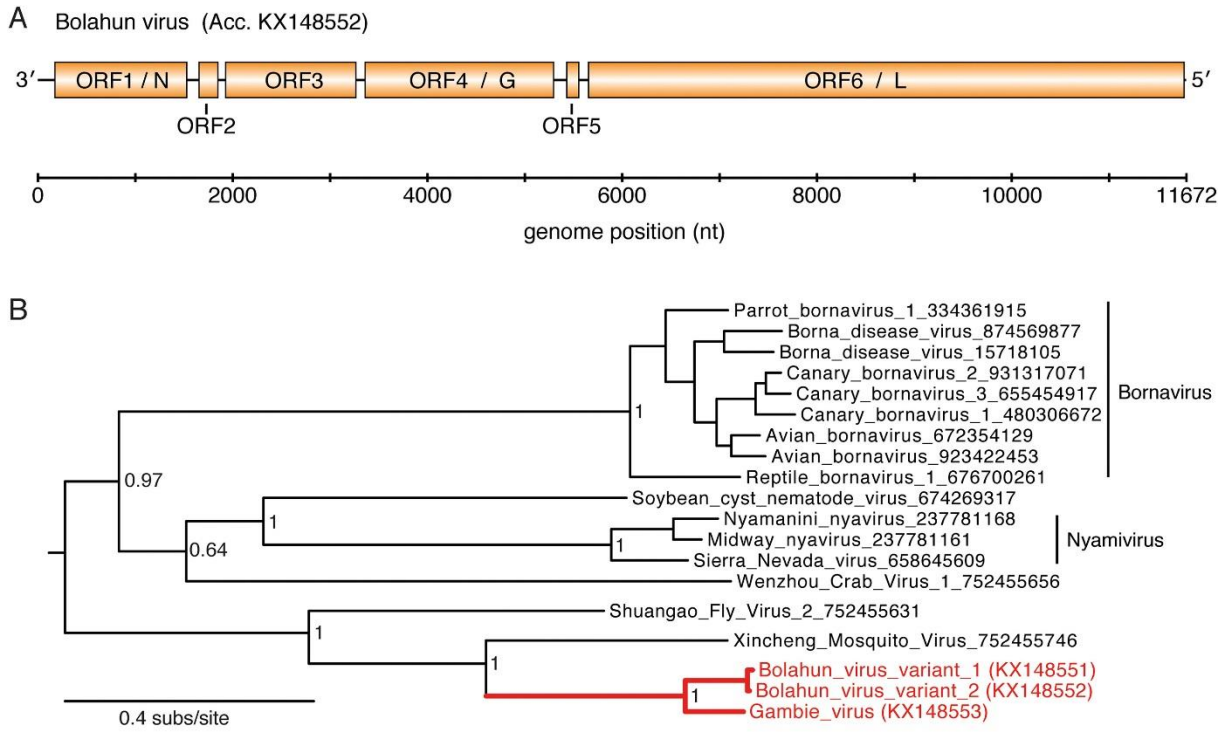

Figure 5.3 Bolahun and Gambie viruses. (A) Genomic organization of Bolahun virus. Predicted coding sequences are indicated. Gambie virus has essentially identical genome organization. (B) Bolahun and Gambie viruses cluster with bornaviruses, nyamiviruses, and arthropod viruses. RdRp-based Bayesian phylogeny. Posterior probabilities of select nodes are indicated. Phylogenies showing all mononegavirus sequences are shown in Supplemental Figure 5.4 **(Appendix 1)** (C) Viruses in this virus clade encode a predicted small zinc finger protein just upstream of the L ORF. Genome cartoons of Bolahun virus, Xincheng mosquito virus, and Shuangao fly virus 2. Open reading frames are labeled according to Li et al. (2015)(262). ORFs not annotated in KM817661 and KM817638 are outlined with dotted lines. Small ORFs predicted to encode Zn finger proteins upstream of L ORFs are colored purple. (D) Multiple alignment of proteins encoded by small ORFs highlighted in (C). Cysteine CXXC motifs characteristic of Zn finger domains are underlined.

Anopheles Totivirus

We identified a totivirus-like sequence in *An. gambiae* mosquitoes in Liberia **(Figure 5.4A)**. Totiviruses were historically only known to infect plant and protist hosts. However a growing number of reports have described totiviruses infecting or associated with arthropod hosts (492-498). Totiviruses have dsRNA genomes that typically have 2 overlapping ORFs that encode the viral capsid and RdRp proteins. We assembled the apparent coding-complete genome of this virus, which we designated Anopheles totivirus (AToV). There are two large ORFs in the AToV genome, but they are in the same reading frame and their translation is unlikely to involve ribosomal frameshifting **(Figure 5.4A)**. The first ORF encodes a predicted protein of 980 AA with similarity to sequences encoded in several dipteran genomes. The second ORF encodes the predicted viral RdRp of 1006 AA. In phylogenies based on alignments of totivirus RdRp sequences, AToV clusters with arthropod-infecting or arthropod-associated totiviruses **(Figure 5.4B)**.

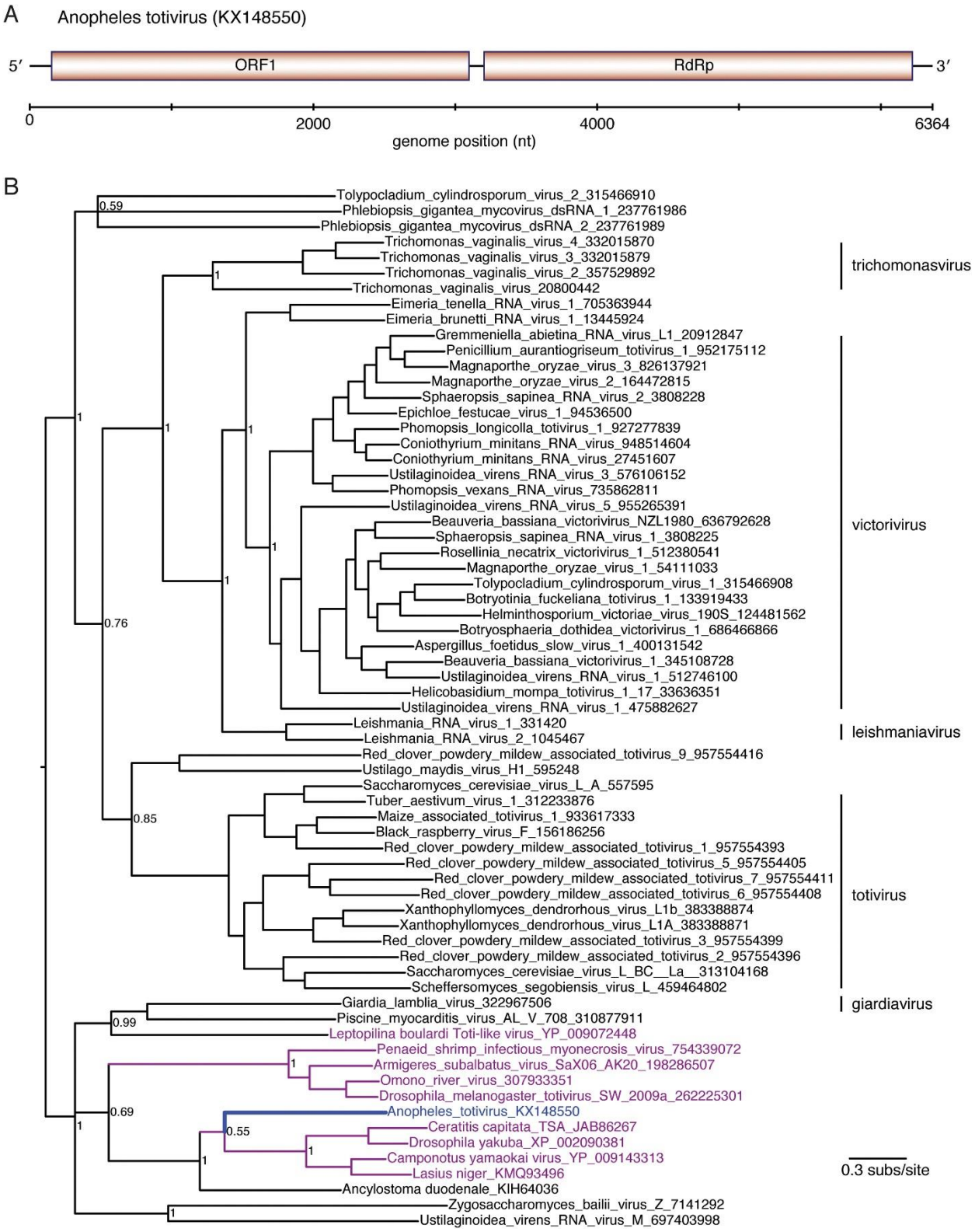

Figure 5.4 (A) Genomic organization of anopheles totivirus. The two large ORFs are indicated. Although the genome is depicted in the positive sense, it is presumed to be dsRNA. (B) Anopheles totivirus clusters with viruses identified in other arthropods. A multiple sequence alignment of totivirus RdRp protein sequences and related

sequences was used to create a Bayesian phylogeny as described in Materials and Methods. Phylogeny is arbitrarily rooted. Posterior probabilities of select nodes are indicated. Sequences associated with arthropods are colored purple. Clades corresponding to ICTV-classified genera are indicated.

Other virus and virus-like sequences

In addition, we identified a number of other putative virus-derived contiguous sequences (contigs) in our datasets **(Table 5.2 and Figure 5.5)**. Some of these are partial sequences and some appear to be coding complete. We restricted our analysis to contigs longer than 500 nt. In addition to the BOAV and GAMV sequences, we identified 5 additional partial mononegavirus sequences from one or more viruses present in various Liberian mosquito pools. Like BOAV and GAMV, the closest related sequences were to various mononegaviruses from Chinese mosquitoes **(Table 5.2)** (262). We also identified 6 sequences most similar to various positive-sense ssRNA viruses identified in wild-caught drosophila. In addition, we identified a number of sequences with similarity to various dsRNA viruses, including partitiviruses, chrysoviruses, and endornaviruses. These sequences were generally closest to sequences from dsRNA viruses identified in arthropods (499-501). The lower coverage and partial nature of these other viral sequences suggests that they might be less prevalent in the mosquito populations that we sampled, that viral nucleic acid is present at lower levels in mosquito midguts, or that sample processing and library preparation protocols may have reduced their representation in sequencing libraries **(Figure 5.5)**.

We also identified virus sequences that likely originated from laboratory contamination. These included West Nile virus (WNV) and Phasi Charoen virus (PCV) sequences, which were nearly identical to database sequences. Our laboratories routinely work with WNV, and PCV has been found in sequencing data from mosquito cell lines in our lab. Two sequencing pools contained reads to PCV sequences, and these were the only pools that had CO1 reads aligning to mosquito genera other than *Anopheles*, corroborating the designation of these sequences as laboratory contaminants.

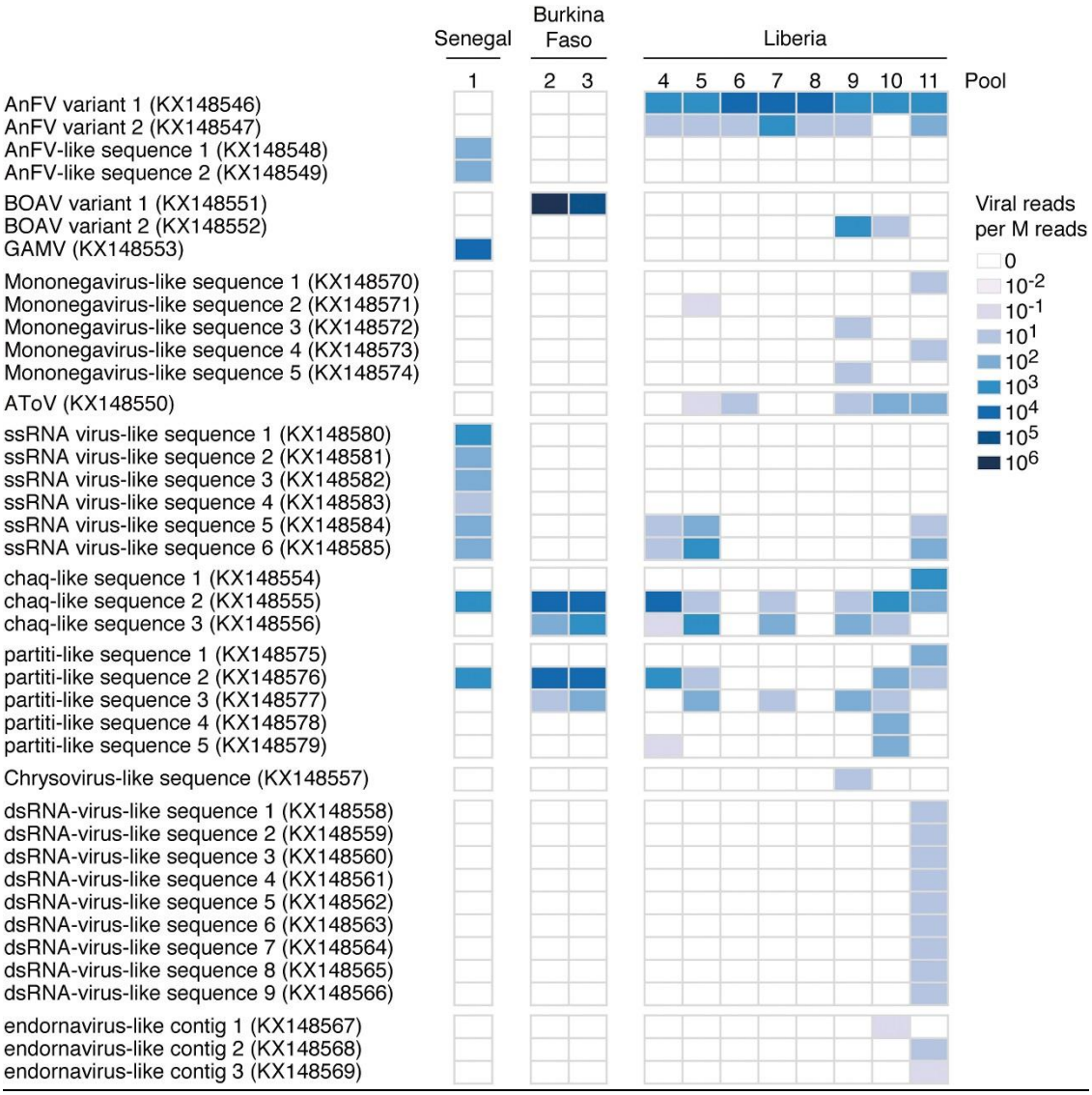

Figure 5.5 Abundance of virus and virus-like sequences in sample sets. The relative abundance of virus-mapping reads in each NGS dataset is shown (see Table 5.2). Virus-mapping reads per million unique filtered reads are shown. Genbank accessions of virus or virus-like sequences are indicated.

Table 5.2 Summary of identified virus sequences

Name [a]	Closest Related Sequence[b]	Seq. Length (nt)	Accession	% Identity[c]	Prelim. Class.
Anopheles flavivirus – variant 1	Palm Creek Virus (AGG76014.1)	10604	KX148546	40	*Flavivirus*
Anopheles flavivirus – variant 2	Palm Creek Virus (AGG76014.1)	10529	KX148547	40	*Flavivirus*
Anopheles flavivirus-like sequence 1	Nienokoue virus (YP_009041466.1)	6063	KX148548	48	
Anopheles flavivirus-like sequence 2	Parramatta River virus (YP_009164029.1)	2730	KX148549	34	
Anopheles totivirus	Camponotus yamaokai virus (YP_009143313.1)	6364	KX148550	30	*Totiviridae*
Bolahun virus – variant 1	Xincheng Mosquito virus (AJG39227.1)	11666	KX148551	39	*Mononegavirales*
Bolahun virus – variant 2	Xincheng Mosquito virus (AJG39227.1)	11672	KX148552	39	*Mononegavirales*
Gambie virus	Xincheng Mosquito virus (AJG39227.1)	11652	KX148553	38	*Mononegavirales*
Chaq virus-like sequence 1	Chaq virus (AKH40308.1)	1361	KX148554	38	
Chaq virus-like sequence 2	Chaq virus (AKH40308.1)	1342	KX148555	32	
Chaq virus-like sequence 3	Chaq virus (AKH40308.1)	1342	KX148556	33	
Partitivirus-like sequence 1	Penicillium aurantiogriseum partiti-like virus (YP_009182157.1)	1418	KX148575	37	
Partitivirus-like sequence 2	Penicillium aurantiogriseum partiti-like virus (YP_009182157.1)	1705	KX148576	38	

Partitivirus-like sequence 3	Penicillium aurantiogriseum partiti-like virus (YP_009182157.1)	1705	KX148577	38	
Partitivirus-like sequence 4	Heterobasidion partitivirus 8 (AFW17811.1)	1955	KX148578	35	
Partitivirus-like sequence 5	Grapevine partitivirus (AFX73019.1)	2078	KX148579	49	
Chrysovirus-like sequence	Anthurium mosaic-associated virus (ACU11563.1)	949	KX148557	39	
ssRNA virus-like sequence 1	Muthill virus (AMO03223.1)	3065	KX148580	32	
ssRNA virus-like sequence 2	Muthill virus (AMO03223.1)	1974	KX148581	38	
ssRNA virus-like sequence 3	Muthill virus (AMO03223.1)	1039	KX148582	29	
ssRNA virus-like sequence 4	Muthill virus (AMO03223.1)	634	KX148583	34	
ssRNA virus-like sequence 5	Blackford virus (AMO03220.1)	4642	KX148584	45	
ssRNA virus-like sequence 6	Buckhurst virus (AMO03221.1)	7413	KX148585	28	
dsRNA virus-like sequence 1	dsRNA virus environmental sample (AJT39583.1)	1886	KX148558	41	
dsRNA virus-like sequence 2	dsRNA virus environmental sample (AJT39584.1)	1434	KX148559	32	
dsRNA virus-like sequence 3	dsRNA virus environmental sample (AJT39583.1)	1230	KX148560	56	
dsRNA virus-like sequence 4	dsRNA virus environmental sample (AJT39584.1)	809	KX148561	44	

dsRNA virus-like sequence 5	dsRNA virus environmental sample (AJT39584.1)	735	KX148562	30	
dsRNA virus-like sequence 6	uncultured RNA virus (AGW51771.1)	664	KX148563	28	
dsRNA virus-like sequence 7	dsRNA virus environmental sample (AJT39583.1)	634	KX148564	62	
dsRNA virus-like sequence 8	dsRNA virus environmental sample (AJT39583.1)	596	KX148565	38	
dsRNA virus-like sequence 9	dsRNA virus environmental sample (AJT39589.1)	588	KX148566	41	
Endornavirus-like sequence 1	Phaseolus vulgairs endornavirus 2 (BAM68540.1)	2858	KX148567	22	
Endornavirus-like sequence 2	Rhizoctonia cerealis endornavirus 1 (YP_008719905.1)	672	KX148568	39	
Endornavirus-like sequence 3	Bell pepper endornavirus (YP_004765011.1)	514	KX148569	39	
Mononegavirus-like sequence 1	Xincheng Mosquito virus (AJG39226.1)	964	KX148570	70	
Mononegavirus-like sequence 2	Xincheng Mosquito virus (AJG39227.1)	948	KX148571	34	
Mononegavirus-like sequence 3	Wuhan Mosquito virus 9 (AJG39215.1)	878	KX148572	56	
Mononegavirus-like sequence 4	Wuhan Mosquito virus 2 (AJG39297.1)	869	KX148573	39	
Mononegavirus-like sequence 5	Wuhan Mosquito virus 2 (AJG39331.1)	550	KX148574	45	

(a) Virus and virus-like contigs with length >= 500 nt are shown.
(b) Closest relative determined by highest scoring alignment from BlastX search of NCBI nr database.
(c) Pairwise percent amino acid identity of BlastX alignment to closest relative

Prevalence of viruses in wild mosquito populations

We performed RT-PCR on an independent set of samples to validate our NGS finding and to measure the prevalence of the viruses we found in Liberian mosquitoes, namely AnFV, AToV, and BOAV **(Table 5.3 and Figure 5.5)**. These samples were collected from additional Liberian villages ~2 years after the initial sample sets were collected **(Table 5.1).** In the villages, prevalence for AnFV and AToV varied between 0-20% and 0-6.7%, respectively. In total, AnFV RNA was detected in 12% of sampled mosquitoes and AToV RNA in 1.3%. BOAV, which was detected in fewer NGS datasets **(Figure 5.5)**, was not detected in any individual M-DBS from this independent set of samples.

Table 5.3 Prevalence of viruses in Liberian mosquitoes

	Village C			Village D			Village E			Village A			Village F			Total
Virus	+[a]	N[b]	% pos	+	N	% pos	+	N	% pos	+	N	% pos	+	N	% pos	
AnFV	2	15	13.3	3	15	20	2	15	13.3	0	15	0	2	15	13.3	12%
BOAV	0	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0%
AToV	1	15	6.7	0	15	0	0	15	0	0	15	0	0	15	0	1.3%

(a) +: number positive blood spots by RT-PCR
(b) N: number individual mosquito blood spots tested

Discussion

In this study we sampled anopheline mosquitoes from Liberia and Senegal and a laboratory colony derived from Burkina Faso. We identified a number of previously undescribed virus and virus-like sequences. For some of these, we detected the same or related virus genotypes in mosquitoes that were collected in multiple countries and in mosquitoes that were sampled up to 2 years apart. Thus, infection by these viruses appears to be geographically widespread and temporally stable in mosquito populations.

It is likely that many of these sequences derive from viruses that genuinely infect these mosquitoes, although this remains to be proven. However, mosquitoes have a diverse microbiome and it cannot be ruled out that some of these sequences are derived from viruses infecting commensal organisms. And, because both male and female mosquitoes feed on plant nectar, and females take blood meals, it is also possible that some of these sequences have a dietary or environmental origin. These sequences do not appear to derive from integrated endogenous viral elements, given their absence in the *An. gambiae* genome, non-uniform presence in mosquitoes, and intact ORFs (502). Several pieces of evidence point to AnFV, BOAV, and GAMV as legitimate mosquito viruses, including their phylogenetic placement and genomic characteristics. While groups of flaviviruses and mononegaviruses are accepted as insect-infecting viruses, totiviruses were classically thought to infect plants and fungi. AToV groups phylogenetically with totiviruses described from various arthropods, however we cannot rule out the possibility that AToV infects commensal fungi or protists of *Anopheles* mosquitoes. In any case, AnFV, BOAV, GAMV, and AToV represent legitimate viral constituents of the microbiome of *Anopheles* species mosquitoes.

Although we identified several virus sequences that likely derive from laboratory contamination, several lines of evidence support the conclusion that the other virus and virus-like sequences we identified were not contaminates. First, we observed sequence variability: for example, the AnFV sequences from the 8 Liberian datasets, which were 99.5-100% identical to the consensus sequence, had distinct sets of single nucleotide variants **(Appendix 1, Supplemental Figure 5.2)**. Second, sequences had variable abundances and non-uniform presence in our datasets **(Figure 5.5)**. Third, we did not detect these sequences in other NGS datasets generated in our labs, nor were they present in online sequence databases.

The AnFV sequences we identified represent the first description of flaviviruses from anopheline mosquitoes. With the exception of tick-borne flaviviruses and no known vector flaviviruses, these

viruses have been described exclusively from culicine mosquitoes (503). The sequence of AnFV is highly divergent from other known flaviviruses, including from other cISFVs (**Figure 5.2C and Appendix 1, Supplemental Figure 5.3**). This is not unexpected, as cISFVs tend to group by host mosquito genera (436). The description of AnFV further exhibits the diversity of the genus *Flavivirus* and supports the hypothesis that flaviviruses are under sampled (504, 505). It remains to be determined how AnFV will contribute to the understanding of the evolution of cISFVs, especially in regards to co-divergence (504). In any case, the additional genomic and phylogenetic information provided by this study will contribute to a more complete picture of flavivirus biology and evolution.

Whether or not any of these viruses impact the biology of *An. gambiae* or other mosquitoes remains to be determined, as does their potential utility as transmission reducing agents. The microbiome of mosquito species has been demonstrated to alter vector competence (428, 506-508). But the effect of ISVs on this is not as well studied and published results are mixed. Kenney et al. demonstrated that co-infection by Nhumirim virus, a dual host associated flavivirus, markedly reduced the production of West Nile virus and other pathogenic flaviviruses in cell culture (270). However, other studies measuring the impact of ISV co-infection have had less promising results (267, 268, 271). The identification of these anopheles viruses will enable researchers to begin to assess their impact on the transmission of *P. falciparum* parasites and other anopheles-vectored human pathogens. Isolation of these viruses in cell culture and will be an important first step in this effort.

Although we found a number of viruses that appear to infect *An. gambiae*, there is little doubt that this represents only a small fraction of such viruses. In fact, Carissimo and co-authors identified a cypovirus and a dicistrovirus in colony *Anopheles* mosquitoes and in wild mosquitoes in Senegal and Cambodia (470). We mainly sampled mosquito midguts and midgut contents from adult mosquitoes from West Africa, and it is possible that sampling of other tissues will reveal viruses with different tropisms. And, although in theory sequencing RNA would detect transcripts from DNA viruses it is

notable that we only identified sequences of RNA viruses. It is likely that sequencing DNA-derived libraries would improve detection of DNA virus sequences. Additional sampling of different mosquito tissues from various developmental stages, and from different geographic locations, as well as utilizing alternative sequencing methods will continue to increase our understanding of the *An. gambiae* virome, and the impact of commensal viruses on mosquito biology.

Chapter 6: Concluding Remarks

Clearly, mosquito-borne diseases, and infectious diseases in general, are not going away. Emerging and re-emerging infectious diseases represent a constant and looming threat. Continued work is necessary in order to combat this threat, starting with the most basic aspect of disease control, knowing the identity of the pathogen in question. Surveillance, preferentially before human cases are being diagnosed, remains the most effective way to determine which pathogens are circulating in a population at any given time.

West Nile virus surveillance and control policy

Conclusions

Through the collection of eight years of routine mosquito/WNV surveillance in the City of Fort Collins, we have determined 1) while fluctuations in mosquito abundance, infection rate, and VI exist within and between seasons, a general trend emerges for both *Cx. tarsalis* and *Cx. pipiens*, 2) the City of Fort Collins is *not* homogenous for the three entomological risk indices measured, and 3) increases in VI are correlated with increases in human WNV cases. These data allow us to determine that trends in mosquito populations are relatively predictable during any given season, however the size of mosquito populations will likely vary substantially. In regards to mosquito control, our data demonstrate that the City of Fort Collins should not be looked at as a single unit, and instead should be divided into more logistically relevant zones. This will affect how mosquito control policy is determined, allowing a more targeted approach to high risk areas within the city. Previous control policy required unattainable risk indices to be achieved in the entirety of the city before emergency action was initiated. These high risk

indices were driven by individual zones and rarely applied to the entire city. This policy resulted in late and ineffective adulticide spraying, possibly putting people at risk for WNV transmission. As well, our data show that VI is correlated with risk of human WNV transmission, and zones with the highest relative risk mirror the zones with the highest entomologic risk.

Future Directions

In addition to continued routine mosquito surveillance within the City of Fort Collins, it should be determined how well the VI predicts risk of WNV transmission to humans. The VI appears to indicate WNV transmission risk to humans with a lag time of about two weeks. In fact, from 2006-2013, 85.2% of the time there was a human case of WNV, there was a VI 2 weeks prior to the onset date (Data Not Shown). This demonstrates that the presence of a VI alone is indicative of WNV transmission to humans, however a more specific VI needs to be determined in order to better inform local policy. As well, we demonstrated that Fort Collins is homogenous based on four operationally relevant zones. Likely, this can be adjusted to be more fluid, and entomological risk indices can be determined week to week on an even finer scale within the city. WNV is endemic in Northern Colorado, so there should be no shortage of data to test this hypothesis.

Xenosurveillance

Conclusions

By a series of laboratory and field based experiments, in combination with previous work conducted in our lab (296), we have characterized xenosurveillance methodology and demonstrated the ability of this technique to identify human pathogens in mosquito blood meals. Laboratory experiments showed that mosquitoes could be fed a “pathogenemic” blood meal at or below clinical concentrations and RNA from those pathogens can be detected for up to 24 hours using species-specific RT-PCR. Our field experiments demonstrated the sensitivity of xenosurveillance. Xenosurveillance can detect the

same pathogens at a similar level compared to a more traditional sampling technique of human finger prick blood. As well, field, molecular, and computational methodologies have been improved throughout the course of these experiments. Importantly, we have shown how essential proper scrutinization of NGS data is for proper data analysis and elimination of false positives. Xenosurveillance has the potential to supplement existing routine biosurveillance programs by contributing a novel stream of data on pathogens circulating in human populations.

Future directions

While we have demonstrated the feasibility and utility of xenosurveillance, it needs to be scaled-up in order to be applicable. Pathogenemia is often transient, and emergence of new pathogens are rare events, meaning the size and scale of a surveillance methodology needs to be increased to detect epidemics. The epidemiological use of xenosurveillance remain to be determined. Current methodology allows us to say a mosquito fed on a person that has detectable levels of pathogen nucleic acid in their blood, but nothing more. Likely that person resides in the house the mosquito was caught, however that is not always the case. At best, we can determine that someone within the vicinity of the home the mosquito was captured in is positive for a pathogen, likely putting the unit at the scale of the village. The above listed technique utilizes NGS and computation biology, luxuries not afforded in areas where xenosurveillance is relevant. Therefore, point-of-care identification needs to be employed to make this technique monetarily realistic. Given the current state of NGS, for example platforms like Oxford Nanopore's MinION (509), the unbiased approach of NGS coupled with extremely reduced cost is on the horizon. These types of technologies will allow xenosurveillance to be implemented throughout the world.

Conclusions

The use of NGS on arthropod samples has revealed an enormous diversity and richness of insect-specific viruses. Data collection from *An. gambiae* mosquitoes from multiple locations in West Africa showed these mosquitoes contain their own diverse array of RNA, insect-specific viruses. We were able to assemble complete coding sequences of 6 viruses and over 25 virus-like segments. Based on library preparation techniques and sequencing strategies, we have likely identified only a small fraction of commensal viruses that make up the *An. gambiae* virome. Interestingly, we have also identified the first Flavivirus genome detected in *An. gambiae*. Insect-specific flaviviruses had previously only been identified in Culicinae mosquitoes, and the discovery of these viruses further demonstrates the wide host range of flaviviruses.

Future directions

As previously stated, these viruses likely only represent a small faction of the virus richness and diversity in *Anopheles* mosquitoes. As well, mosquito midguts were the only tissues sampled. Sampling other tissues and DNA from these tissues may reveal an even greater assortment of viruses infecting this medically important insect. Also, AnFV should be more thoroughly characterized in order to assess the effect of co-infection in *Anopheles* mosquitoes with AnFV and *Plasmodium*. Isolation of this virus will allow these experiments to be conducted, as well as experiments regarding effects on life history and tissue tropism of this virus.

Collectively, this body of work represents an advancement of traditional surveillance techniques, development and validation of novel surveillance techniques, as well as a characterization of the microbiome of *An. gambiae* mosquitoes.

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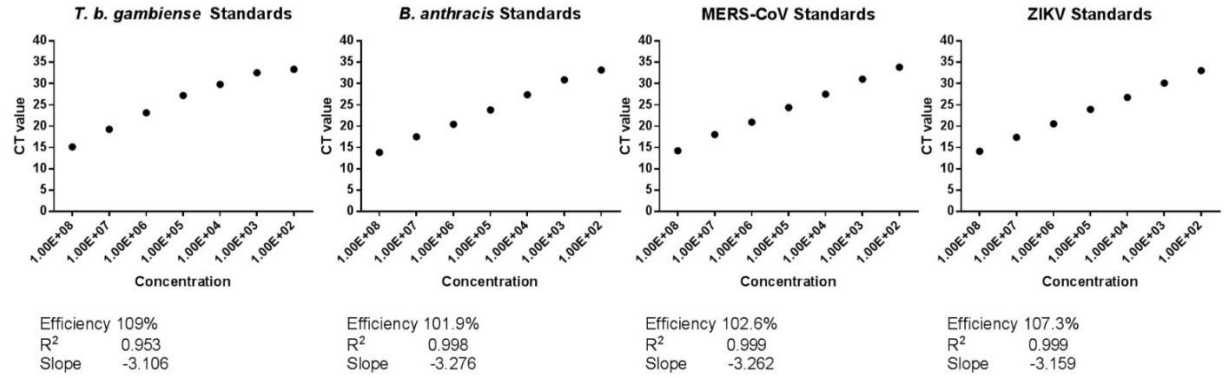
Appendix

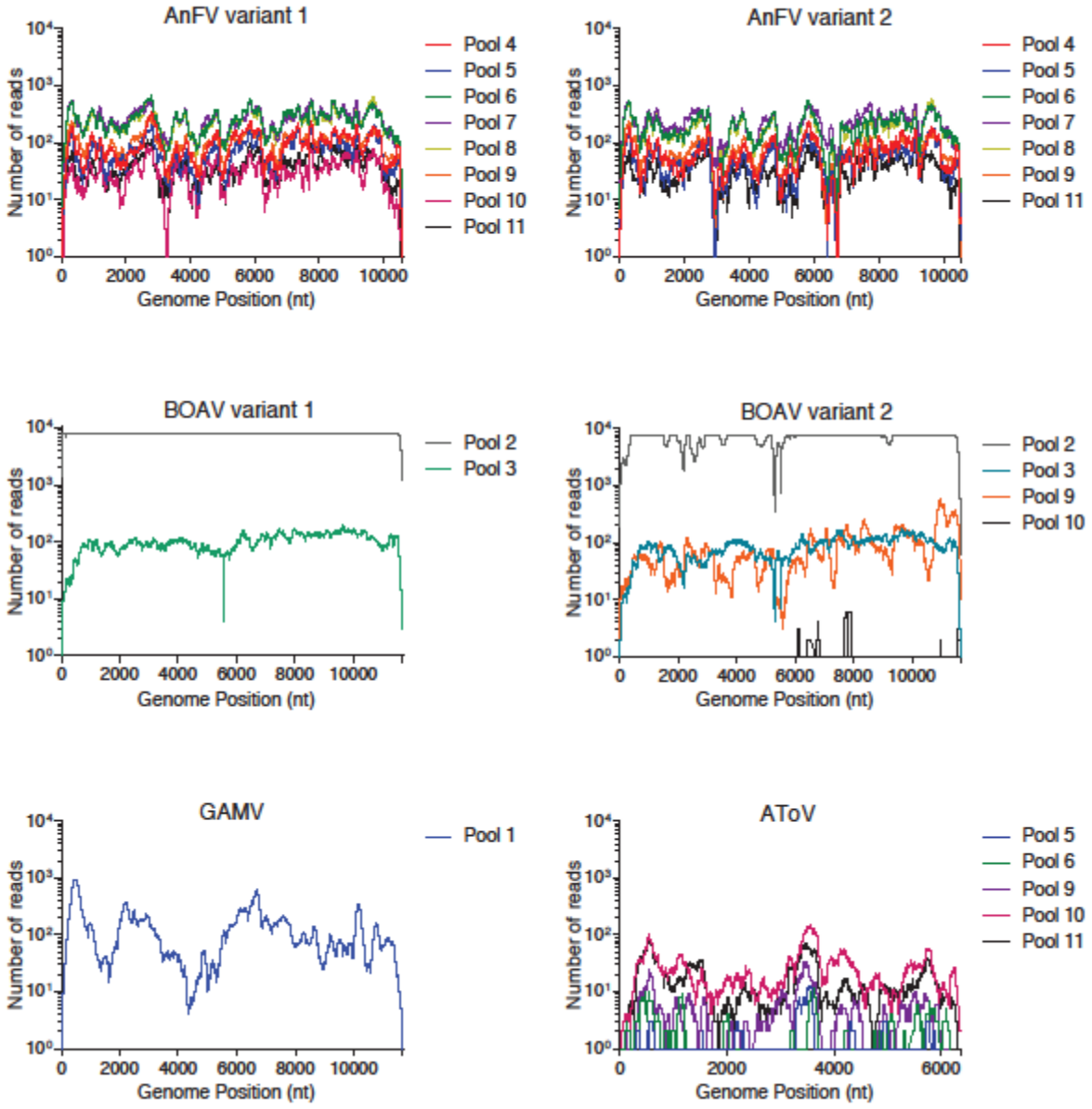
Supplemental materials



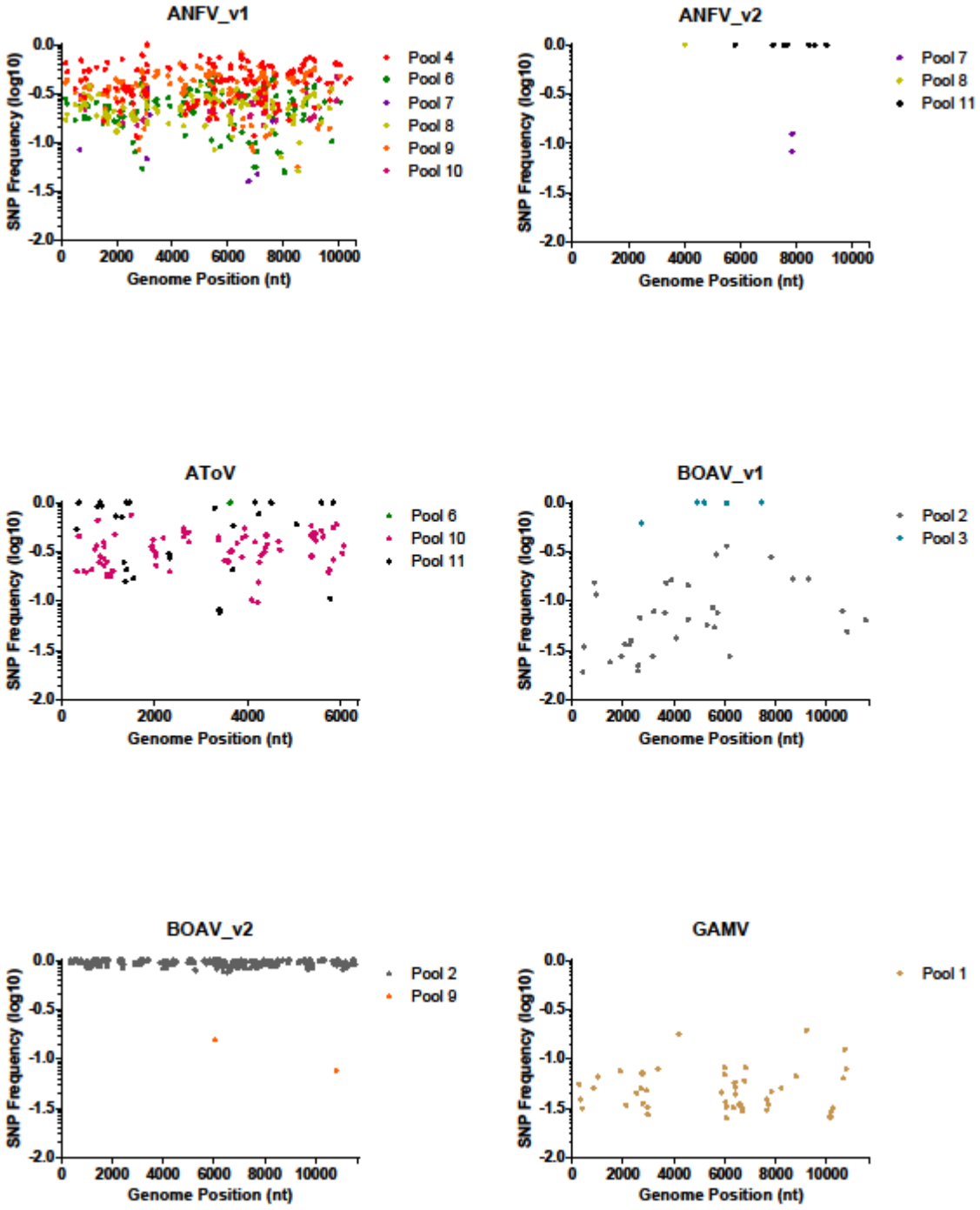
Supplemental Figure 3.1 Standard curves and PCR data for each RT-qPCR assay.

Supplemental table 4.1 Primer Sequences used for PCR confirmation of NGS data

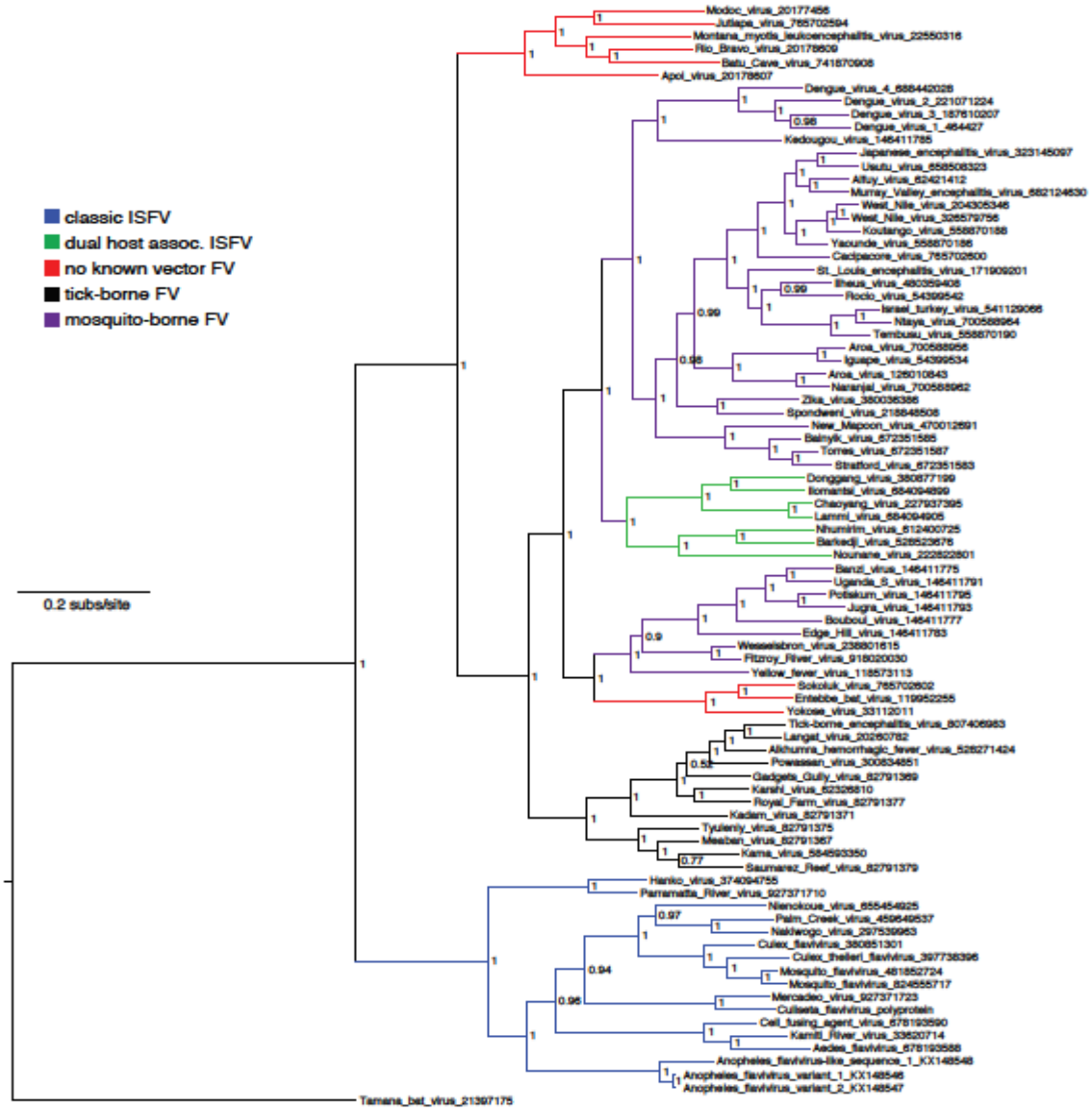
Primer	5'-3' Sequence	Genome Position	Product Size
GBVC_F	GCCAGCAACTGTTTGACCTG	8,204-8,588	384
GBVC_R	TGATGACCCACCGTGTGATG		
HBV_F	AGAGGCAGGTCCCCTAGAAG	2,354-2,414	60
HBV_R	GCGGCGATTGAGATCTTCGT		



Supplemental Figure 5.1 Coverage levels of virus sequences in sequencing datasets. Coverage was calculated by mapping quality filtered, duplicate-collapsed reads to viral sequences using Bowtie2. Coverage was calculated using the mpileup function in SAMTOOLS (407)



Supplemental Figure 5.2 Single nucleotide variant frequency in NGS datasets. The frequency and genome position of SNVs in individual NGS datasets is indicated. Since our datasets derived from mosquito pools, this variation could derive from intra or inter-host variation, or both. Variant frequencies were calculated using the using the call function in LoFreq with parameter -a 0.01 (510)



Supplemental Figure 5.3 AnFV clusters phylogenetically with classic ISFVs. A multiple sequence alignment of flavivirus NS5 protein sequences was used to create a Bayesian phylogeny as described in Materials and Methods. Clades are labeled and colored according to Blitvich and Firth (2015) (436). Posterior probabilties of select nodes are indicated. The phylogeny was rooted on the branch to Tamana bat virus.

Supplemental Figure 5.4 Mononegavirales phylogeny. A multiple sequence alignment of RdRp sequences was used to create a Bayesian phylogeny as described in Materials and Methods. Clades corresponding to select virus families and genera are indicated, as is the position of Bolahun and Gambie viruses. Phylogeny is arbitrarily rooted at midpoint of branch to chuviruses.

Supplemental Table 5.1 Primers used for PCR amplification of *Anopheles* viruses

Primer	5'-3' Sequence	Genome position	Product Size
AnFV NS5 F	CGTATCGGTCGCGTTCTGTA	7565-7902	337
AnFV NS5 R	GGATCGCTTTCGCCAATGTC		
BoAV RDRP F	GCGTCCTGATGATCAAGGTT	6432-6642	211
BoAV RDRP R	TGGTCCAGGAGCATTTCTTC		
AToV F	CGCTGTTGTGTGAGTGGTTG	5312-5637	325
AToV R	ACTCGCCAACGTGTACCATT		