Master of Public Health Field Experience Report

PUBLIC AND ANIMAL HEALTH IMPLICATIONS OF BLOOD FEEDING VECTORS

by

NATHANIEL KAPALDO
MPH Candidate

submitted in partial fulfillment of the requirements for the degree

MASTER OF PUBLIC HEALTH

Graduate Committee:
Lee W. Cohnstaedt, PhD
James W. Carpenter, MS, DVM, Dipl. ACZM
Natalia Cernicchiaro, DVM, MS, PhD
Michael Dryden, DVM, PhD, Dipl. ACVM

Field Experience Site:
United States Department of Agriculture
23 July – 21 August 2015
Department of Public Health Fort Riley Medical Activity
13–23 July 2015

Field Experience Preceptors:
Paul D. Benne, MD, MPH, COL (US Army)
D. Scott McVey, DVM, PhD, Dipl. ACVM

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Summary

Arthropod vectors are capable of transmitting pathogens (e.g., bacteria, helminths, protozoa, and viruses) between mammals which may result in numerous diseases affecting both human and animal populations. Arthropod transmitted pathogens are responsible for 17% of infectious diseases and 20% of emerging infectious diseases worldwide. Recent emergence of traveler-associated vector-borne diseases (VBD) in North America (e.g., Chikungunya and Zika viruses) affecting human health, attention to vectors and to the pathogens that they transmit has greatly increased. Effective and efficient monitoring of vector populations is the logical first step in understanding disease transmission risk and avenues for initiating vector control.

At military installations, service dogs and handlers employed by the United Stated (US) Army, are potentially at an increased risk from VBDs due to protracted entomological exposure during their work and training. These VBDs pose significant risk to the health and welfare of military working dogs (MWD) and additional risk to military personnel through both zoonotic transmission (i.e., animal to human pathogen transmission) and through MWDs acting as pathogen reservoirs able to infect local arthropod vector populations. Monitoring MWD kennel and training areas is currently not employed by the US military and may benefit both the canine handler's and MWD health.

Within zoos, risk of VBD transmission is potentially increased due to unique biodiversity and static animals within customized enclosures. Zoos provide a great diversity of microhabitats that are capable of establishing both highly abundant and diverse populations of disease vectors (e.g., mosquitoes and biting flies). The impact of high biting insects populations on animals kept static in enclosures is unknown; however, recent research eludes to significant entomological risk. Risk may be in the form of both increased risk of disease transmission and increased distress due to significant biting pressures experienced by zoo animals. There is great need for an effective and efficient vector monitoring program that may be fitted to unique zoo environments and may be used annually. Information gained may guide our efforts making future monitoring and control recommendations.

Key words: mosquito surveillance, vector-borne disease, zoonotic, zoo, biting pressure, animal welfare

Table of Contents

Table of Tables

Table of Figures

Chapter 1 - Scope of Work

This report accounts activities performed while completing field experiences and capstone projects at both Fort Riley Department of Public Health (FRDPH) and at the United States Department of Agriculture (USDA) in Manhattan, Kansas. The experiences were completed during the summer of 2015 and the capstone project for the USDA was completed throughout 2015 and through the Spring of 2017. While at FRDPH, I worked under the supervision of Dr. Paul Benne LTC, MC, Chief of the FRDPH, Fort Riley, Kansas. While at USDA, I worked under the supervision of D. Scott McVey, DVM, PhD, Dipl. ACVM who is the Research Leader of the Arthropod-Borne Animal Diseases Research Unit (ABADRU) within the Agriculture Research Service (ARS) of the USDA. The majority of time spent at USDA was under the leadership of Lee Cohnstaedt, PhD, senior research entomologist in ABADRU.

The field experience at FRDPH consisted of rotations through various branches (detailed below). In addition, a project was completed looking into developing mosquito monitoring and population control guidelines surrounding military working dog (MWD) training and kennel areas. The field experience while at USDA consisted of working with entomologists, veterinarians, and a molecular biologist to look at the public and animal health implications of blood feeding insect vectors within a zoological park and in doing so, developing a flexible surveillance protocol for all United States zoological parks.

The purpose of this report is to:

1. Describe the field experience at both FRDPH and USDA.
2. Present the write up for the FRDPH project on vector monitoring and control in regard to MWDs.

3. Present the project write up for developing a mosquito monitoring program for use in a zoological park.

This report will also discuss connections and relevance to the MPH core competency courses and the emphasis area of my field experience (i.e., how the core competencies relate to insect vector monitoring and population control).

Acknowledgements

I would like to thank Dr. Lee Cohnstaedt, Dr James W. Carpenter, Dr. Michael Dryden, Dr. Natalia Cernicchiaro, Dr. Dustin Swanson, Dr. Steve Dritz, Elin Maki, Dr. Paul Benne, Dr. Scott McVey, Megan Cabot, the entire staff of the Sunset Zoo, and the entire Fort Riley Department of Public Health as well as the staff at USDA for allowing me to complete this work and assisting me. I would not have been able to complete this without any one of these individuals.

Chapter 2 - Learning Objectives

Learning objectives for each field experience were detailed and outlined on the Field Experience Agreement Form. The objectives and activities performed as part of each objective are described below:

1. Observe and learn the basic role of each department within the FRDPH, establish a basic understanding of public health methods and applications, and learn about vector-borne diseases of public health importance to MWDs.
    a. Participated in routine mosquito monitoring at the FRPHD's Entomology Service.
2. Use information gained while at FRDPH to help prepare both the Fort Riley MWD write up and the Sunset Zoo Vector study.
    a. A literature review was conducted to review the basics of vector surveillance and control, particularly surrounding canine specific issues (e.g., canine associated vectored pathogens).
3. Establish a basic understanding of insect vector biology and ecology.
    a. Literature review on vector biology and ecology.
    b. Information gained on insect vector biology and ecology was applied to developing a mosquito monitoring program for use in the Sunset Zoo study.
    c. Investigated mosquito community dynamics within the Sunset Zoo.
4. Understand vector surveillance and control methods.
    a. Shadow entomologists at both Fort Riley and the USDA while performing vector surveillance and control methods.

b. Constructed three interceptor barriers used at the Sunset Zoo. These barriers are a common method for vector control in and out of a given area.
c. Learned about attractive toxic-sugar baits (ATSBs) and implement similar methodology to investigate vector movement within the Sunset Zoo.

Chapter 3 - Field Experiences: Fort Riley Department of Public Health and United States Department of Agriculture

Fort Riley Department of Public Health

Field experience at the FRDPH consisted of 40 hours shadowing personnel at different departments. While at Fort Riley, I rotated through the Veterinary Services section, Army Wellness Center, Army Hearing Program, Environmental Health section, Occupational Health section, and the Industrial Hygiene section. Below is a concise review of the field experience activities while at each section.

1. Veterinary Services (VS) – The mission of the US Army Veterinary Corps is to protect the US Soldiers and MWDs and support the National Military Strategy of the US. The VS accomplish their mission through the provision of public health services. The VS is responsible for the inspection and enforcement of food safety codes, laws and regulations. Additionally, their mission is accomplished through veterinary medical and surgical care of both civilian and military working and research animals. Listed below are activities performed during this field experience:
    a. Participated in numerous food sanitation inspections at both food preparation locations in dining facilities, day care facilities, and hospitals. Topics I learned:
        i. Inspection of food products upon receipt and in storage for compliance with food safety codes, laws, and regulations.
        ii. Evaluate packaging, packing, and marketing requirements and if facilities were in compliance.
        iii. Make recommendations regarding any violations observed and halt distribution of food products if determined to be compromised.

b. Animal preventive medicine:

    i. Participated in routine health care of both civilian owned animals and MWDs (e.g., administer vaccinations, assess for infectious and zoonotic disease, and prepare both interstate and international travel certificates).

    ii. Complete canine-human bite report cases in Fort Riley.

    iii. Perform childcare facility inspections for animals kept as classroom pets

2. Army Wellness Center (AWC) – The mission of the AWC is to provide both diagnostics and counseling concerning Soldier's overall health to better improve the strength of the US Army. The following are areas of emphasis covered while at the AWC:

    a. Army Wellness Center's (AWC) goals, standards, and quality assurance rationale.

    b. Health assessments of Soldiers (e.g., explain the purpose of assessments to Soldiers, identify modifiable risks and those that are non-modifiable in their lives, and educate Soldiers on the primary risk factors Soldiers experience in their occupation).

    c. Physical fitness testing of Soldiers (e.g., explain the physical and physiologic benefits of exercising and staying active).

    d. Healthy nutrition and metabolic testing of Soldiers (e.g., discuss basic biologic needs of the human body, discuss basic terms useful in Soldier's tracking their personal health such as obesity, overweight, body mass index, percent body fat, and body composition, and additionally, and explain guidelines for caloric and nutritional intake).

    e. Stress management of Soldiers (e.g., conduct basic assessment of Soldier's stressors within their lives and describe methods for combatting the negative

effects of chronic stress such as the benefits of using relaxation techniques throughout each day).

3. Army Hearing Program – the mission of the Army's hearing program is to ensure relevant regulations held by the Department of the Army are supported within the functional units of the Army.
    a. Review of various products available for Soldiers and programs in place to ensure proper fitting and proper usage of ear protection equipment.
    b. Discuss responsibilities of Army leadership in ensuring these regulations and equipment are in place and available to Soldiers.
4. Environmental Health Section
    a. Complete food sanitation inspections (i.e., human sanitation within facilities).
    b. Water quality surveillance (e.g., test water samples for biologic and heavy metal contaminants on routine schedules).
    c. Vector surveillance (e.g., perform vector monitoring and control throughout Soldier working areas on Fort Riley, discuss protocols in use for vector monitoring and control [e.g., biologic control]).
    d. Complete literature review regarding vector-borne pathogens for which MWDs and their handlers are at potential increased risks of acquiring. Investigate basic control mechanisms of vectors surrounding enclosed animals or training areas (e.g., kennel areas). Investigate the use of barrier type vector control methods for use in both the context of MWDs and a zoological park animal enclosure.

United States Department of Agriculture, Agriculture Research Service - Arthropod-Borne Animal Diseases Research Unit

Chapter 5 will summarize the focus of this project (below); however, other studies not covered in Chapter 5 which I conducted while at ABADRU are as follows:

1. **Using barriers to reduce contact with insects.**[1]

Thirty meter long barriers were constructed and placed in three locations along Sunset Zoo's peripheral fences, as illustrated in Figures 1 and 2. The concept of these barriers are to intercept insects of interest from passing directly through into an area. Regardless of size, insects will not fly through these barriers; however, they will land and either walk up the fence, walk through a hole, and fly off or in the case of larger insects, will walk up the fence to the top. In either case, the short contact time when the barrier is impregnated with pesticides is all that is needed to prevent small insects from moving past the barrier. Additionally, the majority of mammalian host-seeking insects travel within 1.5 m from the ground and thus a barrier of height greater than this will inhibit the majority of insect movement.[2]

For the barriers constructed in this project, a gutter on the top was constructed (i.e., an area of overhanging mesh netting) to inhibit insects from flying away once walking to the top or flying to the top to get over. This gutter was used as a collection area to observe the diversity of insects collected by these nets, and therefore determine potential implications and sensitivity to all insects of treated barriers when employed. These interceptor traps were in place for two 14-day-periods during which they were sampled and observed twice daily. Additional research in the form of a formal study needs to be employed with these barriers to determine the tangible effects and to determine how efficacious these barriers would be in targeted vector control around zoo animal enclosures. Additionally, these vertical barriers were used as an artificial

resting site for blood-engorged mosquitoes. These mosquitoes were used for the project, described in the following chapter, which aimed to identify the blood meal host of origin.

References:

1. Melhorn H. Arthropods as Vectors of Emerging Diseases. In: Impact of Insecticide-Treated Nets on Insects of Medical and Veterinary Relevance. Berlin: Springer; 2012.
2. Gillies MT, Wilkes TJ. The effect of high fences on the dispersal of some West African mosquitoes (Diptera: Culicidae). Bulletin of Entomological Research. 1978 Sep 1;68(03):401-408.

Figure 1. Images A and B above illustrate one of three 30 m long un-treated mesh netting interceptor barriers that were installed along three Sunset Zoo boundaries at the Sunset Zoo. The barriers were used for evaluation of insect diversity that may be affected by the institution of like interceptor traps that were impregnated with a pesticide around either animal enclosures or the Sunset Zoo.

Figure 2. Image above illustrates the locations of three interceptor traps (yellow bars) placed around the Sunset Zoo's boundaries during the summer of 2015. Interceptor trap 1, along the Northern boundary separating the Sunset Zoo from the non-human primate exhibits. Interceptor trap 2, along the Southwestern boundary separating the Sunset Soo from the riverine area from large cat and avian enclosures. Interceptor trap 3, along the Southeastern boundary separating a forested area from ungulate enclosures in the Sunset Zoo.

2. Determining the blood feeding ecology of hematophagous insects within the Sunset Zoo

Numerous hematophagous insects are widely reported as potential vectors and, consequently, their life cycles and feeding behaviors have become the focus of many pathogen transmission studies. Polymerase chain reaction (PCR) amplification of conserved mitochondrial vertebrate genes can be used for host identification of blood meals from engorged mosquitoes.

Mitochondrial Cytochrome oxidase subunit I (COX1) and 12s ribosomal RNA (12s) genes are used to positively identify host species of mosquitoes sampled from the Sunset Zoo (SSZ; Manhattan, KS). This used mitochondrial gene identification to investigate local vector-host preference. Characterizing vector-host interactions in a zoo setting establishes at-risk animals and zoonotic agent reservoirs aiding in preventative veterinary medicine, species conservation, and targeted insect vector control. Proof of concept of the identification of blood meal species origins were conducted; however, due to time constraints full identification of blood meals was unable to be completed.

Field work consisted of the use of the Biogents-Sentinel (BG-S) trap (www.bg-sentinel.com, Regensburg, Germany) with BG-Lure cartridges and the CDC ultra-violet light traps(Trap Model 1212, John W. Hock Company, Gainesville, FL). Interceptor traps of at least 30 m of Vestergaard (Frandsen, Switzerland) untreated netting was cleared by aspiration at sunrise and sunset for a 30 day period the month of June and July. Collections stored in 70% ethanol were sorted with dissecting microscopes to separate out hematophagous insects. Blood fed insects were transferred to 100% ethanol for storage and later identified to genus/species.

Molecular work was completed with the use of DNeasy Tissue Kit (Qiagen) protocol was used for DNA extraction and products quantified with Nanodrop. All samples underwent PCR

amplification of both 12s and CO1 genes.[1,2] The PCR amplification for 12s genes was carried out for 25 cycles (30 s denaturation at 94 °C, 30 s primer annealing at 60 °C, and 1 min extension at 72 °C) with 2min initial denaturation step at 94 °C and a 10 min final extension step at 72 °C.2.[1] The PCR amplification for COX1 genes was carried out for 35 cycles (30 s denaturation at 94 °C, 50 s primer annealing at 50 °C, and 1 min extension at 72 °C) with 1 min initial denaturation step at 95 °C and a 5 min final extension step at 72 °C.3.[2] PCR samples were cleaned (Zymo Research) and sent to the DNA Analysis Facility on Science Hill at Yale University for sequencing. Sequenced nucleotide regions were analyzed against sequenced animal whole blood from SSZ and against reported Genbank sequences using the BLAST function.

Over 110 blood fed mosquitoes were identified over the course of this project. Control mosquito (*Culex tarsalis*) blood meal and whole animal (e.g., cheetah blood [*Acinonyx jubatus*]) blood DNA extraction, yield was quantified with Nanodrop, resulted in pure DNA (260/280 ~1.8) where the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A sample ratio of 1.8 is considered to be relatively pure DNA.[3] However, samples did have evidence of contamination with an absorbance ratio of 260/230 being less than 2.0, where a sample with a ratio of 2.0-2.2 being considered pure nucleic acids and anything less than 2.0 considered to have contaminants present absorbing light at a shorter wave length (e.g., carbohydrates, phenols).[3] Bands indicative of positive 12s and CO1 gene amplification were identified on PCR gel (Figure 3). Purified DNA was sequenced and when searched with the BLAST function on Genbank, the identity of a cheetah and sheep (*Ovis aries*) via extraction of DNA from whole blood was confirmed.

Host identification based on blood meal analysis will potentially reveal species experiencing higher biting pressures, indicating the areas of greatest need for preventive

measures such as, insect vector control or medical treatment. Further investigation must also be made into whole blood DNA extraction, especially in the case of avian species, in order to complete SSZ specific animal gene sequencing.

References:

1. Humair, Pierre-François, et al. "Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker." Journal of Medical Entomology 44.5 (2007): 869-880.
2. Townzen JS, Brower AZ, Judd DD. "Identification of mosquito bloodmeals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences." Medical and Veterinary Entomology 22.4 (2008): 386-393.

   Desjardins P. and Conklin D., 2010. NanoDrop microvolume quantitation of nucleic acids. JoVE (Journal of Visualized Experiments), (45), pp.e2565-e2566

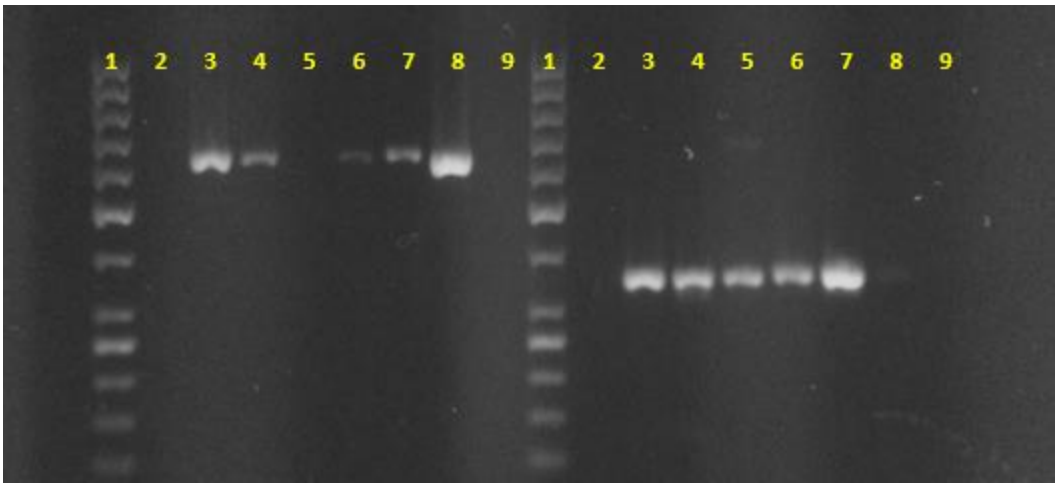

Figure 3. Gel electrophoresis of 12s (left) and COX1_short (right) mitochondrial gene PCR products. The columns are labeled as follows: 1. ladder; 2. non-fed mosquito; 3. 0-day bloodfed mosquito; 4. 1-day bloodfed mosquito; 5. non-fed midge; 6. 0-day bloodfed midge; 7. sheep whole blood; 8. cheetah whole blood; and, 9. negative control.

1. Determining vector movement in and out of the Sunset Zoo.

Attractive toxic-sugar baits (ATSBs) are used globally as a means of controlling populations of vectors (e.g., mosquitoes) known to transmit disease causing pathogens. The concept is that all blood feeding vectors require carbohydrates regardless of their host seeking behaviors (i.e., using proteins and lipids in blood for egg production). By mixing an attractant (e.g., sugar) with water and then a pesticide and spraying this mixture on non-flowering foliage (explained below), the mixture desiccates and crystalizes. When blood feeding vectors (e.g., biting midges, mosquitoes) land on leaves with these sugar crystals and taste them via their taste receptors on their legs (tarsi), they get exposed to the toxin.

This project comprised the use of the sugar water mixture; however, instead of an insecticide, food grade dyes were used (e.g., blue, red, and green). The solutions were sprayed in three locations outside of the zoo (Figure 4). When insects were collected within the CDC or BG-S traps within Sunset Zoo and sorted under dissecting microscope, the presence of dye was observed as a color change (e.g., blue, red, and green) present in their abdomen.
More than 50 insects were observed as having the presence of dye in their gut; however, images were difficult to capture the dye within their exoskeleton which reflected much of the light from any camera. The food dye would fade with exposure to alcohol, therefore the specimens had to be rapidly processed.

The use on non-toxic dyed dugar baits is useful in application as it may expose the source of vector species in a given area. The use of dyed sugar bait as used in this application allowed the discovery that there is movement of vector species from outside of the SSZ to inside the SSZ. While this does not mean that all blood feeding species within the zoo originate from outside of the zoo, it does demonstrate there is a proportion of blood feeding insects within the zoo that do

not originate from solely inside the zoo. This information may be used if making recommendations for control (e.g., treated barriers) of specific vector species if they are identified as originating outside of the zoo. However, additional work would need to be done to clarify sites of breeding of vector species (e.g., within or outside of the zoo) and to clarify the degree to which vector movement from outside to inside the zoo occurs prior to making control recommendations.

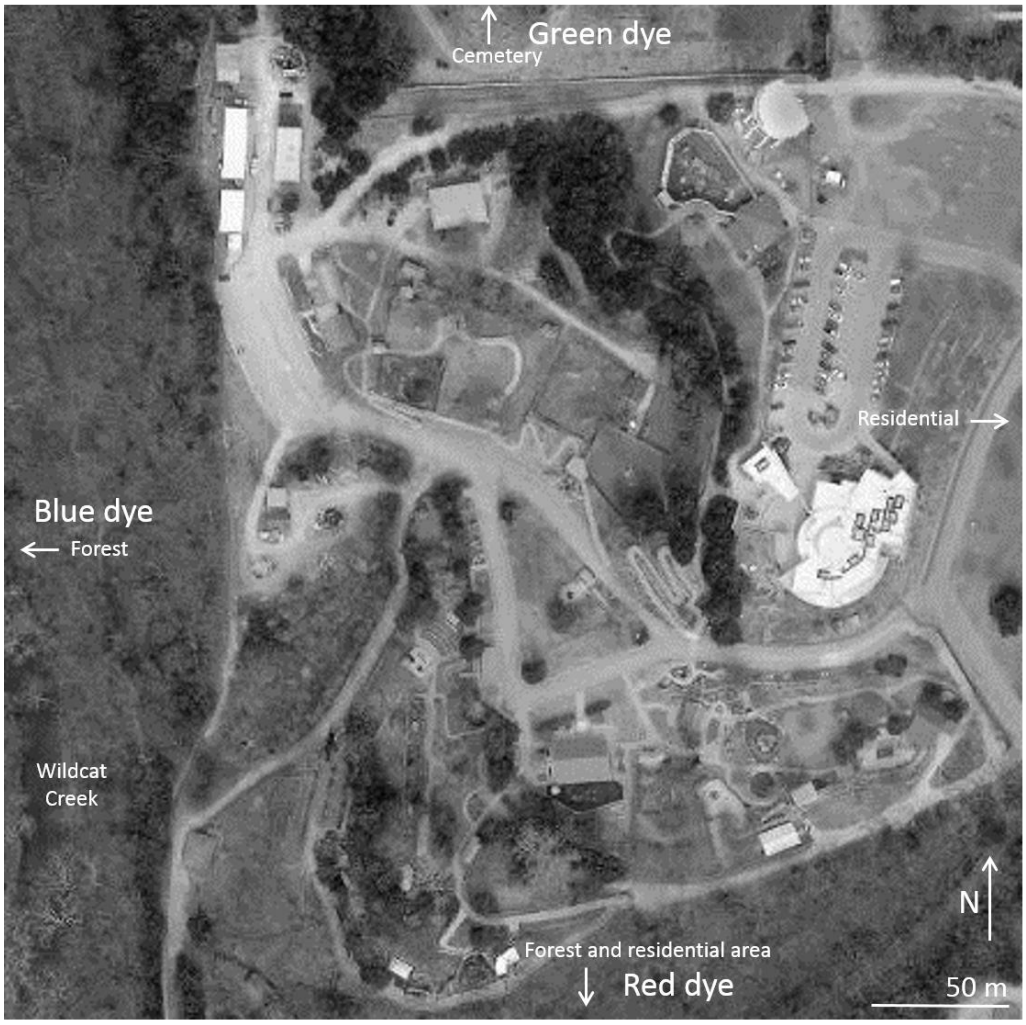

Figure 4. Overlay of the Sunset Zoo illustrates the three areas outside of the Sunset Zoo where dyed sugar-bait was sprayed on non-flowering foliage. Red dye was sprayed outside the Southern boundary in a forested area, blue dye was sprayed outside the Western boundary near a small creek, and green dye was sprayed outside the Northern boundary on foliage present within a local cemetery.

1. **Potential adverse effects of attractive toxic-sugar baits to beneficial insects such as honey bees (*Apis mellifera ligustica* and *Apis mellifera carnica*)**

As mentioned above, the ATSB label restricts its use to non-flowering foliage to reduce the impact on beneficial insects such as honey bees and butterflies. The study aim was to identify if vector species entered the zoo premises from environments outside the zoo using dyed sugar water (e.g., 10 ml food dye, 3.8 L water, and 1 kg sugar) that was sprayed on specifically targeted non-flowering foliage (Figure 5). Despite following the spray protocol, honey from six of eight hives kept within the Sunset Zoo (honey production for sale) were reported to have "blood red" honey in cells by the Master beekeeper. The keeper additionally, reported "bright green" honey within cells as well observed in the hive. Figure 6 illustrates images from multiple hives, more than one month after the initial dyed honey was observed during processing (over 180 pounds were presumably affected).

To the author's knowledge, this is one of the first reports of potential direct implications to beneficial insects from the use of an ATSB system. Reports of bees having dyed honey are not unique; however, bees foraging in non-flowering areas is and represents many concerns about the status of hives and the local environment.[1]

The author attempted to quantitatively prove the presence of dye months after the event. The use of high-performance liquid chromatography (HPLC) was attempted by collaboration with a Kansas State University biochemist. The attempt resulted in the inability to extract enough of the red-dye within the honey to quantitatively prove its presence. The dyes used are light

sensitive and break down over time from UV exposure; therefore, specific HPLC protocols would need to be developed to detect breakdown products of the dyes.

References:

1. Daly M. How bees revealed a pot farm beneath the maraschino cherries. [Internet] The Daily Beast. (2015) Article may be viewed from: http://www.thedailybeast.com/articles/2015/03/03/how-bees-revealed-a-pot-farm-beneath-the-maraschino-cherries.html.

Figure 5. Images above demonstrate representative areas of non-flowering foliage targeted for application of the blue, green, and red dyed sugar-bait at three locations external to the Sunset Zoo during the summer of 2015. These areas were identified due to the absence of any flowering flora species.

Figure 6. Investigation of red-dyed honey in two hives present within the Sunset Zoo. Panel A, break down of a hive for observation of red-dyed. Panels B-D, red-dyed honey cells represented with normal amber colored honey cells adjacent.

Chapter 4 - Capstone Project: Targeted Arthropod Vector Surveillance and Control near Military Working Dog Training and Kennel Areas

Introduction

Arthropod vectors are capable of transmitting pathogens (e.g., bacteria, helminths, protozoa, and viruses) between mammals which may result in numerous diseases affecting both human and animal populations. Service dogs and handlers employed by the United Stated (US) Army are potentially at an increased risk from vector-borne diseases (VBDs) due to protracted entomological exposure during their work and training. With recent unique traveler associated vector-borne viruses (e.g., Chikungunya virus and Zika virus) emerging within the US for the first time, attention to vectors and pathogens they transmit has greatly increased. Arthropod vectored pathogens are responsible for 17% of infectious diseases, making up 20% of emerging infectious diseases worldwide.[1,2] These VBDs pose significant risk to the health and welfare of military working dogs (MWDs) and additional risk to military personnel through both zoonotic transmission (i.e., animal to human pathogen transmission) and through MWDs acting as pathogen reservoirs able to infect local arthropod vector populations.

Military working dogs are used in a multifunctional capacity, trained to respond to numerous sensory stimuli assisting in the detection of various chemicals, narcotics, ammunitions, and mine detection.[3] Service dogs additionally act as vital force multipliers in US-Army ground operations.[3] Training and working locations place MWDs in prolonged exposure to numerous insects both within the continental US (CONUS) and outside the continental US (OCONUS). This extended exposure has been linked to MWDs possessing a higher sero-prevalence of certain VBDs (e.g., ehrlichiosis and anaplasmosis) in the CONUS than both domestic or shelter dogs.[4] Furthermore, socioeconomic priorities of countries or areas in the CONUS MWD's may operate

varies greatly (e.g., emphasis on vector surveillance or control).[5] Baseline data gaps in vector prevalence and distribution (i.e., surveillance data) in areas MWDs are kenneled, trained, or operate may increase risk from arthropod vectors.[5]

Increased exposure of MWDs to VBDs results in (1) MWDs having increased opportunities to develop clinical disease, posing significant risk to the health status of service dogs, and (2) MWDs developing non-clinical (i.e., no overt signs of illness) infections therefore potentially not being identified as needing treatment or acting as reservoirs.[6,7] Introduced VBD pathogens due to infected reservoirs or competent vectors in North America are canine heartworm disease (*Dirofilaria immitis*), leishmaniasis (*Leishmania infantum*), and other VBDs (e.g., West Nile virus).[6,8,9] Trypanosomiasis, which causes Chagas disease, was first found in MWDs in 2007 and impacts on MWDs in the Southern US. Military service dogs in Southern Texas, where *Trypanosoma cruzi* has increased in prevalence, have demonstrated up to 8% exposure based on serum antibodies.[10] Trypanosomiasis in MWDs has resulted in shortened deployments, leaving units without their valuable canine assets.[10]

Arthropod vector identity, prevalence, and distribution within the CONUS and OCONUS environments – surrounding MWD training areas and kennels – will identify public and animal health risks (e.g., disease transmission). Surveillance data are the vital pre-requisites needed for military veterinarians and entomologists to develop arthropod vector control strategies for mitigating disease transmission to MWDs.[7,11] Here we discuss arthropod vectors, their associated pathogens relevant to MWDs focusing on and emphasizing an integrated approach to vector surveillance and control.

Canine Associated Vectors and Pathogens

Arthropod vectors are insects (e.g., fleas, flies, mosquitoes) or ticks capable of pathogen transmission, acting as a biological vector or as a mechanical vector mosquitoes are an iconic example of arthropod vectors capable of transmitting numerous parasitic nematodes and viruses to both MWDs and humans (Table 1). Sandflies are perhaps one of the most significant arthropod vectors to canine populations worldwide, transmitting *Leishmania* spp. (e.g., *L. infantum*; visceral leishmaniasis), resulting in potentially fatal conditions (Table 1).[8,10] Other significant vectors of concern to MWDs include numerous other non-flying vectors such as ticks, triatomine bugs, and fleas as seen in Table 1.[8,10] Arboviruses (i.e., arthropod-borne viruses) rarely produce clinical disease in canine populations. However, arboviral pathogens retain significant importance considering both their impacts on human health and their being vectored by the same species vectoring pathogens affecting MWDs.[12]

Presence of pathogen-competent vectors does not equate to prevalence of clinical illness in a MWD population. Vector presence, population density, and vector competency for pathogens all play a role in the risk of MWDs developing a VBD. Host factors concentrate around MWD's immune-competency, age, and general health status at the time of effective vector-host interaction (i.e., effective pathogen transmission).[8] Environmental factors such as annual rainfall, temperature, regional flora and fauna, are significant in determining geographic and temporal distribution. Numerous factors determine the risks MWDs have in developing VBDs; however, being that presence of pathogen-competent vectors is the necessary component, surveillance is the first step required to any effective vector control program.

Table 1. Arthropod-vectors of concern for military working dogs in North America, pathogens transmitted, resultant diseases, and zoonotic potential.

Vector	Pathogen		Disease/Syndrome	Zoonotic	Ref
Fleas	Helminths	*Dipetalonema reconditum*	Dipetalonemiasis	No	8
*(*Pulicidae*)*		*Dipylidium caninum*	Dipylidiasis	No	7,8
Flies	Bacteria	*Bacillus anthracis*	Anthrax	Yes	7
Calliphoridae,		*Francisella tularensis*	Tularemia	Yes	7
Tabanidae,	Helminths	*Thalazia* spp.	Ocular thalaziasis	No	7,8
Drosphilidae)					
Hard ticks	Bacteria	*Anaplasma* spp.	Anaplasmosis	Yes	7,8
(Ixodidae)		*Bartonella* spp.	Bartonellosis	Yes	7,8
		Borrelia burgdorferi	Lyme disease	Yes	7,8
		Ehrlichia spp.	Ehrlichiosis	Yes	7,8
		Francisella tularensis	Tularemia	Yes	7,8
		Rickettsia rickettsi	Rocky Mountain spotted fever	Yes	7,8
	Protozoa	*Babesia* spp.	Babesiosis	Yes	7,8
		Hepatozoon spp.	Hepatozoonosis	No	7,8
Mosquitoes	Helminths	*Dirofilaria immitis*	Difilariasis	Yes	7,8
(Culicidae)		*Dirofilaria repens*	Difilariasis	Yes	7
	Viruses	Eastern equine encephalitis virus	Viral encephalitis	No	12
		La Cross virus	Viral encephalitis	No	12
		Saint Louis encephalitis virus	Viral encephalitis	No	12
		Venezuelan equine encephalitis virus	Viral encephalitis	No	12
		West Nile virus	Viral encephalitis	No	12
		Western equine encephalitis virus	Viral encephalitis	No	12
Sandflies	Protozoa	*Leishmania* spp.	Leishmaniasis	Yes	7,8
(Psychodidae)					
Triatomines	Protozoa	*Trypanosoma cruzi*	Trypanosomiasis	Yes	8
(Reduviidae)					

Arthropod Vector Surveillance

Vector surveillance aims to continuously or routinely collect, analyze, and interpret arthropod prevalence data to better inform vector control strategies and to assess disease transmission risks.[11] Insect traps take advantage of the common host-seeking behaviors and biological mechanisms of arthropod vectors.[7] Host-seeking behaviors are initiated by visual and kairomone cues. Kairomone are chemicals emitted by one organism but used by another (vector) to their benefit (e.g., locating hosts).[7] Effective surveillance plans target multiple life stages or behaviors (e.g., host-seeking, resting). Surveillance plans generate disease vector abundance and distribution data which is required and fundamental to developing and implementing effective vector control strategies (Figure 7).[11] Vector collection modalities include mechanical traps (e.g., baited & light traps), manual collection methods, or habitat investigation.

Mechanical traps may solely use a visual cue like ultraviolet or white light, for crepuscular and night time feeding species, or may have visual cues such as black on white contrast, in conjunction with a bait. Baits often consist of synthetic kairomones (e.g., lactic acid or pheromones). Trap attraction may be further increased by carbon-dioxide via tank or dry-ice hung above the trap, stimulating flight in vectors beyond the line of site of the trap.

There are two traps commonly used to survey flying host-seeking vectors. The Biogents-Sentinel (BG-S) trap is a mosquito specific trap most effectively used in covered areas (e.g., brush or wood lines) using synthetic baits and visual cues as attractants targeting diurnally active insects.[13] Second, the CDC light trap uses visual cues to non-selectively lure insects (e.g., mosquitoes, sandflies) to the trap, this trap inherently works more effectively at crepuscular and night time and is essentially the gold standard in mosquito collection studies.[14]

Manual collection or habitat investigation may be used for the collection of adult vectors (e.g., triatomine spp. or blood fed mosqutioes) or other life stages including pupae, larvae, or eggs not routinely captured or attracted to tradition traps (e.g., CDC ultra-violet light traps). Tick collection methods target questing-ticks (i.e., host-seeking) by environmental dragging or flagging, a method that may use CO_2 to enhance collections.[15] Triatomine bug collection methods involve visual examination of likely harborage and daily inspection of kennels.[10] Collection of other mosquito life stages may be conducted using dippers in suspected breeding locations (e.g., tree holes, plants, tires etc.).

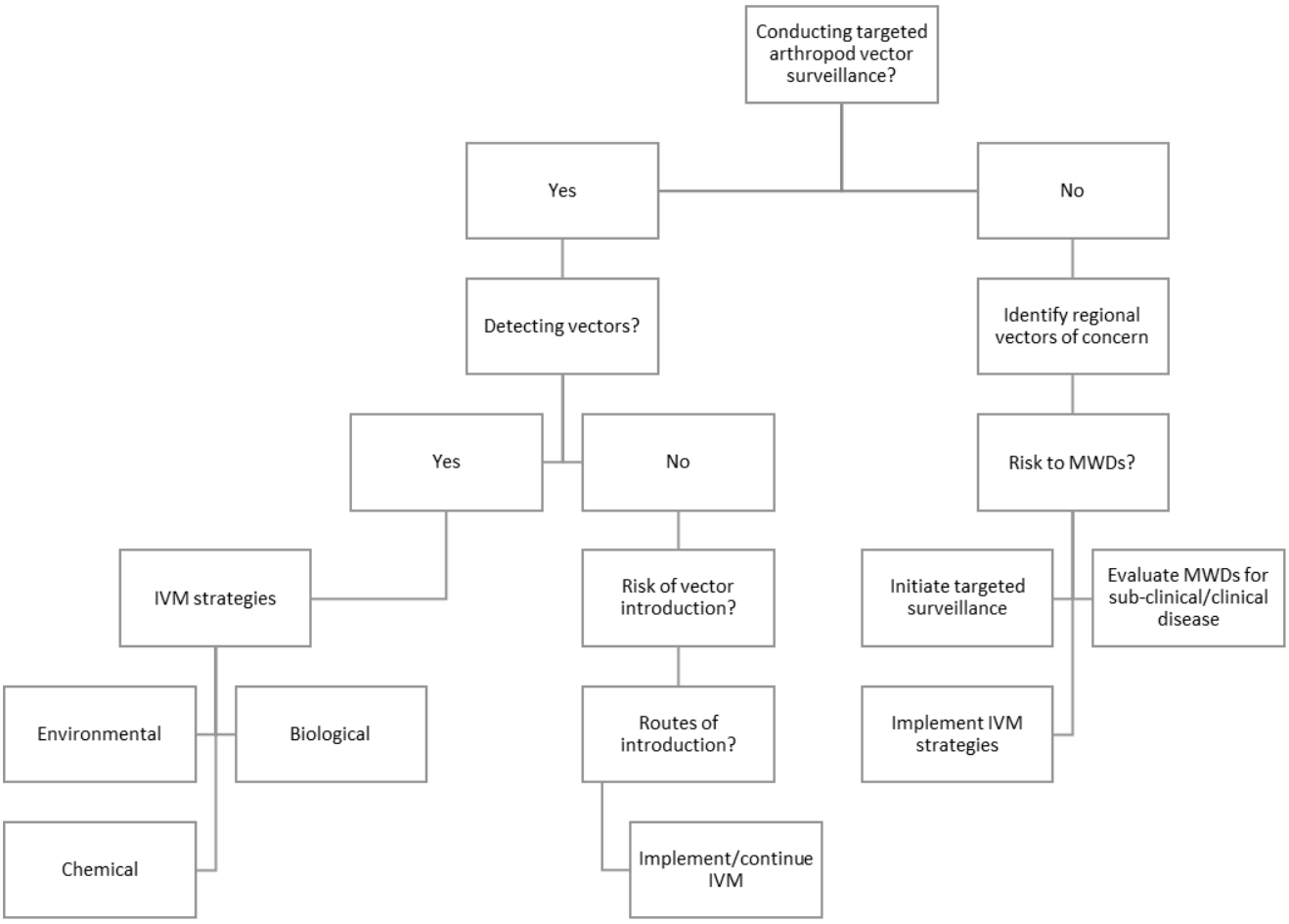

Figure 7. Decision tree for implementing integrated vector management (IVM) strategies for the surveillance and control of arthropod vectors near military working dog kennels, training areas, or operating areas.

Arthropod Vector Control

Effective vector control programs must take an integrated approach to vector control. Integrated vector management (IVM) is not a new concept and has been adopted by the World Health Organization as the primary means by which vector control is implemented. Primarily, IVM aims to increase both resource efficiency and efficacy in controlling arthropod vectors. The principles of IVM are directed towards: 1) understanding the targeted arthropod vectors to effectively reduce adult populations; 2) implementing ecologically and environmentally acceptable control strategies; and 3) taking an evidence-based and collaborative approach to vector control.[16]

When military installations approach initially implementing vector control strategies, leadership should ensure all relevant disciplines are involved. If present a military installation has a Department of Public Health, they should drive the decision to implement vector control and surveillance programs (Figure 7). Veterinary corps officers (VCOs) – responsible for eradicating animal reservoirs and disease vectors on any military post – and installation entomologists or environmental safety officers should be also be involved and take charge if on an installation without a department of public health.[17] In addition, there should be sufficient communication between VCOs, human physicians, and public health officials regarding any vector-borne or zoonotic disease transmission risks.

Vector control strategies themselves fall into three categories: environmental, biological, and chemical. Environmental control focuses on habitat modification targeting breeding and resting sites in the vicinity of MWD areas. Habitat modification may include clearing low growing foliage or emptying standing water. Biological control takes advantage of natural biological antagonists to the vector species of interest. Examples include mosquito fish or

Bacillus thuringiensis sp. *israelensis* being introduced to pools or standing water for mosquito larvae control.

Chemical control strategies may be used in the vicinity of MWD areas in numerous methods. Aerial spraying may be conducted in MWD areas using insecticidal chemicals retaining residual activity. Treated mesh netting impregnated with insecticides (e.g., pyrethroids), that when landed on will non-selectively kill insects, can be used to directly inhibit vector access to MWD (or military horse pastures) kennels on fences.[18] Chemical application may be implemented to produce a push-and-pull system where repellents are used in one vicinity (e.g., near MWD kennels) and attractants used in another vicinity (e.g., away from MWD kennels).[19] Push and pull techniques allow for predictable areas of vector abundance which can then be targeted for vector elimination.[19] It is important to understand that no single approach will prove to be all effective; however, targeted use of many methods to the vector species of interest will prove most effective.

Conclusion and Recommendations

Military service dogs providing invaluable functions to the US Army are at an increased risk to VBDs due to their occupation and in turn require specific considerations to ensure their service and health. Presented here are methods to survey and monitor arthropod vector populations which the data from may be used subsequently in determining risk of disease transmission and help drive vector control strategies near MWD kennel and training areas. Finally, consider the following recommendations in developing any IVM program:

- Recommendation 1. Military installations with MWDs prepare a comprehensive IVM plan involving VCOs, human physicians, entomologists, and other public health officials.

- Recommendation 2. Ensure the preliminary arthropod vector surveillance plan targets both vectors of concern in the region and surveys the environment to identify baseline vector species diversity currently present.
- Recommendation 3. If vectors of concern are identified in MWD kennel and training areas, develop control strategies targeting those vectors and evaluate MWDs for the specific pathogen potentially transmitted by the identified vector species.
- Recommendation 4. Implement effective long-term surveillance strategies to identify shifts in vector populations, the introduction of foreign or emerging vector species of concern, and the evaluation of ongoing control strategies.

References

1. World Health Organization. "The World health report: 2004: Changing history." 2004.
2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL., et al. . (2008). Global trends in emerging infectious diseases. Nature. 2008;451(7181):990-993.
3. Kelley P, ed. Military preventive medicine: mobilization and deployment. Vol. 2. Dept. of the Army, 2006.
4. McCown ME, Alleman A, Sayler KA, Chandrashekar R, Thatcher B, Tyrrell P, et al. Point Prevalence Survey for Tick-Borne Pathogens in Military Working Dogs, Shelter Animals, and Pet Populations in Northern Colombia. J Spec Oper Med. 2013;14(4):81-5.
5. Dantas-Torres F. Canine vector-borne diseases in Brazil. Parasit Vectors. 2008;1(1):25.
6. Enserink M. Epidemiology. Tropical disease follows mosquitoes to Europe. Science (New York, NY). 2007;317(5844):1485.

7. Otranto D, Wall R. New strategies for the control of arthropod vectors of disease in dogs and cats. Med Vet Entomol. 2008;22(4):291-302.
8. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part one. Trends Parasitol. 2009;25(4):157-63.
9. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part two. Trends Parasitol. 2009;25(5):228-35.
10. McPhatter L, Roachell W, Mahmood F, Hoffman L, Lockwood N, Osuna A, et al. Vector surveillance to determine species composition and occurrence of Trypanosoma cruzi infection at three military installations in San Antonio, Texas. US Army Med Dep J. 2012;12-21
11. Cohnstaedt LW, Rochon K, Duehl AJ, Anderson JF, Barrera R, Su NY, et al. Arthropod surveillance programs: basic components, strategies, and analysis. Ann Entomol Soc Am. 2012;105(2):135-49.
12. Sykes JE. Canine and feline infectious diseases. Elsevier Health Sciences, 2013.
13. BioGents. Scientists worldwide use the BG-Sentinel for mosquito surveillance and monitoring. Retrieved from: http://www.bg-sentinel.com/en/mosquito_monitoring.html. Accessed on November 28, 2015.
14. Cohnstaedt L, Gillen JI, Munstermann LE. Light-emitting diode technology improves insect trapping. J Am Mosq Control Assoc. 2008;24(2):331-334.
15. Gherman CM, Mihalca AD, Dumitrache MO, Györke A, Oroian I, Sandor M, et al. CO2 flagging-an improved method for the collection of questing ticks. Parasit Vectors. 2012;5:125.

16. Beier JC, Keating J, Githure JI, Macdonald MB, Impoinvil DE, Novak RJ. Integrated vector management for malaria control. Malar J. 2008 Dec 11;7(1):1.
17. United States Army. Army Regulation 40-905 Veterinary Health Services. 2006. Retrieved from: http://www.apd.army.mil/pdffiles/r40_905.pdf. Accessed: December 21 2015.
18. Melhorn H. Arthropods as Vectors of Emerging Diseases. In: Impact of Insecticide-Treated Nets on Insects of Medical and Veterinary Relevance. Berlin: Springer; 2012.
19. Cook SM, Khan ZR, Pickett JA. The use of push-pull strategies in integrated pest management. Ann Rev Entomol. 2006 Dec 12;52(1):375.

Chapter 5 - Capstone Project: Developing a Mosquito Surveillance Program for Use in a Zoological Park: a Pilot Study

Introduction

Mosquitoes present within zoos are a risk to both human and animal health. In addition to being a nuisance and reducing quality of life, mosquitoes can transmit pathogens such as the West Nile virus (WNV), which was first detected in North America at the New York City metropolitan zoo.[11,47] Mosquitoes from the *Culex* genus are the primary transmission vectors of the WNV within North America. Since being introduced into North America, the WNV has been reported in zoos across the United States (US) with the WNV infection affecting almost half of all native avian species studied and resulting in more than 40,000 human cases representing more than 750 million dollars in health care associated costs alone, not including costs associated with mosquito control or surveillance efforts.[33,36,62,72]

The effects of mosquitoes within zoos is a poorly researched area with numerous potential implications outside of mosquito-borne disease transmission. The environment of zoos is uniquely suited to hosting diverse communities of mosquitoes with inherently biodiverse populations of both native and non-native flora and fauna in close proximity which create numerous artificial microhabitats (both terrestrial and aquatic) and include indoor facilities (e.g. tropical and arctic habitats).[1,20,23,24,39,69] Additionally, zoos globally receive more than 700 million annual visitors globally representing more than 10% of the global population being a part of the zoological environment during a given year.[39]

Disease transmission by mosquitoes is the single most reported impact of mosquitoes within zoos, of which WNV cases are the majority. During the outbreak in the Bronx Zoo (New York City, New York) in 1999, more than 125 species tested positive for serum antibodies with a

case-fatality-rate greater than 70%.[6,47] Numerous other mosquito-borne viral pathogens have been reported in zoos including an outbreak of Eastern equine encephalitis virus (EEEV) in a flock of African penguins (*Spheniscus demersus*) where 64% of the flock was effected resulting in a 4.5% mortality rate.[70] Additional reports include an affected captive harbor seal (*Phoca vitulina*) and flock of captive whooping cranes (*Grus americana*) where the local mosquito vector (*Culiseta melanura*) was also identified in close proximity and confirmed competent for EEEV.[19,50]

Mosquito-associated protozoal infections within zoos predominantly impact penguins with significant mortality events being reported in captive populations. Definitive reasons for penguins being reportedly more susceptible to mosquito-borne protozoans, primarily avian malaria (e.g., *Plasmodium* spp.), are unknown. One hypothesis is that penguins often originate from regions that are cold, arid, and windy leading to limited to no exposure to mosquitoes and mosquito-borne pathogens.[37] Without this suspected co-evolution in their native environments, penguins may lack inherent pathogen-host relationships (e.g., immunity, survival to infection). For this reason, penguins enclosures within zoos have evolved towards closed indoor facilities to mitigate their sensitivity and exposure to mosquito-borne pathogens. In a survey of 40 zoos, 12.5% of zoos at one time diagnosed avian malaria in their collection of penguins including African and Humbolt penguins (*Spheniscus humboldt*).[4,15,37,38]

Confirmed competent mosquito vectors of *Plasmodium* spp. have been implicated as the cause of infection in penguins at the Baltimore Zoo with breeding sites for the mosquitoes surrounding the enclosure.[4] Additional reports of infections with *Plasmodium* spp. include a group of captive Chilean flamingos (*Phoenicopterus chilensis*) in Chicago and group of Magellanic penguins (*Spheniscus magellanicus*) in Iowa.[29,66] Another protozoa primarily

affecting the red blood cells of bird species are hematoprozoan parasites. These mosquito-borne parasites have been reported in numerous avian zoo collections with varying degrees of morbidity and mortality.[12,40,71]

Canine heartworm (e.g., *Dirofilaria immitis*) is a well-documented pathogen primarily affecting domestic canine; however, *D. immitis* also may have significant impacts on domestic feline and has been reported in numerous captive and free-ranging wildlife species worldwide, many of these cases reported for the first time in a species. There are 25 mosquito species known to be competent for *D. immitis* in the US.[46] Cases within captive species include a black-footed cat (*Felis nigripes*), California sea lions (*Zalophus californianus*), Humboldt penguins , leopard (*Panthera pardus pardus*), North American river otters (*Lontra canadensis*), pale-headed saki monkey (*Pithicia pithecia*), raccoon dogs (*Nyctereutes procyonoides*), red pandas (*Ailurus fulgens*), and a snow leopard (*Uncia uncia*).[2,18,31,43,48,49,56,57,58,62]

Many aspects of mosquitoes within zoos have been researched to some degree (e.g., blood-feeding ecology, larval habitats); however, the degree of mosquito abundance (i.e., how many mosquitoes are present) and the potential impact of abundance have been poorly researched. A survey in the Wellington Zoo in Aukland, New Zealand, reported mosquito density within zoos being significantly higher than in surrounding forested areas.[23] Implications of greater mosquito abundance may be significant to captive animals that are unable to flee blood-feeding vectors, leading potentially to increased risk of disease transmission as well as animal welfare concerns from increased insect bites per night or day (i.e., biting pressure).

Despite the impact the WNV and other mosquito transmitted pathogens have had in zoos, there remains no standard practice or guidelines for monitoring vector (e.g., mosquito) populations within zoo institutions.[1,21,51] Following the WNV emergence, collaboration between

the Centers for Disease Control (CDC), Association of Zoos and Aquariums (AZA, accrediting association of zoos and aquariums), and American Association of Zoo Veterinarians (AAZV) resulted in a program aimed at surveillance for the WNV within zoological institutions (McNamara, T.S., unpubl. data); however, the program ran for only 4 years and required no routine surveillance.[51] Additionally, most zoos mitigation strategy for mosquitoes or other arthropods are through commercial pest control vendors, but these services generally do not include insect monitoring. The companies treat for mosquitoes based on complaints by zoo keepers and guests or as a standard treatment of mosquito larval habitats without evaluating efficacy of the treatments.

The objectives of the current study are: (1) to characterize the mosquito abundance and diversity using common mosquito traps in a zoological park, and (2) to develop a customizable and economical mosquito monitoring protocol that may be fitted to any zoo for sustainable mosquito surveillance. This protocol aims to provide information towards what species are of priority for either abundance or disease transmission issues, what the best trapping locations are for each priority species, what the best months for capture are, when during the day the highest risk for biting pressure or disease transmission may be, and what trap would be the ideal trap to use. The protocol optimizes routine monitoring to minimize resources but maximize information from the collections to address biting pressures and disease transmission risk to both animals and humans within a zoo.

Materials and Methods

Study site

The Sunset Zoo (SSZ) is an AZA accredited zoo in the city of Manhattan, Kansas (N 39° 10′, W 96° 35′). The city of Manhattan was built on a flood plain at the junction of the Kansas

River and the Big Blue River in the northeastern region of Kansas. This region is dominated by rolling hills and tall and short grass prairies. The SSZ itself is atop a hill within Manhattan and is surrounded immediately by a city cemetery (North), heavily forested riverine area (West), and residential areas (South and East) (Figure 8). The zoo grounds span 48 acres with more than 100 species representing more than 300 animals.

Collection sites

Eight collection sites were selected and used throughout the study period within the SSZ, from spring through fall of 2015 (Figure 8). Collection sites were selected to achieve sampling across the zoo ground's various habitats in order to demonstrate vector communities in close proximity to enclosures. To ensure collection samples were unique, collection locations were placed no closer than 50 m from each other. Collection locations were positioned near: the Southern boundary and quarantine building; Northern boundary and chimpanzee (*Pan troglodytes*) enclosure; Northwestern boundary and maintenance buildings; between Australian animal enclosures and raptor enclosures; Southwestern boundary and Malaysian Tiger (*Panthera tigris jacksoni*) and red-crown crane (*Grus japonensis*) enclosures; Kansas native animal enclosures; near ungulate enclosures; and near a children's playground (Figure 9).

Collection methods

Mosquitoes were collected using two trap types: Centers for Disease Control (CDC) traps with ultraviolet light (Trap Model 1212, John W. Hock Company, www.johnwhock.com) and the Biogents-Sentinel (BG-S) trap (www.bg-sentinel.com) with BG-Lure cartridges.[13] The CDC traps were suspended approximately 1.5 m from the ground with adequate tree canopy coverage (e.g., >50%). The BG-S traps were placed on the ground abutting low growing woody flora in the same respective location as the CDC traps in all but one location (e.g., location seven), which

had only a CDC trap. A total of eight CDC and seven BG-S traps were used through this study. Each trap site location was supplemented with carbon dioxide (CO_2) via 0.5 kg of dry-ice which was continuously present in 3.8 L insulated Igloo containers (www.Igloocoolers.com) suspended above both traps. All traps were powered by either 6-V (10-, 12-, or 20-amps per hour) (Models PS-6200, PS-6100F1, and PT12B-4; Power Sonic®, www.power-sonic.com) or 12-V (1,100 Ah) or rechargeable recreational vehicle batteries.

Collection periods

Study collections were initiated when ambient temperatures reached greater than 15 °C (i.e., ~59 °F) for a period of 6 h or more per day (e.g., March) and ceased when the opposite occurred (e.g., October). Monthly collections occurred over three consecutive 24 h periods. Collections occurred in the final week of each month with consideration given to appropriate trapping conditions (i.e., avoiding inclement weather). Inclement weather resulted in halting of trapping until conditions were safe for the equipment and collectors. In these situations, traps were run for an additional 12-24 hours to compensate for lost trapping time, if weather allowed. During the collection period, traps were run continuously. Each 24 h of trapping consisted of two periods, one collection capturing peak nocturnal activity (e.g., night period) of the vectors (capturing activity 1 h following sunset and 1 h prior sunrise) and one collection capturing crepuscular and diurnal activity (e.g., day period) from 1 h prior to sunrise to 1 h after sunset. Therefore, a trap day was defined by the time after trap deployment in the morning until trap clearing and re-deployment for the evening collection, and vice versa for night trapping periods. This resulted in approximately 9 hours of night in March, 5 hours in June, and 10 hours in October. Each three 24 hour collection period consisted of six trapping periods (i.e., 3 night and 3 day) for the CDC and BG-S traps.

Collection processing

Insects captured in CDC traps were collected into 70% ethanol from March through July; following which, catches were collected alive prior to processing (this was done as a concurrent study was conducted where catches needed to be preserved in ethanol). Insects caught in BG-S traps were collected alive in the BG-S trap catch bags. Following each trapping period, catches were stored at -20 °C until gross samples were sorted and identified.

Identification of female mosquitoes to species was done using morphological keys.[45] Some specimens were identified to genera level only if damage to specimens occurred during the trapping, handling, or identification process. When appropriate (unknown species or species confirmation), specimens were sent to and identified by the Walter Reed Biosystematics Unit (WRBU) (Smithsonian Institution, Suitland, Maryland, USA 20746).

Analysis

The community of mosquito vectors were assessed using three estimates of species diversity: the Shannon Weaver index, the associated evenness to the Shannon index, and species richness. Diversity was selected to be used to describe the vector communities as increased diversity has been associated with increased risk of infectious disease transmission, when added species are additional sources of infection.[44,55] The Shannon Weaver index is an informational index – rooted in information theory – where an increase in heterogeneity of a population is equated to the uncertainty of a species sampled at random from a community.[60] This index takes into account both the number of species and abundance of relative species, where the more species there are and the more even their representation, the higher the diversity.[60] The Shannon Weaver Index (H) is calculated using Equation 1, where S is the species richness, i is

the species identified in the collection, pi is the proportion of the species represented in the collection, and ln is the natural logarithm.[60]

The Shannon index has an associated evenness (E_H) (Equation 2) value.[60] This value is calculated from the index value and represents the level of equitability among the species in questions used to calculate the Shannon index.[60] Evenness of species is reported from 0 to 1, and 1 is complete equitability among species and lower values represent disproportionate representation of a species within the community in question. Evenness was used in this study to describe the relative abundances of mosquito species present within the SSZ (i.e., describe the relative disproportionate representation of one species in the vector communities present in the SSZ). This information may be used to direct monitoring or control efforts towards species suspected of contributing most heavily towards biting pressures.

$$\text{Equation 1) Shannon Index (H)} = -\sum_{i=1}^{S}(p_i)\ln(p_i)$$

$$\text{Equation 2) Evenness } (E_H) = \frac{H}{\ln(S)}$$

Species richness (i.e., gross number of species) was also used as an index of the vector communities' diversity.[54,60] Counts of species in a community are considered the most simple and practical measure of species richness and diversity in a community.

Trapping effort (i.e., the number of collection days performed) evaluated for effect on interpretation of abundance and diversity data. Abundance values and diversity indexes (e.g., H, E_H, and S) were calculated for one, two, and three consecutive days of collection data. This was conducted to identify if there was a lowest minimum number of collection days needed to achieve monitoring recommendations that would be made from the three consecutive days of

collection data. By finding the lowest number of required collection days, significant resources (e.g., time, financial) may be saved from minimizing trapping effort.

Results

A total of 22,652 mosquitoes (Diptera: Culicidae) were collected over the 8-month active mosquito season at the SSZ. Females accounted for 20,161 (89%), males for 2,042 (9%), and 449 (2%) were damaged and sex could not be determined. Five genera were identified including: *Aedes (Ae)* represented the highest proportion of mosquitoes with 93.47%, *Culex (Cx)* with 4.69%, *Anopheles (An)* with 0.98%, *Culiseta (Cs)* with 0.81%, and *Psorophora (Ps)* with only 0.04%. There were 15,372 females identified to species level representing 20 species (i.e., species richness: S) (Table 2). The six most collected species were *Ae. vexans*, 89.47% (n = 13,791); *Cx. tarsalis*, 3.10% (n = 478); *Ae. nigromaculis*, 1.78% (n = 275); *Cx. restuans*, 1.77% (n = 272), *Cs. inornata*, 1.06% (n = 164); and *An. punctipennis*, 0.78% (n = 120) (Figure 9).

Peak monthly collections for *Ae. vexans* (n = 7070), *Cx. tarsalis* (n = 320), and *Ae. nigromaculis* (n = 160) was noted during June; however, an additional peak in collections occurred in September for *Ae. vexans* (n = 2655) and *Cx. tarsalis* (Figure 9). Collection peaks for *Cs. inornata* were found in April (n = 35) and in October (n = 127) (Figure 9). *Culex restuans* is collected throughout the study with peak collections occurring during September (n = 57) (Figure 9). *Anopheles punctipennis* was found in relatively high numbers throughout the study and peaks in August (n = 29) (Figure 9). Peak species richness was from June through September with the most number of unique species being collected in July (n = 17) representing 85% of unique species identified within the SSZ (Table 2). The most abundant month for mosquito collections was June (n = 10,596).

There were a total of 702 trapping periods throughout the study. These trapping periods consisted of 356 day periods and 346 night periods or 375 CDC periods and 327 BG-S trapping periods, respectively. Each month comprised of between 83 and 90 trapping periods. Differential capture rates for each species are represented in Table 2. Four species (*Ae. vexans*, *An. punctipennis*, *Cx. tarsalis* and *Cx. restuans*) were collected each month throughout the study (Table 2). Mosquitoes collected during night period collections (72.75%, n = 16,479) far exceeded those collected during day period collections (27.06%; n = 6,129) during the study.

Location six had the highest number (n = 3,162) of mosquitoes trapped throughout the study with location three having the fewest (n = 602) (Table 3). The highest diversity (H = 0.96) and evenness (E_H = 0.40) of mosquitoes collected – at the end of three consecutive days of collection – were found at location three (maintenance buildings at the northwestern corner of the SSZ). Location seven (near ungulate enclosures) had the highest richness of species (S = 16), representing proportionally 80% of the unique species collected within the SSZ. The lowest richness was at location three (S = 11) where only 55% of unique species in the SSZ were identified.

The addition of consecutive days of collection (i.e., trapping effort) were also considered (Table 3). Cumulative mosquito counts during one day of collection were highest at location one; however, when adding one or two additional collection days, location six was found to have the highest mosquito counts, respectively. For each location the highest diversity and evenness at each location respectively was found to be at the end of one or two days of collection (Table 3). The diversity and evenness of mosquitoes collected were found to be inversely related to both abundance and richness, at each location respectively. That is, at any given location, when cumulative abundance increased and/or additional unique species were identified (i.e., with the

addition of consecutive collection days), the diversity and evenness values decreased. Species richness at each location was always higher with the addition of one or two consecutive collection days, with the exception of location eight (Table 3). The addition of a second or third consecutive day of collection resulted in up to five additional unique species to be identified at each location. On average, a second day and third day of collection resulted in the addition of 1.6 and 1.4 unique species to be collected at each location, respectively.

Discussion

Surveillance of mosquitoes is currently practiced by few zoo institutions and guidance on protocols for conducting an effective and efficient sampling method within a zoo has significant room for optimization. Literature on mosquito diversity within zoos has increased in the previous decade with much of the work being descriptive in nature describing the unique ecology and diversity contained within zoos.[68,69] The present study's aim was to develop a usable and practical mosquito monitoring protocol that zoos may institute in their preventive medicine programs for both human and animal health. Development of this protocol was facilitated by the use of both diversity and abundance data of mosquitoes within the SSZ and at specific animal enclosures. This data allowed the development of several human and animal health priorities within the zoo and the development of mosquito monitoring goals. Information gained from this monitoring protocol may provide insights into developing recommendations for animal welfare and disease transmission reduction via mosquito population control.

Within the SSZ, mosquito abundance surrounding location six (Kansas native animal enclosures) was as high as 1,749 mosquitoes during the month of June. Highly dense mosquito populations were reported similarly in a survey conducted in New Zealand which demonstrated

similar trends in abundance within their zoos, compared to outside of the zoos.[23] Further significance in this may be interpreted from a study demonstrating similarly employed CO_2-baited traps collected only 10-18% of the target mosquito species' population present within a given area.[14] Additionally, mosquitoes were primarily present during nocturnal hours and so any behavioral impacts of these mosquitoes experienced by the animals may be missed by any zoo staff working around these enclosures during day periods. Therefore, biting pressures were likely 5-10 times higher on the zoo animals than would be expected given trap collections alone.

Presumably, increased mosquito abundance is correlated with increased biting pressure (i.e., number of bites received per night). While the impacts of dense mosquito populations on captive zoo animals are not studied, the effects of biting insects numerous free ranging species is. Animal defensive responses to biting insects involve behaviors such as stomping, wing shakes, and head movements in avian species and ear flicking, muscle twitching, leg stomping, and tail switching in other mammals.[16,26,34,53,54] Animals harassed by biting insects are also observed being more active (e.g., standing or moving) and taking part in microhabitat selection (i.e., choosing a local area with reduced biting pressures).[53,54,61,67] Flocking or herding species may take part in cooperative behaviors such as fleeing an area or taking part in animal grouping behaviors with the goal of diluting their individual biting pressures.[53] Constant harassment and annoyance in animals may lead to decreased food intake and decreased play behaviors in the young of some species.[5,67]

Physiologic effects of biting insects is correlated with energetic costs to the hosts, increased immune system activity, and a hypothalamic-pituitary-adrenal axis response, or stress response, leading to increased circulating corticosteroid (CCS) levels (i.e., commonly associated with stress).[64] Ectoparasitism has been associated with increased circulating CCS levels.[64]

Additionally, the magnitude of CCS increase has been shown to be related to the parasite-host co-evolutionary history, where if this relationship is absent (i.e., naive animals exposed to foreign ectoparasites), the stress response may be greater without any learned defensive behaviors against the biting insects.[64]

Stress in captive animals has been studied on the basis of behavior (i.e., observations on the behaviors induced from biting insect presence, like those mentioned above) and has been attempted to be quantified with measurements of corticosteroids via urine, feces, blood, or saliva.[74] One study looking into the difference between captive and free-ranging cortisol levels in cheetahs (*Acinonyx jubatus*) showed significant indicators of chronic stress. In those captive increased fecal cortisol levels, decreased testosterone and estrogen levels in males and females respectively, morphological changes in the adrenal glands indicating the effect of chronic stress was demonstrated.[65]

Connecting abundance of mosquitoes, stress invoked from biting insects, and inherent stress that may be experienced in many captive species is required if conclusions are to be made from high mosquito densities within zoos. In a recent study, birds with elevated CCS were shown to be twice as likely to be bitten by mosquitoes as those with normal CCS levels.[34] Mosquitoes locate hosts by searching for specific host cues such as body temperature, odor (e.g., sweat), and carbon dioxide output all of which may be effected by increased CCS levels and thus alter mosquito feeding trends towards more stressed animals.[30,34] Additionally, it has been shown that diseased animals retain higher CCS levels leading to implications in clinically unhealthy zoo animals.[25]

The significance of stress in zoo animals and increased mosquito density is something unstudied to date. This area may be vital in elucidating potentially significant levels of biting

pressures captive zoo animals may experience – which may compound potential chronic stress – but also may explain potential increased risk of disease transmission if a pathogen is present in the zoo vector community. Implications of increased zoo animal biting pressures due to inherent elevations in CCS and elevated mosquito densities touch on aspects of zoo animal welfare as well if biting insect do in fact contribute significantly to distress in zoo animals.[65,73,74]

Aside from abundance, species diversity provides a useful descriptor of vector communities. There are currently 54 species of mosquitoes that have been identified within Kansas.[42] Recently a study performed in North-central Kansas over a 280 km^2 area demonstrated 11 unique mosquito species, with collection environments being mixed prairie and crop landscapes.[32] The current study identified 20 unique mosquito species in the SSZ, covering less than 0.15 km^2. The number of species identified within the SSZ may be explained by both the biodiversity (e.g., variable hosts) and by the numerous microhabitats founds within zoos capable of supporting a greater number of mosquito species.[1,68] Habitats outside of zoos simply may not possess the microhabitat heterogeneity capable of supporting the diversity of species like those found within the SSZ. This increased diversity may contribute to increased disease transmission risks within zoological environments. This diversity, with contribution from abundance, is what determines the pathogen transmission risks within as zoo (i.e., competent insect vectors must be present in relative abundance for disease transmission to occur).

Measures of diversity by the use of a diversity index (e.g., Shannon diversity index: H) are useful tools for assessing habitat diversity (or productive mosquito larval habitats).[44,54] The most apparent finding from the SSZ mosquito communities were low relative evenness values (Table 4). This is likely a result of high relative *Ae. vexans* abundance dominating each community. If animals (or humans) are experiencing clinical symptoms of high biting pressures

(e.g., displaying defensive behaviors), than *Ae. vexans* is the likely target for both monitoring and control in the SSZ. *Aedes vexans* is a commonly reported pestiferous mosquito species. While location six, with Kansas native animals, experiences the highest densities of *Ae. vexans*, it cannot be concluded that these animals (e.g., those native to Kansas) will be most affected by high densities; however, the author recommends the highest density location be monitored for evaluating the peak biting pressure with a zoo. However, clinical symptoms and behavioral assessments should be the determining factors in implementing mosquito control. Additionally, knowing the most abundant mosquito indicates which larval habitats should be targeted for treatment as well. Most likely the *Ae. vexans* are coming from the marshy creek area outside the zoo, therefore a barrier treatment (e.g., Interceptor traps) would likely be helpful to reduce immigration into the zoo.

Diversity was greatest at locations three (Northwestern corner near maintenance buildings) and eight (near children's playground). These high diversity values may be explained from both high local microhabitat diversity, supporting both a greater number of species and mosquitoes or simply from relatively low mosquito abundances represented at these two locations. The H and E_H values respectively at these low abundant locations may be explained by the inverse relationship found between H and E_H values and both the abundance and species richness. Communities of mosquitoes like those within the SSZ are dominated primarily by a single species, as such with each additional poorly represented species identified, diversity and the resulting equitability are reduced. Similarly, with increased abundance, the relative relationship among the mosquitoes in the community become more distant (i.e., common species are collected commonly, rare species are collected rarely) resulting in low diversity and evenness

values, respectively. Vice versa, low abundance as seen at locations three and eight may result in closer relationships between species (i.e., higher H and E_H values).

With low abundance in mosquitoes but high diversity of mosquito species, these are areas that should be targeted for mosquito control after or during outbreaks of pathogens. Having a high diversity of mosquitoes means succession of pathogens between mosquito species is likely. For example, *Culiseta* and *Culex* mosquitoes over winter as adult mosquitoes which can harbor pathogens. Because they are present and feeding early in the year, they can infect animals prior to the most abundant (e.g., *Ae. vexans*) mosquitoes being present.[36] Furthermore, having high mosquito diversity indicates a high diversity of larval habitats, which makes larval habitat control more difficult and the zoo will likely have to focus on adult control.

Diversity and evenness values may be used to identify the impact of microenvironment and habitat diversity on mosquito species richness and abundances within the zoo.[44] The author holds that H and E_H are of particular value when initially surveying a zoo as it identifies if certain areas within the zoo have starkly different areas of microhabitat diversity. This will subsequently allow a zoo to target where to monitor and or control, rather than simply monitoring at the most abundant locations (i.e., monitoring the most abundant species). These values may be used in determining priorities of surveillance within the zoo depending on if conducting general mosquito surveillance or targeted disease transmission control, due to a local outbreak.

When evaluating the effect of effort (the addition of consecutive days of collection) on evaluating abundance, H, E_H, and species richness within the SSZ, specific goals require differing levels of effort. When attempting to describe diversity and evenness, no more than 2 days is necessary according to the present data, with additional days diluting the relationships of

the community with either greater abundance or the addition of rare poorly represented species. The author notes that with mosquitoes communities like those at the SSZ (i.e., dominated by a single species), additional days of collection when attempting to describe diversity and evenness are unlikely to provide significantly more data, for reasons explained above. Abundance at collection locations may be best investigated with three days of collection; however, only two days of collection were needed to identify the peak abundance areas within the SSZ and the most abundant month as well. Considering species richness, each additional collection day provided additional information about unique species present.

Species of interest for the SSZ are listed within Table 4 which describes the relative abundance and known pathogens that the respective species in Kansas are known to vector. Determining species of interest should be balanced between relative abundance and species' disease transmission characteristics. Biting pressure from mosquitoes within the SSZ is likely due to *Ae. vexans*, a commonly reported pestiferous species of humans. Many mosquito-borne viral pathogens found within North America, the *Culex* genera are commonly associated with transmission. *Culex tarsalis* is a common and efficient vector for most viral encephalitides in the US whereas *Ae. vexans* may transmit similar pathogens, the mosquito is an inefficient vector but more is often present in more abundant populations.[36] *Culiseta inornata* is an important mosquito to monitor as it is the most abundant cold-adapted species within the SSZ. This species may maintain and transmit viruses such as the WNV during cold weather months while other genera inactive (e.g., *Aedes*, *Culex*).[36] *Anopheles* spp. are a well-known vector for *Plasmodium* spp. and should be particularly monitored and screened for pathogen if penguins are present within a zoo. Much work has been done to identify competent hosts for *D. immitis* and if cases have been confirmed in a zoo institution, known and unknown competent vectors should

be surveyed and even sent to a diagnostic lab to check for the presence of L3-larval antigen as institution prophylaxis may be beneficial.

Identifying species of interest on the basis of known ability to transmit pathogens should not be considered the end all be all. Continued monitoring of mosquito species of interest and assessing for changes in both richness of species and diversity should be considered. Each institution should develop specific action thresholds unique to each priority species or captive species (e.g., increase in biting insect defensive behaviors). When collection rates of a species exceeds the monitoring threshold or behavioral indicators are observed indicating elevated biting pressures, control strategies may be implemented in the form of environmental (e.g., habitat modification), biological (e.g., *Bacillus* spp., mosquito fish), or chemical (e.g., pesticide treated barriers, attractive toxic-sugar baits).

Conclusions

If zoos are looking to conduct mosquito or arthropod-vector surveillance within their premises, a comprehensive survey over one vector season is recommended to capture trends in both cold- and warm-adapted species. This survey should describe the vector community and answer questions such as what species are present within the zoo (i.e., what pathogens may potentially be transmitted), where they predominate (e.g., around specific animal enclosures), whether one species dominates (i.e., the species providing the primary biting pressure), or when peak abundance occurs (e.g., night, day, seasonality). Once this survey has been conducted, annual mosquito monitoring may be targeted and consist of only a few trapping locations during a few months of the year, significantly reducing both the cost and time required. This will allow mosquito surveillance to be more achievable by the zoo.

For the SSZ, the following recommendations were made for successive annual surveillance (where an asterisk indicates prioritized locations for given criteria):

1. Targeting surveillance towards monitoring mosquito abundance (e.g., biting pressure) surrounding animal enclosures. Surveillance targeted in this manner should be prioritized in order from most to least abundant locations. Recommended to the SSZ is monitoring the top two most abundant locations with exceptions to be made at other locations if defensive behaviors in animals are observed towards biting insects:
    a. *Location six (n = 3162) – Adjacent Kansas native animal enclosures
    b. *Location two (n = 2832) – Adjacent chimpanzee enclosure and cemetery to the North
    c. Location five (n = 2422) – Adjacent southwestern forested periphery and Malaysian Tiger and red-crown crane enclosures
    d. Location four (n = 2297) – Adjacent Australian animal enclosures and raptors
    e. Location one (n = 2033) – Southern forested periphery across road from quarantine building
    f. Location seven (n = 1166) – Adjacent small ruminant and ungulate enclosures
    g. Location eight (n = 852) – Adjacent children's playground
    h. Location three (n = 602) – Adjacent western forest periphery and maintenance buildings
2. Targeting for species richness (i.e., relative risk of unique pathogen transmission). Surveillance should be ranked in order from most to least species rich locations:
    a. *Location seven (S = 16)
    b. Location one, four, five, and six (S = 14)

c. Location two and eight (S = 12)

d. Location three (S = 11)

3. Targeting abundance and species richness with consideration to both location and month. Monitoring should attempt to capture the abundance surrounding (+/- a month on either side) the two primary peaks observed (Figure 9) in June and September. Monitoring during these months suggested at locations indicated above for abundance and species richness (asterisk) above.

    a. May through July to capture primary vector peak (June) during summer months.

    b. August through October to capture both late season peak (September) and to capture peak of cold-adapted mosquito species.

4. Specific monitoring for species of interest may be performed with targeted capturing; however, the above recommendations will capture peak activity of species identified as priority species (Table 4).

5. Surveillance may be performed to identify species richness with either trap type employed during this study; however, when abundance of mosquitoes was at its highest, the CDC ultraviolet light trap was most sensitive for capturing changes in abundance. Therefore, the CDC trap was recommended to the SSZ for annual surveillance.

6. Staff education on behaviors and signs of animals expressing defensive behaviors from biting insects should be implemented to allow assessment of animals during peak abundance months.

7. Staff education on known breeding sites for mosquitoes should be implemented with routine control of sites if abundance or specific species of mosquitoes are of concern.

Initiating mosquito surveillance may pose the most significant hurdle for a zoo. Collaboration with regional or local entomologists is key in making collection data useful for the zoo.[13] Equipment is easy to set-up and use by zoo staff and once collections are taken an entomologist may then sort and identify insects to usable information for a zoo.

To the author's knowledge, this is the first study that describes the impacts increased numbers of mosquitoes (e.g., biting pressures) within a zoo may have on both stress and disease transmission in captive zoo animals. Biting pressure being increased towards stressed animals, mosquitoes targeting animals with increased CCS, and the magnitude of mosquito abundance within zoos is an area needing further research. This highlights not only animal (and human) health implications from infectious disease but, with suspected increased biting pressures on zoo animals, highlights aspects of animal welfare as well with the effects of potentially high biting pressures on zoo animals being unknown. Research needs to be done to quantify the biting pressure zoo animals endure while in captivity.

This is also to the author's knowledge, the first study demonstrating the use of a mosquito monitoring program that may potentially be fitted for use in zoological institutions. Zoos possess a significant biodiversity and the capability to be involved in numerous avenues of research including vector-host ecology, disease transmission studies, and research involving animal welfare.

References

1. Adler PH, Tuten HC, Nelder MP. Arthropods of medicoveterinary importance in zoos. Ann Rev Entomol. 2011;56:123-142.

2. Alho AM, Marcelino I, Colella V, Flanagan C, Silva N, Correia JJ, Latrofa MS, Otranto D, de Carvalho LM. *Dirofilaria immitis* in pinnipeds and a new host record. Parasit Vectors. 2017;10(1):142.

3. Ansell J, Hamilton KA, Pinder M, Walraven GE, Lindsay SW. Short-range attractiveness of pregnant women to *Anopheles gambiae* mosquitoes. Trans R Soc Trop Med Hyg. 2002;96(2):113-116.

4. Beier JC, Trpis M. Incrimination of natural culicine vectors which transmit *Plasmodium elongatum* to penguins at the Baltimore Zoo. Can J Zool. 1981;59(3):470-475.

5. Bennett B, Fewell JH. Play frequencies in captive and free-ranging bighorn lambs (*Ovis canadensis canadensis*). Zoo biol. 1987;6(3):237-241.

6. Brenner D, Larsen RS, Wack RF, Agnew D, Imai D. Concurrent West Nile virus and *Mycobacterium avium* infection in a black-necked swan (*Cygnus melanocoryphus*). J Zoo Wildl Med. 2007;38(2):357-362.

7. Bueno MG, Lopez RP, de Menezes RM, de Jesus Costa-Nascimento M, de Castro Lima GF, de Sousa Araújo RA, Guida FJ, Kirchgatter K. Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. Vet Parasitol. 2010;173(1):123-127.

8. Byford RL, Craig ME, Crosby BL. A review of ectoparasites and their effect on cattle production. J Anim Sci. 1992;70(2):597-602.

9. Carlitz EH, Kirschbaum C, Stalder T, van Schaik CP. Hair as a long-term retrospective cortisol calendar in orang-utans (*Pongo* spp.): new perspectives for stress monitoring in captive management and conservation. Gen Comp Endocrinol. 2014;195:151-156.

10. Carlson JS, Walther E, Fryxell TR, Staley S, Tell LA, Sehgal RN, Barker CM, Cornel AJ. Identifying avian malaria vectors: sampling methods influence outcomes. Parasit vectors. 2015;8(1):365.

11. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis--New York, 1999. [Internet] Morbidity and mortality weekly report; c1999.

12. Chagas CR, Valkiūnas G, Guimarães LO, Monteiro EF, Guida FJ, Simões RF, Rodrigues PT, Luna EJ, Kirchgatter K. Diversity and distribution of avian malaria and related haemosporidian parasites in captive birds from a Brazilian megalopolis. Malar J. 2017;16(1):83.

13. Cohnstaedt LW, Rochon K, Duehl AJ, Anderson JF, Barrera R, Su NY, Gerry AC, Obenauer PJ, Campbell JF, Lysyk TJ, Allan SA. Arthropod surveillance programs: basic components, strategies and analysis. Ann Entomol Soc Am. 2012;105(2):135-149.

14. Cooperband MF, Cardé RT. Orientation of *Culex* mosquitoes to carbon dioxide-baited traps: flight manoeuvres and trapping efficiency. Med Vet Entomol. 2006;20(1):11-26.

15. Cranfield MR, Graczyk TK, Beall FB, laleggio DM, Shaw ML, Skjoldager ML. Subclinical avian malaria infections in African black-footed penguins (*Spheniscus demersus*) and induction of parasite recrudescence. J Wildl Dis. 1994;30(3):372-376.

16. Darbro JM, Harrington LC. Avian defensive behavior and blood-feeding success of the West Nile vector mosquito, *Culex pipiens*. Behav Ecol. 2007;18(4):750-757.

17. Darsie RF, Ward RA. Identification and geographical distribution of the mosquitoes of North America, North of Mexico. Walter Reed Army Institute of Research. Washington DC; 1981.

18. Deem SL, Heard DJ, LaRock R. Heartworm (*Dirofilaria immitis*) disease and glomerulonephritis in a black-footed cat (*Felis nigripes*). J Zoo Wildl Med. 1998:199-202.

19. Dein FJ, Carpenter JW, Clark GG, Montali RJ, Crabbs CL, Tsai TF, Docherty DE. Mortality of captive whooping cranes caused by eastern equine encephalitis virus. J Am Vet Med Assoc. 1986;189(9):1006-1010.

20. Derraik JG. A survey of the mosquito (Diptera: Culicidae) fauna of the Auckland Zoological Park. N Z Entomol. 2004;27:51–55.

21. Derraik JG. Recommendations for mosquito control in zoological parks to reduce disease transmission risk. Weta. 2005;29:16–20.

22. Derraik JG, Calisher CH. Is New Zealand prepared to deal with arboviral diseases? Aust N Z J Public Health. 2004;28(1):27-31.

23. Derraik JG, Stanley D, Weinstein P, Lester P, Purdie G. Presence of adult *Ochlerotatus* (Finlaya) *notoscriptus* (Skuse) and *Culex* (*Culex*) *pervigilans* Bergroth (*Diptera*: *Culicidae*) in tree canopy in Wellington, New Zealand. N Z Entomol. 2003;26(1):105-107.

24. Derraik JG. Mosquitoes (*Diptera*: *Culicidae*) breeding in artificial habitats at the Wellington Zoo. Weta. 2004;28:28-31.

25. Dhondt AA, Dobson AP. Stress hormones bring birds, pathogens and mosquitoes together. Trends Parasitol. 2017.

26. Edman JD, Scott TW. Host defensive behaviour and the feeding success of mosquitoes. International Int J Trop Insect Sci. 1987;8(4-5-6):617-622.

27. Epidemiology & Geographic Distribution [Internet] (2016). Center for Disease Control [cited 2017 April 2]. Available from: https://www.cdc.gov/easternequineencephalitis/tech/epi.html Accessed 4/5/2017

28. Ferrell ST, Snowden K, Marlar AB, Garner M, Lung NP. Fatal hemoprotozoal infections in multiple avian species in a zoological park. J Zoo Wildl Med. 2007;38(2):309-316.

29. Fix AS, Waterhouse C, Greiner EC, Stoskopf MK. *Plasmodium relictum* as a cause of avian malaria in wild-caught Magellanic penguins (*Spheniscus magellanicus*). J Wildl Dis. 1988;24(4):610.

30. Foster WA, ED Walker. Mosquitoes (Culicidae) In: Mullen G, Durden L (eds). Medical and veterinary entomology. Academic Press, New York (NY): Academic Press; 2002;207-261

31. Gamble KC, Fried JJ, Rubin GJ. Presumptive dirofilariasis in a pale-headed saki monkey (*Pithecia pithecia*). J Zoo Wildl Med. 1998;1:50-54.

32. Ganser C, Wisely SM. Patterns of spatio-temporal distribution, abundance, and diversity in a mosquito community from the eastern Smoky Hills of Kansas. J Vector Ecol. 2013;38(2):229-236.

33. George TL, Harrigan RJ, LaManna JA, DeSante DF, Saracco JF, Smith TB. Persistent impacts of West Nile virus on North American bird populations. Proc Natl Acad Sci U S A. 2015;112(46):14290-14294.

34. Gervasi SS, Burkett-Cadena N, Burgan SC, Schrey AW, Hassan HK, Unnasch TR, Martin LB. Host stress hormones alter vector feeding preferences, success, and productivity. Roy Soc. 2016; 283(1836),1278.

35. Gervasi SS, Civitello DJ, Kilvitis HJ, Martin LB. The context of host competence: a role for plasticity in host–parasite dynamics. Trends Parasitol. 2015;31(9):419-425..

36. Goddard LB, Roth AE, Reisen WK, Scott TW. Vector competence of California mosquitoes for West Nile virus. J Emerg Infect Dis. 2002;8(12):1385-1391.

37. Grilo ML, Vanstreels RE, Wallace R, García-Párraga D, Braga ÉM, Chitty J, Catão-Dias JL, Madeira de Carvalho LM. Malaria in penguins–current perceptions. Avian Pathol. 2016;45(4):393-407.

38. Griner LA, Sheridan BW. Malaria (*Plasmodium relictum*) in penguins at the San Diego Zoo. Am J Vet Clin Path. 1967;1:7-17.

39. Gusset M, Dick G. The global reach of zoos and aquariums in visitor numbers and conservation expenditures. Zoo Biol. 2011;30(5):566-569.

40. Halpern N, Bennett GF. *Haemoproteus* and *Leucocytozoon* infections in birds of the Oklahoma City Zoo. J Wildl Dis. 1983;19(4):330-332.

41. Harrison BA, Whitt PB, Roberts LF, Lehman JA, Lindsey NP, Nasci RS, Hansen GR. Rapid assessment of mosquitoes and arbovirus activity after floods in southeastern Kansas, 2007. J Am Mosq Control Assoc. 2009;25(3):265-271.

42. Huff CG, Shiroishi T. Natural Infection of Humboldt's penguin with *Plasmodium elongatum*. J Parasitol. 1962;48(3).

43. Janovy J. Epidemiology of *Plasmodium hexamerium* Huff, 1935, in meadowlarks and starlings of the Cheyenne Bottoms, Barton County, Kansas. J Parasitol. 1966:573-578.

44. Keesing F, Holt RD, Ostfeld RS. Effects of species diversity on disease risk. Ecol. Let. 2006;9(4):485-498.

45. Krucik DD, Van Bonn W, Johnson SP. Association between positive canine heartworm (*Dirofilaria immitis*) antigen results and presence of *acanthocheilonema odendhali* microfilaria in california sea lions (*Zalophus californianus*). J Zoo Wildl Med. 2016;47(1):25-28.

46. Ledesma N, Harrington L. Mosquito vectors of dog heartworm in the United States: vector status and factors influencing transmission efficiency. Topics in companion animal medicine. 2011;26(4):178-185.

47. Ludwig GV, Calle PP, Mangiafico JA, Raphael BL, Danner DK, Hile JA, Clippinger TL, Smith JF, Cook RA, McNamara T. An outbreak of West Nile virus in a New York City captive wildlife population. Am J Trop Med Hyg. 2002;67(1):67-75.

48. Matsuda K, Baek BK, Lim CW. Eurasian otter (*Lutra lutra*), a definitive host for *Dirofilaria immitis*. J Zoo Wildl Med. 2003;34(2):200-201.

49. Mazzariol S, Cassini R, Voltan L, Aresu L, di Regalbono AF. Heartworm (*Dirofilaria immitis*) infection in a leopard (*Panthera pardus pardus*) housed in a zoological park in north-eastern Italy. Parasit Vectors. 2010;3(1):25.

50. McBride MP, Sims MA, Cooper RW, Nyaoke AC, Cullion C, Kiupel M, Frasca Jr S, Forrester N, Weaver SC, Weber ES. Eastern equine encephalitis in a captive harbor seal (*Phoca vitulina*). J Zoo Wildl Med. 2008;39(4):631-637.

51. McNamara T. The role of zoos in biosurveillance. Int Zoo Yearb. 2007;41(1):12-15.

52. Meeraus WH, Armistead JS, Arias JR. Field comparison of novel and gold standard traps for collecting *Aedes albopictus* in northern Virginia. J Am Mosq Control Asso. 2008;24(2):244-248.

53. Mooring MS, Fitzpatrick TA, Fraser IC, Benjamin JE, Reisig DD, Nishihira TT. Insect-defense behavior by desert bighorn sheep. Southwest Nat. 2003;48(4):635-643.

54. Mooring MS, Hart BL. Animal grouping for protection from parasites: selfish herd and encounter-dilution effects. Behav. 1992;123(3):173-193.

55. Morris EK, Caruso T, Buscot F, Fischer M, Hancock C, Maier TS, Meiners T, Müller C, Obermaier E, Prati D, Socher SA. Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. Ecol Evol. 2014;4(18):3514-3524.

56. Murata K, Yanai T, Agatsuma T, Shigehiko UN. *Dirofilaria immitis* infection of a snow leopard (*Uncia uncia*) in a Japanese zoo with mitochondrial DNA analysis. J Vet Med Sci. 2003;65(8):945-947.

57. Nakagaki K, Yoshida M, Nogami S, Nakagaki K. Experimental infection of *Dirofilaria immitis* in raccoon dogs. J Parasitol. 2007;93(2):432-434.

58. Neiffer DL, Klein EC, Calle PP, Linn M, Terrell SP, Walker RL, Todd D, Vice CC, Marks SK. Mortality associated with melarsomine dihydrochloride administration in two North American river otters (*Lontra canadensis*) and a red panda (*Ailurus fulgens fulgens*). J Zoo Wildl Med. 2002;33(3):242-248.

59. O'Malley CM. *Aedes vexans* (Meigen): an old foe. In: Proc Annu Meet N J Mosqu Exterm Assoc; 1990. p. 90-95.

60. Peet RK. The measurement of species diversity. Annu Rev Ecol Syst. 1974;5(1):285-307.

61. Ralley WE, Galloway TD, Crow GH. Individual and group behaviour of pastured cattle in response to attack by biting flies. Canad J Zoo. 1993;71(4):725-734.

62. Reisen WK. Ecology of West Nile virus in North America. Virus. 2013;5(9):2079-105.

63. Sano Y, Aoki M, Takahashi H, Miura M, Komatsu M, Abe Y, Kakino J, Itagaki T. The first record of *Dirofilaria immitis* infection in a Humboldt penguin, *Spheniscus humboldti*. J Parasitol. 2005;91(5):1235-1237.

64. St Juliana JR, Khokhlova IS, Wielebnowski N, Kotler BP, Krasnov BR. Ectoparasitism and stress hormones: strategy of host exploitation, common host–parasite history and energetics matter. J Anim Ecol. 2014;83(5):1113-1123.

65. Terio KA, Marker L, Munson L. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J Wildl Dis. 2004;40(2):259-266.

66. Thurber MI, Gamble KC, Krebs B, Goldberg TL. Molecular detection of *Plasmodium* in free-ranging birds and captive flamingos (*Phoenicopterus chilensis*) in Chicago. J Zoo Wildl Med. 2014;45(4):749-754.

67. Toupin B, Huot J, Manseau M. Effect of insect harassment on the behaviour of the Riviere George caribou. Arctic. 1996:375-382.

68. Tuten HC. Habitat characteristics of larval mosquitoes in zoos of South Carolina, USA. J Am Mosq Control Assoc. 2011;27(2):111-119.

69. Tuten HC, Bridges WC, Paul KS, Adler PH. Blood-feeding ecology of mosquitoes in zoos. Med Vet Entomol. 2012;26(4):407-416.

70. Tuttle AD, Andreadis TG, Frasca Jr S, Dunn JL. Eastern equine encephalitis in a flock of African penguins maintained at an aquarium. J Am Vet Med Assoc. 2005;226(12):2059-2062.

71. Valkiūnas G, Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA. Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on *haemosporidian* vector research. Parasitol Res. 2013;112(6):2159-2169.

72. West Nile virus disease cases reported to CDC by state of residence, 1999-2015 [Internet]. Center for Disease Control; c1999-2015 [cited 2017 April 1]. Available from:

https://www.cdc.gov/westnile/resources/pdfs/data/1-WNV-Disease-Cases-by-Year_1999-2015_07072016.pdf

73. Whitham JC, Wielebnowski N. New directions for zoo animal welfare science. Appl Anim Behav Sci. 2013;147(3):247-260.

74. Wielebnowski N. Stress and distress: evaluating their impact for the well-being of zoo animals. J Am Vet Med Assoc. 2003;223(7):973-977.

75. Zar JH. Biostatistical Analysis. 3rd ed. Upper Saddle River (NJ): Prentice-Hall; 1996.

Figure 8. Overlay of the Sunset Zoo grounds with the respective eight mosquito collection locations. Collections locations are labeled 1-8 and each site consists of a CDC-ultraviolet light trap, BG-S trap, cooler with dry ice, and batteries for trap power. Locations of traps are near characteristic enclosures or other zoo facilities as follows: 1, Southern forested periphery across road from quarantine building; 2, adjacent chimpanzee (*Pan troglodytes*) enclosure and cemetery to the North; 3, adjacent western forest periphery and maintenance buildings; 4, between Australian animal enclosures and raptors; 5, adjacent southwestern forested periphery and Malaysian Tiger (*Panthera tigris jacksoni*) and red-crown crane (*Grus japonensis*) enclosures; 6,

adjacent Kansas native animal enclosures; 7, adjacent small ruminant and ungulate enclosures; 8, adjacent children's playground.

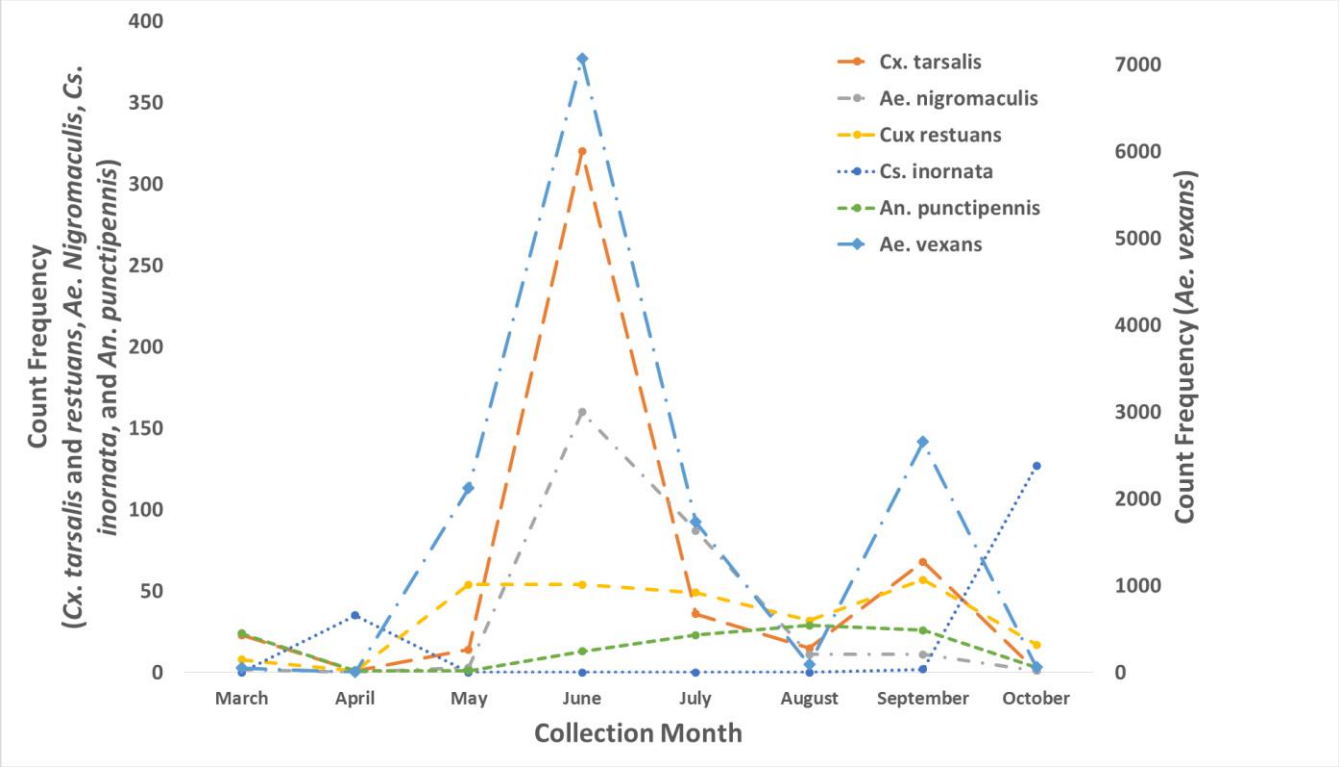

Figure 9. Total monthly collection frequencies of the six most prevalent mosquito species collected throughout 8 months in the Sunset Zoo during 2015. *Aedes vexans'* count frequencies are scaled on the right axis. *Cx. tarsalis* and *restuans*, *Ae. nigromaculis*, *Cs. inornata*, and *An. punctipennis'* count frequencies are scaled with the left axis.

Table 2. Collection totals of mosquitoes identified each month from March through October with differential collection totals between the Biogents-sentinel and Centers for Disease Control ultra-violet traps during 2015 in the Sunset Zoo.[a]

	Month and trap type																
	March		April		May		June		July		August		September		October		Study Total
Species	BG-S	CDC	BG-S	CDC	BG-S	CDC	BG-S	CDC	BG-S	CDC	BG-S	CDC	BG-S	CDC	BG-S	CDC	
Aedes albopictus							7	1	25	14	8	11	8	8	4		86
Aedes canadensis canadensis									2	1							3
Aedes hendersoni								1	2	2				1			6
Aedes nigromaculis		2				3	42	118	23	64	6	5	4	7		1	275
Aedes triseriatus									1	1	3	4	5				14
Aedes trivittatus							3	1	2	9			5	5			25
Aedes vexans	3	51	1	4	599	1523	409	6661	330	1402	23	68	708	1910	31	31	13754
Aedes zoosophus									1								1
Anopheles barberi										3	1	4					8
Anopheles punctipennis		24		1		1		13	1	22	9	20	4	21	1	2	119
Anopheles quadramaculatus								1			7	24	15	14	2		63
Anopheles walkeri										4							4
Culex erraticus									47	9	13	9	9	2			89
Culex quinquefasciatus										1							1
Culex restuans		8		1	6	48	13	41	11	38	9	23	8	49	3	14	272
Culex salinarius									1	1							2
Culex tarsalis	1	22		1	1	13	44	276	6	30	6	9	13	55		1	478
Culiseta inornata			11	24									2		37	90	164
Psorophora ciliata					1	1		1	1	1			1	1			7
Psorophora longipalpus							1										1

[a] table does not represent male specimens or those unable to be identified to species level.

Table 3. Mosquito abundance, Shannon Weaver index (H) for respective location mosquito communities, evenness (E_H), species richness (S), and proportion of S ($S_{proportion}$) for one, two, and three consecutive days of collections for each trapping location throughout study in the Sunset Zoo during 2015.

	Location							
Collection days and Indices [a])	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Day 1								
Abundance	**742**	**477**	**181**	**304**	**501**	**567**	**354**	**198**
H	**0.62**	**0.67**	**0.71**	**0.64**	**0.36**	**0.52**	**0.63**	**1.17**
E_H	**0.28**	**0.28**	**0.32**	**0.28**	**0.15**	**0.21**	**0.26**	**0.51**
S	**9**	**11**	**9**	**10**	**11**	**12**	**11**	**10**
$S_{proportion}$	**45%**	**55%**	**45%**	**50%**	**55%**	**60%**	**60%**	**50%**
Day 1-2								
Abundance	**1241**	**1749**	**442**	**1522**	**1575**	**1915**	**771**	**478**
H	**0.69**	**0.62**	**0.99**	**0.47**	**0.34**	**0.44**	**0.82**	**1.07**
E_H	**0.29**	**0.25**	**0.43**	**0.18**	**0.13**	**0.17**	**0.32**	**0.43**
S	**11**	**12**	**10**	**13**	**13**	**13**	**13**	**12**
$S_{proportion}$	**55%**	**60%**	**50%**	**65%**	**65%**	**65%**	**65%**	**60%**
Day 1-3								
Abundance	**2033**	**2832**	**602**	**2297**	**2422**	**3162**	**1166**	**852**
H	**0.60**	**0.55**	**0.96**	**0.47**	**0.31**	**0.35**	**0.79**	**0.90**
E_H	**0.23**	**0.22**	**0.40**	**0.18**	**0.12**	**0.13**	**0.29**	**0.36**
S	**14**	**12**	**11**	**14**	**14**	**14**	**16**	**12**
$S_{proportion}$	**70%**	**60%**	**55%**	**70%**	**70%**	**70%**	**80%**	**60%**

[a] indicators are abundance (count) of mosquitoes, Shannon diversity index (H), evenness (E_H), species richness (S), and the proportional S compared to total number of species identified during study.

Table 4. Priority mosquito species based on abundance over 8 months of collections and risk for pathogen transmission within the Sunset Zoo based on known pathogens[a] which the priority species are both competent for and are reported in Kansas or surrounding states.

Priority species	Abundance (count)	Known pathogen competency[b] (reported in KS)
Aedes albopictus	86	SLEV[31], WEEV[31], WNV[31]
Aedes triseriatus	14	WNV[31]
Aedes vexans	13,791	*Haemoproteus* spp.[39], *D. immitis*, EEEV[1], SLEV[31], WEEV[31], WNV[31], *Plasmodium* spp.[9,41]
Anopheles punctipennis	120	*Haemoproteus* spp.[39], *D. immitis*, WNV[31],
Culex restuans	272	WEEV[31], WNV[31], *Plasmodium* spp.[9,41]
Culex tarsalis	478	EEEV[1], SLEV[31], WEEV[31], WNV[31], *Plasmodium* spp.[9,41]
Culiseta inornata	164	SLEV[31], WEEV[31], WNV[31]

[a] St. Louis encephalitis virus (SLEV), Western equine encephalitis virus (WEEV), West Nile virus (WNV), and Eastern equine encephalitis virus (EEEV).

[b] pathogens listed are known to be competent by respective listed species with reports of pathogen(s) in Kansas or surrounding states.