ECOLOGICAL INVESTIGATION OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUSES AND OTHER PRIORITY ZOONOTIC VIRUSES IN WILD ANIMALS AT ONE HEALTH INTERFACES IN TANZANIA

APPLICANTS

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APPLYING INSTITUTIONS

1. Sokoine University of Agriculture (SUA): Grant Sub awardee from University of California Davis and main field implementer of wildlife-based study
2. University of California Davis (UCD): Grant Recipient from USAID

Approved budget for SUA:

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1.0 SUMMARY

The continued emergence of high-consequence zoonotic pathogens is a clear and present threat to human and animal health globally. In the past decade alone numerous outbreaks, most notably the 2013-2016 West Africa Ebola virus disease epidemic[1], have demonstrated the severely impacts of these diseases on overall population health, health care workers, and the fundamental economic stability of entire countries and regions[1]. Other viral zoonotic pathogens may have less global notoriety but nonetheless present a high disease burden in endemic areas and have the potential to expand their geographic range in coming years due to human activity, the influences of climate change on disease vectors, and geopolitical instability.

Crimean-Congo hemorrhagic fever virus (CCHFV), a tick-borne virus that can cause fever, bleeding, and high case fatality rates in people. The virus has a complex ecology that remains poorly understood in many areas of the world, particularly in Africa. Ecological investigation of the virus is vital for understanding the distribution and transmission of viruses both at a population and an individual level. Population level surveillance of CCHFV in wildlife, livestock and tick can shed light on patterns of viral circulation and aid in identification of risk factors associated with infection.

In Tanzania, studies on CCHFV have mainly been in human and livestock in the north[3]. Epidemiological information regarding CCHFV and other emerging pathogens is scarce in other parts of the country. Thus, the initial part of this study is designed to establish seroprevalence and genetic diversity of CCHFV in selected humans, livestock, and wildlife interface areas in Tanzania. The findings will contribute to a better understanding of the roles that livestock, wildlife and ticks of different species play in the maintenance and transmission of CCHFV, ultimately aiding in the development of targeted public health interventions and strategies for transmission, prevention and control in endemic regions.

Sampling locations within Tanzania will include Iringa rural and Kilolo Districts (Iringa region), Kilombero , Mlimba and Ulanga Districts (Morogoro region) and Bagamoyo District (Coastal region).

The laboratory testing will employ advanced serological and molecular tools, including ELISA (Enzyme-Linked Immunosorbent Assay), RT-PCR (Real Time Polymerase Chain Reaction), and next-generation sequencing. Testing will be conducted at multiple facilities including the project laboratory at Sokoine University of Agriculture. The lab is a BSL-2 plus equipped with Biosafety Cabinet class II (BSC II). Additional testing, such as next-generation sequencing will be performed at various international facilities as needed.

Biological and Ecological niche models will be developed to characterize virus risk using publicly available and project-derived environmental and virus infection data. Published reports on CCHFV in East Africa will be compiled into a database stratified by host, vector, and location, among other variables, and analyzed to characterize the taxonomic breadth and host plasticity of the viruses. Environmental parameters, extracted from publicly available datasets e.g. WorldClim, and MerraClim will be used. Data on species occurrence and geographical distribution will be obtained from the Global Biodiversity Information Facility (GBIF). Similarly, VectorMap that provides a geospatial distribution of known tick vectors of CCHFV will be used. Overall, the models under this specific objective aims to assess knowledge gaps in the disease ecology of this complex vector-host and virus-reservoir host zoonotic disease systems and inform targeted surveillance strategies.

Dissemination of research findings will be guided by the Director for TAWIRI and COSTECH. The PI will be responsible for seeking advice of plans, logistics and strategies for knowledge/ information sharing to the community/public, scientists through publications in scientific journals, and Government Ministries through Policy Briefs and fact sheets.

2.0 BACKGROUND

Crimean-Congo hemorrhagic fever virus (CCHFV), is a member of the virus Order *Bunyavirales*, which as a group are known for tripartite single-stranded RNA genomes, glycoprotein envelopes, and a propensity to be transmitted by arthropod vectors[4]. In particular, CCHFV is a member of the genus Orthonairovirus, which specifically includes viruses transmitted by ticks. CCHFV is primarily vectored by the tick genus *Hyalomma*, though other genera (such as *Amblyomma*, *Rhipicephalus*, and *Dermacentor*) have been implicated as possible alternative vectors[5-7]. The distribution of CCHFV closely mirrors that of *Hyalomma* ticks, and spans across much of the continent of Africa, throughout the Mediterranean basin, the Balkan states, the Middle East, and as far east as western China and India. Climatic shifts over the coming decades are likely to broaden the range for *Hyalomma* ticks and other suspected vectors[8], which in turn increases the risk that CCHFV will spread into new regions and cause significantly more human infections in the coming decades. For example, the first two cases with CCHFV genotype III (Africa 3) were reported in Spain in 2016; introduction is thought to be due to migratory birds from Western Africa[9-10]. Since then, new genotype V (European) has been detected, perhaps introduced similarly by migratory birds or through animal trades[10]. Migratory birds of eastern Europe fly through Turkey to reach Africa, and CCHFV was first described in Turkey in 2022[9,11]. In 2015, over 9,000 human cases were confirmed. During the COVID-19 pandemic, another CCHFV outbreak occurred, with over 200 confirmed cases and 13 human deaths attributed to CCHFV[11]. Elevating temperatures, humidity, and precipitation may influence survival and reproduction of Hyalomma tick populations carried through the country on migratory birds[12].

Crimean-Congo hemorrhagic fever virus (CCHFV), is maintained in a vector-host-pathogen disease ecologic cycle, where humans are thought to be an incidental and terminal host for the virus[7,13]. Cattle are likely the primary animal host for adult ticks and CCHFV and are an essential component of the virus life cycle, although infected cattle have no clinical signs and sustain a very brief, low-level viremia post-infection. Surprisingly to date, no other non-human animals (aside from limited case reports among ostriches) have been found to sustain infection or show any signs of clinical disease[5,14]. Cattle and other ungulates, such as sheep, goats, camels, deer, and wild boar, also represent a major part of the *Hyalomma* spp. life cycle as the preferred host for feeding adult *Hyalomma* ticks, while small mammals (such as hares and hedgehogs) and passerine birds compose the preferred hosts during the larval and nymph portion of their two-host life cycle[5,7]. *Hyalomma* ticks are persistently infected by CCHFV after becoming infected, with infected female ticks transmitting the virus to new offspring (i.e., transovarial transmission) a lack of virus clearance between life stages (larvae, nymph, to adult, i.e., transstadial transmission)[7]. Given these ecological factors, *Hyalomma* spp. ticks are considered both the primary vector and definitive host for CCHFV[5,13], and the virus may potentially persist indefinitely in *Hyalomma* tick populations into which the virus has been introduced.

The impact of other mammalian wildlife on tick and CCHFV disease ecology is not well understood. A 2023 study in Kenya explored CCHFV dynamics in cattle, donkeys, sheep, and goats along with rodents. After sampling 93 rodents, seroprevalence was determined to be approximately 6.5%. Additionally, CCHFV viral RNA was detected in homogenized rodent (*Rattus rattus* and *Mus musculus*) tissue samples[15]. Exploring species such *Rattus rattus* (black rat), *Mastomys natalensis* (multimammate mouse), *Crocidura* spp. (shrews), and house mice (*Mus* spp.) in unique regions of human-livestock-wildlife interfaces provide further information about viral transmission and the potential role as an amplification host. Finally, rodents can be valuable sentinels of zoonoses present in the environment. In Tanzania, studies on CCHFV has mainly been in human, domestic animals and wildlife in the Northern part of Tanzania. Epidemiological information regarding CCHFV is scarce in other parts of the country.

An additional small mammal of interest is the hare. Hares are the preferred host for the immature stages of *Hyalomma marginatum*, the proposed primary vector of CCHFV. As of 2022, hares have been successfully experimentally infected with CCHFV via tick and are postulated to play a major role as an amplifying host in France[16]. While hares and CCHFV have been explored in Europe, they seem to be less studied in Africa. The African Savanna Hare (*Lepus microtis*) is widely distributed through Tanzania and found in all game reserves and national parks, other than Rubondo National Park[17]. Surveillance of hares would not only provide information on the life cycle of *Hyalomma s*pecies in Africa along with their potential role in CCHFV transmission.

Finally, free-ranging ground-feeding birds offer a similar role as hares in, as immature stages of both *Hyalomma marginatum* and *H. rufipes* feed on birds[18]. In Europe, ticks from

migratory birds from Africa have tested positive for CCHFV viral RNA via PCR20. Migratory and ground-dwelling birds that have been found positive for CCHFV genome or antibodies include a blue-helmeted guinea fowl (experimentally), magpies, red beaked hornbills, and starlings[19-20]. Guinea fowl, pheasants, grouse such as francolin, and hornbills are present in national parks, wildlife management areas, and in the bordering villages. These birds share an environment with both humans, crops, and livestock in regions of Tanzania. Additionally, guinea fowl often interact with domestically raised poultry species, thus living closely with humans and livestock. Furthermore, hornbills represent a migratory bird that, while capable of flying long distances, spends much of its time on the ground, thus offering a unique view of both local and long-distance disease transmission. Therefore, extending surveillance to birds would offer an adept vantage point of both tick lifecycle and viral transmission.

Due to the variety of domestic and wildlife animal species associated with the primary vector of CCHFV, humans who work and live in close proximity to animals—particularly livestock workers, slaughterhouse workers, and individuals in pastoral communities—are at highest risk for exposure to CCHFV[5,6,20]. CCHFV can be transmitted directly through the bite of an infected tick or through exposure to the blood and viscera of infected animals, which accounts for many reported CCHF cases. Although sustained human-human transmission has not been documented, one of the greatest exposure risks for contracting the disease during outbreak scenarios is for hospital or clinic workers attempting to care for patients with severe infection—blood and other bodily fluids from infected patients are able to transmit the virus very effectively, making fomite transmission and nosocomial infections a major risk[5].

3.0 PROBLEM STATEMENT

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne zoonotic pathogen responsible for severe human hemorrhagic fever disease characterized by fever, weakness, myalgia, and hemorrhagic signs. The CCHFV has a broad mammalian host range and circulates primarily in a tick-vertebrate-tick cycle, with *Hyalomma* spp. ticks identified as the natural vector and reservoir. The majority of reported human cases are linked to exposure to *Hyalomma* spp. as the primary tick vector often found on domestic livestock. Our preliminary serological surveys conducted in Iringa and Morogoro regions between 2020-2021 have demonstrated a high CCHFV exposure (approximately 30% in cattle) across these study areas. However, the molecular testing of the primary vector tick genera (*Hyalomma* spp.) collected in areas where cattle herds were tested for CCHFV yielded negative results. To further help understand the ecology of CCHFV transmission in the study areas we extended testing to include *Amblyomma* spp. collected directly from cattle between 2020-2021 by RT-qPCR assays. Despite screening over 900 individual ticks, no CCHFV positive specimens were detected. This data indicates that the ecology of CCHF in this region may be more complex than originally hypothesized, with other tick genera and

other livestock and wildlife species potentially serving as the drivers of CCHFV transmission. Overall, despite CCHFV's widespread impact, especially in endemic regions, the virus's epidemiology and ecology remain poorly understood in wildlife species in particular the rodents, hare and ground feeding birds at the human-livestock-wildlife interface areas. This study therefore aims to evaluate the ecology and genetic diversity of CCHFV in selected wildlife species and ticks from the human-livestock-wildlife interface areas of Tanzania.

4.0 SIGNIFICANCE AND OBJECTIVES

Emerging infectious pathogens are a global concern, and the majority originate in animals. Due to the high risks and consequences of emerging zoonotic pathogen spillover from animals and their vectors (e.g. ticks) to people, there is a need to fill critical gaps in our understanding of ecology for emerging pathogens, including zoonotic viruses. Good examples of these are Crimean-Congo Hemorrhagic Fever Virus (CCHFV), hantaviruses, and other zoonoses and wildlife animal pathogens that may not be zoonotic. Disease surveillance efforts in wildlife and domestic species provide valuable research data to identify animal reservoirs and amplification hosts for emerging pathogens, improve our understanding of pathogen ecology, and characterize animal-human interfaces that facilitate disease spillover, amplification, and spread.

The protocol has been designed to be implemented uniformly in countries around the world. Primary and initial activities will be implemented first in Tanzania (East Africa) with potential for expansion to other areas as needed and funding allows to respond to epidemics and pandemics. The geographic scope of program activities, including those involving the sampling of wildlife and domestic animal populations is dynamic and subject to change.

All research activities will be implemented by local partners from Sokoine University of Agriculture under the guidance of the Principal Investigator (PI) and UC Davis One Health Institute personnel will provide technical and backup support for study design and planning, as well as the technical and analytical support during implementation, along with assuring compliance in target countries. All research procedures will be conducted by local implementing partners in accordance with relevant in-country laws and regulatory permissions.

In addition to the animal surveillance work mandated by award sponsors, this project will conduct research and disease surveillance in a complementary fashion with study protocols designed to assess exposure history and risk among human populations living

near sampled animals in human, livestock wildlife interface areas. This work is described in more detail in UC Davis IRB submission (IRB 2086677-1). Additionally, a NIMRI permit to cover human components has been issued (NIMR Ethics Approval Cert: NIMR / HQ / R.8a / Vol.IX / 4324; IHI IRB ID: 27-2023; and TALIRI permit for complementary livestock sampling (TARI/CC.21/041).

5.0 OBJECTIVES

The research objectives are as follows:

1. To characterize tick population composition and alternate vectors, viral prevalence and genetic lineage, and wildlife exposure to CCHFV in the Iringa, Morogoro, and Coastal regions of Tanzania.
   Hypotheses: Those wild hares, rodents, and ground-feeding birds that interface with livestock will show evidence of higher seroconversion to anti-CCHFV antibodies compared to those with less contact. Additionally, CCHFV prevalence will be elevated in rodents and ground-dwelling birds compared to hares.
2. To determine biological and ecological factors influencing the risk of viral spillover, amplification, and spread to develop and parameterize ecological and epidemiological models to estimate exposure risk for emerging pathogens (e.g., CCHFV).
   Hypotheses: Regions where wildlife and domestic animals interact will demonstrate an elevated risk of viral/CCHFV spillover compared to regions with less interactions. Additionally, those regions associated with higher rainfall and vegetation will have higher numbers of ticks and therefore CCHFV.

6.0 METHODOLOGY

6.1 STUDY LOCATIONS OF SAMPLING

Sampling locations within Tanzania will include the following regions: Iringa regions (Iringa rural districts in villages bordering Ruaha National Park and Kilolo districts in villages bordering Udzungwa National Park and West Kilombero reserve), Morogoro regions (Kilombero District, Ulanga, and Mlimba Districts in villages bordering Udzungwa, Mikumi and Mwalimu Nyerere (Former Selous Game Reserve) National Parks) and the Coastal region (Bagamoyo districts in villages bordering Saadani National Park as well as Wami Mbiki Game Controlled Area).

The Kilombero river valley, which includes Chita receives an average annual rainfall of 1200mm 1800mm and annual temperatures between 25°C and 32°C. This location has been selected to represent wildlife ecosystems in Udzungwa, Mikumi and Mwalimu Nyerere (Former) Selous National Parks. Specifically, sampling will occur in villages bordering National Parks, Game reserves and forests.

The Iringa Rural District, which contains Idodi and Pawaga, is known for its more semi-arid climate, receiving an average annual rainfall of approximately 500 mm and annual temperatures ranging between 20°C–25°C (Council et al., 2013). This location has been selected to represent wildlife ecosystems in Ruaha National Park. Specifically, villages bordering Ruaha National Park will be sampling sites.

Bagamoyo district in the Coastal Region of Tanzania is characterized by a warm, tropical climate. The area experiences an average annual rainfall of approximately 800 mm, with temperatures typically ranging between 25°C and 30°C. The climate is influenced by its proximity to the Indian Ocean, resulting in higher humidity and more consistent rainfall compared to more semi-arid regions like Iringa (NEMC, 2021). This location has been selected to represent wildlife ecosystems in Saadani National Park and Wami Mbiki game-controlled areas. Specifically, sampling will occur in villages bordering Saadani National Park.

6.2 SPECIES

The following taxa/genera are proposed to be sampled with this initial permit, with the possibility of expanding to include migratory birds and other small mammals. Taxa include rodents, lagomorphs (i.e., *Lepus* species), and fowl (i.e., *Numida* species, *Pternistis* species, *Coturnix* species, *Scleroptera* species, *Pterocles* species). Examples of rodents and shrews collected include but are not limited to *Rattus* species, *Mastomys species, Crocidura* species, *Mus* species, and *Pedete* species. The aforementioned species are not all-inclusive of what may be captured. Species captured will be identified in the field during the sampling. An amendment to the permit will be submitted should this be desired. All targeted numbers of animals to sample have been determined using Scalex and ScalaR calculators designed to determine samples sizes necessary to determine disease prevalence[21].

For an expected prevalence of 6.5%, the required sample size of rodents per trapping session is 105 for the margin of error or absolute precision of ±5% in estimating the prevalence with 95% confidence and considering the potential loss/attrition of 10%. With this sample size, the anticipated 95% CI is (1.5%, 11.5%).

For an expected prevalence of 13.6%, the required sample size for lagomorphs is 202 per trapping session for the margin of error or absolute precision of ±5% in estimating the prevalence with 95% confidence and considering the potential loss/attrition of 10%. With this sample size, the anticipated 95% CI is (8.6%, 18.6%).

For an expected prevalence of 5%, the required sample size of guineafowl per trapping session is 82 for the margin of error or absolute precision of ±5% in estimating the prevalence with 95% confidence and considering the potential loss/attrition of 10%.

With an expectation to sample in each of our three sites twice per season (wet and dry) for approximately 3 years, this led to the following targeted numbers for our entire study:

Taxa	**Target Number Per Trapping Session**	**Numbers to not Exceed in 1 Year of Sampling**	**Numbers to not Exceed in 3 Years of Sampling**
Rodents and Shrews	105	650	2000
Lagomorphs/ Small Mammals	50	150-300	450-1000
Birds	82	500	1500

6.3 BRIEF PROCEDURES FOR CAPTURE AND SAMPLING

Animals will be sampled for emerging infectious disease surveillance, including but not limited to Crimean-Congo Hemorrhagic Fever virus. As CCHFV is our initial focus with potential expansion to other emerging infectious diseases, an Appendix 1 is available with additional background on CCHFV.

Wildlife species will be either manually restrained or trapped (+/- chemical restraint) depending on the species. Animal processing will include a brief physical examination, collection of weight and measurements, and sample collection: blood, swabs (oropharyngeal/oral, nasal, urogenital/cloacal, rectal/cloacal, skin), urine, feces, tissue, and external parasites. If anesthesia is utilized, it will be short acting or chemically reversed with appropriate observation. After sampling free-ranging animals will be returned to the immediate vicinity of capture.

In each situation where sampling will occur, all studies will be conducted in a humane, non-lethal manner, while minimizing the impacts on individual animals and animal populations. Where possible, sampling will include non-invasive methods that do not require the capture or restraint of animals. Project field teams will work with UC Davis team members to adapt

our sampling strategy based on new knowledge we obtain on estimated pathogen prevalence in order to minimize the number of animals sampled.

Taxa	Procedures/Treatments
Rodents	Capture, restrain (physical/chemical), sample, and release live animals to collect research specimens, including blood, mucosal swabs (oropharyngeal/oral, nasal, urogenital, rectal), skin or lesion swabs, tissue (skin biopsy/scraping or lesion biopsy), feces, urine, saliva, hair, ectoparasites, and milk (from lactating females). Physical exam, weight, morphometric measurements, and dentition exam. Temporary or permanent marking of individuals. Non-contact sampling and data collection may occur, including urine and feces collected from the ground, beneath roosts, from nests not in use, or latrine areas; imaging and video/audio recordings (e.g., photographs, thermal/IR imaging, LIDAR, camera traps, acoustics recorders); saliva from chewed dental ropes or discarded plant materials, and behavioral observations. Capture will occur via non-lethal rodent traps, e.g., Sherman traps, pit traps, and box traps.
Lagomorphs/Small Mammals	Capture, restrain (physical/chemical), sample, and release live animals to collect research specimens, including blood, mucosal swabs (oropharyngeal/oral, nasal, urogenital, rectal), skin or lesion swabs, tissue (skin biopsy/scraping or lesion biopsy), feces, urine, saliva, hair, ectoparasites, and milk (from lactating females). Physical exam, weight, morphometric measurements, and dentition exam. Temporary or permanent marking of individuals. Non-contact sampling and data collection may occur, including urine and feces collected from the ground, beneath roosts, from nests not in use, or latrine areas; imaging and video/audio recordings (e.g., photographs, thermal/IR imaging, LIDAR, camera traps, acoustics recorders); saliva from chewed dental ropes or discarded plant materials, and behavioral observations. Capture will occur via box trap followed by chemical restraint as needed.
Birds	Capture, restrain (physical/chemical), sample, and release live animals to collect research specimens, including blood, mucosal swabs (oropharyngeal/oral/tracheal, nasal, urogenital, cloacal), skin or lesion swabs, tissue (skin biopsy/scraping or lesion biopsy), feces, urine, feathers, and ectoparasites. Physical exam, weight, morphometric measurements, and oral exam. Temporary or permanent marking of individuals. Non-contact sampling and data collection may occur, including urine and feces collected from the ground, beneath roosts, or from nests not in use; imaging and video/audio recordings (e.g., photographs, thermal/IR imaging, LIDAR, camera traps, acoustics recorders); and behavioral observations. Capture will occur via net (e.g., hand net, throw net, projectile nets, mist nets, boat-assisted net), leg noose, or box trap (for waterfowl).

6.4 SAMPLE STORAGE, TESTING AND ANALYSIS

Collected blood samples will be allowed to clot before having serum extracted on the day of sampling. All Samples will be packed in cryovial and transferred to the HALI laboratory at Sokoine University of Agriculture in cold chain (liquid nitrogen tank) for further storage at minus 80°C prior to specific laboratory testing. Serum samples will be heat treated for a minimum of 120 minutes at 56°C prior to testing. Heat treatment of serum under these

conditions is based on requirements for inactivation of any notifiable viral, bacterial or parasitic pathogens in these samples as well as also being sufficient to inactivate CCHFV 165 (Weidmann et al., 2016). Serum samples will be tested using the ID Screen® CCHF Double Antigen Multi-species ELISA (IDvet, Grabels, France) used according to the manufacturer's directions.

The collected ticks will be identified by experienced laboratory personnel using published morphological keys for African ticks (Walker et al., 2014) . The sample containing tubes will be obtained from -80°C once the tick specimens are dead and immobilized. To each tube of tick specimen 70% ethanol will be added, then caped and inverted approximately 5 times in order to rinse the ticks and remove any dirt or hair. Ethanol will be discarded and immediately rinse the ticks with distilled water. The distilled water will be discarded and tick transferred to a paper towel to briefly dry. Ticks will be transferred into a petri dish for identification and sorting by sex and genera based on the presence or absence of banded legs, coloured or patterned scutum and conscutum, presence or absence of festoons and eyes and shapes of the mouthparts.

The morphologically identified tick species will further be identified in the laboratory using a stereo microscope then properly stored in -80^{0}C freezers and species will be confirmed using polymerase chain reaction targeting Internal Transcribed Spacer 2 (ITS2) as DNA barcoding genes for selected members of species and those which would be difficult to identify to species level morphologically (Lv et al., 2014).

RNA will be extracted from pooled tick samples (consisting of 7–10 individual ticks according to site and species) using the QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. Invitrogen SuperScript™III Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and samples will be tested by qRT- PCR using specific primers. Wildlife serum specimens will be prioritized for CCHF testing following likely transmission and exposure through contact with people and domestic animals. Following CCHF testing, any products identified for sequencing will be cloned and sequenced to enable identification at the genus and species level for CCHF viruses. Findings will be characterized and interpreted to determine their potential to cause disease, along with their genetic and phenotypic properties to better understand their evolution, emergence, host range, and risk for transmission and spread in people or other animal populations. All consensus-based CCHF viral family testing will take place in-country. Interpretation of PCR results, preparing of potential positives for sequencing, and interpretation of obtained sequences will also take place in-country and occasionally for validation purpose abroad at partner UCDavis One Health laboratories.

Tick collection data will be stored in a computer, using Microsoft Excel. Statistical analysis on tick distribution data will be performed using R software to compare the proportion of hard tick abundance in the study areas, compare the proportion of hard tick species burden in cattle in the dry and wet season. A two-way ANOVA will be performed to detect the effect of season and village on the tick species. The obtained DNA sequences will be compared with the sequences on GeneBank (NCBI database) using Basic Local Alignment Search Tool

(BLAST) to obtain sequence similarities.

6.5 ASSESSMENT OF BIOLOGICAL AND ECOLOGICAL FACTORS

Biological and Ecological niche models will be developed to characterize virus risk using publicly available and project-derived environmental and virus infection data. Published reports on CCHFV in East Africa will be compiled into a database stratified by host, vector, and location, among other variables, and analyzed to characterize the taxonomic breadth and host plasticity of the viruses. Environmental parameters, extracted from publicly available datasets e.g. WorldClim, and MerraClim (9) will be used. Data on vertebrate hosts and tick vectors known to carry CCHFV will be actively curated by the team using standardized systematic searches of peer-reviewed scientific literature. Data on species occurrence and geographical distribution will be obtained from the Global Biodiversity Information Facility (GBIF), which currently hosts over 110 million records of spatially referenced animal species occurrences (10). Our team also has curated a dataset of macroecological traits of mammalian species which will be used to develop distribution models along with other known datasets including PanTHERIA (11).

Similarly, VectorMap provides a geospatial distribution of known tick vectors of CCHFV. Using these data streams we will develop boosted regression tree models to create large-scale ecological niche models and predict spatial distribution of CCHFV risk. Specifically, the probability that spillover can occur at a given location will be assessed based on environmental, climatic, land use, host and vector distribution covariates, using boosted regression trees (12). Project-derived data on CCHFV prevalence in ticks and cattle, estimated tick species distributions, and climatic and ecological variables will be used to assess spatial and temporal patterns of CCHFV risk in Tanzania as funding allows.

Project findings will be compared to the ecological niche models and used to resolve relevant questions, e.g. What are the key drivers of species of tick presence or absence? Overall, models from the aim of this objective will assess knowledge gaps in the disease ecology of this complex vector-host and virus-reservoir host zoonotic disease systems and inform targeted surveillance strategies.

7.0 DISSEMINATION OF INFORMATION

Before permission to publicly share results is granted, from in-country authorities, laboratory data from wildlife samples will be stored in a secure, internal database, accessible to a limited number of authorized staff, including the Principal Investigator and in-country laboratory leads. The PI shall work with all project team to complete all required reports (quarterly financial and technical reports, semi-annual report, final report etc.) and respond to all requests for information from the grantor in a timely and professional manner. Project PI shall prepare an official letter to accompany results report for TAWIRI and government ministries responsible for wildlife health.

Specifically, the report will be submitted to the Director of Tanzania Wildlife Research Institute (TAWIRI) and the Director of Tanzania Commission of Science and Technology (COSTECH). The PI will contact the Directors and discuss the plan on logistics and strategies for knowledge/ information sharing to the community/public including taking the lead of manuscripts development for publications in scientific journals.

8. ETHICS

The study protocols, questionnaires, and consent documents will be approved by the Tanzania Wildlife Research Institute (TAWIRI) ethics committees, and Sokoine University of Agriculture Ethics Committee. Written informed consent or assent for sample collection and questionnaire administration will be collected from all participants. Permission to publish will be requested from the Director of TAWIRI

9. CAPACITY STRENGTHENING

9.1 VIRUS DETECTION TRAINING

All testing will be conducted at HALI SUA lab. The lab is a BSL-2 plus laboratory equipped with Biosafety Cabinet class II (BSC II). The lab structurally has ground and first floor. It has four main lab rooms/stations separated from each other by walls. Rooms in ground floor are G1 & G2 while those in first floor are F1 & F2. The stations are positioned to allow unidirectional workflow to easily control contaminations.

The Project team shall expand detection capabilities beyond pathogens detected by previous projects at SUA to include next generation sequencing techniques; the MinIon Nanopore Technologies 1D sequencing to include previously undetected ssRNA CCHV among sampled ticks and livestock hosts. The training on the technology will be provided by UCDAVIS partners.

The PI, Co-PI's shall work with laboratory experts from SUA and University of Calfornia Davis (UCD) partners to provide training to staff and students on PCR-based diagnostics, Nanopore sequencing technology, and metagenomics analysis of detected viruses. Trainings shall be designed to enhance diagnostic capabilities and support broad-based PCR detection of high-consequence emerging CCHV viruses using the aforementioned nucleic acid amplification techniques.

HALI-SUA lab is also mentored with molecular expert from the Department of Veterinary Medicine and public Health of SUA. The technology will markedly improve the technical capacities of SUA and Tanzania as whole to identify and track animal-to-human transmission chains in cases of zoonotic viruses' spill over and sequential outbreaks

10. THE PROJECT WORKPLAN AND BUDGET

The proposed project will be conducted for three years and the activities will spread across different quarters shown below:

10.1 PROPOSED PROJECT WORKPLAN

Table 1: Proposed Project Workplan

TASK	2025				2026				2027			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Permit application for wildlife sampling												
Procurement of materials including supplies and reagents												
Development of animal sampling and lab testing protocols together with data collection tools												
Onsite refresher training of technicians on basic principles on PCR techniques												
Wildlife sampling at huma-animal interface areas												
Laboratory testing for CCHF viruses												
Data analysis												
Publication of research findings												
Report writing												

10.2 PROPOSED BUDGET

Table 2: Proposed budget for the project in US$

S/N	Item	Year 1	Year 2	Year 3	Total
1	Salaries and emoluments	60,876.29	63,920.10	67,116.11	191,912.50
2	Fringe benefits	13,367.02	14,035.37	14,737.14	42,139.53
3	Domestic and International travels	18,170.00	19,078.50	20,032.43	57,280.93
4	Equipment and Supplies	23,631.00	24,812.55	26,053.18	74,496.73
5	Other costs (permits, dissemination)	22,596.24	23,726.05	24,912.35	71,234.65
6	Sub-total	138,640.55	145,572.58	152,851.21	437,064.33
7	10% overhead	13,864	14,557	15,285	43,706.43
8	**Total**	**152,504.61**	**160,129.84**	**168,136.33**	**480,770.77**

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12. APPENDICES

12.1 APPENDIX 1: ANIMAL CAPTURE AND SAMPLE COLLECTION

Live captures will be supervised by experienced project veterinarians or a trained field biologist. Pre-capture safety briefings will be held prior to each capture event so that all participants are informed of the capture plan, location of human first aid equipment, and emergency procedures. In addition, all personnel taking part in project animal capture, handling, and sampling activities will undergo and receive training in protocols prior to initiation of animal sampling activities. Capture methods and manual or chemical restraint will be utilized as appropriate to the species being sampled, as described below for specific taxa. Great care will be taken to minimize disruption to social groups/colonies, and to habitat, whenever wild free-ranging animals are captured. Non-target animals captured in nets or traps will be given a quick health check and then released.

RODENTS AND SHREWS ("RODENTS")

Capture: Free-ranging rodents will be captured through live-traps, including Sherman traps, pit traps, and box traps; captive rodents, including resident free-ranging wild rats/rodents in markets, will be manually captured or captured through live-traps. Traps will be checked a minimum of once daily in the morning. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. When shrews are anticipated to be a target of capture, we will reduce checking time to at least once every 6-12 hours. Traps will include shelter, food, and water, and padding will be added depending on the trap type. Shelter will consist of hay or straw, fine wood chips, leaf litter, or similar material depending on species and site. We will include high-energy food supplements such as water-soaked sunflower seeds, peanut butter and oats, mealworms, wet cat food, etc., and access to water through moist food and fresh fruit like apples (or melon, watermelon, mango, etc.) and/or edible hydration gel (e.g., rodent HydroGel). Pest-rodents (i.e., Ratus rattus or Ratus norvegicus) may be opportunistically sampled if available from pest-control operations at our study sites (i.e., zoos, rehabilitation centers, markets, human dwellings); however, no urban pest rodents will be intentionally captured by the project team unless specifically related to disease outbreak response research.

Restraint: Following capture, small animals will be restrained with a fine mesh bag to minimize entanglement, taking precautions to ensure the animals are not traumatized by the hoop of the net or through net removal. Sampling procedures will involve manual

restraint and anesthesia if necessary. Larger rodents will be restrained for sampling in specialized squeeze-cages, allowing adjustments appropriate to the size of the animal. Squeeze-cages consist of a wooden frame with a plasticized wire bottom and a Plexiglas shield used to press the animal, while ensuring visualization of the animal. Once squeezed, a rod is inserted to keep the plastic shield in place. The box is then inverted, allowing sampling to be conducted through the open wire bottom and abdomen of the animal when the animal is safely immobilized. Anesthesia for small rodents will be conducted using an "open-drop" technique. The animals will be transferred directly from the traps to clear re-sealable plastic container or clear plastic tubes. 0.4 ml of isoflurane will be applied to a cotton ball which will be placed into a metal tea ball (to permit the gas to escape, but prevent contact with the skin) and dropped in the re-sealable bag or plastic tube (nose cone) (0.4 ml is the appropriate dose for a ca. 20g mouse, dose will be adjusted as needed for larger rodents). Where available, isoflurane may be delivered with a vaporizer and facemask at 4-5% for induction and 2% for maintenance. For larger rodents, chemical restraint and anesthesia (ketamine alone 25-50mg/kg, or ketamine combined with xylazine 80-100 mg/kg ketamine + 10-12.5 mg/kg xylazine) will be applied either through the squeeze cages by syringe if applicable, or by blow dart to minimize tissue damage.

Sampling and data collection: Sample types and data collection will follow procedures described above for all taxa groups. In small rodents, a blood sample will be collected using a 3-5.5mm point lancet depending on size on the facial or submandibular vein (http://www.medipoint.com/html/for_use_on_mice.html). Saphenous, femoral, ventral tail vein or jugular venipuncture may be used for larger rodents (e.g., grass cutters). After blood is collected, a clean compress will be placed on the site of puncture to stem blood flow.

WILD BIRDS

Capture:
Capture of free-ranging flighted birds will be done through a variety of methods, depending on habitat, species size and behavior, etc. Generally, methods described in FAO Wild Birds and Avian Influenza: An Introduction To Applied Field Research And Disease Sampling Techniques[26] and The Humane Capture, Handling, and Disposition of Migratory Birds[27] will be followed. Guinea fowl will be captured by hand or via baited drop-net technique. Other methods include mist-netting and traps for shorebirds and passerines; boat-assisted net, leg noose, and corral or box trap capture of waterfowl and other aquatic species; and mist netting and manual capture of larger species such as storks and cranes. Traps will be under frequent observation and animals will be removed immediately after being caught. Mist nets will typically be set at dawn and/or dusk and checked every 15-30 minutes during the capture period. The number of nets will be limited to that which the field crew can effectively

deal with should a large number of birds be caught. Mist nets will not be set in direct sunlight and birds will not be captured during extreme climatic conditions when they may be at increased risk of hypothermia or hyperthermia.

Restraint:

Fowl will be manually restrained and captured either manually or through nets or nooses if necessary. To reduce stress, a breathable fabric or cloth stockinette may be used to cover the head and reduce visual stimulus.

All birds captured will be removed from traps or nets and kept in clean cloth bags or cardboard boxes until they are processed. Larger birds captured individually will be processed directly upon capture. To reduce stress, lightweight dark cloth of breathable fabric or a cloth stockinette may be used to cover the head and reduce visual stimulus. Birds will primarily be physically restrained in a manner that minimizes stress by maintaining control of the bird's head, legs, feet and wings. If necessary, isoflurane anesthetic can be administered at 3% isoflurane for induction and 1-2% maintenance.

Sampling and data collection: Sample types and data collection will follow procedures described above for all taxa groups. Venipuncture from fowl will target the brachial (wing) or jugular veins.

For flighted birds, blood samples will be taken by basilic, metatarsal, or jugular venipuncture, followed by pressure to promote hemostasis. Even though only minimal blood volumes will be collected, in small birds, sterile saline at body temperature may be injected SC to avoid pressure drop, dehydration and reduce recovery time. Collection of samples from roosts and nests will be undertaken with minimization of disturbance time and minimal disruption to structure of nest to reduce nest abandonment. Sampling will be undertaken during times when adult birds are absent from the nest to reduce disturbance.

WILD SMALL MAMMALS/LAGOMORPHS

Capture and restraint: Capture of free-ranging small mammals (e.g. lagomorphs) will be done with box traps. The animals will be restrained and anesthetized with techniques similar to those described for rodents. Briefly, care will be taken to minimize direct handling through the use of plastic tubes and pre-fabricated squeeze cages. If necessary, anesthesia will be conducted with 4-5% isoflurane gas by placing the animal cage into a chamber (e.g., a hard plastic container as appropriate) with isoflurane applied to a cotton ball placed into a metal tea ball or plastic tube that prevents direct contact from the animal. The volume of

isoflurane used will be appropriate to the container volume and desired percentage of gas. Where available, isoflurane may be delivered at 4-5% for induction and 2% for maintenance.

Sampling and data collection: Sample types and data collection will follow procedures described above for all taxa groups. Blood collection site will be selected based on the species, and may include saphenous, femoral, ventral tail vein, ear veins, or jugular venipuncture. After blood is collected, a clean compress will be placed on the site of puncture to stem blood flow.

LIVE ANIMAL SAMPLE COLLECTION

All animals captured will be identified visually or by additional genetic testing as needed. Multiple types of specimens will be collected from each animal routinely, including blood; swabs (urogenital, oropharyngeal/oral, nasal, and rectal) in duplicate; and ectoparasites. When possible, free catch urine and fresh feces will be collected instead of swabs. A skin biopsy/scraping or swab may be collected for genetic testing (barcoding) or to sample a lesion. Milk may be collected from lactating females. Animals may also be marked using temporary or permanent methods, photographed or recorded, weighed, measured, examined, and observed. Senior veterinary scientists will train all field personnel in conducting these procedures. No animal will be euthanized in order to sample it, and our staff will not facilitate or encourage other researchers to collect animals from the wild using euthanasia or any other lethal means.

SWABS: Appropriately sized sterile polyester swabs will be used to collect oropharyngeal/oral, nasal, urogenital, rectal, cloacal, and skin samples. The swabs will be gently inserted into the cavity or placed against the body surface and rubbed in a circular motion for 10 seconds to exfoliate surface cells and collect any body fluids or mucous. As needed, swabs will be lubricated with sterile saline or viral transport media (VTM).

SALIVA: As an alternative to traditional oral swabs, chewed dental ropes[25] or chewed and discarded plant materials[26] may be utilized to collect saliva samples. To encourage animals to chew on offered dental ropes, the ropes will be dipped in a suitable attractant (e.g., jam, honey, or fruit juice) or placed within a species appropriate food item (e.g., banana for primates). Nectar feeding stations may be used to collect saliva samples for some taxa and will resemble hummingbird or oriole feeders with a container for nectar and small openings for feeding. Feeding stations will be cleaned, disinfected, and refilled with fresh nectar daily when in use. Nectar will consist of a sweet fruit juice that animals find palatable (e.g., mango juice, apple juice, or similar), a sugar solution composed of 1 part sugar to 4 parts water, or a commercially available nectar product typically used for attracting birds.

BLOOD: Venipuncture will be used to collect less than 1% of the body mass of blood from each individual (10µL blood per gram of body weight). Animals weighing at least 0.5 kg (500 grams) and above such as Lagomorphs and birds we will safely collect 5.0 mL of blood when possible,as needed for laboratory testing . Only the volume of blood needed for planned testing will be collected, which will result in smaller relative volumes being collected from larger animals. For all species, no more than two attempts to collect blood will be made by a veterinarian or a trained field biologist. If blood cannot be collected, the animal will be released without collecting blood. As appropriate to the species being sampled, collection of blood may be assisted by initially warming the collection site with a water bottle filled with warm water until vein engorgement becomes evident.

FECES AND URINE: Samples of fresh feces and urine will be collected directly from the ground, or by placing tarps underneath roosting sites or areas where animals are congregating and collecting droppings with sterile swabs, forceps, or pipets. Care will be taken to avoid the disruption of roosting animals when placing tarps. Feces and urine may also be collected while animals are restrained for sampling using free-catch methods and may replace rectal and urogenital swabs when it is possible to obtain these sample types.

TISSUE: Skin biopsies will be conducted to collect a small piece of tissue for analyses of lesions or genetic/cellular material when other less invasive sampling methods will not yield sufficient data. A sterile disposable punch biopsy tool or scalpel blade will be used to collect a 2-5mm tissue sample. Alternatively, and as appropriate to the lesion type, a skin scraping will be collected with a sterile scalpel blade, covering an area of no more than 1 squared centimeter in larger animals and 0.5 squared centimeter in smaller animals (e.g. small bats, rodents). Up to 2 samples will be collected per animal.

ECTOPARASITES: If ectoparasites are noted, a sample will be collected and placed in ethanol using appropriate instruments (e.g., forceps or a small comb).

PHYSICAL EXAMINATION AND MORPHOMETRICS: Each animal captured will receive a brief physical examination, noting body condition and any pre-existing injuries or evidence of illness. Weight and measurements will be taken, and dentition may be examined.

IMAGING AND RECORDING: Imaging and video/audio recordings, including the use of photographs, camera traps, thermal/infrared (IR) imaging, light/laser detection and ranging (LIDAR), and acoustics recorders (e.g., Wildlife Acoustics Song or Echo Meter, or similar), may be taken of free ranging animals and of animals during capture and handling events. Cameras/recorders may be automatically triggered by movement or sound, or manually triggered by the researcher. These tools are utilized to confirm species identity, to assess

species density, distribution, diversity, and behavior at a sampling site, and detect and monitor species that are difficult for researchers to directly observe. When illumination is required for imaging, white or red lighting will temporarily used; however, flash photography will be avoided whenever possible to prevent disorientation in the animals being imaged.

ENVIRONMENTAL SAMPLES: Environmental samples of vegetation may occur near animals, including sampling of ticks. Sample collection will occur a safe distance from any animals.

BEHAVIORAL OBSERVATIONS: Behavioral observations of wildlife populations may be undertaken to assess physical health of individual animals, assess social structure, determine behavioral risk factors in acquiring diseases and the effect of disease on movement, behavior, feeding, etc. Care will be taken to minimize the disturbance of individual animals, especially concerning breeding, feeding, caring for young, and rare species.

MARKING ANIMALS: Most individual animals are only targeted for sampling once, yet in some field locations we will be conducting longitudinal sampling, where it will be necessary to apply some form of individual identification. When longitudinal sampling occurs, we will attempt to mark or tag all individuals using a species-appropriate method to avoid duplicate sampling. We will use both temporary and permanent methods to avoid repeated sampling within a short time period and help identify individual recaptures in successive sampling efforts. Short-term methods may include fur clipping, bleaching using hydrogen peroxide or hair dye, marking wing membranes with a permanent marker, or painting claws with nail polish. In some instances where repeated sampling is desired for longitudinal surveillance at one site, animals may be marked by inserting a sterile microchip (for example, Avid, Co. www.avidid.com, or Trovan https://www.trovan.com/) with a unique ID number subcutaneously between the scapulae (see PIT tagging, below). Additionally, and depending on species, site, and need, animals might be permanently marked with tattoos (Animal Identification & Marking Systems, http://www.animalid.com, for example, South Point Surgical, http://www.southpointesurgical.com/lab_animal_tattoo.aspx) on the ear, tail or chest as needed, or tagged with an ear tag. In the case of birds, approved leg or wing bands may be applied by a certified bander, or individuals trained by certified banders. Bands will comply with SAFRING or equivalent ringing certification schemes. For rodents, small ear cuts can be made which allows for both marking and genetic barcoding by collecting and preserving the small tissue specimen. These cuts will be similar to standard rodent hole punches, which are typically 1-1.5 mm with no more than 2 sites per ear. Wild animals will be anesthetized during marking procedures that may cause pain or distress. Some species

such as non-human primates and other large wildlife, can often be identified by photography and unique scarring or other features and therefore do not require additional marking.

PIT TAGGING: Passive Integrated Transponder tags (PIT tags) are tiny electronic radio frequency devices used to facilitate animal tracking without the drawbacks of external devices. PIT tags are typically encased in glass, protecting the electronic components and preventing tissue irritation in the animal. PIT tags will be inserted subcutaneously using an appropriate gauge needle under the animal's skin to provide a reliable unique alpha-numerical code serving as an animal ID. Skin will be disinfected with 70% ethanol prior to tag placement using a plastic syringe with an appropriate gauge single-use sterile needle. After implantation, the puncture hole may be sealed with pharmaceutical grade cyanoacrylate adhesive following label instructions including standard surgical preparation for incision site. For trained personnel, the procedure should take less than 1 min. There are different sized PIT tags available for smaller species. The tags are activated by a handheld reader through radio frequency identification (RFID), through the generation of a close-range electromagnetic field. The size of PIT tags and the location of PIT tag insertion is species dependent. PIT tags will be used by the program only in animals with sufficient body size where tag insertion has negligible impact (generally animals weighing more than 30 grams for 11-12mm standard length tags, or 15 grams for 7-8mm mini tags). Subcutaneous PIT tagging is a minimally invasive procedure that does not require anesthesia; however, if animals are anesthetized for other procedures, PIT tag deployment will be done prior to reversal or recovery. No surgically implanted PIT tags will be used in this study. PIT tags will be used for species where external marking does not allow for individual identification. PIT tags will be used only in cases where mark-recapture is needed for longitudinal studies and/or when sampling is conducted repeatedly at a location over time to avoid duplicate sampling of the same individuals. In these cases, particularly when temporary marking or other marking methods named above will not be sufficient to avoid duplicate sampling, we will attempt to tag all animals captured that are large enough for tag placement. PIT tags will also be deployed when information such as foraging behavior or movement is needed.

RELEASE: Release of the free-ranging animals will occur following sample and data collection, and complete anesthesia recovery if applicable. Animals will be released near the site of capture, away from open nets/traps, water, and other habitat elements that might cause injuries. Under no circumstances will the release site exceed 0.5 kilometer from the capture site. Captive animals will be returned to their enclosure or owner as applicable.

SAMPLE COLLECTION FROM DEAD ANIMALS

ANIMALS FOUND DEAD: In addition to our typical sample set for live animals, samples of bone, carcass, fur, fresh or clotted blood, and tissues may be collected from animals that are found dead. Depending on the condition, carcasses of dead animals will undergo partial or full necropsy. Similarly, if a death occurs during study activities, the standard surveillance samples plus tissues will be collected. After sample collection, the remainder of the carcass will be disposed of in accordance with local permitting, which may include depositing the carcass with a public health authority, natural history museum, or similar institution, or disposal of the carcass (e.g., burial, landfill burial, incineration, rendering, and composting).

EUTHANIZED ANIMALS: In the event that an animal is euthanized due to a situation in which, according to the attending veterinarian or biologist's judgment, the animal will be unlikely to survive if released and cannot be rehabilitated, then a post-mortem examination may be performed and organ tissue samples collected, in addition to other routine study samples and data. After sample collection, the remainder of the carcass will be disposed of in accordance with local permitting, which may include depositing the carcass with a public health authority, natural history museum, or similar institution, or disposal of the carcass (e.g., burial, landfill burial, incineration, rendering, and composting).

12.2 APPENDIX 2: PERSONNEL SAFETY AND TRAINING

All personnel taking part in project surveillance activities will receive training in project protocols prior to initiation of activities. Additionally, safety briefings will be held prior to each surveillance event so that all participants are informed of the activity plan, potential for exposure, the location of human first aid equipment, and emergency procedures. Individuals capturing and sampling animals will wear appropriate PPE to prevent exposure to potential pathogens. Typical PPE ensembles would include: N95 mask, gloves, goggles or face-shield, field covers, or for certain high-risk taxa or environmental interfaces coveralls with hoods (e.g., Tyvek), and rubber boots or foot covers as appropriate to the terrain and activities.

DECONTAMINATION OF DEVICES AND PPE: All multi-use instruments and devices will be decontaminated between uses at different sites to prevent inadvertent pathogen spread. Additionally, all field gear and instruments used to handle or measure animals will be decontaminated between contact with individuals as appropriate. Depending on the specific device and its sensitivity to chemicals, one of the following disinfectants will be used following manufacturer recommendations for application and contact times: 70% ethanol, 10% bleach, or 1% Virkon solution. Similarly, all reusable PPE will be decontaminated between uses with one of these methods. To minimize the need to decontaminate sensitive devices, devices may be covered with plastic bags or sheeting that can be easily disinfected, whenever doing so does not interfere with operation of the device. Devices that cannot withstand contact with these disinfectants and cannot be suitably covered will be treated with vaporized hydrogen peroxide, ethylene oxide gas, an ultraviolet sterilization lamp, or other suitable cold-sterilization technique as appropriate.

12.3 APPENDIX 3: DRUGS AND COMPOUNDS TO BE ADMINISTERED

Anesthetic drugs chosen are with the intention of short periods of restraint for our non-painful sampling procedures as needed (i.e., blood and tick collection, oral swabs, urogenital/cloacal/rectal swabs, etc.). If appropriate restraint can be maintained with minimal stress to the animal, anesthetics will not be used. Due to the flexibility necessary of anesthetizing animals (i.e., drug availability, individual species differences, individual animal metabolisms), an Appendix 7 of all possible anesthetic drugs used is available.

Taxa	**Drug**	**Dose**	**Route**	**When and how often will it be given?**
Wild bird	Isoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously as needed. 4-5% induction, 1-2% maintenance.
	Ketamine + Xylazine + Midazolam	15 mg/kg 2.5 mg/kg 0.3 mgkg	Intravenous (IV) or Intramuscular (IM)	Specific to guineafowl with possibility to supplement with 10 mg/kg ketamine
	Ketamine + Xylazine	25 mg/kg 1 mg/kg	Intravenous (IV) or Intramuscular (IM)	
Rodent	Isoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously or incrementally as needed. Passive diffusion method or via vaporizer and mask as appropriate for species. 4-5% induction, 1-2% maintenance.
Lagomorphs	Isoflurane/Sevoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously or incrementally as needed. Passive diffusion method or via vaporizer and mask as appropriate for species. 4-5% induction, 1-2% maintenance. Requires additional sedation usually.

	Ketamine + Acepromazine + Dexmedetomidine	10 mg/kg 0.25 mg/kg 0.0125 mg/kg	Intramuscular (IM)	Specific for European Brown Hare, can be supplemented with 6 mg/kg ketamine + 0.15 mg/kg acepromzaine + 0.0075 mg/kg dexmedetomidine
All Species	Atipamezole	0.1 mg/kg	Intramuscular (IM)	Reversal for medetomidine/dexmedetomidine, given at 5x the anesthetic dose for rodents or an equal volume for lagomorphs

Anesthesia monitoring: An experienced wildlife veterinarian will oversee animal anesthesia events. All sampling procedures are not expected to take more than 10 minutes per individual for small animals and 20-30 minutes for larger animals, and thus the total duration of anesthesia will be brief and limited to safe handling and sampling needs. Administration of anesthetic drugs and monitoring will be done according to "Zoo Animal and Wildlife Immobilization and Anesthesia"[22]. Briefly, after drug delivery, animals will be monitored for initial signs of drug effect including slight behavior changes, standing still, moving away from other animals, lowering the head, disorientation and staggering. For animals that are anesthetized prior to any handling, animals will not be approached until they are recumbent and unable to raise their head. Animals showing no effects or still exhibiting voluntary movements (not repetitive stereotypical movements associated with opioid agents) or which are able to lift their head, will receive a supplemental dose of the appropriate immobilization drug before being handled. Ear twitch, pedal reflexes, swallow reflex, palpebral reflex, pupil dilation, eye position, and respiration quality will be assessed to monitor anesthetic depth, noting that swallow and blink reflex are usually preserved when using ketamine or tiletamine. For certain species where this is practical, temperature, heart rate, respiration rate and quality, capillary refill time, mucous membrane color, and oxygen saturation (via pulse oximeter) will be monitored every 5 minutes. Animals will be placed on a warmed surface as needed to maintain body temperature.

For safe and rapid recovery reversal agents will be used as indicated when available. Reversal drugs (e.g., atipamezole, yohimbine, flumazenil) will be administered 1/2 intravenously and 1/2 intramuscularly when venous access is reasonably achievable, to provide both immediate and longer lasting effect (i.e., prevent anesthetic relapse after

apparent recovery). Personnel will position the animal in the optimal orientation for recovery, administer the recovery agents and back away to a safe distance. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings.

Post-Anesthetic Monitoring: Post anesthesia monitoring will be done according to "Zoo Animal and Wildlife Immobilization and Anesthesia" [22]. When possible and safe to do so, temperature, heart rate, and respiration rate and quality will be monitored every 5 minutes during the post-anesthetic recovery period. Animals will be observed for the following signs of recovery: ability to hold up their head, ability to maintain a sternal position, ability to stand, and ability to ambulate normally. Recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals receiving chemical reversing agents and animals recovering from gas anesthesia will likely recover very quickly, thus we expect the post-anesthetic monitoring period to be short for most species.

EUTHANASIA: Euthanasia will only be implemented under exceptional conditions such as the case of a severely injured animal. Euthanasia methods will vary depending on species and wild captive/domestic vs. free-ranging wild; however, methods to be used will not deviate from the American Association of Zoo Veterinarians (AAZV) "Guidelines for the Euthanasia of Nondomestic Animals," and the American Veterinary Medical Association (AVMA) "Guidelines for the Euthanasia of Animals"[29-30.] In general, animals requiring euthanasia will be immobilized and anesthetized as described above (via injectable anesthesia or isoflurane via face mask), then euthanized with pentobarbital IV, or IP, when IV access is not possible (e.g. small rodents and other small animals, severe dehydration, low blood volume) or by isoflurane overdose when appropriate to the species. When feasible, a post-mortem examination will be performed and organ tissue samples collected to maximize information collected from deceased animals. After sample collection, the remainder of the carcass will be disposed of in accordance with local permitting, which may include depositing the carcass with a public health authority, natural history museum, or similar institution, or disposal of the carcass (e.g., burial, landfill burial, incineration, rendering, and composting). There is considerable variation in what methods may be applicable.

No animals will be deliberately subjected to euthanasia as part of this study. All animals sampled as part of this study are intended for immediate release to their wild or captive respective environments following sampling. However, if an animal does sustain severe injury and cannot be transferred to a wildlife rehabilitation facility, the animal will be humanely euthanized by an experienced veterinarian according to best practices outlined in the AAZV's "Guidelines for the Euthanasia of Nondomestic Animals" or AVMA "Guidelines for the Euthanasia of Animals"[29-30.] Any adverse events resulting in euthanasia will be

reported to UC Davis staff for evaluation. across sites and countries, partly as a result of legal requirements and availability of equipment and necessary drugs. Also, the use of chemical euthanasia options may preclude some methods. Therefore, chemical methods will only be applied when proper carcass disposal is achievable and all efforts are made to limit risk of potential for relay toxicity.

Euthanasia Drugs:

Species Group	Method	Drug	Dose	Route
Wild bird	Overdose to effect	Isoflurane	Per AVMA/AAZV	Inhalation
Rodent/Lagomorph	Overdose (≥100mg/kg)	Pentobarbital	Per AVMA/AAZV	Intravenous (IV), Intraperitoneal (IP)
	Overdose to effect	Isoflurane	Per AVMA/AAZV	Inhalation

12.4 APPENDIX 4: HANDLING ADVERSE EFFECTS

NON-CONTACT PROCEDURES: Adverse effects are expected to be minimal during sampling of animals that do not require handling or contact of any kind. Potential effects include the disturbance of natural behaviors and stress associated with the presence of personnel and equipment during surveillance activities.

CAPTURE AND SAMPLING PROCEDURES: Adverse effects are expected to be minimal during the capture and sampling of individuals. Potential effects include injury during capture, hematoma formation during blood collection, reactions to stress and capture (e.g., hyperthermia, shock, capture myopathy), and reactions to anesthesia (e.g., hypothermia, regurgitation/aspiration, respiratory depression, cardiac arrest, bloat, seizures). Some of the adverse effects listed here may result in death in sensitive wildlife species. This is most likely to occur in species that stress easily when captured. Stressful capture events may also lead to the abortion of an early-stage pregnancy in small mammals. We anticipate deaths may occur in healthy wild individuals no more than once per 100 capture events. Mortality rates may be higher when sampling individuals in poor health due to disease, as these animals may be emaciated, dehydrated, or otherwise compromised.

Animals will only be captured and handled by experienced personnel to avoid injury to the animal(s). If an animal does sustain even a mild injury, veterinary care will be readily available and supplied as applicable.

NON-CONTACT PROCEDURES: During all non-contact surveillance techniques, animals will be visually monitored for evidence of disturbance and care will be taken to minimize any potential disturbance. If a significant disturbance occurs, as indicated by an unexpected increase in activity near where surveillance is occurring, personnel will physically move away from animal areas and cease operation of any equipment until animal activity levels return to normal. Procedures will be adjusted to avoid any activities that result in behaviors that indicate disturbance.

CAPTURE AND SAMPLING PROCEDURES: Animals will be observed during the capture event and promptly extracted from any capture device. In species that stress easily, any pregnant females inadvertently captured will be immediately released. Animals will be observed to identify individuals experiencing elevated levels of stress or distress, as indicated by excessive struggling movements or vocalization, increased respiration, labored breathing, or rigid or limp body posture. Wildlife captures will be scheduled to avoid inclement weather and extreme temperatures and will be carried out in terrain suitable for safely immobilizing and observing the animals as applicable. Animals are briefly examined

to identify any potential injuries that may have occurred during capture. If an animal does sustain an injury, veterinary care will be readily available and provided as applicable.

RELEASE: Animals will be released near the site of capture, away from open nets/traps, water, and other habitat elements that might cause injuries.

The potential for adverse effects during study procedures will be minimized by having all procedures conducted by experienced wildlife veterinarians and staff.

CAPTURE AND HANDLING: To minimize stress and potential injury during capture, animals will be observed during the capture event when possible and promptly extracted from capture devices as described under the procedures section. Animals will only be handled for the minimum amount of time needed to collect samples and data outlined in this protocol. Wounds that occur during capture will be treated as described below.

SAMPLING: Sampling will be conducted efficiently to minimize the duration of any discomfort. Wildlife species will be effectively physically restrained or immobilized to reduce stress and eliminate discomfort. For small animals not requiring chemical immobilization, blockage of visual stimulus, handling in cloth bags, and other methods will be employed to minimize stress.

12.5 APPENDIX 5: ANAESTHETIC DRUG LIST

Taxa	Drug	Dose	Route	When and how often will it be given?
Wild bird	Isoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously as needed. 4-5% induction, 1-2% maintenance.
	Ketamine + Xylazine + Midazolam	15 mg/kg 2.5 mg/kg 0.3 mgkg	Intravenous (IV) or Intramuscular (IM)	Specific to guineafowl with possibility to supplement with 10 mg/kg ketamine
	Ketamine + Xylazine	25 mg/kg 1 mg/kg	Intravenous (IV) or Intramuscular (IM)	
Rodents	Ketamine	25-50 mg/kg	Intramuscular (IM)	Induction and brief anesthesia. Dosages will follow cited sources, depending on species and whether the animal is free-ranging or captive.
	Ketamine + Xylazine	100 mg/kg _ 12.5 mg/kg	Intramuscular (IM)	Used for induction and brief anesthesia. 80-100 mg/kg ketamine + 10-12.g mg/kg xylazine.
	Isoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously or incrementally as needed.

				Passive diffusion method or via vaporizer and mask as appropriate for species. 4-5% induction, 1-2% maintenance.
	Dexmedetomidine	0.1 mg/kg	Intramuscular (IM)	Substitute for medetomidine if not available. Used for induction and brief anesthesia. Given one time.
	Ketamine + Medetomidine	50 mg/kg 1 mg/kg	Intramuscular (IM)	General guidance for rats.
Lagomorphs	Isoflurane	1-5%	Inhalation	Gas anesthesia delivered continuously or incrementally as needed. Passive diffusion method or via vaporizer and mask as appropriate for species. 4-5% induction, 1-2% maintenance. Requires additional sedation usually.
	Sevoflurane	1-5%	Inhalation	Usually a quiet option without the need for additional sedation.

	Butorphanol + Acepromazine	0.2 mg/kg 0.75 mg/kg	Intramuscular (IM)	May be mixed int he same syringe. Laryngeal reflexes preserved.
	Dexmedetomidine	0.05-0.2 mg/kg	Intramuscular (IM)	Profound drop in heart rate possible.
	Ketamine Xylazine Acepromazine	20-35 mg/kg 3 mg/kg 0.75 mg/kg	Intramuscular (IM)	First inject acepromazine and xylazine mixed in same syringe, then inject ketamine into different muscle.
	Ketamine Midazolam	25 mg/kg 0.05-2 mg/kg	Intramuscular (IM)	Can be mixed in the same syringe.
	Ketamine + Acepromazine + Dexmedetomidine	10 mg/kg 0.25 mg/kg 0.0125 mg/kg	Intramuscular (IM)	Specific for European Brown Hare, can be supplemented with 6 mg/kg ketamine + 0.15 mg/kg acepromzaine + 0.0075 mg/kg dexmedetomidine
All species	Diazepam	0.5-1mg/kg	Intravenous (IV)	Treat seizures during anesthesia. Dosages will follow cited sources, depending on species.
	Doxapram	2 mg/kg	Intravenous (IV)	Respiratory stimulant for emergencies in

				anesthesia. Dosages will follow cited sources depending on species.
	Lidocaine	2 mg/kg	Intravenous (IV)	Respiratory stimulant for emergencies in anesthesia. Dosages will cited sources depending on species.
	Epinephrine	0.01 mg/kg	Intravenous (IV)	Cardiac stimulant for emergencies in anesthesia. Dosages will follow cited sources, depending on species
	Atropine	0.5 mg/kg	Subcutaneous (SC)	To prevent excess salivation and choking. Dosages will follow cited sources, depending on species.
	Dexamethasone	2 mg/kg	Intravenous (IV)	Treat shock during anesthesia. Dosages will follow cited sources, depending on species.
	Atipamezole	0.1 mg/kg	Intramuscular (IM)	Reversal for medetomidine/ dexmedetomidi

				ne, given at 5x the anesthetic dose for rodents or an equal volume for lagomorphs

12.6 APPENDIX 6: TAXA JUSTIFICATION

Refinement, Replacement, and Reduction: The "three R's" in animal research aim to minimize pain and distress for animals used in research while maintaining scientific integrity.

Refinement: The objectives of this project are to monitor the interface between wildlife, humans, and domestic animals for emerging pathogens that could emerge and spread in human populations. To do so, we will need to restrain or capture, sample, and release live animals to collect research specimens, including blood, swabs, tissue, hair, feces, urine, and ectoparasites, to determine the potential for and risk factors for disease emergence. The live animal samples collected are minimally invasive and handling times are minimized to prevent unnecessary stress. There are no alternative methods to achieving these objectives for live animal sampling, although, when possible, we will collect samples that do not require the capture of individuals (feces below roosts, chewed material, etc.) when they are reasonable substitutes for other required sample types. The capture, anesthetic, and sampling techniques proposed for this study have been published in peer-reviewed and edited journals and volumes, including "Zoo Animal and Wildlife Immobilization and Anesthesia"[22], "Handbook of Wildlife Chemical Immobilization"[23] and the Field Manual for Wildlife Diseases[24].

Replacement (Species Rationale): Wild animals and their associated arthropod vectors are known to be the source of multiple emerging pathogens affecting humans, oftentimes via domestic animals, and the study of wild and domestic animals and their vectors where they are in contact is one practical way to determine the pathogens of these species and their potential transmission pathways to humans. The proximity of pets and domestic animals to humans creates circumstances where these species can play an important role in disease transmission from domestic animal populations to human populations or from wild animal populations to human populations.

1. Rodents and shrews. The importance of this taxa for a number of diseases is well known (e.g. plague, Lassa Fever, hantaviruses) or has been suggested. Given the diversity of the species in this group and the difficulty in identifying most of the species, very little is known about their ecology or the pathogens they carry and their potential impacts on public health. Species: Wild free-ranging animals such as mice and rats, resident rodents in households, markets, peri-urban and urban areas, areas adjacent to National Parks, rodents caught by subsistence hunters if those animals are available for sampling live, and non-native rodents caught for pest control.

2. Lagomorphs. Hares around the world can play a role in the transmission of zoonotic diseases such as *Francisella tularensis*, plague, *Encephalitozoon cuniculi*, leptospirosis, ringworm, and more along with diseases important to domestic animals and agriculture such as rabbit hemorrhagic diseases virus and myxomatosis. Specifically for CCHF and emerging infectious diseases, hares are the preferred hosts for immature *Hyalomma* species, the proposed primary vector of CCHFV. Additionally, they have been found to be amplifying hosts of CCHF in France. This information has not been explored in Tanzania. Species: Wild free-ranging animals, such as the African Savanna Hare and Scrub Hare, dwelling near households, markets, peri-urban and urban areas, those caught by subsistence hunters if those animals are available for live sampling, and in villages bordering National Parks.

3. Wild birds. Birds play an important role in the transmission of a number of zoonotic diseases such as West Nile virus and avian influenza and are also capable of dissemination of arthropod vectors across great geographic distances during migration. Specifically, ground-feeding guinea fowl, which represent a host for immature ticks and are in close proximity with humans and domestic animals. The global diversity of birds and the interactions between wild and domestic birds indicates the potential for cross species transmission of pathogens.
Species: Initial sampling plans are focused on ground-feeding guinea fowl dwelling near households, markets, peri-urban and urban areas, areas adjacent to national parks, and

potentially those caught by subsistence hunters if available for live sampling. Expansion of the project could potentially include migratory birds such as wild free-ranging birds and birds in the wildlife trade, including waterfowl, shorebirds, storks, cranes, passerines, and parrots, as well as other bird species in markets and already housed as pets, if available for sampling.

Reduction: The purpose of this study is to conduct long term disease surveillance across multiple countries in large populations of animals with the goal of detecting emerging high-consequence zoonotic viruses and other pathogens that may pose a risk to the health of both humans and animals. The detection of virus shedding in animal populations is difficult, as shedding may only occur sporadically for a short duration and some diseases may cause no obvious clinical symptoms to indicate sampling efforts are needed in our species of interest. For certain viruses, such as CCHFV, infected livestock have very short infective windows with low levels of viremia, and are required hosts for the ticks which primarily vector the virus; thus, in order to potentially detect viral molecular components from ticks or characterize serological evidence of infection in livestock, robust sample numbers will be required. Further, not all disease hosts are known at the species level, thus we often need to focus more broadly on sampling within taxa groups where some members are known or suspected to be hosts. In addition to testing collected specimens for viruses, we are adapting serological assays to perform well on small blood samples from multiple species to be able to indicate past exposure to known pathogens and characterize risk in the absence of virus shedding.

From previous work, we understand that the number of individuals within a species that will test positive is highly correlated with number of individuals sampled, and thus a robust sampling effort is required to meet project objectives. Current projected sample sizes for taxa of interest are based on 1) the estimated rate of virus shedding for rare pathogens, with < 1% shedding prevalence in a target host population during a sampling period; 2) the estimated likelihood of encountering a previously undetected pathogen (for pathogen discovery and characterization efforts), which will only represent a subset of all virus detections; and 3) our interest in characterizing pathogen presence or absence while accounting for expected variation in shedding among the species affected and the ecological context, and multiple regional emerging disease hotspots of interest. Thus, we project that fewer than 1 in 100 animals sampled will be shedding a virus of interest and only a subset of those positives will include a newly detected virus of interest. To evaluate and characterize the risk of viral spillover, amplification, and spread, multiple detections of each virus of interest is often needed.

12.7 ANNEX 7: TICK SPECIMEN DATA FORM

Data recorded by: _____ **Specimens identified by:** _____

Date: (dd)_____(mm)_____(yyyy)_____

Animal ID (CXXXX):_____

	Adult		**Larvae/Nymph**	
	Male	**Female (engorged)**		**# of Tubes**
Amblyomma				
Hyalomma				
Haemaphysalis				
Boophilus				
Rhipicephalus				
Unidentifiable				
Totals:				

Section II - Tick Collection

Materials:

Labelled 15 ml conical

50 ml conical

Blunt ended forceps or other tick removal tool

Forceps (to manipulate ticks for identification)

Ethanol (70%)

Labelled 2ml cryovial tubes

Cryoboxes

Fully charged dry shippers (LN2)

Petri Dishes (10 cm)

Distilled water

Stereo/Dissecting microscope

Tick Key (for species identification)

Wet ice or ice packs

Labelled 2 ml cryovial tubes

Hand lens

Folding table (sample processing in field)

Procedure for Tick Collection:

1. Before sample collection, ensure that the animal is effectively restrained to avoid injury to the animal and/or study personnel.

2. Visually inspect the animal for ticks. Take photos to document the presence of ticks in each of the following locations: Head (pay particular attention to ears), Flank/Abdomen, Legs, and Perianal region (perineum and tail). For sample photos see below. Estimate and document the overall tick burden using the attached guide

3. Remove ticks from animal using blunt ended forceps or tick removal instrument. Secure the forceps/instrument around the tick as close to the skin as possible and pull upward with steady, even pressure.

*Be careful to avoid crushing engorged ticks.

*Be cautious that some species of ticks can cause localized pain to the animal during removal, which may cause the animal to jerk or kick.

4. After removal, quickly transfer ticks into an empty labeled 15 ml conical and place the thumb from your non-dominant hand over the mouth of the conical to temporarily seal the opening while you are collecting additional ticks. (Engorged ticks may be too large for storage in a 15 ml conical. If this is the case, use a separate labeled 50 ml conical to store engorged ticks.) All of the ticks collected from a single livestock animal can be pooled/stored together (in the same 15 ml conical(s)) until identification/sorting.

5. The tick burden on individual livestock animals will vary. It is not necessary to collect ALL ticks from each animal. Please use the following as a guide:

- If an animal has <25 ticks, please collect all tick specimens.
- If an animal has 25 or more ticks, please collect all ticks from the perianal region and 5-10 ticks from each other body region outlined above: 1) flank/abdomen 2) head 3) legs.

  Also, please attempt to collect Hyalomma and/or Amblyomma ticks as the priority (detailed in tick identification guide, easily identified by their striped legs).

6. Be sure to utilize a newly labeled 15 or 50 ml conical for each livestock animal sampled. Ensure that outer latex gloves are wiped down with germicidal wipes or sprayed with 70% ETOH, then removed, placed in a biohazard waste bag, and replaced between sampling of individual livestock animals.

7. Store ticks (15 & 50 ml conical) in a cool dry place (cooler box) until all livestock sampling has been completed for the day. At the end of each day, ticks should be identified and sorted.

NOTE: Only a face shield and a single pair of nitrile gloves are necessary during tick sorting/identification procedures.

8. In order to identify and sort ticks, first, sample collection tubes should be placed at -80°C or below (please use dry shipper while in the field) for 10-15 minutes or until tick specimens are dead and immobilized.

9. Once ticks are immobilized/dead, remove from the dry shipper. Add 70% ETOH to each tube of tick specimens, then cap and invert the tube approximately 5 times in order to rinse the ticks and remove any obvious dirt or hair. Discard/pour off the ETOH and immediately rinse the ticks with DI water following the same procedure described above. Pour off DI water and transfer ticks to a paper/absorbent towel to briefly pat them dry.

10. Dead ticks should then be transferred to a petri dish for identification and sorting (by sex and genera). Ticks collected from different animals should be sorted and pooled separately. It is essential to keep the petri dish on ice/ice packs during identification and sorting to keep the ticks cold.

11. For each livestock animal, first separate ticks into the following groups: 1) male 2) female 3) engorged female.

12. Next, identify the genus of each tick, starting with the male specimens. Please see attached detailed tick identification guide. Utilize a hand lens to aid in genus identification if necessary.

13. Transfer ticks into appropriately labelled 2ml cryovials, separated by their sex and genera.

14. Transfer tick sample tubes into cryoboxes and store in fully charged dry shippers for cold transport in the field.