TOS SCIENCE DESIGN FOR VECTORS AND PATHOGENS

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1 DESCRIPTION

1.1 Purpose

NEON design documents are required to define the scientific strategy leading to high-level protocols for NEON subsystem components, linking NEON Grand Challenges and science questions to specific measurements. Many NEON *in situ* measurements can be made in specific ways to enable continental-scale science rather than in ways that limit their use to more local or ecosystem-specific questions. NEON strives to make measurements in ways that enable continental-scale science to address the Grand Challenges. Design Documents flow from questions and goals defined in the NEON Science Strategy document, and inform the more detailed procedures described in Level 0 (L0; raw data) protocol and procedure documents, algorithm specifications, and Calibration/Validation (CalVal) and maintenance plans.

1.2 Scope

This document defines the rationale and requirements for vector and pathogen sampling in the NEON Science Design.

1.3 Acknowledgements

The design for each module was reviewed by and refined with input from technical working groups consisting of academic and public health researchers with relevant expertise:

Tick and tick-borne pathogens working group: Drs. Brian Allan, Ben Beard, Lorenza Beati, Dustin Brisson, Maria Diuk-Wasser, Rebecca Eisen, Holly Gaff, Sarah Hamer, Nicholas Ogden, Rick Ostfeld, Joe Piesman, Daniel Sonenshine, Andrea Swei, Michael Yabsley

Mosquito working group (combined for abundance/diversity/phenology and mosquito-borne pathogen designs): Drs. Chris Barker, Roberto Barrera, Mark Blackmore, William Bradshaw, Desmond Foley, Howard Ginsberg, Mary Hayden, Chris Holzapfel, Steve Juliano, Laura Kramer, Shannon LaDeau, Chet Moore, Roger Nasci, Bill Reisen, Harry Savage

Rodent-borne pathogens working group: Drs. Brian Amman, Mike Begon, Charles Calisher, James Childs, Richard Douglass, Janet Foley, Scott Gardner, Gregory Glass, Brian Hjelle, Amy Kuenzi, Jim Mills, Serge Morand, Robert Parmenter

Additionally, Dr. David Hoekman made significant contributions to the mosquito-borne pathogen sampling design, Dr. Cara Gibson contributed to early versions of the design for mosquito sampling, and Dr. Paul Duffy offered invaluable assistance with and input on statistical matters.

2 RELATED DOCUMENTS AND ACRONYMS

2.1 Applicable Documents

Applicable documents contain information that is applied in the current document. Examples are higher level requirements documents, standards, rules and regulations.

AD[01]	NEON.DOC.000001	NEON Observatory Design
AD[02]	NEON.DOC.001282	Introduction to the TOS Science Designs
AD[03]	NEON.DOC.000913	NEON TOS Science Design for Spatial Sampling
AD[04]	NEON.DOC.014045	Field and Lab Protocol: Tick and Tick-borne Pathogen Sampling
AD[05]	NEON.DOC.014049	Field and Lab Protocol: Mosquito Sampling
AD[06]	NEON.DOC.000481	Field and Lab Protocol: Small Mammal Sampling
AD[07]	NEON.DOC.005003	NEON Scientific Data Products Catalog
AD[08]	NEON.DOC.000910	NEON TOS Science Design for Mosquito Abundance, Diversity, and Phenology
AD[09]	NEON.DOC.000915	NEON TOS Science Design for Small Mammal Abundance and Diversity
AD[10]	NEON.DOC.014015	Fundamental Sentinel Unit Bioarchive Facility Design

2.2 Reference Documents

Reference documents contain information complementing, explaining, detailing, or otherwise supporting the information included in the current document.

RD[01]	NEON.DOC.000008	NEON Acronym List
RD[02]	NEON.DOC.000243	NEON Glossary of Terms
RD[03]		
RD[04]		

2.3 External References

External references contain information pertinent to this document, but are not NEON configuration-controlled. Examples include manuals, brochures, technical notes, and external websites.

ER[01]	
ER[02]	
ER[03]	

2.4 Acronyms

All acronyms used in this document are defined in RD[01].

3 INTRODUCTION

3.1 Overview of the Observatory

The National Ecological Observatory Network (NEON) is a continental-scale ecological observation platform for understanding and forecasting the impacts of climate change, land use change, and invasive species on ecology. NEON is designed to enable users, including scientists, planners and policy makers, educators, and the general public, to address the major areas in environmental sciences, known as the Grand Challenges (Figure 1). NEON infrastructure and data products are strategically aimed at those aspects of the Grand Challenges for which a coordinated national program of standardized observations and experiments is particularly effective. The open access approach to the Observatory's data and information products will enable users to explore NEON data in order to map, understand, and predict the effects of humans on the earth and understand and effectively address critical ecological questions and issues. Detailed information on the NEON design can be found in AD[01] and AD[02].

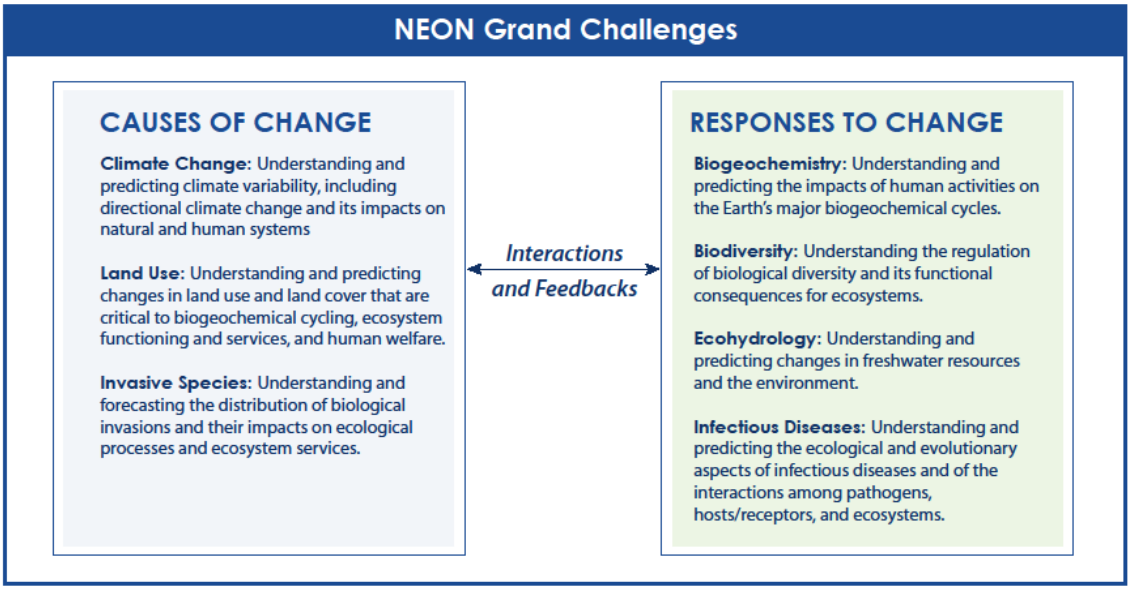

Figure 1. The seven Grand Challenges defined by the National Research Council (2001)

3.2 Components of the Observatory

There are five components of the Observatory, the Airborne Observation Platform (AOP), Terrestrial Instrument System (TIS), Aquatic Observation System (AOS), Aquatic Instrument System (AIS), and Terrestrial Observation System (TOS). Collocation of measurements associated with each of these components will allow for linkage and comparison of data products. For example, remote sensing data provided by the Airborne Observation Platform (AOP) will link diversity and productivity data collected on individual plants and stands by the Terrestrial Observation System (TOS) and flux data captured by instruments on the tower (TIS) to that of satellite-based remote sensing. For additional information on these systems, see Keller et al. 2008, Schimel et al. 2011.

3.3 The Terrestrial Observation System (TOS)

The NEON TOS will quantify the impacts of climate change, land use, and biological invasions on terrestrial populations and processes by sampling key groups of organisms (sentinel taxa), infectious disease, soil, and nutrient fluxes across system interfaces (air, land, and water) (AD[01], AD[02]). The sentinel taxa were selected to include organisms with varying life spans and generation times, and wide geographic distributions to allow for standardized comparisons across the continent. Many of the biological measurements will enable inference at regional and continental scales using statistical or process-based modeling approaches. The TOS sampling design captures heterogeneity representative of each site to facilitate this inference when possible. Plot and organism-scale measurements will also be coordinated with the larger-scale airborne measurements, which provide a set of synergistic biological data products at the regional scale. Details of these design elements and algorithms can be found in individual design documents available through the NEON website (www.NEONinc.org).

The standardization of protocols across all sites is key to the success of NEON (and its novelty) and must be maintained at all sites through time. Thus, although specific techniques may be required at some sites (e.g., due to different vegetation types), protocols have been developed to ensure data comparability. These details can also be found in individual design documents available through the NEON website (www.NEONinc.org).

The TOS Science Designs define the scientific strategies leading to high-level sampling designs for NEON sentinel taxa, terrestrial biogeochemistry, and infectious disease, linking NEON Grand Challenges and science questions to specific measurements (AD[02]). The TOS Spatial Sampling Design document describes the sampling design that collocates observations of the components of the TOS (AD[03]). TOS Science Design documents were developed following input from the scientific community, including module-specific Technical Working Groups, and the National Science Foundation (AD[02]). Science Designs will be reviewed periodically to ensure that the data collected by NEON are those best suited to meet the requirements of the observatory (AD[01]), are (to the extent possible) consistent with standards used by the scientific community, and fit within the scope of NEON. Additional information on the development and review process can be found in AD[02].

4 INTRODUCTION TO THE VECTOR AND PATHOGEN SAMPLING DESIGN

4.1 General Background

Parasites and pathogens (hereafter, pathogens) are important drivers of ecological and evolutionary changes in natural, agricultural, and urban ecosystems and have exerted significant effects on the demography and culture of human populations throughout history (Dobson and Grenfell 1995, Dobson and Carper 1996, Harvell et al. 1999, Swabe 1999, Daszak et al. 2000, Taylor et al. 2001, Hudson et al. 2002, Strange and Scott 2005, Alexander 2010, Brown and Gilfoyle 2010, McNeill 2010, Brooks and Hoberg 2013). In recent decades the number of emerging and re-emerging pathogens and associated infectious diseases, many of which are vector- and reservoir-borne zoonoses of significant public health concern, has increased dramatically (Garnett and Holmes 1996, Dobson and Foufopoulos 2001, Taylor et al. 2001, Anderson et al. 2004, Morens et al. 2004, Woolhouse and Gowtage-Sequeria 2005, Jones et al. 2008, but see Rosenberg et al. 2013). This trend is believed to reflect both the growing epidemiological connectivity between natural and human-associated systems as well as anthropogenic environmental modification: changes in climate, land- and resource-use practices, and patterns of human trade and travel have enormous potential to alter patterns of infection and disease dynamics (Coakley et al. 1999, Patz et al. 2000a, Gubler et al. 2001, Harvell et al. 2002, Patz et al. 2004, Weiss and McMichael 2004, Wolfe et al. 2005, Wolfe et al. 2007, Lafferty 2009, Rohr et al. 2011, Altizer et al. 2013, Perry et al. 2013). In some cases, increases in the frequency and severity of pathogen outbreaks, particularly those arising from cross-system infection events (e.g., zoonoses, anthroponoses), are predicted (Marano et al. 2007, Myers et al. 2013). In others, environmental change may result in pathogen losses, some of which will have important public health or economic consequences (Gomez and Nichols 2013). In both scenarios, changes in the abundance of vectors, reservoirs, or pathogens, and in the epizootiology (study of the causes and outcomes of diseases in animal populations) and epidemiology (study of diseases in human populations) of associated diseases, may have important implications for the health of human and livestock populations and the conservation of wildlife (Gubler 1998, Binder et al. 1999, Daszak et al. 2000, Cleaveland et al. 2001, Daszak et al. 2001, Strange and Scott 2005, Thompson et al. 2010). These changes could also have unanticipated effects on ecological communities at large, particularly when individuals of these species play influential roles in community-level interactions or ecosystem function (Mitchell and Power 2003, Hudson et al. 2006, Hatcher et al. 2014).

In light of these patterns and predictions, there is a clear need for increased pathogen surveillance efforts that bridge the historical divisions among human, domesticated animal, and wildlife diseases (e.g., epidemiology of human and domesticated animal diseases versus epizootiology and ecology of wildlife diseases). For instance, the field of conservation medicine and the OneHealth initiative are both predicated on a synthetic approach that emphasizes the linkages between environmental change and the health of human and wildlife populations (Daszak et al. 2004, Kaplan et al. 2009, Rock et al. 2009, Atlas et al. 2010, Coker et al. 2011, Aguirre et al. 2012). The importance of understanding the particular links between the dynamics of natural ecosystems and human health was highlighted as a critical

priority by the National Research Council (National Research Council 2001, 2003) and the Millennium Ecosystem Assessment (Patz et al. 2005). Given the broad spatiotemporal extents over which many important changes in disease dynamics are likely to occur, and the myriad factors that could underlie those changes, and the broad spatiotemporal scales over which some of those patterns (e.g., climate- and land use-driven) are likely to become apparent, the value of multifaceted surveillance efforts is increasingly recognized (Altizer et al. 2013). As the size and scope of surveillance efforts expands, appropriate sampling design and methodological standardization greatly facilitate comparisons across both datasets and scales. Although logistically challenging, such large-scale efforts are critical to characterize regional, continental, and multi-decadal patterns of disease dynamics. Insights gleaned from such projects hold promise for informing efforts to promote human health and wildlife conservation while furthering our fundamental understanding of the ecology and evolution of host-pathogen interactions (Kovats et al. 2001, Crowl et al. 2008).

In 2012, the United States National Science Foundation began funding the construction of the National Ecological Observatory Network (NEON) with the goal of creating the first continental-scale ecological monitoring system (Keller et al. 2008). Using standardized methods implemented at sixty sites for up to thirty years, NEON will provide insights into the effects of global change drivers (e.g., climate and land-use change) on the physical and ecological environment across multiple spatial and temporal scales (www.NEONinc.org, Schimel et al. 2011). As part of NEON's terrestrial observation system (Kao et al. 2012), sampling of pathogens and associated vector and reservoir species will be conducted to elucidate the changing ecology of a suite of tick-, mosquito-, and rodent-borne pathogens. Among these are the etiological agents of important human diseases including Lyme borreliosis, Hantavirus pulmonary syndrome, and West Nile virus disease. The sampling also has the potential to provide baselines for detecting future epizootiological shifts in other pathogens not currently known to be of importance to human or animal health. Measures of vector/reservoir abundance and pathogen prevalence will be made simultaneously with a suite of environmental and organismal information collected at NEON sites. These include a variety of measurements of abiotic environmental conditions, remotely-sensed data on vegetation and biogeochemistry, and phenological, demographic, and biodiversity information on sentinel taxa that include soil microbes, plants, insects, birds, and small mammals. To promote the advancement of open-access science, NEON data and archived physical samples will be freely available for additional analyses by members of the research community. Given its broad spatiotemporal scope, emphasis on methodological standardization, numerous and varied eco-environmental foci, and open-access policies, the NEON project will advance our current understanding of and ability to predict changes in host/pathogen interactions and associated disease dynamics in novel, inter-disciplinary, and collaborative ways.

NEON's vector and pathogen sampling broadly targets tick, mosquito, and small mammal (specifically, rodent) populations and associated pathogens. These vector/reservoir taxa were selected as sampling targets for two reasons. First, because of their physiology, ecology, and human associations, individuals and populations of these taxa are likely to respond quickly and measurably to changes in climate and

land-use practices. Second, they play important roles in the transmission of a diverse suite of pathogens, many of which are of public health significance. In contrast to a focus on particular pathogens, this "vector-borne" framework should increase the breadth of sampling and enhance its long-term flexibility to accommodate previously uncharacterized and/or emerging pathogens. It also mirrors the organizational structure and operational approach employed by most public health agencies for vector-borne disease surveillance. The framework accommodates NEON's organizational focus on sentinel taxa and encompasses pathogens of interest identified in the early plans for the Observatory: West Nile virus and dengue viruses, *Borrelia burgdorferi* (primary etiological agent of Lyme borreliosis in North America), and Sin Nombre virus (an etiological agent of hantavirus pulmonary syndrome). Generally speaking, sampling will involve the collection of individuals of target vector/reservoir taxa to quantify their abundance, analysis of associated samples (tissues or whole organisms) to estimate the prevalence of infection by pathogens, and archive of some or all remaining samples for additional use by other members of the research community.

4.1.1 Ticks and Tick-Borne Pathogens

Ticks transmit a variety of pathogens, many of which are zoonotic and have considerable public health significance (Spach et al. 1993, Sonenshine 1994, Sonenshine and Roe 2014). In northern latitudes, tick-borne pathogens are responsible for the majority of cases of vector-borne diseases in humans (Randolph 2001) and Lyme borreliosis is the most frequently reported vector-borne disease in the United States (Centers for Disease Control and Prevention 2008). First recorded in the U.S. in 1975 (Steere et al. 1977), the reported number of human cases of Lyme borreliosis within the country has increased over 200% since 1992 (http://www.cdc.gov/lyme/stats/index.html, accessed on May 1, 2014). According to a recent analysis, up to 300,000 human infections may occur annually (Mead et al. 2013). Other zoonotic tick-borne pathogens in North America (e.g., *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp.) have exhibited similar patterns of emergence in terms of increases in prevalence and spatial extent over the past two decades (Childs and Paddock 2003, Doudier et al. 2010, Centers for Disease Control and Prevention 2012a). Tick-borne pathogens are associated with 13% of the infectious diseases currently classified by the CDC as nationally notifiable (http://wwwn.cdc.gov/nndss/, accessed on May 1, 2014, Centers for Disease Control and Prevention 2010).

In addition to the public health significance of diseases associated with tick-borne pathogens, the physiology and ecology of ticks make them an ideal target for NEON sampling. Among arthropods, ticks are particularly sensitive to abiotic environmental conditions (Sauer and Hair 1986, Needham and Teel 1991, Stafford 1994, Dister et al. 1997, Jones and Kitron 2000, Teel et al. 2010, Sonenshine and Roe 2014) and associated physiological constraints make it highly likely that the demography and biogeography of ticks of many species, and the pathogens they transmit, will be affected by climate change (Estrada-Pena 2009, Gatewood et al. 2009, Diuk-Wasser et al. 2012, Leger et al. 2013, Medlock et al. 2013, Ogden et al. 2013). Further, the multi-host life cycles of ticks of most species increase their ecological connectivity and sensitivity to community-level perturbations associated with changes in

human land- and resource-use patterns. These anthropogenic effects can manifest as direct alterations to the physical environment (Barbour and Fish 1993) or changes in community structure that include the abundance and diversity of available hosts (Childs and Paddock 2003, LoGiudice et al. 2003, Paddock and Yabsley 2007, Allan et al. 2010, Keesing et al. 2010).

4.1.2 Mosquito-Borne Pathogens

The tremendous human health burden attributable to pathogens transmitted by mosquitoes is unsurpassed among vector-borne diseases. Globally it is estimated that in 2004 alone there were over 1.8 million human malaria deaths (Murray et al. 2012), and 96 million people are thought to experience disease associated with infection by dengue viruses each year (Bhatt et al. 2013). Moreover, mosquito-borne pathogens can also cause substantial reductions in populations of livestock and wildlife, with potentially important repercussions for human health, economic productivity, and the structure and function of ecological communities (e.g., Daubney et al. 1931, van Riper et al. 1986, Morris 1989, Scott and Weaver 1989, LaDeau et al. 2007, Paweska and van Vuren 2014).

Forecasts of potential ecological and public health consequences of climate change often focus on mosquitoes and the pathogens they transmit (Shope 1992, Reeves et al. 1994, Sutherst 2004). Although mosquitoes are found worldwide in all areas that are not permanently frozen, they are most consistently abundant in localities with tropical or moderately temperate climes where relatively warm and wet conditions prevail (although populations of some species in subarctic and alpine regions reach extremely high abundance during parts of the year). As a result, increases in temperature or precipitation at higher latitudes or elevations due to climate change may promote the range expansion of mosquitoes currently confined to tropical areas (Epstein et al. 1998, Patz et al. 2000b, Caminade et al. 2012, Eisen and Moore 2013, but see Reiter, 2001). This geographic spread could be facilitated by the incidental and periodic long-distance transport of mosquitoes that often occurs as part of human travel and international commerce (Lounibos 2002, Tatem et al. 2006). In North America and Europe, the potential introduction and establishment of dengue viruses, chikungunya virus, yellow fever virus, and Rift Valley fever virus are of particular concern (Gould and Higgs 2009, Weaver and Reisen 2010). Additionally, there is abundant evidence that changing climatic conditions will significantly affect mosquito demography and processes associated with the transmission of mosquito-borne pathogens (Mordecai et al. 2013). For example, changes in ambient temperature are predicted to alter mosquito vectorial capacity (Watts et al. 1987, Reisen et al. 2006, Paaijmans et al. 2012) and biting rates (Lardeux et al. 2008), and may in some cases catalyze host range shifts in arboviruses (Brault and Reisen 2013).

4.1.3 Rodent-Borne Pathogens

Small mammals, and rodents in particular, are common and influential members of most ecological communities and play central roles in the epidemiology and epizootiology of numerous pathogens. Rodentia is the most diverse order of the class Mammalia, including roughly 40% of extant species (Huchon et al. 2002). Rodents of some species are opportunistic foragers that have been synanthropic for thousands of years (e.g., Matisoo-Smith et al. 1998). Others inhabit the urban/wildland interface and may serve as epidemiological links between humans and infectious agents endemic to natural ecosystems (Kuenzi et al. 2001, Douglass et al. 2006). As a result of these intimate associations, and perhaps in part because of their shared mammalian pedigree, a variety of pathogens can be transmitted from rodents to humans (Meerburg et al. 2009). One of the most notable examples is the bacterium *Yersinia pestis*, the etiological agent of plague. The bacterium is usually transmitted to humans via the bite of a flea that has fed on an infected rodent (Gage and Kosoy 2005). Over the course of recorded history plague has been responsible for multiple human pandemics and tens of millions of fatalities (Gage et al. 2008). More recently, human infection by rodent-borne hantaviruses (Mills et al. 2010) has received considerable attention following an outbreak of hantavirus pulmonary syndrome caused by Sin Nombre virus in the American southwest (Nichol et al. 1993, Centers for Disease Control and Prevention 2012b). Of the 17 hantaviruses identified in North America, six are known to cause HPS, which has a case fatality rate of roughly 36% (Mills et al. 2010, MacNeil et al. 2011). In addition to these highly publicized examples, rodents are known reservoirs for more than 35 bacterial and viral pathogens worldwide (http://www.cdc.gov/rodents/index.html, accessed May 1, 2014), many of which cause human diseases including Lyme borreliosis, typhus, babesiosis, and Rocky Mountain spotted fever (Meerburg et al. 2009).

Rodents and the pathogens they carry may also be model systems for investigating the effects of climate and land use changes on the epizootiology and epidemiology of zoonotic diseases. The often large sizes and high densities of rodent populations may favor the maintenance and spread of pathogens, and frequent contact between some rodent species and both domestic animals and human populations increases the likelihood of pathogen spillover or zoonotic transmission. Additionally, populations of many species of rodents that serve as reservoirs have high reproductive potential and turnover, attributes that promote rapid demographic responses to environmental changes with cascading effects on infection dynamics and the risk of human disease (Yates et al. 2002, Luis et al. 2010). Modifications of ecosystems by human activities, including urbanization and agricultural development, may also affect the structure of rodent communities in ways that alter the relative abundance of reservoir-competent species (Mills 2006, Clay et al. 2009).

4.2 NEON's Contribution

Data associated with NEON's vector and pathogen sampling modules will provide rare long-term datasets on disease dynamics at locations that span a range of anthropogenic impacts, from wildland areas to agricultural and urban sites. These datasets will be additionally unique and valuable in that they will be generated using standardized methods across multiple sites that collectively span a variety of ecological systems. Datasets based on this spatially-replicated, long-term, methodologically standardized approach are exceedingly uncommon and should prove highly valuable in extending current understanding of the ecology of host/pathogen interactions and the effects of global change phenomena on associated transmission and disease dynamics. Vector and pathogen samples collected by NEON can further this understanding by providing the materials necessary for additional studies on topics including the biogeography, phylogeography, evolution, and coevolution of vectors, reservoirs, and pathogens.

4.3 Purpose and Scope

This document outlines the sampling design plans and associated rationale underlying NEON's three vector and pathogen sampling modules: tick-borne pathogens, mosquito-borne pathogens, and rodent-borne pathogens. It also provides cursory information about associated protocols. Associated details can be found in Field and Lab Protocol: Tick and Tick-borne Pathogne Sampling (AD[04]), Field and Lab Protocol: Mosquito Sampling (AD[05]), and Field and Lab Protocol: Small Mammal Sampling ([AD06]).

5 SAMPLING FRAMEWORK

The sampling designs for NEON's tick-, mosquito-, and rodent-borne pathogen sampling modules are described below. Detailed information on the specific conception of and plans for each individual module is preceded by a discussion of the general design elements common across modules. Because sampling protocols flow from these designs, an understanding of the priorities and strategic decisions underlying the latter is integral to contextualizing when, where, and how the data and samples will be collected within NEON. This understanding should facilitate the use of NEON-generated resources and the replication (or modification) of NEON methods by other researchers or organizations attempting to integrate their work with or extend that being conducted by NEON.

5.1 Science Requirements

This science design is based on Observatory science requirements that reside in NEON's Dynamic Object-Oriented Requirements System (DOORS). Copies of approved science requirements have been exported from DOORS and are available in NEON's document repository, or upon request.

5.2 Data Products

Execution of the protocols that stem from this science design procures samples and/or generates raw data satisfying NEON Observatory scientific requirements. These data and samples are used to create NEON data products, and are documented in the NEON Scientific Data Products Catalog (AD[07]).

5.3 Priorities and Challenges for Vector and Pathogen Sampling

5.3.1 Priorities and Challenges Common to All Three Sampling Modules

5.3.1.1 Prioritization of Time Series Data

Sampling designs emphasize a high frequency of intraannual sampling to generate fine-scale time series data on changes in vector/reservoir abundance and pathogen prevalence through time. When implemented over the relatively long lifespan of the observatory (up to 30 years at core sites), this approach should allow changes in sampled vector, reservoir, and pathogen populations to be characterized at two temporal scales. Of primary interest are interannual changes in the seasonal mean or maximum of vector/reservoir abundance and pathogen prevalence (Figure 2A). Given a sufficiently high sampling frequency, sampling should secondarily elucidate changes in aspects of interannual phenology of sampled populations (e.g., timing of onset and duration of seasonal cycles) (Figure 2B). The secondary focus is particularly important since the seasonal phenology of many vector, reservoir, and pathogen populations is expected to be sensitive to changes in climate and land-use practices (Altizer et al. 2013). Resulting phenological shifts could have profound implications for the maintenance, abundance, and spread of pathogens (Altizer et al. 2006, Fisman 2007).

The emphasis on fine-scale time series data is driven largely by fundamental constraints on the spatial resolution of data generated by this sampling. Because NEON sites were not selected based on considerations of local vector or reservoir abundance or pathogen prevalence, not all sites will be productive in terms of sampling yields. Within sites, the number of sampling plots will generally be insufficient to characterize or measure changes in local habitat associations of targeted taxa. In light of these limitations, the designs seek to characterize changes in vector/reservoir abundance and pathogen prevalence at the level of the site rather than of individual sampling plots. While plot-level field data will be available (e.g. number of adult ticks of species A collected at plot B within site C during sampling event D), data and samples will be aggregated across plots within sampling events at each site to make inferences at the site level (e.g., adult ticks of species A collected at all sampling plots within site C during sampling event D).

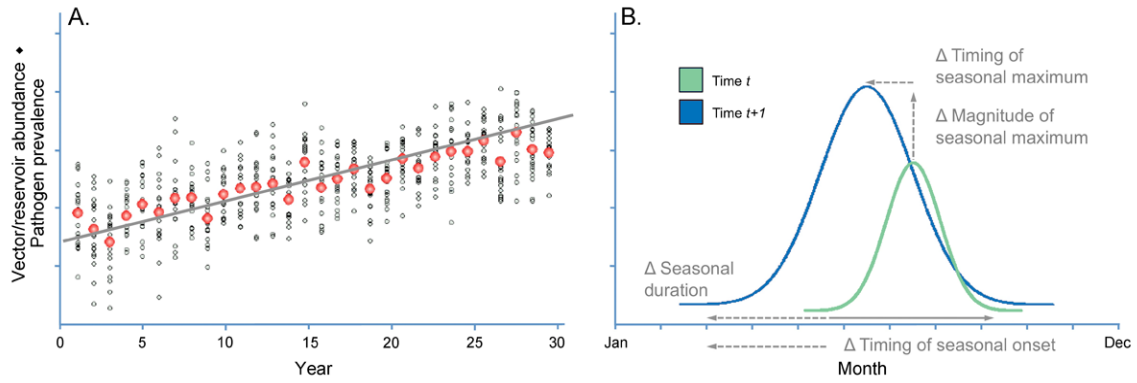

Figure 2. Hypothetical data illustrating detection of long-term (A) and seasonal (B) changes in vector/reservoir abundance and pathogen prevalence.

The allocation of sampling effort (e.g., number of sampling plots, frequency of sampling events) captured in module specific sampling designs reflects the prioritization of high resolution time series data. In addition, designs attempt to incorporate considerations of anticipated resource availability, logistic constraints, and levels of effort commonly reported or deemed reasonable for surveys of tick-, mosquito-, and rodent-borne pathogens. Evaluating the adequacy of these plans through traditional power analyses is not wholly appropriate given that this approach is typically used to assess the design of studies motivated by one or a few specific research questions. Nevertheless, the module-specific sections below include results of power analyses evaluating the ability of proposed sampling plans to detect pathogens, and interannual trends in the seasonal mean prevalence of pathogens, at a NEON site. These analyses highlight just two of the many ways in which data collected by NEON vector/pathogen sampling could be used to test hypotheses about the dynamics of host/pathogen interactions and the epizootiology and epidemiology of associated diseases. More exhaustive statistical exploration of the power of NEON data when used in these various analyses is beyond the scope of this document.

Science requirements associated with vector and pathogen sampling prescribe that the prevalence of infection by pathogens (or immunological indications thereof) must be quantified. This requires statistical power that is adequate relative to at least two criteria. First, sampling should be able to detect pathogens when they are present at biologically meaningful prevalence. Results of power analyses reported in the module-specific sections below provide quantitative estimates of detection probabilities under a variety of sampling scenarios. Second, sampling should be able to estimate prevalence with acceptable precision. In power analyses reported in the module-specific sections below, this is estimated as the ability to detect temporal changes in prevalence under a variety of sampling scenarios (notably, variation in trend magnitudes). A higher power to detect trends of smaller magnitudes is evidenced by smaller confidence intervals around the prevalence estimate at a given time point.

Because NEON will conduct tick- and rodent-borne pathogen testing through analysis of individual samples (i.e., not pooled), associated power analyses for trend detection used the negative binomial distribution. Due to the expectation of relatively low rates of infection, mosquito-borne pathogen testing will involve pooled samples (20-50 individual mosquitoes physically homogenized and the resulting homogenate tested for parasites). Because of this approach power analyses for trend detection used the binomial distribution and the complementary log-log (CLL) link function, which accommodates for group testing within a GLM framework (Farrington 1992). Trend magnitudes were specified in terms of an annual increase in prevalence (values varied by module) and trend detection periods were set at either 10 or 25 years (approximations of the lifespans of NEON relocatable and core sites, respectively). Year-zero infection prevalence was parameterized using values typically reported in the literature and/or commonly associated with the types of pathogens under consideration. Two combinations of type I error rate (α) and power ($1-\beta=1-p_{(\text{type II error})}$) were considered: a higher confidence scenario involving $\alpha=0.05$ and power=0.9 and a lower confidence scenario in which $\alpha=0.1$ and power=0.8. Calculations were performed both with and without a temporal autocorrelation term (e^{-1} at a half year). This value was chosen to represent a moderate magnitude of temporal correlation to provide contrast with respect to the case with temporal independence. While it is anticipated that there will be some temporal autocorrelation, the magnitude and lag will only be clarified through several years of sampling. Sampling frequencies considered in the analyses included those proposed at core and relocatable sites for each sampling module as well as lower frequencies (e.g., one, two, three, and four times per year) to generate continuous power curves in accompanying figures. In these figures, green boxes are used to represent regions of sampling space where designs had sufficient power to detect trends of a specified magnitude. Unless otherwise noted in the figure captions, these boxes are bounded along the x-axis by sampling frequencies proposed for core and relocatable sites and on the y-axis by anticipated levels of replication (i.e., number of field-collected samples or laboratory tests) associated with a single sampling event. Scripts used to perform these power calculations in R (R Core Team 2013) are available in Appendix A.

In addition to the power analyses involving inter-annual trends in pathogen prevalence, methods detailed in Gu and Novak (2004) were used to quantify the ability of the proposed designs simply to detect a pathogen when it is present at a site. Two approaches were used. First, the relationship between the number of samples tested and the probability of detecting a pathogen at various levels of infection prevalence was evaluated. Second, the relationship between infection prevalence and number of samples that would need to be tested to detect a pathogen with varying levels of statistical confidence was characterized.

5.3.1.2 Selection of Sampling and Testing Methods

Designs prioritize field sampling methods that meet three criteria. First, the methods must provide an effective means of collecting individuals of targeted vector/reservoir taxa. Second, utilization of well-established, widely employed methods should promote use of NEON data by the research community and the integration of these and similar data collected by other groups including private and academic researchers, local and state-level organizations, and federal agencies such as the U.S. Centers for Disease Control and Prevention (CDC) or U.S. Department of Agriculture (USDA). As an example, the CDC miniature CO_2 light trap has been used for decades in locations around the world to collect a variety of mosquitoes important in arbovirus transmission (Sudia and Chamberlain 1962, Newhouse et al. 1966, Pfuntner 1979). Analogous methods for the collection of ticks and small mammals include drag sampling (Milne 1943) and mark-recapture live trapping, respectively. Third, because standardization within the Observatory is emphasized to facilitate comparability of data across sites and through time, methods should be applicable across the spectrum of site-specific environmental conditions and ecology of sampled populations at NEON sites. While designs prioritize methods that can be used under a wide range of circumstances, they also reflect the reality that site-specific modifications may be desirable or necessary in some cases. For example, the primary design may be augmented through use of additional and supplementary methods (e.g., trap types) to increase sampling success at sites where vector/ reservoir species are rare or not effectively sampled by methods employed in the primary design.

The majority of post-collection sample processing, including most taxonomic identification of vector/reservoir samples and all pathogen testing, will be performed on a contract basis by experts at external facilities. To increase cost efficiency, testing methods that can simultaneously detect more than one pathogen species are prioritized over the use of multiple pathogen-specific tests (e.g., family-specific assay for mosquito-borne flaviviruses rather than separate tests specific for West Nile virus, St. Louis encephalitis virus, and dengue viruses). Broadly reactive screens will be followed up with more specific assays to identify the pathogen(s) present in samples that test positive.

5.3.1.3 Selection of Sampling Plots

A fixed plot design will be used for vector/reservoir and pathogen field sampling. Selecting the locations of sampling plots at each site is complicated by the fact that in many cases, individuals of target taxa are likely to exhibit clustered patterns of distribution in space. This aggregation can arise from factors such as abiotic tolerances, specific habitat associations, and/or patterns of host-mediated dispersal. The productivity of sampling would be increased by locating at least a portion of sampling plots in or near areas of aggregation (e.g., adaptive cluster sampling, Thompson 1990, Brown et al. 2013). Identifying these within-site locations in a systematic way that can be applied across the observatory, however, is complicated by at least three issues. First, site-specific data on these locations are generally unavailable for most sites prior to the start of NEON sampling. Second, inferring patterns of local spatial distribution using regional data or expertise is problematic since the quality and availability of these resources vary considerably among sites and regions. Finally, because populations of target species often exhibit considerable interannual fluctuations in size,

multiple years of within-site sampling would be required to empirically characterize patterns of site-specific distribution and/or local habitat associations with confidence.

Given these complications in systematically gathering accurate information on the within-site distribution of individuals of target taxa, sampling plots will be established at random locations within sites. This approach has a number of general strengths. First, because the goal of the sampling is to make site-level inferences about local vector, reservoir, and pathogen populations, the random distribution of sampling plots within sites allows for spatially unbiased site-level estimates of parameters of interest. Second, given that local patterns of distribution of individuals of target species may change over the lifespan of the observatory, plot locations based on current patterns of distribution might not be optimal in future years. A strategy of randomized plot distribution would be more robust to these changes. Finally, using the same approach to selecting plot locations as other NEON TOS sampling modules should facilitate statistical analyses and modeling involving multiple NEON data streams.

The design of each sampling module specifies a standard number of sampling plots per site. As detailed in the NEON TOS Science Design for Spatial Sampling (AD[03]), a stratified random approach will be used to select plot locations within the dominant vegetation types ($\geq$5% total cover) at each site. The number of sampling plots per type will be proportional to the percent cover of each type. Data collected at these plots during the first few years of sampling will be used to statistically evaluate whether the proposed number of plots within sites is sufficient to characterize parameters of interest with desired confidence. If it is determined that more data and/or samples are needed to achieve this confidence, additional sampling plots will be added as resources and logistics permit. Changes in the location of fixed plots can be made in consultation with members of the relevant technical working group(s), the Assistant Director of Terrestrial Ecology, and the Observatory Director.

5.3.2 Priorities and Challenges for Mosquito-Borne Pathogen Sampling

The mosquito-borne pathogen sampling design was developed in conjunction with the design of sampling for mosquito abundance and diversity (for additional details see the NEON TOS Science Design for Mosquito Abundance, Diversity, and Phenology, AD[08]). Because these two sampling efforts differ fundamentally in their objectives, they would be optimized using different strategies if designed independently. Pathogen-related sampling seeks to collect and test large numbers of particular mosquito species to quantify rare phenomena (infection by pathogens). In contrast, abundance and diversity sampling should involve spatiotemporally and taxonomically broad sampling to characterize the community of mosquitoes present at a site. Although a combined sampling plan driven by considerations of pathogen-related sampling would not be suitable for robust sampling of mosquito abundance and diversity, a plan based upon the sampling priorities of abundance and diversity sampling would also collect some of the mosquitoes needed for pathogen testing. The sampling design therefore represents a combination of the two sampling efforts into a unified approach (hereafter, mosquito sampling) that is driven largely by priorities of abundance and diversity sampling and that can be augmented as needed to meet requirements of pathogen-related sampling.

5.3.3 Priorities and Challenges for Rodent-Borne Pathogen Sampling

As with mosquito sampling, rodent-borne pathogen sampling occurs coincidently with NEON small mammal abundance and diversity sampling (for additional details see the NEON TOS Science Design for Small Mammal Abundance and Diversity, AD[09]). The proposed sampling design for the former is structured in part by design priorities and considerations that are central to the latter (e.g., number and location of sampling plots within sites), referred to hereafter as small mammal sampling.

6 SAMPLING DESIGN FOR VECTOR AND PATHOGEN SAMPLING

6.1 Sampling Design for Tick and Tick-Borne Pathogen Sampling

Sampling for ticks and tick-borne pathogens (hereafter, tick sampling) will broadly target hard ticks (family Ixodidae). Six species are of particular interest for tick-borne pathogen testing: *Ixodes scapularis* (black-legged tick), *I. pacificus* (western black-legged tick), *Amblyomma americanum* (lone star tick), *A. maculatum* (Gulf coast tick), *Dermacentor andersoni* (Rocky mountain wood tick), and *D. variabilis* (American dog tick). Collectively, ticks of these species transmit a large and taxonomically-diverse suite of pathogens, many of which are zoonotic and of public health concern (http://www.cdc.gov/ticks/diseases/, accessed on May 1, 2014, Spach et al. 1993).. Ticks of other species collected through this sampling will be tested for pathogens as resources permit and sample sizes warrant. Pathogen testing will broadly target bacteria. Additional details of the implementation of NEON tick sampling can be found in Field and Lab Protocol: Tick and Tick-borne Pathogen Sampling (AD[04]).

6.1.1 Sampling Methods

6.1.1.1 Sample Collection in the Field

Ticks will be sampled using the dragging method, which is arguably the most commonly used method to sample ticks and is particularly effective for ticks of species that exhibit questing behavior (i.e., sit-and-wait) (Milne 1943, Falco and Fish 1992). Among tick sampling methods, dragging also most closely approximates the human risk of picking up hard ticks from the environment. During drag sampling, a cloth of standardized size is pulled along the ground at a slow pace. The cloth is periodically examined for attached ticks, which are typically removed with forceps or tape. Ticks that become attached to the clothing of sampling personnel during drag sampling can also be collected when the drag cloth is examined. The distance covered during a drag is generally standardized and recorded for use in calculating tick density. In instances where thick vegetation prevents continuous drag sampling, the flagging method can be used as an alternative or in conjunction with dragging (Ginsberg and Ewing 1989). Flag sampling essentially involves using the drag cloth held in the hand and slowly waved over or underneath vegetation rather than pulled along the ground (Rulison et al. 2013).

While their use is not currently planned, two additional methods of tick sampling could be considered to supplement drag sampling as resources permit. Collection of ticks using dry-ice baited CO_2 traps is well suited to sample ticks of species such as *A. americanum* that exhibit active hunting behavior and locate hosts by following carbon dioxide plumes (Garcia 1962, Falco and Fish 1989, Kinzer et al. 1990). Ticks attracted to a CO_2 trap are captured on tape attached to the edge of the trap's base platform. A drag/flag cloth can be used to collect additional ticks from the ground and vegetation in the vicinity of the trap. If/when used, CO_2 traps will utilize a vented 1.9L insulated cooler containing approximately 1.5kg of dry ice and be deployed in the center of each sampling plot for 24 hours following the completion of drag and/or flag sampling during a sampling event.

The removal of ticks from vertebrate hosts is another commonly used method of sampling ticks (Luckhart et al. 1992, Clark et al. 1998, Kollars et al. 2000). Results of sampling using this method can provide important insights into the host associations of ticks and by extension, the transmission cycles of associated pathogens (Clark et al. 2001, Eisen et al. 2004). Deer are final hosts for ticks of many species, and researchers often remove ticks from deer killed by sportsmen (i.e., at hunter check stations) or from road kill (Luckhart et al. 1992, Kollars et al. 2000). Because NEON will not be sampling deer this is not a methodological option. Although NEON will conduct regular sampling of small mammals, including species that are important hosts for ticks and play significant roles as reservoirs of many tick-borne pathogens (Donahue et al. 1987, Ostfeld et al. 1996), removal of ticks from captured small mammals is not currently planned. This decision is driven by the need to limit small mammal handling time in the face of other prioritized data and sample collection requirements. Design-related details on tick sampling from rodents can be found in the rodent-borne pathogen section of this document. Any associated protocol details will be captured in Field and Lab Protocol: Small Mammal Sampling (AD[06]).

In 2012, tick sampling using the drag method was prototyped at all three sites in NEON Domain 03: Ordway Swisher Biological Station (core), Jones Ecological Research Center (relocatable), and Disney Wilderness Preserve (relocatable). Results demonstrate the utility of drag sampling to elucidate spatial variation in tick abundance within and among sites. The lone star tick (*A. americanum*) is known to be established throughout central and northern Florida and southern Georgia and often reaches levels of extremely high abundance where it occurs (Springer et al. 2014). Based on this regional information it could be assumed that individuals of this species would be present in abundance at all three NEON sites in Domain 03. While results of tick sampling at the core site in June, 2012 confirmed a high abundance of ticks, concurrent sampling of nearly 16,000m^2 (collectively) at the two associated relocatables sites resulted in the collection of only five ticks total (Table 1). These results may indicate that local conditions at these sites are not conducive to the maintenance of large tick populations. Regular vegetation burning at both relocatable sites likely contributed to this outcome, demonstrating the impact of land-use practices on tick demography. Results of iterative sampling that will occur during Observatory observations could shed light on the temporal duration of such demographic effects (i.e., rates of recovery of tick populations after controlled burns at a site).

Ticks were found in all three vegetation types sampled at Ordway Swisher Biological Station, where results provided evidence supporting spatial clustering of ticks among vegetation types. Ticks were one to two orders of magnitude more abundant in deciduous closed tree canopy habitats compared to evergreen open tree canopy and perennial graminoid grassland habitats (Table 1). This pattern was apparent in spite of the greater sampling effort (number of plots and average distance covered during drag and walking sampling) associated with the latter two vegetation types. Further, although sample sizes were low, ticks were present in every plot sampled within deciduous closed tree canopy plots. In contrast, ticks were absent in 85% and 60% of sampled plots located in evergreen open tree canopy and perennial graminoid grassland habitats, respectively.

Table 1. Results of year-one spatial sampling of ticks in NEON Domain 03, spring and summer 2012.

Site	Vegetation type	# plots sampled	Tick abundance per plot (mean±stdev)	Total # ticks* collected	Max # ticks* per plot	# plots with no ticks*	Total area sampled (m^2)
Disney Wilderness Preserve	Evergreen open tree canopy	9	0.3±0.95	3	3	8	2,700
	Mixed evergreen / deciduous open tree canopy	1	0.0±0.0	0		1	300
	Perennial graminoid grassland	7	0.0±0.0	0		7	2,100
Total		17	0.2±0.7	3	3	16	5,100
Jones Ecological Research Center	Annual graminoid or forb	6	0.0±0.0	0		6	1,958
	Evergreen open tree canopy	5	0.4±0.89	2	2	4	5,973
	Mixed evergreen / deciduous open tree canopy	4	0.0±0.0	0		4	1,481
	Perennial graminoid grassland	4	0.0±0.0	0		4	1,401
Total		19	0.1±0.5	2	2	18	10,813
Ordway Swisher Biological Station	Deciduous closed tree canopy	3	20.0±17.1	60	39	0	811
	Evergreen open tree canopy	14	0.2±0.6	3	2	12	6,156
	Perennial graminoid grassland	5	1.2±1.8	6	4	3	1,615
Total		22	3.1±8.7	69	39	15	8,582
Overall		58	1.2±5.4	74	39	49	24,495

* only nymphal and adult ticks considered

6.1.1.2 Sampling Event

An event of tick sampling at a site will involve drag sampling around the perimeter of each of six 1600m^2 square sampling plots per site. During a sampling event, a 1m^2 piece of white, cotton flannel cloth will be pulled along the ground at a slow pace and examined at five to ten meter intervals along the drag path (Figure 3). Ticks found attached to the drag cloth and field technician clothing will be removed using forceps (nymphal and adult ticks) or reusable lint rollers (larval ticks, Savage et al. 2013). The distance of each drag will be recorded for calculations of tick density. Flagging will be used along any portions of the sampling path where thick vegetation prevents continuous drag sampling.

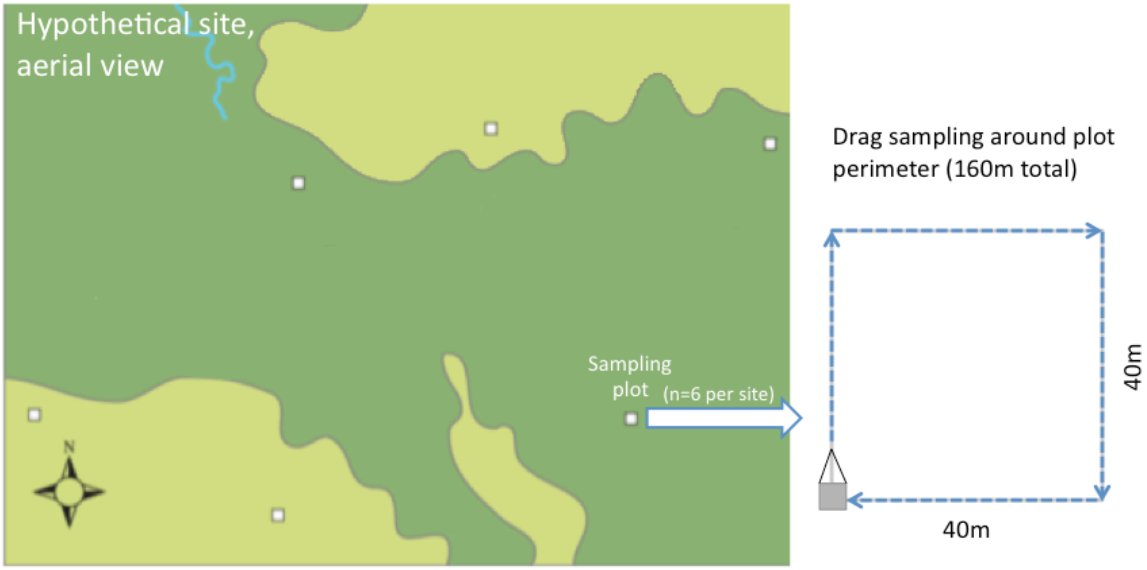

Figure 3. Schematic illustrating an event of tick sampling.

In the field, collected nymphal and adult ticks will be transferred into labeled vials containing 95% ethanol. Larvae collected using resusable lint rollers will be washed into filter paper using water for subsequent transfer into vials (Savage et al. 2013). Samples will be transported on ice in portable coolers to a NEON domain lab. For each sampling event/plot combination, collected ticks will be enumerated by life stage and then transferred into cold storage at <4°C (ideally -20°C).

6.1.1.3 Taxonomic Identification of Samples

NEON technicians will not perform any taxonomic identification of collected ticks. Collected ticks will be sent to one or more external facilities for taxonomic identification and pathogen testing. Nymphal and adult ticks will be identified to species based on visual examination of external morphology (e.g., Cooley and Kohls 1944, Cooley and Kohls 1945, Keirans and Litwak 1989) and enumerated by species and life stage for each sampling event/plot combination (e.g. number of nymphs of species A collected at plot B within site C during sampling event D). Uncertain identifications can be verified as necessary or desired through examination by a secondary ID facility or using genetic methods such as DNA barcoding (Hebert et al. 2003, Pons et al. 2006, Mukherjee et al. 2014). Larval ticks will not be identified but will be counted and archived.

6.1.1.4 Pathogen Testing

Following identification, ticks will be combined by species, lifestage, site, and event (e.g., all nymphs of species A collected at site C during sampling event D) for pathogen testing. A subset of species-identified nymphal and adult ticks will be tested for infection by pathogens. A minimum of 10 and maximum of 100 ticks per species/life stage combination will be analyzed for each sampling event. Actual sample sizes for pathogen testing will depend on collection success and analytical costs. Ticks will be tested individually using next-generation sequencing with barcoded, universal 16S rRNA primers (Carpi et al. 2011, Budachetri et al. 2014). This method will allow for the detection of a wide range of pathogens including individuals in the genera *Anaplasma*, *Borrelia*, *Ehrlichia*, *Francisella*, and *Rickettsia*. The method will also return information on other prokaryotic endosymbionts of ticks (e.g. gut bacteria) and may provide data associated with tick taxonomy akin to DNA barcoding. To quantify the likelihood that a given pathogen is present within a tested tick, sequences generated during pathogen testing will be screened against known pathogen sequences in a library to be created by NEON and based on published sequences (e.g., available through the National Center for Biotechnology Information, Acland et al. 2013). Pathogens in the reference library will initially include species in the genera *Anaplasma (*e.g., *Anaplasma phagocytophilum)*, *Borrelia (*e.g., *Borrelia burgdorferi*, *Borrelia miyamotoi*), *Ehrlichia (*e.g., *Ehrlichia ewingii*, *Ehrlichia chaffeensis*) *Francisella (*e.g., *Francisella tularensis)*, and *Rickettsia (*e.g., *Rickettsia rickettsii, Rickettsia parkeri)*. Pathogens will be considered present if the degree of alignment between test-generated and reference sequences exceeds designated match percentage threshold(s) (e.g., Altschul et al. 1990).

6.1.1.5 Archive

Species-identified but untested nymphal and adult-stage ticks, all unidentified larval, nymphal and adult-stage ticks, and products generated during pathogen testing (e.g., genomic extractions, PCR products) will be archived in existing facilities. Archiving plans, including details on accessing samples for private investigation, are detailed in the Fundamental Sentinel Unit Bioarchive Facility Design (AD[10]).

6.1.2 Spatial Distribution of Sampling

As described in the general introduction and in the NEON TOS Science Design for Spatial Sampling (AD[03]), a stratified random approach will be used to select six tick sampling plots located within the dominant vegetation types ($\geq$5% total cover) at each site. The number of plots per type will be proportional to the percent cover of each type. Once a set of long-term sampling plots is selected, NEON will maintain a fixed plots design, sampling those plots irrespective of changes in the presence or abundance of ticks and/or associated pathogens.

6.1.3 Temporal Distribution of Sampling

Sampling at each site will begin at a frequency of one sampling event every six weeks. This frequency will be maintained until one or more ticks are collected during a sampling event, a threshold that will trigger an increase in sampling frequency to one event every three weeks. This frequency will be maintained for the remaining lifespan of the site irrespective of subsequent sampling success.

Sampling will occur from March through December, but during this period a given sampling event will only be performed if the high temperature on the day prior to planned sampling was >0°C and the mean high temperature in the five days prior to planned sampling was >7°C. These temperatures represent conservative thresholds below which ticks are generally not active (Duffy and Campbell 1994, Clark 1995, Vail and Smith 1998). Sampling will be postponed whenever the ground is wet (e.g., shortly after a rain event or when dew is heavy) and avoided during the hottest and/or driest periods of the day (relative to typical temperatures at any given site).

6.1.3.1 Power Analyses Evaluating Sampling Frequencies

Evaluation of power to detect prevalence trends

For power analyses to evaluate the ability of sampling and testing to detect long-term trends in tick-borne pathogen infection prevalence, year-zero prevalence values were set to 5% (rare pathogen) or 15% (common pathogen) based on results of studies of *B. burgdorferi* infection in *I. scapularis* populations (Piesman et al. 1986, Ginsberg et al. 2004, Ogden et al. 2007, Diuk-Wasser et al. 2012). Sampling event frequencies of one, two, three and four times per year, as well as every three or six weeks, were considered. Given plans to test between 10 and 100 ticks per species/life stage combination per sampling event, a conservative sample size threshold of 40 ticks tested for pathogens per sampling event was used. Hence, a sampling frequency was deemed adequate if 40 or fewer tested ticks per event were sufficient to detect a pathogen prevalence trend of a given magnitude. Note that while these calculations assume that sample sizes for pathogen testing will be dictated mostly by analytical costs and statistical considerations, ticks may be uncommon at many sites and/or during some periods, and the number available to be tested for pathogens may fall below 40 per event.

When power analyses were run with a temporal autocorrelation term associated with the prevalence data, the proposed design was only capable of detecting annual prevalence increases of $\leq$1.0% for a common pathogen at a core site (i.e., over 25 years). In this scenario, the design can detect an annual increase of 0.75% with higher confidence (α=0.05, power=0.9) (Figure 4A). In the absence of temporal autocorrelation in the data, the design can detect an annual increase of 0.25% with high confidence at sampling frequencies of one event every three and six weeks. At these sampling frequencies the design can detect an annual increase of 0.50% for a rare pathogen at a core site with higher confidence (Figure 4B), and an annual increase of 0.25% with lower confidence (α=0.1, power=0.8) but only at a sampling frequency of one event every three weeks. The power of the design was weaker at relocatable sites (i.e.,

over 10 years). There, for common pathogens and at a sampling frequency of one event every six weeks, annual increases of 0.75% (Figure 4C) and 1.0% can be detected with lower and higher confidence, respectively. For rare pathogens, an annual increase of 1.0% can only be detected with lower confidence at a sampling frequency of one event every three weeks (Figure 4D).

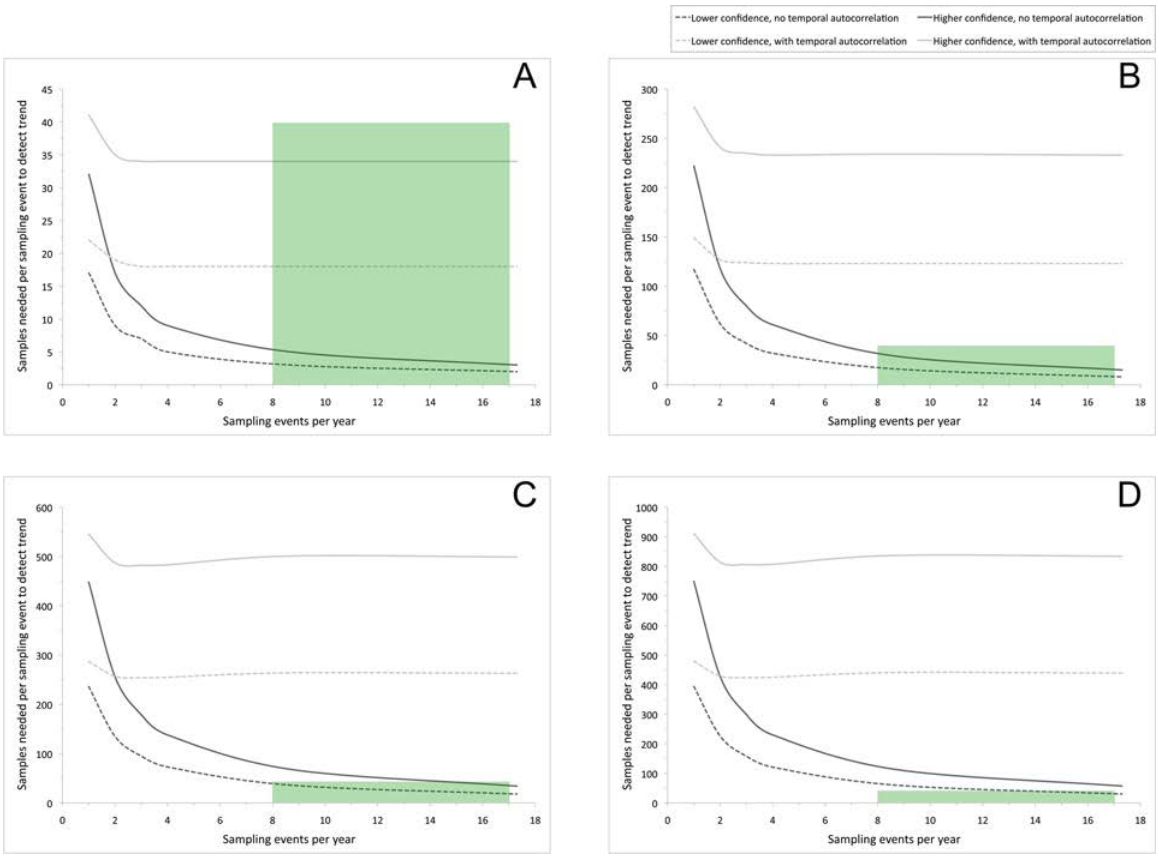

Figure 4. Results of power analyses for a common tick-borne pathogens (prevalence=15%) at a core (i.e., 25 years, A) and a relocatable (i.e., 10 years, B) site and a rare tick-borne pathogen (prevalence=15%) at a core (C) and relocatable (D) site. Each analysis includes results for all combinations of low (α=0.1, power=0.8) and high (α=0.05, power=0.9) confidence and with and without a temporal autocorrelation term (e-1 at a half year). Green boxes indicate levels of sampling effort (event frequency and number samples/event to be tested for pathogens) specified in the design.

Evaluation of power to detect pathogen(s)

The design has a ~100% probability of detecting a common pathogen and an 87% probability of detecting a rare pathogen when 40 or more ticks per species/lifestage combination are tested per sampling event (Figure 5A). For very rare pathogens (1% prevalence) the probability is 33%. When analytical sampling effort is reduced by 50% (20 ticks per species/lifestage combination tested per sampling event), these probabilities fall to 96%, 64%, and 18%, respectively. To achieve a 50% probability of detecting a pathogen, four, 14, and 68 ticks must be tested under scenarios when the pathogen is common, rare, or very rare, respectively (Figure 5B). These sample sizes must be increased to 10, 31, and 160 ticks to achieve an 80% detection probability.

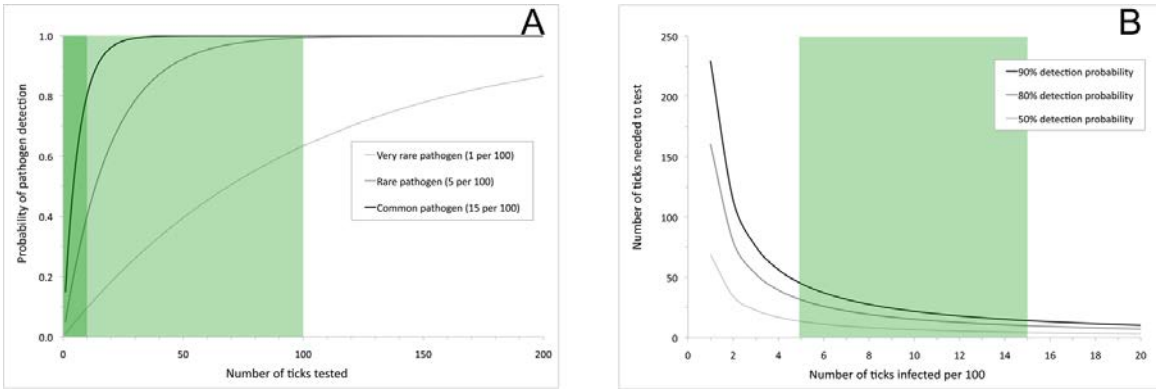

Figure 5. (A) Relationship between the number of samples tested for pathogens and the probability of detecting tick-borne pathogens. Green boxes indicated upper and lower estimates of number of samples to be tested per sampling event. (B) Minimum number of samples required for specified detection probabilities at various levels of infection prevalence for tick-borne pathogens. Green box indicates estimated prevalence of rare and common pathogens at NEON sites.

6.1.4 Logistics and Adaptability

To promote high temporal resolution time series data in the face of potential financial and logistic constraints, the current sampling strategy prioritizes intensive within-site sampling. In the event that field sampling effort must be reduced, a relatively high level of sampling effort will be maintained at NEON core sites, and effort reduced at relocatable sites, to preserve the longer time series data associated with the former. If analytical cost savings are required, field sampling efforts specified in the sampling designs (e.g., frequency of sampling events, number of plots per site) will be maintained and cost savings realized through reductions in the number of samples that are tested for pathogens. Foregoing the testing of all samples collected during selected sampling events, rather than reducing the number of samples tested for every event, will facilitate data comparability by maintaining more consistent levels of analytical sampling effort and associated uncertainty through time. Remaining samples (i.e., untested or residual) can be archived for processing at a later date, either by NEON or through agreements with other members of the research community.

The development of technologies associated with the tick-borne pathogen testing method may soon make it possible to conduct multiplex PCR using additional primers to test for an even broader suite of pathogens. For example, detection of eukaryotic parasites (e.g., *Babesia* spp.) is desirable but would require use of 18S rRNA primers. Such modified tests, including multiplex assays involving both 16S and 18S rRNA primers, will be considered as technological advances and resources permit.

6.2 Sampling Design for Mosquito-Borne Pathogen Sampling

Mosquito sampling will broadly target all members of the family Culicidae. For mosquito-borne pathogen testing, samples will be screened for infection by arboviruses (Calisher 1994). Mosquitoes in the genera *Aedes* and *Culex* are of particular interest because they collectively transmit a variety of viral pathogens (Moore et al. 1993, Turell et al. 2005). Mosquitoes of other species will be tested for pathogens as resources permit and sample sizes warrant. Female mosquitoes, pooled by species, will be

tested for the presence of alphaviruses, bunyaviruses, and flaviviruses using PCR-based and/or Vero cell culture methods. Some or all virus-positive pools will be subjected to additional pathogen-specific tests to identify the pathogen(s) present within them. Additional details of the design and implementation of NEON mosquito sampling can be found in the NEON TOS Science Design for Mosquito Abundance, Diversity and Phenology (AD[08]) and in Field and Lab Protocol: Mosquito Sampling (AD[05]).

6.2.1 Sampling Methods

6.2.1.1 Sample Collection in the Field

Mosquito sampling will be conducted using CDC miniature CO_2 light traps (Sudia and Chamberlain 1962). Among commonly used mosquito traps this type arguably collects the greatest diversity of mosquitoes across a broad range of environmental conditions and is regularly deployed as part of arbovirus surveillance (Service 1993). As such, use of this trap for NEON sampling should facilitate the integration of NEON mosquito data with similar data collected by many public health and mosquito control agencies. CDC miniature CO_2 light traps attract mosquitoes through the emission of light and release of carbon dioxide (e.g., from sublimating dry ice or compressed gas cylinders), the latter being a component of vertebrate exhalation that female mosquitoes use to locate hosts. Mosquitoes attracted to a trap are drawn into a specimen catch cup by the trap's fan.

6.2.1.2 Sampling Event

An event of mosquito sampling will involve the deployment of one dry-ice baited CDC miniature CO_2 light trap at each of 10 sampling plots per site. During a sampling event, each trap will be deployed continuously for roughly 40 hours beginning at dusk on the first day. Traps will be checked (collected mosquitoes retrieved, dry ice replenished) at dawn following both nights of deployment and at dusk on the second day. This results in each sampling event being split into three trapping periods: two trap nights and the intervening day. Traps will be hung at a height of roughly two meters and baited with approximately 1.5kg of dry ice during each trapping period. Light bulbs will be turned off during deployment to reduce bycatch and conserve battery life. Additional details about mosquito sampling can be found in the NEON TOS Science Design for Mosquito Abundance, Diversity, and Phenology (AD[08]).

Following their collection from traps, sampling personnel will transports catch cups containing mosquitoes to a NEON domain lab in portable coolers containing dry ice. At the domain lab, mosquitoes will be flash frozen and transferred into labeled vials. Sample vials will be stored at -80°C at the domain lab until they are sent to one or more external facilities for mosquito taxonomic identification and pathogen testing.

6.2.1.3 Taxonomic Identification of Samples

Taxonomic identification will be based on visual examination of external morphology (e.g., Darsie and Ward 1981) with some confirmation using DNA barcoding (Gibson et al. 2012). From among mosquitoes collected during each trapping period (three periods per sampling event: two trap nights and the intervening day), NEON will either identify a set proportion of the total catch or a fixed number of mosquitoes. This decision will ultimately be based on catch rates observed during the first few years of sampling. The initial plan involves the latter approach: up to 200 mosquitoes per trapping period will be identified and enumerated by species and sex (e.g. number of females of species A collected at plot B within site C during night one of sampling event D). When more than 200 mosquitoes are collected in a trapping period, a representative subsample of ~200 individuals will be identified. All processing will be conducted in a manner that maintains the cold chain and prevents freeze/thaw cycles that could compromise the quality of samples for pathogen testing. Following identification, mosquitoes will be combined by species, sex, site, and event (e.g., all females of species A collected at site C during sampling event D) for pathogen testing. Any remaining unidentified mosquitoes will be bulked at the site/event level. See the NEON TOS Science Design for Mosquito Abundance, Diversity, and Phenology (AD[08]) for more details on taxonomic identification, DNA barcoding, preservation of pinned NEON mosquito samples, and handling and archive of bycatch.

6.2.1.4 Pathogen Testing

A subset of the species-identified female mosquitoes collected during each event of sampling will be tested for pathogens. Mosquitoes will be tested in pools of 20-50 individuals grouped by species at the site level (catches combined across all trapping periods and plots sampled during the event). Actual sample sizes for pathogen testing will depend on collection success and analytical costs. Because the prevalence of arboviruses in mosquitoes is generally very low (e.g., 1-5 infected individuals per 1,000 individuals, Andreadis et al. 2004, Gu and Novak 2004, Gu et al. 2008, Kwan et al. 2010), it is desirable to maximize the number of mosquitos tested for pathogens. In instances where only a portion of mosquitoes collected during a sampling event are identified, NEON may pursue the identification and removal of additional individuals of target vector species from the unidentified bulk lot to increase analytical sample sizes for pathogen testing. This would occur at the end of the sampling season. Target species will include known vectors of arboviral pathogens within the NEON purview (Moore et al. 1993, Turell et al. 2005):

1. Species in the genus *Aedes* including *Ae. aegypti*, *Ae. albopictus*, *and Ae. triseriatus.* These species collectively transmit dengue viruses and La Crosse encephalitis virus.
2. Species in the genus *Culex* including *Cx. pipiens*, *Cx. p. quinquefasciatus, Cx. tarsalis*, *Cx. salinarius*, *Cx. nigripalpus*, and *Cx. restuans*. These species are important vectors of West Nile virus and St. Louis encephalitis virus.
3. Mosquitoes of other taxa including *Culiseta melanura* and *Coquillettidia perturbans* (vectors of eastern equine encephalitis virus) or confirmed vectors of arboviral pathogens within NEON domains.

Each mosquito pool will first be tested for the presence of alphaviruses, bunyaviruses, and flaviviruses. This can be accomplished using various methods (or combinations thereof) including Vero cell screening, RT-PCR using specific or general (i.e., broadly-reactive, family specific) primers, and melt curve assays for viral RNA (Earley et al. 1967, Kuno et al. 1996, Kuno 1998, Lanciotti et al. 2000, Sanchez-Seco et al. 2001, Nasci et al. 2002, Naze et al. 2009). Because West Nile virus and dengue viruses are flaviviral pathogens of particular interest, any flavivirus-positive pools will be further assayed with pathogen-specific tests to identify the particular pathogen(s) present within them. Alphavirus- and bunyavirus-positive pools will be subjected to similar secondary tests as resources allow.

6.2.1.5 Archive

A small number of species-identified but untested mosquitoes will be used to develop a mosquito DNA barcode library and pointed for archiving. Remaining species-identified and untested mosquitoes, all or a subset of unidentified mosquitoes, and products generated by mosquito-borne pathogen testing (e.g., genomic extractions, RT-PCR products) will be archived in vials in existing facilities. Archiving plans, including details on accessing samples for private investigation are detailed in the Fundamental Sentinel Unit Bioarchive Facility Design (AD[10).

6.2.2 Spatial Distribution of Sampling

As described in the general introduction and in the NEON TOS Science Design for Spatial Sampling (AD[03]), a stratified random approach will be used to select 10 mosquito sampling plots within the dominant vegetation types ($\geq$5% total cover) at each site. The number of plots per type will be proportional to the percent cover of each type. Plot locations will additionally be constrained to fall within 30m of roads because of a need to reduce sampling-associated travel times to and from plots. Once a set of long-term field season sampling plots is selected, NEON will maintain a fixed plots design, sampling those plots irrespective of changes in the presence or abundance of mosquitoes and/or associated pathogens.

6.2.3 Temporal Distribution of Sampling

Sampling will be conducted at a frequency of one sampling event every two weeks at core sites and every four weeks at relocatable sites. These frequencies will be maintained for the lifespan of each site irrespective of sampling success. Sampling will occur year round as long as mosquitoes are being collected (see below for description of off season sampling), but a given sampling event will only be performed if the mean daily high temperature for the five days prior to the first day of the event is $\geq$4°C. This temperature is a conservative threshold below which mosquitoes are generally not active (e.g., Bailey et al. 1965, Corbet and Danks 1973, Almeida and Gorla 1995, Ciota et al. 2011).

Mosquito sampling at some higher latitude NEON sites can be discontinued for part of each year when environmental conditions are unfavorable for mosquito activity. At these sites, a program of off-season sampling will be implemented to empirically detect the onset and conclusion of annual mosquito activity

cycles. Within a NEON domain, off-season sampling will commence following three consecutive zero-catch sampling events at the core site. Off-season sampling will involve weekly deployment of one CDC miniature CO_2 light trap at each of three sampling plots for a single night of trapping at the core site. Sampling will transition back to the regular season plan (10 sampling plots with events every two or four weeks) following the collection of at least one mosquito during an off-season sampling event. See the NEON TOS Science Design for Mosquito Abundance, Diversity, and Phenology (AD[08]) for additional details on off-season mosquito sampling.

6.2.3.1 Power Analyses Evaluating Sampling Frequencies

Power analyses associated with mosquito-borne pathogen sampling are complicated by the tremendous spatiotemporal variability in mosquito abundance and associated difficulty in estimating the catch size for a "typical" sampling event. Based on current cost and budget estimates we anticipate sufficient funding for the taxonomic identification of up to 600 mosquitoes per trap per sampling event (up to 200 in each of the three trapping periods per event). Given the plan to deploy one trap at each of ten sampling plots at a site during an event, this yields a maximum of 6,000 taxonomically identified mosquitoes per site/event combination. Because catches during the diurnal portion of sampling events are likely to be relatively small, and nocturnal catches will not consistently exceed 200 mosquitoes per trap/trapping event at most sites, we assume this maximum can be reduced to a more conservative value of 2,000, the majority of which will be females. This catch success seems reasonable at sites where mosquitoes are abundant but could regularly be lower by an order of magnitude or more at sites where they are rare. The number of resulting analytical pools of 50 that can be generated from these 200-2,000 mosquitoes will vary depending on the relative abundance of species at a site. For power analyses we assumed sample size thresholds of 15-20 pools per species/sampling event combination on the high end and 1-4 pools on the low end. Year-zero prevalence values were set to 0.1% (rare pathogen) and 0.5% (common pathogen) based on typical rates of arboviral infection in mosquitoes (Andreadis et al. 2004, Gu and Novak 2004, Gu et al. 2008, Kwan et al. 2010). In addition to evaluating the power associated with sampling event frequencies of every two and four weeks, frequencies of one, two, three and four times per year were evaluated to generate power curves.

Evaluation of power to detect prevalence trends

In the absence of temporal autocorrelation, the design can detect an annual prevalence increase of 2.0% for a common pathogen at a core site (i.e., over 25 years) with higher confidence (α=0.05, power=0.9) (Figure 6A), and an annual increase of 1.5% with lower confidence (α=0.1, power=0.8). Assuming one sampling event every two weeks at core sites, a minimum of eight and 30 pools must be tested per event to detect annual prevalence increases of 1.0% and 0.5%, respectively, with lower confidence. When the pathogen is rare at a core site an annual increase of 3.0% can be detected with lower confidence. At relocatable sites (i.e., over 10 years) the design cannot detect an annual prevalence increase of $\leq$5.0% when the pathogen is common or rare (Figure 6B).

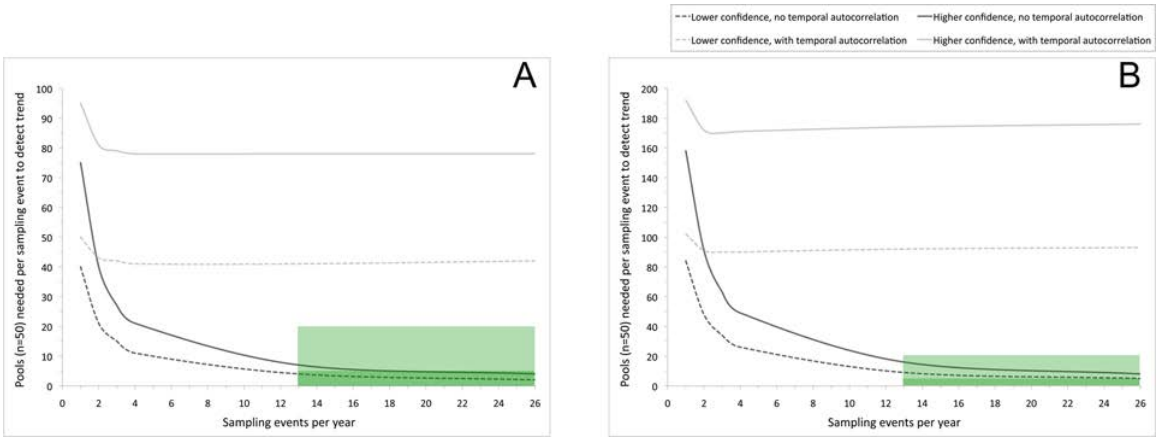

Figure 6. Results of power analyses for a common mosquito-borne pathogens (prevalence=0.5%) at a core (i.e., 25 years, A) and a relocatable (i.e., 10 years, B) site. Each analysis includes results for all combinations of low (α=0.1, power=0.8) and high (α=0.05, power=0.9) confidence and with and without a temporal autorcorrelation term (e-1 at a half year). Green boxes indicate levels of sampling effort (event frequency and number samples/event to be tested for pathogens) specified in the design.

Evaluation of power to detect pathogen(s)

Sampling has high power to detect the presence of pathogens at Observatory sites. The design has a >90% probability of detecting a common pathogen when 450 or more mosquitoes (nine or more pools of 50) are tested (Figure 7A). This probability is between 22% and 63% when analytical sample sizes are lower (between one and four pools of 50, respectively). Power is lower for rare pathogens: the detection probability exceeds 50% when roughly 685 mosquitoes are tested, and probabilities are between 5% and 18% when analytical sample sizes are very low (one and four pools of 50, respectively). For reference, a minimum of 1,609 and 2,301 mosquitoes would need to be tested to detect a rare pathogen with 80% and 90% confidence, respectively (Figure 7B). For a common pathogen these values are 321 and 459. Fifty percent detection probabilities require the testing of 693 mosquitoes when the pathogen is rare and 138 mosquitoes when the pathogen is common. Detection would be more likely during epizootics when arboviral infection prevalence is higher (e.g., 20-25 infected mosquitoes per thousand). In these scenarios, 50%, 80% and 90% detection probabilities require testing a minimum of 34, 80, and 114 mosquitoes, respectively.

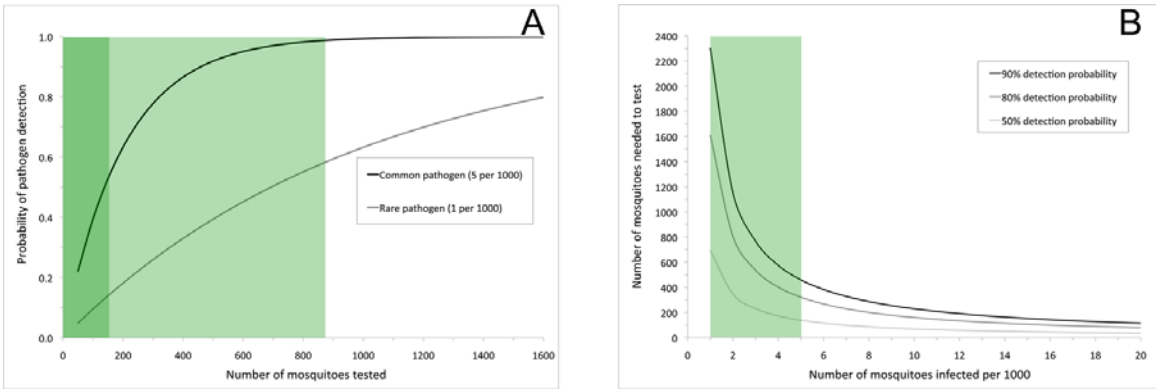

Figure 7. (A) Relationship between the number of samples tested for pathogens and the probability of detecting mosquito-borne pathogens. Green boxes indicated upper and lower estimates of number of samples to be tested per sampling event. (B) Minimum number of samples required for specified detection probabilities at various levels of infection prevalence for mosquito-borne pathogens. Green box indicates estimated prevalence of rare and common pathogens at NEON sites.

6.2.4 Logistics and Adaptability

In the event that sampling effort must be reduced, the sampling design (e.g., frequency of sampling events, number of plots per site) should be maintained, and cost savings realized through reductions in the number of samples that are tested for pathogens. Foregoing the testing of all samples collected during selected sampling events, rather than reducing the number of samples tested for every event, will facilitate data comparability by maintaining more consistent levels of analytical sampling effort and associated uncertainty through time. Remaining samples (i.e., untested or residual) can be archived for processing at a later date, either by NEON or through agreements with other members of the research community.

The CDC miniature CO_2 light trap has known sampling biases and limitations, at least two of which are relevant for mosquito-borne pathogen sampling. First, these traps are relatively ineffective at sampling the gravid or previously blood-fed mosquitoes that are the preferred targets for pathogen testing. Because gravid female mosquitoes are previously blood fed, their inclusion in testing pools enhances the likelihood of detecting pathogens when they are present at a site. Gravid traps represent an attractive supplement to CDC miniature CO_2 light traps because they target gravid mosquitoes through baiting with fetid water associated with oviposition sites (Reiter 1983). Gravid female mosquitoes of foul water-breeding species seeking a site to lay their eggs approach the trap and are sucked into a specimen catch cup by the trap's fan. While the use of gravid traps would increase mosquito catch rates (especially for gravid females) and the likelihood of detecting pathogens, the logistic challenges associated with standardizing and transporting the fetid water bait greatly complicate the use of gravid traps for NEON mosquito sampling. Additionally, the efficacy of these traps is often relatively low in rural or wildland settings where many NEON sites are located. Another alternative and more easily standardized method involves resting box traps (Williams and Gingrich 2007). These resting shelters offer cool, shaded environments that many mosquitoes seek out during daylight hours (Burkett-Cadena et al. 2008).

Mosquitoes alighting on an interior surface of a resting trap can be collected by vacuum aspiration or by a fan assembly integrated into the trap (Panella et al. 2011). For some mosquitoes these traps may represent a relatively inexpensive and easily standardizable method of collecting gravid females (Komar et al. 1995). When catch rates are deemed insufficient, NEON will initially pursue deployment of additional CDC miniature CO_2 light traps to increase trapping success. The deployment of resting or gravid traps alongside CDC miniature CO_2 light traps may be considered as resources allow and as issues related to methodological standardization can be satisfactorily addressed.

The second notable limitation of the CDC miniature CO_2 light trap is that it does not effectively sample mosquitoes of certain species. Notable among these are species in the genus *Aedes* that are important vectors of multiple arboviruses including dengue viruses (Hoel et al. 2009). The BG sentinel trap represents an alternative trapping option for addressing this taxonomic sampling deficiency (Krockel et al. 2006, Pialoux et al. 2007, Meeraus et al. 2008). After several years of data collection at sites where *Ae. aegypti* and *Ae. albopictus* mosquitoes (the principle vectors of dengue viruses) are known or suspected to be present, NEON will evaluate whether sampling utilizing CDC miniature CO_2 light traps has adequately sampled these species. If capture rates are low, NEON will evaluate the feasibility of deploying BG sentinel traps alongside CDC miniature CO_2 light traps to collect these species for use in associated pathogen testing.

6.3 Sampling Design for Rodent-Borne Pathogen Sampling

Sampling for rodent-borne pathogens will primarily target rodents in the family Cricetidae. Associated species are often abundant and ecologically important members of rodent communities and individuals tend to be physiological tolerant of blood sample collection by commonly used methods. Species in the genus *Peromyscus*, especially *P. maniculatus* (deer mouse) and *P. leucopus* (white-footed mouse) are of particular interest since they are broadly distributed, often present at high abundance, and are known reservoirs for hantaviruses. All blood samples will be tested using enzyme-linked immunosorbent assay (ELISA) tests to detect antibodies reactive against hantaviruses and, if resources permit, against arenaviruses. Blood samples will be collected from individuals of other rodent taxa when sampling does not significantly increase their morbidity or mortality. The number of attached larval and nymphal ticks will also be recorded. As mentioned in the tick and tick-borne pathogen sampling design section, removal of ticks from captured small mammals is not currently planned. This decision reflects the need to limit handling time in the face of other prioritized data and sample collection requirements. Data on tick burdens will be used to estimate the additional handling time that would be required to remove ticks from sampled rodents. These estimates will allow the feasibility of incorporating tick collection into the NEON small mammal sampling protocol to be evaluated. Additional details of the design and implementation of NEON small mammal sampling can be found in the NEON TOS Science Design for Small Mammal Abundance and Diversity (AD[09]) and in Field and Lab Protocol: Small Mammal Sampling (AD[06]).

6.3.1 Sampling Methods

6.3.1.1 Sample Collection in the Field

Blood samples to be used for pathogen testing can be collected using a variety of methods. In selecting one or more methods for use by NEON, important considerations include taxonomic breadth of applicability, effects on sampled animals, need for associated anesthesia, volume of resulting samples, and required personal protective equipment (Mills et al. 1995). Methods vary in the degree to which they are restricted for use with animals of particular taxa due to anatomical incompatibility or physiological sensitivity. Related to this, some methods require the use of anesthesia, which increases handling time and may have deleterious health effects for individuals of particular species and/or under certain conditions (e.g., extreme temperatures or when the dosage of anesthetic is difficult to control) (Kosek et al. 1972). Any sampling-related increase in morbidity and mortality of sampled animals has the potential to bias data generated through NEON small mammal mark/recapture sampling and should be avoided. Finally, sampling methods differ in the volume of the blood that they generate because of variation in the size of and pressure in the blood vessel(s) involved. Given the desire to archive NEON samples for additional analyses by other members of the research community, larger sample volumes are preferable. Taken together, the ideal sampling method could be used on animals of a wide range of taxa, generate samples of relatively large volume, and have minimal deleterious impacts on sampled animals and risk for sampling personnel.

Based on these considerations, NEON will initially conduct blood collection using either the retororbital method or the submandibular method. Experiences and results generated during the first few years of sampling will be evaluated to select a single method to be used throughout the observatory. The retroorbital method has been commonly used by field ecologists studying rodent/hantavirus interactions and is considered by many to be the preferred technique for blood collection (Joslin 2009, Auffray et al. 2011, Sikes et al. 2011). A microhematocrit tube is inserted behind one of the rodent's eyes and used to puncture the retroorbital sinus, a highly vascularized region at the back of the orbit (Figure 8A). The procedure can be applied humanely, yields a large volume of blood relative to other methods, and does not require the use of needles or lancets that may cause injury to animals and sampling personnel during handling. Field studies have shown that when used properly, the retroorbital method does not increase handling mortality or decrease recapture rates of sampled animals (Swann et al. 1997, Parmenter et al. 1998). While these results have been produced even without the use of anesthesia (Douglass et al. 2000), sampled animals are generally anesthetized via inhalation of isoflurane prior to blood collection. This precaution reduces the risks of injury to animals and of bites or scratches to sampling personnel. Anesthetization will be included in the NEON retroorbital bleeding protocol as blood samples will sometimes be collected by sampling personnel with little if any prior experience handling small mammals.

Given concerns about deleterious effects of anesthesia on sampled animals, and the anticipation of growing resistance among institutional animal care and use committees to authorizing use of the

retroorbital method, NEON will also pursue use of the submandibular method. The submandibular method involves using a disposable metal lancet to puncture the submandibular vein that runs below and behind a rodent's mandible (Figure 8B). The method yields blood samples of variable but generally comparable volume to those generated by the retroorbital method but can be applied with little or no need for anesthesia. The submandibular method has proven highly effective with laboratory mice (Golde et al. 2005) but to date, has been used relatively rarely in field studies. NEON will utilize this method where possible the first few years of sampling to evaluate the efficacy and safety of this method when used with wild rodents under field conditions.

Other methods of blood collection, including tail and saphenous vein bleeding methods (Hem et al. 1998, Abatan et al. 2008) are not as attractive as the retroorbital and submandibular methods because they generate samples of relatively small volumes.

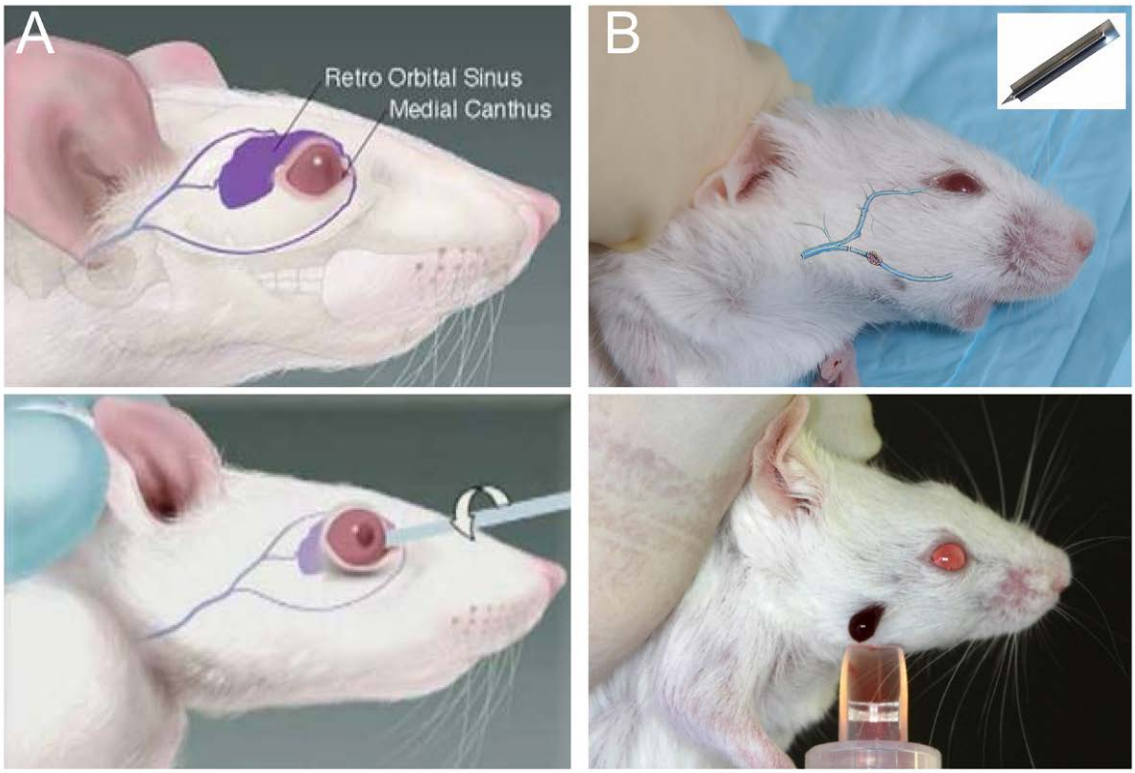

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Figure 8. Diagrams illustrating retroorbital (A) and submandibular (B) bleeding methods.

To minimize increases in sampling-related morbidity and mortality, NEON will only collect blood from animals that meet five criteria. First, the animal must be a member of a taxonomic group for which the sampling does not cause significant stress or injury. For both the retroorbital and submandibular methods, this includes most species of rodents in the family Cricetidae (e.g., *Peromyscus* spp.) and other taxa for which the methods are anatomically suitable and their application (including use of anesthesia)

is not unduly stressful. For example, Heteromyids are not included. Second, NEON must have a permit to handle and collect blood from individuals of the species in question. Third, the animal must weigh at least ten grams. Fourth, the animal should appear to be in good health and not show signs of pronounced or physically debilitating injury (e.g., blindness in or damage to one or both eyes, one or more broken or deformed limbs). Finally, the animal can only be bled once during any given sampling event. Upon capture, an animal meeting these criteria will be anesthetized as needed and bled.

Methods for capturing small mammals are described in the NEON TOS Science Design for Small Mammal Abundance and Diversity (AD[09]) and Field and Lab Protocol: Small Mammal Sampling (AD[06]). In the field, blood samples will be collected into labeled cryovials and immediately frozen on dry ice in portable coolers. Coolers will be transported to a NEON domain lab, where cryovials will be stored at -80°C until they are sent to one or more external facilities for pathogen testing.

In 2012 rodent-borne pathogen sampling was prototyped at Rocky Mountain National Park in NEON Domain 10. The goals of the exercise were to test the feasibility of training and sampling using the retroorbital bleeding method. Training of field staff, many of whom had no prior experience handling rodents or collecting blood samples, was conducted during a one day, hands-on course at the animal care facility of Colorado State University (Fort Collins, CO). Experiences during field sampling and resulting data demonstrated the feasibility of the proposed design and sampling protocol and provided a dataset that will be used to test rodent-borne pathogen ATBDs. Small mammal abundance and diversity sampling was conducted monthly on ten 100-trap grids for four months. Coincident with this activity, blood samples were collected from a total of 272 rodents (Table 2). These samples were sent to and analyzed by the lab of Dr. Brian Hjelle at the University of New Mexico. Results of strip immunoblot assay tests indicated that of the four rodent species tested, evidence of hantavirus (presumably Sin Nombre virus) infection was only apparent in deer mice populations, which had an estimated infection prevalence of 10.3% at the site level and across the sampling period.

Table 2. Results of rodent-borne pathogen sampling in Rocky Mountain National Park (NEON Domain 10, spring and summer 2012).

Species	# individuals tested	# individuals positive	Seroprevalence (%)
Long-tailed vole *(Microtus longicaudus)*	16	0	0.0
Montane vole (*Microtus montanus)*	8	0	0.0
Southern red-backed vole *(Myodes gapperi)*	15	0	0.0
Deer mouse *(Peromyscus maniculatus)*	233	24	10.3
	272	24	8.8

Many rodents play important roles as intermediate hosts for ticks and reservoirs for tick-borne pathogens. Because of the very large size of many rodent populations and the fact that individuals of particular species are highly competent pathogen reservoirs, rodents make significant contributions to demography of ticks and the epizootiology of tick-borne pathogens (LoGiudice et al. 2003, Ostfeld et al. 2006). In recognition of these relationships there is strong interest in quantifying and collecting larval and nymphal ticks attached to rodents captured as part of NEON small mammal sampling. Nevertheless, removal of ticks from captured small mammals is not currently planned. This decision is driven by the need to limit handling time in the face of other prioritized data and sample collection requirements (e.g., length and weight measurements, blood collection). If time permits during processing of captured small mammals, NEON technicians will inspect and count attached larval and nymphal ticks. This approach has been shown to generate reliable estimates of absolute tick burdens for some cricetid and sciurid rodents (Brunner and Ostfeld 2008). Protocol details associated with any observational sampling of ticks on small mammals will be captured in Field and Lab Protocol: Small Mammal Sampling (AD[06]).

6.3.1.2 Pathogen Testing

All collected blood samples will be tested for serum antibodies reactive against hantaviruses and arenaviruses (the latter only if resources permit) using ELISAs. Test results will indicate past exposure to or infection by pathogens of interest (Elgh et al. 1997). External analytical facilities will report the sensitivity and specificity of their testing methods to NEON whenever possible. Samples in which select agents have been identified will be handled in accordance with state and/or federal regulations

6.3.1.3 Archive

Blood samples remaining after NEON pathogen testing will be archived in existing facilities. Archiving plans, including details on accessing samples for private investigation are detailed in the Fundamental Sentinel Unit Bioarchive Facility Design (AD[10]).

6.3.2 Spatial Distribution of Sampling

A stratified random approach will be used to select three to eight small mammal sampling grids within the dominant vegetation types ($\geq$5% total cover) at each site (NEON TOS Science Design for Spatial Sampling (AD[03]). The number of plots per type will be proportional to the percent cover of each type. Plot locations will additionally be constrained to fall within 50m of roads because of a need to facilitate deployment and retrieval of sampling equipment. Vehicular access to plots during winter months may also be considered.

During the first year of sampling, blood samples for use in pathogen testing will be collected from animals captured on up to three of the sampling grids at each site. Grids with the highest abundance of the rodent taxa targeted for blood collection will be selected. Following the first year of sampling, grid-level data on the abundance and diversity of small mammals collected during the first year of sampling will be used to select up to three long-term bleeding grids to be sampled beginning in the second year of

sampling. Long-term bleeding grids will be chosen such that they collectively span the range of small mammal communities (i.e. levels of diversity and abundance) documented in the first year of sampling. Once a set of long-term bleed grids is selected, NEON will maintain a fixed plots design, sampling those grids irrespective of changes in the presence or abundance of rodents and/or associated pathogens.

6.3.3 Temporal Distribution of Sampling

Samples events will occur at a frequency of once every lunar cycle at core sites and once every second lunar cycles at relocatable sites. The sampling frequency proposed for relocatable sites represents a minimum as lower frequencies have been shown to significantly increase error in estimates of both rodent abundance and prevalence of serum antibodies reactive against pathogens (Carver et al. 2010). Sampling will occur year-round during any months when sampling personnel can access plots and weather conditions are safe for personnel and captured animals.

6.3.3.1 Power Analyses Evaluating Sampling Frequencies

Evaluation of power to detect prevalence trends

For power analyses evaluating the ability of sampling and testing to detect long-term trends in prevalence of serum antibodies reactive against rodent-borne hantaviruses and potentially arenaviruses, year-zero antibody prevalence values were set to 2.5% (rare pathogen) or 10% (common pathogen) based on results of long-term studies of Sin Nombre virus infection in deer mice populations (Douglass et al. 2001, Calisher et al. 2007). In addition to evaluating the power associated with sampling event frequencies of every four and eight weeks, frequencies of one, two, three and four times per year were evaluated to generate power curves. Given an estimated capture success of 10-20% (based on data published in Thibault et al. (2011)) and an anticipated sampling effort of between one and three 100-trap trapping grids per site, between 30 and 60 rodents are likely to be sampled per site/sampling event combination. Based on this, a conservative per-event sample size threshold of 40 rodents bled and tested for antibodies reactive against pathogens was used. Thus, a sampling frequency was deemed adequate if 40 or fewer samples per event were sufficient to detect an antibody prevalence trend of a given magnitude.

When power analyses were run with a term depicting temporal autocorrelation in the antibody prevalence data, the proposed design was only capable of detecting annual prevalence increases of $\leq$1.0% for a common pathogen at a core site (i.e., over 25 years). In this scenario, the design can detect an annual increase of 1.0% with higher confidence (α=0.05, power=0.9) and an increase of 0.75% with lower confidence (α=0.1, power=0.8) (Figure 9A). In the absence of temporal autocorrelation in the data, the design can detect annual increases of 0.5% and 0.25% with high confidence, but the latter only at a sampling frequency of one event every four weeks. Similarly, for a rare pathogen at a core site, the design can detect annual increases of 0.75% and 0.5% with high confidence, but the latter only at the one event every four weeks sampling frequency (Figure 9B). In contrast, the ability of the design to

detect annual prevalence increases of <1.0% at relocatable sites (i.e., over 10 years) is considerably lower. When the pathogen is common the design can detect an annual prevalence increase of 1.0% with higher confidence, but only at the highest sampling frequency (one event every four weeks) (Figure 9C). When the pathogen is rare the design cannot detect an annual increase of <1.0% irrespective of the level of confidence or sampling frequency (Figure 9D). While the power of rodent-borne pathogen sampling to detect interannual infection trends for rare pathogens is limited, the probability of detecting these pathogens when they are present at a site is still reasonably high. For example, using the same methods as employed for similar mosquito-borne pathogen calculations, the design has a 64% probability of detecting a rare rodent-borne pathogen (2.5% antibody prevalence) in a particular sampling event when 40 rodents are tested. At a mean prevalence of 4% that probability rises to 80%.

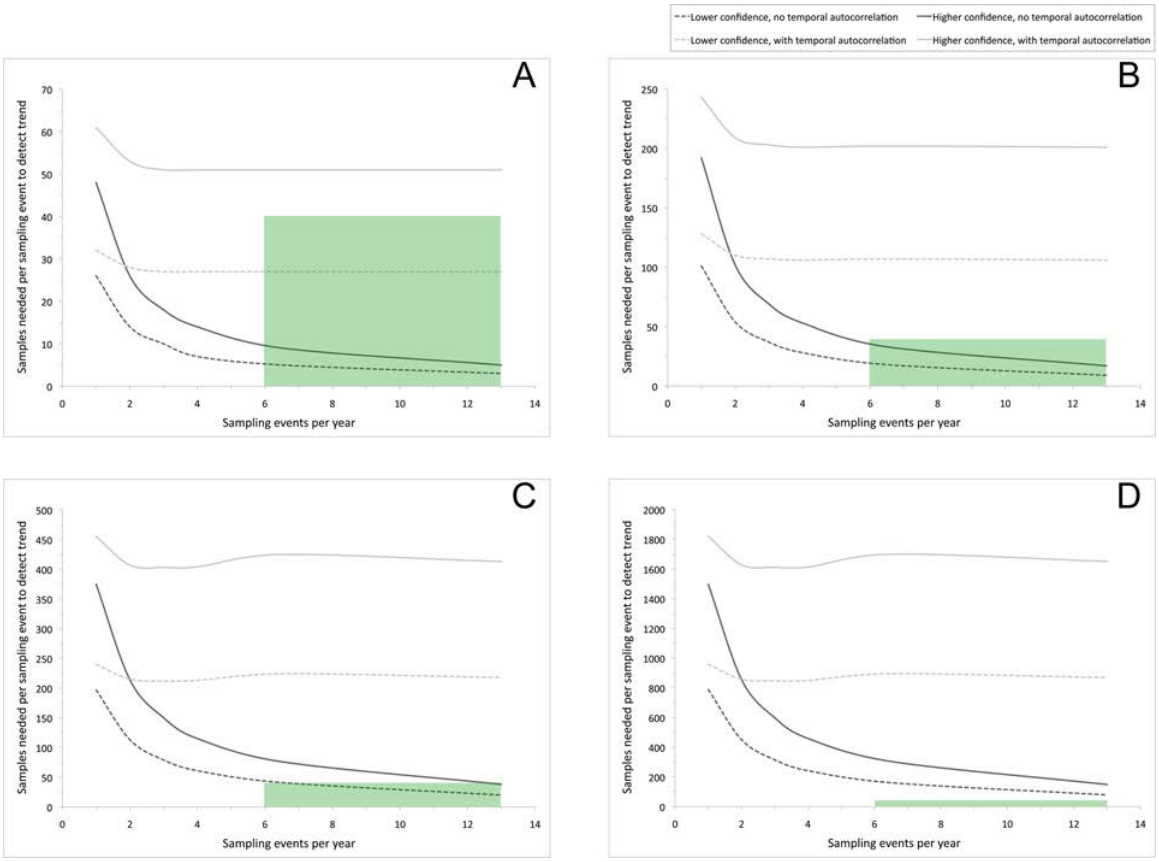

Figure 9. Results of power analyses for a common rodent-borne pathogen (prevalence=10%) at a core (i.e., 25 years, A) and relocatable (i.e., 10 years, B) site, and a rare rodent-borne pathogen (prevalence=2.5%) at a core (C) and relocatable (D) site. Each analysis includes results for all combinations of low (α=0.1, power=0.8) and high (α=0.05, power=0.9) confidence and with and without a temporal autorcorrelation term (e-1 at a half year). Green boxes indicate levels of sampling effort (event frequency and number samples/event to be tested for pathogens) specified in the design.

Evaluation of power to detect pathogen(s)

In terms of simply detecting antibodies reactive against hantaviruses or arenaviruses (i.e., presence/absence of infection) in a sampled reservoir population, the design has a 99% probability of detecting a common pathogen and a 64% probability of detecting a rare pathogen when 40 or more rodents per species are tested per sampling event (Figure 10A). That probability falls to 33% when infection is very rare (1% prevalence). When analytical sampling effort is reduced to 20 rodents tested per sampling event, these probabilities fall to 88%, 40% and 18%, respectively. A 50% probability of detecting infection requires testing 7, 29, or 60 rodents per sampling bout, respectively (Figure 10B). These sample sizes must be increased to 15, 67, and 160 rodents to raise the detection probability to 80%.

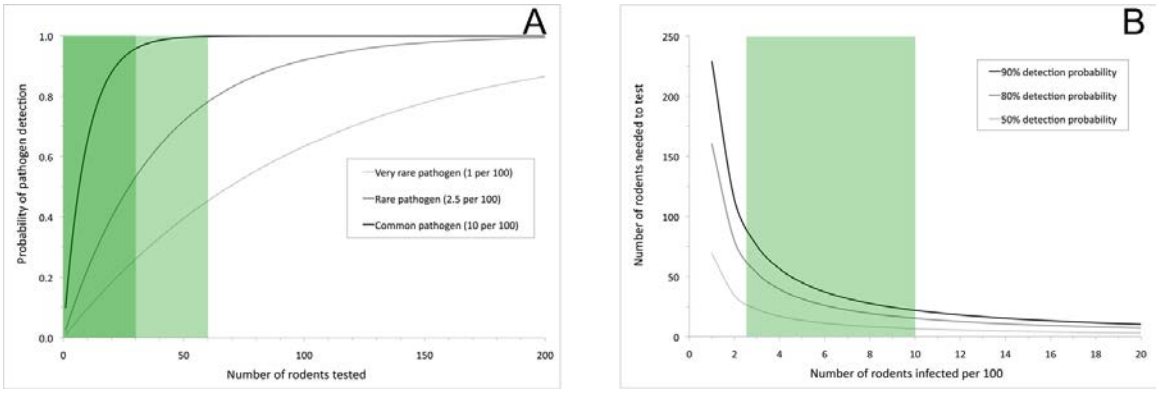

Figure 10. (A) Relationship between the number of samples tested for pathogens and the probability of detecting rodent-borne pathogens. Green boxes indicated upper and lower estimates of number of samples to be tested per sampling event. (B) Minimum number of samples required for specified detection probabilities at various levels of infection prevalence for rodent-borne pathogens. Green box indicates estimated prevalence of rare and common pathogens at NEON sites.

6.3.4 Logistics and Adaptability

In the event that sampling effort must be reduced, the sampling design (e.g., frequency of sampling events, number of plots per site) should be maintained, and cost savings realized through reductions in the number of samples that are tested for pathogens. Foregoing the testing of all samples collected during selected sampling events, rather than reducing the number of samples tested for every event, will facilitate data comparability by maintaining more consistent levels of analytical sampling effort and associated uncertainty through time. Remaining samples (i.e., untested or residual) can be archived for processing at a later date, either by NEON or through agreements with other members of the research community.

As an alternative to blood-based testing, many rodent-borne viral pathogens (including hantaviruses and arenaviruses) can be detected in fecal samples using PCR-based methods (Phan et al. 2011). This approach is attractive for a number of reasons. The collection of fecal samples does not require anesthetization and is minimally invasive for sampled rodents. Additionally, use of fecal samples would

allow for the sampling of rodents that could not be bled due to their small size. This would expand rodent-borne pathogen sampling to include younger rodents as well as adults of species that are anatomically and/or physiologically not amendable to blood collection. Among these are shrews, known reservoirs of hantaviruses (Arai et al. 2008) that typically experience high and often fatal levels of stress associated with handling and blood sample collection. PCR-based analyses of tissue (including fecal) samples for rodent-borne pathogens are currently much more expensive than enzyme-based tests and are therefore less attractive as fewer samples can be tested for a given level of funding. Additionally, it is not always possible to collect fecal samples on demand. NEON may consider the use of PCR-based tests in the future if/when associated costs of PCR-based testing decline. NEON will collect (but not analyze) fecal samples from captured rodents and store/archive these in a manner that will preserve them for use in such pathogen testing (frozen at -80C).

Data on tick burdens collected as part of small mammal sampling could be used to estimate the additional handling time that would be required to remove ticks from sampled small mammals. These estimates would allow the feasibility of incorporating tick collection into the NEON small mammal sampling protocol to be evaluated.

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APPENDIX A CODE FOR POWER ANALYSES

Code written by P. Duffy (Neptune Inc.) for NEON

Code for functions

```
# Function to compute the sample size for detecting a linear trend when the response
# is binomial for samples to be taken uniformly-spaced in time
# Result is the per-sampling-period sample size
# p0 = baseline (year 0) probability
# b1 = annual increase in log-odds [parameter of interest for inference]
# maxtime = total number of years of sampling,
# sampfreq = time interval between samples (in years), e.g. 1 = once per year, 0.5 = twice per year, etc
# timerange = time interval at which temporal correlation drops to exp(-1)
# note: if timerange = 0, then no temporal correlation is included
# sigLevel = significance level or type I error rate
# power = power (1 minus acceptable type II error rate) to detect trend at the level specified by b1
# pooled = logical. If True, multiple organisms are homogenized prior to analysis
# poolsize = number of organisms in the pool if pooled =T

binomialsampsize = function( p0, b1, maxtime, sampfreq, timerange=0,
          sigLevel=0.05, power=0.9, pooled=TRUE,
          poolsize=50){
# p0 is vector of baseline rates or probabilities
# b1 is vector of trend values
# sigLevel = significance level or type I error rate
# power = power (1 minus acceptable type II error rate) to detect trend at the level specified by b1
# maxtime is a vector of different maximum lengths of time (years)
# sampfreq is the number of sampling intervals per unit of time 1=once per year,
# 0.5=twice per year, 0.3333=three times per year, 0.25=four times per year,
# 0.07692=every 4 weeks, 0.03846=every 2 weeks)
# timerange is an exponential-decay correlation parameter
# pooled indicates whether the samples are pooled
# poolsize is the number of samples in each pool
# p0 = 0.1; b1 = 0.05; maxtime = 10; sampfreq = 0.5; timerange=0; sigLevel=0.05;
#   power=0.9; pooled = TRUE; poolsize = 50
# Convert p0 to b0
if(!pooled){
b0=log(p0/(1-p0))
}
if(pooled){ # See Farrington article for derivation.
b0 = log((-1)*log(1-p0))
# An approximation of this for small p0 is b0 = log(p0)
}
# Precalculate re-used quantity
zpre=qnorm(1-sigLevel)+qnorm(power)
# Set up storage
narr=array(dim=c(length(sampfreq),length(maxtime),length(p0),length(b1)))
# Loop through different specified sampling frequencies
```

```
for(i in 1:length(sampfreq)){
# Loop through different specified time limits
for(j in 1:length(maxtime)){
# Set up sequence of sampling times
tm=seq(0,maxtime[j],sampfreq[i])
# Compute temporal correlation matrix
if( timerange==0){
cormat=diag(1,length(tm))
} else {
tmp=as.matrix(dist(tm,upper=TRUE,diag=TRUE))
cormat=exp(-tmp/timerange)
}
# Construct design matrix for regression
if(!pooled) xmat=cbind(1,tm)
if(pooled) xmat=cbind(log(poolsize),1,tm)
# Loop through different specified intercept terms
for(k in 1:length(p0)){
# Loop through different specified slop terms
for(m in 1:length(b1)){
# Compute regression curve
if(!pooled) regrFits=as.vector(xmat%*%c(b0[k],b1[m]))
if(pooled) regrFits=as.vector(xmat%*%c(1,b0[k],b1[m]))
# Convert to sampling mean
if(!pooled){
tmp = exp(regrFits)
p = tmp/(1+tmp)
}
if(pooled){
p=1-exp((-1)*exp(regrFits))
}
# Construct variance and standard deviation matrix
wmat=diag(p*(1-p))
wrootmat=diag(sqrt(p*(1-p)))
# Compute standard error of estimate based on
# sample size of 1
if(!pooled){
xtxinv=solve(t(xmat)%*%wmat%*%xmat)
ses=xtxinv%*%t(xmat)%*%wrootmat%*%cormat%*%wrootmat%*%xmat%*%xtxinv
}
if(pooled){
xtxinv=solve(t(xmat[,-1])%*%wmat%*%xmat[,-1])
ses=xtxinv%*%t(xmat[,-1])%*%wrootmat%*%cormat%*%wrootmat%*%xmat[,-1]%*%xtxinv
}
# Compute required sample size
narr[i,j,k,m] = ceiling((zpre/b1[m])^2*ses[2,2])
}
}
}
}
# Label output and return
```

```
dimnames(narr)=list(sampfreq,maxtime,p0,b1)
return(narr)
}

 ############
# Function to compute the sample size for detecting a linear
# trend when the response is negative binomial for samples
# to be taken uniformly-spaced in time
# Result is the per-sampling-period number of "successes" to be sampled
# m0 = the mean under the baseline condition (year 0)
# p1 = annual percent increase (decrease) in the mean [parameter of
# interest for inference], related to slope: b1 = log( 1 + p1/100 )
# dispersion = dispersion parameter of negative binomial
# variance = mu + mu^2/dispersion [higher dispersion -> closer to poisson]
# maxtime = total number of years of sampling
# sampfreq = time interval between samples (in years)
# scale = scale parameter for negative binomial (poisson over-dispersal)
# timerange = time interval at which temporal correlation drops to exp(-1)
# if timerange=0, then no temporal correlation is included
# sigLevel = significance level or type I error rate
# power = power (1 minus acceptable type II error rate) to detect trend at the level specified by b1

negbinomialsampsize = function( m0, p1, dispersion, maxtime, sampfreq,
timerange=0, sigLevel=0.05, power=0.9 ){
# Precalculate re-used quantity
zpre=qnorm(1-sigLevel)+qnorm(power)
# Set up storage
narr=array(dim=c(length(sampfreq),length(maxtime),length(dispersion),
length(m0),length(p1)))
# Loop through different specified sampling frequencies
for(i in 1:length(sampfreq)){
# Loop through different specified time limits
for(j in 1:length(maxtime)){
# Set up sequence of sampling times
tm=seq(0,maxtime[j],sampfreq[i])
# Compute temporal correlation matrix
if(timerange==0){
cormat=diag(1,length(tm))
}else{
tmp=as.matrix(dist(tm,upper=TRUE,diag=TRUE))
cormat=exp(-tmp/timerange)
}
# Construct design matrix for regression
xmat=cbind(1,tm)
# Loop through different specified dispersion parameters
for(d in 1:length(dispersion)){
# Loop through different specified intercept terms
for(k in 1:length(m0)){
# Loop through different specified slop terms
for(m in 1:length(p1)){
```

```
# Compute regression curve
logmu=as.vector(xmat%*%c(log(m0[k]),log(1+p1[m]/100)))
# Construct variance and standard deviation matrix
mu=exp(logmu)
wmat=diag(mu)
wrootmat=diag(sqrt(dispersion[d]*mu))
# Compute standard error of estimate based on
# sample size of 1
xtxinv=solve(t(xmat)%*%wmat%*%xmat)
ses=xtxinv%*%t(xmat)%*%wrootmat%*%cormat%*%wrootmat%*%xmat%*%xtxinv
# Compute required sample size
narr[i,j,d,k,m] = ceiling((zpre/log(1+p1[m]/100))^2*ses[2,2])
}
}
}
}
}
# Label output and return
dimnames(narr) = list(sampfreq,maxtime,dispersion,m0,p1)
return(narr)
}
```

Additional code to add for tick-borne pathogens

```
# Code to generate tables
# csv files will be created in the current R directory
# Create tables

m0=c(2,6)
# for m0 we are assuming a max testing number of 100 and an associated conservative
# testing number of 40 and so for the starting #values an m0 of 2 translates to 5% and
# an m0 of 6 translates to 15%
p1=c(0.25,0.5,0.75,1)
# for p1 we are assuming annual prevalence increases of 0.25%, 0.5%, 0.75%, and 1%
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.1154,0.05769)
nbin1s = negbinomialsampsize(m0,p1,2,maxtime,sampfreq)[,,1,,]
cat(",",file="tick_negbi_notemp.csv")
for(i in 1:length(m0)){
cat("Baseline Mean = ",m0[i],",",file="tick_negbi_notemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="tick_negbi_notemp.csv",append=T)
}
}
cat("\n",file="tick_negbi_notemp.csv",append=T)
for(i in 1:length(p1)){
cat(",",file="tick_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
cat("Annual % Increase = ",p1[i],",",file="tick_negbi_notemp.csv",append=T)
```

```
for(k in 2:length(maxtime)){
cat(",",file="tick_negbi_notemp.csv",append=T)
}
}
cat("\n,",file="tick_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="tick_negbi_notemp.csv",append=T)
}
}
cat("\n",file="tick_negbi_notemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="tick_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(nbin1s[m,k,j,i],",",file="tick_negbi_notemp.csv",append=T)
}
}
cat("\n",file="tick_negbi_notemp.csv",append=T)
}
}

m0=c(2,6)
# for m0 we are assuming a max testing number of 100 and an associated conservative testing number of 40 and
so for the starting values an m0 of 2 translates to 5% and an m0 of 6 translates to 15%
p1=c(0.25,0.5,0.75,1)
# for p1 we are assuming annual prevalence increases of 0.25%, 0.5%, 0.75%, and 1%
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.1154,0.05769)
nbin1c = negbinomialsampsize(m0,p1,2,maxtime,sampfreq,timerange=0.5)[,,1,,]
cat(",",file="tick_negbi_withtemp.csv")
for(i in 1:length(m0)){
cat("Baseline Mean = ",m0[i],",",file="tick_negbi_withtemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="tick_negbi_withtemp.csv",append=T)
}
}
cat("\n",file="tick_negbi_withtemp.csv",append=T)
for(i in 1:length(p1)){
cat(",",file="tick_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
cat("Annual % Increase = ",p1[i],",",file="tick_negbi_withtemp.csv",append=T)
for(k in 2:length(maxtime)){
cat(",",file="tick_negbi_withtemp.csv",append=T)
}
}
cat("\n,",file="tick_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="tick_negbi_withtemp.csv",append=T)
```

```
}
}
cat("\n",file="tick_negbi_withtemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="tick_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(nbin1c[m,k,j,i],",",file="tick_negbi_withtemp.csv",append=T)
}
}
cat("\n",file="tick_negbi_withtemp.csv",append=T)
}
}
```

Additional code to add for mosquito-borne pathogens

```
# Code to generate tables
# csv files will be created in the current R directory
# Create tables
p0=c(.001,.005,.01)
b1=c(.005,.01,.015,.02,.03,.04,.05)
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.07692,0.03846)
binom = binomialsampsize(p0,b1,maxtime,sampfreq,timerange=0.5)
cat(",",file="Mosquito_withtemp.csv")
for(i in 1:length(p0)){
cat("Baseline Probability = ",p0[i],",",file="Mosquito_withtemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="Mosquito_withtemp.csv",append=T)
}
}
cat("\n",file="Mosquito_withtemp.csv",append=T)
for(i in 1:length(b1)){
cat(",",file="Mosquito_withtemp.csv",append=T)
for(j in 1:length(p0)){
cat("beta1 = ",b1[i],",",file="Mosquito_withtemp.csv",append=T)
for(k in 2:length(maxtime)){
cat(",",file="Mosquito_withtemp.csv",append=T)
}
}
cat("\n,",file="Mosquito_withtemp.csv",append=T)
for(j in 1:length(p0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="Mosquito_withtemp.csv",append=T)
}
}
cat("\n",file="Mosquito_withtemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="Mosquito_withtemp.csv",append=T)
```

```
for(j in 1:length(p0)){
for(k in 1:length(maxtime)){
cat(binom[m,k,j,i],",",file="Mosquito_withtemp.csv",append=T)
}
}
cat("\n",file="Mosquito_withtemp.csv",append=T)
}
}

# Code to generate tables
# csv files will be created in the current R directory
# Create tables
p0=c(.001,.005,.01)
b1=c(.005,.01,.015,.02,.03,.04,.05)
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.07692,0.03846)
binom = binomialsampsize(p0,b1,maxtime,sampfreq,)
cat(",",file="Mosquito_notemp.csv")
for(i in 1:length(p0)){
cat("Baseline Probability = ",p0[i],",",file="Mosquito_notemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="Mosquito_notemp.csv",append=T)
}
}
cat("\n",file="Mosquito_notemp.csv",append=T)
for(i in 1:length(b1)){
cat(",",file="Mosquito_notemp.csv",append=T)
for(j in 1:length(p0)){
cat("beta1 = ",b1[i],",",file="Mosquito_notemp.csv",append=T)
for(k in 2:length(maxtime)){
cat(",",file="Mosquito_notemp.csv",append=T)
}
}
cat("\n,",file="Mosquito_notemp.csv",append=T)
for(j in 1:length(p0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="Mosquito_notemp.csv",append=T)
}
}
cat("\n",file="Mosquito_notemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="Mosquito_notemp.csv",append=T)
for(j in 1:length(p0)){
for(k in 1:length(maxtime)){
cat(binom[m,k,j,i],",",file="Mosquito_notemp.csv",append=T)
}
}
cat("\n",file="Mosquito_notemp.csv",append=T)
}
}
```

Additional code to add for rodent-borne pathogens

```
m0=c(1,4)
# for m0 we are assuming a catch rate per bout of 40 animals and so for the starting values
# an m0 of 1 translates to 2.5% and an m0 # of 4 translates to 10%
p1=c(0.25,0.5,0.75,1)
# for p1 we are assuming annual prevalence increases of 0.25%, 0.5%, 0.75%, and 1%
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.1539,0.07692)
nbin1s = negbinomialsampsize(m0,p1,2,maxtime,sampfreq)[,,1,,]
cat(",",file="rodent_negbi_notemp.csv")
for(i in 1:length(m0)){
cat("Baseline Mean = ",m0[i],",",file="rodent_negbi_notemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="rodent_negbi_notemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_notemp.csv",append=T)
for(i in 1:length(p1)){
cat(",",file="rodent_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
cat("Annual % Increase = ",p1[i],",",file="rodent_negbi_notemp.csv",append=T)
for(k in 2:length(maxtime)){
cat(",",file="rodent_negbi_notemp.csv",append=T)
}
}
cat("\n,",file="rodent_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="rodent_negbi_notemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_notemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="rodent_negbi_notemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(nbin1s[m,k,j,i],",",file="rodent_negbi_notemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_notemp.csv",append=T)
}
}
m0=c(1,4)
# for m0 we are assuming a catch rate per bout of 40 animals and so for the
# starting values an m0 of 1 translates to 2.5% and an m0 of 4 translates to 10%
p1=c(0.25,0.5,0.75,1)
# for p1 we are assuming annual prevalence increases of 0.25%, 0.5%, 0.75%, and 1%
```

```
maxtime=c(10,25)
sampfreq=c(1,0.5,0.3333,0.25,0.1539,0.07692)
nbin1c = negbinomialsampsize(m0,p1,2,maxtime,sampfreq,timerange=0.5)[,,1,,]
cat(",",file="rodent_negbi_withtemp.csv")
for(i in 1:length(m0)){
cat("Baseline Mean = ",m0[i],",",file="rodent_negbi_withtemp.csv",append=T)
for(j in 2:length(maxtime)){
cat(",",file="rodent_negbi_withtemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_withtemp.csv",append=T)
for(i in 1:length(p1)){
cat(",",file="rodent_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
cat("Annual % Increase = ",p1[i],",",file="rodent_negbi_withtemp.csv",append=T)
for(k in 2:length(maxtime)){
cat(",",file="rodent_negbi_withtemp.csv",append=T)
}
}
cat("\n,",file="rodent_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(maxtime[k],",",file="rodent_negbi_withtemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_withtemp.csv",append=T)
for(m in 1:length(sampfreq)){
cat(sampfreq[m],",",file="rodent_negbi_withtemp.csv",append=T)
for(j in 1:length(m0)){
for(k in 1:length(maxtime)){
cat(nbin1c[m,k,j,i],",",file="rodent_negbi_withtemp.csv",append=T)
}
}
cat("\n",file="rodent_negbi_withtemp.csv",append=T)
}
}
```