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ECOSPHERE

SPECIAL FEATURE: NEON DESIGN

Tick-, mosquito-, and rodent-borne parasite sampling designs for the National Ecological Observatory Network

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Abstract. Parasites and pathogens are increasingly recognized as significant drivers of ecological and evolutionary change in natural ecosystems. Concurrently, transmission of infectious agents among human, livestock, and wildlife populations represents a growing threat to veterinary and human health. In light of these trends and the scarcity of long-term time series data on infection rates among vectors and reservoirs, the National Ecological Observatory Network (NEON) will collect measurements and samples of a suite of tick-, mosquito-, and rodent-borne parasites through a continental-scale surveillance program. Here, we describe the sampling designs for these efforts, highlighting sampling priorities, field and analytical methods, and the data as well as archived samples to be made available to the research community. Insights generated by this sampling will advance current understanding of and ability to predict changes in infection and disease dynamics in novel, interdisciplinary, and collaborative ways.

Key words: infectious disease; mosquito; National Ecological Observatory Network; NEON design; parasite; pathogen; reservoir; rodent; sampling design; Special Feature: NEON Design; tick; vector; zoonoses.

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Introduction

Parasites and pathogens (hereafter, parasites) can be 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 1999, Swabe 1999, Hudson et al. 2002, Alexander 2010, Brown and Gilfoyle 2010, Brooks and Hoberg 2013). In recent decades, the number of reportedly emerging and re-emerging parasites—many of which are vector- and reservoir-borne zoonoses of 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 systems and human-associated environments 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. 2000*a*, Gubler et al. 2001, Harvell et al. 2002, Patz 2004, Weiss and McMichael 2004, Wolfe et al. 2005, 2007, Patz et al. 2008, Lafferty 2009, Mills et al. 2010*b*, Rohr et al. 2011, Altizer et al. 2013, Perry et al. 2013). In some cases, increases in vector and reservoir abundance, rates of parasite transmission, and the severity of disease outbreaks—particularly those arising from cross-system infection events (e.g., zoonoses)—are predicted (Marano et al. 2007, Myers et al. 2013, Harrigan et al. 2014). In others, environmental change may result in parasite 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 parasites, and in the epidemiology 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 constituent 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 and growing need for increased parasite surveillance efforts that bridge the historical divisions among studies of human, domesticated animal, and 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 these linkages was highlighted as a critical priority in both the National Research Council's Grand Challenges (National Research Council 2001, 2003) and the Millennium Ecosystem Assessment (Patz 2005). Given the broad spatiotemporal extents over which many important changes in infection and disease dynamics are likely to occur, and the myriad factors that could underlie those changes, the value of large-scale, multifaceted surveillance efforts is increasingly recognized (Altizer et al. 2013). As the size and scope of surveillance efforts expand, appropriate sampling design and methodological standardization will greatly facilitate comparisons across data sets and scales. Although logistically challenging, such large-scale, standardized sampling efforts are critical to characterize regional, continental, and multidecadal 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–parasite interactions in natural systems (Kovats et al. 2001, Crowl et al. 2008).

In 2012, the U.S. National Science Foundation (NSF) 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 as many as 60 sites for up to 30 yr, NEON will

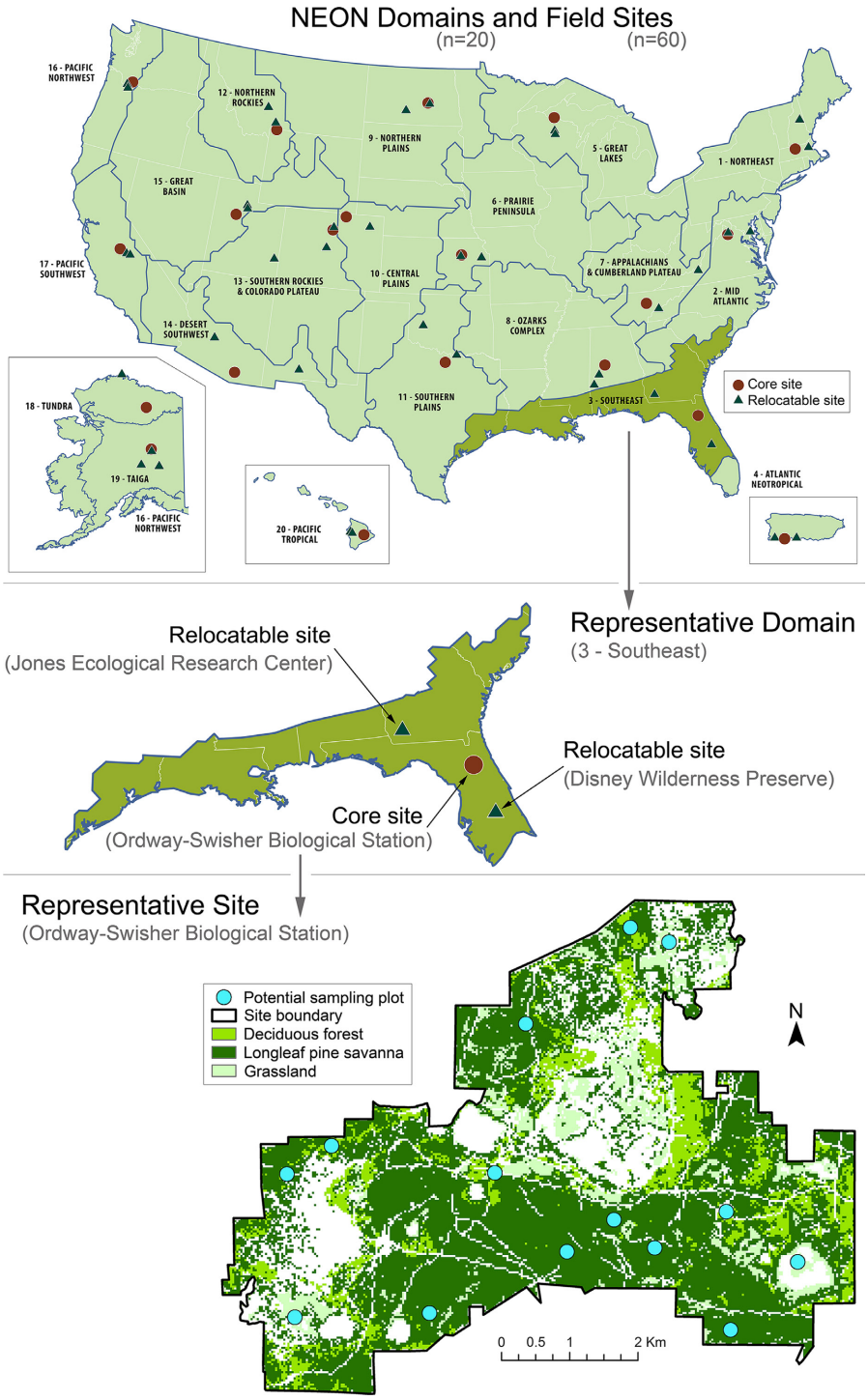


Fig. 1. Overview of the hierarchical spatial structure of the National Ecological Observatory Network. Panels show (from top to bottom) the boundaries of the 20 ecoclimatic domains and locations of associated sites, the distribution of one core site and two relocatable sites in a representative domain, and multiple sampling plots distributed among vegetation types at a representative site.

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 (Schimel 2011). Observatory sites will be distributed throughout the continental United States, Alaska, Hawaii, and Puerto Rico. Statistical methods were used to partition this area into 20 ecoclimatic regions termed domains (Hargrove and Hoffman 2004, Fig. 1) that collectively span the range of climate and vegetation communities found within the United States. Multiple Observatory sites are planned in each domain: one core site (location fixed for 30-yr lifespan of the Observatory) and between one and three (typically two) relocatable sites (location potentially dynamic within the domain, with intended relocation occurring every 6–10 yr). Core sites are located in wildland areas and will provide baseline measurements of the changing biotic and abiotic characteristics of associated domains. In many cases, relocatable site locations have been (and will be) selected to represent points along ecological, climatic, or land-use gradients of interest (e.g., urban/rural/undeveloped, active silva- or agriculture/discontinued harvest/uncultivated, highly invaded/minimally invaded/pristine). Other considerations for selecting site locations include anticipated extent and duration of access and previous or ongoing research efforts and infrastructure. The sizes of currently selected sites vary according to administrative boundaries and logistical constraints; core sites range from 11 to 214 km^2 while relocatable sites are typically smaller and more variable in size, ranging from small agricultural sites (~5 km^2) to larger wildland locations (up to 50 km^2).

As part of NEON's terrestrial observation system (Kao 2012), sampling of parasites and associated vector (organisms that transmit parasites between hosts) and reservoir (organisms in which parasites can live, multiply, and disperse from) species will be conducted to elucidate the changing ecology of a suite of tick-, mosquito-, and rodent-borne parasites. Among these are the etiological agents of important human illnesses including Lyme borreliosis, West Nile virus disease, and Hantavirus pulmonary syndrome (HPS) (Fig. 2). Others cause diseases in wildlife species that can have important effects on the ecology of natural systems and may represent threats to veterinary health. Unlike most surveillance programs, sampling of vectors, res-

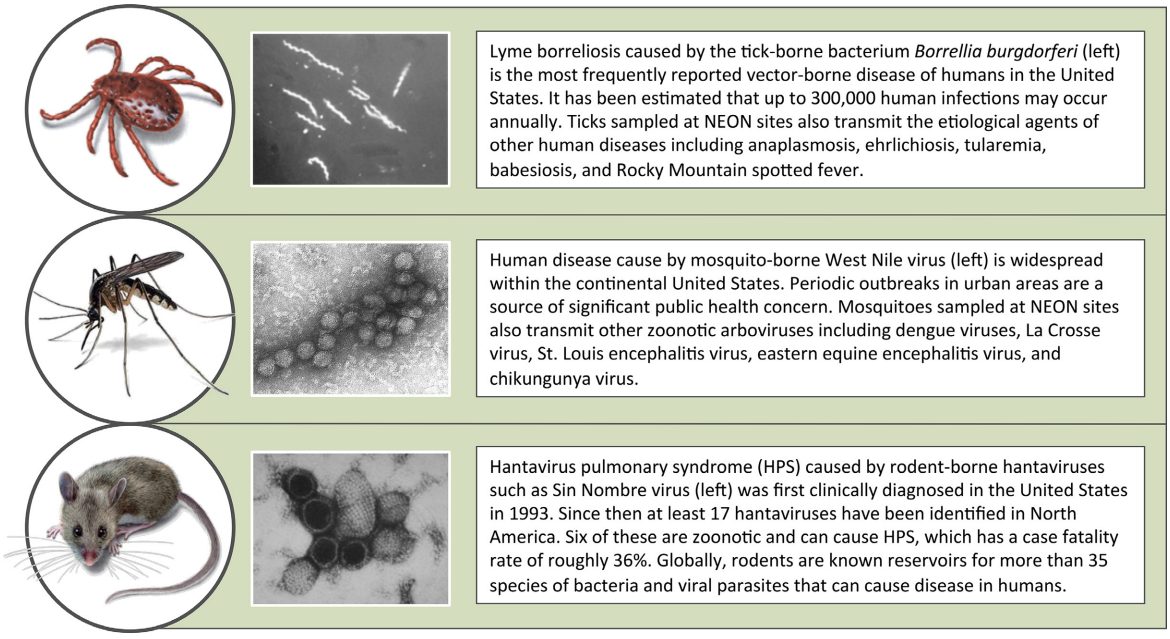


Fig. 2. Overview of targets of National Ecological Observatory Network vector, reservoir, and parasite sampling. Parasite images were obtained from the U.S. Centers for Disease Control and Prevention Public Health Image Library.

ervoirs, and parasites within NEON will not be focused in space and time on acute human health events (e.g., localized in populated areas during short-lived outbreaks of human disease). Instead, sampling will occur continuously over multiple decades and at numerous and ecologically varied sites. Associated data sets will provide unique baselines of and insights into host–parasite and disease dynamics. Additionally, NEON measures of vector/reservoir abundance and parasite prevalence will be made simultaneously with a suite of environmental and organismal sampling. This will include measurements of numerous attributes of the physical environment, remotely sensed data on vegetation and biogeochemistry, and phenological, demographic, and biodiversity sampling of sentinel taxa that include soil microbes, plants, insects, birds, and small mammals (Kao 2012). To promote the advancement of open-access science, NEON data and archived samples will be freely available for additional analyses by other members of the research community. Given its broad spatiotemporal scope, varied eco-environmental targets, emphasis on methodological standardization, and open-access policies, NEON will advance our understanding of and ability to predict changes in host–parasite interactions and associated disease dynamics in novel, interdisciplinary, and collaborative ways.

Here, we detail the sampling designs for NEON's tick-, mosquito-, and rodent-borne parasite sampling modules. The sampling designs formalize specific approaches to achieve the general research and monitoring objectives codified in NEON's high-level science requirements. These requirements were generated through discussions among experts in the ecological community at workshops convened by NSF during the conception phase of the Observatory (e.g., American Institute of Biological Sciences 2004) and subsequently formalized by NSF and NEON, Inc., the independent 501(c)(3) (not-for-profit) corporation that was managing the NEON project at the time these sampling designs were created (Schimel 2011). We begin with a brief discussion of the general considerations and priorities common across modules and then provide specific information on the formulation and details of the sampling design for each individual module. We based designs on established methods and approaches, particularly those that have been used by other surveillance programs, and sought to maximize the breadth (number of measurement and sample types) and temporal frequency of sampling within the bounds of currently anticipated logistical and financial constraints. For each sampling module we report results of power analyses to quantitatively evaluate the ability of the design to detect parasites and interannual changes in infection prevalence in vectors and reservoirs. 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 measurements and samples will be collected. This understanding should facilitate the use of NEON-generated resources and the replication or modification of NEON methods by others attempting to integrate their work with or extend that being conducted within NEON. All associated protocols for NEON field sampling, laboratory analyses, and sample archiving are or will be available online from NEON, Inc. (http://www.neoninc.org/) or the organization managing the NEON project (the identifiers NEON, NEON, Inc., and "the Observatory" are used interchangeably herein). Finally, note that these designs reflect the general priorities, background information, methodological options, and logistic and budgetary constraints associated with NEON sampling and known to the authors when this article was written. It is important to acknowledge that the designs will likely evolve over the lifespan of the Observatory as a result of changes in one or more of these or other factors that facilitate, limit, or otherwise guide-associated sampling plans.

General Design Considerations and Priorities

Targets

Sampling within the NEON will broadly target tick, mosquito, and small mammal (specifically, rodent) populations and associated parasites (Appendix S1). 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 have the potential to respond quickly and measurably to changes in climate and land-use practices. Second, these taxa play important roles in the amplification and transmission of a diverse suite of parasites, some

of which are of public health significance. In contrast to a focus on particular parasites, this approach aims to increase the breadth of sampling and enhance its long-term flexibility to accommodate previously uncharacterized and/or emerging parasites. 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 target parasites, and archiving of some or all remaining samples for additional use by other members of the research community.

Ticks and tick-borne parasites.—Ticks transmit a variety of parasites, 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 parasites 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 (U.S. Centers for Disease Control and Prevention 2008). First recorded in the United States in 1975 (Steere et al. 1977), the reported number of human cases of Lyme borreliosis within the United States more than doubled between 1995 and 2013 (http://www.cdc.gov/lyme/stats/index.html, accessed on September 15, 2015). According to a recent analysis, up to 300 000 human infections may occur annually (Kuehn 2013). Other zoonotic tick-borne parasites 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 2012*a*). Tick-borne parasites are associated with 8% of the infectious conditions in humans currently classified by the U.S. Centers for Disease Control and Prevention (CDC) as nationally notifiable (http://wwwn.cdc.gov/nndss/conditions/notifiable/2015/, accessed on September 15, 2015).

In addition to the public health significance of diseases associated with tick-borne parasites, the physiology and ecology of ticks make them an ideal target for sampling within NEON. 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), suggesting that the demography and biogeography of ticks of many species—and the parasites they transmit—will be affected by climate change (Estrada-Pena 2009, Gatewood et al. 2009, Diuk-Wasser 2012, Leger et al. 2013, Medlock 2013, Ogden et al. 2013). Further, the multihost life cycles of ticks of many 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 affect the abundance or diversity of available hosts (Childs and Paddock 2003, LoGiudice et al. 2003, Paddock and Yabsley 2007, Allan et al. 2010*a*, Keesing 2010).

Mosquitoes and mosquito-borne parasites.—Worldwide, mosquito-borne parasites are currently responsible for a human health burden unsurpassed among vector-borne diseases. In 2004 alone over 1.8 million human deaths were attributed to malaria (Murray et al. 2012), and 96 million people are estimated to experience disease associated with infection by dengue viruses each year (Bhatt 2013). Moreover, mosquito-borne parasites 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 parasites they transmit (Shope 1992, Reeves et al. 1994, Sutherst 2004, Harrigan et al. 2014). Although mosquitoes occur worldwide, 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 could promote range expansions of mosquitoes currently confined to tropical areas (Epstein et al. 1998, Patz et al. 2000*b*, Caminade et al. 2012, Eisen and Moore 2013, but see Reiter 2001). This geographic spread could be facilitated by the periodic long-distance transport of mosquitoes that can occur incidentally as part of human travel and international commerce (Lounibos 2002, Tatem et al. 2006). Establishment and spread of mosquito species into new localities creates the potential for associated parasites to be concurrently introduced (e.g., Gould and Higgs 2009, Weaver and Reisen 2010). Additionally, there is evidence that changing climatic conditions will significantly affect mosquito demography and processes associated with the transmission of mosquito-borne parasites (Mordecai 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 could potentially catalyze arboviral host range shifts (Brault and Reisen 2013). Because of their extensive geographic distribution, ecological and epidemiological significance, and sensitivity to processes associated with climate and land-use change, mosquitoes and the parasites they transmit are natural targets for sampling within NEON.

Rodents and rodent-borne parasites.—Small mammals, and rodents in particular, are common and influential members of most terrestrial ecological communities and play central roles in the amplification and transmission of numerous parasites. 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 lived in close symbioses with humans 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 because of their shared mammalian pedigree, a variety of parasites can be transmitted from rodents to humans (Meerburg et al. 2009). One of the most notable examples is *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*a*) has received considerable attention following outbreaks of HPS caused by Sin Nombre virus (Nichol et al. 1993, Centers for Disease Control and Prevention 2012*b*). 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*a*, MacNeil et al. 2011). In addition to these highly publicized examples, rodents are known reservoirs for the etiological agents of over 35 human diseases worldwide (http://www.cdc.gov/rodents/index.html, accessed on September 15, 2015) including Lyme borreliosis, typhus, babesiosis, and Rocky Mountain spotted fever (Meerburg et al. 2009).

Rodents and the parasites they carry may also be model systems for investigating the effects of climate and land use changes on the transmission of zoonotic parasites and epidemiology of associated diseases. The often large sizes and high densities of rodent populations may favor the maintenance and spread of parasites, and frequent contact between some rodent species and both domestic animals and human populations increases the likelihood of parasite 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).

Methods

Our sampling designs prioritize field methods that meet three criteria. First, the method(s) must provide an effective means of collecting individuals of targeted vector/reservoir taxa. Second, utilization of well-established, widely employed methods will promote use of NEON data by

other members of 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 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 mosquitoes of a variety of species that play important roles 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, methods must be widely applicable across the spectrum of site-specific logistic constraints, environmental conditions, and ecology of sampled populations at NEON sites.

The NEON will contract with experts at external facilities to perform the majority of sample processing, including most taxonomic identification of vector/reservoir samples and all parasite-related testing. To increase cost efficiency, our sampling designs prioritize testing methods that can simultaneously detect more than one parasite species over the use of multiple, parasite-specific tests (e.g., a single, general assay for mosquito-borne arboviruses or flaviviruses rather than a panel of individual tests specific to West Nile virus, St. Louis encephalitis virus, and dengue viruses). Broadly reactive screens will be followed up with more specific assays to identify the parasite(s) present in samples that test positive. NEON will apply quality assurance and control processes to both field and laboratory sampling methods whenever possible (plans still in development), and report associated error metrics with raw and processed data. Samples that test positive for parasites classified as select agents (see http://www.selectagents.gov/) will be handled in accordance with State and/or Federal regulations as appropriate.

Emphases

Our sampling designs prioritize a high frequency of intra-annual sampling to generate fine-scale time series data. When implemented over the relatively long lifespan of the Observatory (up to 30 yr at core sites), this approach will allow changes in sampled vector, reservoir, and parasite 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 parasite prevalence (Fig. 3a). Given a sufficiently high sampling frequency, sampling could secondarily elucidate changes in aspects of intra-annual phenology of sampled populations (e.g., timing of onset and duration of seasonal cycles, Fig. 3b). The secondary focus is particularly important because the seasonal phenology of many vector, reservoir, and parasite populations is expected to be sensitive to changes in climate and land-use practices

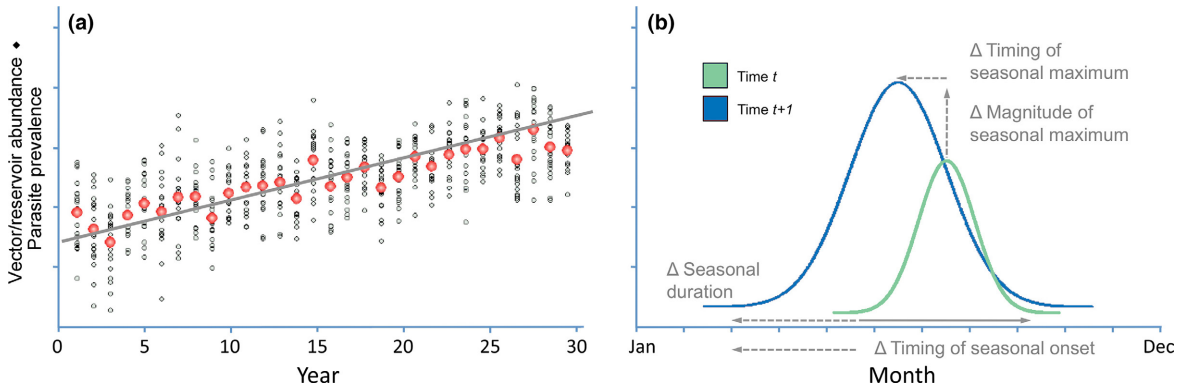


Fig. 3. Hypothetical data illustrating interannual (a) and intraannual (b) changes in vector/reservoir abundance and parasite prevalence. In general, sampling according to the designs proposed herein is intended to quantify changes in sampled vector, reservoir, and parasite populations at these two temporal scales.

(Altizer et al. 2013). Resulting phenological shifts could have important consequences for the maintenance, abundance, and spread of parasites (Altizer et al. 2006, Fisman 2007).

To maximize the frequency, consistency, and temporal extent of time series data, our sampling designs include two general prescriptions for instances in which financial or logistical constraints necessitate reductions in field sampling effort (i.e., frequency of sampling events). First, if reductions in field sampling effort are required, NEON will maintain the specified level of sampling effort at core sites while scaling-back effort at relocatable sites. This will ensure the temporal consistency and continuity of sampling at sites with longer lifespans. Second, when faced with general budgetary constraints, NEON will attempt to maintain levels of field sampling efforts specified in the designs and realize cost savings through reductions in the number of collected samples that are tested for parasites. 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 consistent levels of analytical sampling effort and associated uncertainty through time. Untested samples can be archived for processing at a later date, either by NEON or other members of the research community.

Our emphasis on fine-scale time series data is driven largely by fundamental constraints on the spatial resolution of NEON sampling. Because the selection of NEON sites was and will be made with little if any consideration of local vector/reservoir abundance or parasite prevalence, not all sites will be productive in terms of sampling yields. Within sites, the number of sampling plots will generally be too low to characterize or measure changes in local habitat associations of target taxa. In light of these limitations, our designs seek to characterize changes in vector/reservoir abundance and parasite prevalence at the level of the site rather than the individual sampling plot. While NEON will make plot-level field data available, our designs are predicated on the combination of data and samples across plots within sampling events to make site-level inferences.

Determining and evaluating sampling effort

Given NEON's open-access policy, our sampling designs attempt to maximize the number of hypotheses, comparisons, and analyses that can be evaluated using NEON data and samples. The diversity of possibilities complicates decisions related to the spatiotemporal distribution of sampling effort. In general, the proposed allocation of effort (e.g., frequency of sampling events, number of plots per site) reflects a balance between anticipated resource availability and logistical constraints and levels of sampling effort commonly reported or deemed reasonable for surveys of tick, mosquito, and rodent populations and associated parasites. 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, in each module-specific section we present results of power analyses evaluating the ability of sampling designs to detect interannual trends in the seasonal mean prevalence of a parasite at a NEON site. These results provide quantitative insight into just one of the many possible analytical applications of NEON data and are not intended as an evaluation of the project as a whole.

Because NEON will conduct tick- and rodent-borne parasite testing through analysis of individual samples (i.e., not pooled), we performed power analyses for trend detection using the negative binomial distribution. Due to the expectation of relatively low rates of infection, mosquito-borne parasite testing will involve pooled samples (20–50 individual mosquitoes physically homogenized and the resulting homogenate tested for parasites). Because of this approach, we performed power analyses for trend detection using the binomial distribution and the complementary log–log (CLL) link function, which accommodates for group testing within a GLM framework (Farrington 1992). We specified trend magnitudes in terms of an interannual increase in parasite prevalence (values varied by module) and set trend detection periods at either 10 or 25 yr (approximations of the lifespans of NEON relocatable and core sites, respectively). Year-zero prevalence was parameterized using values typically reported in the literature or commonly associated with the types of parasites under consideration. We evaluated two combinations of type I error rate (α) and power ($1 - \beta = 1 - p_{(\text{type II error})}$): 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. We performed analyses both with and without a temporal autocorrelation term. A correlation of approximately 0.37 ($\rho = 1/e$) at 6 months was chosen to represent a moderate magnitude of temporal correlation to provide contrast with respect to the case with temporal independence. We considered year-round sampling at frequencies proposed for core and relocatable sites in the design for each sampling module as well as for lower frequencies (e.g., one, two, three, and four times per year) in order to generate continuous power curves in accompanying figures. In these figures, we used green boxes to represent regions of sampling space where designs had sufficient power to detect trends of a specified magnitude. Unless otherwise specified in the figure caption, 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 provided in Appendix S2.

In addition to these power analyses, we used two approaches involving methods detailed in Gu and Novak (2004) to quantify the ability of the sampling designs to simply detect a parasite when it is present at a site. First, we characterized the relationship between the number of samples tested and the probability of detecting a parasite at various levels of parasite infection prevalence. Second, we quantified the relationship between infection prevalence and number of samples that would need to be tested to detect a parasite with varying levels of statistical confidence.

Plot selection

Our sampling designs are predicated on a fixed plot approach to field sampling. In many cases, individuals of target taxa are likely to exhibit relatively clustered patterns of distribution in space. This aggregation can arise from factors such as abiotic tolerances, habitat preferences, and/or host-mediated dispersal. The productivity of sampling would be increased by locating at least a portion of sampling plots in areas of aggregation (e.g., adaptive cluster sampling, Thompson 1990, Brown et al. 2013), but identifying these within-site locations in a systematic way that can be applied across the Observatory is complicated by at least three issues. First, site-specific data on these productive 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, our designs involve the establishment of sampling plots 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 vector, reservoir, and parasite 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 terrestrial sampling modules will facilitate statistical analyses and modeling involving these data.

For each sampling module, our designs specify a standard number of sampling plots per site. As described in the NEON spatial sampling design (Barnett et al., *unpublished manuscript*), NEON will use a stratified random approach to select plot locations within the dominant vegetation types (≥5% total cover) at each site. The number of plots per vegetation type will be roughly proportional to the percent cover of each type. NEON will use data collected at these plots during the first few years of sampling to statistically evaluate whether the specified number of plots is sufficient to characterize parameters of interest with desired confidence at each site. If analyses of these results indicate that more data

and/or samples are required to achieve this confidence, NEON will add additional sampling plots as needed.

Sample archiving

The NEON will archive a subset of field-collected and laboratory-generated samples and make them available for use by other members of the research community. These archival collections will include one or more of the following: untested samples (e.g., whole ticks), samples remaining after parasite testing has been conducted (e.g., residual volumes of rodent blood samples or frozen tissues), and products generated by this testing (e.g., DNA/RNA extractions or (RT-)PCR products). NEON is currently developing sample archive plans and will adopt associated methods that maximize the lifespan of these samples. Frozen samples will be aliquoted prior to storage to minimize the number of freeze/thaw cycles associated with their use. Additionally, voucher specimens will be archived to allow verification of taxonomic identifications.

Tick and Tick-Borne Parasite Sampling

Targets

Sampling for ticks and tick-borne parasites (hereafter, tick sampling) will broadly target hard ticks (family Ixodidae). Six species are of particular interest for tick-borne parasite testing: *Ixodes scapularis* (black-legged tick), *Ixodes pacificus* (western black-legged tick), *Amblyomma americanum* (lone star tick), *Amblyomma maculatum* (Gulf Coast tick), *Dermacentor andersoni* (Rocky Mountain wood tick), and *Dermacentor variabilis* (American dog tick). Collectively, ticks of these species transmit a large and taxonomically diverse suite of parasites, many of which are zoonotic and of public health concern (http://www.cdc.gov/ticks/diseases/, accessed on September 15, 2015, Spach et al. 1993). Ticks of other species collected through this sampling will be tested for parasites as resources permit and sample sizes warrant. Parasite testing will broadly target bacteria.

Sampling methods

The NEON will sample ticks 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). 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. We recommend that steps be taken to standardize clothing worn by sampling personnel. The distance covered during a drag is generally standardized or 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*a*). 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 NEON does not currently plan to use them, two additional methods of tick sampling will be considered to supplement drag sampling as resources permit. Collection of ticks using dry ice-baited CO_2 traps is well suited for sampling 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 collected additional ticks from the ground and vegetation in the vicinity of the trap. If used, CO_2 traps will consist of a vented 1.9-L insulated cooler containing approximately 1.5 kg of dry ice and be deployed in the center of each sampling plot for 24 h following the completion of drag and/or flag sampling.

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 parasites (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). This is not a methodological option for NEON as large mammals are not being sampled within the Observatory. In contrast, NEON will conduct regular sampling of small mammals (rodents), including species that are important hosts for ticks and play significant roles as reservoirs of many tick-borne parasites (Donahue et al. 1987, Ostfeld et al. 1996). Because of a need to limit small mammal handling time in the face of other prioritized data and sample collection requirements, NEON is not currently planning to remove ticks from captured small mammals. Instead, NEON will quantify the tick burdens of captured small mammals using visual counts of attached ticks. This latter approach has been shown to generate reliable estimates of absolute tick burdens for some cricetid and sciurid rodents (Brunner and Ostfeld 2008). If these handling constraints are relaxed, we recommend that NEON collect ticks from captured small mammals.

Field sampling plan

An event of tick sampling at a site will involve drag sampling around the perimeter of each of six 1600 m^2 square sampling plots per site. As described in the general introduction and the NEON spatial sampling design (Barnett et al. in prep), a stratified random approach will be used to select plot locations within the dominant vegetation types (≥5% total cover) at each site. The number of plots per type will be proportional to the percent cover of each type. During a sampling event, a 1-m^2 piece of white, cotton flannel cloth will be pulled along the ground at a pace not to exceed ~0.3 m/s (i.e., approximately 50 s to sample 15 m) and examined at 5–10 m intervals along the drag path. Ticks found attached to the drag cloth and clothing of sampling personnel will be removed using forceps (nymphs 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 vegetation prevents continuous drag sampling.

Sampling at each site will begin at a frequency of one sampling event every 6 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 3 weeks. This frequency will be maintained for the remaining lifespan of the site irrespective of subsequent sampling success. This mixed-frequency sampling approach is implemented to reduce sampling effort in areas of the NEON purview where ticks are not present. While we recommend that sampling be conducted year-round, logistic constraints will likely limit sampling to the March–December portion of each year. 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 5 d 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 this temperature threshold is not met, or when the ground is wet (e.g., after a rain event or when dew is heavy). Additionally, sampling will be avoided during the hottest and/or driest periods of the day (relative to typical temperatures at any given site).

Sample processing

In the field, collected nymphal and adult ticks will be transferred into labeled vials containing 95% ethanol. Larvae will be collected using reusable lint rollers and washed into filter paper for subsequent transfer into these vials (Savage et al. 2013). Samples will be transported on ice in portable coolers to a NEON domain lab (one per domain, generally in the vicinity of the core site and <3 h by car from each relocatable site). For each sampling event/plot combination, NEON will enumerate collected ticks by life stage and then transfer them into cold storage at <4°C (−20°C recommended).

The NEON will send collected ticks to one or more external facilities for taxonomic identification and parasite testing. Nymphal and adult ticks will be identified to species based on visual examination of external morphology (e.g., Cooley and Kohls 1944, 1945, Keirans and Litwak 1989) and enumerated by species and life stage for each sampling event/plot combination. 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 taxonomically identified but will be counted and archived.

Following identification, ticks will be combined by species, life stage, site (pooling across plots), and sampling event for parasite testing. NEON will test a subset of species-identified nymphal and adult ticks collected during each sampling event for infection by parasites. Actual sample sizes for parasite testing will depend on collection success, analytical costs, and desired levels of statistical power associated with prevalence estimates. For any species/life stage combination tested, a minimum of 10 and a target of 100 individuals per site/sampling event combination are recommended. 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 parasites 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. The development of associated technologies may eventually make it possible to conduct multiplex PCR using additional primers to test for an even broader suite of parasites. 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, are recommended and will be considered by NEON as technological advances and resources permit. To quantify the likelihood that a given parasite is present within a tested tick, sequences generated during parasite testing will be screened against known parasite 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 2013).

Design evaluation

For power analyses to evaluate the ability of sampling and testing to detect interannual trends in the prevalence of tick-borne parasites at a site, we set year-zero prevalence values to 5% (rare parasite) or 15% (common parasite) based on results of studies of B. burgdorferi infection in I. scapularis populations (e.g., Piesman et al. 1986, Ginsberg and Ewing 1989b, Ogden et al. 2007, Diuk-Wasser 2012). Given plans to test between 10 and 100 ticks of a given species/life stage combination per sampling event, we used a conservative sample size threshold of 40 ticks tested. Hence, a sampling frequency was deemed adequate if 40 or fewer tested ticks per event were sufficient to detect an interannual increase in parasite prevalence of a given magnitude at a site.

When power analyses included a temporal autocorrelation term, the design (sampling events every 3 or 6 weeks) could detect an interannual increase in prevalence of 0.75% for a common parasite at a core site (i.e., over 25 yr) with higher confidence ($\alpha = 0.05$, power = 0.9) (Fig. 4a). This was the only scenario in which an interannual increase in <1.0% could be detected by the design given temporal autocorrelation in parasite prevalence. In the absence of temporal autocorrelation, the design could detect an interannual increase in 0.25% for a common parasite at a core site with higher confidence. For a rare parasite at a core site the design could detect an interannual increase in 0.50% with higher confidence (Fig. 4b). An increase in 0.25% could be detected with lower confidence ($\alpha = 0.1$, power = 0.8), but only at a sampling frequency of one event every 3 weeks. For a common parasite at a relocatable site (i.e., over 10 yr), the design could detect interannual increases of 0.75% (Fig. 4c) and 1.0% with lower and higher confidence, respectively. For a rare parasite at a relocatable site, an interannual increase in 1.0% could only be detected with lower confidence at a sampling frequency of one event every 3 weeks (Fig. 4d).

The design essentially had a 100% probability of detecting a common parasite and an 87% probability of detecting a rare parasite at a site when 40 or more ticks per species/life stage were tested per sampling event (Fig. 5a). For very rare parasites (1% prevalence) the probability of detection was 33%. When analytical sampling effort was reduced by half (to 20 ticks tested) these probabilities fell to 96%, 64%, and 18%, respectively. To achieve a 50% probability of detecting a parasite, four, 14, and 68 ticks had to be tested under scenarios when the parasite was common, rare, or very rare, respectively (Fig. 5b). These sample sizes had to be increased to 10, 31, and 160 ticks to achieve an 80% detection probability.

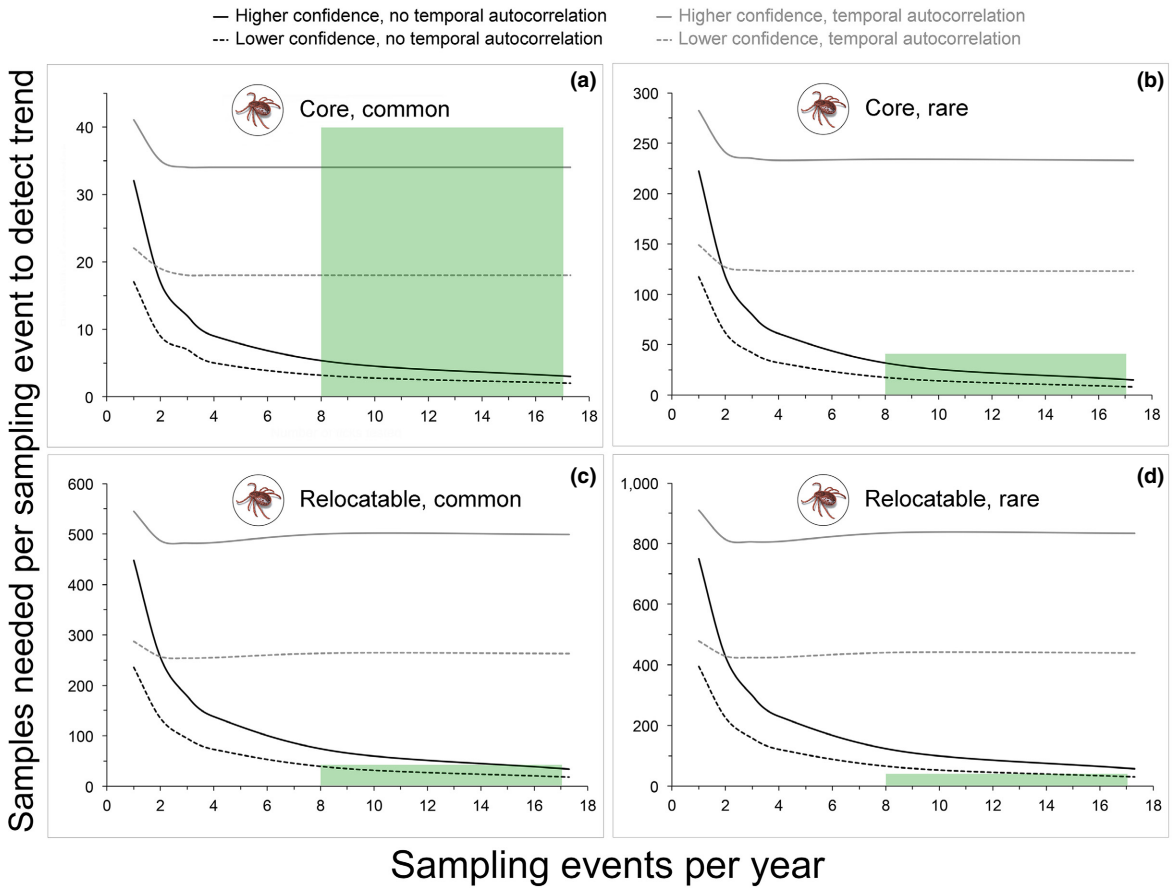


Fig. 4. Power analyses to evaluate the ability of sampling to detect interannual trends in tick-borne parasite prevalence. Each panel present results of power analyses involving a different combination of National Ecological Observatory Network site type (core site = modeled at 25 yr duration, relocatable site = modeled at 10 yr duration) and year-zero parasite infection prevalence (rare = 5%, common = 15%). Results for both low ($\alpha = 0.1$, power = 0.8) and high ($\alpha = 0.05$, power = 0.9) confidence scenarios, each with and without a temporal autocorrelation term (~0.37 at 6 months), are provided in each panel. Green boxes indicate levels of sampling effort (sampling event frequency and number of samples per event to be tested for parasites) specified in the associated design.

Data reporting

The following data generated by (or relevant to) tick sampling or associated parasite testing will be made available through an online data portal maintained by NEON, Inc. (http://www.neoninc.org/data-resources/get-data) or the organization managing the NEON project. Data will become available as they are collected, processed (including quality control), and posted to the data portal.

At the spatiotemporal scales of the sampling plot/sampling event and the site/sampling event

- Tick abundance: the numbers of nymphal and adult ticks (both by species, also by sex for adults) and larval ticks (cumulative across species) collected.
- Sampling effort: the distances covered in meters during drag and/or flag sampling.
- Tick density: the densities of ticks (by species and/or life stage) estimated using the quotient of number of ticks collected and distance covered during sampling.

For each tick tested for evidence of infection by parasites

- Tick information: species, life stage (nymph or adult), sex (for adults), and date and location of collection.

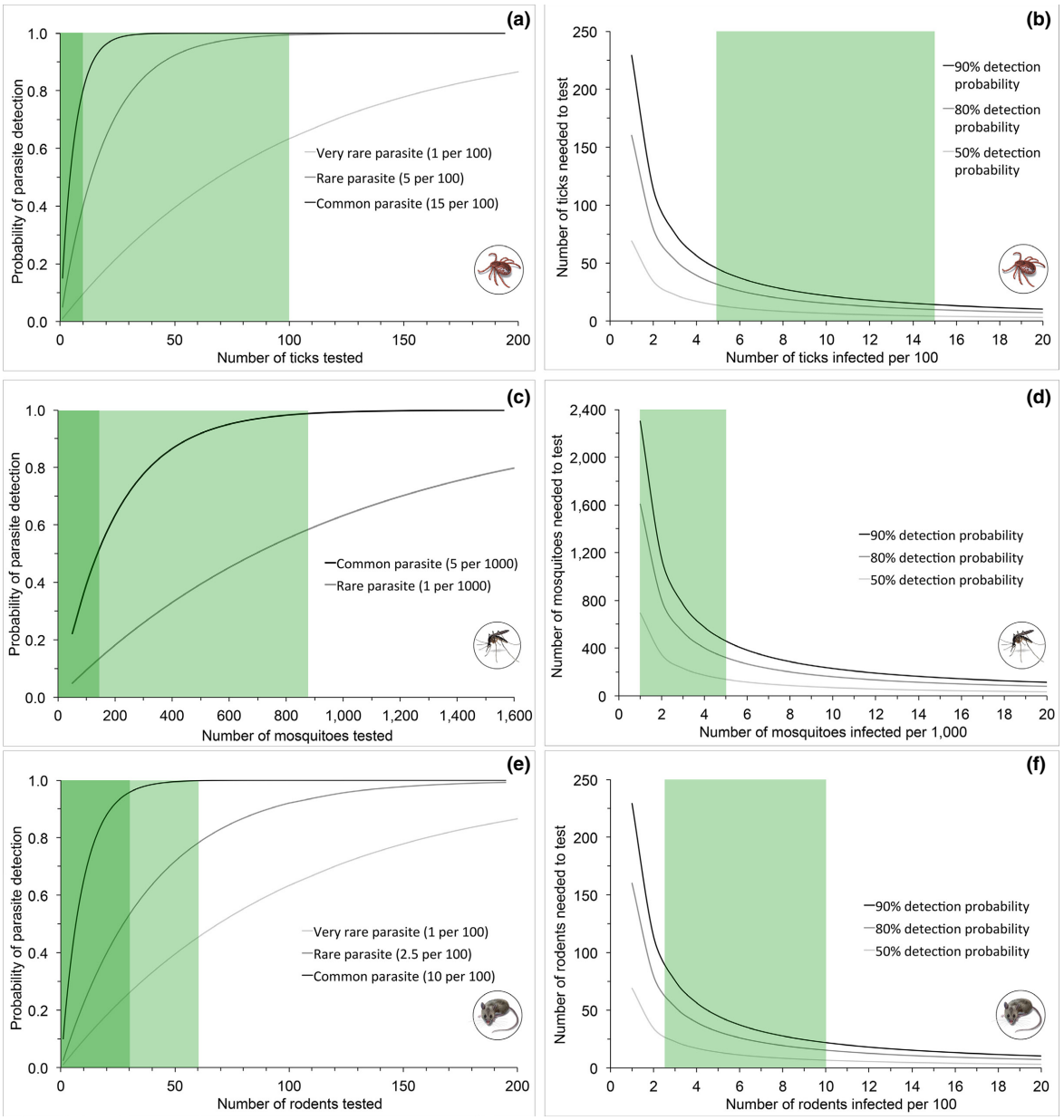


Fig. 5. Power analyses to evaluate the ability of sampling to detect parasites when they are present. Panels on the left (a, c, e) depict relationships between the number of samples tested for parasites and the probability of parasite detection. Green boxes represent upper (light green) and lower (dark green) estimates of number of organisms likely to be tested per site/sampling event combination, as reported in the text (e.g., between 10 and 100 ticks per sampling site/event combination). Panels on the right (b, d, f) depict relationships between the minimum number of samples required for specified detection probabilities at various levels of parasite prevalence. Green boxes span the range of estimated prevalence for rare and common parasites at National Ecological Observatory Network sites, as indicated in legends of corresponding panels on the left. Target taxa are indicated symbolically.

- Sequence data: all raw genomic sequences generated using next-generation sequencing with barcoded 16S rRNA primers.
- Infection status: the presence/absence of each parasite in the NEON reference library based on alignment of testing-generated and library reference sequences above one or more match percentage thresholds (e.g., Altschul et al. 1990). Parasites in the reference library will initially include one or more species in the genera *Anaplasma*, *Borrelia*, *Ehrlichia*, *Francisella*, and *Rickettsia.*

For each combination of tick species/life stage tested for evidence of infection by parasites, at the spatiotemporal scale of the site/sampling event

- Testing effort: the numbers of ticks tested for parasites.
- Testing results: the numbers of ticks that tested positive for each parasite in the NEON reference library.
- Prevalence estimates: the prevalence of infection by each parasite in the NEON reference library estimated using the quotient of number of ticks that tested positive and the total number of ticks tested. Prevalence estimates will be reported with Wilson 90% confidence intervals as error estimates (Zelmer 2013).

The type(s), number(s), and availability of archived samples

These will include: whole untested ticks and genomic extractions, and PCR and sequencing products from tested ticks.

Mosquito-Borne Parasite Sampling

Targets

Mosquito sampling will broadly target all members of the family Culicidae. For mosquito-borne parasite testing, NEON will screen samples 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 parasites (Moore et al. 1993, Turell et al. 2005). NEON will test female mosquitoes (pooled by species within sampling events) for the presence of alphaviruses, bunyaviruses, and flaviviruses using molecular methods (e.g., RT-PCR, melt curve analysis) and/or Vero cell culture methods. Virus-positive pools will be subjected to additional parasite-specific tests as needed to identify the parasite(s) present within them.

The mosquito-borne parasite sampling design was formulated in conjunction with the design for NEON mosquito abundance and diversity sampling (Hoekman et al., *in press*). Because these two sampling efforts differ fundamentally in their objectives, they would be optimized using different strategies if designed independently. Parasite-related sampling seeks to collect and test large numbers of mosquitoes of particular species to quantify rare phenomena (infection by parasites). In contrast, abundance and diversity sampling aims to characterize the community of mosquitoes at a site using spatiotemporally and taxonomically broad sampling. Although a combined sampling plan driven by considerations of parasite-related sampling would not be suitable for robust sampling of mosquito abundance and diversity, a plan based upon the priorities of abundance and diversity sampling would also collect some of the mosquitoes needed for parasite testing. The 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 (e.g., collection of additional samples) to meet requirements of mosquito-borne parasite sampling.

Sampling methods

The NEON will sample mosquitoes using CDC miniature CO_2 light traps (Sudia and Chamberlain 1962), which are one of the most commonly used types of mosquito trap and arguably collect the greatest diversity of mosquitoes across a broad range of environmental conditions (Service 1993). Traps of this type are regularly deployed as part of arboviral surveillance by public health and vector control organizations. As such, use of this trap type will facilitate the integration of NEON mosquito data with similar data collected by other groups. CDC miniature CO_2 light traps primarily attract mosquitoes through the release of carbon dioxide (e.g., from sublimating dry ice or compressed gas cylinders), a component of vertebrate exhalation that female mosquitoes

use to locate hosts. In addition, traps may be configured with a light bulb to attract individuals of phototactic species. Mosquitoes attracted to a trap are drawn into a catch cup by the trap's fan.

The CDC miniature CO_2 light trap has known sampling biases and limitations, at least two of which are relevant for mosquito-borne parasite sampling. First, these traps are relatively ineffective at sampling the gravid or previously blood-fed mosquitoes often targeted for parasite testing. Because gravid female mosquitoes are previously blood fed, their inclusion in testing pools enhances the likelihood of detecting parasites 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 catch cup by the trap's fan. While the use of gravid traps in conjunction with CO_2 light traps would increase in the number of collected mosquitoes (especially for gravid females) and the likelihood of detecting parasites, NEON is not currently planning to sample using this method due to the logistical challenges associated with standardizing the composition of and transporting the fetid water used as bait. Additionally, the efficacy of these traps is often relatively low in rural or wildland settings where many NEON sites are and will be located. Another alternative and more easily standardized method involves resting box traps (Williams and Gingrich 2007). These artificial 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). These traps may represent a relatively inexpensive and easily standardizable method of collecting gravid female mosquitoes of some species (Komar et al. 1995). If mosquito sampling success at a site is insufficient to attain desired statistical confidence in parasite prevalence estimates, NEON will deploy CDC miniature CO_2 light traps at additional plots at all or a subset of sites. If sampling success is still too low following this change, we recommend that NEON further augment sampling via inclusion of gravid and/or resting traps (provided that 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 individuals of some species in the genus *Aedes* that are important vectors of multiple arboviruses (Hoel et al. 2009). The BG sentinel trap represents an attractive option for addressing this taxonomic sampling deficiency (Krockel et al. 2006, Pialoux et al. 2007, Meeraus et al. 2008). When *Aedes aegypti* and *Aedes albopictus* mosquitoes, the principal vectors of dengue viruses, are known or suspected to be present at a site, NEON will consider deploying BG sentinel traps alongside CDC miniature CO_2 light traps to collect these species for use in associated parasite testing. While the BG sentinel trap may perform as well or better than other trap types (including the CDC miniature CO_2 light trap) in some circumstances (e.g., Lühken et al. 2014), the proprietary nature of the trap's chemical lure is problematic for maintaining methodological standardization within the Observatory since the manufacturer could change lure chemistry in unspecified ways at will. Because the CDC miniature CO_2 light trap offers greater control over and ability to maintain consistent sampling effort, its use as the primary mosquito sampling method within the Observatory should enhance comparability of NEON data through time.

Field sampling plan

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. As described in the general introduction and the NEON spatial sampling design (Barnett et al, *unpublished manuscript*), a stratified random approach will be used to select plot locations within the dominant vegetation types (≥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 30 m of roads because of a need to reduce sampling-associated travel times to and from mosquito sampling plots. During a sampling event, each trap will be deployed continuously for roughly

40 h beginning at dusk on the first day. NEON sampling personnel will check traps (retrieve collected mosquitoes, replenish dry ice, and replace trap batteries as needed) at dawn following both nights of deployment and at dusk on the second day. Consequently, each sampling event will be split into three trapping periods: two trap nights and the intervening day. Traps will be hung at a height of roughly 2 m and baited with approximately 1.4 kg 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 design for NEON mosquito abundance and diversity sampling (Hoekman et al., *in press*).

The NEON will conduct sampling at a frequency of one sampling event every 2 weeks at core sites and every 4 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 5 d prior to the first day of the event was ≥4 °C. This temperature represents 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).

The NEON will discontinue mosquito sampling at some higher latitude sites during 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 field season plan (10 sampling plots with events every 2 or 4 weeks) following the collection of at least one mosquito during an off-season sampling event. Additional details about off-season sampling can be found in the design for NEON mosquito abundance and diversity sampling (Hoekman et al., *in press*).

Sample processing

NEON sampling personnel will transport catch cups containing mosquitoes to a NEON domain lab (one per domain, generally in the vicinity of the core site and <3 h by car from each relocatable site) 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 parasite testing.

Taxonomic identification will be based on visual examination of external morphology (e.g., Darsie and Ward 1981) with additional confirmation using DNA barcoding (Gibson et al. 2012) for a subset of samples from each site/year combination. 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. During these initial years of sampling, up to 200 mosquitoes per trapping period will be identified and enumerated by species and sex. When more than 200 mosquitoes are collected in a trapping period, a representative subsample of ~200 individuals will be identified and the proportion of the sample that was not examined will be estimated and reported. 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 parasite testing. Following identification, mosquitoes will be combined by species, sex, site (across plots), and sampling event for parasite testing. Any remaining unidentified mosquitoes will be combined into a bulk lot at the site/event level. Additional details on taxonomic identification, DNA barcoding, preservation of pinned NEON mosquito samples, and handling and archive of bycatch are provided in the design for NEON mosquito abundance and diversity sampling (Hoekman et al., *in press*).

A subset of the species-identified female mosquitoes collected during each event of sampling and sorted by species will be tested for parasites. 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 parasite 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 1000 individuals, Monath 1980, Andreadis et al. 2004, Gu and Novak 2004, Gu et al. 2008, Kwan et al. 2010), it is desirable to maximize the number of mosquitoes tested for parasites. In instances where only a portion of mosquitoes collected during a sampling event are identified, NEON will pursue the identification and removal of additional individuals of target vector species from the unidentified bulk lot to increase analytical sample sizes for parasite testing whenever possible. Target species will include known vectors of arboviral parasites within the NEON purview (Moore et al. 1993, Turell et al. 2005):

- Species in the genus *Aedes* including *Ae. aegypti*, *Ae. albopictus*, and *Ae. triseriatus*. Mosquitoes of these species collectively transmit dengue viruses, La Crosse virus, and chikungunya virus.
- Species in the genus *Culex* including *Cx. nigripalpus*, *Cx. pipiens*, *Cx. p. quinquefasciatus*, *Cx. restuans, Cx. salinarius*, and *Cx. tarsalis*. Mosquitoes of these species are important vectors of West Nile virus and St. Louis encephalitis virus.
- Mosquitoes of other taxa including *Culiseta melanura* and *Coquillettidia perturbans* (vectors of eastern equine encephalitis virus).

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 RT-PCR using broadly reactive, family-specific primers, melt curve assays for viral RNA, and Vero cell screening (Earley et al. 1967, Kuno et al. 1996, Kuno 1998, Lanciotti 2000, Sanchez-Seco et al. 2001, Nasci et al. 2002, Naze et al. 2009). Any pool testing positive will be subject to additional analyses (e.g., sequencing) to identify the specific parasite(s) present within it.

Design evaluation

Evaluating the ability of the design to detect interannual trends in the prevalence of mosquito-borne parasites was complicated by difficulty in estimating likely average sample sizes. Because mosquito abundance tends to exhibit tremendous spatiotemporal variability, any single value representing the number of mosquitoes collected during a "typical" sampling event will inherently be imprecise. Based on cost and budget estimates at the time of design formulation, our analyses assumed 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 10 sampling plots at a site during an event, this yields a maximum of 6000 taxonomically identified mosquitoes per site/event combination, the majority of which will be female. Because catches during the diurnal portion of sampling events will generally be small, and nocturnal catches will not consistently exceed 200 mosquitoes per trap/trapping event at most sites, we assumed this maximum can be reduced to a more conservative value of 2000 mosquitoes per site/event combination. 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 mosquitoes are rare. The number of resulting analytical pools of 50 mosquitoes per species that can be generated from these 200–2000 collected mosquitoes will additionally 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 parasite) and 0.5% (common parasite) 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).

Results of power analyses suggested that sampling at the simulated levels of effort generally provides insufficient power to detect interannual increases in parasite prevalence of biologically realistic magnitudes. This is consistent with the assertion that sample sizes of more than 1000 mosquitoes are needed to estimate arboviral infection prevalence with confidence, particularly when prevalence is low (Bernard 2001, Nasci et al. 2002). In the absence of temporal autocorrelation in prevalence, the design (sampling events every 2 weeks at core sites and every 4 weeks at relocatable sites) could detect a 2.0% interannual in-

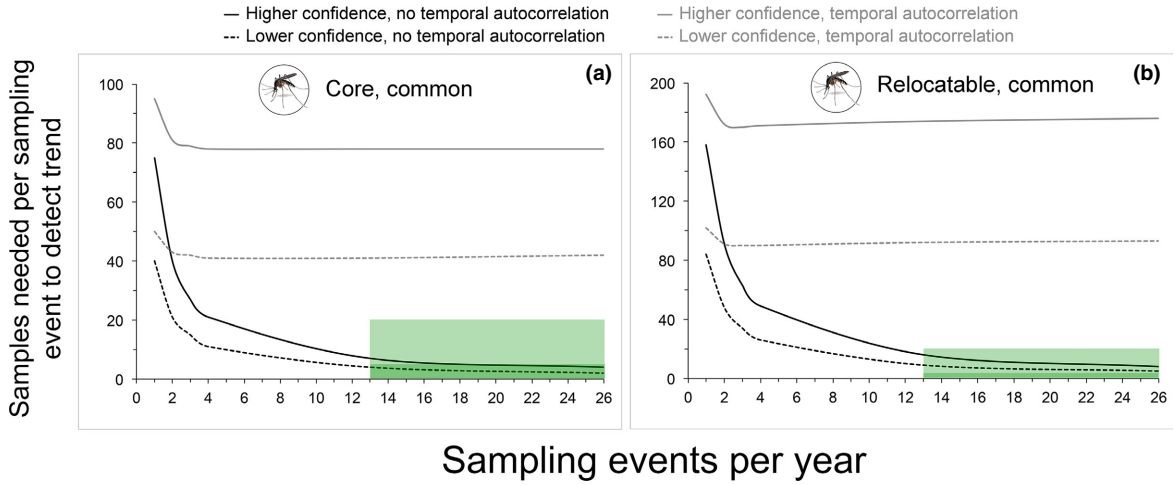


Fig. 6. Power analyses to evaluate the ability of sampling to detect interannual trends in mosquito-borne parasite prevalence. Panels present results of power analyses involving a common mosquito-borne parasite (year-zero parasite infection prevalence = 0.5%) at both types of National Ecological Observatory Network sites (core site = modeled at 25-yr duration, relocatable site = modeled at 10-yr duration). Results for both low ($\alpha = 0.1$, power = 0.8) and high ($\alpha = 0.05$, power = 0.9) confidence scenarios, each with and without a temporal autocorrelation term (~0.37 at 6 months), are provided in both panels. Green boxes indicate levels of sampling effort (sampling event frequency and number of samples per event to be tested for parasites) specified in the associated design: light green boxes correspond to 20 analytical pools (50 female mosquitoes each) per species/sampling event combination, dark green boxes correspond to four analytical pools (50 female mosquitoes each) per species/sampling event combination.

crease in prevalence for a common parasite at a core site (i.e., over 25 yr) with higher confidence ($\alpha = 0.05$, power = 0.9) (Fig. 6a) and a 1.5% increase with lower confidence ($\alpha = 0.1$, power = 0.8). At a sampling frequency of one event every 2 weeks, minimums of 8 and 30 pools of 50 mosquitoes per species per sampling event would need to be tested to detect interannual increases of 1.0% and 0.5%, respectively, with lower confidence. For a rare parasite at a core site, this sampling frequency could detect interannual increases of 2.0% and 1.5% with higher and lower confidence, respectively. At relocatable sites (i.e., over 10 yr) the design could not reliably detect an interannual increase of <5.0% irrespective of whether the parasite was common or rare (Fig. 6b). As an example, for established parasites at relocatable sites sampled every 4 weeks, 56 and 229 pools per event would need to be tested to detect annual prevalence increases of 2.0% and 1.0%, respectively.

While the power of the design to detect interannual trends in mosquito-borne parasite prevalence was limited, sampling had reasonable power to detect the presence of parasites. The design had a >90% probability of detecting a common parasite when 450 or more mosquitoes per species (nine or more pools of 50) were tested (Fig. 5c). This probability fell to between 22% and 63% when analytical sample sizes fell to 1–4 pools of 50, respectively. For rare parasites, the detection probability only exceeded 50% when roughly 685 mosquitoes were tested, and probabilities were between 5% and 18% when sample sizes fell to 1–4 pools of 50, respectively. For reference, a minimum of 1609 and 2301 mosquitoes would need to be tested to detect a rare parasite with 80% and 90% confidence, respectively (Fig. 5d). For a common parasite these values were 321 and 459. A fifty percent detection probability required the testing of 693 mosquitoes when the parasite was rare and 138 mosquitoes when the parasite was common. Detection would be more likely during epizootics when the parasite prevalence is higher (e.g., for arboviruses, 15 or more infected mosquitoes per thousand, Barker et al. 2009). In these scenarios, 50%, 80%, and 90% detection probabilities required testing a minimum of 46, 106, and 152 mosquitoes, respectively.

Data reporting

The following data generated by (or relevant to) mosquito sampling or associated parasite testing will be made available through an online data portal maintained by NEON, Inc. (http://www.neoninc.org/data-resources/get-data) or the organization managing the NEON project. Data will become available as they are collected, processed (including quality control), and posted to the data portal.

At the spatiotemporal scales of the sampling plot/sampling event period and/or the site/sampling event

- Mosquito abundance: the numbers of mosquitoes collected, by species/sex combination.
- Sampling effort: the duration of trap deployment in hours/minutes.
- Mosquito diversity: various diversity measures described in the design for NEON mosquito abundance and diversity sampling (Hoekman et al., *in press*).

For each pool of female mosquitoes tested for evidence of infection by parasites

- Pool information: species, date(s) and location(s) of collection, and number of mosquitoes included (20–50).
- Infection status: the presence/absence of parasites. This will include the results of (1) general tests for the presence of alphaviruses, bunyaviruses, and flaviviruses, and (2) specific tests to identify the parasite(s) present within pools that tested positive in general tests. Target parasites, each of which is vectored by mosquitoes of particular species, will include West Nile virus, dengue viruses, La Crosse virus, St. Louis encephalitis virus, eastern equine encephalitis virus, and any other arbovirus(es) detected through sampling by NEON or other members of the research community at Observatory sites.

For each species of mosquito tested for evidence of infection by parasites, at the spatiotemporal scale of the site/sampling event

- Testing effort: the numbers and sizes of mosquito pools tested for parasites.
- Testing results: the numbers and sizes of mosquito pools that tested positive for (1) alphaviruses, bunyaviruses, and/or flaviviruses, and (2) any parasite(s) for which specific tests were conducted.
- Prevalence estimates: the prevalence of infection of the aforementioned specific parasites (in mosquitoes of associated vector species) estimated using both minimum infection rate (MIR) and maximum likelihood estimator (MLE) statistics (Chiang and Reeves 1962, Biggerstaff 2005) and reported with appropriate confidence intervals (Biggerstaff 2008).

The type(s), number(s), and availability of archived samples.—These will include: whole untested mosquitoes and genomic extractions and (RT-)PCR products from parasite-positive pools.

Rodent-Borne Parasite Sampling

Targets

Sampling for rodent-borne parasites 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 *Peromyscus maniculatus* (deer mouse) and *Peromyscus leucopus* (white-footed mouse) are of particular interest since they have broad geographic distributions, are 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. Samples will additionally be tested for arenaviruses if resources permit. NEON will collect blood samples from individuals of other rodent taxa when sampling does not significantly increase their morbidity or mortality. Additionally, NEON sampling personnel will inspect captured small mammals and count attached larval and nymphal ticks. As explained in the tick and tick-borne parasite sampling design section, NEON is not currently planning to remove ticks from captured small mammals, and will instead

estimate tick burdens through visual examination of each captured rodent. Data on tick burdens will be used to estimate the additional handling time that would be required for tick removal and thereby gauge the feasibility of incorporating this recommended procedure into the NEON small mammal sampling protocol.

As with mosquito sampling, rodent-borne parasite sampling occurs coincidently with NEON small mammal abundance and diversity sampling. 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). Additional details about NEON small mammal abundance and diversity sampling (hereafter, small mammal sampling) are provided in the NEON design for small mammal abundance and diversity sampling (Thibault et al., *unpublished manuscript*).

Sampling methods

Blood samples for rodent-borne parasite testing can be collected using a variety of methods. In selecting 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 rodents 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 on rodents 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 will 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 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 rodents 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 conduct blood collection using either the retro-orbital 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 retro-orbital 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, Herbreteau et al. 2011, Sikes et al. 2011). A microhematocrit tube is inserted behind one of the rodent's eyes and used to puncture the retro-orbital sinus, a highly vascularized region at the back of the orbit. 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 can cause injury to animals and sampling personnel during handling. Field studies have shown that when used properly, the retro-orbital 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 retro-orbital 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 use of the retro-orbital method, NEON will also consider the submandibular method to collect rodent blood samples. The submandibular method involves use of a disposable metal lancet to puncture the submandibular vein that runs below and behind a rodent's mandible. The method yields blood samples of variable but generally comparable volume to those generated by the retro-

orbital method, but can be applied with little or no need for anesthesia. It may also require less hands-on training to reach proficiency than the retro-orbital method. The submandibular method has proven highly effective with laboratory mice (Golde et al. 2005) but has been used relatively rarely in field studies to date. NEON is currently conducting prototyping trials to evaluate the efficacy and safety of this method when used with wild rodents under field conditions.

Field sampling plan

An event of small mammal sampling will involve mark/recapture live trapping using Sherman traps at each of three to eight sampling plots per site. Each plot will consist of a square, 100-trap grid, with traps spaced every 10 m. A subset of these plots (generally three but occasionally more if capture rates are low) will be designated as bleeding plots. In general, bleed plots at each site will be selected such that they collectively span/represent the range of small mammal abundance and diversity observed at the site during the first 2–3 yr of sampling. Blood samples for parasite testing will only be collected from rodents captured in these plots. As described in the general introduction and the NEON spatial sampling design (Barnett et al., *unpublished manuscript*), a stratified random approach will be used to select plot locations within the dominant vegetation types (≥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 300 m of roads because of a need to reduce travel time to/from plots and facilitate deployment and retrieval of sampling equipment. Vehicular access during winter months may also be considered as part of plot selection. During a sampling event, trapping will be conducted for three consecutive nights around the new moon. Captured animals will be processed and released on the morning following each night of trapping. The implementation of small mammal sampling, as well as the duration of and manner in which captured animals are processed, will be dictated in part by ambient temperature thresholds. Additional details about small mammal sampling are provided in the NEON design for small mammal abundance and diversity sampling (Thibault et al. in prep).

Processing of a captured animal will begin with blood collection. 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 the retro-orbital method, this includes individuals in the family Cricetidae (e.g., *Peromyscus* spp.) and other taxa (e.g., family Muridae, genus *Mus*) for which the method is anatomically suitable and its application (including use of anesthesia) is not unduly stressful. Suitable target species for the submandibular method will be identified as part of the aforementioned prototyping trials. Use of this method should allow blood samples to be collected from individual small mammals associated with taxa not well disposed to the retro-orbital method (e.g., families Heteromyidae and Dipodidae). 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 10 g. 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, in the event of recapture, the animal can only be bled once per sampling event (here, equivalent to a maximum frequency of once per month). Upon capture, an animal meeting these criteria will be anesthetized as needed and bled. When the submandibular method is used, the pelt around the puncture site will be surface sterilized with an alcohol swab prior to blood collection. NEON will collect a volume of blood not to exceed 1% of the animal's body weight (Sikes et al. 2011).

Following any blood collection, captured animals will be processed according to the NEON design for small mammal abundance and diversity sampling (Thibault et al., *unpublished manuscript*). Numbered metal ear tags and/or subcutaneously implanted radio frequency identification (RFID) tags will be used to uniquely mark individual animals. Sampling personnel will collect data on taxonomic ID, sex, size, and reproductive condition, and counts of attached ticks. In addition to blood, NEON will collect hair, whiskers, feces, and ear punch samples from all or a subset of captured animals. Although feces and ear punch samples can both be used for

parasite testing (e.g., Sinsky and Piesman 1989, Phan et al. 2011), NEON will not conduct any parasite testing using these samples and will instead archive them for use by other members of the research community.

To minimize the probability of sampling-associated parasite transmission among captured animals, all nonconsumable sampling equipment (e.g., traps, scales, RFID tag needles) that comes into contact with a captured animal or associated bodily fluids, or that is handled during processing of an animal, will be disinfected using quaternary ammonia before being reused. Contaminated consumables (e.g., microhematocrit tubes, gauze) will be disposed of in biohazard containers. Sampling personnel will wear appropriate personal protective equipment (e.g., half-face respirators with P100 filters, eye protection, double latex gloves) as specified by NEON Environmental Health and Safety. Latex gloves will be disinfected between each processed animal.

Based on previous long-term studies of hantavirus epidemiology in wild rodent populations (e.g., Calisher et al. 2007, Douglass et al. 2007, Carver et al. 2011), NEON will conduct small mammal trapping and associated rodent-borne parasite sampling at frequency of one event every lunar cycle (roughly once every 4 weeks). If this sampling frequency cannot be achieved, we recommend that NEON not reduce the sampling frequency below one event every other lunar cycle. Lower sampling frequencies have been shown to significantly increase error in estimates of both rodent abundance and prevalence of serum antibodies reactive against parasites (Carver et al. 2010). Due to resource constraints, NEON is currently planning to conduct rodent-borne parasite once per lunar cycle at core sites and every second lunar cycle at relocatable sites. Sampling will be conducted year-round during any months when weather conditions are safe for sampling personnel and captured animals and when a sufficient number of staff are available.

During the first year of sampling at a site, NEON will bleed rodents captured in the plots with the highest abundance of the rodent taxa targeted for parasite sampling. Following the first 1 or 2 yr of sampling, plot-level data on the abundance and diversity of small mammals will be used to select the three or more long-term bleeding plots. Ideally, these will collectively span the range of small mammal communities (i.e., levels of diversity and abundance) documented at the site during the first 1–2 yr of sampling and be distributed among the different vegetation types present at the site.

Sample processing

In the field, sampling personnel will collect blood samples into labeled cryovials and immediately freeze them on dry ice in portable coolers. Coolers will be transported to a NEON domain lab (one per domain, generally in the vicinity of the core site and <3 h by car from each relocatable site), where cryovials will be stored at −80°C until they are sent to one or more external facilities for parasite testing. All collected blood samples will be tested individually for serum antibodies reactive against hantaviruses using ELISAs. If resources are available we recommend that samples also be tested for serum antibodies reactive against arenaviruses using similar methods. Test results will indicate past exposure to or infection by parasites of interest (Elgh 1997).

Design evaluation

For power analyses to evaluate the ability of sampling and testing to detect interannual trends in the prevalence of serum antibodies reactive against rodent-borne parasites, we set year-zero antibody prevalence values to 2.5% (rare parasite) or 10% (common parasite) based on results of studies of Sin Nombre virus infection in *P. maniculatus* populations (e.g., Douglass et al. 2001, Calisher et al. 2007). Given an anticipated sampling effort of between one and three 100-trap plots per site, and a likely capture success of 10–20% (estimated using data in Thibault et al. 2011), we estimated that between 30 and 60 rodents are likely to be captured per site/sampling event combination. Based on this, we used a conservative sample size threshold of 40 rodents tested per sampling event. The number of rodents of a given species tested during any single event may be lower depending on patterns of local small mammal diversity and relative abundance.

When power analyses included temporal autocorrelation, the design (sampling events roughly every 4 or 8 weeks) could detect an in-

terannual increase in prevalence of 1.0% for a common parasite at a core site (i.e., over 25 yr) with higher confidence (α = 0.05, power = 0.9) and an increase in 0.75% with lower confidence (α = 0.1, power = 0.8) (Fig. 7a). These were the only scenarios in which an interannual increase in <1.0% could be detected by the design given temporal autocorrelation in infection prevalence. In the absence of temporal autocorrelation, the design could detect interannual increases in 0.5% and 0.25% with higher confidence, but the latter only at a sampling frequency of one event every 4 weeks. For a rare parasite at a core site, the design could detect interannual increases of 0.75% and 0.5% with higher confidence, but the latter only at a frequency of one sampling event every 4 weeks (Fig. 7b). For a common parasite at a relocatable site (i.e., over 10 yr), an interannual increase in 1.0% could be detected with higher confidence, but only at a sampling frequency of one event every 4 weeks (Fig. 7c). This result provides incentive for implementing this recommended sampling frequency at all NEON sites. For a rare parasite at a relocatable site, the design could not detect an interannual increase in ≤1.0% irrespective of the level of confidence or sampling frequency (Fig. 7d).

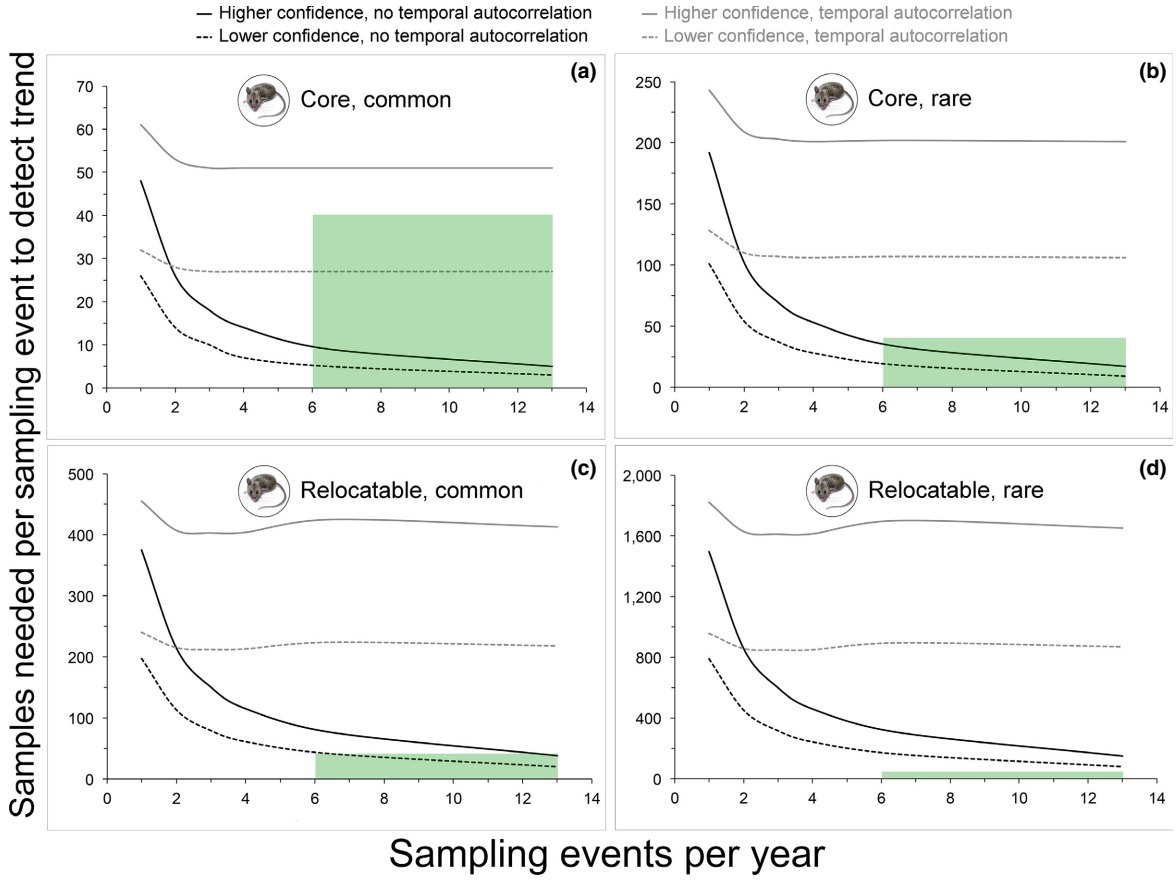


Fig. 7. Power analyses to evaluate the ability of sampling to detect interannual trends in rodent-borne parasite prevalence. Each panel present results of power analyses involving a different combination of National Ecological Observatory Network site type (core site = modeled at 25-yr duration, relocatable site = modeled at 10-yr duration) and year-zero parasite infection prevalence (rare = 2.5%, common = 10%). Results for both low (α = 0.1, power = 0.8) and high (α = 0.05, power = 0.9) confidence scenarios, each with and without a temporal autocorrelation term (~0.37 at 6 months), are provided in each panel. Green boxes indicate levels of sampling effort (sampling event frequency and number of samples per event to be tested for parasites) specified in the associated design.

The design had a 99% probability of detecting a common parasite and a 64% probability of detecting a rare parasite when 40 or more rodents per species were tested per sampling event (Fig. 5e). This detection probability fell to 33% when infection was very rare (1% prevalence). When analytical sampling effort was reduced to 20 rodents per species tested per sampling event, these probabilities were reduced to 88%, 40%, and 18%, respectively. A 50% probability of detecting infection required testing 7, 29, or 60 rodents per species per sampling bout, when infection was common, rare, or very rare, respectively (Fig. 5f). These sample sizes would have to increase to 15, 67, and 160 rodents tested to raise the detection probability to 80%.

Data reporting

The following data generated by (or relevant to) small mammal sampling or rodent-borne parasite testing will be made available through an online data portal maintained by NEON, Inc. (http://www.neoninc.org/data-resources/get-data) or the organization managing the NEON project. Data will become available as they are collected, processed (including quality control), and posted to the data portal.

At the spatiotemporal scales of the sampling plot/sampling event and/or the site/sampling event

- Rodent abundance: the numbers rodents collected, by species/sex combination.
- Sampling effort: the number of trap nights.
- Rodent density: the densities of rodents (by species) estimated using methods described in the NEON design for small mammal abundance and diversity sampling (Thibault et al., *unpublished manuscript*).
- Small mammal diversity: various diversity measures described in the NEON design for small mammal abundance and diversity sampling (Thibault et al., *unpublished manuscript*).

For each captured rodent at the scale of a capture event

- Data collected for small mammal abundance and diversity sampling: species, sex, length/weight, reproductive condition, date and location of capture, and type(s) of tissue samples collected.
- Tick burden: the numbers of attached ticks (by life stage).

For each blood sample tested for evidence of infection by parasites

- Rodent information: species, sex, length/weight, and reproductive condition of animal at time of blood sample collection, date and location of blood sample collection.
- Infection status: the presence/absence of serum antibodies reactive against hantaviruses (and arenaviruses if resources permit) based on results of ELISA test(s).

For each rodent species tested for evidence of infection by parasites, at the spatiotemporal scale of the sampling plot/sampling event and the site/sampling event

- Testing effort: the numbers of blood samples tested for antibodies.
- Testing results: the numbers of blood samples that tested positive for serum antibodies reactive against hantaviruses (and arenaviruses if resources permit).
- Prevalence estimates: the prevalence of serum antibodies reactive against hantaviruses (and arenaviruses if resources permit) estimated using the quotient of number of blood samples that tested positive and the total number of blood samples tested. Prevalence estimates will be reported with Wilson 90% confidence intervals as error estimates (Zelmer 2013).

The type(s), number(s), and availability of archived samples.—These will include: residual blood samples as well as hair, whiskers, feces, and ear punch samples.

Opportunities

The NEON will collect data and test samples in order to estimate the abundance and diversity of vector/reservoirs and prevalence of infection by parasites at Observatory sites. While these measures on their own will constitute important long-term time series data, the greatest advances in understanding of host–parasite dynamics will likely be made through additional, complementary sampling and/or testing efforts undertaken

by external investigators. NEON's open-access policy is intended to make data and samples available for use in a variety of such efforts. Furthermore, we hope that many investigators will integrate their research with sampling undertaken by NEON to expand the scope of work being conducted within the Observatory. Here, we provide a few examples of ways in which members of the research community can extend the work done by NEON. We divide examples among three categories of opportunities based on the manner in which Observatory resources are engaged in these efforts: use of NEON data, use of NEON samples, and extensions of NEON sampling.

Using NEON data

The NEON will make available all raw data and calculated data products generated as part of sampling and testing (e.g., tick abundance and density, arboviral infection prevalence in mosquitoes, small mammal diversity). These data can be used alone or paired with those generated by NEON's other sampling modules, or by other members of the research community (e.g., vector control agencies, academic researchers), to empirically explore topics related to the ecology of vectors, reservoirs, and parasites.

Characterizing patterns of temporal variability

Prior longitudinal studies of vector, reservoir, and parasite populations have demonstrated that rates of infection are often highly variable in time, and that local patterns of rate fluctuations can vary considerably among sites. These conclusions are exemplified by findings of long-term studies of Sin Nombre virus conducted between 1994 and 2013 at sites distributed across four States (Mills et al. 1999*a*,*b*, 2010*a*). Yet most research with these foci is considerably more limited in both spatial and temporal breadth. In some cases, data collection is motivated by acute public health crises that are localized in space and time. In others, studies are restricted in their duration or coverage by financial and/or logistical constraints. NEON data will provide a rare opportunity to quantify variability in vector/reservoir abundance and parasite prevalence over multidecadal timescales at multiple sites. This will be particularly relevant for phenomena that occur gradually (e.g., driven by climate change) and are ideally investigated through long-term studies. The use of space-for-time comparisons (e.g., studying the effects of long-term climate change through short-term studies along elevation gradients, Pickett 1989) can provide proxies for long-term data collection, but the planned 30-yr lifespan of NEON will allow changes to be monitored as they unfold at their natural rate. For example, some sites that exist in a relatively wild state in the early years of NEON operations may become more human-influenced over the life of the Observatory due to encroachment of adjacent developed areas.

Tracking invasion and establishment

The long lifespan of many NEON sites may occasionally allow the demographic and ecological changes associated with the invasion of vector, reservoir, or parasite species to be observed. Simultaneously tracking this establishment while monitoring shifts in environmental and ecological attributes of associated systems could provide insights into the processes by which vector, reservoir, and parasite species become endemic in novel environments. Data collected by local vector control efforts, which collectively have much greater geographic sampling resolution than the Observatory, could provide early warning of the pending arrival of novel species at NEON sites.

Exploring ecological connectivity

Infection dynamics are generally the result of complex biotic and abiotic interactions, yet few studies of host–parasite interactions are able to integrate multiple lines of relevant monitoring across broad spatial and temporal extents. Such multifaceted studies have the potential to capture a more complete picture of associated drivers and responses. For each module of NEON vector/reservoir/parasite sampling, data on target species will be paired with abiotic environmental measurements made by NEON sensors at each site, and remotely sensed data collected by annual site flyovers by NEON aircraft. Demographic, phenological, and diversity data on other taxa that may directly or indirectly influence the ecology of target species will also be collected. For example, data on plant communities will provide insights into habitat attributes, birds are hosts for many

arboviruses, and insects represent an important food source for both birds and small mammals. In many cases, data collected by other projects (e.g., citizen science projects such as the annual Christmas Bird Count and Project BudBurst) can also be integrated into analyses that explore the variety of factors that influence infection dynamics beyond direct interactions between hosts and parasites.

Modeling infection dynamics

The multifaceted data sets generated by NEON vector/reservoir/parasite sampling will inform the construction and parameterization of models to forecast infection dynamics. Specifically, there is a need for long-term (interannual) models that predict the impact(s) of climate and associated ecological changes on the incidence and distribution of vector-borne and zoonotic diseases. Additionally, the development of short-term (intra-annual) models could be used to guide public health preparedness and response to seasonal outbreaks of sporadic diseases such as West Nile virus infection or Lyme disease. Because of the multidecadal duration of sampling within the Observatory, NEON data will allow predictions of many of these models to be evaluated in real time. Related to this, members of the research community could use remotely sensed data collected by NEON's airborne operations platform, including LiDAR and hyperspectral imaging (Kampe et al. 2010, Krause et al. 2013), to develop and refine the use of remotely sensed metrics to predict changes in vector/reservoir abundance and parasite prevalence (e.g., Dister et al. 1997, Glass 2000, Loehman et al. 2012).

Using NEON Samples

In addition to streaming field and lab-generated data, physical samples (tissues and whole organisms) collected during vector/reservoir/parasite sampling will be archived and available to members of the research community for use in a variety of additional analyses, including the following:

Testing for additional parasites

While NEON will endeavor to use testing methods capable of detecting multiple parasites in the collected samples, these represent only a fraction of the parasites associated with target vector and reservoir species. Archived samples could be used to conduct tests for additional parasites including viral and protozoan parasites in ticks, helminth, and protozoan parasites in mosquitoes, and helminth parasites in rodent fecal samples. Archived samples could may also prove valuable in retrospective analyses for newly discovered parasites (e.g., Heartland virus in ticks, Savage et al. 2013). These "novel" or "emerging" parasites are usually detected only when they are implicated as the cause of human disease. The archived materials collected through NEON sampling will be useful for determining the extent to which these parasites may have existed cryptically within vectors and reservoirs prior to being implicated in human disease. The conditions under which such parasites came to infect humans can then be studied. Information on the identity of and rates at which different parasites infect individuals of the same vector or reservoir species could additionally provide insights into the ecological and evolutionary dynamics of parasite coinfection.

Testing for nonparasitic targets

Archived samples could additionally be used for analyses related to vector and reservoir ecology. For example, blood meal analysis of ticks and mosquitoes can provide insights into patterns of host feeding preferences that have important implications for vector demography and parasite transmission (e.g., Kilpatrick et al. 2006*a*, Hamer et al. 2009, Allan et al. 2010*b*, Brunner et al. 2011). Isotopic analyses of rodent tissue samples can elucidate changes in diet, metabolic rates, and movement (Crawford et al. 2008), all of which influence the dynamics of small mammal populations and associated parasites.

Studying parasite evolution

Parasite genomic material isolated from samples can help advance molecular studies of the phylogenetic relationships within parasite lineages and the biogeographic structure of parasite populations. Further, archived samples could be used to explore genetic changes in parasite populations through time such as those that occurred in West Nile virus following its introduction to and initial epidemic spread across North America (Davis 2005).

Extending NEON Sampling

The types of methods, measurements, and samples that form the basis of the NEON vector/reservoir/parasite sampling designs are by no means exhaustive. As with other large research platforms (e.g., telescopes, ships), the Observatory is intended to provide a framework within which other members of the research community can conduct additional investigations. These can build upon baseline data provided by NEON to further expand the breadth and/or depth of insights into vector, reservoir, and parasite ecology. General options for this integration include the following:

Increasing sampling effort

Supplementary sampling to increase the number and/or types of organisms collected could expand both the variety of sample analyses performed or the level of associated replication. For example, the mark/recapture method that will be used for NEON small mammal sampling limits the number and variety of physical samples that can be collected. Establishment of small mammal removal grids set up in parallel with NEON's mark/recapture grids would allow whole animals collected on these grids to be examined for a wider range of ecto- and endoparasites. Associated blood samples could also be tested for evidence of infection by hantaviruses and arenaviruses.

Expanding spatiotemporal breadth

Supplementary sampling could be conducted in parallel with that performed by NEON to investigate patterns in un- or poorly sampled locations (e.g., rare vegetation types) or along environmental gradients of interest (e.g., locations with varying levels of human impact). Whether carried out at additional plots at Observatory sites or at alternate locations, both efforts increase the spatial resolution of NEON sampling and potentially broaden the range of hypotheses that can be addressed. Similarly, replicating NEON sampling at additional times could increase sampling frequency and/or the portion of the year when sampling is conducted. For example, "shoulder season" sampling just before and after the annual period when vectors, reservoirs, and hosts are present or abundant will be important in detecting phenological shifts that may have important consequences for vector/reservoir population sizes and rates of parasite transmission (Kilpatrick et al. 2006*b*, Altizer et al. 2013). As both effects could influence rates of infection in humans (when parasites are zoonotic), phenological data could provide insights into changes in seasonal patterns of human risk and inform associated public health recommendations for risk reduction. Because the predicted rate of phenological change is modest for many taxa (Parmesan 2007), additional sampling events, particularly during the shoulder seasons, may be necessary to detect and accurately characterize these temporal shifts. Collaboration with external investigators represents a promising means of accomplishing this.

Adding sampling targets

Supplementary sampling employing methods other than those used by NEON (e.g., pheromone-baited mosquito traps) could expand the taxonomic breadth of vector, reservoir, and parasite taxa sampled within the Observatory. Alternatively, surveys or sampling of host taxa not targeted by NEON (e.g., large mammals such as deer that serve as final hosts for ticks of many species) or those that influence the distribution and abundance of target taxa through trophic relationships (e.g., mesopredators such as coyotes that feed on rodents) could provide additional insights into the diversity, demography, and epidemiology of target taxa.

Incorporating experimental manipulations

With proper coordination and integration, researchers could conduct manipulative experiments in concert with NEON sampling, the latter providing baseline and control data to compare against data generated by the former. The opportunity to integrate experimental studies with NEON efforts will allow specific hypotheses to be evaluated in a focused way, thereby providing greater context for interpretation of observed patterns and associated forecasting (LaDeau et al. 2011).

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Supporting Information

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