Prevalence of *Borrelia burgdorferi* and *Borrelia miyamotoi* in *Ixodes scapularis* Ticks in Southwest Pennsylvania

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Prevalence of *Borrelia burgdorferi* and *Borrelia miyamotoi* in *Ixodes scapularis* Ticks in Southwest Pennsylvania

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Background: Tick-borne illnesses are increasing across the United States (US), especially in northeastern states like Pennsylvania (PA). Lyme disease, caused by *Borrelia burgdorferi*, is the most prevalent and well-known tick-borne illness presently. A common vector of tick-borne pathogens in the US is *Ixodes scapularis*, known as the black-legged (deer) tick. *Ixodes scapularis* can spread multiple tick-borne pathogens simultaneously, including *Borrelia miyamotoi* (the causative agent of tick-borne relapsing fever). Despite the importance of knowing the prevalence of *Borrelia* species in nymphal ticks to gauge public health risk of tick-borne illness, the baseline prevalence of tick-borne pathogens in southwest Pennsylvania's *I. scapularis* population is not well documented. This study addressed this gap in the literature by providing comprehensive *B. burgdorferi* and *B. miyamotoi* prevalence data from *I. scapularis* nymphs collected across southwest Pennsylvania over two consecutive years. Overall, 28.8% of all nymphal *I. scapularis* tested positive for *B. burgdorferi* and 0.7% tested positive for *B. miyamotoi.* Co-infection with *B. burgdorferi* and *B. miyamotoi* only occurred in 0.1% of ticks sampled (n=2). Across suburban, urban, and rural sites, the prevalence of *B. burgdorferi* differed (F (2, 3) = 27.65, p = 0.01), and was higher in rural and urban sites compared to suburban sites. While the prevalence of *B. burgdorferi*, *B. miyamotoi*, and *Borrelia* co-infection increased between 2021 and 2022, this change was not significant. This change was the most pronounced in suburban and rural areas, with a higher abundance of herbaceous plant life and shrubs. Furthermore, this data provides

insight into the richness and abundance of *B. burgdorferi* and *B. miyamotoi* throughout southwestern PA and contributes to ongoing surveillance efforts that inform public health outreach and physician education that are regionally appropriate and specific.

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Preface

This thesis was made possible with the guidance, ideas, and support of advisor and committee members, Dr. Danielle Tufts, Dr. Joshua Mattila, and Dr. Jill Hennings. I am fortunate to be mentored by Dr. Danielle Tufts in these research endeavors, and thankful for her encouragement and guidance throughout my degree. The work of Deanna Dailey, Emily Bache, Audrey Burch, and Zoe Weaver were also essential to this project, and I am grateful for the aid of 2021 and 2022 field research teams. I owe many thanks to Josee Kahambwe and EJ Young, who made this thesis possible. Lastly, I must thank my partner, Zachary, for his unwavering support throughout my degree.

1.0 Introduction

In the United States, approximately 35,000 new cases of Lyme disease were reported in 2019, following a 10-year trend of over 30,000 new cases occurring in the United States (US) annually (CDC, 2022a). However, the true number of new cases of Lyme disease annually is estimated to be 10 times higher due to underreporting and underdiagnosis of the disease (Kugeler et al., 2021). Lyme disease, caused by *Borrelia burgdorferi* bacteria in the US, accounts for more than 60% of all tick-borne disease cases in the US (CDC, 2022b). Lyme disease is spread by *Ixodes scapularis* ticks, also known as black-legged ticks or deer ticks, in the northeastern United States where Lyme disease cases are highest. Pennsylvania (PA), for example, reported an average of 62.3 new cases of Lyme disease per every 100,000 people from 2016 – 2019 and reported nearly 9,000 of the 34,945 confirmed and probable cases of Lyme disease in 2019 (CDC, 2021). Given the high burden of Lyme disease in PA, the state is a critical location for tick-borne pathogen research.

While Lyme disease is the most prevalent and well-known tick-borne disease presently, *I. scapularis* can spread multiple tick-borne pathogens simultaneously (Boyer et al., 2022). Tick-borne pathogens that may be present alongside *B. burgdorferi* can include *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia mayonii*, *Borrelia miyamotoi*, rickettsial diseases, and arboviruses (Powassan virus, among others) (Kocan et al., 2015; Westblade et al., 2017; Eisen & Eisen, 2018; Wolf et al., 2020; Boyer et al., 2022). The relationships between *B. burgdorferi* and other tick-borne pathogens are ultimately not well understood. The relationship between *B. burgdorferi* and a closely related pathogen *B. miyamotoi*, for example, is still an area of research that is on-going as *B. miyamotoi* spreads across the US (Xu et al., 2021; Swanson et al., 2023).

The prevalence of *B. miyamotoi* in *I. scapularis* nymphs is not well documented in PA. Southwestern PA in particular acts as a junction between tick populations in the northeast and the northwest, thus acting as a pivotal location for understanding tick-borne disease dynamics. Despite the importance of this region in tick-borne pathogen transmission and spread, information on tick-borne pathogen prevalence in southwest PA is relatively sparse, even when compared to the rest of the state (Diuk-Wasser et al., 2010; Hutchinson et al., 2015).

The prevalence of tick-borne pathogens in the tick vector is often used to assess the public health risk of disease (Diuk-Wasser et al., 2014). By gathering information on the prevalence of pathogens in ticks themselves, we can better ascertain the possible human exposure to tick-borne pathogens if they are bitten. Given the diversity in development and population across southwest PA moving from urban to rural environments, capturing the abundance of tick-borne pathogens within ticks is essential to understanding which communities may be at the greatest risk of exposure to tick-borne disease.

1.1 *Ixodes scapularis* as a Vector for Multiple Pathogens

Ixodes scapularis ticks are the primary vectors of tick-borne pathogens in the northeastern United States (Shapiro, 2014; Eisen & Eisen, 2018). *Ixodes scapularis* depend on a combination of environmental and host factors to sustain a robust population of ticks to ensure success for each life stage across the tick life span (Margos et al., 2012). Adult female *I. scapularis* will lay a clutch of eggs which will hatch into larvae (Margos et al., 2012; Kocan et al., 2015; Stewart et al., 2020). After hatching, *I. scapularis* larvae will seek a blood meal (Kocan et al., 2015; Stewart et al., 2020). The larvae may feed on any available host in the environment but regularly feed on small

mammals, such as *Peromyscus leucopus* (Levin & Fish, 2000; LoGiudice et al., 2003). *Peromyscus leucopus*, known as white-footed mice, are competent reservoir hosts for several tick-borne pathogens, including *Borrelia* species (Levin & Fish, 2000; Diuk-Wasser et al., 2016). Larval *I. scapularis*, after taking a first blood meal, will molt into a nymph the following spring and take an additional blood meal before further molting into an adult (Radolf, et al., 2021). Nymphal *I. scapularis* will feed upon a broad range of hosts, preferentially small mammals like *P. leucopus* (Levin & Fish, 2000; Diuk-Wasser et al., 2016). However, they can feed on deer and larger mammals as well, which can facilitate co-feeding with adult ticks (Voordouw, 2015). Adult *I. scapularis* almost exclusively feed on deer and other large mammals, where they will take blood meals and mate with another adult *I. scapularis* (Kocan et al., 2015). However, many large mammals are poor reservoir hosts for pathogens such as *B. burgdorferi*, and thus are more important to the life cycle of the vector than the life cycle of the pathogen the tick may spread (Margos et al., 2012; Eisen & Eisen, 2018). Without a suitable habitat for both ticks and their blood meal host, the life cycle of *I. scapularis* could not be sustained.

When ticks bite their host, they can acquire pathogens in two ways, through co-feeding or through the host themselves (Randolph et al., 1996; Voordouw 2015; States et al., 2017). With co-feeding, ticks from different life stages feed spatiotemporally, thus they can transfer pathogens directly to other ticks (Voordouw, 2015; States, et al., 2017). This allows for a *B. burgdorferi*-naïve larvae to acquire spirochetes from a nearby feeding nymph or adult tick (Voordouw, 2015). However, co-feeding requires tick synchrony, such that multiple life stages emerge and feed simultaneously (States et al., 2016). *Ixodes scapularis* synchrony is generally found only in the upper Midwest, so this is likely not a large contributing factor to circulating tick-borne pathogens in southwest PA (States et al., 2017). The ticks in southwest PA likely acquire pathogens more

traditionally, directly from a reservoir host (Hersh et al., 2014; Radolf et al., 2021). *Ixodes scapularis* may acquire pathogens from competent reservoir hosts when they take blood meals throughout their lifespan, which increases their risk of acquiring pathogens over time (Jaquet et al., 2017). That said, there is evidence to suggest that some tick-borne pathogens such as *B. miyamotoi* may use transovarial transmission in *I. scapularis*, meaning that adult females can transmit the pathogen to their eggs resulting in infected larval ticks (Krause et al., 2015; Eisen & Eisen, 2018; Fleshman et al., 2022). Thus, multiple means of pathogen transmission provide several opportunities for pathogen acquisition in ticks and may increase the overall pathogen abundance in different regions (Voordouw, 2015; Jaquet et al., 2016; Cutler et al., 2021).

Nymphal *I. scapularis* play a substantial role in facilitating tick-borne pathogen transmission to humans, as they can evade detection by human hosts due to their small size and emerge in the spring when human outdoor activity increases in the US (Kurokawa et al., 2020; Radolf et al., 2021). Given resource and time limitations to testing human populations for tick-borne pathogens, our study uses nymphal *I. scapularis* ticks as a proxy to ascertain pathogen prevalence and risk for humans. *Borrelia burgdorferi* prevalence in ticks can be used to predict disease incidence in humans, thus supporting the use of *I. scapularis* nymphs to measure human risk (Barbour & Fish, 1993). Previous studies show that Lyme disease reports are highly correlated with higher entomological risk index scores, which are a calculation of the relative abundance of ticks with the prevalence of *B. burgdorferi* infection in that same area per unit of time (Mather et al., 1996; Diuk-Wasser et al., 2012). These studies lead to the widescale adoption of using pathogen prevalence in ticks to assess human tick-borne disease risk (Mather et al., 1996; Diuk-Wasser et al., 2012).

1.2 *Borrelia burgdorferi* and *Borrelia miyamotoi* Infection in Humans

Borrelia burgdorferi is a spirochetal bacterium that causes Lyme disease and is the most common tick-borne pathogen in the United States (Mead, 2015; Eisen & Eisen, 2018; Radolf et al., 2021). The incidence of Lyme disease is increasing annually, and with this, treatment delays are still a major cause of worsened outcomes of Lyme disease (Hirsh et al., 2017). A major concern regarding worsened Lyme disease outcomes surrounds the neurological manifestations of Lyme disease, such as meningitis and cranial neuritis can increase the risk of poor healthcare outcomes and increase the fatality risk in patients (Roos, 2021). Alongside *B. burgdorferi*, *B. miyamotoi* causing significant concern across the United States as it can co-exist easily with *B. burgdorferi* in the tick vector (Krause et al., 2015; Fleshman et al., 2022; Cleveland et al., 2023). *Borrelia miyamotoi* is the causative agent of hard tick-borne relapsing fever, which may cause illness with similar symptoms to Lyme disease but is set apart by its ability to cause acute, relapsing febrile illness in humans (Krause, et al., 2015; Cleveland, et al., 2023). In severe cases, *B. miyamotoi* can cause neurological symptoms such as meningoencephalitis (Krause et al., 2015). *Borrelia miyamotoi* was first discovered in Japan in 1995 but is relatively new to the US as the first documented human cases in the US were found in 2013 (Krause et al., 2015; Molloy et al., 2015; Cleveland et al., 2023). While the reported incidence of hard tick relapsing fever is low, molecular assays for *B. miyamotoi* may cross-react with Lyme disease tests and thus make cases of *B. miyamotoi* harder to identify (Krause et al., 2015). Given the severity of *B. miyamotoi* infections and the possible cross-reactivity of *B. miyamotoi* and *B. burgdorferi* in clinical assays, knowing the prevalence of both *B. burgdorferi* and *B. miyamotoi* in *I. scapularis* nymphs is essential for appropriately educating physicians on testing and treatment (Krause et al., 2015).

Ultimately, *Borrelia* species co-infections can make treating patients more challenging, especially given the compounding risk of complications that each individual tick-borne pathogen can cause (Wolf et al., 2020; Schwartz et al., 2022). There is a growing body of research suggesting that co-infection with multiple tick-borne pathogens may worsen Lyme disease outcomes, as well (Krause et al., 1996; Bhanot & Parveen, 2019; Djokic et al., 2019). Co-infected individuals may experience worsened symptoms and slower recovery of Lyme disease compared to their *B. burgdorferi*-only infected counterparts (Krause et al., 1996; Bhanot & Parveen, 2019). Consequently, this may exacerbate the symptoms of Lyme disease, including Lyme arthritis and neuroborreliosis (Goldstein et al., 2001; Knapp & Rice, 2015). While the symptoms of many tick-borne illnesses may overlap Lyme disease overlap substantially, the treatment for these conditions may not (Pruthi et al., 1995; Bhanot & Parveen, 2019). Therefore, if co-infection is not identified, patients can experience continued infection after treatment for Lyme disease is discontinued (Sanchez et al., 2016, Schwartz et al., 2022). While the complex relationship between these tick-borne pathogens is still being explored, having the knowledge of pathogen prevalence can be empowering for communities to ensure the best possible public health outcomes. This is particularly important in rural communities that may lack healthcare access or in urban communities that are unaware of their risk of tick-borne illness. Thus, knowing the prevalence of *Borrelia* species in local communities and across an urban-to-rural landscape is critical for effective public health interventions (Swanson et al., 2006; Hutchinson et al., 2015).

1.3 Pathogen Prevalence Across Suburban, Urban, and Rural Sites

Overall, the social and medical implications of differing pathogen prevalence in ticks across an urban-to-rural gradient are sufficient to warrant concern among public health professionals. This concern is also shared among disease ecologists, given the possible ecological drivers of pathogen spread across urban, suburban, and rural landscapes (Diuk-Wasser et al., 2020; Combs et al., 2022). Ecological factors, such as forest fragmentation, forest composition, and patchiness, are theorized to influence co-infection rates in vectors (Adalsteinsson et al., 2018; Plowright et al., 2008). Fragmented forests are defined by the loss of continuous forest cover by splitting the forest into smaller blocks, resulting in differing levels of patchiness (Plowright et al., 2021). Vegetation types, forest fragmentation, and patch dynamics are discussed widely when examining tick-borne disease risk in urban and suburban settings (Combs et al., 2021). Predominantly, forest fragmentation may provide greater space for humans and wildlife to co-exist and interact (Combs et al., 2021; Alberti 2005). Suburban and urban areas may have greater forest fragmentation, but it is unclear how that impacts pathogen richness and abundance in local tick populations.

Suburban areas are often marked by land consumption mixed with forest fragments to create commercial buildings, residential housing, and community resources such as parks. This fragmentation can provide recreational spaces for communities, but at the risk of increasing human interaction with ticks (Falco & Fish, 1989). With this, there is concern that microclimate variation and changes in forest composition introduced through the development of green space alongside forest patches in suburban areas may increase the risk of many zoonotic pathogens, including *B. burgdorferi* (Ostfeld et al., 2005; Combs et al., 2021; Diuk-Wasser et al., 2021). Forest composition, predominantly the abundance and diversity of different herbaceous plant life, may

play a role in maintaining robust tick populations in a local area. For example, Japanese barberry and other similar shrubs may provide suitable habitats for *P. leucopus*, and the control of such shrubbery is linked to reduced *B. burgdorferi* infection prevalence among ticks (Prusinski et al., 2006; Williams et al., 2009; Williams et al., 2010 Reaser et al., 2021). Additionally, these environments may be preferable for ticks: providing a more suitable microclimate for tick survival and questing by increasing humidity, which could in turn reduce desiccation-reduced mortality (Williams et al., 2010). Thus, variation in forest composition that may be observed between suburban, urban, and rural landscapes may impact pathogen prevalence given the practical advantages that more shrubbery and grassland may play on *I. scapularis* and reservoir hosts of *B. burgdorferi*.

Forest fragmentation and forest composition both can act as ecological drivers that alter mammalian biodiversity in a forest ecosystem (Allan, et al., 2003; LoGiudice, et al., 2021). Mammal biodiversity is important in providing a wider variety of hosts for ticks to feed upon, including mammals that are poor reservoirs for pathogens (Schmidt & Ostfeld, 2001; Keesing et al., 2010). Pathogen abundance is dependent on competent reservoir hosts that will allow pathogens to be transmitted effectively to a vector or another host (Martin et al., 2016). In settings that lack diversity in host competency for tick-borne pathogens, ticks may have a higher likelihood of feeding on hosts that are highly competent for preserving and spreading pathogens like *B. burgdorferi* (Schmidt & Ostfeld, 2001; Ostfeld, 2009). For example, *Sorex* spp. shrews, *Tamias striatus* (eastern chipmunk), and various squirrels are less competent for *B. burgdorferi* when compared to *P. leucopus* (Keesing et al., 2006; LoGiudice et al., 2008). *Peromyscus leucopus* are robust hosts for tick-borne pathogens such as *B. burgdorferi* and *B. miyamotoi*, among other pathogens such as *B. microti* and *A. phagocytophilum* (LoGiudice et al., 2008; Krause et al., 2015).

More biodiversity yields a lower risk that ticks will consistently feed on highly pathogen-competent hosts (such as *P. leucopus)* for every blood meal, thus reducing the likelihood of high pathogen burden in a population and co-infection among ticks (LoGiudice et al, 2008). Tick dependence on mice alone for their bloodmeals is postulated to be a contributing factor for increasing *B. burgdorferi* circulating among mice (Allan et al., 2003). Since forest fragmentation is affiliated with higher mouse density, this causes concern for tick-borne pathogen prevalence in suburban areas (Allan et al., 2003).

In this study, population density is used as a proxy for forest fragmentation. Forest fragmentation is largely contingent on forest disruption by developed areas, creating "patchiness" in the landscape (Piedmonte et al., 2018). The aim of analyzing forest fragmentation by means of defining suburban, urban, and rural sites is to determine how human intervention may impact pathogen prevalence by altering the landscape in which vectors and hosts live. For example, forest fragmentation may decrease the risk of tick-borne illness by lowering the density of ticks, as higher patchiness and forest fragmentation can reduce the available leaf litter that is necessary for *I. scapularis* molting (Falco & Fish, 1989). Additionally, it may lead to previously mentioned reductions in host abundance, thus limiting the number of ticks feeding and residing in those areas (Ogden & Tsao, 2009). This, alongside human interventions like city park maintenance, can lead to a lower tick density overall (Combs et al., 2021). Following this logic, we would expect an overall decrease in ticks, and therefore a reduced pathogen richness and abundance in highly urbanized areas (Falco & Fish, 1989). However, some previous research tick-borne pathogen prevalence conflicts with this rationale, as noted above (Schmidt & Ostfeld, 2001; Allan et al., 2003; LoGiudice et al, 2008; Ostfeld, 2009). Forest fragmentation and patchiness, therefore, generate complex dynamics that create a need for regionally specific disease ecology research. For

this research, southwest PA's urban sprawl is an ideal location to explore how complex environments can alter the risk of tick-borne illness, even over the span of a few miles.

1.4 Public Health Relevance

As aforementioned, PA experiences a high incidence of Lyme disease in humans annually (CDC, 2021). The infection prevalence of *B. burgdorferi* in adult *I. scapularis* was reported to be as high as 47.4% on average across PA, but only 39% of samples collected in southwest PA tested positive for Lyme disease, well below the average as indicated in that same study (Hutchinson et al., 2015). Recent research completed by the Pennsylvania Department of Environmental Protection (2022) indicated that southwestern PA had the highest prevalence of *B. burgdorferi* in their nymphal *I. scapularis* population, with 30.1% of the nymphs testing positive for the pathogen. However, most studies of tick-borne pathogens in PA, including studies by the Department of Health and Department of Environmental Protection and previous research groups, only focus on adult *I. scapularis* due to the timing and availability of resources to collect ticks. One such study, for example, suggested that *B. miyamotoi* may be found in as many as 0.3% of adult ticks (Livengood et al., 2020). While this information can confirm the presence of pathogens in PA, the infection rates observed in this study likely do not reflect the prevalence of *B. miyamotoi* in nymphs, and thus cannot be used to gauge the risk of tick-borne pathogen infection. In this context, pathogen prevalence in *I. scapularis* nymphs is critical as we observe changes in the human incidence of Lyme disease in PA and the surrounding states.

The east-to-westward expansion of *I. scapularis* across PA and into Ohio is of major interest to researchers since little research is conducted between the Mississippi River Valley and

Allegheny Mountains in western PA (Eisen et al., 2016). Given the robust tick expansion across the northeastern United States in the mid to late 20th century, multi-year studies are necessary for improving our understanding of annual changes in pathogen prevalence among ticks and predicting future pathogen prevalence (Barbour & Fish, 1999; Eisen & Eisen, 2016; Eisen & Paddock, 2021). Exploring the overall differences in tick-borne pathogen prevalence between rural, suburban, and urban sites is critical to ascertaining local community risk for physicians and patients alike. For example, if *B. miyamotoi* prevalence is low in a rural area, medical professionals may not immediately test for it, which may be a cost-saving measure for rural health clinics and patients that may already struggle with the cost of medical care (Hirsh et al., 2014). In areas where testing may be difficult, empirical treatment may also be risky due to the potential for antimicrobial resistance development. That said, comprehensive testing of tick-borne pathogens is needed in areas with great pathogen richness and abundance. In communities with high rates of Lyme disease, more physician education may be needed to ensure infections are not misdiagnosed or improperly treated (Hirsh et al. 2014). For example, if patients are aware of the high prevalence of Lyme disease and other tick-borne pathogens locally, they may be more willing to seek care for rashes or other symptoms they would otherwise ignore. Misattribution of symptoms as a possible other disease with similar manifestations (i.e. influenza) as well as misunderstandings of Lyme disease symptoms can cause a major delay in seeking treatment (Hirsh et al., 2017). A study of pediatric Lyme disease cases from rural zip codes in PA noted that a higher percentage of cases from rural zip codes complained of joint pain and swelling compared to their non-rural counterparts but reported fewer erythema migrans (EM) rashes (Eddens et al., 2019). Additionally, patients in rural zip codes sought care for symptoms of late Lyme disease more often than patients in non-rural zip codes, indicating that healthcare access and decision-making in rural communities

may differ based on zip code (Eddens et al., 2019). Given the potential for the development of long-term symptoms like neuroborreliosis, prompt medical care may be sought more readily if people are aware of their local risk of tick-borne illness. Notably, from 2003 to 2013, there was a positive trend in Lyme disease cases diagnosed in children living in non-rural communities, including within the urban city of Pittsburgh in western PA (Eddens et al., 2019). However, it is unclear if this increase is due to healthcare access decreases in rural communities, or a true increase in tick-borne illness in urban communities. Testing of nymphal *I. scapularis* can help elucidate if the prevalence of tick-borne pathogens in rural, suburban, and urban communities is truly changing over time, which can lead to better educational efforts or better allotment of healthcare resources. Without information on pathogen prevalence across these communities, many human cases of Lyme disease or hard tick relapsing fever may go unnoticed, leading to worse healthcare outcomes in communities that may unknowingly be at exceptionally high risk of tick-borne disease.

A study by Diuk-Wasser et al. (2010), indicated that research and previous models attempting to describe *B. burgdorferi* prevalence in western PA may be insufficient to depict the overall prevalence of single and multiple pathogen prevalence in ticks in southwest PA. As Hutchinson et al. (2015) explains, the regional prevalence of tick-borne pathogens in PA is not well elucidated as little research is available on the subject. In this context, our study addresses a critical gap in knowledge regarding *B. burgdorferi* and *B. miyamotoi* prevalence across a diversely developed area over multiple years. With knowledge of pathogen prevalence and annual change, more effective, regionally appropriate interventions can be introduced to better address tick-borne disease risk across southwest PA's suburban, urban, and rural communities.

1.5 Project Scope and Aims

To address deficits in the knowledge regarding the abundance and prevalence of *B. burgdorferi* and *B. miyamotoi* in southwest Pennsylvania, this study will focus on the southwest region of PA and uses molecular assays to explore the prevalence of *Borrelia* species in questing *I. scapularis* nymphs (Diuk-Wasser et al., 2014). Our central hypothesis was that the prevalence of *Borrelia* species will be higher in suburban areas, as they provide fragmented forest spaces that maintain steady populations of high-competency host animals, such as white-footed mice. These suburban forest fragments, therefore, allow ticks to feed on consistently infected reservoir hosts, thus perpetuating the cycle of infection. Given the interface that suburban parks and forests establish between humans and wildlife, understanding the prevalence of pathogens in tick populations is critical for public health and education efforts.

Through this research, we aimed to (1) examine the dynamics of *B. burgdorferi*, *B. miyamotoi*, and *Borrelia* species co-infection in *I. scapularis* nymphs by comparing the prevalence of each pathogen in ticks collected from 2021 and 2022, and (2) examine differences in pathogen prevalence between sites classified as rural, suburban, and urban. To address these aims, we collected questing nymph *I. scapularis* ticks through standard dragging methods across southwest PA for two consecutive years. We then analyzed questing *I. scapularis* nymphs from 2021 and 2022 using DNA extraction and subsequent pathogen screening by quantitative real-time polymerase chain reaction (qPCR). This study affords us the unique opportunity to examine the regional prevalence of tick-borne pathogens in southwest PA, an area that is highlighted by past studies as needing additional testing and surveillance (Diuk-Wasser et al., 2010; Hutchinson et al., 2015).

2.0 Methods

2.1 Sample Collection

Six sampling sites were selected for this study by identifying public land and green space using Google Maps and ground surveying for suitable host and vector habitat. Each site was surveyed prior to dragging, to ensure the site was accessible and included appropriate vegetation for collecting ticks (i.e., semi-forested areas not recently developed). Sites selected include Schenley Park (SCH) [40°43’21.1”N, 79°94’03.5”W], Riverview Park (RVP) [40°48’80.8”N, 80°02’19.6”W], and Boyce Mayview Park (BOM) [40°33’65”N, 80°10’15.6”W] in Allegheny County, Crooked Creek Horse Park (CCH) [40°70’38.1”N, 79°52’37.9”W] in Armstrong County, State Game Lands #232 [40°18’21.7”N, 80°48’97.4”W] in Washington County, and State Game Lands #42 [40°40’97.8”N, 79°00’29”W] in Westmoreland County. Sites were visited on a biweekly basis from May to August in 2021 and 2022, resulting in seven and eight separate tick collection events at each site, respectively. Grids were composed of six 100 m transects and organized by placing flags every 10 m, with a total of 60 flags at each location. Grids spanned 600 m^2 and were established based on landscape and topography of each location.

Ticks were collected by dragging a standard 1 m^2 corduroy cloth on the ground between flags and every 10 m (from one flag to the next) the cloth was flipped to be checked for attached ticks. Ticks were preserved in a 1.5 mL microcentrifuge tube filled with 95-100% EtOH. Each tube was labeled for the specific site, grid line, and date, thus allowing for close tracking.

2.2 DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Each collected tick was morphologically identified and *I. scapularis* nymphs were separated individually into 1.5 mL microcentrifuge tubes. When ready for processing, alcohol was removed from each tube and each nymph was air-dried for 30 s. To weaken the hard exoskeleton of the nymph and to aid in the crushing process, the tube was dipped into liquid nitrogen for approximately 5 s. Each tick was crushed using a sterile pestle in the tubes and genomic DNA was extracted using the QIAcube HT robotic workstation (Qiagen) through the DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504), following manufacturer's protocol. Samples were frozen at -80 ^{o}C until further analysis could be completed.

All nymphal I. scapularis collected from the grid sites were tested for B. burgdorferi and B. miyamotoi. Extracted DNA was screened using quantitative PCR (qPCR) assays specific for Borrelia species. Samples were screened in duplicate for B. burgdorferi and B. miyamotoi as a duplex, targeting a 69 bp segment unique to the bacterium's 16S rRNA gene, with forward primer (5'-GGC GGC ACA CTT AAC ACG TTA G-3'), reverse primer (5'-GCT GTA AAC GAT GCA CAC TTG GT-3'), B. burgdorferi probe (6FAM-TTC GGT ACT AAC TTT TAG TTA A-MGBNFQ), and B. miyamotoi probe (VIC-CGG TAC TAA CCT TTC GAT TA-MGNFQ) (Barbour, et al., 2004; Barbour, et al., 2009). Samples were screened on a ViiA 7 real-time PCR system (Applied Biosystems®, ThermoFisher Scientific, Waltham, WA, USA). Cycling conditions all assays consisted of: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds with data acquisition in the FAM and VIC channels occurring during the final step in each cycle. Positive control samples were kindly provided by the Pennsylvania Department of Environment Protection. Samples were considered positive if duplicate samples had a cycling threshold value under 40. Thus, if any samples presented

conflicting results, the sample was re-run in duplicate to ascertain if the sample was truly positive or truly negative. A serial dilution of B. burgdorferi and B. miyamotoi respectively (starting with 10^6 copies of DNA to one copy of DNA in duplicate) were used as positive controls. There were additionally two negative controls used to ensure that no contamination occurred.

2.3 Suburban, Urban, or Rural Site Classification and Correlation Analysis

Sites were classified as suburban, urban, and rural through analysis with ArcGIS Pro. To determine urban, suburban, and rural site classifications, one geospatial positioning system point (GPS point) was selected at random for each site visited. Around each grid site (n = 6) buffers were generated at 300 m, 600 m, 900 m, and 1200 m. Multiple buffer sizes were analyzed to ensure the buffer zone selected for this analysis represented the site accurately based on the data from the National Land Cover database as well as matched visual site observations regarding land use and distribution of buildings from each location. These buffers were then enriched with population density data (persons per square mile) from the US Census 2022. Given that the National Land Cover Database and other geospatial information databases analyze land in 30 m^2 pixels, buffer regions were selected to coincide with pixel measurement dimensions. Enriched buffer data was then exported into CSV and Excel files for analysis. This analysis uses population density and proximity to an urban center as our criteria for rural, suburban, and urban classifications. This protocol was derived from methodology employed by a 2018 Pew Research Center report on urban, suburban, and rural communities.

Urban locations were defined as locations within 12 miles of a city or urban center, with a population density of 1314 households per square mile (Pew Research Center, 2018). We used

population density per square mile as a metric, given the variable household size throughout the region in question. The urban center selected for this study was identified as Pittsburgh, PA. Three 12-mile buffers were created at the east, west, and north-most points within the city boundaries, as defined by local census information. Suburban locations were defined as locations within 12 miles of the city, with a population density of fewer than 1314 households, or locations with between 107 and 1314 people per square mile (Pew Research Center, 2018). This distinction categorizes large urban areas more accurately, even if distant from the urban center (Pittsburgh). Locations not within 12 miles of a city with a population density of fewer than 107 people per square mile were classified as rural (Pew Research Center, 2018).

Thus, this study utilizes a population density-based classification system to determine if sites were suburban, urban, or rural. While both population density and household density are common variables to use while sorting sites into their respective rural-urban category classifications, these variables alone may be insufficient to classify an area as rural, urban, or suburban. Some studies suggest that measures involving forest fragmentation and development percentage may create more accurate analyses of land use (Diuk-Wasser et al., 2020; Baldwin et al., 2022). To address concerns regarding forest cover and development percentage, these variables were collected from the National Land Cover Database and were analyzed to support the classification system selected. Namely, forest cover and development percentage were analyzed alongside population density to ascertain if these variables were correlated, and to what degree. If these variables were correlated, using population density as a proxy for these variables may be more justified.

Along with forest cover, forest composition may play an important role in pathogen prevalence among *I. scapularis ticks* (Adalsteinsson et al., 2012; Combs et al., 2021). Thus, any

correlations between pathogen prevalence and forest composition, namely the percent of land covered by shrub, scrub, grassland, or herbaceous plant life as well as the percent of pasture/hay/cultivated crops, were also analyzed. Given that suburban, rural, and urban sites may have different forest composition, any correlations may assist in establishing what factors may impact pathogen prevalence.

2.3 Statistical Analyses

The prevalence of each pathogen was calculated by dividing the total number of positive nymphs by the total number of nymphs collected for a given year, a given site, or a given site classification as appropriate. Nymphs positive for co-infection are represented both in the number of samples positive for co-infection, as well as within each individual pathogen prevalence. Statistical analyses were performed using GraphPad Prism 9.5.1. Fisher's exact tests were used to compare prevalence at each site, as well as overall and by site classification, from 2021 to 2022. A Pearson *r* correlation of data was used to explore the relationships between population density, forest cover, forest composition, and pathogen prevalence. One-way ANOVAs were used to compare pathogen prevalence in ticks from urban, suburban, and rural sites. Based on the one-way ANOVA results, two-sample t-tests were used to compare pathogen prevalence between urban and rural; suburban and urban; and urban and rural sites as needed. Figures were created using GraphPad Prism 9.5.1. Maps were created using ArcGIS, University of Pittsburgh license.

3.0 Results

3.1.1 *Ixodes scapularis* Collection and *Borrelia spp.* Prevalence Summary

In total, 1,812 nymphal *I. scapularis* were collected between 2021 and 2022. Of these, 913 were collected in 2021, and the remaining 899 were collected in 2022 (**Table 1**). Thus, 51% of the nymphs tested in this study were from 2021 and 49% were from 2022. The greatest number of *I. scapularis* nymphs in 2021, 2022, and overall were collected from SGL #42 (**Fig. 1A**). The smallest number of nymphs collected from one site overall was 228 at SGL #232, equating to approximately 13% of the nymphs collected overall (**Table 1**, **Fig. 1**). The greatest disparity in *I. scapularis* nymphs collected in 2021 and 2022 was found in SCH, as 176 (73.9%) of nymphs collected from the park were found in 2021 alone (**Table 1**, **Fig. 1A**).

All 1,812 *I. scapularis* nymphs were analyzed for *B. burgdorferi* and *B. miyamotoi*. Of these ticks, 28.8% were infected with *B. burgdorferi* (n = 521) and 0.8% were infected with *B. miyamotoi* (n = 14) and 0.1% were co-infected with *B. burgdorferi* and *B. miyamotoi* (n = 2) (**Table 1**). Of nymphs collected in 2021, 27.6% were infected with *B. burgdorferi* (n = 252), and 0.5% were infected with *B. miyamotoi* (n = 5) (**Table 1, Fig. 2**). None of the *I. scapularis* nymphs collected in 2021 were co-infected with *B. miyamotoi* and *B. burgdorferi*. Of nymphs collected in 2022, 29.9% were infected with *B. burgdorferi* (n = 269), and 1.0% were infected with *B. miyamotoi* (n = 9). Of these, 0.1% were co-infected with *B. burgdorferi* and *B. miyamotoi* (n = 2) (**Table 1, Fig. 2**). Furthermore, *B. burgdorferi* and *B. miyamotoi* prevalence increased from 2021 to 2022, although not significantly (p = 0.27 and p = 0.29, respectively) (**Table 1**).

Table 1. *Borrelia burgdorferi*, *Borrelia miyamotoi*, and *Borrelia* species co-infection prevalence in *Ixodes scapularis* nymphs collected by site in 2021 and 2022.

		BOM	CCH	RVP	SCH	SGL #232	SGL #42	Total
2021	No. Nymphs Collected	131	140	133	176	144	189	913
	#No. Positive *B. burgdorferi* (%)	26 (19.8%)	25 (17.9%)	36 (27.1%)	65 (36.9%)	44 (30.6%)	56 (29.6%)	252 (27.6%)
	#No. Positive *B. miyamotoi* (%)	1 (0.8%)	0 (0.0%)	2 (1.5%)	0 (0.0%)	1 (0.7%)	1 (0.5%)	5 (0.5%)
2022	No. Nymphs Collected	126	110	244	62	84	273	899
	#No. Positive *B. burgdorferi* (%)	26 (20.6%)	26 (23.6%)	76 (31.1%)	23 (37.1%)	40 (47.7%)	78 (28.6%)	269 (29.9%)
	#No. Positive *B. miyamotoi* (%)	0 (0.0%)	2 (1.8%)	3 (1.2%)	1 (1.6%)	0 (0.0%)	3 (1.1%)	9 (1.0%)
	#No. Positive for *Borrelia* Co-infection (%)	0 (0.0%)	0 (0.0%)	1 (0.3%)	0 (0.0%)	0 (0.0%)	1 (0.2%)	2 (0.2%)
Overall	No. Nymphs Collected	257	250	377	238	228	462	1812
	#No. Positive *B. burgdorferi* (%)	52 (20.2%)	51 (20.4%)	112 (29.7%)	88 (36.9%)	84 (36.8%)	134 (29.0%)	521 (28.8%)
	#No. Positive *B. miyamotoi* (%)	1 (0.3%)	2 (0.8%)	5 (1.3%)	1 (0.4%)	1 (0.4%)	4 (0.9%)	14 (0.8%)
	#No. Positive for *Borrelia* Co-infection (%)	0 (0.0%)	0 (0.0%)	1 (0.4%)	0 (0.0%)	0 (0.0%)	1 (0.4%)	2 (0.1%)
Change from 2021 to 2022	*B. burgdorferi* Prevalence	0.8%	5.8%	4.1%	0.2%	17.1%*	-1.0%	2.3%
	B. miyamotoi Prevalence	-0.8%	1.8%	-0.3%	1.6%	-0.7%	0.6%	0.5%
	Borrelia Co-infection Prevalence	0.0%	0.0%	0.4%	0.0%	0.0%	0.4%	0.2%

*p = 0.01. BOM: Boyce Mayview Park, CCH: Crooked Creek Horse Park, RVP: Riverview Park, SCH: Schenley Park, SGL #232: State Game Lands #232, SGL #42: State Game Lands #42

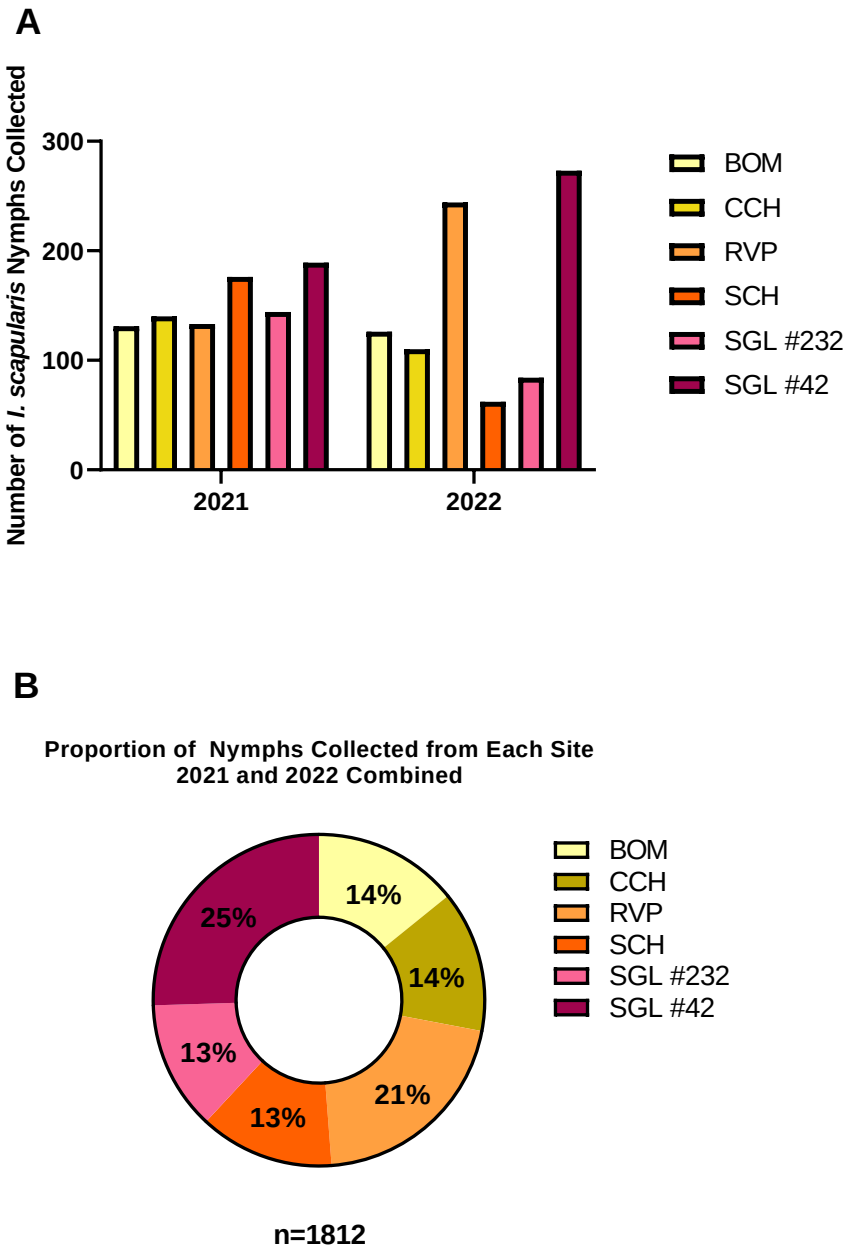

Figure 1. *Ixodes scapularis* nymphs collected by site, 2021 and 2022. A) The number of *I. scapularis* nymphs collected by site. B) The proportion of ticks contributed to the total number of nymphs collected in both 2021 and 2022 (*n* = 1812) by site.

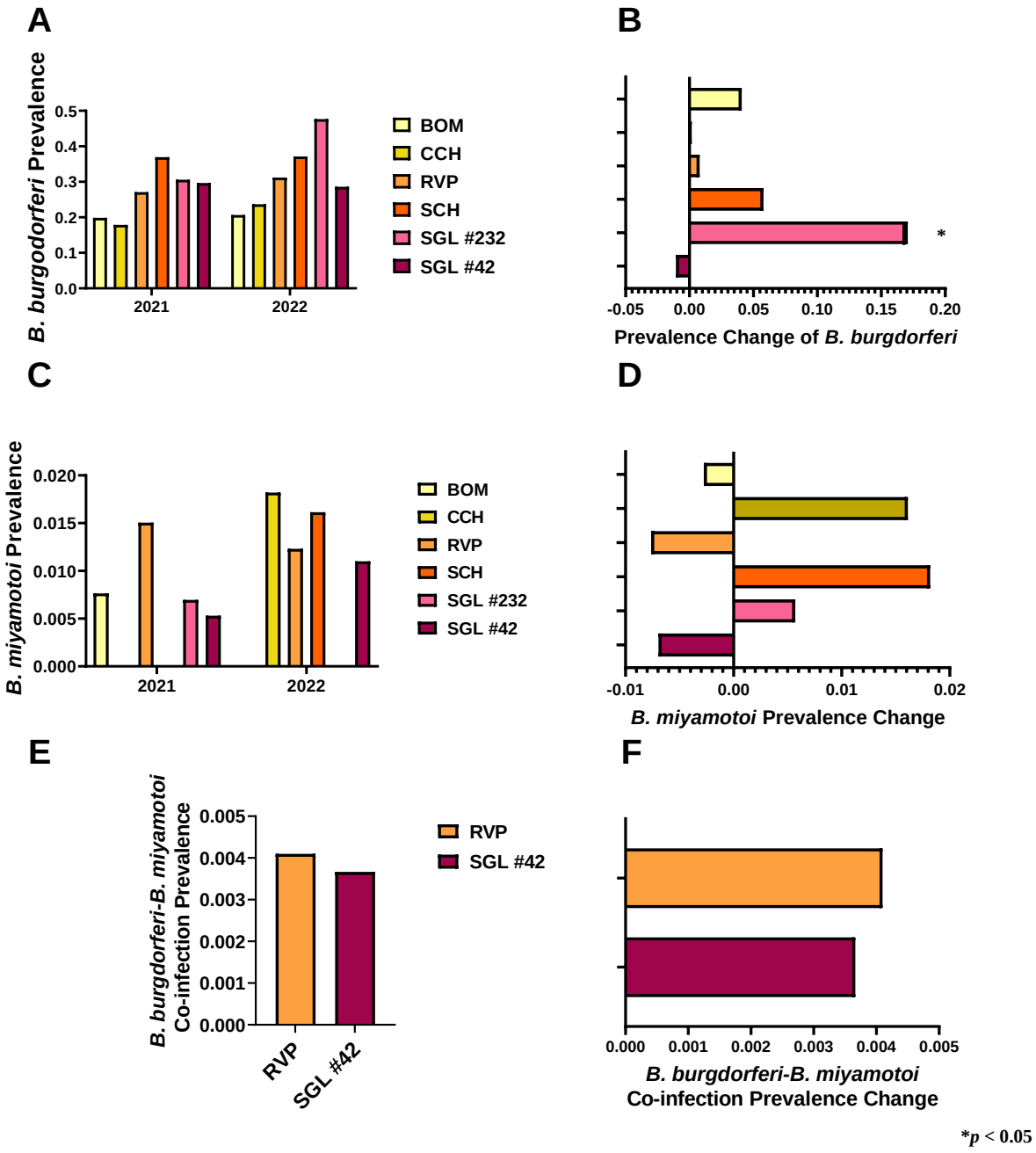

Figure 2. Pathogen prevalence in *Ixodes scapularis* nymphs by site. A) The prevalence of *B. burgdorferi* in *I. scapularis* nymphs collected by site. B) *B. burgdorferi* prevalence change from 2021 to 2022 by site. C) The prevalence of *B. miyamotoi* in *I. scapularis* nymphs collected by site classification. D) The *B. miyamotoi* prevalence change from 2021 to 2022 by site. E) *B. burgdorferi-B. miyamotoi* co-infection prevalence by site classification. Only sites where co-

infected nymphs were found are listed. F) *B. burgdorferi-B. miyamotoi* co-infection prevalence change by site. Only sites where co-infected nymphs were found are listed.

3.1.2 *Ixodes scapularis* Collection and *Borrelia spp.* Prevalence by Site

At an individual site level, there was no significant change between 2021 and 2022 in the prevalence of *B. burgdorferi* 2021 to 2022, except for SGL #232. At SGL #232, the prevalence of *B. burgdorferi* increased from 30.6% in 2021 to 47.7% in 2022 ($p = 0.01$). The prevalence of *B. burgdorferi* increased at all sites except for SGL #42, which experienced a 1.0% decrease in *B. burgdorferi* prevalence from 2021 to 2022 (**Table 1, Fig. 2**). *Borrelia miyamotoi* was found at every location sampled, thus indicating its presence throughout southwest Pennsylvania. Sites to the west of the city of Pittsburgh all experienced decreases in the prevalence of *B. miyamotoi*, while sites to the east of Pittsburgh experienced an increase in *B. miyamotoi* prevalence from 2021 to 2022 (**Table 1, Fig. 2**). *Borrelia burgdorferi* and *B. miyamotoi* co-infection was observed only at two parks, RVP and SGL #42 (**Table 1**).

3.1.3 Rural, Suburban, and Urban Site Classification

When selecting the size buffers for this analysis, caution was taken to ensure that buffers would most accurately represent each site. Large buffers (900 m and 1200 m) captured too much information, thus inappropriately skewed the population density calculations, and captured other land features that did not impact the site itself. Conversely, buffers that were too small (300 m) also misrepresented the area due to excluding important nearby housing complexes. Grid sites visited in this study were 600 m^2, and thus, a 600 m buffer was selected for this analysis. Overall,

two locations each were determined to be rural, suburban, and urban (**Table 2, Fig. 3**). Both State Game Land sites were identified as rural, while BOM and CCH were identified as suburban. SCH and RVP were identified as urban parks, both with well over 1314 people per square mile and within Pittsburgh city limits.

Table 2. 2022 U.S. Census population density and status as within 12 miles of an urban center (Pittsburgh) at each site.

Site	**Population Density (people/mile^2)**	**Within 12 Miles of Urban Center**	**Classification**
BOM	1248.1	Yes	Suburban
CCH	112.4	Yes	Suburban
RVP	2955	Yes	Urban
SCH	2766.9	Yes	Urban
SGL #232	0	No	Rural
SGL #42	55.1	No	Rural

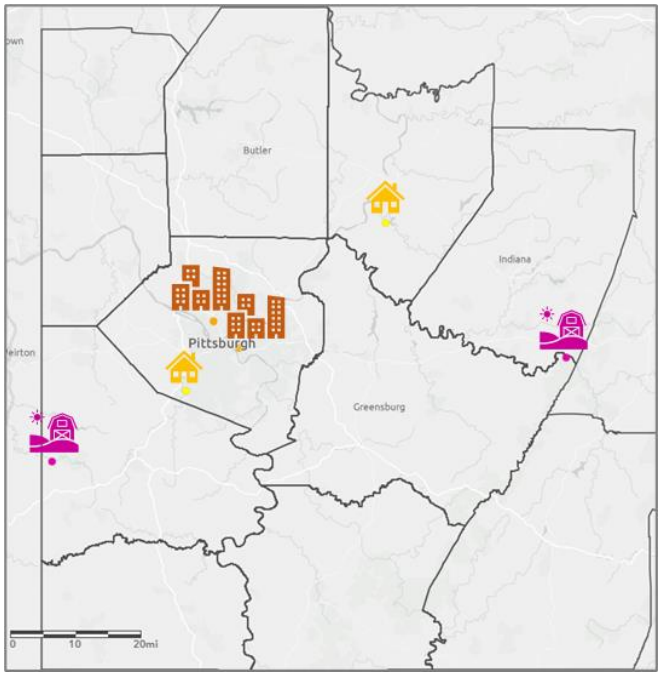

Figure 3. Map of sites. Suburban sites (BOM and CCH) are labeled in yellow, urban sites (RVP and SCH) are labeled in orange, and rural sites (SGL #232 and SGL #42) are labeled in pink.

3.1.4 Site Classification Support and Factor Correlation Analysis

Population density was the primary factor which led to the classification of sites as suburban, urban, and rural. While some studies suggest that forest cover may be a better metric to use when analyzing pathogen dynamics in ticks across a diversely developed landscape, there is a strong negative correlation between population density and percent forest cover (r (6) = -0.91, p = 0.01) (**Fig. 4**). Population density and development percentage (r (6) = 0.95, p = 0.003) are strongly positively correlated to one another and can be used in tandem to gather more multi-dimensional information on sites (**Fig. 4**). Like population density, development percentage and percent forest cover are negatively correlated as well (r (6) = -0.97, p = 0.002) (**Fig. 4**). Thus, population density

may act as a similar metric or proxy for forest cover percentage and development percentage, supporting its use in this study.

Population density was negatively correlated with *B. burgdorferi* prevalence change, although not significantly (r (6) = -0.39, p = 0.45) (**Fig. 4**). Population density was weakly correlated with *B. burgdorferi* prevalence overall (r (6) = 0.26, p = 0.62) as well as *B. miyamotoi* prevalence overall (r (6) = 0.26, p = 0.62) although additionally not significant (**Fig. 4**). However, percentage of shrub, scrub, grassland, or herbaceous plant life was strongly positively correlated with *B. burgdorferi* prevalence change (r (6) = 0.84, p = 0.04) and, while not significant, moderately positively correlated with *B. burgdorferi* prevalence overall (r (6) = 0.44, p = 0.38). On average, the rural sites had a higher percentage of shrubs, scrub, and grasslands compared to rural and suburban sites. Interestingly, the percentage of pasture, hay, or cultivated crops was positively correlated with *B. burgdorferi* prevalence change (r (6) = 0.77, p = 0.07), but weakly negatively correlated with *B. burgdorferi* prevalence overall (r (6) = -0.15, p = 0.77) although not significantly.

B. burgdorferi prevalence change was positively correlated with *B. burgdorferi* prevalence overall (r (6) = 0.34, p = 0.51) but weakly negatively correlated with both *B. miyamotoi* prevalence change (r (6) = -0.36, p = 0.49) and *B. miyamotoi* prevalence overall (r (6) = -0.16, p = 0.76) (**Fig. 4**). While not significant, the relationship between these two pathogens should be continually monitored as more data becomes available from these sites.

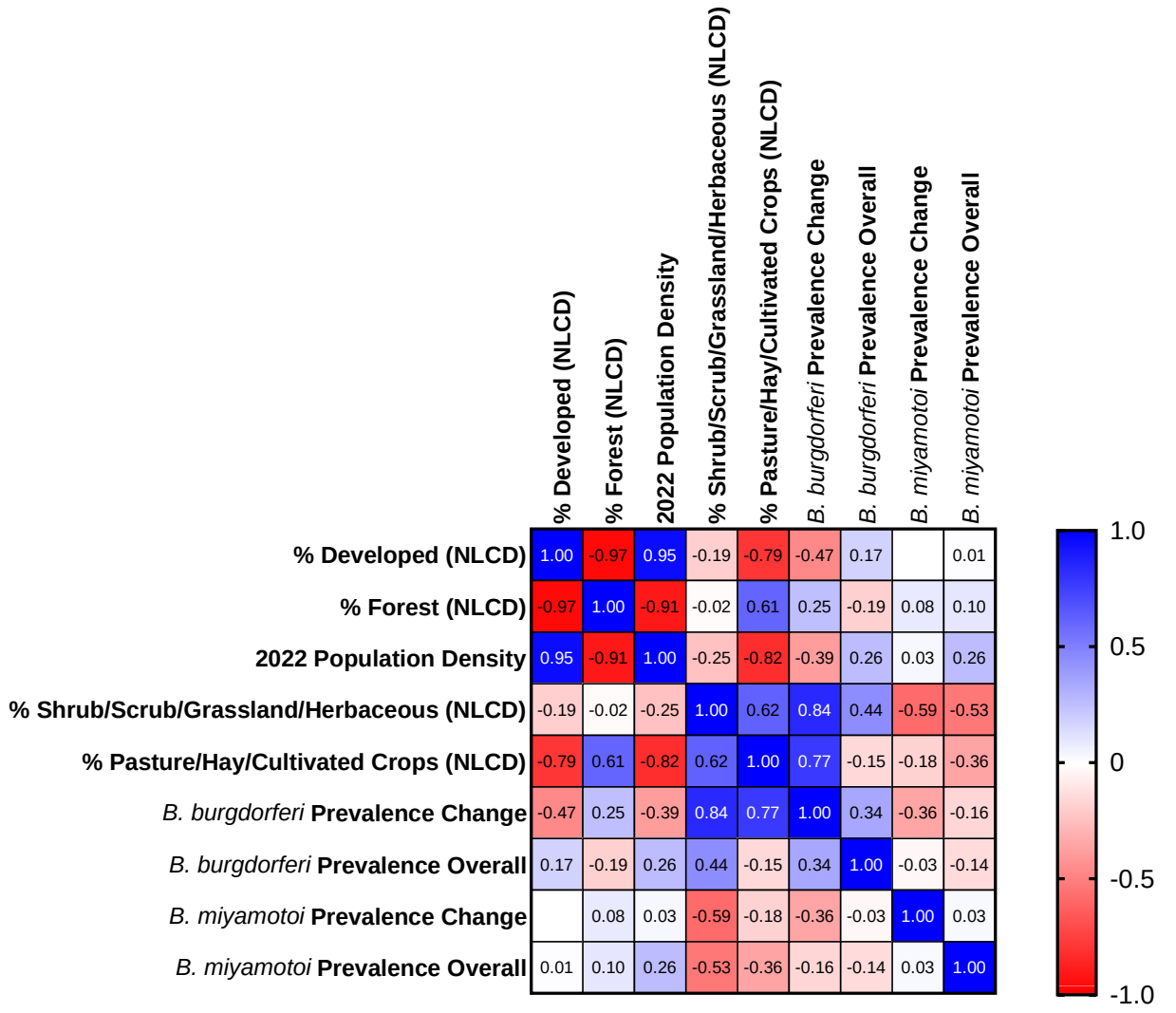

Figure 4. Pearson *r* correlation of data. Data includes National Land Cover Database (NCLD) development percentage, forest composition, and forest cover percentage as well as US 2022 Census data on population density. Positive correlations may indicate that there may be a linear relationship between two variables such that if one variable increases, the other does as well. Conversely, negative correlations may indicate that as one variable increases, the other decreases. Correlations with a value of zero indicate that there is no correlation between the variables.

3.1.5 Infection Prevalence in Rural, Suburban, and Urban Sites

The suburban parks in this study, BOM and CCH, included 507 nymphs tested in this study, or 28.0% of the nymphs tested overall (**Fig. 5**). In total, 271 (53.5%) nymphs were collected in 2021, and 236 (46.5%) nymphs were collected in 2022 (**Table 3**). Of these ticks, 20.3% were infected with *B. burgdorferi* (n = 103) and 0.6% were infected with *B. miyamotoi* (n = 3) (**Table 3, Fig. 6**). No ticks were co-infected with *B. burgdorferi and B. miyamotoi* in suburban parks sampled. The urban parks in this study, RVP and SCH, included 615 nymphs tested in this study, or 34.0% of the nymphs tested overall (**Fig. 5**). In total, 309 (50.2%) nymphs were collected in 2021, and 306 (49.8%) nymphs were collected in 2022 (**Table 3**). Of these ticks, 32.5% were infected with *B. burgdorferi* (n = 200) and 1.0% were infected with *B. miyamotoi* (n = 6) (**Table 3, Fig. 6**). Only one nymph (0.2%) was found to be co-infected with *B. burgdorferi and B. miyamotoi.* The rural parks in this study, SGL #232 and SGL #42, included 690 nymphs tested in this study, or 38.0% of the nymphs tested overall (**Fig. 5**). In total, 333 (48.3%) nymphs were collected in 2021, and 357 (51.7%) nymphs were collected in 2022 (**Fig. 5**). Of these ticks, 31.6% were infected with *B. burgdorferi* (n = 218) and 0.9% were infected with *B. miyamotoi* (n = 5) (**Table 3, Fig. 6**). Only one nymph (0.1%) was found to be co-infected with *B. burgdorferi* and *B. miyamotoi* (**Table 3**). Overall, there was no significant difference in the prevalence of *Borrelia* species co-infection between urban and rural sites (**Table 3, Fig. 6**). Through one-way ANOVA, it was determined that *B. burgdorferi* prevalence did significantly differ between site classification (F (2, 3) = 27.65, p = 0.01), whereas *B. miyamotoi* prevalence did not (F (2, 3) = 0.59, p = 0.61). Specifically, there was a significant difference in *B. burgdorferi* prevalence between rural and suburban sites (t (2) = 5.04, p = 0.04) and urban and suburban sites (t (2) = 7.48, p = 0.02). The

difference in *B. burgdorferi* prevalence was not significant between urban and rural sites (t (2) = 0.64, p = 0.59).

Overall, an increase in the prevalence of both *Borrelia* species from 2021 to 2022 was observed. *Borrelia burgdorferi* prevalence increased by 2.3% (p = 0.2297), while *B. miyamotoi* prevalence increased by 0.5% (p > 0.99) (**Table 3**). While neither increase was significant, the cumulative effect of annual infection prevalence increases may result in much higher pathogen prevalence over time. In 2022, co-infection with both *Borrelia* species was observed in two of the sampled areas for the first time (n = 2). Albeit not significant, the greatest prevalence changes of *Borrelia* species were observed in rural parks with the prevalence of *B. miyamotoi* increasing from 0.6% in 2021 to 0.8% in 2022 (p > 0.99) and the prevalence of *B. burgdorferi* increasing from 30.0% in 2021 to 33.0% in 2022 (p = 0.41). The suburban and urban parks sampled both saw decreases in the overall prevalence of *B. miyamotoi* from 2021 to 2022 (p = 0.61 and p = 0.45, respectively). However, establishing the prevalence of *B. miyamotoi* will act as a baseline to monitor future changes in *B. miyamotoi* prevalence throughout western PA. While the prevalence of *B. burgdorferi* remained stable in urban parks from 2021 to 2022 (at 32.7% and 32.4% respectively, p = 0.93), the prevalence of *B. burgdorferi* increased in suburban parks from 18.8% in 2021 to 22.0% in 2022 (p = 0.38).

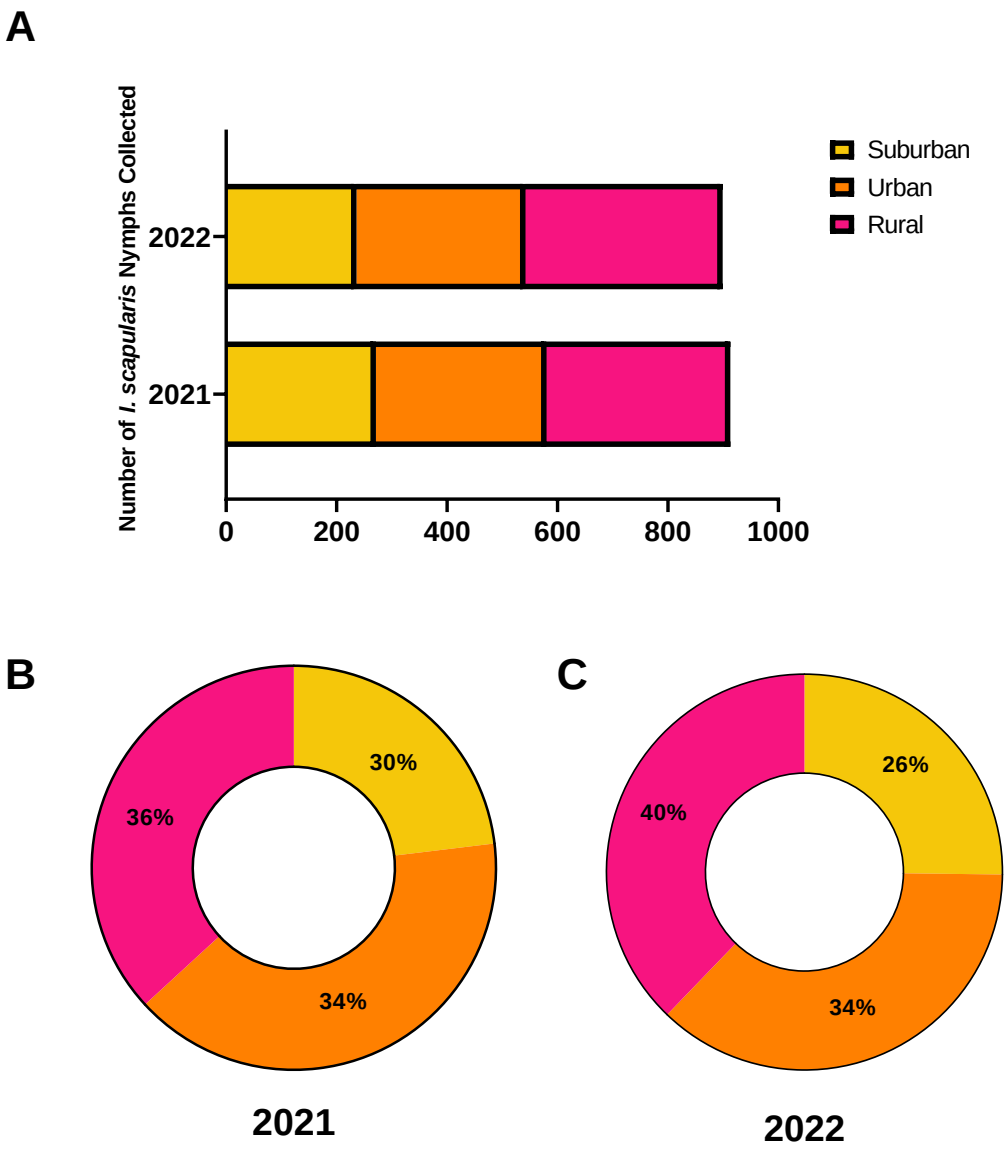

Figure 5. Summary of *Ixodes scapularis* nymphs collected by site classification. A) The number of *I. scapularis* nymphs collected by site classification: suburban (yellow), urban (orange), and rural (pink) sites, 2021 to 2022. B) The proportion of ticks contributed to the total number of nymphs collected in 2021 (*n* = 912) by site classification. C) The proportion of ticks contributed to the total number of nymphs collected in 2022 (*n* = 899), by site classification.

Table 3. *Borrelia burgdorferi*, *Borrelia miyamotoi*, and *Borrelia* species co-infection prevalence in *Ixodes scapularis* nymphs collected by site classification, 2021 to 2022.

		Suburban	Urban	Rural	Total
2021	No. Nymphs Collected	271	309	333	913
	#No. Positive *B. burgdorferi* (%)	51 (18.8%)	101 (32.7%)	100 (30.0%)	252 (27.6%)
	#No. Positive *B. miyamotoi* (%)	1 (0.4%)	2 (0.6%)	2 (0.6%)	5 (0.5%)
2022	No. Nymphs Collected	236	306	357	899
	#No. Positive *B. burgdorferi* (%)	52 (22.0%)	99 (32.4%)	118 (33.0%)	269 (29.9%)
	#No. Positive *B. miyamotoi* (%)	2 (0.8%)	4 (1.3%)	3 (0.8%)	9 (1.0%)
	#No. Positive for *Borrelia* Co-infection (%)	0 (0.0%)	1 (0.3%)	1 (0.3%)	2 (0.2%)
Overall	No. Nymphs Collected	507	615	690	1812
	#No. Positive *B. burgdorferi* (%)	103 (20.3%)	200 (32.5%)	218 (31.6%)	521 (28.8%)
	#No. Positive *B. miyamotoi* (%)	3 (0.6%)	6 (1.0%)	5 (0.7%)	14 (0.7%)
	#No. Positive for *Borrelia* Co-infection (%)	0 (0.0%)	1 (0.2%)	1 (0.1%)	2 (0.1%)
Change from 2021 to 2022	*B. burgdorferi* Prevalence Change	3.2%	-0.3%	3.0%	2.3%
	B. miyamotoi Prevalence Change	0.4%	0.7%	0.2%	0.5%
	Borrelia Co-infection Prevalence Change	0.0%	0.3%	0.3%	0.2%

BOM: Boyce Mayview Park, CCH: Crooked Creek Horse Park, RVP: Riverview Park, SCH: Schenley Park, SGL #232: State Game Lands #232, SGL #42: State Game Lands #42

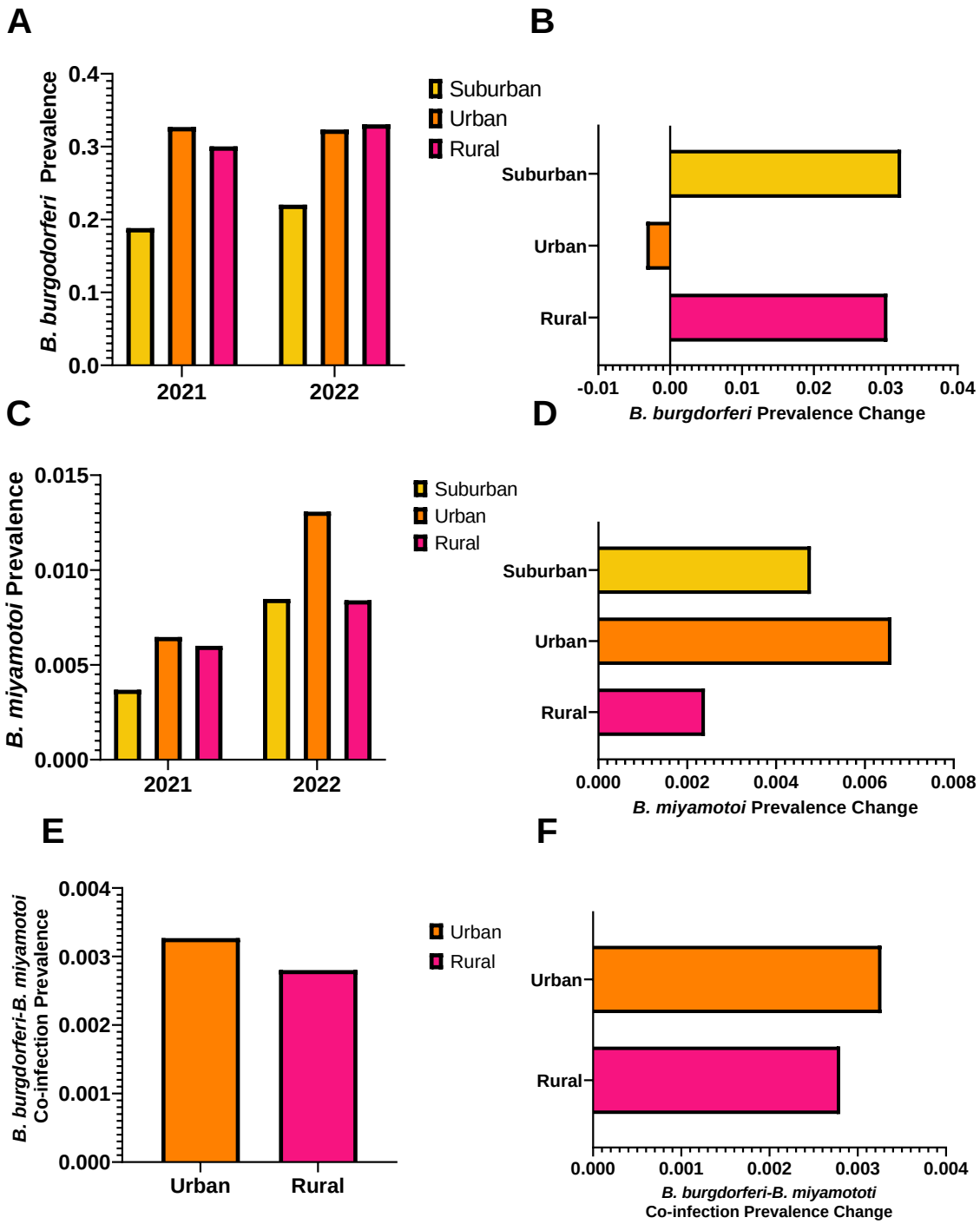

Figure 6. Pathogen prevalence in *Ixodes scapularis* nymphs by site classification, comparing 2021 and 2022. A) The prevalence of *B. burgdorferi* in *I. scapularis* nymphs collected by site classification: suburban (yellow), urban (orange), and rural (pink) sites, 2021 and 2022. B) The *B. burgdorferi* prevalence change from 2021 to 2022 by site classfication. C) The prevalence of

B. miyamotoi in *I. scapularis* nymphs collected by site classification. D) The *B. miyamotoi* prevalence change from 2021 to 2022 by site classfication. E) *B. burgdorferi-B. miyamotoi* co-infection prevalence by site classification. No co-infected nymphs were found in suburban sites. F) *B. burgdorferi-B. miyamotoi* co-infection prevalence change by site classification. No co-infected nymphs were found in suburban sites.

4.0 Discussion

4.1 Pathogen Prevalence Across Suburban, Urban, and Rural Locations

This study examined the prevalence of medically important tick-borne pathogens in *I. scapularis* nymphs collected across a multiyear longitudinal study in southwest PA. We aimed to better understand the change in infection prevalence from 2021 to 2022, as well as explore the differences in *Borrelia* species prevalence among suburban, urban, and rural locations. Additionally, we aimed to explore factors that may influence *Borrelia* species prevalence across suburban, urban, and rural locations and determine if using a population density-based approach would be suitable for defining site classification in the future.

The pathogen prevalence observed between suburban, urban, and rural locations provides evidence to suggest that the rate in which co-infection occurs and changes annually is different between location classifications. Thus, individuals living near urban parks and rural locations may be at higher risk of *B. burgdorferi*, *B. miyamotoi*, and *Borrelia* co-infection when compared to suburban areas. As aforementioned, the percentage of shrub, scrub, grassland, or herbaceous plant life as well as the percentage of pasture, hay, and cultivated crops were strongly positively correlated with *B. burgdorferi* prevalence change. On average, the rural sites had a higher percentage of shrubs, scrub, and grasslands compared to suburban and urban sites. Rural and suburban sites also had a higher percentage of pasture, hay, and cultivated crops. Given the relatively stable prevalence of *B. burgdorferi* in urban locations compared to the increase in *B. burgdorferi* prevalence in rural and suburban sites from 2021 to 2022, it is possible that forest composition is a major factor influencing the prevalence change of *B. burgdorferi* in *I. scapularis*

nymphs. This may impact *B. burgdorferi* prevalence change by altering the suitability of the environment for highly competent reservoir hosts (Prusinski et al., 2006; Adalsteinsson et al., 2018; Reaser et al., 2021). As aforementioned, the abundance of shrubs like Japanese barberry may alter *B. burgdorferi* infection prevalence among ticks by providing the ideal habitat for reservoir hosts such as *P. leucopus* (Prusinski et al., 2006; Williams et al., 2009; Williams et al., 2010 Reaser et al., 2021). It is possible that the higher presence of herbaceous plant life is indeed sustaining a greater abundance of pathogen-competent reservoir hosts, which allow for *I. scapularis* nymphs and larvae to continually feed upon infected hosts for each blood meal. This would particularly explain the increasing pathogen prevalence over time, as a highly suitable habitat for mammals like *P. leucopus* would permit *B. burgdorferi* to be sustained in both the tick and mouse populations more readily. Abundant herbaceous growth was observed at BOM and SGL #232, where *B. burgdorferi* prevalence increased from 2021 to 2022. Thus, these locations may feature plentiful species of shrubs such as Japanese barberry, which could support a robust small mammal population and propagate *B. burgdorferi* infection in ticks.

In the urban locations sampled, there is a stable threat of tick-borne illness that poses risk to possibly several hundred people daily during the summer months. Suburban parks featured a significantly lower prevalence of *B. burgdorferi*-positive nymphs on average at 20.3%, and these sites yielded the lowest number of ticks as well (only 507 *I. scapularis* nymphs compared to 615 found in urban parks and 690 found in rural areas). We originally hypothesized that the abundance of competent hosts would be the primary driving factor in pathogen prevalence, resulting in greater pathogen prevalence in suburban areas where *Borrelia* species can be sustained among several populations that live in nearby forest patches. However, it is possible that suburban parks in southwest Pennsylvania host smaller white-tailed deer (*Odocoileus virginianus*) populations,

which is a factor theorized to limit *I. scapularis* tick populations by reducing the overall number of hosts that adult *I. scapularis* can feed upon (Kilpatrick et al., 2014). Ultimately, the composition of hosts across these sites may impact the overall prevalence and prevalence change of these pathogens. For example, it is possible that *B. miyamotoi* prevalence across southwest PA could be facilitated by host animal migration. This study, alongside others researching *Borrelia spp.* prevalence found that the overall rate of *B. miyamotoi* is much lower than that of *B. burgdorferi* annually (Barbour et al., 2009; Crowder et al., 2014; Eisen & Eisen, 2018). In 2010, studies of *I. scapularis* nymphs across PA suggested that *B. miyamotoi* prevalence may range from 0-0.3% (Barbour et al., 2010). More recent studies of *B. miyamotoi* prevalence in Pennsylvania focus on adult *I. scapularis*, and therefore, there was previously no established baseline of *B. miyamotoi* prevalence in *I. scapularis* nymphs in southwest PA. Our data provides baseline *B. miyamotoi* prevalence that will allow for future studies on the changes in *B. miyamotoi* prevalence and will assist in research endeavors to continue to explore how the pathogen dynamics change over time and geographical location. For example, the occurrence of co-infection with *B. miyamotoi* in both an urban park and a rural site may suggest that a highly mobile host organism, such as a bird, could assist in dispersing ticks containing *B. miyamotoi* into novel areas (Majerova et al., 2020; Keesing et al., 2021). Birds, including American robins and gray catbirds, are cited as possible hosts for *B. miyamotoi* alongside small mammals such as mice and shrews (Keesing et al., 2021). The two locations with *Borrelia* co-infected nymphs were sites where shrews such as *Sorex* species and *Blarina brevicauda* (short-tailed shrews) were captured (unpublished data). The overall number of shrews captured at those sites was higher in 2021 than in 2022, as well (unpublished data). *Blarina brevicauda* was found in New York to be a possible major host of *B. miyamotoi*, with 34% of short-tailed shrews sampled testing positive for the pathogen (Keesing et al., 2021). That said,

current research suggests that *B. miyamotoi* is present across the Northeast and upper Midwest in the United States in growing numbers (Xu et al., 2021; Fleshman et al., 2022). These factors may play a role in the overall changes of *B. miyamotoi* prevalence over time, which is now more readily observable as baseline data is established. This may help with contextualizing human cases of hard tick relapsing fever in the future across southwest PA.

As aforementioned, a study published in 2019 suggested that long-term or severe Lyme disease symptoms may be more prevalent in rural areas due to differences in the ability to access or seek care (Eddens et al., 2019). This same study also suggested that rates of pediatric Lyme disease cases were increasing in non-rural areas while decreasing in rural areas (Eddens et al., 2019). Previous data collected in 2016 from RVP and SCH suggested a lower prevalence of *B. burgdorferi* in *I. scapularis* nymphs, at 26.2% and 19.3% respectively (Simmons et al., 2020). Our data suggests the prevalence of *B. burgdorferi* in nymphs collected from RVP and SCH has risen to 31.1% and 37.1% respectively in 2022, suggesting that previous assertions regarding an increase in Lyme disease cases in urban locations may indeed be reflected in the vector species. The urban sites sampled in this study both feature population densities of over 2760 households within 600 m, and thus there is potentially a large population at-risk of *B. burgdorferi* infection alone. However, the rate at which the prevalence of *B. burgdorferi* in nymphs is growing in rural areas (3.0% between 2021 to 2022) suggests that decreasing numbers of pediatric Lyme disease cases in rural communities may be a result of reduced healthcare access or a behavioral change (Eddens et al., 2019). The results of this research, when combined with pre-existing knowledge surrounding Lyme disease in humans in southwest PA, suggest that the increasing prevalence of Lyme disease may be shared between rural and urban communities, but rural communities lack the ability to access proper testing or treatment for the disease.

The high prevalence of *B. burgdorferi* in rural communities could be facilitated by the presence of other tick-borne pathogens in nymphs as well, such as *B. microti*. *Borrelia burgdorferi* infection can occur in tandem with *B. microti*, an intraerythrocytic, apicomplexan protozoan that causes babesiosis in humans (Westblade et al., 2017). *Babesia microti* exhibits vertical transmission in mice, leading to successive generations of *P. leucopus* infecting ticks without needing prior direct contact with an infected tick (Tufts & Diuk-Wasser, 2018; Tufts & Diuk-Wasser, 2021). Thus, *B. microti* circulation can continue in mouse populations in the absence of ticks and can reintroduce *B. microti* to previously naïve tick populations (Tufts & Diuk-Wasser, 2018). Since *B. microti* is cited as possibly facilitating *B. burgdorferi* infection in ticks, the high and rising prevalence of *B. burgdorferi* infection could be related to circulating *B. microti* in the host population (Moro et al., 2002; Dunn et al., 2014). Thus, any location, regardless of being rural or non-rural, that maintains robust tick communities with a suitable environment for highly competent reservoir hosts may display a high prevalence of *B. burgdorferi*.

The Pennsylvania Department of Health stated in reports published in both 2020 and 2021 that cases of Lyme disease reported in 2020 and 2021 are likely underestimates of the disease due to testing limitations that arose during the COVID-19 pandemic (Pennsylvania Department of Health, 2021; Pennsylvania Department of Health, 2022). In 2021, only 2,900 confirmed and probable cases of Lyme disease were reported in PA, a substantial decrease in cases when compared to the 9,008 cases of Lyme disease reported in PA in 2019 (Pennsylvania Department of Health, 2021; Pennsylvania Department of Health, 2023). Therefore, it is unclear the overall prevalence and incidence of Lyme disease in human populations in 2020 and 2021 given the challenges presented by the COVID-19 pandemic. Additionally, as of January 1, 2022, states with a high incidence of Lyme disease are now required to only count and report cases of the disease

with laboratory evidence (Pennsylvania Department of Health, 2023). This may result in an artificial decrease in the Lyme disease cases reported in rural areas, since healthcare access and testing can be limited. Thus, the burden of Lyme disease in rural communities may be inadequately represented and may not emphasize the need for education and resources to be allocated to certain regions appropriately. By researching the prevalence of pathogens such as *B. burgdorferi* in *I. scapularis* nymphs in rural areas, we may be able to ascertain if any decreases in the number of human cases are reflective of the negative effects of case reporting changes, or reflective of a true change in the prevalence of Lyme disease in rural communities.

Furthermore, our data provides critical insight into the continued education and public health intervention that is needed to prevent tick-borne illness throughout southwest Pennsylvania. Given the growing risk overall of *Borrelia* species and co-infection particularly in rural communities, public health efforts should focus on meeting the healthcare needs and building medical trust within some of these rural and remote communities. Educational efforts to prevent tick bites should be universally promoted, with particular emphasis in urban parks which feature high rates of *B. burgdorferi* in *I. scapularis* nymphs. By continuing to promote Lyme disease and hard tick relapsing fever awareness, communities at large can mitigate their risk as policies to improve human tick-borne disease reporting is better established throughout PA.

5.0 Limitations and Future Directions

This research was conducted across six grid sites, which provided insight into southwestern PA's tick population. However, there are some factors that limited the scope of this analysis.

Unfortunately, there is no standard protocol for identifying sites as rural, suburban, or urban. The 2018 Pew Research Study's suburban, urban, and rural definitions were, as previously mentioned, based on the number of households per square mile. However, this metric can be incredibly challenging to define across large areas because the average household size varies regionally. Thus, population density was used as a substitute metric. While this deviation from the original definitions was practical for this study, altering this metric may have unintended effects in different regions, should it be replicated. Additionally, data on population density was derived from 2022 alone and was not recalculated to adjust for any changes from 2021 to 2022. Future research should account for the annual changes in development percentage and population density. Additionally, future studies should utilize multiple points from each site to better analyze the development and population density surrounding a site, such as that used in Baldwin et al., 2022. However, utilizing too many data points may also skew the classification process, and therefore was not pursued in this current study.

Our study includes a multi-year analysis; however, this only includes data from 2021 and 2022 alone. This limits the ability to gauge if any changes seen in pathogen prevalence are within expectations regionally, especially by site classification. While the overall changes in prevalence from 2021 to 2022 at suburban, urban, and rural sites were not significant, the potential for annual increasing pathogen prevalence may result in a cumulation of higher pathogen prevalence overall.

Continued monitoring of pathogens across these sites is crucial to growing our understanding of how landscape may impact pathogen dynamics.

Additionally, our analysis did not include more granular analyses of the kind of foliage species present in each site. While we did include analysis of the general kinds of foliage present (i.e. the percentage of shrub, scrub, grassland, or herbaceous plant life as well as the percentage of pasture, hay, and cultivated crops), we did not include species-level identification of shrubs and other plant life in each location. Future research will include more detailed information regarding the diversity and abundance of plant life in each site. However, the overall strong and significant relationship between shrub, scrub, grassland, or herbaceous plant life with *B. burgdorferi* prevalence change opens the possibility for research that aims to model and predict pathogen prevalence changes across southwest Pennsylvania based on environmental data such as forest composition.

The prevalence of *B. microti* could not be ascertained for the ticks sampled in this study but is a central focus of future research endeavors. *Borrelia burgdorferi* and *B. microti* are the most common combination of tick-borne pathogen co-infection in humans, occurring in up to 81% of co-infection cases in New England (Swanson et al., 2006; Hersh et al., 2014). However, given the lack of reporting requirements for human cases of babesiosis, the actual incidence of human babesiosis in PA is not clear (Liu et al., 2019). In a mouse model, it appears that *B. microti* may enhance *B. burgdorferi* colonization in certain tissues (Moro et al., 2002; Dunn et al., 2014). This suggests that *B. microti* could increase *B. burgdorferi* colonization in human tissues, which could lead to worsened symptoms of Lyme disease (Moro et al., 2002). Given the possible interactions of *B. burgdorferi* and *B. microti* in both human and mammalian hosts, future research will include *B. microti* testing to ascertain the possible risk of *B. microti* alongside *Borrelia* species.

Additionally, *A. phagocytophilum*, *B. mayonii*, rickettsial organisms, and Powassan virus are tick-borne pathogens that are becoming an increasing threat to public health in PA. How these pathogens interact with *Borrelia* species is not well understood. Thus, future research endeavors will include testing for additional pathogens such as *A. phagocytophilum* and arboviruses, given the sparse knowledge on co-infection rates in consideration of non-*Borrelia* pathogens.

It has been suggested that larval ticks may play a greater role in facilitating *B. miyamotoi* transmission due to the transovarial transmission of the pathogen when compared to nymphal ticks (Eisen & Eisen, 2018). *Borrelia miyamotoi* may not persist as long in reservoir hosts when compared to *B. burgdorferi*, thereby limiting the role of reservoir hosts in pathogen amplification (Barbour et al., 2009; Crowder et al., 2014). Future research endeavors should focus on testing larvae alongside nymphs to determine if there is a true difference in *B. miyamotoi* prevalence between the two life stages, as a result. Additionally, the small number of *B. miyamotoi*-infected nymphs is a limitation in this study, as it limits the strength of the conclusions we can draw from this data. Given the specificity of this assay to detect *B. miyamotoi*, it is likely that any positive results observed are truly positive for *B. miyamotoi*, rather than false positives.

This study did not explore the possible blood meal sources of nymphs collected (Goethert et al., 2021). Additional information on possible reservoir hosts that ticks are feeding upon could strengthen, or refute, assertions that suburban, urban, and rural locations may observe differences in pathogen prevalence in ticks due to reservoir host availability. With this, collecting ticks directly from host organisms may be helpful in elucidating the role of these host species, as well as the presence of those host species, in the richness and abundance of pathogens in areas studied (Goethert et al., 2021). This is particularly important in the context of *B. miyamotoi*, as the host

range may include shrews and birds that were previously implicated as stronger reservoir hosts for *B. miyamotoi* than *P. leucopus* in previous studies (Keesing et al., 2021).

There is evidence to suggest that some genetic variants of *B. burgdorferi* may be more pathogenic or cause more specific symptoms than others. A study by Eddens et al. (2019) demonstrated human Lyme disease cases are expanding across the western half of the state. A stark increase in the cases of Lyme disease was found in non-rural zip codes, as well as in the main urban center of Pittsburgh in southwest PA. However, symptoms did differ among cases in rural versus non-rural zip codes, which neurological and rheumatological manifestations of Lyme disease occurring more frequently in rural zip codes (Eddens et al., 2019). This may be related to human factors or may be related to more pathogenic strains of *B. burgdorferi* circulating in rural zip codes. Understanding the genotypic diversity of *B. burgdorferi* in local *I. scapularis* populations would be helpful for future public health interventions, and thus will be explored in future studies. Additionally, human subject data should be supplemented with, but not replaced by, pathogen prevalence research in the main vector species. In this context, the data presented in Eddens et al., 2019 should not be directly compared to nymphal *I. scapularis* pathogen prevalence data. Our data is only intended to illuminate trends seen in human subject data and cannot fully explain results found in human research. It should be noted, however, that our data describes the prevalence of *B. burgdorferi* several years following the period in which the human data was collected in Eddens et al., 2019. There is no comprehensive data on *B. burgdorferi* prevalence in *I. scapularis* nymphs in western PA during the study period (2003 – 2013). The lack of data on pathogen prevalence in nymphal *I. scapularis* in those years emphasizes the importance of data on pathogen prevalence in the primary vector of disease alongside human data in future studies.

The human incidence of Lyme disease during this period was not available. This information may inform if rates of Lyme disease and hard tick-borne relapsing fever are still changing in rural and non-rural communities, which would help construct more comprehensive public health efforts. In the future, we hope to integrate human infection data into this research to further explore how disease incidence may change over time in respect to suburban, urban, and rural communities.

While these limitations are important to recognize, the overall objective of this research was to improve our understanding of *Borrelia* species in ticks in southwest PA, which was accomplished. Limitations presented in this study provide new directions for future research endeavors, both within southwest PA and elsewhere. Overall, future studies aim to gain greater insight into additional tick-borne pathogens, explore genetic variation in tick-borne pathogens including *B. burgdorferi*, and refine spatial and topographical analyses of sites sampled.

6.0 Conclusion

The prevalence of *B. burgdorferi* is increasing in *I. scapularis* ticks in southwest PA, and thus provides a new rationale for increasing health provider and public awareness on the risk of both Lyme disease and hard tick relapsing fever. This prevalence change appears higher in suburban and rural areas. This study also ultimately provides insight into the abundance of tick-borne pathogens in especially urban areas in southwestern PA, providing evidence that there may be a true increase in the presence of *Borrelia* species and co-infection occurring in a major urban center.

Being equipped with the knowledge of the overall prevalence of medically important pathogens ticks in an area can empower providers to conduct different testing and treatment regimens for their patients, and aid in creating more impactful public health education and outreach. Future monitoring of *Borrelia* species prevalence is needed to ensure that accurate information is provided to state and local public health entities, thus emphasizing the necessity for continued research in this domain. Moreover, this study supports previous research endeavors suggesting that Southwestern PA tick-borne pathogen dynamics should be further studied to protect public health efforts. Thus, we conclude with recommendations to continue surveilling tick populations in southwestern PA annually, especially in urban and rural areas.

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