Seasonal Dynamics and Glycerol-3-Phosphate Dehydrogenase Variability in *Ixodes scapularis* Tick Population in Eastern Texas

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Introduction

Ticks are responsible for most of the vector-borne diseases cases within the United States (Parola and Raoult, 2001). Among these, Lyme disease is the most common and annual case rates have been increasing (Yanagihara and Masuzawa, 1997; Stanek et al. 2012; Mead, 2015). *Ixodes scapularis* tick is the primary vector for this illness in the eastern United States and reports indicate that its range has been expanding (Hahn et al., 2016). The midwestern and northeastern regions of the United States have the highest incidences of Lyme disease, which coincides with the greater population density of *I. scapularis* in these areas (Hahn et al., 2016). While *I. scapularis* may have a large range, there are variations in its population dynamics throughout it, such as population density, population distribution, and seasonality. Research into understanding what biotic and abiotic factors correlate with these variations can allow us to better control the risk of human contact with *I. scapularis* ticks and the causative agents of disease they can transmit. Although population dynamics of *I. scapularis* ticks are well described in the midwestern and northeastern parts of United States, there is minimal data on this topic available from the southern United States, including eastern Texas. This project will investigate the seasonal population dynamics of *I. scapularis* ticks in eastern Texas by flag sampling ticks in a locality where their presence has been previously confirmed. In addition, the variability of glycerol-3-phosphate dehydrogenase (GPDH) in collected *I. scapularis* ticks will be explored. Previous studies indicate that GPDH variability could be an adaptation in *Ixodes ricinus* ticks in response to fluctuations of environmental factors in Europe (Healy et al, 2004; Radulovic, et al 2012). This research will try to answer if a similar pattern of GPDH variability is present in *I. scapularis* populations. Collected GPDH variability data will be compared to the population

dynamics of *I. scapularis* to investigate possible correlations between GPDH variability and host-seeking activity.

Objectives

1. Document seasonal population dynamics of *I. scapularis* in eastern Texas.
2. Explore variability of GPDH in *I. scapularis* ticks in eastern Texas and relate data to its population dynamics.

Literature Review

Taxonomy of *I. scapularis*

Ticks are classified into the order Ixodida (Sonenshine and Roe, 2014). *Argasidae, Ixodidae,* and *Nuttalliedidae* are the only extant tick families (Oliver, 1989; Horak et al., 2002). Over 850 species of ticks have been identified; however, there is still debate over the classification of some genera and species (Horak et al., 2002; Guglielmone et al., 2010). The ticks in the *Ixodidae* family are considered "hard ticks" because they possess scutum, a sclerotized dorsal shield, while members of *Argasidae* are known as "soft ticks" and do not possess a scutum (Oliver, 1989; Estrada-Peña and de la Fuente, 2014). *Ixodidae* is the largest tick family and contains about 700 species of ticks (Oliver, 1989; Guglielmone et al., 2010). Ticks in *Ixodidae* family are further divided into two groups, Metastriata and Prostriata (Oliver, 1989; Black and Piesman, 1994). These groups can be differentiated based on the position of the anal groove; the anal groove in Prostriata is located anterior to the anus, while in Metastriata it is found posterior to the anus or can be absent (Beati and Keirans, 2001; Nicholson et al., 2009). Besides morphological differences, there are some physiological differences between these two groups. One of the most important differences is male Prostriata ticks' ability to start

spermatogenesis before taking a blood meal, which allows mating in this group to occur off of the host. Alternatively, Metastriata male ticks begin spermatogenesis while taking a blood meal and mating in this group occurrs on their hosts. Prostriata encompasses the genus *Ixodes*, while the remaining genera are classified in Metastriata group (Table 1) (Oliver, 1989; Black and Piesman, 1994).

Table 1. – The genera of *Ixodidae* ticks categorized into Prostriata and Metastriata (Guglielmone et al., 2010).

***Ixodidae* Group**	**Genera Contained Within the Group**
Prostriata	*Ixodes*
Metastriata	*Amblyomma, Bothriocroton, Haemaphysalis, Hyalomma, Nosomma, Anomalohimalaya, Cosmiomma, Dermacentor, Margaropus, Rhipicentor, Rhipicephalus,*

Morphology and Physiology of *I. scapularis*

Ixodes scapularis ticks undergo four life stages in their development: eggs, larvae, nymphs, and adults (Figure 1) (Oliver, 1989). All stages follow the basic morphological patterns of higher taxonomic groups. Their body is divided into the capitulum, idiosoma, and the legs (Sonenshine and Roe, 2014). Larval ticks, including those of *I. scapularis*, have three pairs of legs; however, nymphs and adults have four pairs of legs (Kierans et al., 1996). Adult females are typically larger than males, but they are mainly distinguished by the size of their scutum (Kierans et al., 1996). The scutum on *I. scapularis* males covers the entirety of their dorsal region but is reduced in females, covering only a third of their dorsal region anteriorly (Kierans et al., 1996). The reduction of the scutum allows females to become engorged when they feed,

since the remaining part of dorsal side is covered by soft and elastic covering known as alloscutum (Sonenshine and Roe, 2014).

Figure 1. – The different life stage of *Ixodes scapularis* on a centimeter scale. From left to right: adult female, adult male, nymph, and larva. Image provided by Winnebago County Health Department.

The period of development of each tick stage can be divided into three phases: free-living development, host finding, and parasitic (Sunhurst et al., 1978). During host finding, unfed ticks search for hosts to parasitize, while in the parasitic phase, ticks are attached and feed on the host (Sunhurst et al.,1978). Unfed ticks, also known as flat ticks, use specialized mouthparts to attach to the skin of a host and feed on their blood (Stanek et al., 2012). Ticks are able to locate and feed on host by responding to different stimuli, including higher CO_2 concentrations, vibrations, odor, touch, and radiant heat (Buczek, 1999). By sensing these changes, ticks can minimize their

metabolic loss during host seeking because the can more efficiently find hosts (Lenovich, 2013). Sensory organs, such as Haller's organ, palpal organ, and integumentary sensilla, aid the tick by detecting these differences in their environment (Leal et al., 2020). The Haller's organ is a chem-mechanoreceptor found on the dorsal side of the tarsus on each foreleg and is strongly associated with the detection of heat, humidity, and odors (Lees 1948; Wilkinson, 1953; Mitchell et al. 2017). This organ can detect odors associated with hosts, such as CO_2 and ammonia, and the hairs in the anterior pit and posterior capsule detect infrared radiation and environmental cues (Lees 1948; Sonenshine and Roe 2014; Mitchell et al., 2017). The palpal organ is assumed to be closely tied to feeding stimuli during host attachment and while attached (Lees, 1948; Wilkinson, 1953). The integumentary system of ixodid ticks is composed of many sensilla; the life stage and species of an individual determines the number and receptor type of sensilla (Leal et al., 2020).

Feeding can have a significant impact on the development of ixodids. The oogenesis and oviposition of ixodid females is regulated by feeding (Oliver, 1989). Blood is consumed gradually throughout the female's attachment until the final day of attachment, during which blood is consumed at a notably accelerated rate (Oliver, 1989). When feeding, females can increase their weight by 80 to 120 times; however, they do not become fully engorged unless they have mated (Oliver, 1989). Ixodids have a single gonotrophic cycle, most of the blood consumed by females is used in egg production for a single, large oviposition (Oliver, 1989). Prostriata ticks are different from Metastriata in their ability to produce spermatids without the need of a blood meal (Oliver, 1989). Despite *I. scapularis* ticks' ability to mate off-host, mating usually occurs on-host during female blood feeding.

Life Cycle of *I. scapularis*

Ticks are obligate temporary parasites of vertebrate animals with a complex life cycle (Estrada-Peña and de la Fuente, 2014). *Ixodidae* only have one nymphal instar, unlike *Argasidae*, which have multiple nymphal instars (Oliver, 1989). *I. scapularis* is a three-host tick, which means that there is a different host for each active stage (Yuval and Spielman, 1990). Ixodid ticks feed once during each active stage and require multiple days to feed at each stage (Oliver, 1989; Stanek et al., 2012). *I. scapularis* is indiscriminate in host selection during larval, nymphal, and adult stages (Oliver, 1989). This species has been found on 119 host species (Anderson and Magnarelli, 1994). Adults have been recorded on 27 mammalian species and 1 lizard species, while larvae and nymphs have been documented on 41 mammalian, 57 avian, and 14 reptilian hosts (Kierans et al., 1996).

After hatching, larvae search for hosts, feed on a host, and drop to the ground to molt and become a nymph (Estrada-Peña and de la Fuente, 2014). The nymphal stage repeats this process and molts into an adult (Estrada-Peña and de la Fuente, 2014). Adults mate before or during feeding; most males die soon afterward (Throughton and Levin 2007). Females take a single blood meal, mate before or during blood feeding, drop of a host, produce a single, large egg mass, and die (Oliver, 1989; Estrada-Peña and de la Fuente, 2014). Males do not typically feed and never gorge (Stanek et al., 2012). Engorged females, after dropping from a host, lay their eggs in protected sites where high relative humidity will prevent their desiccation and die after this process is completed (Estrada-Peña and de la Fuente, 2014). The production of larvae from engorged adult females is thought to be the most sensitive stage in the *I. scapularis* life cycle (Yuval and Spielman 1990).

Seasonality of *I. scapularis*

The life cycle of *I. scapularis* is typically two years (Figure 2), but it can be extended for four years if hosts are relatively scarce (Piesman and Gern, 2004; Yuval and Spielman, 1990). Seasonal abundance could potentially be determined by cold-induced torpor, desiccation, and photoperiodic responses (Yuval and Spielman, 1990). Ambient temperature conditions also seem to determine developmental rates (Ogden et al., 2004). In order for *I. scapularis* to efficiently undergo its life cycle, adequate warming during the summer months might be necessary (Piesman and Gern, 2004).

According to Yuval and Spielman (1990), larvae feed during late summer, nymphs feed during spring and early summer, and adults feed between September and May. When ticks drop off of their host, they relocate onto or near the soil surface; they require a high humidity in order to survive being off-host (Stanek et al., 2012). Ixodids spend most of their life cycle off-host and take several months to develop into their next developmental stage (Needham and Teel, 1991; Stanek et al., 2012). Females tend to lay eggs in early summer after having previously fed (Yuval and Spielman, 1990).

This generalization of *I. scapularis* seasonal patterns does not consider the significant climatic differences in this species' broad range. Model simulation have indicated that larvae may be active earlier in the year in the southeastern United States compared to the northeastern region (Ogden et al. 2008). Larvae occur later in the year than nymphs; nymphs would be more likely to be active in autumn if larvae activity is also earlier (Ogden et al., 2008; Stanek et al., 2012). In a study by Diuk-Wasser et al. (2006), peak nymphal density occurred later in the most northern sites than in the southern sites. In southern areas, adult activity can peak during February instead of pausing during the winter (Goddard, 1992). In an Alabama study site,

nymphs were found to be the most abundant in spring and larval activity was highest between May-June (Ogden et al., 2018).

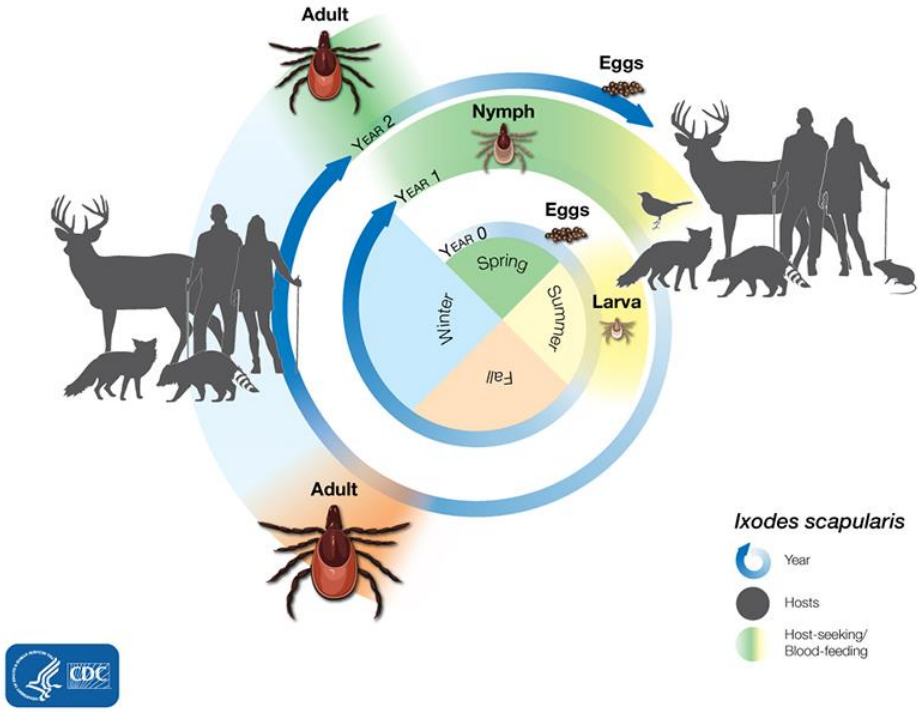

Figure 2. – The typical two-year life cycle of *I. scapularis*. Image provided by the Centers for Disease Control and Prevention.

Host-Seeking Activity of Ticks

There are two categories of host-seeking behavior in ticks, nidicolous and non-nidicolous (Tietjen et al., 2019). Nidicolous ticks are found in the nests and burrows of their hosts, but non-nidicolous ticks actively seek out their hosts in open environments (Sonenshine and Roe 2014b; Tietjen et al., 2019). *I. scapularis* is considered a non-nidicolous tick because larvae, nymphs, and adults can all be collected from vegetation when sampled in its northern range; however, in its southern range it is more difficult to collect both larval and nymphal stages from vegetation (Tietjen et al., 2019). *I. scapularis* displays a type of host-seeking behavior called questing

(Tietjen et al., 2020). During questing, ticks ascend plants, extend their forelegs, and wait for a host to latch onto (Figure 3) (Leal et al., 2020; Tietjen et al., 2020).

Figure 3. – Female *I. scapularis* questing. Image provided by Dr. Richard Gerhold.

Many exogenous and endogenous factors can affect host-seeking in ticks, such as humidity, sunlight, endogenous rhythms, and presence of vertebrate hosts (Sonenshine and Roe, 2014b). Compared to other ticks, the members of genera *Ixodes* are more sensitive to temperature and relative humidity (Knulle and Rudloph, 1982; Sonenshine and Roe 2014). In a study conducted by Vail and Smith (1998), temperature and relative humidity accounted for 33% and 44%, respectively, of nymphal activity variation.

Temperature can affect many aspects of host seeking. In a study by Vail and Smith (2002), temperature seemed to influence the mean distance moved during host-seeking and the percentage of time in questing posture. The temperature threshold for questing activity in *I. scapularis* appears to be 4°C (Duffy and Campbell, 1994). Activity in adults was positively correlated with temperatures over 4°C (Daniels et al., 1989). However, some studies have shown this threshold to be somewhat higher (Clark, 1995). In Mississippi, *I. scapularis* ticks were most often caught around 20°C (Goddard, 1992).

Due to their susceptibility to desiccation, humidity also significantly dictates the activity of *I. scapularis*. Larvae of this species are particularly vulnerable to mesic conditions while seeking hosts as a result of their low water mass and water retention inefficiency (Stafford, 1994). Relative humidity could affect the activity of *I. scapularis* by influencing the height at which ticks quest (Vail and Smith, 2002). Humidity also affects the number of daily visits individuals take to the leaf litter layer in order to rehydrate and prevent desiccation (Randolph and Storey, 1999).

Habitat and Distribution of *I. scapularis*

I. scapularis is distributed across eastern North America (Figure 4) (Piesman and Gern, 2004). In the United States, it is found along the eastern coast from Florida to Maine and as far west as central Texas (Piesman and Gern, 2004). These ticks are typically found in hardwood forests with an abundant leaf litter; however, they can also be found in pine forests that are surrounded by hardwoods (Schulze et al., 1998; Piesman and Gern, 2004). Leaf litter provides cover from desiccation and protection during snowfall; the removal of leaf litter can decrease *I. scapularis* populations by 72-100% (Schulze et al., 1998; Piesman and Gern, 2004). This species also associated with moist, well-drained soils (Piesman and Gern, 2004).

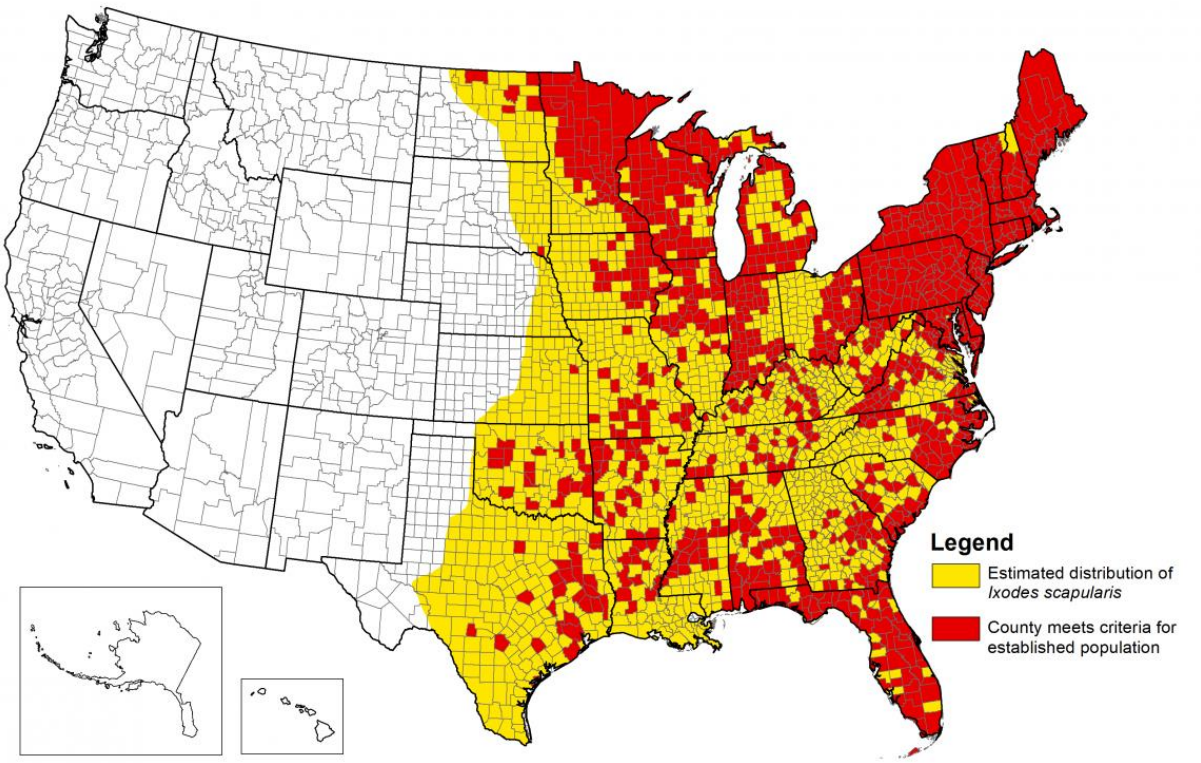

Figure 4. – Estimated and established distribution of *I. scapularis* within the United States. Image provided by the Centers for Disease Control and Prevention.

The distribution and abundance of *I. scapularis* can be restrained by climatic conditions. Climatic conditions affect tick survival the most during non-parasitic periods of life (Ogden et al. 2004). Developing and host-seeking ticks are vulnerable to desiccation and require microclimates with relative humidity of over 80% for extended amounts of time (Gray, 1998). Higher temperatures and lower relative humidity cause the decline of survival, developmental rate, oviposition, and the hatching of ticks (Needham and Teel, 1991).

Populations of *I. scapularis* in North America are spatially disconnected and patchy (Wilson, 1998). This may be because ticks do not typically enter new territories by themselves, instead, their hosts move while being parasitized and introduce them to new areas (Estrada-Peña and de la Fuente 2014). The introduction of *I. scapularis* into new areas seems to happen often;

however, the colonization of these sites is not always successful (Wilson, 1998). The location in which engorged females detach and oviposit is critical factor to establishing a new population (Wilson, 1998).

The distribution of *I. scapularis* has increased throughout the years. The amount of counties in which *I. scapularis* is considered to be established has more than doubled between 1996 and 2015 (Eisen et al. 2016). Some models, along with the continuous expansion of this species, indicate that there is a significant amount of potentially suitable habitat in which *I. scapularis* can survive and reproduce with no established population (Hahn et al., 2016). This could indicate that populations are under-reported or that this species has the ability to further increase its range (Hahn et al., 2016). Global climatic changes may also promote the expansion of *I. scapularis* by increasing the amount of suitable habitat available.

Variability of Glycerol-3-Phosphate Dehydrogenase in Tick Populations

Unfed ixodid ticks have a limited amount of reserve material for host seeking activity, which is available from previous developmental stage's blood meal. Thus, efficient utilization of reserve material greatly impacts their success in completion of life cycle (Radulovic et al., 2010). Studies in different arthropods have indicated that variability of *Gpdh,* the gene encoding glycerol-3-phosphate dehydrogenase (GPDH), is associated with efficient use of reserve materials in response to fluctuation of environmental conditions. GPDH is a significant component in glucose and lipid metabolism, which catalyzes reversible conversion of glycerol-3-phosphae to dihydroxyacetone using NAD as a cofactor. This is one of the key steps in cellular respiration, because it allows regeneration of NAD (Young and Pace, 1958). The rate of NAD

regeneration depends on GPDH efficiency and the efficiency of different variants of this enzyme in arthropods has been shown to be temperature dependent. Consequently, GPDH variability has been suggested to have an adaptive role in populations of some *Drosophila* and *Collias* species (Johnson, 1976; Alahiotis et al., 1977). Research has indicated that temperature fluctuations can determine differential allozyme activity in *Drosophila melanogaster* (Miller et al., 1975). Interactions between environmental temperature and GPDH allozymes were also shown to impact flight ability in *D. melanogaster* (Barnes and Laurie-Ahlberg, 1986).

Healy et al. (2004), suggested that the biochemical processes for movement in insects could be fundamentally similar to the processes initiating and maintaining movement in ticks. A study linked differential mobility at low temperatures in *I. ricinus* ticks to different GPDH genotypes in individuals (Healy, 1979). Variability of GPDH has been indicated as the main factor for activity variation of ticks in response to temperature (Healy et al., 2004). Current research suggests that the polymorphism of *Gpdh* is an adaptive feature in *I. ricinus* to environmental temperature fluctuations (Healy et al, 2004; Radulovic, et al 2006).

Polymorphism of GPDH has been identified in *I. ricinus* populations throughout Europe (Healy, 1979; Delaye et al., 1997; Radulovic et al., 2006). Four alleles of the *Gpdh* locus have been detected by allozyme electrophoresis and designated as very fast (VF), fast (F), slow (S), and very slow (S) (Healy, 1979; Delaye et al., 1997; Radulovic et al., 2006). Radulovic et al. (2010) amplified and sequenced 36 coding sequences, 1023 base pairs long, of the *Gpdh* gene in *I. ricinus* and the resulting nucleotide alignments showed 40 polymorphic nucleotide sites. Of these polymorphic nucleotides, 34 were silent and the remaining six change the amino acid sequence of the enzyme subunit (Radulovic et al., 2010).

Radulovic et al. (2012) studied the seasonal and spatial distribution of *Gpdh* variability in Serbian population of *I. ricinus*. Their results showed significant seasonal variations of *Gpdh* related haplotype and genotype frequencies in open habitats; however, there was no seasonal variation found in forested sites. This could be caused by the greater seasonal and daily variation of environmental condition in open habitats contributing to more diversity in the genetic composition of those populations (Radulovic et al, 2012).

Medical Importance of *I. scapularis*

Ticks are obligate hematophagous ectoparasites and during blood feeding they can transmit a variety of pathogens to vertebrate hosts (de la Fuente et al., 2008; Radulovic et al., 2012; Estrada-Peña and de la Fuente, 2014). Around 10% of tick species are considered medically significant as a result of this trait (Oliver, 1989). The pathogens that can be transmitted include viruses, bacteria, protozoa, and helminthes (de la Fuente et al., 2008). Ticks are vectors for the most types of microorganisms when compared to other arthropod taxa (Hoogstraal, 1985). Behind mosquitoes, ticks are one of the most important vectors for human diseases worldwide (de la Fuente et al., 2008). They are also considered to be the most important disease vector in domestic and wild animals (de la Fuente et al., 2008).

Within North America, ticks are regarded as the most important vectors of pathogens (Parola and Raoult, 2001). About 50,000 cases of locally acquired vector-borne disease cases are reported to the Centers of Disease Control and Prevention, CDC, annually, and almost 95% of them are pathogens transmitted by ticks (Adams et al., 2016). Increase in range and an abundance of *I. scapularis* ticks, as a result of reforestation in the northeast, led to an increase of tick-borne disease cases in the United States during the 1980s and 1990s (Throughton and Levin

2007). There has also been an increase in the number of pathogens associated with *I. scapularis;* six *I. scapularis*-borne human pathogens have been described since *Babesia mircoti* was first reported in 1970 (Table 2) (Eisen and Eisen, 2018).

Table 2. – Pathogens transmitted by *Ixodes scapularis* as reported by the Centers for Disease Control and Prevention (2021).

Pathogen	**Infectious Disease**
Anaplasma phagocytophilum (bacterium)	Human granulocytic anaplasmosis
Babesia microti (protozoa)	Human babesiosis
Borrelia burgdorferis sensu stricto (bacterium)	Lyme disease
Borrelia mayonii (bacterium)	Lyme disease
Borrelia miyamotoi (bacterium)	*Borrelia miyamotoi* disease
Ehrilichia muris eauclariensis (bacterium)	Ehrlichiosis
Powassan virus (virus)	Powassan disease

Lyme disease is the most common tick-borne disease in North America and the northern hemisphere (Yanagihara and Masuzawa, 1997; Stanek et al. 2012). In the United State, the spirochete bacteria *Borrelia burgdorferi* sensu stricto and *Borrellia mayonii* cause Lyme disease (Tietjen et al., 2020). The amount of reported cases of Lyme disease in the United States has almost tripled since the late 1990s (Mead, 2015). According to the CDC there were 16,455 confirmed cases of Lyme disease in 1996; however, the National Surveillance case definition was revised in 2008 to include probable cases. In 2019, the number of confirmed cases was 23,453 and the number of probable cases was 11,492.

I. scapularis is the principle vector of Lyme diseases in the eastern half of North America and *Ixodes pacificus* is the principle vector on the western region of the continent (Piesman and Gern, 2004). *Borrelia* is transmitted through the injection of tick saliva into a host during feeding (Stanek et al., 2012). In order for *B. burgdorgeri* to be efficiently transmitted, ticks must be attached to the host for over 48 hours (Piesman et al. 1987b; des Vignes et al. 2001). The majority of Lyme disease cases in North America are a result of nymphal *I. scapularis* bites (Piesman and Gern, 2004; Duffy and Campbell, 1994). Nymphs of *I. scapularis* are often able to avoid detection due their small size and remain attached to their host long enough to transmit Lyme disease (Piesman 1987, Falco et al. 1996). As previously mentioned, the range of *I. scapularis* has expanded, which has likely contributed to the current scale of Lyme disease throughout the northeastern, north-central, and mid-Atlantic regions of the United States (Hahn et al., 2016)

Significance

I. scapularis is a significant vector of diseases within the United States. Information on the host-seeking behavior and seasonality of *I. scapularis* is important for determining periods of risk. Despite this, there is minimal information on the seasonality of *I. scapularis* in the southern United States and none in eastern Texas. In regard to glycerol-3-phosphate dehydrogenase, understanding its variability and connection to seasonality further increases our understanding of tick seasonality and the factors that determine host-seeking activities. This project is particularly important for public health and may allow for more accurate preventative measures to be designed, but also provides insight into the environmental adaptations in this tick species.

Methodology

Tick Collection

Ticks will be collected every two weeks at Jack Creek Loop, a trail that is about 1.3 km long and located within the Stephen F. Austin Experimental Forest; this forest is a mixed hardwood forest. The coordinates for the trailhead of Jack Creek Loop are -94.762110° and 31.498194° and the trail is at an elevation of about 77.07 m. The forest is located 13 km southwest of Nacogdoches, TX. Two people will conduct flag sampling during each collection event; one person will be on each side of the trail. The flags used will be 1 m^2 in size and will be checked for ticks every 5-10 meters along the trail. When ticks are found on the flag, they will be placed inside a 5 mL tube with either a wet paper or a blade of grass to prevent the ticks' desiccation. All species and life stages will be collected and identified afterwards in the laboratory. However, only individuals in the adult and nymph life stage of *I. scapularis* while be used for this study. *I. scapularis* ticks will be kept alive through the prevention of dessication until RNA extraction for a maximum of 4 days to prevent RNA degradation.

Seasonal Dynamic Data

During collection events, environmental and temporal data will be collected. The date, time, temperature, and humidity during the collection will be recorded. Data from the National Weather Service and a Kestrel Drop D2 data logger will be used to record temperature and humidity. Tick collection is currently underway and will continue for a total length of two years. Possible correlation between temperature, humidity, and tick activity will then be analyzed through a regression analyses using MS Excel (Microsoft), Prism (GraphPad), and SAS.

RNA Extraction

I. scapularis ticks will be washed once in 70% ethanol and thrice in nuclease free water to remove external contaminants. Once washed, ticks will be individually processed in TRIzol (Thermo Fisher Scientific) according to manufacturer's protocol. Briefly, ticks will be placed in a 1.5 ml tube filled with 500 µl of TRIzol, and then cut into small pieces using sterile scissors for each sample. Samples will then be frozen at -80°C for a minimum of 2 hours, and then thawed on ice, vortexed, and incubated at room temperature for 5-10 minutes. A total of 100 µl of chloroform will be added to each sample, followed by vortexing for 15 seconds and incubation at room temperature for 3 minutes. The samples will then be centrifuged at 12,000xg at 4°C for 15 minutes. The top, colorless aqueous phase, where the RNA is located, will be carefully transferred into a new 1.5 ml tube and 250 µl of isopropanol will be added to the aqueous phase, vortexed for 15 seconds, and incubated for 10 minutes at room temperature to allow RNA precipitation. Following this, RNA will be pelleted by centrifugation at 12,000xg at 4°C for 15 minutes. Supernatant will be removed, and sample will be kept on ice before washing the pellet by adding 500 µl of 75% ethanol, diluted with DEPC water. The sample will then be centrifuged at 7,500xg at 4°C for 5 minutes and the supernatant will once again be removed. The pellet will be dried by opening the tubes inside the fume hood for 4 minutes as suggested by manufacturer's protocol. Afterwards the tubes will be checked for remaining liquid and left open for longer if liquid is found; caution will be taken to not over dry the RNA pellet because if the pellet is over-dried it will not be able to be resuspended. Once dry, the pellets will be resuspended using 10 µl of DEPC water. The samples will be briefly vortexed for a few seconds and incubated at 55°C for 10 minutes to denature secondary structures of RNA. The concentration of RNA will be

checked using NanoValue Spectrophotometer (GE). Finally, the RNA samples will be stored at -80°C until further use.

cDNA Synthesis

The Verso cDNA Synthesis Kit (Thermo Fisher Scientific) will be used for the cDNA synthesis. cDNA will be synthesized in 10 µl reactions that contain 2 µl 5X cDNA Buffer, 1 µl of dNTP mix, 0.375 µl of random hexamer primer, 0.125 µl oligo dT primer, 0.5 µl of RT enhancer, and 0.5 µl of enzyme mix. A total of 500 ng of RNA will be used per sample; however, if RNA concentration in a sample is too low to achieve desired amount of RNA in cDNA synthesis reaction, the maximal allowed volume of RNA sample will be added. The ratio of used primers in reaction will be changed by increasing the amount of anchored oligo (d)T primers in samples where synthesis of cDNA initially is not appropriate. Reverse transcription reaction will be performed at 42°C for 30 minutes, followed by step of enzyme inactivation at 95°C for 2 minutes, and hold of the samples at 12°C until they are permanently stored at -20°C. The quality of synthesized cDNA will be checked via PCR amplification of tick actin gene sequence using 2XGoTaq Green Mater Mix (Promega) and primers 5'GGACAGCTACGTGGGCGACGAGG3' and 5'CGATTTCACGCTCAGCCGTGGTGG3'. A total of 1 µL of prepared cDNA will be used as a template. PCR program will include initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute, concluding with final extension at 72°C for 7 minutes. PCR products will be run on 2% agarose gel and checked for presence of a band around 500 bp.

PCR Amplification of *Gpdh* Coding Region

In order to amplify and sequence the full open reading frame of *Gpdh* gene, primers are designed in 5' and 3'UTR regions of this gene. Two forward and two reverse primers are designed using the sequence of *I. scapularis* mRNA originating from the *Gpdh* gene, which is deposited in the GenBank under accession number XM_029993338.3. Primer sequences are presented in Table 3.

A 40 µl PCR will be prepared for each sample. The reaction will consist of 20 µl of 2xGoTaq Green Master mix (Promega), 1 µl of synthesized cDNA, 2 µl of each forward and reverse primer solutions (10 µM), and 15 µl of nuclease free water. Initially, in-house primers IscapGPDH-F and –R will be used. PCR program will include initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute and 45 seconds. Afterwards, final extension step at 72°C for 7 minutes will be performed. PCR products will be run on 1.5% agarose gel and expected size of products is around 1,200 bp.

If amplification is not successful, alternative PCR methods will be used, including gradient PCR, use of other two pairs of in-house designed primers (Table 3) and combination of designed forward and reverse primers, as well as nested or semi-nested PCR, having in mind that IsOuterGPDH-F and –R primers are positioned to be used in the first step of nested-PCR. After successful amplification of *Gpdh* region by PCR, sequencing reactions will be prepared.

Table 3. – Primer sequences that will be used for *Ixodes scapularis* glycerol-3-phopate dehydrogenase coding region amplification. Corresponding pairs of primers are separated by a dotted line.

Primer Name	Sequence 5'→ 3'	Sense
IsOuterGPDH_F	CCCTGGTACAATCTGCGTCC	Forward
IsOuterGPDH_R	GCGAGTATTGAGAAGCTGCG	Reverse
IscapGPDH-F	TTCCAGTGCCGTATGTTCGT	Forward
IscapGPDH-R	ACATGGCGTTTCAGTGCGA	Reverse

Sequencing of the Coding Region of Glycerol-3-Phosphate Dehydrogenase

PCR products of *Gpdh* coding sequence amplification will be resolved on 1.5% agarose gel. The appropriate band will be excised from the gel and DNA will be extracted and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to manufacturer's protocol. Briefly, excised gel slices will be mixed with the appropriate amount of provided membrane binding buffer and incubated with mixing at 60°C until gel is completely dissolved. The solution will be moved to a provided spin column positioned on a collection tube and centrifuged at 16,000xg for 1 minute. Flowthrough from collection tube will be discarded and spin column will be repositioned on the top, filled with 500 µL of provided membrane wash solution, and centrifugation at 16,000xg for 1 minute will be performed. After removal of flowthrough from the collection tube, one more washing step with 500 µL of membrane washing solution will be performed. In order to dry the membrane at the bottom of spin column, where

DNA is bound, a 3-minute centrifugation at maximal speed will be performed. After this step, spin column will be carefully removed from collection tube, placed on a sterile 1.5 mL Eppendorf tube, and 50 µL of nuclease-free water will be applied directly to the membrane on the bottom of spin column. After 1 minute incubation at room temperature, in order to allow release of DNA molecules from the membrane, centrifugation at 16,000xg for 1 minute will be performed and DNA in aqueous solution will be collected in 1.5 mL tube. Samples will be stored at -20°C until further use.

Concentration and quality of purified PCR products will be checked on NanoValue Spectrophotometer (GE). If sufficient concentration and purity of DNA is present in the sample, preparation of sequencing reactions will be performed. For each sample two sequencing reactions will be prepared, one with forward and one with reverse primer. Eurofins Genomics sequencing service will be used, and sequencing reactions will be prepared by mixing recommended amounts of purified PCR product and primer and sent for sequencing. If concentration of purified PCR product is too low for sequencing, or alcohol contamination is observed in a sample, DNA from the sample will be dried by using SpeedVac vacuum concentrator and reconstituted in 20 µl of nuclease free water. In this way concentration of DNA will be increased and all remaining alcohol from the sample will be eliminated.

Resolving Glycerol-3-Phosphate Dehydrogenase Allele Sequences

Coding sequence of *Gpdh* gene for each tick will be resolved by analysis of results of two sequencing reactions, one performed with forward and the other performed with reverse primer. Alignment of these two sequences, by using a free BioEdit software, will provide us with a consensus sequence for each analyzed individual.

Chromatograms obtained as a part of sequencing results will be manually examined for each sequencing reaction, in order to detect the presence of polymorphic nucleotide positions. Polymorphic nucleotide positions are represented by double peaks on the chromatograms (Figure 5). Polymorphic nucleotide positions will be compared between the forward and reverse sequences to confirm their presence. If an individual does not show polymorphic nucleotide positions, it will be considered as homozygous, while individuals with polymorphic nucleotide positions will be considered heterozygous for *Gpdh* locus. If heterozygous individuals exhibit one polymorphic nucleotide position, sequences of two present alleles will be obvious. However, if more than one polymorphic nucleotide position is identified in an individual, cloning will be performed in order to determine present allele sequences.

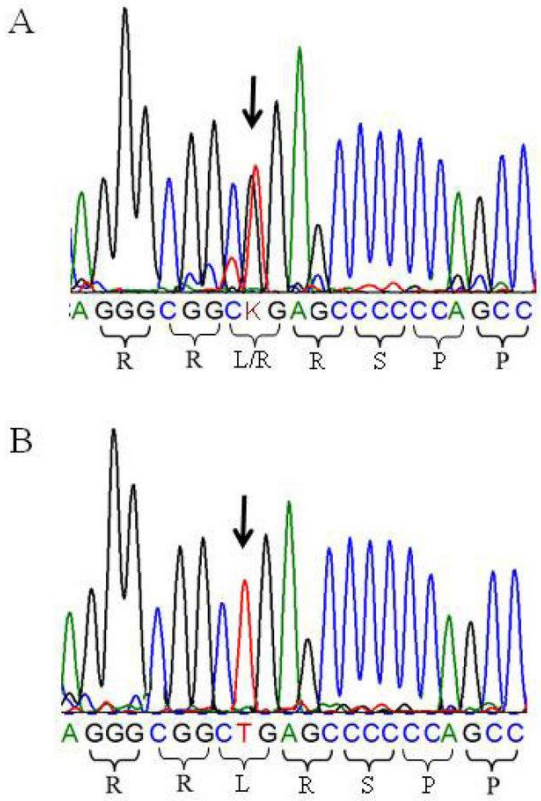

Figure 5. –Two chromatograms resulting from sequencing the same in two individuals. A. The arrow depicts a polymorphism, in which two different nucleotides were detected in the same position in DNA. B. Depicts a section of DNA without a polymorphism.

TA Cloning

The pGEM®-T Easy Vector System with JM 109 competent cells (Promega) will be used for TA cloning. The ligation reaction will be prepared by addition of an appropriate amount of purified PCR product to 2.5 µL of provided 2x Rapid Ligation Buffer, 0.5 µL of T4 DNA ligase (1.5 Weiss units), and 0.5 µL of pGEM-T easy vector (2.5 mg) in total of 5 µL. Ligation reaction will be incubated at 4°C overnight. Around 2.5 µL of ligation reaction will be added to 40 µL of provided JM 109 competent cells thawed on ice, mixed by tapping the bottom of the tube, and incubated on ice for 20 minutes. The mixture will be then exposed to heat shock at 42°C for 45 seconds and placed back on ice for 2 minutes. Around 500 µL of pre-warmed SOC medium will be added to the mixture and incubated at 37°C for 1 hour with shaking 225 rpm. Around 100 µL of culture will be plated on LB agar plates with ampicillin (100 µg/mL) and incubated at 37°C overnight. At least five colonies per plate will be selected for insert-check colony PCR, to inspect if appropriate sequence is incorporated into pGEM-T easy vector. A 40 µL reactions will be prepared by mixing 20 µL of 2x GoTaq Green Master Mix, 2 µL of each 10 µM primers related to the vector (SP6 and T7), and 16 µL of nuclease-free water. As a template, a small portion of colony will be introduced into prepared reaction by using a sterile pipette tip. PCR program will start with 5-minute initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 40°C for 30 seconds, and extension at 72°C for 2 minutes, and final extension at 72°C for 7 minutes. PCR products will be run on 1.5% agarose gel and bands of expected size of around 1300 bp will be excised. Initially, 2 PCR products will be further processed as previously described in order to resolve allele sequences in heterozygous individuals, but additional PCR products and colonies could be analyzed if it is necessary.

Analysis of *Gpdh* Variability in Studied Population

Analysis of *Gpdh* variability in studied *I. scapularis* population will start with identification of all present alleles, with a focus on distinguishing polymorphic nucleotide positions that are result of missense mutations. In this way allele groups that encode GPDH enzymes with different amino acid sequences will be identified. Frequencies of alleles, allele groups, and genotypes, will be analyzed in the whole sample, as well as in subsamples that are related to seasonal dynamic of the population, sex, and developmental stage of analyzed ticks. Regression analyses will be run on SAS to determine correlations between the categories previously described.

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