1-1-1999

The effect of Borrelia burgdorferi on the cold-hardiness of Ixodes scapularis and comparative cold-hardiness of Ixodes scapularis and Dermacentor varabilis

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The effect of *Borrelia burgdorferi* on the cold-hardiness of *Ixodes scapularis* and comparative cold-hardiness of *Ixodes scapularis* and *Dermacentor varabilis*

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Majors: Entomology; Veterinary Microbiology
Major Professors: Wayne A. Rowley and Kenneth B. Platt

Iowa State University
Ames, Iowa
1999

Graduate College
Iowa State University

This is to certify that the Master's thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

Dedication

This work is dedicated to mother, Susan. M. White, who shaped me into the strong, independent, self-reliant person that I am today. And to my husband, Frank A.Elsbecker, who loves me despite the fact.

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CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This thesis is composed of a general introduction, a literature review of the natural history of Lyme disease, detection of *Borrelia burgdorferi* Johnson, Hyde, Schmid, Steigerwalt and Brenner, vector ecology of *Ixodes scapularis* Say and *Dermacentor variabilis* (Say), cold-hardiness, three manuscripts, and a section summarizing experimental results and their implications. The manuscripts, as well as part of the literature review, will be submitted for publication. The references cited in the literature review and the three papers appear at the ends of their respective chapters.

Research Summary

All of the studies described in this thesis were designed to evaluate the cold-hardiness of Ixodid ticks and to determine if infection with *B. burgdorferi* had an effect on cold-hardening. Initial studies with adult field collected *I. scapularis* determined an LT_{50} of -15.3 ± 0.01°C and did not suggest that *B. burgdorferi* affected the survival of adult ticks exposed to cold temperatures. After finding the *B. burgdorferi* did not affect naturally

infected adults, it was of interest to determine if *B. burgdorferi* would have an effect on laboratory reared immature ticks. Both *I. scapularis* and *D. variabilis* were experimentally infected with *B. burgdorferi* and then subject to cold-hardiness testing. There was no effect of *B. burgdorferi* on the cold-hardiness of immature *I. scapularis* ticks. However, the LT_{50} of each immature life stage was determined. Flat larvae were the least cold-hardy with an LT_{50} –12.77 ± 0.09°C, followed by engorged larvae that had an LT_{50} of –14.27 ± 0.03°C. Flat nymphs were the most cold-hardy and had an LT_{50} of –17.83 ± 0.05°C.

Dermacentor variabilis did not successfully harbor *B. burgdorferi* and as a result the effect of infection on cold-hardiness could not be assessed. Cold-hardiness testing did, however, show that immature *D. variabilis* ticks were much more cold-hardy than *I. scapularis*. *Dermacentor variabilis* engorged larvae were the most cold-hardy with an estimated LT_{50} of –40.17 ± 0.01°C, ~26°C colder than *I. scapularis* engorged larvae. Flat larvae had an LT_{50} of –21.96 ± 0.01°C, which was very similar to flat nymphs at –21.63 ± 0.02°C.

CHAPTER 2. LITERATURE REVIEW: NATURAL HISTORY OF LYME DISEASE, VECTOR ECOLOGY AND COLD-HARDINESS

Introduction

Many people have heard of Lyme disease, but know little about its cause, its vector or how to prevent the disease. Lyme disease, is a multisystem disease transmitted by ticks of the *Ixodes ricinus* (Hoogstraal 1981) complex. It is a zoonotic disease, a disease of animals that can be transmitted to man, with humans as an incidental tick host. Domestic animals are also at risk. Dogs are the most commonly afflicted domestic animal due to their greater exposure to the outdoors and the vector. There is evidence that cats, cows, horses and sheep are affected, although there has not been extensive research into these cases (Gibson et al. 1995).

According to the Centers for Disease Control (CDC), Lyme disease accounts for over 95% of all reported vector-borne illnesses reported in the United States. Since the recognition of the tick-borne illness in the U.S. in the 1960's, the known range of the disease continues to spread geographically due to various biological and ecological factors tied to the vector. The disease is often misdiagnosed because of the range of symptoms and questioned reliability of available diagnostic testing. It is suspected that Lyme disease is grossly under-reported (Coyle et al. 1996).

This research project was designed to evaluate cold-hardiness differences between *Ixodes scapularis* and *Dermacentor variabilis*, and more importantly the cold-hardiness differences of *Borrelia burgdorferi* infected and uninfected ticks. Cold-hardiness is the ability of an organism to survive at extreme cold temperatures. Cold-hardiness is measured by calculating the lowest lethal temperature (LT_{50}) values. Since there is no real 'forecasting' method for predicting tick populations, using the level of survivorship of overwintering stages might be important in determining future populations and as a consequence, aid in Lyme disease risk assessment. By ascertaining if there is a difference in the cold-hardiness of *B. burgdorferi* infected and uninfected ticks, the ground work is laid for further research. Future research could use this information and climatic information to make functional models, possibly predicting the proportion of infected ticks within a given population.

History of Lyme Disease

Contrary to popular belief, Lyme disease was initially discovered in Europe. Its earliest reference was in 1883 by a German physician, Dr. A. Buchwald, who first described a diffuse skin lesion of unknown cause, which was later named in 1902 acrodermatitis chronica atrophicans, ACA (Burgdorfer et al. 1982). In 1909, a Swedish physician, Dr. Arvid Afzelius

demonstrated a ring-like lesion measuring 1/2 to 2 cm in diameter in a woman who had been bitten by a sheep tick, *I. ricinus*. He noticed that the lesion migrated with a clearing in the center. Remembering a similar case in 1908, he termed the lesion erythema migrans, EM (Coyle et al. 1996). In Vienna, Austria, Dr. B. Lipschutz had seen the described lesions, but the lesions in his patients often lasted more than seven months, thus he termed the lesion erythema chronica migrans, ECM (Coyle et al. 1996). The cause of the rash, other than a tick bite, was hypothesized to be a virus or toxin transmitted by the tick (Coyle et al. 1996).

For the next 60 years, a variety of physicians from all over Europe and Russia became involved with the mystery of ECM. In 1930, Dr. S. Hellerstrom in Stockholm, Sweden also recognized ECM in conjunction with nervous system involvement. These reported conditions that resulted from a tick bite became known as Bannwarth's syndrome (Coyle et al. 1996). Physicians were now realizing that ticks were the cohesive factor in all reported cases of ECM. In 1936, Dr. H. Askani from the Dermatology Clinic of The University of Heidelberg, reviewed the significance of ticks as vectors of human and animal pathogens. He concluded that ECM was caused by a toxin or by a living pathogen from the tick's salivary glands (Coyle et al. 1996). From 1909 to 1936, no further progress was made in determining the agent of the disease. In fact, it took roughly 30 years to accept that ticks were the factor (vector) in most of the documented cases of ACA, ECM,

and EM, which are the same dermatological manifestation of the disease.

Hellerstrom addressed the 43rd Annual Meeting of Southern Medical Association in Cincinnati in 1949 with a talk entitled, "ECM Afzelius with Meningitis" (Coyle et al. 1996). He reviewed several cases of EM and meningocerebrospinal symptoms that occurred after a tick bite, but in addition, he was the first to report successful treatment with penicillin (Coyle et al. 1996). Hellerstrom further discussed the notion that EM could be a result of spirochetes harbored by *I. ricinus*. This hypothesis was never tested, as spirochetes had never been found in association with Ixodid ticks. Spirochetes had been found in other ticks in the past but were thought to be restricted to other genera (Burgdorfer 1993). Binder (Coyle et al. 1996) and his colleagues made a breakthrough in the biology of EM in 1955. He was able to show that an infectious agent caused EM after transplanting a peripheral piece of skin from an EM patient to an uninfected volunteer. He tried this experiment three times, and in each case, each volunteer exhibited the characteristic EM within one to three weeks of the transplant. In addition to showing the infectious nature of the agent, like Hollstrom he was able to show susceptibility of the agent to penicillin (Coyle et al. 1996). Moreover, it was commonly accepted that EM with meningitis was caused by a viral agent found in *I. ricinus* (Coyle et al. 1996). In 1974, this was disproved with the successful treatment of meningitis with ECM by penicillin (Coyle et al. 1996), thus proving the agent to be bacterial.

In 1969, EM debuted in the United States. A physician hunting in north central Wisconsin was bitten by a tick. Following removal of the tick, a red, ring-like rash appeared. The attending physician was familiar with the European cases of EM and promptly treated the patient with penicillin (Scrimenti 1950). During that same year, Polly Murray and Judith Mensch were perplexed by all of the diagnosed cases of juvenile rheumatoid arthritis occurring in their hometown of Old Lyme, Connecticut. All cases exhibited the same symptoms: severe headache, skin lesions, subsequent recurring arthritis and neurologic involvement. Upon discussion of the cases and considering most were children, the women contacted the state health department (Burgdorfer 1993). The Connecticut State Health Department sought help from Dr. Allen Steere of the Rheumatology Department at Yale University Medical School. In 1975, Dr. Steere started a retrospective study, which led to the description of Lyme arthritis (Stafford 1992). Lyme arthritis was considered a new complex, multisystem disorder of an unknown cause (Stafford 1992). Steere's study considered the agent of the disease at large since arthritis had never been described with EM (Stafford 1992).

The discovery of the etiologic agent of EM and the other manifestations took place in the fall of 1981 during an investigation of Rocky Mountain Spotted Fever on the east coast (Coyle et al. 1996). *Dermacentor variabilis*, the American dog tick, was the suspected vector, but

after examination proved not to be the vector. William Burgdorfer and colleagues thought that other tick species, specifically Ixodes, could be responsible for carrying the pathogen. Upon inspection of collected *I. scapularis* ticks from Long Island, NY, Burgdorfer saw coiled spirochetes in 60% of the ticks collected (Burgdorfer 1993). Recalling Hellerstrom's earlier address hypothesizing a spirochete as the cause of EM, Burgdorfer wondered if the organism he found was the organism of question in both EM and Lyme arthritis (Burgdorfer 1993). He successfully cultured the bacteria and through collaborative work was able to show that the organism was present in *I. ricinus*, which was implicated in the European disease, and was present in *Ixodes pacificus* which had been implicated in the western coastal states in the United States since 1975. Soon after the discovery of the same agent in suspect ticks both in Europe and in the United States, the organism was cultured from EM and blood of ECM patients (Burgdorfer 1993, Burgdorfer et al. 1977, Burgdorfer et al. 1983). This organism came to be called *B. burgdorferi* (Johnson et al. 1984). With this discovery, Steere could put the pieces of the Old Lyme, Connecticut mystery together and Lyme arthritis became Lyme disease.

Borrelia burgdorferi

Etiology

Borrelia burgdorferi sensu lato, is the causative agent of Lyme disease, was first described in 1982 by William Burgdorfer (Burgdorfer et al. 1982). *Borrelia burgdorferi* is a gram negative spirochete that measures 10 to 30 microns in length and 0.2 to 0.5 microns in width (Barbour 1986, Burgdorfer 1993, Hovid-Hougen 1984). It is motile, microaerophilic, very fastidious and slow growing. The organism readily stains with Geimsa. Unstained, the organism is visible with phase contrast and dark field microscopy (Barbour 1988, Burgdorfer 1993, Johnson et al. 1984). The primary species in North America is *B. burgdorferi* sensu stricto. The European and Asian strains are *Borrelia afzelii, Borrelia garinii* and *Borrelia japonica* (Barbour 1996). In nature, these spirochetes are only found in association with the arthropod vector or the mammalian hosts. The organisms survive naturally through an infectious cycle between its arthropod vector and wild animal host.

Detection

Before the advent of molecular techniques, scientists used staining and immunochemical reactions to detect bacterial infections in arthropods (Higgins and Azad 1995). Although both methods are problematic, primarily relying on the physical characteristics of the organism, they are still used today for many different applications. The gold-standard for isolating *B. burgdorferi* is direct culture. However, direct culture has problems; it is time intensive and will only detect viable organisms. The development of polymerase chain reaction (PCR) has allowed scientists a quick and more reliable technique to detect *B. burgdorferi* both in clinical and research settings.

Polymerase chain reaction is a very powerful tool that allows for direct detection of DNA (Mullis and Faloona 1987). PCR is an enzymatic reaction that amplifies minute amounts of DNA or RNA to a sufficient level that the product can be detected (Pershing 1991). The technique relies on the fact that infectious agents have unique DNA sequences and thus can be molecularly identified based on their genetic code. Due to the unique target of the test, DNA, many of the obstacles faced by the alternative methods mentioned above are no longer a problem.

The first PCR assay described for the amplification of *B. burgdorferi* was by Rosa and Schwan in 1989. Since then the use of PCR for *B.*

burgdorferi detection has been extensively researched and used in both clinical and research settings.

Many different primers are currently used either based on chromosomal or ribosomal gene sequences. Most commonly used primers are directed to chromosomal outer surface protein A (*Osp A*) or the flagellin gene (*fla*). Ribosomal primers are directed against the 16S rRNA that is universal to bacteria and can be tailored to a specific organism or used in conjunction with chromosomal primers to increase the specificity of the PCR (Higgins and Azad 1995).

Due to the tough, chitinous exoskeleton of arthropods, careful sample preparation is necessary to provide a good template for use in PCR. As a result, various methods of sample preparation have evolved; dissecting the tick and removing the midgut, lancing the tick with a needle and boiling for 10 minutes (Higgins and Azad 1995), and collecting hemolymph and processing with trituration and heating (Stich et al, 1993). For convenience, ticks can be pooled, frozen, physically smashed with a hammer, washed and then extracted with phenol and anhydrous ethyl ether (Johnson et al. 1993).

It was first noted by Higuchi in 1989 that blood inhibits the PCR. Ticks are hematophagous and must have taken at least one blood meal to become infected with *B. burgdorferi*. Schwartz et al. (1997) studied the inhibition of PCR of *B. burgdorferi* in blood fed ticks. Unengorged infected nymphs were tested by PCR then 500 prelysed *B. burgdorferi* cells were

spiked into each tick lysate and retested. Of the unspiked, 19% of the ticks were positive by PCR and 97% of the spiked tick lysates were positive. None of the engorged infected ticks tested positive. Interestingly, when engorged tick lysates were spiked with *B. burgdorferi*, still no ticks tested positive. This data was highly suggestive that the presence of blood interfered with amplification, which was indeed confirmed by the addition of spikes. To overcome this hurdle, a commercially available DNA extraction kit (Isoquick, ORGA Research, Bothell, WA) was used. In addition, clinical researchers reported that host DNA from skin samples interfered with *B. burgdorferi* detection and that a purification step of protein digestion and denaturation was necessary. It was hypothesized that host DNA, if present in high concentrations, was able to competitively inhibit primer-template hybridization, therefore inhibiting the detection of *B. burgdorferi* (Cogswell et al. 1996).

In an effort to make PCR more sensitive, nested PCR was developed. Nested PCR uses two amplification steps and four different primers. The product of the first set of primers produces a target sequence for the second set of primers during the second amplification step. Therefore, the final amplicon is a product of both amplification steps, making the test more sensitive. If DNA is not amplified in the first round, no template will be present for the second round. It has been published that a second round of amplification cycles can increase the sensitivity of a test up to 10^3 or 10^7

times (Haff 1994, Valsangiacoma et al. 1996). Although this technique is preferred over standard PCR, it is not without its pitfalls. With such increased sensitivity it is very prone to contamination and with two amplification steps, most reactions require an entire day to be completed.

Research done in the United Kingdom by Livesley and colleagues has shown that tick homogenates that were directly cultured for 2 weeks, then analyzed by PCR, yielded less false negative results versus direct PCR analysis of the homogenate. Twelve tick homogenates were tested by PCR and subjected to culture. When directly tested by PCR, 75% were positive. After 2 weeks in culture, all but one of the tick homogenates was PCR positive. There are two possible reasons for the increase of positive samples; spirochetes replicated in the culture medium and/or an inhibitor was destroyed by the culture conditions. Successive weeks in culture, 4-8 weeks, proved to have fewer positives and pelleting cultures after 10 weeks in culture prior to subjecting to PCR produced only 50% positive. The researchers of this study believe that as a result of this study, the standard methods for isolating and identifying *B. burgdorferi* need to be re-evaluated (Livesley et al. 1994).

The PCR methodology used in this research was based on a technique adapted by Dr. Mike Loeffelholtz at The University of Iowa's Hygienic Laboratory. Briefly, the nested technique was modeled after Pershing et al. (1990), using chromosomal flagellin gene primers established by Lebech et

al. in 1995.

Ticks

Vector Ecology

Ticks (phylum Arthropoda) are not insects, but are closely related to spiders and mites. Unlike insects, they lack wings and mandibulate mouthparts. They lack antennae and have four pairs of legs as adults. All ticks are obligate ectoparasites and hematophagous. As a result, they can easily become a reservoir for microorganisms and pass on the infection due to their hematophagous nature.

The largest and most economically important family of ticks is the *Ixodidae*, commonly called hard ticks due to their characteristic tough scutum. The family contains 13 genera and approximately 650 different species (Sonenshine 1991). The two most medically important species are *I. scapularis* and *D. variabilis*. *Ixodes scapularis* is the vector of *B. burgdorferi*, the Lyme disease agent, in the upper midwest. *Dermacentor variabilis* is primarily associated with the rickettsial disease, Rocky Mountain Spotted Fever, but has been implicated in the transmission of *B. burgdorferi* (Piesman and Sinsky 1988).

Life Cycle

The life cycle of these ticks requires three hosts and takes two years to complete (Yuval et al. 1990, Platt et al. 1995). Ticks are hemimetabolous, thus their life cycle is composed of three successive life stages: larva, nymph and adult, all of which resemble each other in morphology, but only increase in size with each successive molt to the next life stage. Central to this metamorphosis is the intake of a blood meal that is required for each successive molt. In general, ticks must feed to repletion, or engorgement, drop off the host, and in 25 to 30 days molt to the following life stage (Krinsky 1979).

Female ticks lay their eggs in the summer, June to July. Larvae generally hatch in late July to early August. Two weeks after hatching, larvae commence questing, the process of finding a host for a blood meal (Steere et al. 1977). Larvae that feed before September usually molt to nymphs before winter and do not feed until spring. Those that feed late in September overwinter engorged and molt to nymphs in the spring. Larvae that fail to feed, overwinter and continue questing the following spring (Yuval et al. 1990).

The resulting summer has two nymphal populations, one of which molted the previous summer and another that molted during the current season. Nymphs start questing in early spring and successful nymphs

usually molt to adults during the summer season. Those that do not molt will overwinter engorged and molt in late August of the following season (Yuval et al. 1990).

Adults emerge from late July to September and seek large vertebrate hosts. Adults that do not feed will overwinter and continue searching the following spring. At this point in the life cycle, a blood meal is required of the female to attract a mate so she can breed and subsequently lay eggs for the next generation. Only female ticks feed to engorgement and the adult males feed intermittently and only to partial engorgement (Yuval et al. 1990).

Due to the biology of the tick vector, the ticks have three chances of coming into contact with *B. burgdorferi.* Larvae and nymphs feed predominately on small mammals, in particular the white-footed mouse, *Peromyscus leucopus* (Rafinesque), which is considered the most important natural host for maintaining *B. burgdorferi* transmission. *Peromyscus leucopus* contribute most *B. burgdorferi* infection to natural tick populations (Mather et al. 1989). As stated earlier, nymphs quest and feed in the spring, where larvae do not become active until later in the summer. Since *B. burgdorferi* is transtadialy transmitted (maintained from one life stage to the next), the potentially infective nymphs are able to transmit *B. burgdorferi* to the rodents that then serve as host to the larvae later in the summer. Thus these ticks maintain an infected 'pool' of rodents for subsequent generations

to feed upon. Adult ticks prefer large vertebrate hosts, generally the white-tailed deer, *Odocoileus virginianus* (Zimmermann) (Magnarelli et al. 1986). Deer are incompetent reservoirs of *B. burgdorferi* but aid in the completion of the tick life cycle by providing females with a blood meal. Eggs laid by the females represent the next generation that will be part of the next *Borrelia* cycle. Deer seroconvert but do not contribute to the transmission of the agent (Magnarelli et al. 1986).

Once ticks have ingested spirochetes, the spirochetes travel to the midgut where they establish infection (Schwan 1996). Spirochetes are maintained in the midgut through the molts to the next life stages. In unfed ticks the organism appears to be restricted to the midgut. As ticks feed, the organism becomes more active, penetrating the midgut and traveling to the salivary glands where the organism can be transmitted via saliva that is discharged during feeding (Schwan 1996).

Organism Transmission

Borrelia burgdorferi transmission to humans and other domestic animals is through an infected tick bite. Nymphs are most likely to be implicated in transmission of *B. burgdorferi* resulting in Lyme disease (Barbour 1996, Coyle 1993, Kazmierczack et al. 1995, National Center for Infectious Disease 1996). Human outdoor activity greatly increases during

the spring and summer months as does the activity of the potentially infective nymph. The incidental human is less likely to detect the nymphal tick, because of its very small size (approximately the size of a pinhead). Due to the lack of detection of the parasite, the tick can feed until repletion, which increases the chance of successful *B. burgdorferi* transmission (Piesman et al. 1987). Adult ticks that are active in late summer and fall, are also involved in *B. burgdorferi* transmission to humans and animals, but are more likely to be detected before feeding to repletion due to the increased size of the tick due to blood consumption.

Contact transmission is not commonly accepted but has been reported in Europe under unusual circumstances. The first case reported involved a research assistant who dropped a laboratory infected tick onto a light source, the tick exploded and tick gut flew into the researcher's eye, resulting in infection. The second case involved a woman, who routinely pulled ticks off her dog and smashed the ticks between her fingers to kill them. As in the first case, tick gut spurted into her eye and she became infected (Angelov 1995).

Geographic Distribution

Lyme disease has a wide distribution in northern temperate regions of the world. In the U.S., the highest incidence is in the northeast, north

central states and on the west coast. A range of ecological and biological factors easily explains the distribution, i.e. the spread of the vector and its inter-related elements (Barbour 1996, Center for Disease Control 1996, Coyle 1993, Kazmierczack et al. 1995). During the past years, deer populations have greatly increased along with forested areas bringing with them a cornucopia of small mammals. As the deer herds have grown and natural predators have fallen, the tick population has expanded. In the northeast and north central areas, *I. scapularis*, commonly called the deer or bear tick is the primary vector. The western blacklegged tick, *I. pacificus*, is the primary vector in the far western states. Endemic states are Connecticut, Rhode Island, New York, New Jersey, Delaware, Pennsylvania, Maryland and Wisconsin. (National Center for Infectious Disease 1996).

Lyme Disease

Frequency of Occurrence

The Center for Disease Control, CDC, reported that 1998 was a record year for Lyme disease with a total of 15,934 cases reported from 45 states. This was a 25% increase over 1997 where 12,801 cases were reported. As expected most of the cases were from endemic areas, northeastern, north-central and Pacific coastal areas, and accounted for 91% of the reported

cases in 1998.

Iowa

The Iowa State Department of Public Health reported 19 cases in 1996 with Wapello, Scott and Polk counties contributing over 10% of the cases. In 1997 there were only 8 cases reported, with Polk county the leader and eastern counties following. Iowa had a record year in 1998, with 27 reported cases of Lyme disease. This was almost a 350% increase over 1997 (National Center for Infectious Disease 1999). A point of interest is that cases are reported in the residence county and does not account for individual travel. Another interesting fact is that The University of Iowa's Hygienic Lab that does Lyme disease diagnostic testing as solicited by clinicians and other institutions throughout the state, reported 26 positive and 15 presumptive positives via serology testing in 1996 (Loeffelholtz, personal communication). This large discrepancy leads one to think there is a weak link somewhere in diagnosing and reporting the disease. This is probably the case throughout the nation.

Prevention and Control

The CDC started surveillance for Lyme disease in 1982. Lyme disease became a nationally noticeable disease in 1991 (CDC 1996). White-tailed deer are used in national surveillance studies due to the ease of collecting blood samples through hunter-killed deer (Kazmierczack et al. 1995, NCID 1996). These samples are used to monitor *B. burgdorferi* activity throughout the nation. State surveillance, as national surveillance, is passive (Russell W. Currier, Iowa Department of Public Health, personal communication). States rely heavily on physicians, who can use their own diagnostic testing and testing preferences to identify *B. burgdorferi*. All counties in the state of Iowa have a public health nurse that is responsible for following up on all presumptive cases of Lyme disease.

Personal prevention

One should try to avoid tick habitats, like wooded areas and outdoor areas with considerable ground brush and grass, especially during the summer months. While hiking, camping or going outdoors in suspected habitats, one can take several precautions that reduce the chance of a tick bite. Individuals should reduce the amount of skin exposed by wearing long pants and shirts. Pants should be tucked into socks or boots to reduce

areas of tick entry to the body. Exterior clothing and any exposed skin should then be sprayed with an insect repellant containing DEET (NCID 1996). After spending time outdoors, all clothing should be washed and dried in high temperatures. All individuals and animals should be inspected for ticks. If a tick is found, it should be removed with tweezers and placed in alcohol for identification.

Tick Control

Tick control is possible but relies heavily on habitat modification and host management efforts. Habitat modification can reduce the number of ticks in hiking areas, backyards and other public places, especially in endemic areas. Leaves, brush and tall grasses should be cleared around the periphery of yards, gardens and other outdoor recreation paths. Applying commercially available acaricides, chemicals that kill ticks, in addition to clearing away ground clutter will serve as a secondary barrier to the tick and provide greater protection to humans and domestic animals. Host management, i.e. controlling the numbers of rodents and deer can also greatly reduce the abundance of ticks (National Center for Infectious Disease 1996).

Cold-Hardiness

Environmental conditions are very important to the distribution and survival of arthropod vectors. Temperature and humidity of microclimate, host availability and intrinsic tick factors all contribute to the conditions that determine vector survival. Several papers have examined cold-hardiness, a measure of cold injury, in *I. scapularis* but none have given scrutiny to *B. burdorferi* infected ticks and explored the nature of the parasitism in relation to cold survival.

Cold-hardiness, simply defined by Bale 1987, is the ability of an organism to survive at low temperatures; a mechanism to prevent freezing. It is a characteristic of all insects (arthropods) that need to survive portions of the year or their life cycle at temperatures below 0°C.

Since there is no accurate forecasting system for determining tick populations, scientists must look at both biotic and abiotic parameters that a tick encounters through its life cycle. Biotic factors include predation, disease, competition and parasitism, which are density dependent. The primary abiotic parameter is temperature, and according to Bale, is the most important density independent variable determining survival (Bale 1987).

Most of the tick's life is spent off-host and due to the ixodid tick's life cycle, any life stage may overwinter. How the life stage responds to the cold

in terms of cold-hardening is important to winter survival and future population densities. Since *B. burgdorferi* is transstadially transmitted, the level of survivorship of overwintering stages could play a major role in determing future risk (Bale 1987).

Arthropods are classified into two groups, either freeze tolerant or freeze intolerant based on their ability to survive extracelluar ice formation (Baust 1982, Salt 1961). Ticks, according to Somme (1981), are freeze intolerant.

The main strategy that ticks use to overcome this hurdle is the production of cryoprotectants, consisting of polyols, sugars and antifreeze proteins (Bale 1987). These are biochemical factors that are cued primarily by temperature and the amount of daylight. The cryoprotectants literally lower the freezing temperature of the organism.

Estimates of cold-hardiness can be measured in a number of different ways. The super-cooling point, SCP, a measure of the temperature at which crystals of ice first develop (Bale 1989). Insects also have a lethal temperature, LT_{50}, or the temperature at which 50% of the population survives. Bale was able to show that the LT_{50} is often well above the SCP. Therefore by measuring the LT_{50}, a more accurate portrayal of an organism's ability to withstand cold is possible (Bale 1987).

The cold-hardiness of *I. scapularis* has been evaluated by determining super-coolings points and estimating the LT_{50}. Schmid (1992) reported the

SCP of adults at –16°C based on early spring and late fall observations and as –8°C in the late spring and early fall. These results suggest that cold-hardiness can vary within a species depending on conditions. In laboratory reared ticks, Burks et al. (1996) was able to show that ticks acclimated to cold by being held at 4°C for seven weeks prior to exposure survived at a temperature 10°C colder than those that were not acclimated. More thorough studies by VanDyk et al. (1996), showed that a 2 hour acclimation at 0°C did not have a significant effect on cold-hardiness.

The lower lethal temperature, LT_{50}, for laboratory reared *I. scapularis* was reported by VanDyk et al. (1996). The LT_{50} was determined by a 2 hour chilling phase followed by 2 hours of re-acclimation. Survival was determined on the basis of movement after re-acclimation. Flat larvae had an LT_{50} of –11.57 $\pm$ 1.59°C. The LT_{50} for engorged larvae was -11.78 $\pm$ 0.48°C, while that of flat nymphs was -18.47 $\pm$ 0.67°C. Respectively, engorged nymphs and flat adults had an LT_{50} of -14.21 $\pm$ 1.94°C and -12.38 $\pm$ 0.14°C.

The effect of *B. burgdorferi* on the cold-hardiness of *I. scapularis* has not been exclusively examined. In 1992, Schmidt reported that infection did not seem to have an effect on the super-cooling point. However, in the same year Sharon et al. (1992) reported a decrease in infection rates and number of organisms per tick in overwintering field ticks in Wisconsin. Since gut content is very important to overwinter survival, it is important to know

what effect *B. burgdorferi* is having on the tick's ability to mount a biochemical response to cold.

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CHAPTER 3. THE EFFECT OF *BORRELIA BURGDORFERI* ON THE COLD-HARDINESS OF ADULT FIELD COLLECTED *IXODES SCAPULARIS*

A paper submitted to the Journal of Medical Entomology

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ABSTRACT

This study was designed to evaluate the cold-hardiness of adult field collected ticks and to determine if there is a difference between *B. burgdorferi* infected and uninfected ticks. Field ticks were collected from Fort McCoy, Wisconsin during the fall of 1997 and 1998. The experimental design separated ticks by sex and exposed each to a range of subzero temperatures, -10 through −18˚C. After survival assessment, ticks were tested for the presence or absence of *B. burgdorferi* using nested set PCR. Survival comparisons between the infected and uninfected do not suggest that *B. burgdorferi* has an effect on cold-hardiness.

INTRODUCTION

Ixodes scapularis Say is the vector of *Borrelia burgdorferi* Johnson, Hyde, Schmid, Steigerwalt and Brenner (Burgdorfer et al. 1982, Anderson et al. 1983, Piesman et al. 1986, Burgdorfer and Gage 1986), the etiologic agent of Lyme disease. *Ixodes scapularis* have a two-year life cycle in which both nymphs and adults overwinter (Yuval et al. 1990, Platt et al. 1992). Environmental conditions are important to the distribution and survival of arthropod vectors. Temperature and humidity of the microhabitat, host availability and intrinsic tick factors all contribute to conditions that determine vector survival.

The cold-hardiness of *I. scapularis* has been investigated by Burks et al. (1996) who reported super cooling points (SCP) of adults suggesting that cold-hardiness can vary within a species depending on conditions. They also examined the role of cold acclimation in laboratory reared ticks and reported that cold-acclimation enabled ticks to survive at temperatures 10°C colder than the unacclilmated. More thorough studies by VanDyk et al. (1996) reviewed the cold-hardiness of each lifestage of laboratory reared *I. scapularis* in both the flat and engorged states.

The effect of *B. burgdorferi* on the cold-hardiness of *I. scapularis* has not been exclusively examined. In 1992, Schmidt reported that infection did not seem to have an effect on the super-cooling point. However, in the same

year Sharon et al. (1992) reported a decrease in infection rates and number of organisms per tick in overwintering field ticks in Wisconsin.

For ticks to survive exposure to cold they must be able to produce cryogenic proteins and alcohols that serve as natural antifreeze. These compounds are vital for winter survival. *Borrelia burgdorferi* infection in ticks does not affect the day to day functioning of *I. scapularis* ticks. Whether parasitism interferes with the tick's ability to produce cryogenic compounds and mount a cold defense is unknown.

The objective of this project was to asses the cold-hardiness of adult field collected ticks and determine if there is a difference in the response of infected and uninfected *I. scapularis*. To date, infection with *B. burgdorferi* has not proven to be deleterious to tick activity and survival.

EXPERIMENTAL DESIGN

TICKS

Ixodes scapularis

Adult ticks were collected in the fall of 1997 and 1998 off hunter-killed deer and by conventional flagging techniques at Fort McCoy, Wisconsin. Fort McCoy and the surrounding area are a known focus for *B. burgdorferi* infected ticks.

Flat adult ticks were separated by sex and housed five per 5 ml tube lined with a damp tissue. Tubes were capped with a 3 cm^2 piece of cheesecloth secured with a rubberband. Tubes were placed in clear plastic crispers with a 50 ml reservoir of potassium phosphate to maintain $\geq$ 95% relative humidity (VanDyk et al. 1996). Crispers were kept on the bench top at room temperature.

COLD-HARDINESS

Cold-hardiness was measured by estimating the LT_{50}, the temperature at which 50% of the population survive. Ticks were directly chilled by exposure to rapidly decreasing temperatures, 1°C min –1 (Salt 1966). Three trials were conducted each year. Each trial consisted of a 4°C control, where 100% survival was expected, followed by 3 treatment temperatures. A range of temperatures, -10 to –14°C, was selected based on previous studies by VanDyk et al. (1996). Temperatures were adjusted as needed for 50% mortality. Each temperature trial consisted of 10 males and 10 females when tick numbers allowed. Results were evaluated based on percent survival, on day 7 post treatment, at each treatment temperature. Unaveraged data were statistically analyzed using ANOVA and linear regression. Resulting regression equations were used to estimate the LT_{50}. The data from the 4°C controls were not included in any analyses as the expected survival rate, 100%, would inaccurately skew the data.

Ixodes scapularis ticks were separated by sex and placed in two 20 ml glass scintillation vials filled with ~25 g of sand as a ballast. The sand was covered with a fitted circular piece of white filter paper to prevent ticks from burrowing. Ten ticks were placed in each vial atop the filter paper and vial lids were sealed with a generous amount of petroleum jelly (VanDyk et al. 1996). Vials were then placed within a refrigerated circulating bath (Fisher Scientific, Pittsburgh, PA) filled with 75% methanol at room temperature. The 2 hr cooling period began when the circulating bath reached the target temperature as shown by the circulator's digital display. Following the 2 hr cold treatment, ticks were reacclimated; 1 hr at 4°C and 1 hr at room temperature, approximately 23°C, and then examined for survival. Survival was based on 2 criteria: response to breath and movement within 10 min. If no response was elicited by exposure to human breath, the tick was set aside and given 10 min. to move from a designated spot. If no movement was noted, the tick was considered dead and scored accordingly. Ticks were scored on a modified injury scale established by Needham et al. (1996). The scale ranged from 0 – 3 and was modified as stated below. Briefly, a tick that displayed walking/questing behavior and responded to breath scored a 0. A score of 1 was given if the tick responded to breath but did not display walking/questing behavior. If no walking/questing behavior was observed and the tick did not respond to breath, but its legs were outstretched and otherwise appeared to be alive, it was scored a 2. A score of 3 was given to

ticks that did not exhibit any walking/questing behavior, did not respond to breath, and legs were curled up under abdomen. For statistical analyses, a score of 0 and 1 was considered alive, 2 and 3 were considered dead.

SPIROCHETE DETECTION

DNA Extraction

All specimens were prepared according to Qiagen Insect preparation guidelines as found in QIAmp Blood Kit and QIAmp Tissue Kit Handbook 01/97. Briefly, single ticks were placed in 1.5 ml microcentrifuge tubes containing 180 ul PBS and cut into small pieces with a 20-gauge needle. DNA was extracted using the blood and body fluid protocol as set forth in QIAmp Blood Kit and QIAmp Tissue Kit Handbook 01/97. A positive control of 1:40 stock *B. burgdorferi* culture was processed with each lot of extractions. Ticks were randomly tested in a blind fashion.

DNA Amplification

The nested PCR protocol used was set forth by Dr. Mike Loeffelholtz of The University of Iowa's Hygienic laboratory. The nested technique was modeled after Pershing et al. (1990), using chromosomal flagellin gene primers established by Lebech et al. in 1995.

Oligonucleotides were synthesized by the DNA facility at Iowa State University. The target of the first round of primers, F1 and F3 were (5'-ATTAACGCTGCTAATCTTAGT) and (5'-GTACTATTCTTTATAGATTC). The second round primers were F6 (5'-TTCAGGGTCTCAAGCGTCTTGGACT) and F8 (5'-GCATTTTCAATTTTAGCAAGTGATG). The resulting amplicon was 320bp. Nucleotides (100mM stocks) and *Taq* polymerase from Promega (Madison, WI) were used to amplify target DNA in an automated DNA thermal cycler (Perkin Elmer-Cetus).

DNA detection

The agarose gel was made with NuSieve/Seakeam 3:1 and TBE buffer with ethidium bromide. PCR products were loaded with 10x glycerol buffer with bromophenol blue. Gels were electrophorised at 110 volts for approximatley 20–25 minutes.

RESULTS

1997

Field collections from 1997 yielded 189 ticks, 69 male and 120 female. Treatment temperatures were –10 (n=60), -12 (n=72) and –14°C (n=57). Fifty-two percent were positive for *B. burgdorferi*. Of that 52%

positive, 33% were male and 67% were female. Day 7 survival assessment was statistically different from day 0. Sex of the tick was significant (p=0.0005) where 43% of males survived and 80% of females survived (See Figure 3.2). Treatment temperatures and infection status did not affect survival. The survival rate for the 4°C controls was 98%.

1998

In 1998, 156 male and 156 female ticks were collected. Ticks were tested at –14 (n=72), -16 (n=120) and –18°C (n=120). Thirty-three percent tested positive for *B. burgdorferi* and of that 33%, both sexes were equally represented. Day 7 post-treatment survival assessment was significantly different from Day 0. Sex of the tick was not significant to survival nor was the infection status.

Linear regression analysis of day 7 data indicated an LT_{50} of –15.3 ± 0.0108°C (equation: y=0.16x ± 2.9) and the R^2 of 0.99. The survival of control ticks (4°C) was 96%.

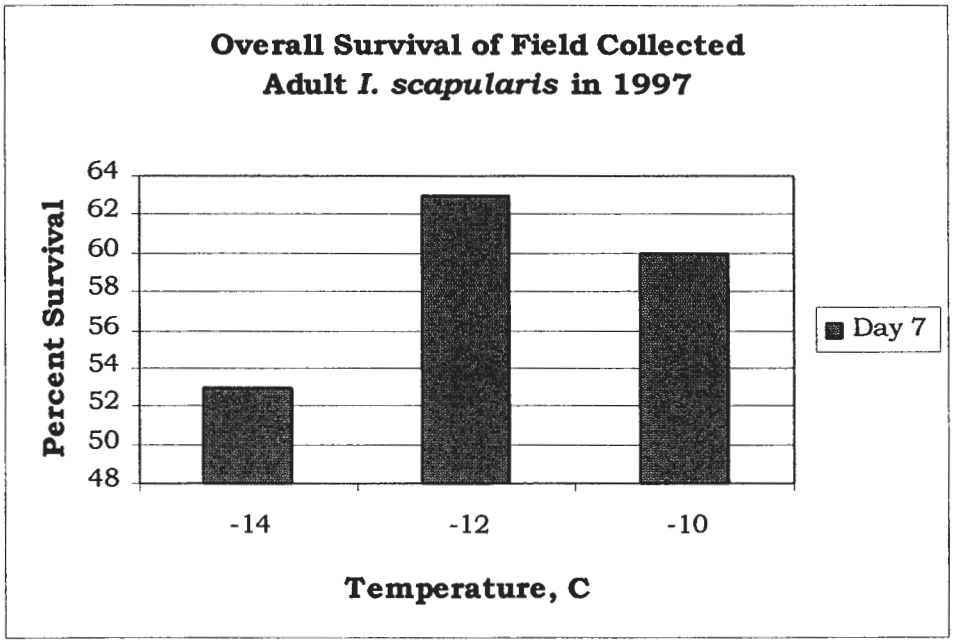

Figure 3.1 Cold-hardiness of Adult, field collected *I. scapularis*, 1997. Each value represents the average of three replicates: -10 (n=60), -12 (n=72), -14˚C (n=57).

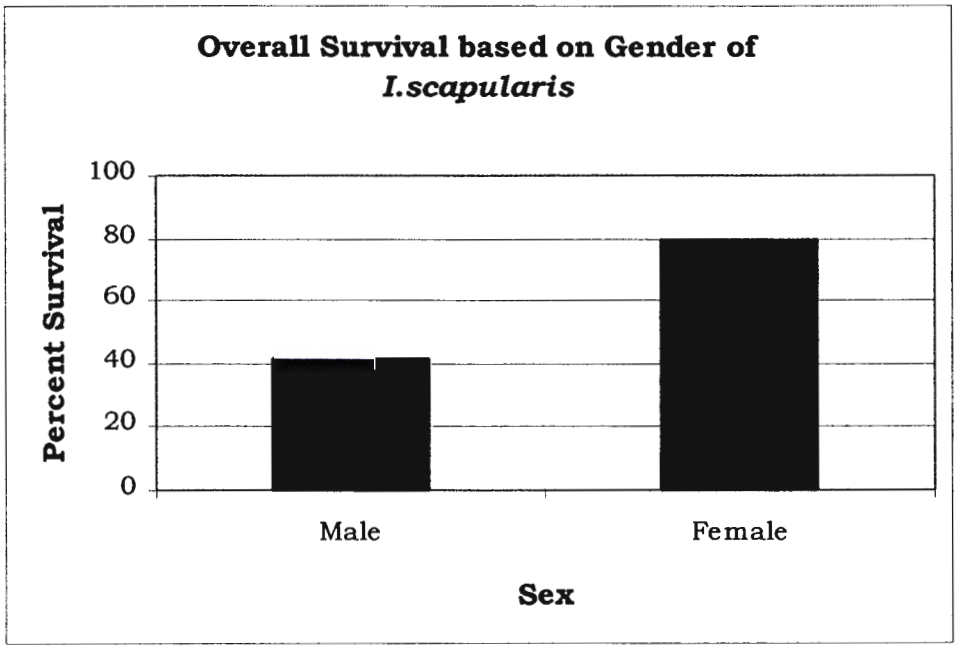

Figure 3.2 Overall cold-hardiness survival based on gender of tick. Values represent 69 males and 120 females.

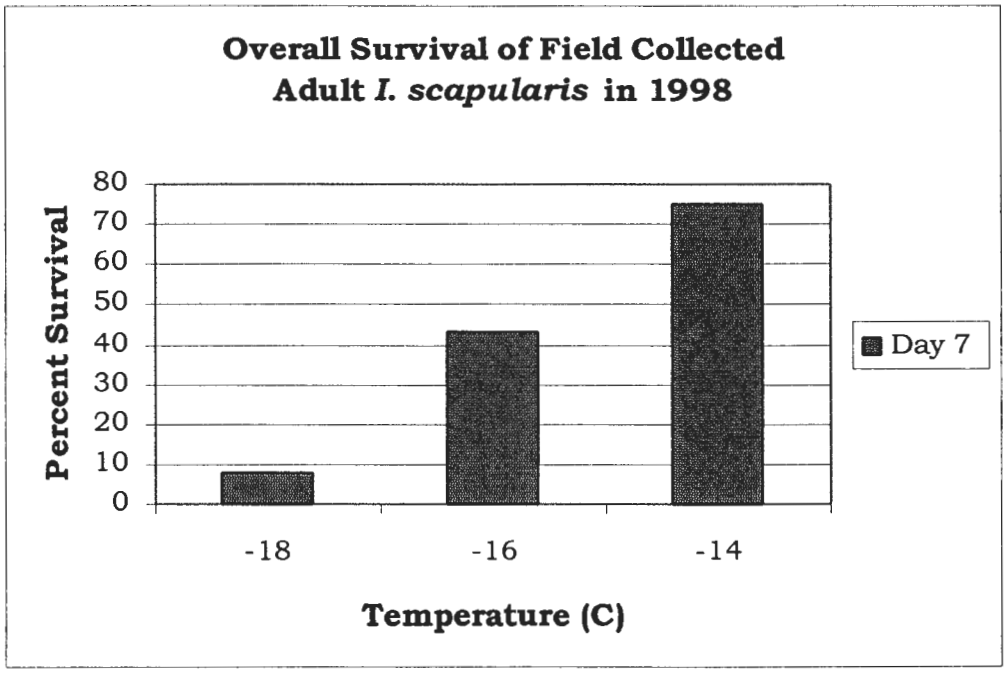

Figure 3.3 Cold-hardiness of adult, field collected *I. scapularis*, 1998. Each value represents three replicates:-14 (n=72), -16 (n=120), -18˚C (n=120).

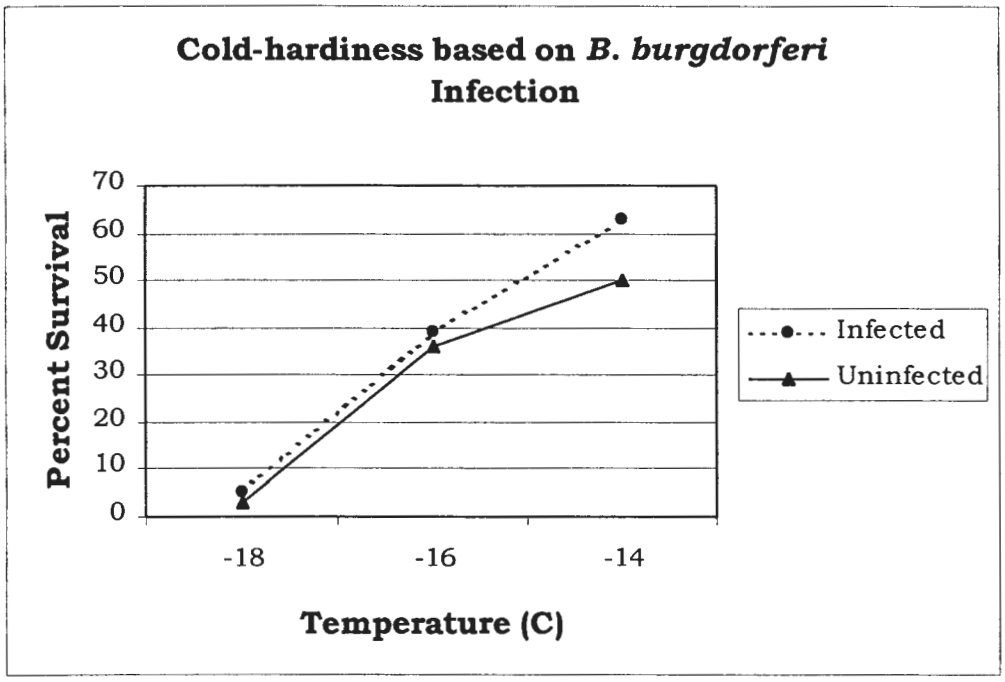

Figure 3.4 Cold-hardiness response based on infection status. Thirty-three percent of ticks were positive for *B. burgdorferi*.

DISCUSSION

1997

An LT_{50} was not calculated for ticks collected in 1997 because treatment temperatures did not have a significant effect on tick survival. Temperatures, -10 to −14°C, were selected based on previous research by VanDyk et al. (1996). He reported an LT_{50} of −12.38 ± 0.14°C, based on day 0 data. However, ticks in this experiment did not reach 50% mortality at those temperatures.

An increase in ticks judged alive on day 7 post-treatment indicate the significance of time in the assessment of survival and a trend that 'survival' changes over time, thus demonstrating a need to evaluate ticks over a longer period of time to more accurately determine cold-hardiness.

1998

Survival assessment based on post-treatment time indicated that survival rate increases with time. Overall survival was only 11% on day 0, while on day 7, 37% were determined to be alive. Clearly, there is no way an organism can be dead at day 0 and alive at day 7. This phenomenon can be explained by 'chill coma' (Lee and Baust 1987). Chill coma is commonly

referred to as the uncoordinated activity threshold, which is defined as the temperature below which a tick can no longer seek a host in a coordinated fashion (Clark 1995).

Based on this study there is no evidence to suggest that *B. burgdorferi* has any effect on the cold-hardiness of adult ticks. However, Figure 3.3 does indicate that infected ticks have a slightly higher survival rate than uninfected.

The LT_{50} (-15.3 ± 0.01°C) was based on survival on day 7. This temperature is ~3°C colder than the LT_{50} reported by VanDyk et al. (1996). Field collected ticks were definitely more cold-hardy than laboratory reared adults.

CONCLUSION

Survival comparisons of infected and uninfected adult field collected *I. scapularis* do not suggest that *B. burgdorferi* has an affect on cold-hardiness. However, the difference in LT_{50} between laboratory reared adults and field collected adults warrant further investigation with the possibility of incorporating field studies of the microhabitat to more accurately understand the effect of temperature on overwintering adult ticks. Moreover, it is unknown whether infected ticks exposed to cold are capable of transmiting *B. burgdorferi*. A study by Shih et al. (1995) indicated that

ticks exposed to temperatures greater than 27°C were not competent vectors of *B. burgdorferi*. Whether direct chilling has the same effect is not known.

ACKNOWLEDGEMENTS

The authors are grateful to Mike Loeffelholtz who graciously lent his knowledge of PCR to this study. Gracious thanks to Laura Franck, Angie Poole and Frank Elsbecker for their many contributions to this project.

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CHAPTER 4. THE EFFECT OF *BORRELIA BURGDORFERI* ON THE COLD-HARDINESS OF IMMATURE LABORATORY REARED *IXODES SCAPULARIS*

A paper submitted to the Journal of Medical Entomology

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ABSTRACT

This study was designed to evaluate the difference in cold-hardiness of *Borrelia burgdorferi* infected and uninfected immature *Ixodes scapularis* ticks. Engorged female ticks were collected from Fort McCoy, Wisconsin and their offspring were reared in the laboratory. Ticks were fed on *B. burgdorferi* infected and uninfected *Peromyscus leucopus*. Each immature life stage was subjected to rapid cold hardening for 2 hrs with a 2 hr re-acclimation period. Ticks were evaluated for survival three times: after cold treatment, 4 days post-treatment and 7 days post-treatment. Each tick was tested for the presence of *B. burgorferi* using nested set PCR. As a result of this study, *Borrelia burgdorferi* had no apparent affect on the cold-hardiness.

INTRODUCTION

Ixodes scapularis is the primary vector of *B. burgdorferi* in the upper midwest. *Ixodes scapularis* has a two-year life cycle in which both immatures and adults overwinter in either a flat or engorged state (Yuval et al. 1990, Platt et al. 1992). Ticks become infected with *B. burgdorferi* when they feed on infected animals. *Peromyscus. leucopus* is the primary reservoir responsible for tick infection. Infected ticks then transmit the organism to future hosts while feeding.

The affect of *B. burgdorferi* on cold-hardiness of *I. scapularis* was examined by Schmid. (1992) who reported that infection did not seem to have an affect on the super-cooling point. However, Sharon et al. (1992) reported a decrease in infection rates and number of organisms per tick in overwintering field ticks in Wisconsin. Thus, suggesting that cold does have an affect on tick infection and survival.

The objectives of this study were to characterize the growth of *B. burgdorferi* both in culture and in reservoir mice, infect laboratory reared ticks and compare cold-hardiness survival of infected and uninfected ticks of the various tick life stages.

EXPERIMENTAL DESIGN

BORRELIA BURGDORFERI

Growth curve

Borrelia burgdorferi, virulent strain B31, in-vitro passage four, was kindly obtained from Dr. Mark Hanson at MedImmune Corp. The initial culture was enumerated using dark field microscopy and a Petroff-Hauser counting chamber to determine the number of cells per ml. Two tubes of 10 ml BSK-H medium, supplemented with 6% rabbit serum, were inoculated with approximately 10^8 bacterial cells. Cultures were incubated at 37°C with 5% CO_2. Cultures were sampled daily for ten consecutive days. An aliquot of each day's culture was sampled, diluted 1:10 with sterile phosphate buffer saline (pH 7.0) and enumerated as previously mentioned. Each sample was counted 3 times and averaged.

Mouse infection and In-vivo spirochete growth kinetics

Eight adult female *P. leucopus* were obtained from the *Peromyscus* Stock Center at The University of South Carolina. Mice were housed separately and randomly assigned to the treatment (*B. burgdorferi* infected)

or control group (n=4). All mice were anesthetized with ketamine and xylazine, as set forth by Care and Handling of Laboratory Animals, 1995, prior to inoculations and samplings. Mice in the treatment group were infected with 0.4 ml of 10^8 log phase virulent B31. The inoculum was prepared from a 4 day culture and diluted as necessary with sterile PBS for the proper concentration of 10^8 cells. Control mice were inoculated with 0.4 ml BSK-H media. Blood and aural tissue samples were taken daily for the first 10 days and every two weeks thereafter up to 72 days post-infection. For humane reasons, only half of the mice were sampled per day to allow for one day of recovery before being sampled again. Blood samples were obtained through the suborbital sinus using microhematocrit tubes. The procedure for blood collection in mice and rats as set forth by the Care and Handling of Laboratory Animals, 1995 was used. A maximum of 250 ul of blood was taken from each mouse. To track the number of organisms present, a 10-fold serial dilution of each blood sample was cultured. The aural tissue sample was taken by using a commercially (Fisher Scientific, Pittsburgh, PA) available ear punch used for tagging small animals. The resulting sample was approximately 1 mm in diameter. Tissue samples were rinsed in 1:1 ethanol, 10% bleach solution then macerated using a sterile scalpel blade. Both blood and tissue samples were placed into 2 ml of BSK-H medium with 6% rabbit serum and an antibiotic mixture of phosphomycin, rifampicin and amphotercin B (Sigma, 1997). All cultures

were placed in a 37°C, 5% CO_2 incubator for 4 days and then examined for presence or absence of spirochetes. If no spirochetes were detected, the cultures were placed back in the incubator and examined at 4-day intervals with a maximum of 3 weeks given for each culture.

TICK ACQUISTION AND REARING

Engorged *I. scapularis* were collected in 1997 and 1998 off hunter-killed deer at Fort McCoy, Wisconsin. Engorged female ticks were stored singly in 15 ml tubes lined with a damp tissue. Each tube was capped with a piece of cheesecloth secured with a rubberband. These tubes were placed in clear plastic crispers. Each crisper had a small reservoir, ~50 ml, of potassium phosphate to maintain $\geq$ 95% relative humidity (VanDyk et al. 1996).

After oviposition, females were removed and eggs were left in the tubes to hatch. Resulting larvae were fed and used in subsequent experiments.

Approximately 100-150 larval ticks were brushed onto the head, neck and back of mice. Mice were monitored daily and checked for replete ticks. Approximately 80 ticks per feeding cycle were used in cold-hardiness experiments.

COLD-HARDINESS

Cold-hardiness was measured by estimating the LT_{50}, the temperature at which 50% of the population survive. Ticks were directly chilled by exposure to rapidly decreasing temperatures, 1°C min –1 (Salt 1966). Three trials were conducted. Each trial consisted of a 4°C control, where 100% survival was expected, followed by the 3 treatment temperatures. A range of temperatures were selected for each lifestage based on previous studies by VanDyk et al. (1996). Temperatures were adjusted as needed for 50% mortality. Each temperature trial consisted of 10 *B. burgdorferi* exposed and 10 unexposed ticks when numbers allowed. Results were evaluated based on percent survival, on day 7 post treatment, at each treatment temperature. Unaveraged data were statistically analyzed using ANOVA and linear regression. Resulting regression equations were used to estimate the LT_{50}. The data from the 4°C controls were not included in any analyses as the expected survival rate, 100%, would inaccurately skew the data.

Ixodes scapularis ticks were separated by lifestage and placed in two 20 ml glass scintillation vials filled with ~25 g of sand as a ballast. The sand was covered with a fitted circular piece of white filter paper to prevent ticks from burrowing. Ten ticks were placed in each vial atop the filter paper and vial lids were sealed with a generous amount of petroleum jelly (VanDyk et al. 1996). Vials were then placed within a refrigerated circulating

bath (Fisher Scientific, Pittsburgh, PA) filled with 75% methanol at room temperature. The 2 hr cooling period began when the circulating bath reached the target temperature as shown by the circulator's digital display. Following the 2 hr cold treatment, ticks were reacclimated; 1 hour at 4°C and 1 hour at room temperature, approximately 23°C, and then examined for survival. Survival was based on 2 criteria: response to breath and movement within 10 minutes. If no response was elicited by exposure to human breath, the tick was set aside and given 10 minutes to move from a designated spot. If no movement was noted, the tick was considered dead and scored accordingly. Ticks were scored on a modified injury scale established by Needham et al. (1996). The scale ranged from 0 – 3 and was modified as stated below. Briefly, a tick that displayed walking/questing behavior and responded to breath scored a 0. A score of 1 was given if the tick responded to breath but did not display walking/questing behavior. If no walking/questing behavior was observed and the tick did not respond to breath, but its legs were outstretched and it otherwise appeared to be alive, it was scored a 2. A score of 3 was given to ticks that did not exhibit any walking/questing behavior, did not respond to breath, and legs were curled up under abdomen. For statistical analyses, a score of 0 or 1 was considered alive, 2 and 3 were considered dead.

SPIROCHETE DECTECTION

DNA extraction

All specimens were prepared according to Qiagen Insect preparation guidelines as found in QIAmp Blood Kit and QIAmp Tissue Kit Handbook 01/97. Briefly, single ticks were placed in 1.5 ml microcentrifuge tubes containing 180 ul PBS and cut into small pieces with a 20-gauge needle. All specimens were then put through a commercial extraction process according to the blood and body fluid protocol as set forth in QIAmp Blood Kit and QIAmp Tissue Kit Handbook 01/97. A positive control of 1:40 stock *B. burgdorferi* culture was processed with each lot of extractions. Tick were processed and tested in a blind fashion.

DNA amplification

The nested PCR protocol used was set forth by Dr. Mike Loeffelholtz of The University of Iowa's Hygienic laboratory. The nested technique was modeled after Pershing et al. (1990), using chromosomal flagellin gene primers established by Lebech et al. (1995).

Oligonucleotides were synthesized by the DNA facility at Iowa State University. The target of the first round of primers, F1 and F3 were (5'-ATTAACGCTGCTAATCTTAGT) and (5'-GTACTATTCTTTATAGATTC). The

second round primers were F6 (5'-TTCAGGGTCTCAAGCGTCTTGGACT) and F8 (5'-GCATTTTCAATTTTAGCAAGTGATG). The resulting amplicon was 320bp. Nucleotides (100mM stocks) and *Taq* polymerase from Promega (Madison, WI) were used to amplify target DNA in an automated DNA thermal cycler (Perkin Elmer-Cetus).

DNA detection

The agarose gel was made with NuSieve/Seakeam 3:1 and TBE buffer with ethidium bromide. PCR products were loaded with 10x glycerol buffer with bromophenol blue. Gels were electrophorised at 110 volts for approximately 20–25 minutes.

RESULTS

BORRELIA BURGDORFERI

In-vitro growth curve

The growth of B. burgdorferi demonstrated the expected bacterial growth curve. Each data point was the average of two cultures, each sampled 3 times. Day 4 cultures yielded peak numbers of organisms.

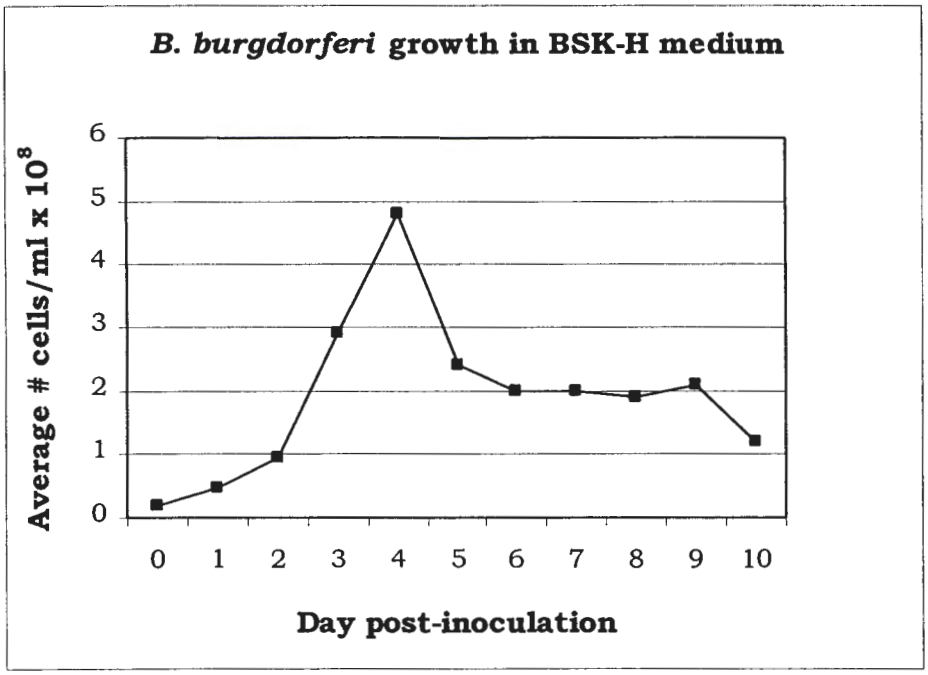

Figure 4.1 Growth of *B. burgdorferi* in BSK-H medium supplemented with 6% rabbit serum. Each value represents an average of six direct counts.

In-vivo growth curve

For the first 9 days post inoculation, *B. burgdorferi* was isolated from blood samples but was absent in tissue cultures. At days 10-77, spirochetes were detected in tissue samples, but from days 21-77 spirochetes were rarely detected in culture. Based on these data, ticks were fed on mice at least 10 days post-infection.

Infection of ticks

Borrelia burgdorferi infection in ticks was not a consideration for flat larvae as it has been well established that the organism is not transovarilly transmitted (Burgdorferi et al. 1967, Lane et al. 1991).

The rate of infection for exposed, engorged larvae was 85%. Of the 105 exposed, 90 tested positive for *B. burgdorferi* with nested PCR. Flat nymphs, that were exposed during their larval feeding, had an infection rate of 100%.

COLD-HARDINESS

Flat larvae

A total of 540 flat larvae were tested at –10, -12 and –14˚C. A total of 180 ticks were tested at each temperature. Day 7 survival assesmenet was

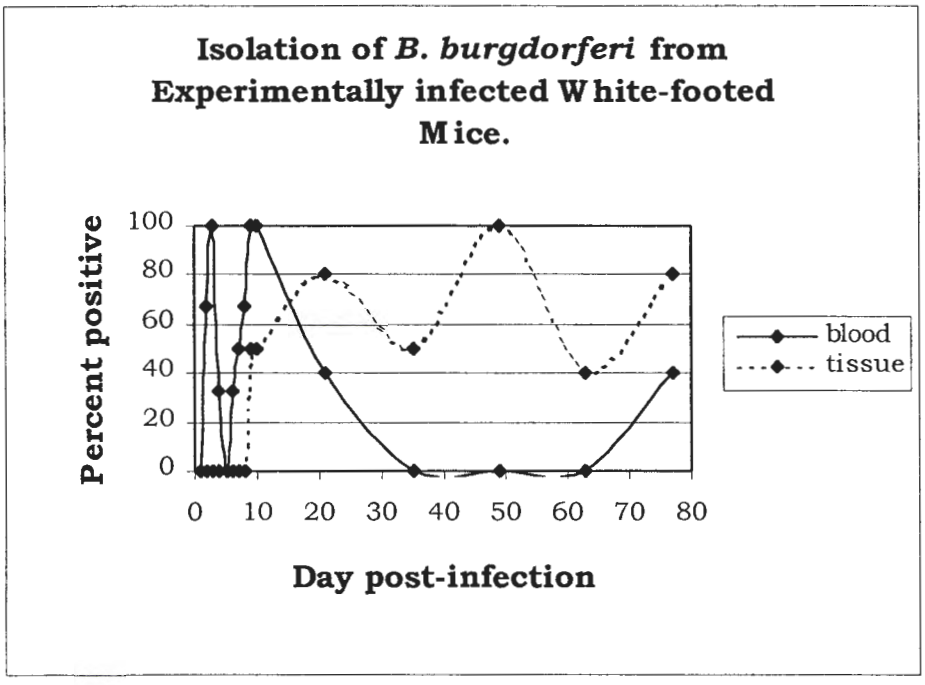

Figure 4.2 Average isolation of *B. borrelia* within the animal model, (n=4)

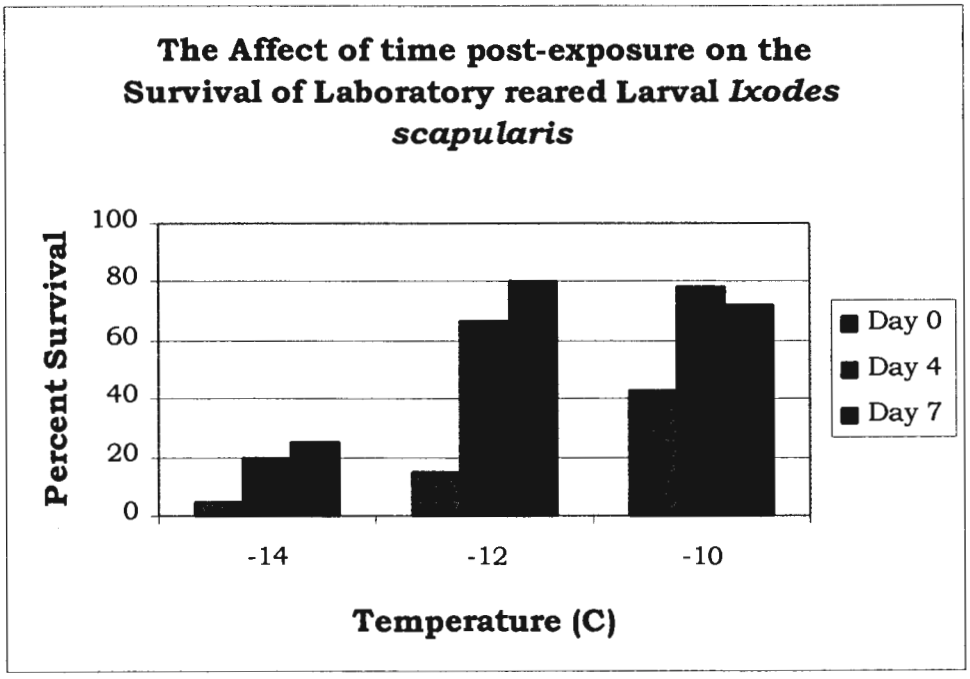

Figure 4.3 Cold-hardiness of larval *I. scapularis* as assessed over time. The same ticks were examined at each time period for all three temperatures. Each value represents 180 ticks.

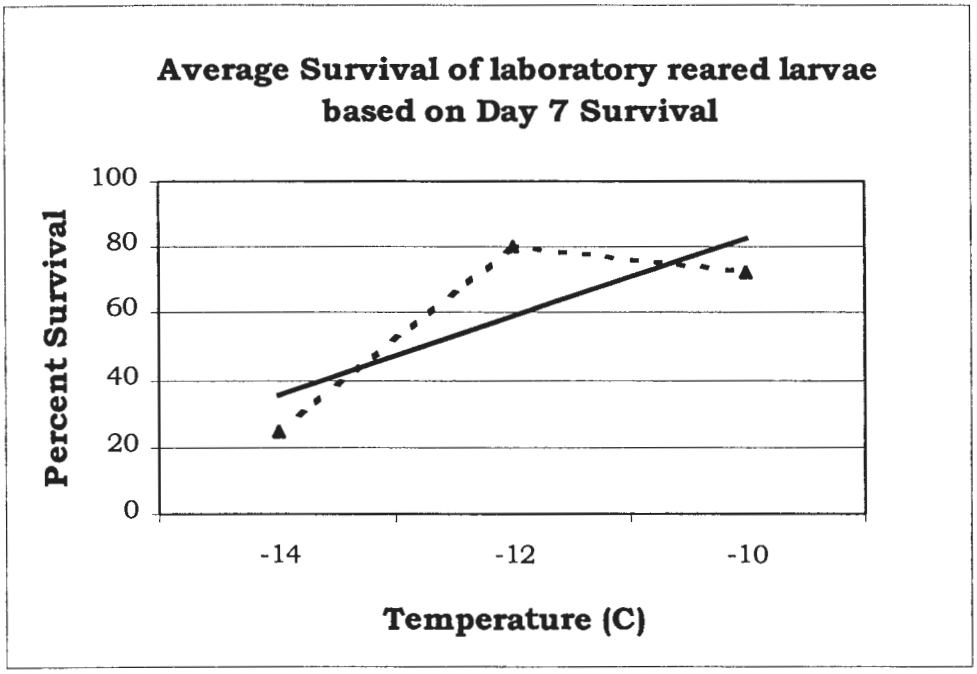

Figure 4.4 Estimation of LT_{50} by linear regression analysis.

significantly different than day 0. See figure 4.3. Figure 4.4 shows the mean survival of flat larvae on day 7 post-treatment. Day 7 survival means were used in linear regression analyses to determine the LT_{50}.

Engorged larvae

Two hundred and ten engorged larvae were surveyed at –10 (n=66), -12 (n=84) and –14°C (n=60). Day of survival assessment was significant (p=0.0293), indicating that there is an increase in survival over time. Figure 4.5. shows the mean survival of engorged larvae at day 7 post-treatment. Fifty percent of ticks tested were exposed to *B. burgdorferi*, 90 ticks tested positive, giving an 85% infection rate. Infection status was not a significant factor in survival (p=0.4321). The LT_{50} was calculated using linear regression estimates using day 7 survival means.

Flat nymphs

Flat nymphs were tested at –14 (n=99), -16 (n=126) and –18°C (n=66) for a total of 291 ticks. The day of survival assesment was determined significant (p=0.0001), indicating an increase in survival over time. Figure 4.7. shows the average survival of flat nymphs based on day 7 survival

assessment. The estimate for the LT_{50} was modeled by the linear regression shown in Figure 4.8. Of the 291 ticks, 50% had previously fed on a *B. burgdorferi* infected mouse. One hundred fifty-nine tested positive for the organism. Based on the ANOVA, infection status was not a significant factor in experimental cold survival (p=0.3404).

DISCUSSION

BORRELIA BURGDORFERI GROWTH

The in-vivo growth curve, Figure 4.2., showed that spirochete isolation declined markedly after day 9 post-infection. Thereafter, the organism was primarily isolated from the tissue cultures.

Our studies indicate that *B. burgdorferi* is in the pheripheral blood stream approximately 9 days after inoculation. After day 9 it is extensively disseminated to the epidermal tissues. Ticks feed in the capillary beds of the epidermis, therefore they were placed on infected mice for feeding at least 10 days post-infection.

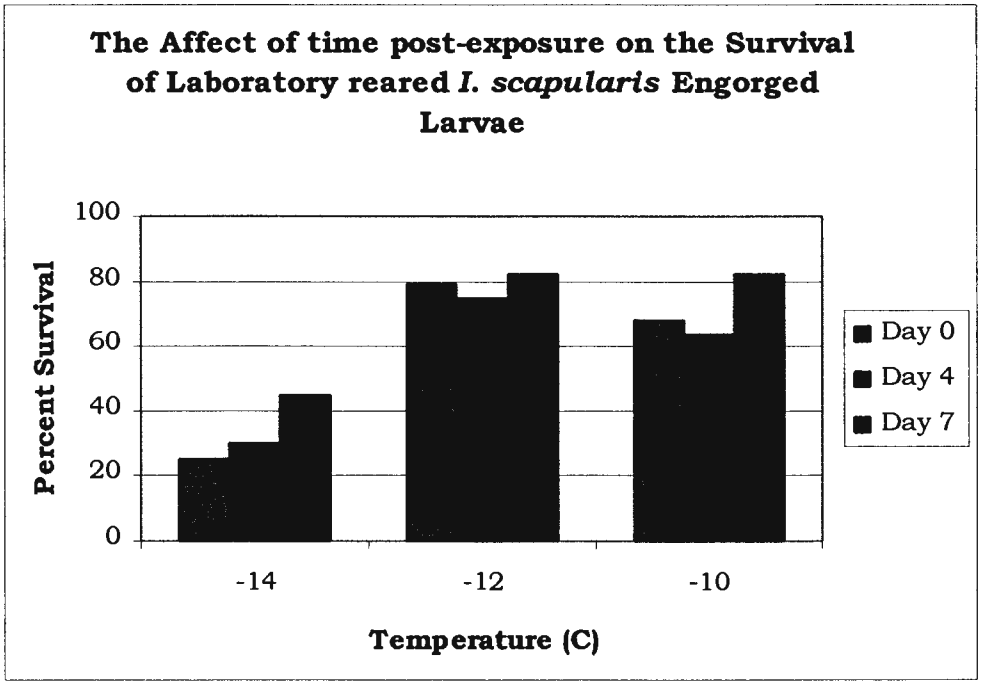

Figure 4.5 The response of *I. scapularis* engorged larvae to cold.

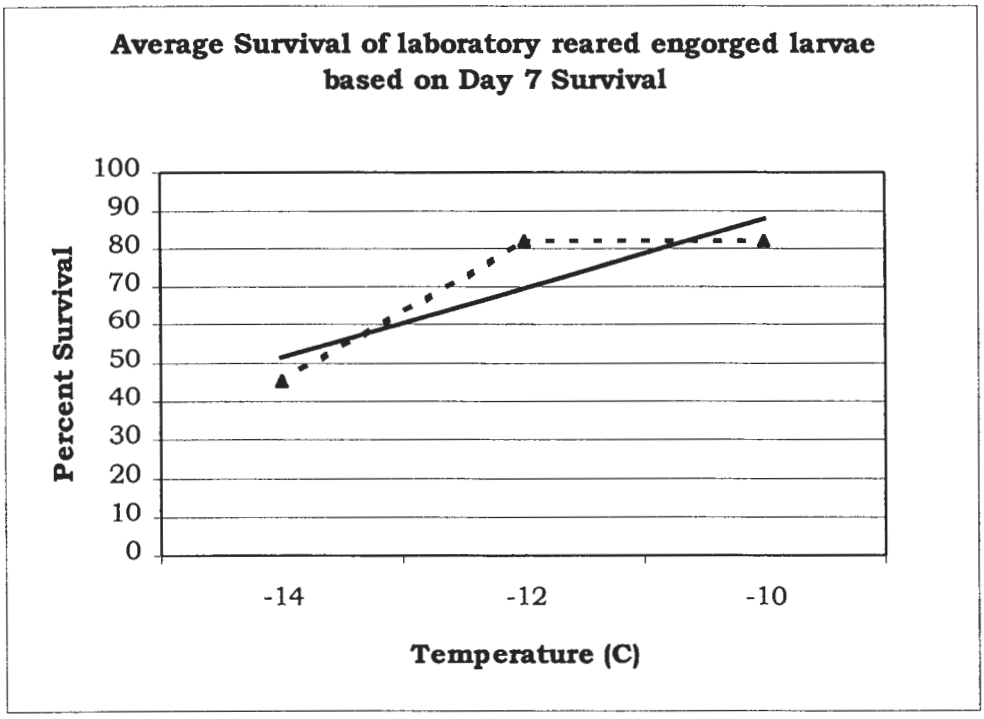

Figure 4.6 Linear regression analyses of cold response by engorged larvae.

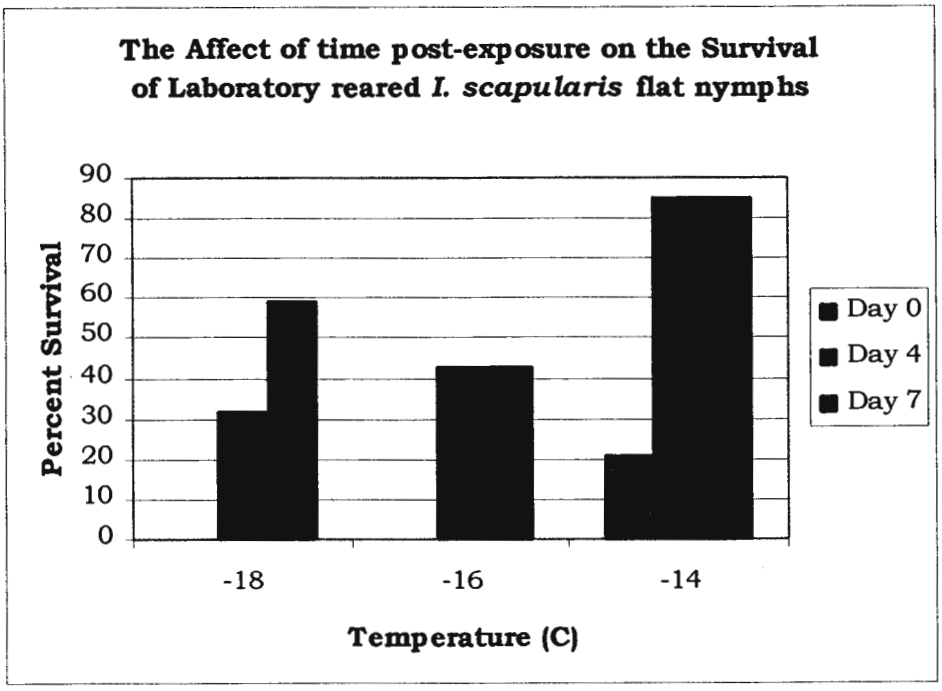

Figure 4.7 The response of *I. scapularis* flat nymphs to cold exposure.

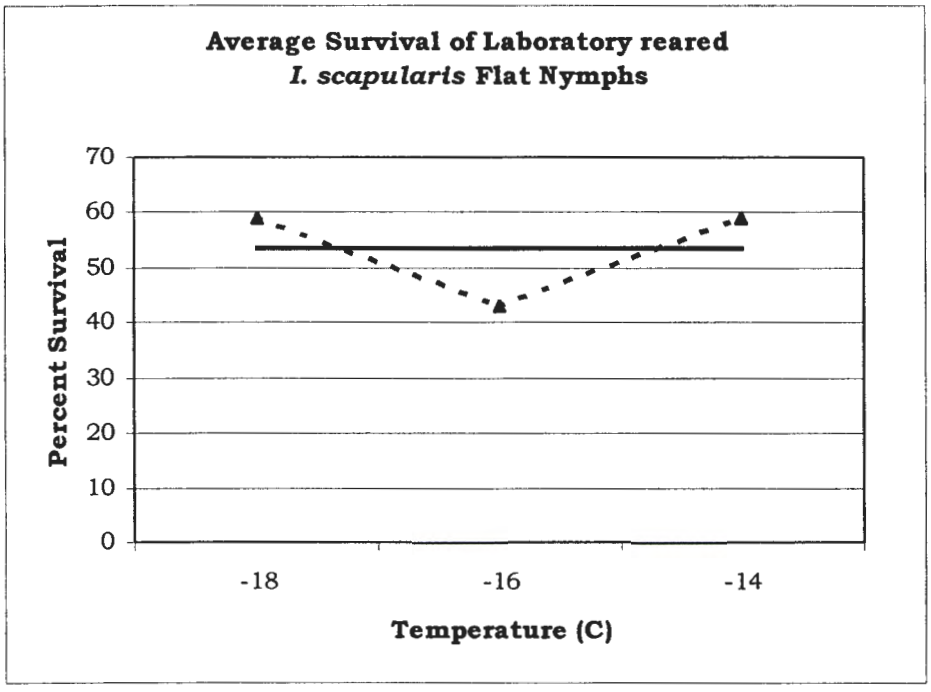

Figure 4.8 Linear regression results for *I. scapularis* flat nymphs.

TICKS

Flat *I. scapularis* larvae had an LT_{50} of -12.77 ± 0.09°C. This is approximately 1°C lower than that reported by VanDyk et al. (1996). Engorged larvae had a lower LT_{50}, –14.27 ± 0.03°C, than previously reported by VanDyk et al (1996) at –11.78 ± 0.48°C This difference could be due to the time differences in determining survival in the two studies. Survival on day 0 in this study, resulted in an LT_{50} of –12.38 ± 0.08°C, which is similar to the results of VanDyk et al. (1996). The LT_{50} of flat nymphs,–17.83°C, was slightly higher than that reported by VanDyk et al. (1996).

These studies indicate that there is no evidence to suggest that the presence of *B. burgdorferi* has a detrimental effect on immature ticks mounting a cold response. Cold-hardiness survival should be monitored for at least 7 days post-treatment to obtain an accurate portrayal of survivorship. Future cold-hardiness studies should examine microclimate conditions as well as temperature. This would help to determine a more accurate picture of how ticks actually overwinter.

ACKNOWLEDGEMENTS

Kind thanks go out to Dr. Mark Hanson who supplied the virulent strain of *B. burgdorferi* and to Dr. Mike Loeffelholz whose skill and patience taught me the art of PCR. And finally, a huge token of appreciation goes to the lab support, Laura L. Franck, Angie Poole and Nikki Youngblut.

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CHAPTER 5. COMPARATIVE COLD-HARDINESS OF *IXODES SCAPULARIS* AND *DERMACENTOR VARIABILIS*

A paper submitted to the Journal of Medical Entomology

J.L. White, W.A. Rowley and K.B. Platt

ABSTRACT

This study was designed to evaluate whether or not differences in rapid cold-hardiness of immature *Ixodes scapularis* and *Dermacentor variabilis* exist and to determine whether the two species could potentially fit into the same predictive model to asses winter survivorship based on temperature. The experimental design consisted of ticks separated by species and lifestage and subjected to rapid cold-hardening for 2 hours, after which they were exposed to a 2 hour re-acclimation period. Survival was assessed by direct examination through response to breath and observation of movement. LT_{50}s, the lowest temperature at which 50% of the population survived, was estimated using linear regression analyses on the average survivorship over the range of exposure temperatures. *Dermacentor variabilis* ticks were markedly more cold-hardy than the *I. scapularis*. The two species responded differently to cold and would need separate attention

if a predictive model was developed to assess winter survivorship based on cold temperature.

INTRODUCTION

Cold-hardiness is a characteristic of arthropods that enables their survival at certain times of the year or stage of their life cycle when temperatures may be below 0°C (Bale 1989). Cold-hardiness can differ between and within a species depending on the life stage. The measurement of cold-hardiness was the lower lethal temperature (LT_{50}), which is the calculated temperature at which 50% of the population survives.

There is limited literature available on the cold-hardiness of *I. scapularis* and *D. variabilis*. VanDyk et al. (1996) published the first paper defining the LT_{50}s, using a wide range of temperatures, for each stage of *I. scapularis*. Burks et al. (1996) looked at super-cooling points, the temperature at which ice forms, and the affect of cold acclimation on cold-hardiness. However, one has explored the small range of temperatures that further define the critical temperature range that determines survivorship. Previous studies, addressed the super-cooling point and the affect of cold acclimation on *D. variabilis* (Dautel and Knulle, 1996; Burks et al. 1996).

The intent of this paper is two fold; to explore the small range of temperatures deemed critical to tick survival, as set forth by VanDyk et al. (1996), and to compare the survival responses of both *I. scapularis* and *D. variabilis* for potential use in a population predicitive model based on temperature.

EXPERIMENTAL DESIGN

TICKS

Ixodes scapularis

Engorged female *I. scapularis* were collected in 1997 and 1998 from hunter-killed deer in Fort McCoy, Wisconsin. Engorged female ticks were stored singly in 15 ml tubes lined with damp tissue. Each tube was capped with a piece of cheesecloth secured with a rubberband. Tubes were placed in clear plastic crispers containing a small reservoir, ~50 ml, of saturated potassium phosphate solution to maintain $\geq$ 95% relative humidity (VanDyk et al. 1996).

Following oviposition, females were removed and eggs were left in the tubes to hatch. Resulting larvae were fed and used in subsequent experiments.

Approximately 100-150 larval ticks were brushed onto the head, neck and back of *Peromyscus leucopus*. Mice were monitored daily and checked

for replete ticks. Eighty ticks per feeding cycle were used in cold-hardiness experiments. Remaining ticks were used in continuing cold-hardiness studies.

Dermacentor variabilis

An engorged female *Dermacentor variabilis* was submitted by a local veterinarian for identification and was retained to start a laboratory colony. The tick and subsequent offspring were housed, maintained and feed in the same manner as the *I. scapularis*.

COLD-HARDINESS

Cold-hardiness was measured by estimating the LT_{50}, the temperature at which 50% of the population survive. Ticks were directly chilled by exposure to rapidly decreasing temperatures, 1°C min –1 (Salt 1966). Three trials were conducted with each life stage: flat larve, engorged larvae and flat nymphs. Each trial consisted of a 4°C control, where 100% survival was expected, followed by the 3 treatment temperatures. Each temperature trial consisted of 20 ticks. Results were evaluated based on percent survival, on day 4 post treatment, at each treatment temperature. Unaveraged data were statistically analyzed using ANOVA and linear regression. Resulting regression equations were used to estimate the predicted LT_{50}.

Ixodes scapularis

Ixodes scapularis ticks were separated by life stage and engorgement state and placed in two 20 ml glass scintillation vials filled with ~25 g of sand as a ballast. The sand was covered with a fitted circular piece of white filter paper to prevent ticks from burrowing. Ten ticks were placed in each vial atop the filter paper and vial lids were sealed with a generous amount of petroleum jelly (VanDyk et al. 1996). Vials were then placed within a refrigerated circulating bath (Fisher Scientific, Pittsburgh, PA) filled with 75% methanol at room temperature. The 2 hr cooling period began when the circulating bath reached the target temperature as shown by the circulator's digital display. Following the 2 hr cold treatment, ticks were re-acclimated; 1 hr at 4°C and 1 hr at room temperature, ~23°C, and then examined for survival. Survival was based on 2 criteria: response to breath and movement within 10 min. If no response was elicited by exposure to human breath, the tick was set aside and given 10 min. to move from a designated spot. If no movement was noted, the tick was considered dead and scored accordingly. Ticks were scored on a modified injury scale established by Needham et al. (1996). The scale ranged from 0 – 3 and was modified as stated below. Briefly, a tick that displayed walking/questing behavior and responded to breath scored a 0. A score of 1 would be given if the tick responded to breath but did not display walking/questing behavior.

If no walking/questing behavior was observed and the tick did not respond to breath, but its legs were outstretched and otherwise appeared to be alive, it was scored a 2. A score of 3 was given to ticks that did not exhibit any walking/questing behavior, did not respond to breath, and legs were curled up under abdomen. For statistical analyses, a score of 0 or 1 was considered alive, 2 and 3 were considered dead.

The range of treatment temperatures chosen was just above and below those reported LT_{50} by Van Dyk et al. (1996). Flat and engorged larvae were tested at -10, -12 and -14°C, while flat nymphs were tested at -14, -16 and -18°C.

Dermacentor variabilis

Dermacentor variabilis ticks were treated in the same manner as *I. scapularis* except for the few modifications mentioned. Preliminary experiments, not shown, using temperatures set forth for *I. scapularis*, yielded greater than 90% survival . As a result, the treatment temperature range was modified, within the limits of the refrigerated circulating bath, to achieve 50% mortality. The final range of temperatures tested and analyzed were –18, -20 and –22°C. In addition, engorged *D. variabilis* larvae were assessed for survival by successful molt to a nymph. This modification was made due to engorged larvae undergoing rapid metamorphosis therefore

an accurate score, according to the damage scale, could not be applied. If the tick successfully molted within 14 days post-treatment it was a zero (deemed alive), and if the tick did not survive the process it was scored a 3 (dead).

RESULTS

FLAT LARVAE

A total of 498 *D. variabilis* and 540 *I. scapularis* were tested. Figure 5.1 shows the difference in cold-hardiness between the species. *Dermacentor variabilis* flat larvae had an LT_{50} of –21.10 ± 0.01°C, while *I. scapularis* flat larvae had an LT_{50} of –12.77 ± 0.09°C.

Figure 5.1 indicates the average percent tick survival at the range of temperatures selected to best estimate the LT_{50}. The response of *I. scapularis* does not show a definite linear response to temperature. The reason for the increased survival at –12°C is unknown. Graphically, 50% survival lies between –12 and –14°C. Linear regression analysis estimated the LT_{50} value of -12.77°C for *I. scapularis* flat larvae. In comparison, *D. variabilis* flat larvae had a strong linear survival response to cold. However, based on averages, the temperature at which 50% of the treated population survived was never achieved (due to the lowest temperature attainable by

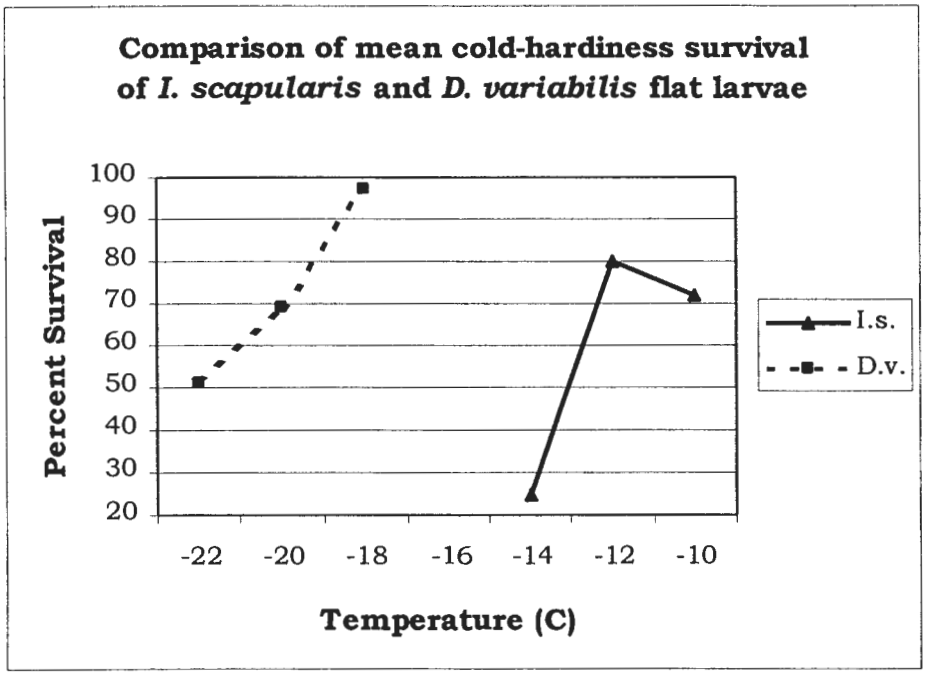

Figure 5.1 Cold-hardiness of *I. scapularis* and *D. variabilis* flat larvae.

the circulating bath being –22°C). This said, when a straight line was fit to the data, the estimated LT_{50} was –21°C.

ENGORGED LARVAE

Figure 5.2 indicates the comparative survival of the two different species as engorged larvae. For the 272 *D. variabilis* tested, the LT_{50} was estimated at –40.17 ± 0.01°C, while engorged *I. scapularis* (n=210) had an LT_{50} of –14.27 ± 0.03°C.

There are major differences in the two species and their response to cold. The *Ixodes* ticks seem to have a critical temperature tolerance of –12°C. With a -2°C shift, survival drastically drops from 80% to 45%. The estimated LT_{50} was –14.27°C. Alternatively, the *D. variabilis* ticks were barely affected by exposure to cold. At the coldest temperature tested, -22°C, 85% of ticks survived the treatment. Based on the data available, an LT_{50} was estimated at –40°C.

FLAT NYMPHS

The response of *D. variabilis* (n=534) and *I. scapularis* (n=120) flat nymphs to cold is shown in figure 5.3. *Ixodes scapularis* had an LT_{50} of –17.83 ± 0.05°C which was only ~4°C warmer than the *D. variabilis* LT_{50} of –21.63 ± 0.02°C.

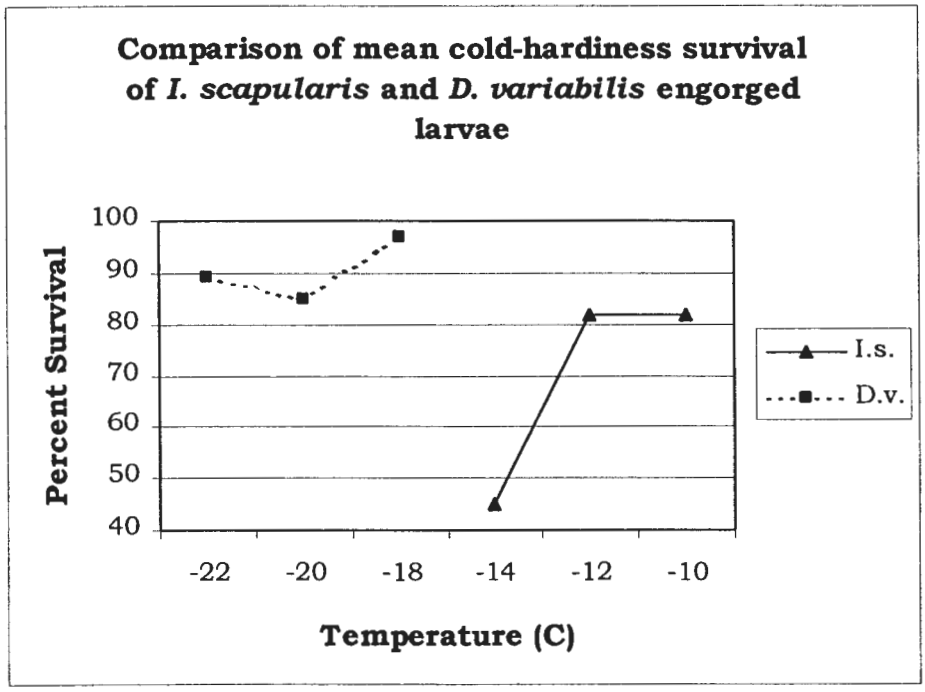

Figure 5.2 Cold-hardiness of *I. scapularis* and *D. variabilis* engorged larvae

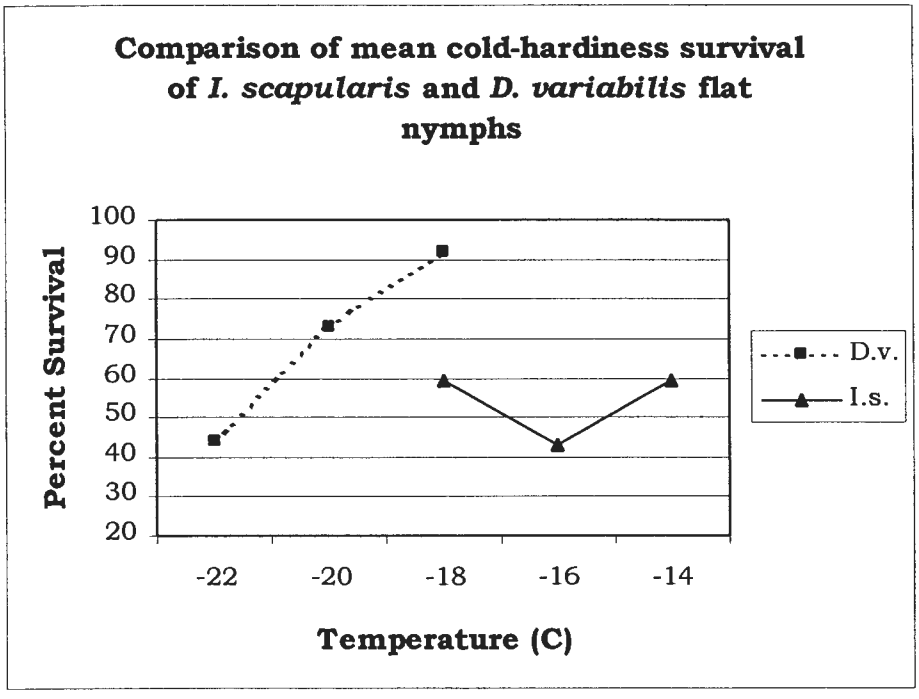

Figure 5.3 Comparison of *I. scapularis* and *D. variabilis* flat nymphs

Ixodes ticks had a fluctuating response to cold. From –14 to –16°C, there is a 15% reduction in survivorship, however at –18°C the 15% reduction was regained making the percent survival at –14°C and –18°C approximately the same, of about 60%. In contrast, *D. variabilis* flat larvae had a linear response to cold, with a 50% survival temperature estimated at –21°C.

DISCUSSION

Dermacentor variabilis was significantly more cold-hardy than *Ixodes scapularis* in both the flat and engorged larval stages. The LT_{50} for flat *D. variabilis* larvae was -21.10°C, approximately 8°C colder than the LT_{50} temperature for *I. scapularis* flat larvae. *Dermacentor variabilis* flat nymphs had an LT_{50} of –21.63°C, essentially the same as flat larvae and very similar to the estimate for *I. scapularis* flat nymphs at -17.83°C. Engorged larvae of the *D. variabilis* were the most cold-hardy with an LT_{50} of -40.17°C, an incredible -26°C difference over *I. scapularis* engorged larvae.

In light of these data, there were consistent fluctuations in the response of *I. scapularis* to cold treatment. In all three lifestages, higher survival percentages occurred at lower treatment temperatures. However, the LT_{50}s for *I. scapularis* in this study were similar to the experimental

values reported by VanDyk et al. (1996).

D.variabilis ticks were extremely tolerant of cold. Both flat larvae and flat nymphs had a LT_{50} of –21°C, which is similar to a reported super-cooling point of –22°C for unfed adult *D. variabilis* (Dautel and Knulle, 1996). Conversely, a report by Burks et al. (1996) stated the LLT_{2h} (another method of denoting the LT_{50}) as being –14°C for nymphal *D. variabilis* (n=120 to 144). Surprisingly, the LT_{50} of engorged *D. variabilis* larvae was –40°C. Treatment temperatures ranged from –18 to –22°C, with survivorship not falling below 85%. Whether the increased survival was due to engorgement is unknown. There are conflicting reports both supporting and disputing the benefit of engorgement in increasing cold-hardiness (Lee and Baust, 1987; Needham et al. 1996; VanDyk et al. 1996).

Immatures of *I. scapularis* and *D. variabilis* have different survival rates in response to rapid cold-exposure. Further research is needed to investigate the microclimate of over-wintering ticks and the actual temperatures experienced within a region before applying critical temperatures to population predictive models.

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CHAPTER 6. SUMMARY AND GENERAL CONCLUSIONS

Summary of Experimental Results

The most significant finding was *Borrelia burgdorferi* infection did not effect cold-hardiness. This was true of both field collected adult *Ixodes scapularis* and those that were laboratory reared and experimentally infected. Whether ticks infected in nature overwinter as well as uninfected ticks has yet to be unexamined. A report in 1992 by Sharon and others found that there were more *B. burgorferi* infected ticks in the fall than the following spring, suggesting that winter may have an effect on infected ticks. However, field studies have not been conducted to look at the overall winter survival of infected ticks.

Repeatedly, the day of assessment was statistically significant. The design of these studies monitored the same ticks up to 14 days post exposure. The common trend seen in all studies was that perceived survival increased over time, due to the chill coma affect. Survival scoring at day 0, right after cold treatment, consistently gave data that reported a much lower survival rate. When the same ticks were again scored at 4 and 7 days post-exposure, it was found that most of the ticks previously recorded as 'dead' were alive.

The LT_{50} values for *I. scapularis* indicate that flat nymphs (laboratory reared) were the most cold-hardy (LT_{50} –17.83°C), followed by field collected adults with an LT_{50} of –15.3°C. Laboratory reared engorged larvae had an LT_{50} of –14.27°C, while the least cold-hardy were flat larvae with an LT_{50} of –12.77°C. These were similar to VanDyk et al. (1996), who reported an LT_{50} of –12.38°C for adults, –18.47°C for flat nymphs, -11.78°C for engorged larvae and –11.57°C for flat larvae, all of which were laboratory reared.

Dermacentor variabilis was much more cold-hardy than *I. scapularis*. Engorged larvae of *D. variabilis* were the most cold-hardy with an LT_{50} estimate of –40.17°C. This value was ~14°C cooler than the LT_{50} for flat larvae where the LT_{50} was –26.25°C. The least cold-hardy were the flat nymphs with an LT_{50} of –22.81°C, however this value was well below the 50% mortality temperatures of *I. scapularis*.

Recommendations for Future Research

In order to increase our understanding of pathobiology of *Borrelia burgdorferi* on host ticks, a closer look at the molecular relationship between an organism and host needs to be taken. Since the ability to cold-harden is a biochemical process, it would be interesting to investigate the different biochemical components of cold-hardiness in both *B. burgdorferi* infected and uninfected ticks.

Future cold-hardiness studies should couple information from

laboratory studies with that of the tick's microhabitat. Tracking winter temperatures, along with humidity and soil temperatures may give a more accurate account of what a tick must endure to survive a winter. These parameters could then be tracked over time (years) and used to determine if laboratory estimated LT_{50} temperatures are pertinent to current climatic conditions. This type of information would have epidemiological importance as a possible predictive tool for future tick populations as tick population has been linked to climatic conditions.

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