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Identification and Characterization of a Relish-type NF-kB, DvRelish in Dermacentor variabilis, the American Dog Tick

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IDENTIFICATION AND CHARACTERIZATION OF A RELISH-TYPE NF-κB, *DVRELISH*, IN *DERMACENTOR VARIABILIS*, THE AMERICAN DOG TICK

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ABSTRACT

Ticks are important worldwide as vectors of bacteria, viruses, and parasites. Pathogenic and non-pathogenic spotted fever group (SFG) *Rickettsia* are maintained and transmitted by ticks with specific hard tick-*Rickettsia* pairings evident in nature. The pathogenic SFG *Rickettsia rickettsii* is transmitted by the hard tick *Dermacentor variabilis*. In response to infection, *D. variabilis* is known to differentially respond to SFG *Rickettsia* infection. The mechanisms of differential immune induction are currently unknown, and are likely involved in the establishment of specific tick-SFG *Rickettsia* pairings. It was hypothesized that the level of response by *D. variabilis* to SFG *Rickettsia* occurs in a species-specific manner, and that this response drives vector competence. To this end, we report the isolation of an mRNA transcript, *dvrelish*, using RACE-PCR. Conserved domain analysis of *dvrelish* identified a Rel-homolgy domain, allowing for its identification as a putative Relish-type NF-κB. DvRelish was identified via Western blot, immunofluorescence assay and MALDI-TOF/TOF mass-spectrometry. Tick infection assays were performed using microinjection and capillary feeding technique methodologies to identify *dvrelish* expression in response to SFG *Rickettsia* infection. Microinjection of 10^7 *R. rickettsii* induced an increased expression of *dvrelish* in hemocytes at 1 hour post injection, and in combined tissues at 6 hours post injected. Injection with similar and lower doses of *Pseudomonas aeruginosa* and *Rickettsia parkeri* did not significantly change *dvrelish* expression. When capillary fed *R. rickettsii*, *dvrelish* expression increased in hemocytes after 1 hour exposure and decreased after a 3 hour exposure. Together, the expression of *dvrelish* was dose- and tissue- specific in response to SFG *Rickettsia* challenge. Understanding the molecular regulation of immunological response to rickettsial infection in ticks may better define the mechanisms of vector competence and the epidemiology of SFG rickettsioses.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Ticks

Ticks are members of the phylum Arthropoda, class Arachnida, subclass Acari, and order Parisitiformes. Ticks are further divided into three families: Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae (Nicholson et al. 2009). Ixodidae are categorized as Prostriata (genus *Ixodes*) or Metastriata based on the position of a ventral grove anterior or posterior to the anus, respectively (Klompen 2005). Ixodidae consists of over 660 characterized species of hard ticks which accounts for 80% of all known tick species. Conversely, Argasidae consists of fewer than 200 recognized species (Nicholson et al. 2009). Nuttalliellidae contains only one recognized species of no known medical importance (Klompen 2005). Both hard and soft ticks have a great impact on human and animal health through direct effects of blood feeding as well as by the transmission of disease causing agents including bacteria, protozoa, and viruses (Table 1.1).

1.1.1 Ixodid ticks (Family Ixodidae)

Ixodid ticks are characterized by the presence of a rigid scutum on the dorsal body surface of both adult males and females. The scutum is greatly reduced in size in females allowing for extensive engorgement during blood-feeding (Nicholson et al. 2009). Males, with a larger scutum, are relatively restricted on the expansion of the cuticle during blood-feeding. Hard (Ixodid) ticks have two body sections: the capitulum and the idiosoma. The capitulum includes the mouthparts, palps, and chelicera. The idiosoma includes the legs, and core organs, and is further divided into the podosoma (includes the legs and genital pore) and the opithosoma (includes the region behind the coxae, spiracles, and anus). Adult and nymphal ticks have 4 pairs of legs, while larva only have 3 pairs (Nicholson et al. 2009). Legs are divided into six segments and the first leg pair contains Haller's sensory organ. Haller's organ has been associated with

Table 1.1 Major tick-borne diseases of humans. Disease causing agents, primary tick vectors, and vertebrate host.

Disease	Causative agent	Primary tick vector species
Human Babesiosis	*Babesia microti, B. divergens, B. major*	*Ixodes scapularis, I. ricinus,*
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor variabilis, D. andersoni, Rhipicephalus sanguineus*
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*A. americanum, D. variabils*
Human anaplasmosis	*Anaplasma phagocytophilum*	*I. scapularis, I. pacificus, I. ricinus*
Q fever	*Coxiella burnetii*	Many tick species
Lyme disease	*Borrelia burgdorferi, B. afzelii, B. garinii, B. bissettii*	*I. scapularis, I. ricinus, I. pacificus, I. persulcatus,* others
Tick-borne relapsing fever	*Borrelia* spp.	*Ornithodoros* spp.
Tularemia	*Francisella tularensis*	*D. variabilis, D. andersoni, D. reticulutus, A. americanum, I. apronophorus, I. ricinus* complex, *Haemaphysalis leporispalustris*
Powassan encephalitis	Flavivirus; family Reoviridae	*Ixodes, Dermacentor*, and *Haemaphysalis* spp.
Colorado tick fever	Coltiivirus; family Reoviridae	*D. andersoni*
Crimean-Congo hemorrhagic fever	Nairovirus; family Bunyaviridae	*Hyalomma m. marginatum, H. m. rufipes*

(Goodman et al. 2005)

thermosensory, gustatory and mechanosensory functions (Nicholson et al. 2009).

As bloodmeals are an essential component of the tick life cycle, finding a vertebrate host is critical. Ixodid ticks have a hemimetabolous life cycle consisting of four life stages including the egg, larva, nymph and adult (Figure 1.1) (Sonenshine and Roe 2014). Post eclosion from the egg, each life cycle stage of the tick acquires a single bloodmeal to allow molting to the next stage. Adult females will feed repletion and oviposit thousands of eggs before dying. *Dermacentor* ticks are known to oviposit upwards of 5,000 eggs per clutch, however the average maximum number of eggs is species dependent (Nicholson et al. 2009). After the eggs have hatched, the larva must find a host. Once bloodfeeding and molting to the nymphal stage are

completed, the host seeking and bloodfeeding process is repeated. After molting and sexual differentiation, adult males and females will mate and feed for egg production, continuing the life cycle (Sonenshine and Roe 2014).

Suitable hosts are recognized through cues including body heat, carbon dioxide, vibrations and odors from sweat, urine and other wastes; these cues lead to increased questing behaviors. Shadows from movement may also be visualized and vibrations in the local environment from potential host movement may be perceived. The combination of long-range

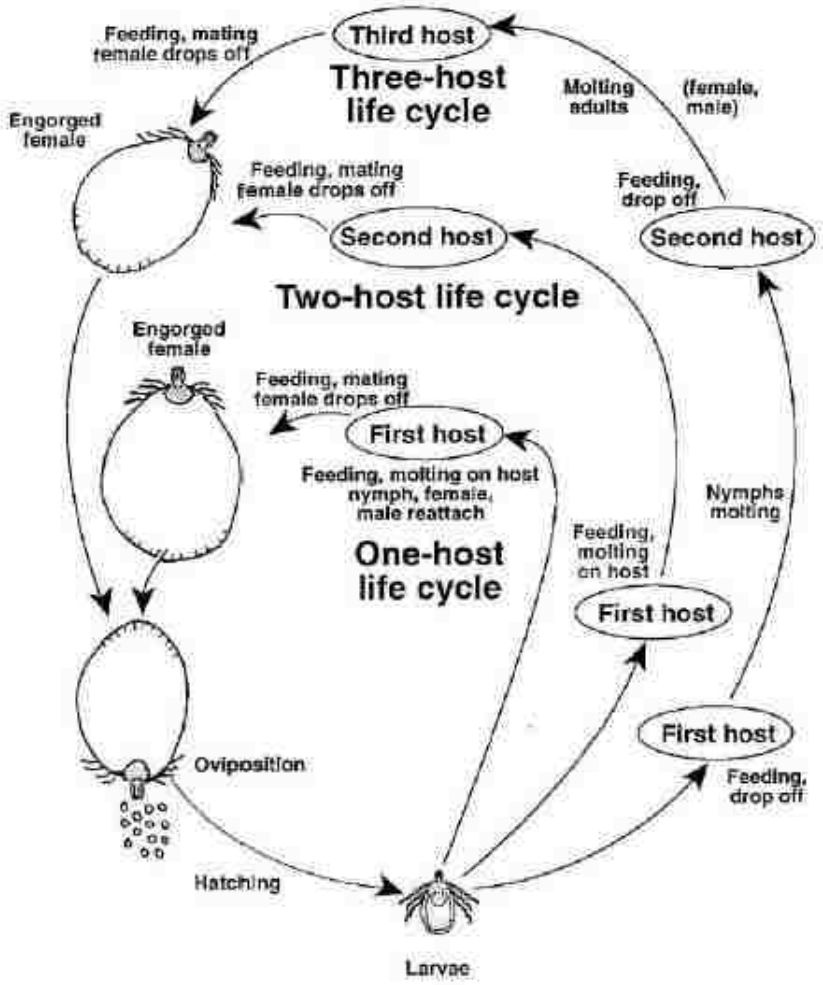

Figure 1.1 Generalized life cycle of three-host, two-host, and one-host ticks (Nicholson et al. 2009).

cues, such as vibrations, and shorter-range cues like odors initiate questing behaviors and aid in the recognition of both suitable hosts and attachment sites (Sonenshine and Roe 2013). There are two strategies for host seeking: the hunter strategy where ticks actively move towards hosts when host cues are sensed and the ambush method where ticks wait for passing hosts. One example of use of the hunter strategy is by the camel tick, *Hyalomma dromedarii,* which is known to emerge from sand or rocks and move quickly towards hosts. The ambush strategy is used by most non-nidiculous ticks which wait on vegetation for passing hosts (Apanaskevich and Oliver Jr. 2014).

The duration of feeding for each life cycle stage varies slightly in ixodid ticks. Under laboratory conditions between 22-24°C, 90% relative humidity, and with a photoperiod of 16:8 (light:dark) hours, Troughton and Levin (2007) determined the life cycles of seven species of ticks in colony. The entire life cycle of *D. variabilis* can be completed in as little as 19 weeks in the laboratory, but generally takes 25 to 27 weeks to complete. Larval *D. variabilis* feed for 2 to 8 days with the majority detaching at day 4 and require between 2 to 3 weeks to molt. Nymphs feed for up to 11 days with the majority detaching at day 5. Nymphs require between 3.5 and 5 weeks to molt. Adult females require 7 to 10 days to feed to repletion. Females will lay an egg clutch 1.5 to 3 weeks after engorgement and the eggs will hatch 5 to 8 weeks after oviposition. Unfed *D. variabilis* larva are viable without a blood meal for up to 6 months, nymphs for 2 months, and adults for up to 8 months (Troughton and Levin 2007).

Adult ticks attach to their host via the hypostome, a structure containing the food canal and rows of recurved barbs which aid in attachment. Some ixodid ticks produce a cement-like secretion to reinforce attachment to the host. Surrounding the hypostome are two chelicerae used to cut through skin, and two palps which enclose the salivary ducts. Feeding occurs through the alternating periods of blood sucking and secretion of saliva (Sonenshine and Anderson 2014).

Tick saliva contains many bioactive compounds which facilitate the long-term feeding style of ixodid ticks. These molecules include anti-hemostatic factors, anti-inflammatory factors, complement inhibitors, and bioactive compounds which modulate host immunity by inducing a Th2 type response (Alarcon-Chaidez 2014).

There are two phases to feeding by ixodid adult females. During the first few days of attachment, ixodid adult females will feed only slightly to allow synthesis of chitin required for further engorgement. In order to progress to the second stage of engorgement where the females will imbibe blood at a much quicker rate, mating must occur. In contrast, males bloodfeed intermittently with spermatogenesis stimulated by bloodfeeding. A male tick can then inseminate multiple females, triggering rapid phase engorgement. Without insemination females will not rapidly feed. For some *Ixodes* species mating occurs off host in burrows or nests (Nicholson et al. 2009).

1.2 Tick species of medical importance in the United States

1.2.1 *Dermacentor* species

Dermacentor spp. are three-host, metastriate ticks taking bloodmeals from separate hosts which allows molting to the subsequent life stages. These ticks have an ornamented scutum with short wide mouthparts. In tropical climates with abundant rainfall, *Dermacentor* ticks can continue to develop year round. However, these ticks are also tolerant to desiccation, and lessen host questing behaviors during periods of low humidity. In the northern part of its distribution, *Dermacentor* larva and adults enter diapause during the coldest part of the winter, resulting in a prolonged life cycle over a two year period (Nicholson et al. 2009). Several species of

Figure 1.2 Approximate distribution *Dermacentor variabilis* in the United States (Distribution map courtesy of Centers of Disease Control and Prevention).

Dermacentor ticks are found throughout North and Central America, Europe, Asia, and Africa. Species of importance in North America include *Dermacentor variabilis*, the American dog tick (Figure 1.2), and *Dermacentor andersoni*, the Rocky Mountain wood tick (Figure 1.3) (www.cdc.gov). These ticks are the vectors for many pathogens including the etiologic agents of Rocky Mountain spotted fever (RMSF) and Colorado tick fever (Nicholson et al. 2009). The major route of transmission of disease causing agents is horizontally through bloodfeeding, but infected ticks may also transmit vertically to subsequent life stages (transstadial) and their progeny (transovarial) (Goodman et al. 2005). In North America, the vectors of *Rickettsia rickettsii* include *D. variabilis* and *D. andersoni*. Horizontal transmission of *Anaplasma marginale* and *Franciscella tularensis* has also been observed in *Dermacentor* species.

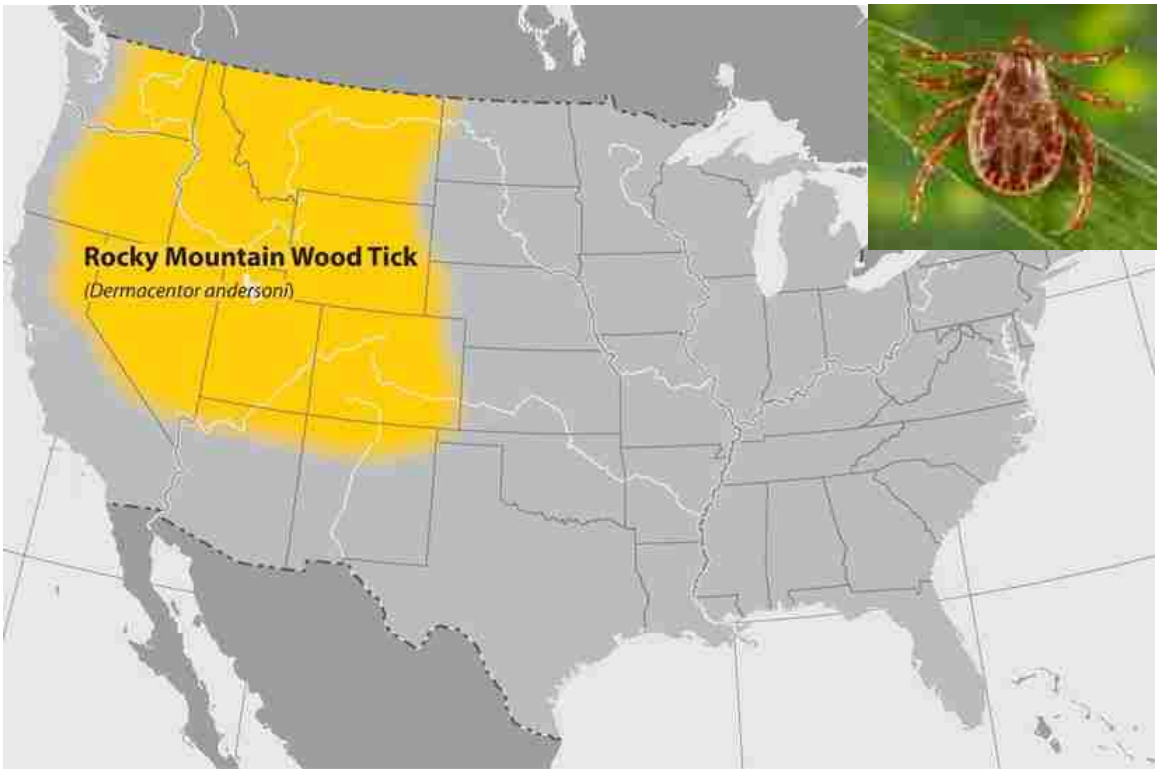

Figure 1.3 Approximate distribution of Dermacentor andersoni in the United States (Distribution map courtesy of the Centers for Disease Control and Prevention.

In addition to pathogenic bacteria, *Dermacentor* ticks are also known to harbor nonpathogenic bacteria including *Rickettsia montanensis* and *Rickettsia peacockii* (Azad and Beard 1998).

1.2.2 *Rhipicephalus* species

Rhipicephalus are found across the Unites States and worldwide (Figure 1.4) and are easily recognizable by the hexagonal shape of the basis capituli. A species of importance is *Rhipicephalus sanguineus*, the brown dog tick, which transmits the etiologic agent of Mediterranean spotted fever, *Rickettsia conorii.* These ticks have a cosmopolitan distribution with increased activity in the summer. All life stages of this 3-host tick species feed on dogs, and also can feed on small wildlife and humans. (Nicholson et al. 2009).

Interestingly, after an increase in incidence of RMSF in the southern United States, *R. sanguineus* was also implicated as a vector of *R. rickettsii.* RMSF cases were recognized on

Figure 1.4 Approximate distribution *Rhipicephalus sanguineus* in the United States (Distribution map courtesy of Centers of Disease Control and Prevention).

Native American reservations in areas with increased contact with heavily *R. sanguineus*-infested dogs near households (Demma et al. 2005). Other *Rhipicephalus* ticks of great veterinary importance include *Rhipicephalus annulatus* and *Rhipicephalus microplus*, which are vectors of *Babesia begimina* and *Babesia bovis*, respectively. The 1-host tick *R. microplus* can also transmit *Anaplasma marginale*, the causative agent of anaplasmosis (Nicholson et al. 2009). In addition to the transmission of pathogenic agents, *R. microplus* can infest cattle at very high levels causing weight loss (Nicholson et al. 2009).

1.2.3 *Amblyomma* species

Amblyomma species of medical and veterinary importance in the United States include the lone star tick, *Amblyomma americanum*, and the Gulf Coast tick, *Amblyomma maculatum*. Lone star adult females are easily identifiable by the singular white spot on their scutum with long mouthparts (Nicholson et al. 2009). On the other hand, the Gulf Coast tick are more

Figure 1.5 Approximate distribution *Amblyomma americanum* in the United States (Distribution map courtesy of Centers of Disease Control and Prevention).

difficult to identify, with similar ornamentation to *D. variabilis*. The primary observable difference between the two species is the relatively longer mouthparts of *Amblyomma*. While *A. americanum* are found throughout the Southern United States (Figure 1.5), its distribution has been expanding north (Dahlgren et al. 2016). *A. americanum* are known vectors of *Franciscella tularensis* and *Ehrlichia* spp (Nicholson et al. 2009). Preferred hosts include wildlife, livestock, and humans. Seasonally, nymphs and adults are active during the late spring with larva active during the summer.

The Gulf Coast tick has a more southern distribution (Figure 1.6), and is also found in Central America (www.cdc.gov). Adults feed mainly on ruminants but all life stages will readily feed on birds and other mammals. The transmission of *Ehrlichia ruminatum*, the causative agent of heartwater, and *Rickettsia parkeri*, the causative agent of *R. parkeri* rickettsiosis, is attributed to *A. maculatum* (Nicholson et al. 2009).

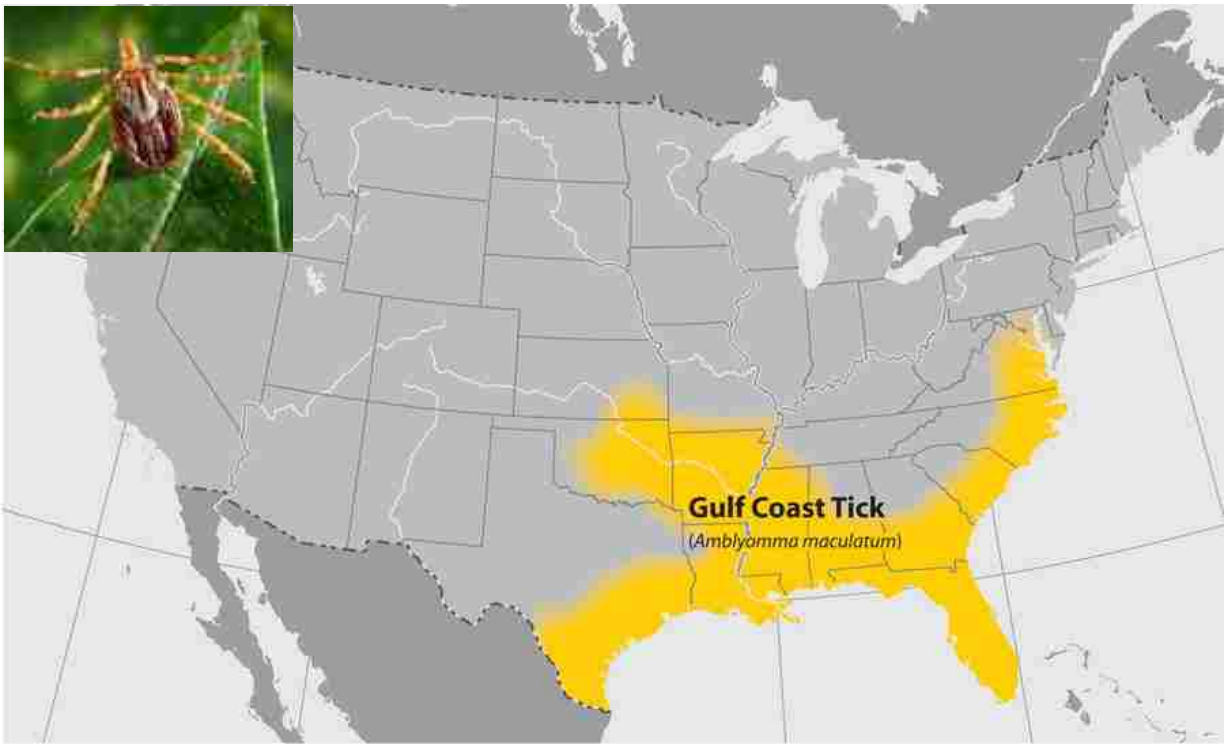

Figure 1.6 Approximate distribution *Amblyomma maculatum* in the United States (Distribution map courtesy of Centers of Disease Control and Prevention).

1.2.4 *Ixodes* species

The blacklegged tick, *Ixodes scapularis*, is the main vector of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. Distributed throughout the eastern and southern United States (Figure 1.7), *I. scapularis* are highly desiccation intolerant and are usually found in humid, shady forested areas (Nicholson et al. 2009).

As three host ticks, larval and nymphal *Ixodes* are known to feed on small mammals, birds and lizards, whereas adults mostly feed on white-tailed deer. Moreover, nymphs are responsible for transmission of *B. burgdorferi* to humans, which occurs during the spring and summer when the nymphs are active (Pal and Fikrig 2003). *Ixodes* ticks are also vectors of *Babesia microti* and *Anaplasma phagocytophilum*, the agents of human babesiosis, and human granulocytic anaplasmosis, respectively (Beaty and Marquardt 1996).

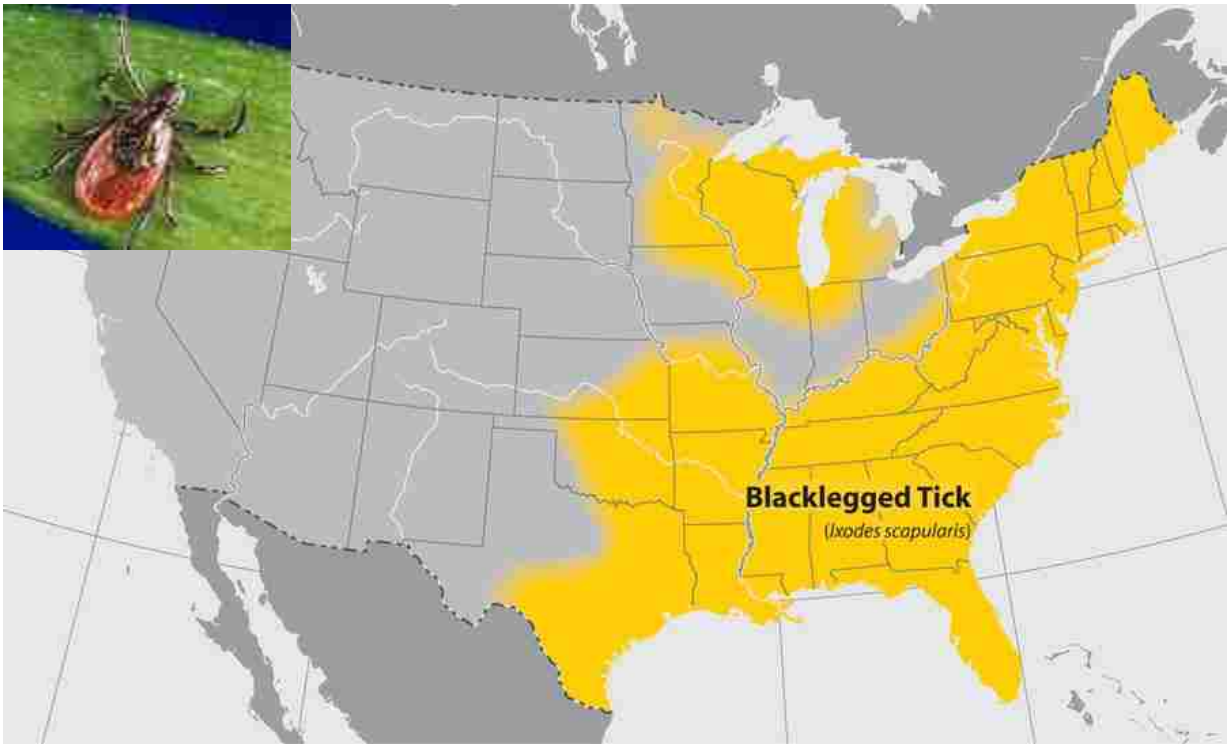

Figure 1.7 Approximate distribution *Ixodes scapularis* in the United States (Distribution map courtesy of Centers of Disease Control and Prevention).

1.3 Tick-borne rickettsioses

Rickettsia are obligate intracellular α-proteobacteria transmitted by hematophagous arthropods including fleas, lice, mites, and ticks. (Azad and Beard 1998). These bacteria are transmitted horizontally through the bloodfeeding by infected arthropods and vertically by transovarial and transstadial transmission. This section will describe relevant rickettsial features, including classifications, pathogenicity, and current epidemiology.

1.3.1 History of tick-borne rickettsioses

The clinical manifestations of RMSF were first described in 1899 in a publication by Edward E. Maxey (Parola et al. 2005). These descriptions were followed by more detailed reports by Howard T. Ricketts in 1906. Ricketts moved to Montana for the purpose of studying RMSF (Ricketts 1906a, Ricketts 1906b, Ricketts 1907b, Ricketts 1907a). Ricketts publications

from 1906 to 1907 identified the tick as the vector for RMSF. These reports describe an organism that was identified through xenodiagnosis. The organism was transmissible through the exchange of blood from a patient to an experimental animal, as well as by *Dermacentor andersoni* ticks (Ricketts 1906a, Ricketts 1906b, Ricketts 1907b). Ticks were suspected as a mode of transmission of RMSF as persons infected were not contagious and diagnoses peaked from May through June in males who worked outside and were exposed to ticks. Ricketts described the maintenance of *R. rickettsii* in *D. andersoni*, with evidence supporting transovarial and transstadial transmission. The organisms could be found in blood and was not culturable (Ricketts 1907a). Ricketts' work in Montana allowed for the development of an animal model of infection in male Guinea pigs which results in fever, rash, and scrotal swelling (Ricketts 1907a).

1.3.2 *Rickettsia* characteristics and classification

Rickettsia are Gram-negative, small, polymorphic coccobacilli that are between 0.8 to 0.2 µm in length and 0.3 to 0.5 µm in width (Hackstadt 1996). Rickettsiae are of the class α-proteobacteria, order Rickettsiales, family Rickettsiacea, and genus *Rickettsia*. Electron microscopy revealed an organism with a trilaminar cell membrane and macrocapsular slime layer (Hayes and Burgdorfer 1982). *Rickettsia* reside in the cytoplasm of cells, but can also be found infecting nuclei (Burgdorfer et al. 1968).

Rickettsia are classified into groups based on antigenic, biological, and genetic characteristics. They have been recently classified into four groups: spotted fever group (SFG), typhus group, ancestral group, and transitional group (Figure 1.8) (Gillespie et al. 2007, Walker and Ismail 2008). Recent phylogenic analyses consider both housekeeping genes and genes which are under evolutionary pressure, allowing for better resolution and classification of the relationships between *Rickettsia* species (Gillespie et al. 2007) (Figure 1.8). While these efforts

have improved upon our understanding of *Rickettsia* classification, there is still much controversy about the determination of new species as there is no universal set of characteristics outlined.

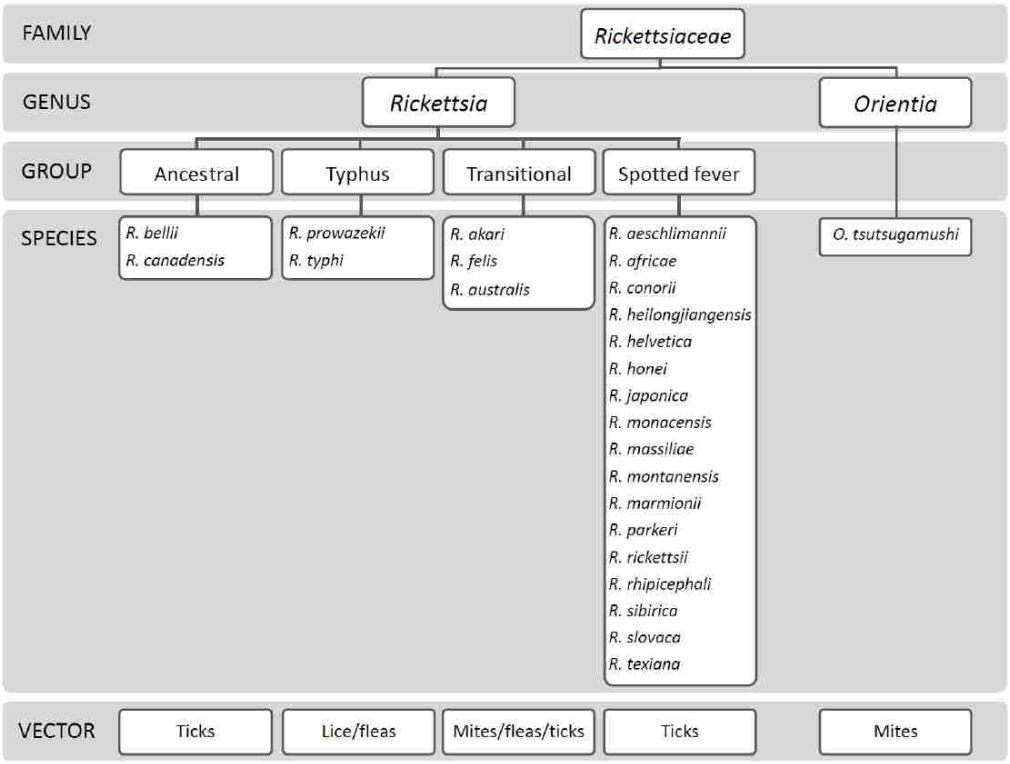

Figure 1.8 Rickettsial species classifications (Sunyakumthorn 2011).

1.3.3 Pathogenesis and pathogenicity

There are varying levels of pathogenicity associated with SFG *Rickettsia*, ranging from pathogenic to mild and non-pathogenic. These classifications have been classically determined by the recognition of human disease, or through infection in animal models such as in Guinea pigs. *R. rickettsii*, *R. parkeri, R. conorrii and R. africae* are considered highly pathogenic as they cause disease in humans. Other SFG *Rickettsia*, including *Rickettsia montanensis, Rickettsia peacockii, Rickettsia rhipicephali and Rickettsia amblyommii*, have not been identified as causing disease in humans and are therefore considered non-pathogenic. Additional strain variation within species may affect the pathogenicity of the *Rickettsia* (Walker and Ismail 2008).

RMSF is considered one of the most severe rickettsial diseases. The current case fatality rate is less than 1% with antibiotic treatment and has been as high as 20-25% in the pre-antibiotic era (Drexler et al. 2016). Clinical symptoms include fever, headache and rash. The characteristic rash begins on the extremities and moves to the trunk. More extreme cases may also include encephalitis, respiratory syndrome and coagulothapies (Walker and Ismail 2008). The incubation period after infection via tick bite is between 2 and 14 days with a rash occurring in most patients 3 to 5 days after the onset of fever (Lin and Decker 2012). Treatment with doxycycline is effective; however, if left untreated death can occur 7 to 15 days after symptoms begin (Lin and Decker 2012).

Efforts to elucidate definitive virulence factors for SFG *Rickettsia* have not been successful. As such, the molecular basis of rickettsial pathogenicity is undefined. Potential virulence determinants have been identified through comparative genomics of pathogenic and non-pathogenic rickettsial species. Outermembrane proteins, such as the SFG-specific OmpA, were identified as potential virulence factors; however the targeted knock down OmpA did not result in attenuated *Rickettsia*. This result suggests the existence of multiple and redundant virulence factors contributing to pathogenicity (Noriea et al. 2015)

As obligate intracellular pathogens, *Rickettsia* induce phagocytosis into host cells where they evade cellular degradation and live freely in the cytoplasm. In vertebrate hosts, SFG *Rickettsia* are biologically transmitted through the bite of an infected tick. Transmitted *Rickettsia* enter into host cells via interaction between the rickettsial outer membrane protein B (OmpB) and a host DNA-dependent protein kinase, Ku70 (Martinez et al. 2005). This interaction induces phagocytosis and once engulfed in the phagolysosome, *Rickettsia* escape to the cytosol of the host cell where they grow, divide and subsequently disseminate to neighboring cells.

Dissemination is most likely facilitated by direct cell-to-cell spread and actin polymerization (Martinez et al. 2005).

Rickettsial infections are characterized by vascular injury resulting from disseminated endothelial infection (Walker and Ismail 2008). The growth and dissemination of *Rickettsia* in endothelial cells induces vascular injury characterized by increased vascular permeability, vascular inflammation and the release of pro-inflammatory products, such as cytokines and pro-coagulation factors (Sahni and Rydkina 2009). Culture of *Rickettsia* infected endothelial cells results in increased cytokine production including IL-1, IL-6, IL-8, increased E-selectin and von Willebrand factor (Teysseire et al. 1992, Elghetany and Walker 1999). As a mechanism to reduce vascular injury, infected endothelial cells regulate cyclooxygenase expression and activity resulting in a decreased prostaglandin expression. Additional anti-inflammatory products are expressed, including antioxidant enzymes such as heme oxygenase (Rydkina et al. 2002).

SFG *Rickettsia* transmission relies on the survival and dissemination of rickettsiae within competent tick vectors (Beaty and Marquardt 1996). Dissemination within the tick host occurs by the escape of SFG *Rickettsia* from midgut to the hemolymph. Subsequent dissemination to distal organs including the salivary glands and the ovaries are required for successful horizontal transmission via bloodfeeding and vertical transmission to progeny. The gut is the first site of infection, and the first site of interaction with the tick immune system. Rickettsiae must evade the tick immune responses at the midgut for dissemination to occur. Furthermore, the hemolymph and organs also respond to disseminating rickettsia through production of antimicrobial products. Infection of the salivary glands is a key component for horizontal transmission through the tick bite and, presumably, *Rickettsia* which are not able to infect the salivary glands will not be transmitted via feeding (Beaty and Marquardt 1996). One such

example is *R. peacockii* which infects *D. andersoni* ticks. *R. peacockii* is not transmitted horizontally, localizing only in the ovaries, and resulting in transovarial (vertical) transmission, but not horizontal transmission (Niebylski et al. 1999). Thus, the mechanisms of rickettsial pathogenesis and dissemination in the tick vector are important considerations in the ecology and epidemiology of tick-borne rickettsial diseases.

1.3.4 Epidemiology-current significance

Rickettsia rickettsii can be found in North America, South America and Central America. In the United States, *Dermacentor* ticks are historically responsible for *R. rickettsii* transmission, specifically *D. variabilis* in the eastern United States and *D. andersoni* in the western United States (Figure 1.9). Recently, *R. sanguineus* has been implicated in the transmission of *R. rickettsii* in areas of the southwest United States where no *D. variabilis* or *D. andersoni* were found, but where cases of RMSF occurred (Demma et al. 2005). Transmission of *R. rickettsii* by *Amblyomma cajennenese* and *Amblyomma aerulatum* occurs in countries of

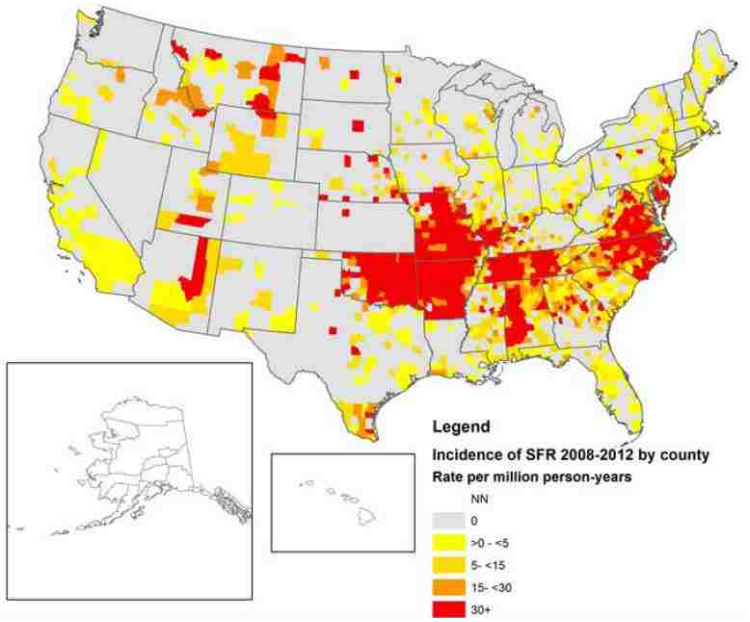

Figure 1.9 Incidence of RMSF in the United States in from 2008-2012 by county (Drexler et al. 2016).

Central and South America including Brazil, Argentina, Colombia and Panama (Macaluso and Paddock 2014).

The incidence of RMSF is closely tied with the feeding habits of its tick vectors, as they act as both a vector and reservoir of *Rickettsia*. RMSF incidence increases in the late spring and summer when ticks are most active. One field study identified that less than 0.1% of *Dermacentor* ticks surveyed were infected with *R. rickettsii.* Moreover, less than 4% of ticks surveyed were infected with a SFG *Rickettsia*, including *R. montanensis* or *R. amblyomii* (Stromdahl et al. 2010). Thus, low incidence of SFG *Rickettsia* infection in ticks suggests that some rickettsial species may require an amplification host.

In the United States, RMSF is a reportable disease with surveillance data collected as far back as 1920 (Openshaw et al. 2010). From 2000-2014, the yearly incidence of RMSF increased dramatically from 1.7 cases per million people per year to a record peak of 14 cases per million people per year (Openshaw et al. 2010, Drexler et al. 2016) Prior to 2000, the incidence fluctuated between 1-5 cases per million people per year (Figure 1.10). Infections tend to be focal; states with the greatest incidence include Oklahoma, Missouri, Arkansas, Tennessee, North Carolina. These states accounted for over 60% of the cases of RMSF in 2010 (Drexler et al. 2016).

The cause of the increase in RMSF cases beginning in 2000 may be multifactorial. One such factor included the recognition of *R. sanguineus* as a vector (Demma et al. 2005). Increases in reporting and changes to the case definition also contributed to increased cases of RMSF beginning in 2000 (Openshaw et al. 2010). The change in case definition allowed for the

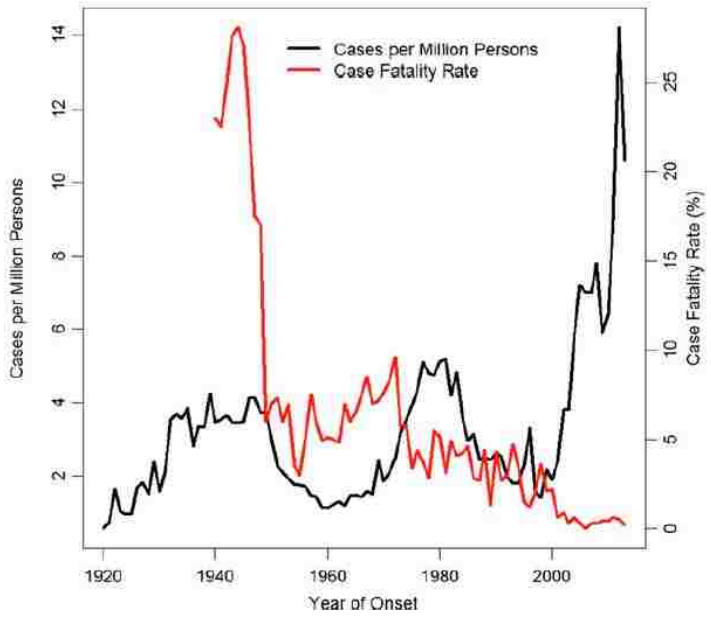

Figure 1.10 Incidence and case fatality rate of RMSF from 1920 to 2013 (Dahlgren et al. 2016).

categorization of probable cases based on increased antibody titers via enzyme-linked immunosorbent assays (ELISA). It has since been recognized that antigen cross-reactivity with other SFG *Rickettsia* pathogens which are found in the United States, including *R. parkeri, R. massilae,* and *Rickettsia* spp. 364D, may affect differential ELISA diagnostics (Openshaw et al. 2010). Decreasing case fatality rates suggest an increase in recognized infections of other SFG *Rickettsia* pathogens with less severe presentations, such as *R. parkeri* rickettsioses (Drexler et al. 2016). As of 2009, the reportable category case definition changed to "spotted fever group rickettsioses" to better reflect the probable spectrum of rickettsial infections reported (Drexler et al. 2016).

1.3.5 Transmission

SFG *Rickettsia* are transmitted by ixodid ticks, which can act as both a vector and a reservoir (Figure 1.11) (Azad and Beard 1998). There are multiple transmission routes observed for tick-borne *Rickettsia*, including horizontal and vertical transmission. Horizontal

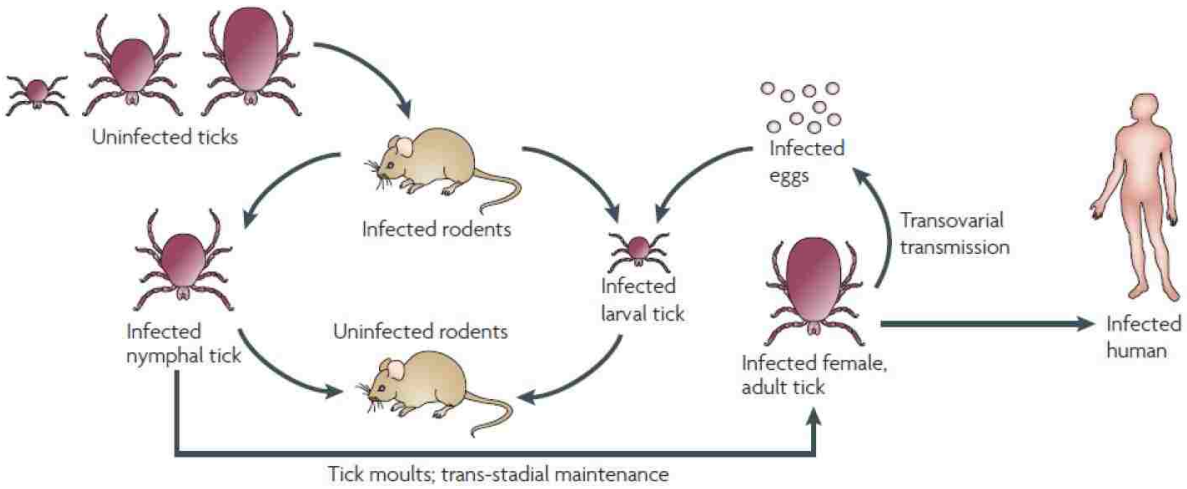

Figure 1.11 Transmission routes of tick-borne bacteria including vertical and horizontal transmission (Walker and Ismail 2008).

transmission describes the transmission from the tick to a vertebrate host through bloodfeeding. Bloodfeeding may result in the infection of subsequent ticks if the infected animal becomes rickettsiemic. Vertical transmission describes the infection of subsequent life stages (transstadial) and the infection of progeny (transovarial). Vertical transmission may not drive the continuation of populations of infected ticks in nature. In such cases, vertical transmission is likely supplemented by horizontal transmission (Azad and Beard 1998).

Most SFG *Rickettsia* infect all tissues of the tick host; however, there is natural variability in the number of SFG *Rickettsia* identified infecting either field-caught or laboratory ticks (Zanettii et al. 2008). Field caught *R. amblyommii*-infected *A. americanum* were found to have a light infection in the ovaries, malphigan tubules, and hemocytes, however, all tissues were infected (Burgdorfer et al. 1981). Moreover, *A. americanum* ticks infected with *R. rickettsii* have been identified as carrying infection loads of 10^6-10^7 via qPCR, with no information regarding life stage, or feeding status reported (Eremeeva et al. 2003). In another case, a laboratory strain of *A. americanum* were infected in all organs, with a combined total of 10^5 *R. amblyommii* per tick (Zanettii et al. 2008). Interestingly, the amount of *R. amblyommii* did not change in this

laboratory colony during bloodfeeding, or mating. The mechanisms driving the infection of tissues and level of infection are unknown, but may rely on SFG *Rickettsia*-derived and tick-derived factors (Zanettii et al. 2008).

1.3.6 Vector competence

Vector competence describes the ability of a species of vector to become infected by a pathogen that it can then subsequently transmit to new susceptible hosts (Beaty and Marquardt 1996). In the case of tick-borne *Rickettsia*, after bloodfeeding on a rickettsemic host the imbibed *Rickettsia* must break through many barriers to infection. Successfully escaping the barriers to infection will result in disseminated infection of the salivary glands where the *Rickettsia*, facilitating horizontally through saliva, or dissemination to the ovaries facilitating vertical transmission to offspring. These barriers include disseminating from the midgut to the hemocoel, from the hemocoel to the salivary glands and ovaries, and then escaping the salivary glands to be secreted in saliva or infecting the eggs. The most important barrier to infection is considered to be the midgut, as this is the first site of contact for pathogens during bloodfeeding (Nicholson et al. 2009). The molecular interactions which facilitate barrier escape, or result in clearance of bacteria are undefined; thus, characterization of mechanisms directly or indirectly affecting *Rickettsia* maintenance in ticks may better explain rickettsial epidemiology.

1.4 Immune response of insects

As little is known regarding the mechanisms of signaling in non-model arthropods, such as *D. variabilis*, the paradigms described in insects can be used as a model for understanding the tick immune response. The response of vectors to pathogens was once overlooked; however, studies focused on the interactions of vector hosts with transmitted pathogens revealed mechanisms of recognition, signal transduction, and varied pathogen specific effector responses

(Beaty and Marquardt 1996). Specific understanding of how the insect immune system is controlled and responds to microorganisms has been greatly influenced by seminal work describing the immune response of the fruit fly *D. melanogaster* (Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009). Studies have examined both the response at the arthropod level, as well as using *Drosophila* cell lines to characterize the molecular mechanisms controlling insect immune responses. Interestingly, as *Drosophila* cell lines have been instrumental in the past in understanding immunological mechanisms, *Drosophila* cell lines also have been introduced into the study of rickettsial infection (Von Ohlen et al. 2012, Luce-Fedrow et al. 2014). Known mechanisms of immune response in *Drosophila* have served as the basis for identifying immune related proteins in other arthropods, especially arthropods for which limited genomic information is available. This section outlines the major immune signaling pathways and effector mechanisms previously characterized in *Drosophila* (Figure 1.12), with an emphasis on responses to bacteria.

1.4.1 Microbial recognition by *Drosophila* and immune response initiation

The immune response of *Drosophila* is initiated by the recognition of microbial pathogen associated molecular patterns (PAMPS) through pattern recognition receptors. These receptors are varied in the molecules they recognize as well as in their spatial distribution in the cell. Microbial patterns recognized include peptidoglycan (PGN), lipopolysaccharide (LPS), teichoic acids, flagellin, glucans and nucleic acids (Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009). The recognition of PAMPs trigger the induction of pathways that respond to the particular type of microorganism encountered allowing for the induction of immune responsive genes necessary for an effective host defense. These include specific pathways for Gram-negative, Gram-positive, fungi, yeast and viruses (Lemaitre and Hoffmann 2007).

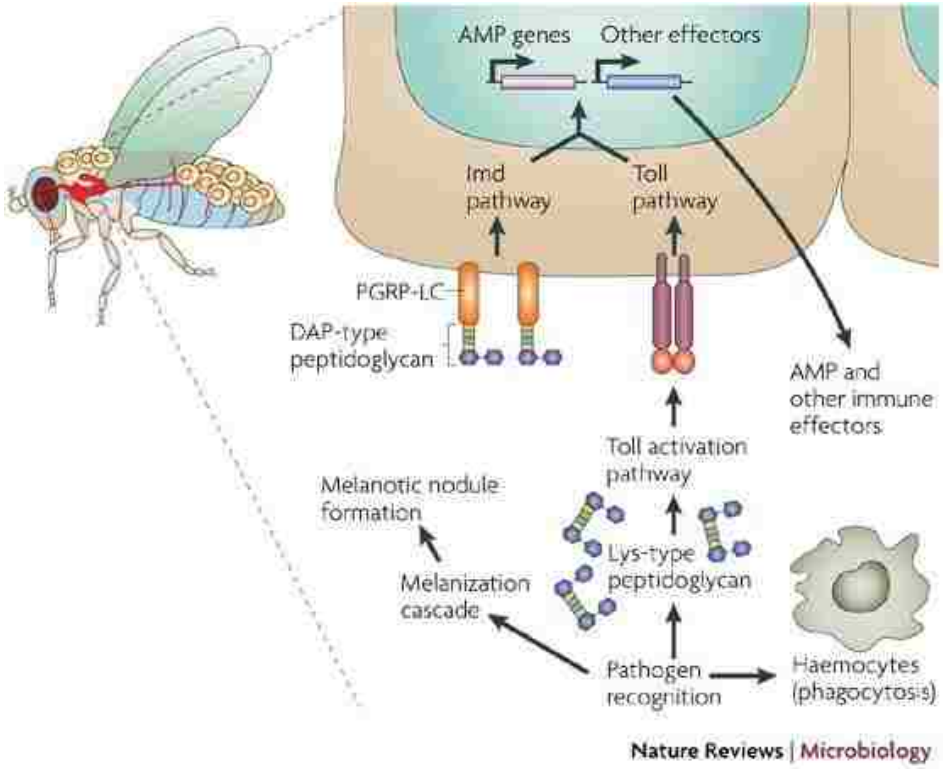

Figure 1.12 *Drosophila* immune response to microbes. Pathogen recognition induces the activation of the signaling cascades which results in the production of AMPs and other effectors (Vallet-Gely et al. 2008).

During bacterial infections, the insect cell primarily recognizes the presence of meso-diaminopimelic-acid type (DAP) PGN and lysine-type PGN, which are characteristic of Gram-negative and Gram-positive PGN, respectively (Hetru and Hoffmann 2009). PGN is recognized by peptidoglycan-recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) which are localized in both the outer membrane of cells and also in the cytoplasm of cells in soluble forms (Valanne et al. 2011, Kleino and Silverman 2014). GNBPs activate the Toll pathway and most PGRPs activate the immune deficiency (IMD) pathway (Kurata 2014). Additionally, a small proportion of known PGRPs are also capable of activating the Toll pathway (Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009).

1.4.1a Toll pathway receptors, signaling, and effector functions

The Toll pathway (Figure 1.13) is activated through recognition of lysine-type PGN by GNBPs in response to Gram-positive bacterium or fungi (Hetru and Hoffmann 2009). These proteins subsequently activate a protein cascade that culminates in activating the cytokine Spatzle. This protein, in turn, binds to the Toll receptor on the *Drosophila* cell membrane. Specifically, there are three encoded GNBPs in the *Drosophila* genome and one additional protein, PGRP-SA, which recognizes Lys-type PGN (Hetru and Hoffmann 2009). These GNBPs activate the proteases Grass and Spirit to bind and activate Spatzle, inducing its dimerization as a transmembrane receptor.

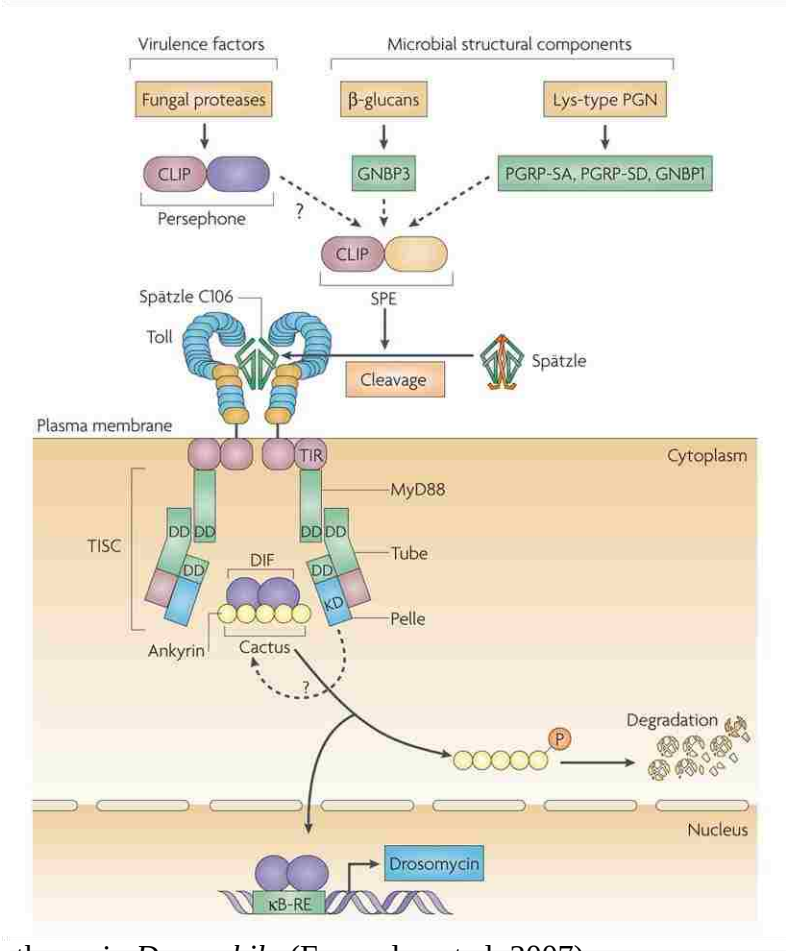

Figure 1.13 Toll pathway in *Drosophila* (Ferrandon et al. 2007).

The Toll receptor is a transmembrane protein with external leucine rich repeats and internal domain similar to mammalian Toll/IL-1R (TIR) domains found on Toll-like receptors (Valanne et al. 2011). Once Toll is activated and dimerized, three proteins which contain death domains, Pelle, Tube, and dMyD88, form a complex. Pelle, which has kinase activity, phosphorylates the inhibitory IκB protein Cactus that is bound to cytoplasmic Dorsal and Dif. Once Cactus is degraded, Dif and Dorsal can then translocate into the nucleus and bind upstream of immune responsive genes (Hetru and Hoffmann 2009, Valanne et al. 2011).

The major function of Toll signaling is to upregulate immune responsive genes whose products aid in the immune response to Gram-positive bacterial infection. This signaling leads to the expression of many characterized antimicrobial peptides (AMPs) including Cecropin, Defensin, and Metchnikowin. These proteins are detectable in the hemolymph of *Drosophila* within 2 hours of infection (Uttenweiler-Joseph et al. 1998) and are produced by both hemocytes and the fat body in response to a systemic infection. Interestingly, the transcription of many AMP genes is downregulated at 24 hours. It was suggested that transient activation is crucial for keeping the response from causing harm to insect tissues (Kim et al. 2006).

1.4.1b IMD pathway receptor, signaling and effectors

The IMD pathway (Figure 1.14) is induced in the presence of DAP-type PGN (Hetru and Hoffmann 2009). In contrast to the Toll pathway, the IMD pathway is induced through direct contact of extracellular DAP-type PGN with the transmembrane PGRPs of the PGRP-LC receptor family (Gottar et al. 2002). The receptors dimerize after PGN recognition and induce downstream signaling. In the case of intracellular Gram-negative bacterium, a different receptor, PGRP-LE, which is a soluble cytoplasmic receptor, can initiate the signaling cascade

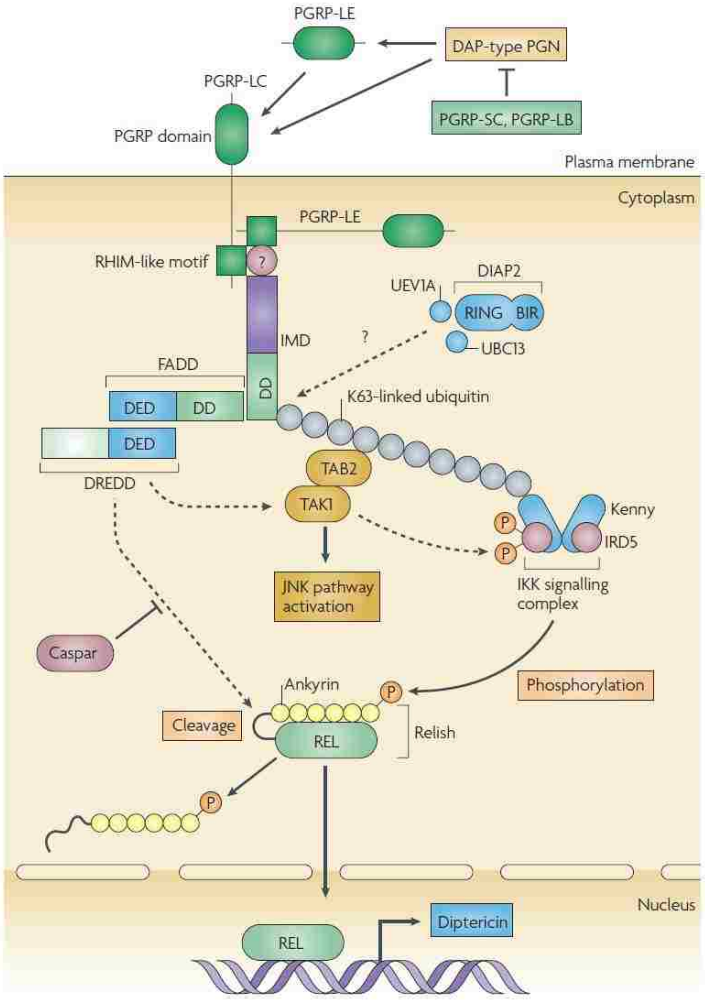

Figure 1.14 IMD pathway in *Drosophila* (Ferrandon et al. 2007).

(Takehana et al. 2002). Once the PGRP receptors have dimerized, the IMD protein will be recruited and bind to the intracellular domain of the receptors via death domains (Lemaitre et al. 1995).

Two additional proteins, DREDD and FADD, join the complex and then activate the MAP kinase TAK1. TAB2 then interacts with TAK2 and is K36 polyubiquitinated (Hetru and Hoffmann 2009). The TAK1/TAB2 complex activates two signaling cascades. One pathway results in Relish activation and subsequent nuclear translocation. The second results in JNK pathway activation, and is discussed below (Hetru and Hoffmann 2009). The Relish arm of the

IMD pathway continues with the TAK1/TAB2 complex activating the IKK complex. The IKK complex phosphorylates specific serines in Relish and the inhibitory ankyrin repeat containing carboxy-terminal portion of the protein is cleaved, revealing a nuclear localization sequence (Stoven et al. 2003). The amino-terminal portion of Relish then moves to the nucleus where it binds DNA upstream of immune responsive genes.

The transcription of IMD controlled genes occurs much earlier than that of the Toll pathway, and can be as early as 6 hours post infection (Lemaitre et al. 1997). AMPs such as Andropin, Attacin, Diptericin, and Drosocin are induced in response to Gram-negative infection (Uvell and Engstrom 2007).

1.4.1c JNK pathway and effectors

The JNK pathway begins with the TAK1 activating a kinase Hemipterous, which subsequently phosphorylates the kinase Basket and activates *Drosophila* transcription factor AP-1. This offshoot of the IMD pathway is known to control transcription of genes regulating cytoskeletal proteins and aids in proapoptosis signaling (Delaney et al. 2006). Some AMPs are also induced through IMD-derived JNK pathway induction (Boutros et al. 2002). This pathway has also been implicated in the induction of the production of opsonins and cytokines, and in hemocyte differentiation (Lemaitre and Hoffmann 2007).

1.4.1d JAK/STAT pathway and effectors

The JAK/STAT pathway consists of three proteins: the receptor Domeless, the Janus Kinase Hopscotch, and the STAT transcription factor. The accumulation of dimerized STAT in the nucleus occurs in response to viral infection and tissue damage. JAK/STAT pathway induction induces the expression of AMPs including thioester-containing proteins (TEP) and

other stress-response activated genes. A full understanding of this pathway and its importance in the immune response of insects has yet to be defined (Lemaitre and Hoffmann 2007)

1.4.2 Barrier defense

The barrier defense of *Drosophila* consists of the basal expression of AMPs in epithelia including the reproductive tract, trachea, and gut. The expression of AMPs appears to be tissue specific and AMPs that are constitutively expressed in certain tissues may be inducible in others (Uvell and Engstrom 2007). The upregulation of inducible AMPs, particularly in the gut, has been shown to enhance survival of insects during oral infection. Interestingly, immature stages of *Drosophila* are also capable of a robust induction of AMPs such as Cecropin A in response to bacterial infection and abrasion wounding (Onfelt Tingvall et al. 2001). Constitutive AMP expression appears to be regulated through the NF-κB factor Dorsal, a transcription factor that also controls the development and differentiation of insects. In contrast, inducible expression of AMPs is controlled through the NF-κB factors Dorsal-related immunity factor (Dif) and Relish (Uvell and Engstrom 2007).

1.4.3 Cellular defenses

The cellular defenses of *Drosophila* are controlled by the three types of fully differentiated hemocytes: plasmatocytes, crystal cells, and lamellocytes (Evans et al. 2003). The main functions of these cells are phagocytosis, encapsulation and clotting. Phagocytosis of pathogens occurs through receptor-mediated recognition, engulfment, and maturation of a phagolysosome. Encapsulation is a process reserved for larger organisms of which phagocytosis is not possible, and may include both melanization and induction of the phenoloxidase cascade. Clotting occurs as response to wounding forming through the deposition of hemocytes in a fibrous matrix (Vlisidou and Wood 2015).

1.4.3a Phagocytosis

Phagocytosis of pathogens by insect cells is initiated by the recognition of microbial PAMPs by soluble and membrane associated receptors. Receptors, such as the Nimrod family, directly recognize molecular patterns including peptidoglycan, and glucans; however, scavenger receptors such as the Peste family can also indirectly recognize microbial products through opsonization (Vlisidou and Wood 2015). Previously identified and characterized receptors are known to have multiple isoforms. Receptor diversity increases the potentially recognized proteins inducing phagocytosis of microbial products. Insects are also known to produce proteins with homology to human α2-macroglobulins and c3/c4/c5 complement proteins called TEPs. These complement-like proteins have been implicated as opsonins leading to increased phagocytosis and are constitutively expressed but also inducible in all life stages and tissues (Bou Aoun et al. 2011). Specifically, phagocytosis has been shown to be increased in *Drosophila* S2 cells infected with *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* by the activity of TEPII, TEP III, and TEPIV (Stroschein-Stevenson et al. 2006).

1.4.3b Encapsulation

Encapsulation occurs after the recognition of foreign particles and microbes that are too large to be phagocytosed by a single hemocyte (Vlisidou and Wood 2015). Lamellocytes and plasmatocytes are utilized for encapsulation (Russo et al. 1996) and are effective on large organisms such as parasitic wasp larvae, tumors, or dead tissues. This process has been best characterized during infection of parasitic wasp eggs in *Drosophila.* Encapsulation in *Drosophila* requires integrins to bind to specific RGD-containing proteins. Other proteins including extracellular matrix proteins, laminin, and β-integrins also contribute to encapsulation. After

enough hemocytes have attached to the side of encapsulation, melanization may also occur as an effect of degranulation, helping to kill the parasite (Russo et al. 1996, Hillyer 2015).

1.4.3c Nodulation

The nodulation of organisms too large to be phagocytosed, such as large aggregates of bacteria occurs through the accumulation of hemocytes, including granulocytes, which bind to one another to form a barrier (Hillyer 2015). Plasmatocytes then bind to the granulocyte layer, further reinforcing the structure and leading to melanization. These processes require the protein Noduler, which is a component of the extracellular matrix. Throughout nodulation, granulocytes release the contents of their granules in an effort to destroy the object or organism within the nodule (Hillyer 2015).

1.4.3d Melanization

Melanization is the blackening of hemolymph in response to immune challenge or wounding caused by the synthesis of melanin (Hillyer 2015). In *Drosophila*, crystal cells are responsible for most melanization and melanin production which localizes to foreign microbes. Melanization triggers the induction of reactive oxygen and nitrogen species as well as inducing encapsulation and nodulation where phagocytosis are not possible. Hemocytes, including crystal cells, contain prophenoloxidase which when released during the rupturing of hemocytes catalyzes the production of melanin (Vlisidou and Wood 2015). The production of melanin, the induction of the prophenoloxidase cascade, and the production of reactive species lead also to the activation of a systemic immune response through Janus-kinase dependent pathways at tissues away from the site of melanization (Nam et al. 2012).

1.4.4 Secretion of soluble factors

In insects, secreted molecules such as AMPs, opsonins, complement-like factors, and prophenoloxidases aid in immune defenses (Hillyer 2015). These secreted factors work in concert with the cellular mechanism to control microbial infections. Some of these factors, such as AMPs, can directly kill pathogens. Other soluble factors may increase the actions of hemocytes, opsonizing and increasing recognition and phagocytic activity (Lemaitre and Hoffmann 2007, Bou Aoun et al. 2011, Bonnay et al. 2014, Hillyer 2015).

1.4.4a AMPs

AMPs are secreted by the hemocytes and fatbody of insects in response to infection. These proteins are both constitutively expressed, and expressed in response to the type of recognized microbe (Vlisidou and Wood 2015). Hemocytes respond to infections and also contribute to the induction of immune responses in other tissues. Recently, hemocytes have been implicated in transferring the signal of immune response from the gut to the fat body of *Drosophila* infected with *Erwinia carotovora*. (Basset et al. 2000, Vlisidou and Wood 2015). In this way, hemocytes are an important in the induction of systemic immune responses in insects.

1.4.4b Opsonins

Opsonins are proteins which bind to pathogens and foreign objects marking them for recognition by phagocytic cells (Vlisidou and Wood 2015). Opsonins have not been widely studied in *Drosophila*, but candidate opsonins include TEPs and Down-syndrome cell adhesion molecules (DSCAMs). Members of the TEP family of proteins have been shown to be required for efficient phagocytosis of Gram-negative bacteria by *Drosophila* S2 cells, but is not required for the phagocytosis of *Candida albicans* (Stroschein-Stevenson et al. 2006). DSCAMs are members of the Ig-superfamily and have the potential to express many isoforms. The binding of

secreted forms of DSCAMs have been suggested as another potential mechanism of opsonization in *Drosophila,* aiding in the efficient phagocytosis of *E. coli* (Watson et al. 2005).

1.4.4c Phenoloxidase

Phenoloxidases are produced in insects as proenzymes. In response to immune challenge or wounding, these zymogens are activated leading to the production of quinones and subsequently melanin (Nappi and Christensen 2005). The induction of the prophenoloxidase cascade and melanization have been observed in both the nodulation and encapsulation responses of insects. Prophenoloxides are found in the hemolymph of insects and are most likely synthesized primarily in hemocytes (Cerenius and Soderhall 2004). The deposition of melanin as a result of prophenoloxidase activation is thought to play a role in immune defense through blocking nutrient absorption. Additionally, byproducts of the phenoloxidase cascade produce reactive nitrogen and oxygen species which aid in direct killing of invading microorganisms (Cerenius and Soderhall 2004).

1.5 Immune response of ticks

While *Drosophila* has provided a model for the study of the immune response of insects to pathogens, they are not hematophagous organisms or vectors of disease. As such, an understanding how pathogens are controlled by bloodfeeding arthropods is best determined through studies of the vector and disease agent together (Beaty and Marquardt 1996). Ticks elicit a much stronger immune response to atypical bacterial infection, in comparison to typical bacterial infection (Munderloh and Kurtti 1995). The relationship between ticks and their pathogens is unique and understanding the immune response of the tick will give insight into the mechanisms which facilitate infection of vectors with atypical or typical pathogens and endosymbiont as well as the transmission of pathogens to vertebrates. The balance of tick-

derived immune responses and *Rickettsia*-derived immune evasion mechanisms results in the establishment of infection. Alternatively, the response of tick to endosymbionts must allow for the establishment of infection as a result of immune tolerance mechanism. The effector mechanisms of ticks have been previously described (Figure 1.15) (Sonenshine and Hynes 2008, Hynes 2014) and recent studies have focused on understanding the signaling required for an effective immune response. The following section describes the tick effector response to infection and the current effort to describe the signaling pathways which orchestrate these responses. Although there are overlapping tick distributions, specific hard tick-SFG *Rickettsia* pairings emerge in nature (Table 1.2). Thus, the identification of mechanisms which control the differential response of ticks to pathogens and non-pathogens, including typical and atypical infections, is of great interest and will lead to a better understanding of vector competence.

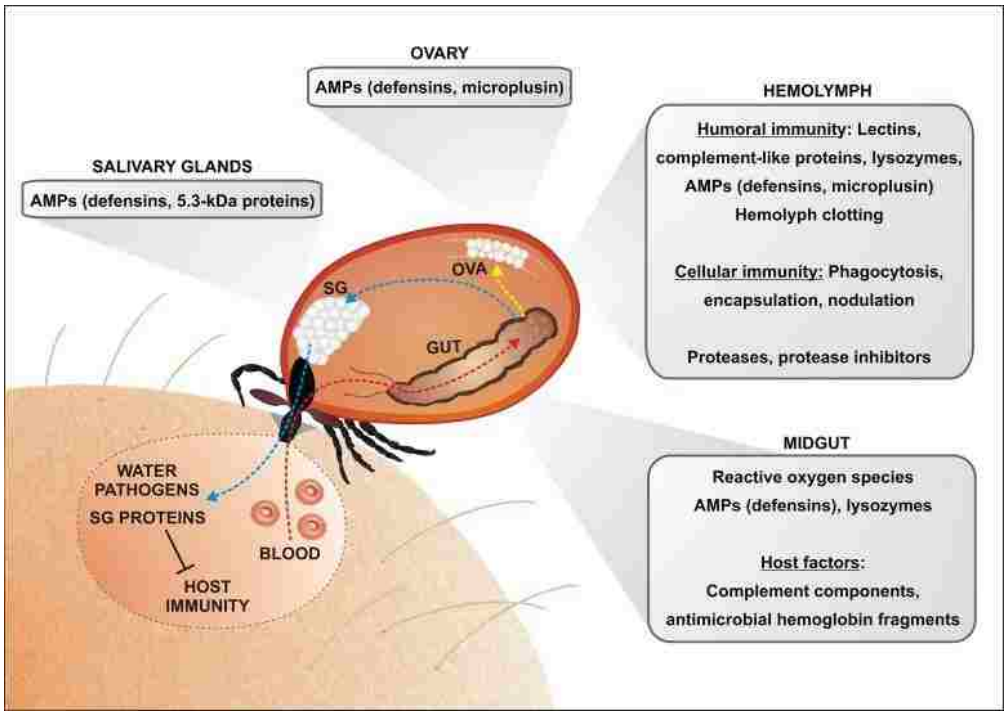

Figure 1.15 Tick immune mechanisms in response to pathogen infection (Hajdušek et al. 2013).

Table 1.2 Established SFG *Rickettsia*-tick pairings, including demonstrated pathogenicity in animals.

Rickettsia species, strain, or candidate species	Pathogenicity	Tick species infected with *Rickettsia* species in nature
R. rickettsii	Pathogenic	*Dermacentor variabilis* *Dermacentor andersoni* *Rhipicephalus sanguineus*
R. montanensis	Non-pathogenic	*Dermacentor variabilis* *Dermacentor andersoni*
R. peacockii	Non-pathogenic	*Dermacentor andersoni*
R. parkeri	Pathogenic	*Amblyomma maculatum* *Amblyomma americanum*
Candidatus R. amblyomii	Non-pathogenic	*Amblyomma maculatum* *Amblyomma americanum*
Candidatus R. andeanae	Non-pathogenic	*Amblyomma maculatum*
Rickettsial Endosymbiont of *Ixodes scapularis*	Non-pathogenic	*Ixodes scapularis*

Modified from (Macaluso and Paddock 2014).

1.5.1 Barrier Defense

Ticks have multiple forms of barrier defense, both externally and internally. The most effective external defense mechanism of the tick is the chitinous cuticle which encases the tick and is reinforced with a waxy outer layer. Together, the waxy cuticle keeps many pathogens at bay, but the presence of pores and glands exposed to the outside environment does allow for the possibility of infection with pathogens. Invasion of airways leading to trachea would be unlikely though, as the trachea are also lined with cuticle (Hynes 2014).

During bloodfeeding, the midgut can be challenged with bacteria or other pathogens. The midgut consists of an epithelial cell layer over a muscle layer (Sonenshine and Anderson 2014). A peritrophic membrane can be formed in some tick species during the initial phases of bloodfeeding, acting as a protective layer and interfering with the adherence and penetration of

the gut (Munderloh and Kurtti 1995). Interestingly, blood is digested slowly and intracellularly by midgut cells in ticks, with a notable absence of digestive enzymes in the midgut. This can be a supportive environment for the uptake of intracellular bacteria, as the endosomes of the digestive cells of the midgut do not immediately fuse with the lysosome (Hynes 2014).

The normal flora of ticks varies by species and life stage and consists mainly on non-pathogenic, symbiotic, and commensal bacteria with only a small proportion representing pathogens (Clay et al. 2008). Non-pathogenic SFG *Rickettsia* have been shown to play a role in transmission of *Rickettsia* through the colonization of ticks which can interfere with the establishment and transmission of subsequent *Rickettsia* (Burgdorfer and Brinton 1975, Macaluso et al. 2002). Infection with *R. peacockii* is the best known example of the interference phenomenon where stable infection of the tick *D. andersoni* inhibits the vertical transmission of pathogenic *R. rickettsii.* This relationship is the foundation for the spatial distribution of high rates of *R. rickettsii* infection in the west side of the Bitteroot Valley and the recognition of the East Side Agent, *R. peacockii,* in *D. andersoni* ticks on the east side of the Valley (Burgdorfer and Brinton 1975). Interference was additionally described in *D. variabilis* where nonpathogenic *Rickettsi*a blocked the transovarial transmission of other rickettsial species (Macaluso et al. 2002). The mechanisms of prevention of secondary rickettsial infection and vertical transmission are currently unknown but suggest that cellular changes in oocytes of SFG *Rickettsia*-infected ticks renders them refractory to secondary infection (Macaluso et al. 2002). Of note, the exclusion of pathogenic *R. rickettsii* infection in *Dermacentor* ticks may be of benefit to the ticks as *R. rickettsii* is known to be pathogenic to the tick (Niebylski et al. 1999). This interference phenomenon does not affect the potential coinfection of ticks by other tick-transmitted pathogens such as *B. microti*, *A. phagocytophilum*, and *B. burgdorferi* (Swanson et al. 2006).

1.5.2 Cellular defenses

The organs of the tick are bathed in hemolymph which consists of plasma and hemocytes. The hemocytes are involved in many immune processes including nodulation, encapsulation, phagocytosis, and the secretion of soluble factors (Hynes 2014). Hemocytes can be characterized into 4 types: plasmatocytes, granulocytes, spherulocytes, and prohemocytes (Grubhoffer et al. 2014). One of the first responses to injury is the coagulation of hemocytes, which leads to a walling-off of the damaged area and decreased spread of introduced microbes (Hynes 2014). However, when pathogens are able to escape these barriers to infection, ticks respond by increasing hemocyte proliferation. Infected *D. variabilis* are known to produce up to 6.4 times the number of hemocytes after infection with an atypical bacteria such as *Bacillus subtilis* (Johns et al. 1998). Interestingly, when infected with the tick-transmitted, but atypical *B. burgdorferi*, the hemocyte proliferation in *D. variabilis* is more rapid but returns to normal within 24 hours of infection (Johns et al. 2000, Johns et al. 2001). These experiments highlight the induction of differing responses to pathogens. Such a quick induction of hemocyte proliferation following infection points to the importance of hemocytes in the immune response of ticks to bacterial infection.

1.5.2a Nodulation

Nodulation is defined as the aggregation of hemocytes to surround invading microbes. While the process of nodulation in insects is better characterized, the events which trigger and control nodulation in ticks are less understood. In insects, nodulation is lectin-mediated and includes both melanization and induction of the prophenoloxidase cascade. Lectins are present in ticks and have been shown in *D. variabilis* to recruit hemocytes to bacterial pathogens (Ceraul et al. 2002). In insects, recognition of pathogen associated molecular patterns such as

lipopolysaccharide leads to the induction of the prophenoloxidase cascade triggering production of melanin (Hillyer 2015). Ticks do not produce melanin during nodulation (Ceraul et al. 2002) and prophenoloxidase activity has not been identified in hard ticks, including *D. variabilis* (Zhioua et al. 1997).

1.5.2b Encapsulation

Encapsulation is a response to large organisms such as nematodes or protozoa, occurring through the binding of hemocytes. In insects, this process involves melanization, after the accumulation of hemocytes in concentric circles (Hynes 2014). In ticks there is no involvement of melanization, but the formation of concentric hemocytes was observed (Eggenberger et al. 1990). Interestingly, *D. variabilis* are known to encapsulate foreign beads, suggesting that the encapsulation process may be a PAMP-independent process triggered by foreign objects of sufficient size (Eggenberger et al. 1990). The mechanisms of regulation of encapsulation are still unknown, but warrant further study.

1.5.2c Phagocytosis

While phagocytosis is less understood in ticks, studies in insects indicate the importance of recognition and signal transduction events (Marmaras and Lampropoulou 2009). The surface receptors responsible for pathogen recognition in tick cells is still unknown. Downstream signaling proteins such as FAK/src and MAP kinase are known to be important in immune activation in insects and have recently been shown to function in the invasion and phagocytosis of *Rickettsia* in tick cells *in vitro* (Petchampai et al. 2015). The hard tick *Ixodes ricinus* has been utilized for studies focused on proteins such as α_2-macroglobulin, C3-like proteins, and TEPs which may act as opsonins increasing the phagocytosis of foreign microbes (Buresova et al. 2009, Buresova et al. 2011). α_2-macroglobulin has been implicated in the inactivation of proteins

and inactivation of such α_2-macroglobulin proteins results in decreased phagocytosis of bacterial infections by hemocytes (Buresova et al. 2009). Additionally, expression of a C3-like proteins has been shown to be specific to hemocytes, suggesting a role in the immune response (Buresova et al. 2011). Moreover, TEP protein was determined to be specific to the salivary glands suggesting a role outside of the immune response (Buresova et al. 2011)

1.5.3 Soluble defense

The secretion of antimicrobial factors has been previously studied in ticks (Grubhoffer et al. 2014, Hynes 2014). Proteins which are recognized to have antimicrobial activities include defensins, varisins, lysozyme, lectins, protease inhibitors, and oxidative stress products (Sonenshine and Hynes 2008). These products have varying effects on microbes and have the potential to both inhibit and kill these pathogens in the organs of the tick, including the hemolymph. As a barrier to infection, a robust soluble response in the hemolymph, in concert with the cellular response, may affect the dissemination of foreign microbes to the ovaries and salivary glands, thereby preventing transmission events (Beaty and Marquardt 1996).

1.5.3a AMPs

AMPs are small proteins produced by multiple organs in arthropods, including the hemocytes, with tissue- and pathogen-specific AMP expression (Sonenshine and Hynes 2008). While the fat body of insects is a primary source of AMP production, the hemocytes of ticks perform this function, as ticks do not have one collective fat body organ (Sonenshine and Hynes 2008). One well-described AMP family in ticks are the defensins. Over 20 defensins have been identified in both ixodid and argasid ticks (Sonenshine and Hynes 2008, Grubhoffer et al. 2014, Hynes 2014). These proteins are less than 6kDa, contain 8 cysteins, and a have characteristic defensin folds which are created by the presence of disulfide bridges (Ganz 2003). Defensins

disrupt membranes of foreign microorganisms through pore formation leading to cell death. Most defensins have conserved active regions, which have been crucial to the identification of novel defensins in the past. Multiple isoforms of defensins have also been identified in the soft tick *Ornithodoros moubata* where tissue and pathogen specific expression was identified in response to both infection and bloodfeeding alone (Nakajima et al. 2001, Nakajima et al. 2002). Different isoforms of defensins, known as varisins, have been identified in *D. variabilis* (Ceraul et al. 2007), yet little is known of their function. Defensins of *D. variabilis* has been shown to be active against *B. burgdorferi*, and are upregulated and releases during tick infection in under 1 hour (Johns et al. 2001). This immediate induction suggests the importance of defensins in the quick clearing of *Borrelia* by *D. variabilis*. An increase in transcription of defensin genes after infection has been observed, specifically in the midgut of *D. variabilis* after bloodfeeding and injection with *R. montanensis* (Ceraul et al. 2007). Of note, while *I. scapularis* encode defensins, no defensin protein could be identified in these ticks after infection with *B. burgdorferi*. These results suggest either an alternative non-immune related function of defensin in these ticks, or potentially a reduced immune response to a spirochete in the competent vector.

1.5.3b Lysozymes

Lysozymes are proteins which hydrolyze the bonds between the N-acetyl-muramic acid and N-acetyl-D-glucosamine found in bacterial PGN. The effects of lysozyme have been studied in *D. variabilis* where upregulation of lysozyme in response to rickettsial infection was observed. The highest transcription of lysozyme mRNA was localized to hemocytes, but was not induced in the midgut (Simser et al. 2004a, Ceraul et al. 2007). Additionally, a synergistic effect of lysozyme when added to hemolymph expressing defensin increased the *in vitro* killing of bacteria (Johns et al. 2001). Interestingly, in *D. andersoni* cell lines, infection with the

endosymbiont *R. peacockii* was not sufficient to induce the expression of lysozyme, indicating a differential immune response of ticks to pathogens and non-pathogens (Mattila et al. 2007).

1.5.3c Proteases and protease inhibitors

Proteases, such as factor D-like serine protease has been isolated and are shown to increase in response to *E. coli* infection in *D. variabilis* (Simser et al. 2004b). These serine proteases have a high similarity to those identified in other arthropods such as the horseshoe crab and mosquito. The activity of serine proteases is speculated to play a role the inability of some malaria parasites to establish disseminated infections in the mosquito (Rodrigues et al. 2007). Proteases activity has also been identified in the hemolymph of ticks, specifically in the small granules of hemocytes indicating its activity may impact the immune response of the tick to pathogen infection (Inoue et al. 2001).

Ticks encoding genes for many protease inhibitors including all known members of the α_2-macroglobulin family, including TEPs and C3-like proteins (Kopacek et al. 2010), and Kunitz-protease inhibitors (KPIs) (Ceraul et al. 2008). α_2-macroglobulins are a family of broad protease inhibitors that entrap and inactivate proteases by proteolytic cleavage (Armstrong and Quigley 1999). These proteins have been identified in *O. moubata* and *I. scapularis* and have been shown to inhibit proteases such as trypsin (Valenzuela et al. 2002, Saravanan et al. 2003). *D. variabilis* KPIs present in the hemolymph, salivary glands, and midgut of ticks are known inhibitors of blood products including thrombin, and factor X (Ceraul et al. 2008). While not fully characterized, mRNA sequence encoding a putative α_2-macroglobulin has also been isolated for *D. variabilis* and was upregulated during *R. montanensis* infection (Mulenga et al. 2003). However, the functional importance of these proteins in the immune response of the ticks to SFG *Rickettsia* infection is unknown.

1.5.3d Oxidative stress products

While reactive oxygen and nitrogen species are produced to combat pathogens, opposing proteins such as antioxidants must additionally be produced by the arthropod to protect its tissues from damage due to infection induced oxidative stress (Sonenshine and Hynes 2008). In ticks, glutathione S-transferase (GST) transcripts are produced in the midgut of fed *D. variabilis* but are decreased during infection with *E. coli* (Dreher-Lesnick et al. 2006). In contrast, GST isoforms were found to be upregulated in chronically *R. montanensis*-infected *D. variabilis* (Mulenga et al. 2003). Interestingly, infection with *R. montanensis* and *R. amblyommii* in *D. variabilis* resulted in differential expression of DvGST organs over time. This result indicated that there is a balance of expression which changes in response to *R. montanensis* or *R. amblyommii* infection, type of organ infected, and the duration of infection (Sunyakumthorn et al. 2013). Taken together, the effects of oxidative stress products are varied during tick infection.

1.5.4 Identification of immune responsive genes in *D. variabilis*

The mechanisms of immune signal transduction in ticks has been less widely studied than the effector responses outlined above. A lack of understanding of the molecular events at the tick-*Rickettsia* interface, specifically mechanisms controlling disseminated infection and transmission events has fueled research in this area of study. Efforts to elucidate immune responsive genes in the ovaries of SFG *Rickettsia* infected *D. variabilis* began with methodologies including subtractive hybridization and differential display PCR (Macaluso et al. 2003, Mulenga et al. 2003). Genes including α_2-macroglobulins, and IgE-dependent histamine release factor were among the immune-related proteins determine to be upregulated by *R. montanensis* infection further reinforcing their potential as tick defense proteins (Mulenga et al. 2003). However, the mechanisms controlling their expression are still understudied.

Studies in recent past have begun to characterize these immune mechanisms using high-throughput sequencing methodologies, focusing on the transcriptomes of various ticks and their tissues under different conditions (Jaworski et al. 2010a, Bissinger et al. 2011, Sonenshine et al. 2011, Ribeiro et al. 2012, Galletti et al. 2013, Heekin et al. 2013, Kotsyfakis et al. 2015). Genome assemblies have been released and are publicly available for *Ixodes scapularis, Rhipicephalus microplus, and Ixodes ricinus.* Recently, an effort to better annotate the *I. scapularis* genome for immune related genes revealed genes with high similarity to the proteins of the *Drosophila* Toll, IMD, and JAK/STAT pathway (Figure 1.16) (Smith and Pal 2014). In the absence of a sequenced genome, transcriptomes are a good alternative for identifying immune proteins through the annotation of resultant reads. The identification of globally transcribed genes has been the focus of recent efforts in ticks under many physiological conditions including during bloodfeeding and in specific tick tissues including the salivary glands (Bissinger et al. 2011, Sonenshine et al. 2011, Ribeiro et al. 2012, Galletti et al. 2013, Heekin et al. 2013, Kotsyfakis et al. 2015), and in ticks infected with agents they transmit (Jaworski et al. 2010a, Heekin et al. 2013). For *D. variabilis*, research efforts in infected ticks have focused on transcription characterization to identify immune responsive factors and tissue specific transmission by ticks infected with *A. marginale*, *E. coli*, *B. subtilis, Micrococcus luteus, C. albicans,* or *Sacchromyces cerevisiae.* Pooled total RNA yielded a transcriptome which included an array of immune responsive transcripts including cytochrome p450, serpins, TEPs, α_2-macroglobulins, and a novel defensin (Jaworski et al. 2010a). Interestingly, seven immune responsive transcripts when assayed by qPCR showed modest upregulation of transcription in response to an orally acquired *A. marginale* infection and a significantly higher upregulation to

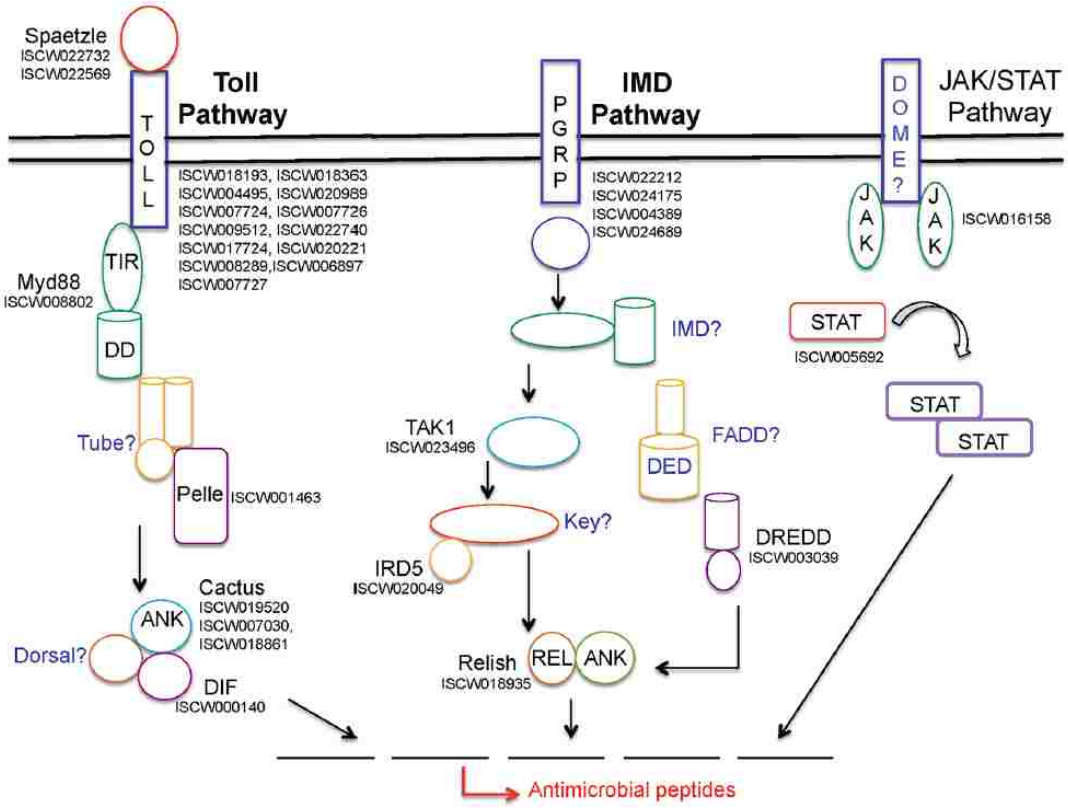

Figure 1.16 Identification of *Ixodes* genes with sequence homology to members of the NF-κB signaling pathway. Listed accession numbers represent entries from the *I. scapularis* genome.

needle inoculations of the bacteria tested (Jaworski et al. 2010b). Additional targeted transcriptomic sequencing from *D. variabilis* synganglion (Bissinger et al. 2011) and male reproductive organs (Sonenshine et al. 2011) has added to the available coding sequence. Transcriptomes allow for more rapid discovery of previously unknown proteins in organisms without fully sequenced genomes and are invaluable for the discovery and annotation of immune responsive genes in ticks.

Sequencing of transcriptomes presumably sequences the majority of transcripts present at the time of RNA isolation, including those encoding proteins which aid in the signaling and control of the immune responsive genes, including those of the NF-κB signaling pathways.

NF-κB proteins have been annotated within the genome of *I. scapularis* including a Dorsal-type NF-κB (accession: DS612897) and a Relish-type NF-κB p105 subunit (accession: DS737890). Of these two proteins, the Relish-type NF-κB has been characterized as a transcription factor which interacts with another regulatory transcription factor, subolesin (Naranjo et al. 2013). The interaction of these two regulatory transcription factors was characterized using RNAi, qPCR, flow cytometry, commercially available ELISA, and electromobility shift assay kits with activity against human, mouse, and rat NF-κB proteins. These assays described the presence of NF-κB proteins in *I. scapularis* and described their putative role in regulating subolesin (Naranjo et al. 2013). The role of NF-κB family proteins in vectors, including hard ticks infected with SFG *Rickettsia* warrants further study.

1.6 Broad hypothesis and objectives

The experimental focus of this dissertation research is to identify and define the role of the Relish-type NF-κB transcription factors in the immune response of *D. variabilis*. The experiments reported in the subsequent chapters aimed to define the response of *D. variabilis* to SFG *Rickettsia*, beginning with the identification and characterization of a Relish-type NF-κB, DvRelish. As Relish-type NF-κB molecules are major immune responsive transcription factor in vertebrates, invertebrates, and other vector species in response to Gram-negative bacteria infection, the studies focused on this transcription factor. The overarching goal of the experiments was to elucidate the uncharacterized immune signaling mechanisms in ticks to facilitate a deeper understanding of the tick response to SFG *Rickettsia*. More specifically, it was hypothesized that the level of response by *D. variabilis* to SFG *Rickettsia* occurs in a species-specific manner, and that this response drives vector competence. This study used *D. variabilis* infected with two SFG *Rickettsia*, *R. rickettsii* and *R. parkeri*, to identify differences in the

transcription, and expression of a Relish-NF-κB protein, DvRelish. This hypothesis was addressed through three aims: 1) identify Relish-type NF-κB transcript through homologue cloning; 2) identify basal Relish-type NF-κB protein expression; and 3) assess the expression of Relish-type NF-κB in response to rickettsial infection.

Towards these aims, a putative Relish-type NF-κB encoding transcript was isolated from *D. variabilis*. Conserved domain searches characterized the presence of four prominent domains in *dvrelish*: a Rel-homology domain, an immunoglobulin/plexin/transcription factor domain, a nuclear localization sequence and multiple ankyrin repeats. Subsequent analyses identified DvRelish and the activated N-terminal DvRelish in *D. variabilis* tissues and hemocytes via Western blot and mass-spectrometric analysis. SFG *Rickettsia* infection assays were performed to characterize the expression of *dvrelish* in response to microinjection, identifying a significant increase in *dvrelish* transcription in the hemocytes after 1 hour, and in combined tick tissues after 6 hours. No increase in DvRelish protein was identified by Western blot. Capillary feeding resulted in either the upregulation or downregulation of *dvrelish* transcription in the tick gut in response to 1 or 3 hour *R. rickettsii* exposures, respectively. Overall, increased *dvrelish* expression occurred after *D. variabilis* exposure with the associated pathogen *R. rickettsii*, but not with *R. parkeri* or *P. aeruginosa*. Together, this dissertation identified a Gram-negative-responsive Relish-type NF-κB molecule in *D. variabilis* and characterized of differential *dvrelish* expression in response to SFG *Rickettsia* infection for the purpose of better understanding the immune mechanisms controlling the infection of SFG *Rickettsia* in ticks.

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CHAPTER 2
ISOLATION OF *DVRELISH*, A TRANSCRIPT ENCODING A PUTATIVE RELISH-TYPE NF-κB PROTEIN, IN THE AMERICAN DOG TICK, *DERMACENTOR VARIABILIS*

2.1 Introduction

Ticks are both the reservoirs and vectors of multiple pathogens including bacteria, protozoa, and viruses (Nicholson et al. 2009). As such, ticks have a unique relationship with the pathogens they transmit. Transmission of SFG *Rickettsia* in ticks via the establishment of a disseminated infection in organs is required for successful transmission, such as in the salivary glands and ovaries. Dissemination depends on either the ability of the pathogens to evade the immune response of the tick, or for the tick to modulate the immune response to different pathogens to serve as competent vector or host. However, SFG *Rickettsia* are recognized as pathogens of ticks capable of inducing deleterious effects, as well as endosymbionts of ticks with the potential provision of beneficial effects for the vector. Understanding the tick effector responses and signaling events mediating specific tick-SFG *Rickettsia* pairings are crucial to understanding vector competence and rickettsial ecology.

The tick immune response to invading pathogens consists of two arms, the cellular and the soluble response. The cellular immune response is characterized by the phagocytosis, encapsulation, and nodulation of invading microbes by the hemocytes of ticks. The beginning of the cell mediated response is triggered by recognition of pathogen associated molecular patterns and the coagulation of hemolymph at the site of infection, followed by an increase in hemocyte proliferation (Sonenshine and Hynes 2008). The humoral immune response is controlled by the secretion of proteins with antimicrobial properties which are produced by most cell types in the tick. As ticks do not have a centralized fat body, the main site of antimicrobial peptide (AMP) production is by hemocytes in the hemolymph of the tick (Grubhoffer et al. 2014) and includes

proteins such as defensins, enzymes such as lysozymes, proteases, protease inhibitors, and oxidative stress products (Sonenshine and Hynes 2008). These soluble factors function during ingestion of the bloodmeal in the midgut and during dissemination of bacteria throughout the migration to the hemolymph, salivary glands, and ovaries. In other arthropods the expression of AMPs in response to infection is primarily controlled by the NF-κB transcription factors Dorsal and Relish (Hetru and Hoffmann 2009). However, the mechanisms controlling effector responses have not been examined in ticks.

The regulatory elements of the tick innate immune system are not as well characterized as the soluble and cell-mediated response effector proteins and cells (Sonenshine and Hynes 2008). Evidence of potential recognition receptors has been realized through the sequencing of the *Ixodes scapularis* genome as well as transcriptomes from other tick species. For example, the genome of *I. scapularis* contains partial sequences comprising portions of the Toll and IMD pathways, including a toll like receptor with leucine-rich repeats, a Dorsal-type NF-κB and its regulating partner Cactus, a Relish-type NF-κB, and Caspar a negative regulator of the IMD pathway (Smith and Pal 2014).

As ticks are the only vectors of SFG *Rickettsia*, the response of ticks to Gram-negative, intracellular bacterium is of interest. *Dermacentor variabilis* is a recognized vector of *Rickettsia rickettsii*, a highly pathogenic SFG *Rickettsia* in both humans and the tick vector. Specific tick-SFG *Rickettsia* pairings predominate in field studies, but the mechanisms driving such parings are unknown. Understanding the signaling and induction of differential effector responses of hard ticks to typical or atypical SFG *Rickettsia* will aid in the understanding of rickettsial ecology. Typical SFG *Rickettsia* comprise of species typically identified by field studies to be present in a particular tick species; whereas, atypical SFG *Rickettsia* describes species rarely or

never associated with a particular tick species. Utilizing the characterization of immune signaling pathways in model arthropods allows for the identification of immune molecules in organisms, including *D. variabilis*, for which there are no currently annotated or released genes which correspond to the immune signaling pathways. A search of the Genbank databases returned no previously sequenced and annotated NF-κB gene transcript or protein sequence for *D. variabilis*. Three 454 pyrosequencing unannotated datasets were previously released to NCBI's Sequence Read Archive (SRX018179, SRX001955, and SRX001954) which consisted of transcripts isolated from *D. variabilis* infected with various bacterial pathogens, and from different organs of uninfected ticks. Interestingly, with the release of the *I. scapularis* genome, a NF-κB protein was putatively identified and labeled as a p105-like subunit (accession: XM_002434459.1), but the molecule was lacking the canonical inhibitory domain containing ankyrin repeats (Smith and Pal 2014). While *Ixodes* and *Dermacentor* are both hard ticks, they are each classified into the prostriate and metastriate groups, respectively (Klompen 2005). Thus, immune molecules and signaling mechanisms may not be conserved between tick species.

In an effort to better understand the relationship between SFG *Rickettsia* and their vector hard ticks, this study was designed to identify and annotate an NF-κB protein in *D. variabilis*. As Relish-type NF-κB proteins are the major Gram-negative responsive transcription factor in other arthropods (Kleino and Silverman 2014), we hypothesized that the *D. variabilis* genome would encode a Relish-type NF-κB gene. Homologue cloning and bioinformatic analyses were used to amplify and molecularly characterize a transcript encoding *dvrelish*, a putative Relish-type NF-κB transcription factor in *D. variabilis*.

2.2 Methods and Materials

2.2.1 Identification of a partial *dvrelish* transcript using previously published high-throughput sequencing databases

For the purpose of identifying previously unidentified *D. variabilis* transcripts with homology to Relish-type NF-κB proteins, a homology cloning approach was designed to include conserved domain searches of previously sequenced high-throughput sequence datasets and rapid amplification of cDNA ends-PCR (RACE-PCR) (Figure 2.1). A thorough Blast search of the Genbank databases (6/2013) using the characterized Relish sequences from *Drosophila melanogaster* (accession: Q94527), *Aedes aegypti* (QMV44), and *Carcinoscorpius rotundicauda* (accession: ABC75034) as the query sequence returned no previously sequenced and annotated NF-κB gene transcript or protein sequence for *D. variabilis*. Three 454 pyrosequencing databases from published studies were previously released to NCBI's Sequence Read Archive (Jaworski et al. 2010, Bissinger et al. 2011, Sonenshine et al. 2011) and consisted of unannotated partial transcripts isolated and sequenced from uninfected *D. variabilis* tissues, whole *D. variabilis*

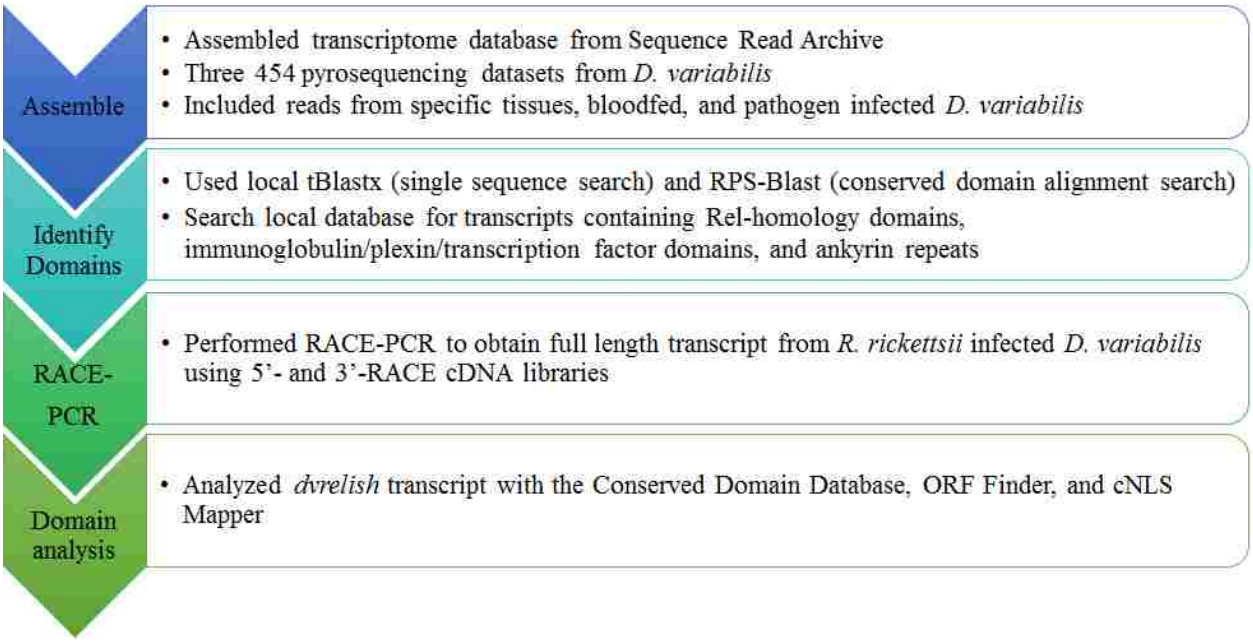


Figure 2.1 Homolog cloning strategy utilized for identification of NF-κB encoding transcripts in *D. variabilis*.

injected with Gram-negative and Gram-positive bacteria, fungi, and ticks infected with the intracellular *Anaplasma phagocytophilum* via feeding on an infected animal (accessions: SRX001954, SRX001955, SRX018179). The sequencing datasets were combined and served as a local database for Blast (v2.2.27). The presence of partial transcripts containing domains characteristic of Relish-type NF-κB proteins was identified using the following domain alignments from the Conserved Domain Database (NCBI): 1) Rel-homology domains (RHD) (cd07++884 RHD-n_Relish); 2) Immunoglobulin/plexin/ transcription factor (IPT) domains (cd01177 IPT_NFkappaB); and 3) ankyrin repeats (cd00204 ANK). A reverse position specific-Blast (RPS-Blast) was performed using conserved domain database alignments for each of the canonical domains described above as the query. Identified partial transcripts with were then used for primer design, transcript isolation, and cDNA library synthesis using the SMARTer RACE 5'/3' cDNA synthesis kit (Clontech).

2.2.2 Infection of *D. variabilis* with *R. rickettsii* and sample preparation

A colony of *Rickettsia*-free *D. variabilis* was maintained on rats, guinea pigs, and rabbits, as previously described (Macaluso et al. 2001). Rickettsiae were maintained and propagated in Vero E6 cells with Dulbecco's modified medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone). Cells were maintained in a 34°C incubator with 5% CO_2. For rickettsial isolation, bacteria was partially purified after the host cells were identified as highly infected (80% or greater) via cytospin (Wescor) and Diff-Quik staining (Siemens) (Sunyakumthorn et al. 2008). Cells were lifted from a single infected T-75 flask and Vero E6 cells were lysed with 10 passages through a 27 gauge needle. The resultant lysate was then centrifuged for 10 minutes at 275 x g at 4°C. The supernatant, which contained rickettsiae, was then passed through a 2 µm filter to remove host cell debris. High-speed centrifugation at 16,000

x g was performed to concentrate the *Rickettsia*. Bacterial viability was determined using the Baclight viability staining kit (Invitrogen). Rickettsiae were enumerated with a Petroff-Hausser bacterial counting chamber on a Leica fluorescent microscope. Enumerated *Rickettsia* (2.5×10^8) were subsequently resuspended in 10 µl of sterile phosphate buffered saline (PBS). Unfed, virgin female adults were injected with *R. rickettsii* (str. Shelia Smith). Prior to injection with rickettsiae, ticks were surface sterilized with 5 minute incubations of 0.1 bleach, 70% ethanol (3 times), and distilled water. Ticks were immobilized with tape, dorsal side down and injected into the hemocoel cavity via the coxae of the third left leg. Five unfed, adult females were injected with 2 µl of *Rickettsia*-solution with a 27 gauge needle. Ticks were maintained in a humidified environmental chamber at 27°C. One hour post exposure, ticks were removed from the incubator and dissected with a scalpel blade in sterile PBS. Additionally, 5 uninfected, surface sterilized ticks were dissected. Salivary glands, gut, ovaries and hemolymph from infected ticks, and separately tissues from uninfected ticks, were combined and collected into 50 µl of PBS and placed in RTL buffer for RNA isolation with the RNeasy kit (Qiagen). Prior to RNA isolation, tissues were homogenized using a TissueLyzer and 3-mm borosilicate glass beads (Sigma) in a 1.7 ml microcentrifuge tube for 4 minutes at 25hz/sec. RNA was isolated as per manufacturer's instructions, and stored at -80°C until used. Total RNA (1 µg) was treated with 2 units of Turbo DNase (Ambion) before cleanup and concentration with the Clean and Concentrator-5 kit (Zymo). Total RNA was subsequently reverse transcribed using the iScript cDNA synthesis kit, including no reverse transcriptase reactions to identify DNA contamination.

2.2.3 RACE-cDNA library synthesis, RACE-PCR, cloning, and sequencing

Total RNA (1 µg) was used for 5'- and 3'-enriched RACE-cDNA library synthesis using the SMARTer RACE 5'/3' kit (Clontech) as per manufacturer's protocols. Primers were

Table 2.1 Primers used for isolation of full length *dvrelish* transcript.

Primer Name	Primer Sequence (5'-3')	Purpose
IPTDV_43F	TGCACATCTGACTCCTGGAA	Initial Isolation
IPTDV_233R	ACAAAGGCTGGAAAGCTCAG	Initial Isolation
IPTLeggo211>5'	GACTATGGCCACCTGATGGT	5' RACE-PCR
RelishLeggo1925>3'	TGCCTTGTGACCCTTCTGA	3' RACE-PCR
RelishLeggo1247>3'	TGCAAGGCGGATACTCTACC	Sequencing
RelishLeggo1797>3'	TGCTGACCTTTCACTTGTGG	Sequencing
RelishLeggo2358>3'	CGGTCAAAAGTGGTGGAAGT	Sequencing

designed using Primer3 (Koressaar and Remm 2007, Untergasser et al. 2012) from a partial *D. variabilis* transcript identified through RPS-blast with homology to Relish-type RHD, and are listed in Table 2.1. Each specific primer was combined with the Universal Primer Mix (Clontech) which amplifies the 5' or 3' adaptor in each library for PCR amplification. Traditional PCR with an additional round of cycling was performed with each RACE-PCR library and the appropriate direction-specific and transcript-specific primer. RACE-PCR was performed using the Advantage cDNA PCR kit (Clontech) with 1 µl of each library as template in separate PCR reactions. The thermocycling conditions consisted of: 1 cycle at 95°C for 10 minutes, amplification for 40 cycles with denaturing at 95°C for 30 seconds, annealing at 45°C for 1 minute, and extension at 72°C for 3 minutes. A final extension was performed for 10 minutes at 72°C. For the additional rounds of PCR, 0.5 µl of the previous reaction was used as the template for the next reaction. PCR reactions were visualized with a 1.5% agarose gel (GenePure) and SybrSafe DNA gel stain (Invitrogen). All bands amplified were cloned using the TOPO TA Cloning kit with pCR4-TOPO (Invitrogen) per manufacturer's instructions. Plasmids were isolated using the Fast Plasmid Mini kit (Eppendorf) according to manufacturer's instructions. Plasmid inserts were sequenced in using the dye terminator method on an Applied Biosystems 3130 Genetic Analyzer in GeneLab at Louisiana State University. Inserts were

analyzed with MacVector (v14.5.0) and aligned with the isolated partial transcript sequence derived from traditional PCR with Clustal W. If the RACE-PCR was not successful after cloning all amplicons, the annealing temperature was varied between 65°C and 45°C or the amount of cDNA was varied, independently. RACE-PCR, cloning, and sequencing was performed until a full transcript sequence was isolated.

2.2.4 Analysis of isolated *dvrelish* transcript

The full-length transcript, *dvrelish*, was aligned to previously isolated Relish-type NF-κB transcript sequences in other model organisms including the fruit fly *Drosophila melanogaster* (accession: Q94527), the mosquito *Aedes aegypti* (accession: Q8MV44), and the horseshoe crab *Carcinoscorpius rotundicauda* (accession: ABC75034) using MacVector (v14.5.0). Nucleotide and translated amino acid alignments were used to determine percent identities. A conserved domain search was performed using the Conserved Domain Database (NCBI) to identify all domains present on the transcript. The Open Reading Frame Finder (NCBI) was used to determine the correct open reading frame of the transcript. The cNLS mapper (Kosugi et al. 2009) was used to determine the presence of a nuclear localization sequence.

2.3 Results

2.3.1 Isolation of a partial dvrelish transcript and completion of full length transcript via RACE-PCR.

The RPS-blast of the *D. variabilis* 454 pyrosequencing database resulted in one partial transcript with a RHD, one partial transcript with an IPT domain, and two partial transcripts with ankyrin repeats. The sequences for RHD-containing and IPT-containing transcripts were utilized for primer design for traditional PCR. Both primer sets were used in traditional PCR with cDNA from uninfected ticks, and *D. variabilis* infected with *R. rickettsii*. Resultant amplicons were

cloned and sequenced. No partial transcripts with homology to previously identified Relish-type NF-κB were identified in cDNA libraries from uninfected *D. variabilis*. Traditional PCR using cDNA from *R. rickettsii*-infected *D. variabilis* as template resulted in the amplification of two partial transcripts with identity to known NF-κB transcripts. However, only the primer set specific for IPT-domains was successful in amplifying the intended target, whereas the RHD-specific primers instead amplified an alternative RHD characteristic of another RHD-containing NF-κB protein, Dorsal. Primers for RACE-PCR with both 5' and 3' enriched libraries were then designed with at least 100 nucleotides for overlap of RACE-PCR amplicons with traditional PCR amplicons. These primers were paired with the universal primer mix (UPM) primers specific to the 5' or 3' adaptor which was ligated during the RACE-library preparation. Amplicons were not immediately visualized with 40 cycles of PCR, so 0.5 µl of first round reactions were used as the template for a second 40 cycles of PCR. All amplicons visualized were cloned into pCR4-TOPO and sequenced. Amplification of the 5'-end of the *dvrelish* transcript occurred with additional rounds of RACE-PCR with primer IPTLeggo211>5'. A single band overlapped with the known partial transcript after sequencing with M13 Forward and M13 Reverse primers and completed the 5' sequencing of the *dvrelish* transcript. Amplification of the 3'-end of *dvrelish* transcript occurred with RelishLeggo1925>3' and the UPM with additional rounds of RACE-PCR and sequencing of all amplicons. One large amplicon of approximately 2,500 base pairs (bp) overlapped with the previously known sequence. Complete sequencing of the cloned amplicon was performed with primer walking. Primers RelishLeggo1247>3', RelishLeggo1797>3', and RelishLeggo2358>3' were used for the sequencing and completing the 3'-end of the transcript. The full-length transcript was deposited into Genbank under the accession KJ484815.

2.3.2 Analysis of isolated *dvrelish* transcript

A schematic representing the domain architecture was determined through searches with the Conserved Domain Database, ORF finder, and cNLS mapper for the putative translated transcript is presented in Figure 2.2. The full-length *dvrelish* transcript was 3,138 nucleotides in length with an ORF that starts at base 409 through the stop codon beginning at base 3031. The putative translated ORF is 873 amino acids long. The conserved domain search determined the presence of a Rel-homology domain (amino acids 20-193), an IPT domain (amino acids 197-300), a nuclear localization sequence (amino acids 307-317), and 5 ankyrin repeats (amino acids 520-751).

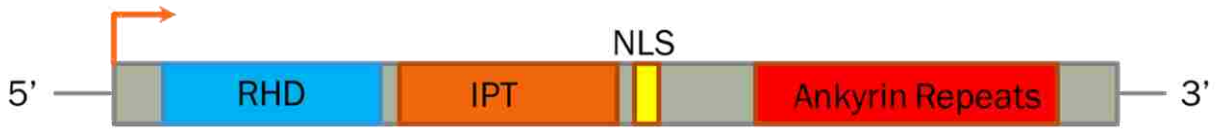


Figure 2.2 Schematic representation of the *dvrelish* transcript. RHD represents the Rel-Homology domain, IPT represents the Immunoglobulin, plexin, transcription factor domain, NLS represents nuclear localization sequence.

The percent identities for the nucleotide alignment and translated amino acid alignment are listed in Table 2.2. In general, the nucleotides align slightly better than the amino acid sequences. The closest nucleotide and amino acid sequence was from the horseshoe crab, *C. rotundicauda*, with nucleotide and amino acid identities of 36.1% and 23.3%, respectively. Compared to the *I. scapularis* p105-like transcript (ISCW018935) which does not contain the canonical inhibitory ankyrin repeats, there is 58.4% nucleotide identity and 35.8% translated amino acid identity across the conserved regions. The nucleotide sequence and translated amino acid sequence of the transcript are aligned to Relish-type NF-κB proteins of other model organisms in Figure 2.3 and Figure 2.4, respectively. The nucleotide sequence is minimally conserved with other arthropods, including the vector mosquito *A. aegypti*. The

Table 2.2 Percent identity of isolated *dvrelish* transcript and putative translated DvRelish protein as compared to the Relish-type NF-κB of other model organisms.

Organism	Percent nucleotide identity	Percent amino acid identity
Drosophila melanogaster	33.2	18.7
Aedes aegypti	35.7	18.5
Carcinoscorpius rotundicauda	36.1	23.3

transcript sequence encoding the *Drosophila* NF-κB contains multiple stretches of nucleotides within the RHD and IPT-domains that are not encoded in the mosquito or horseshoe crab Relish NF-κB transcripts. Additionally, the horseshoe crab Relish-type NF-κB transcript encodes numerous additional stretches of nucleotides present in the C-terminal ankyrin repeat domains that were previously identified as linker sequence (Fan et al. 2008). In stark contrast, the transcript encoding *dvrelish* contains only two linker sequences.

Interestingly, while the putative translated amino acid sequence of *dvrelish* has minimal amino acid identity to other arthropods, the conserved domain search reveals the RHD, IPT and ankyrin repeats are highly conserved (Figure 2.4). The recognized domains and their specific amino acid sequence correspond to structures which are integral to the function of Relish-type NF-κB proteins. DNA binding sites and ankyrin repeat binding sites throughout the RHD and IPT were conserved in *dvrelish*.

Figure 2.3a-d (Following page). Multiple sequence comparison of Relish-type NF-κB mRNA. *dvrelish* transcript nucleotide sequence was aligned to Relish-type NF-κB molecules of *Drosophila melanogaster* (accession: Q94527), *Aedes aegypti* (accession: Q8MV44), and *Carcinoscorpus rotundicauda* (accession: ABC75034). Shaded nucleotides represent base identity across aligned sequences.

Figure 2.3a Continued from previous page.

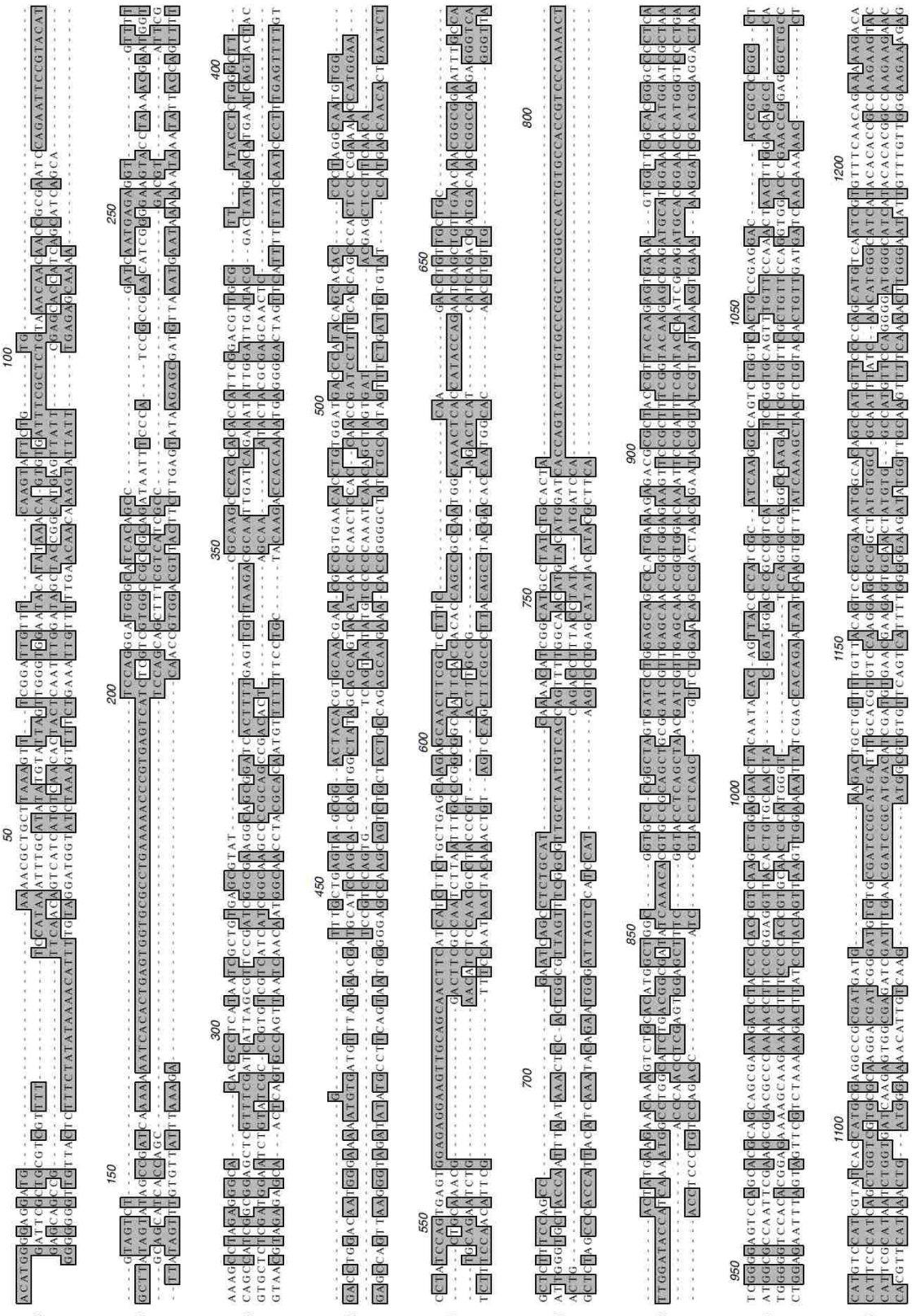
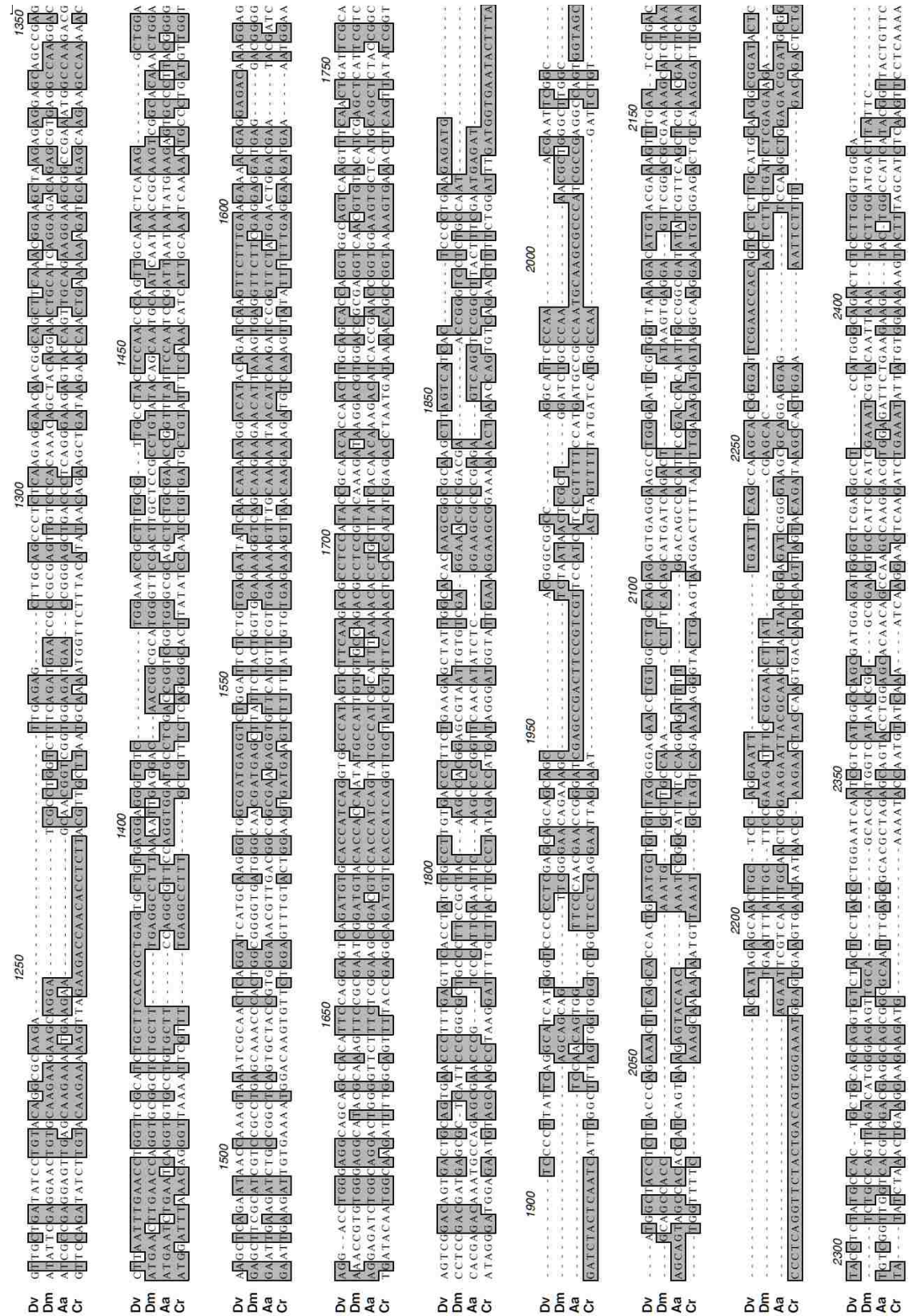


Figure 2.3b Continued from previous page.



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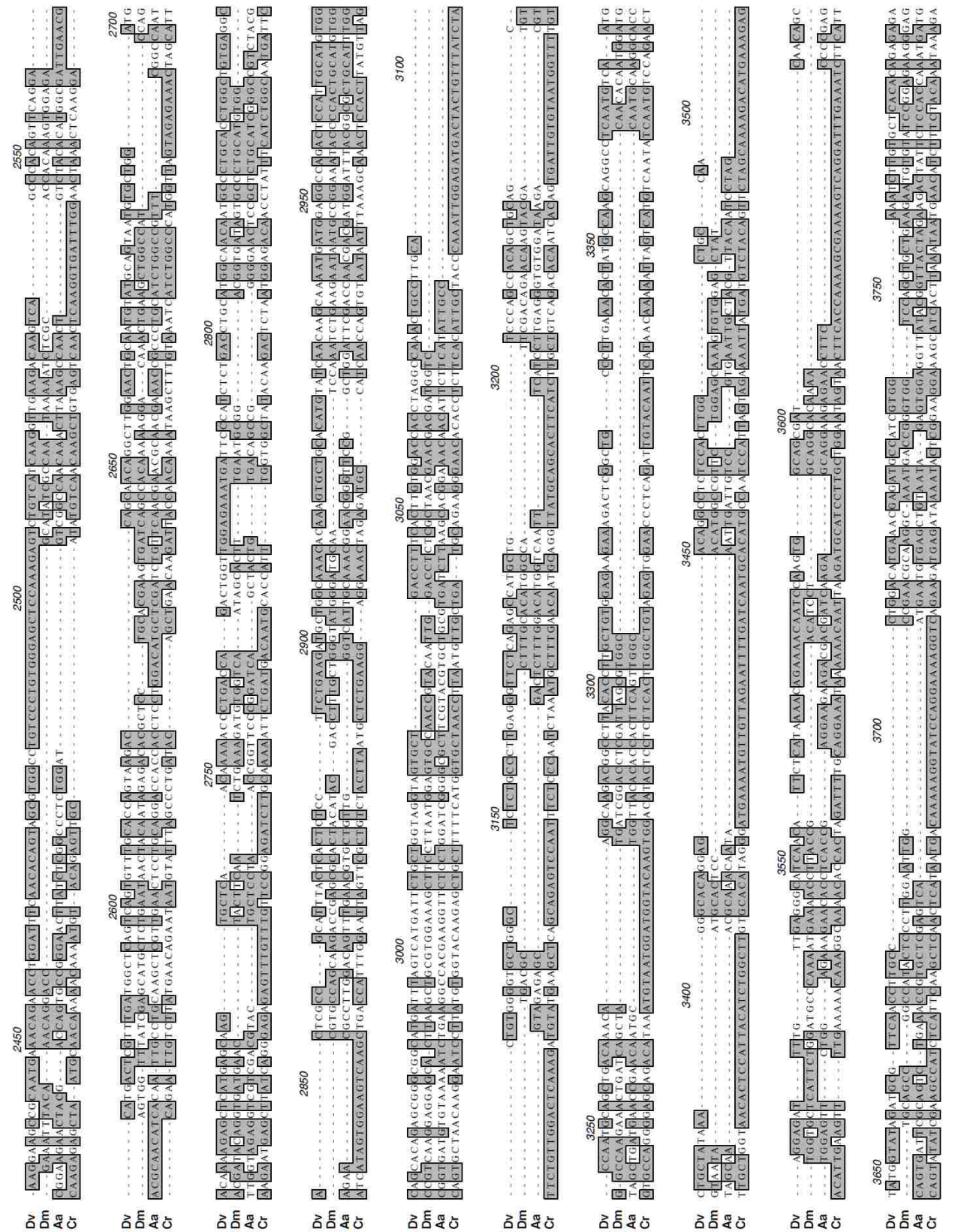

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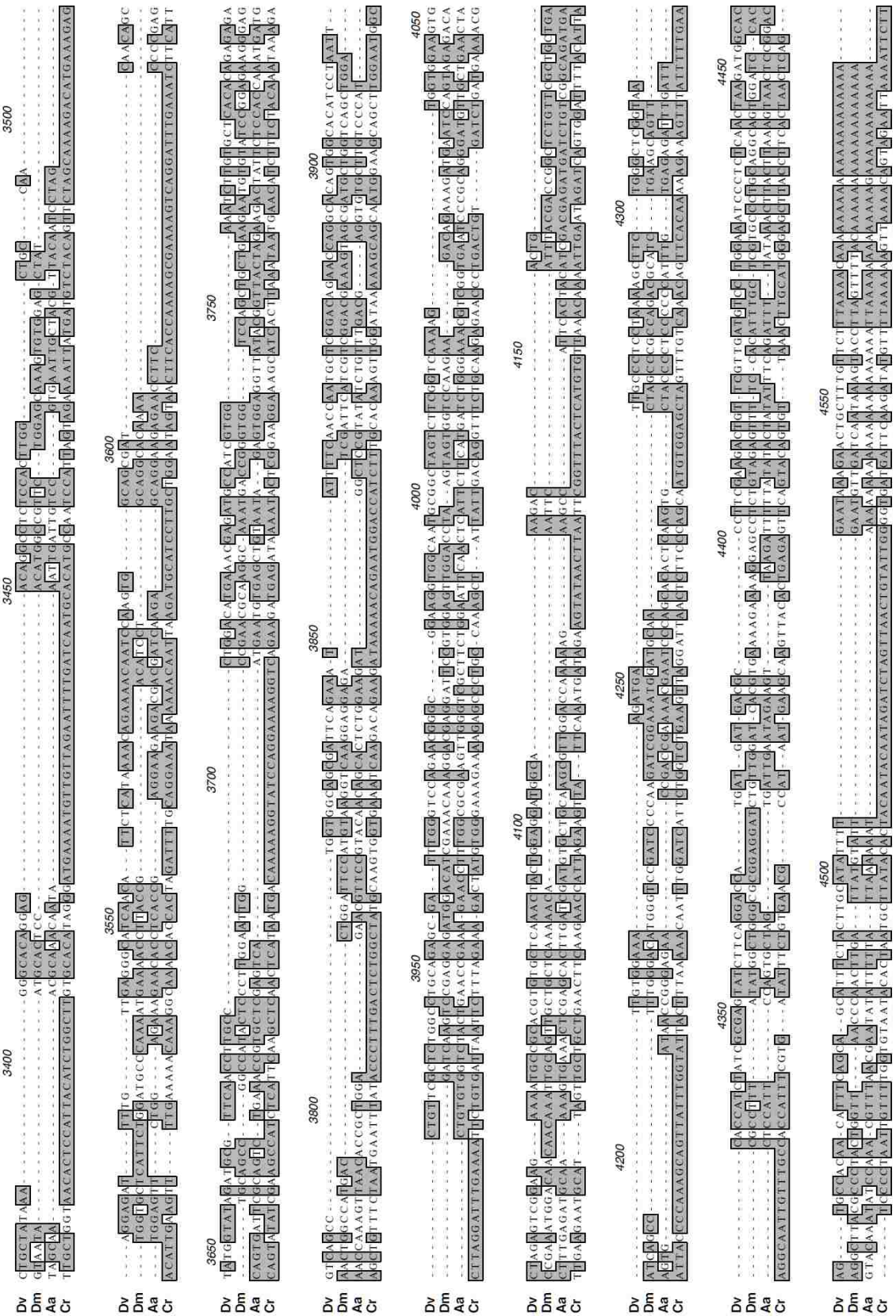


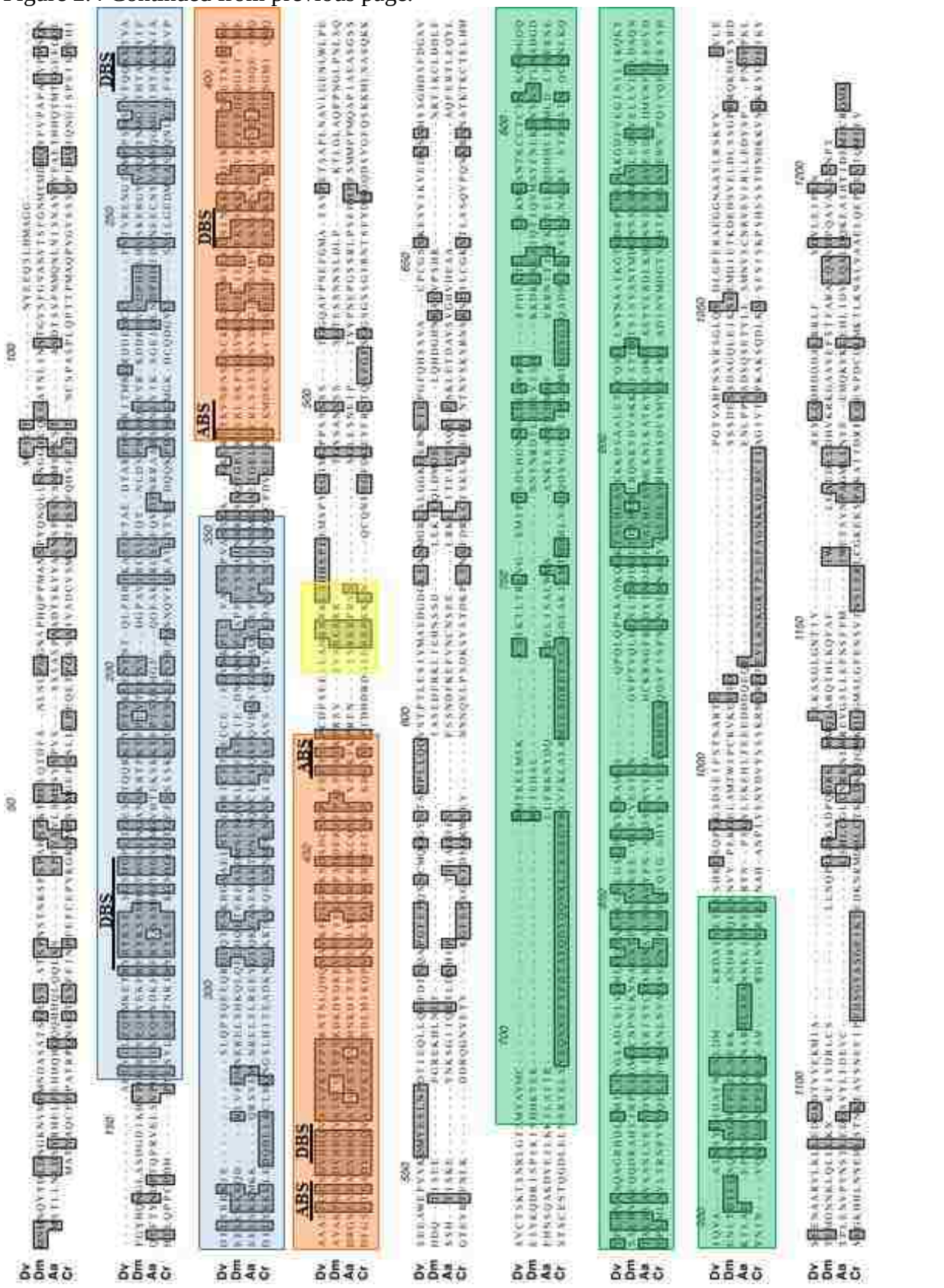
2.4 Discussion

Dermacentor variabilis is the vector of multiple tick-borne pathogens, including *R. rickettsii*, the etiologic agent of Rocky Mountain spotted fever. The relationship between hard ticks and SFG *Rickettsia* is unique, as hard ticks are hosts and vectors for pathogenic, non-pathogenic and endosymbiotic SFG *Rickettsia* (Azad and Beard 1998). From the infection of the tick through horizontal transmission to a subsequent vertebrate host or vertical transmission to tick progeny, the tick immune system must control the infection. While it is known that *R. rickettsii* has deleterious effects on tick host fecundity, *D. variabilis* are able to survive a disseminated infection required for horizontal and vertical transmission (Niebylski et al. 1999). It is understanding the mechanisms that control the balance between establishment of infection and clearance that is important for eventual development novel approaches to control. The immune system of the tick is characterized by cellular and soluble defenses which include the actions of hemocytes such as phagocytosis, and proteins such as AMPs (Sonenshine and Hynes 2008). Together, these defenses are expressed in accordance to the type of pathogen recognized after challenge (Johns et al. 1998, Johns et al. 2000, Johns et al. 2001).

Understanding mechanisms of recognition and signaling in hard ticks has been challenging without available genomic sequence. The release of the *I. scapularis* genome allowed for the annotation of many components of Toll and IMD pathways (Smith and Pal 2014)

Figure 2.4 (Following page). Multiple sequence comparison of Relish-type NF-κB translated amino acid sequence. *dvrelish* was translated and aligned to Relish-type NF-κB molecules of *Drosophila melanogaster* (accession: Q94527), *Aedes aegypti* (accession: Q8MV44), and *Carcinoscorpus rotundicauda* (accession: ABC75034). Conserved domains are represented on the alignment. The blue box represents the Rel-homology domain, the orange box represents the IPT domain, the yellow box represents nuclear localization sequence, and the green box represents the ankyrin repeat domain. Shading indicates identities across the aligned sequences. DBS represents regions corresponding to DNA binding sites. ABS represents regions corresponding to ankyrin binding sites.

Figure 2.4 Continued from previous page.



However, *I. scapularis* and *D. variabilis* are genetically divergent; as such, their genes may not be conserved. While there are differences between ticks, previously released and annotated genes, combined with the genomic and transcriptomic data of other model organisms and hematophagous arthropods, can serve as the basis for the molecular identification of homologous immune molecules in organisms without an available genome.

In this chapter, a transcript encoding a putative Relish-type NF-κB protein was identified in the American dog tick, *D. variabilis*. The successful isolation of a full-length transcript of *dvrelish* marks the first complete sequence for a Relish-type, NF-κB protein in *D. variabilis*. The *dvrelish* transcript is of low abundance, as evidenced by the necessity of increasing the traditional and RACE-PCR cycling parameters to include additional rounds of PCR. Moreover, isolation of *dvrelish* with cDNA libraries from uninfected *D. variabilis* was unsuccessful. The cDNA from *R. rickettsii*-infected *D. variabilis* contained detectable amounts of *dvrelish* transcript, transcribed in response to the infection of the tick. It has been previously recognized that infection in arthropods induces the increased transcription of immune related genes, including those encoding NF-κB proteins (Stöven et al. 2000, Meister et al. 2005, Tanaka et al. 2007, Antonova et al. 2009). The detection of the target transcript allowed for visualization of partial *dvrelish* with additional rounds of traditional PCR. The partial isolation of *dvrelish* coupled with RACE-PCR resulted in the isolation of a full *dvrelish* transcript.

While another NF-κB protein has been annotated in the *I. scapularis* genome, the annotated sequence does not contain a critical canonical domain of these type of proteins, the ankyrin repeats. Dorsal-type NF-κB proteins are unique in having a separate inhibitory protein which sequesters the protein in the cytoplasm; however, Relish proteins have encoded ankyrin repeats which act as the inhibitory domain (Hetru and Hoffmann 2009). Once activated, the

inhibitory N-terminal portion of the protein is cleaved, the nuclear localization sequence is exposed, and the N-terminal portion is translocated into the nucleus of the cell. The presence of encoded ankyrin repeats supports the classification of *dvrelish* as encoding a putative Relish-type NF-κB protein.

Conserved domain searches parse out the RHD of *dvrelish* as Relish-type, as opposed to Dorsal/Dif-type. While the nucleotide and amino acid identities are quite low in comparison to other arthropods, the amino acids responsible for the protein function of Relish-type NF-κB proteins are highly conserved in the RHD, IPT and ankyrin repeats. The conservation of amino acids in these domains characterized in *Drosophila* NF-κB molecules allows for binding to DNA, dimerization, and the binding of the encoded ankyrin repeats for sequestration in the cytoplasm of cells. Nucleotide Blast searches alone were not able to identify the partial transcripts from *D. variabilis* transcriptomes as NF-κB encoding molecules. However, conserved domain searches take into account the overall domain architecture through the use of multiple sequence alignments for identification. Hence, conserved domain searches are better suited for the identification low identity transcripts with domains of high structural homology, such as *dvrelish*. This difference is annotation methodology likely explains how *dvrelish* was not recognized in the transcriptional studies from which the starting partial transcripts originated.

Previous research was focused on the effector responses of the tick (Sonenshine and Hynes 2008), without developing molecular tools and assays to study the signaling events leading to and controlling differential effector response. The study of how the immune responses of the tick vector is differentially expressed after infection with differing bacterial infections will aid in the understanding of the mechanisms underlying the vector competence of *D. variabilis* for specific rickettsial pathogens.

2.5 Reference List

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CHAPTER 3
EXPRESSION OF DVRELISH, A PUTATIVE RELISH-TYPE NF-κB PROTEIN IN *DERMACENTOR VARIABILIS*

3.1 Introduction

As vectors of spotted fever group (SFG) *Rickettsia,* hard ticks are recognized to have a unique relationship with this group of intracellular bacteria (Azad and Beard 1998). Specific SFG *Rickettsia* and tick associations have been supported by field studies, even considering that many hard tick vectors have overlapping distributions and share vertebrate hosts (Macaluso and Paddock 2014). Members of the SFG *Rickettsia* are varied in their pathogenicity to their vertebrate and tick hosts (Walker and Ismail 2008). As an example, SFG *Rickettsia* are also recognized as endosymbionts of hard ticks as there are no detrimental fitness effects associated with infection with some species (Niebylski et al. 1997, Baldridge et al. 2007, Gillespie et al. 2012, Paddock et al. 2015).

SFG *Rickettsia* are able to survive within their specific vector hosts, indicating the presence of mechanisms favoring bacterial survival within specific tick-*Rickettsia* pairings. However, instances of tick host fitness costs associated with infection have been documented (Niebylski et al. 1999). For example, the association of *Rickettsia rickettsii* with its vector *Dermacentor variabilis* results in both the development disseminated infections required for transovarial and horizontal transmission and documented negative fitness effects such as reduced fecundity (Ricketts 1907, Burgdorfer and Brinton 1975, Schumacher et al. 2016). Such mechanisms favoring bacterial survival likely include *Rickettsia*-derived factors driving immune evasion within the tick host; and, conversely, tick-derived factors facilitating immune tolerance required for such pairings to emerge. Both the *Rickettsia*-derived and tick-derived factors enabling specific associations are currently unknown.

The immune response of vector ticks may factor greatly in rickettsial ecology, specifically the potential to persist or be cleared defines vector competence. If ticks are controlling microbial infections (unwanted pathogens) then the immune response is likely the mechanism involved. However, the mechanisms of recognition, signal transduction, and transcriptional control of the immune response is largely unexplored in ticks without sequenced genomes, including *D. variabilis*. Characterization of the immune response of vector ticks has been examined with emphasis on describing effector mechanisms (Sonenshine and Hynes 2008). The response to bacterial infection in arthropods is best described in the non-vector model organism, *Drosophila melanogaster* (Lemaitre and Hoffmann 2007). Known effector responses, such as antimicrobial peptide (AMP) production, are controlled in a pathogen-specific manner via transcription factor-dependent regulation (Hetru and Hoffmann 2009). Dorsal and Relish NF-κB proteins from *D. melanogaster* are expressed and activated in response to Gram-positive and Gram-negative bacteria, respectively (Valanne et al. 2011, Kleino and Silverman 2014). Relish-type NF-κB protein encoding genes have been previously identified in *Ixodes scapularis* (Naranjo et al. 2013); however, the divergent gene sequence does not encode inhibitory ankyrin repeat domains. Given that NF-κB proteins control the response to Gram-negative bacteria in model arthropods, the expression of homologous proteins in *D. variabilis* was probable.

The characterization of Relish-type transcription factors in arthropods has been assessed subsequent to infection with bacteria, viruses, or protozoa in mosquitoes and typical protozoa in tsetse flies (Lemaitre et al. 1995, Hu and Aksoy 2006, Costa et al. 2009, Cirimotich et al. 2011). Gram-negative bacterial infections in *Drosophila* result in an increase in Relish expression with a peak at 3 hours (Dushay et al. 1996). This phenotype was additionally noted in a horseshoe crab infection model (Wang et al. 2006). The temporal patterns of NF-κB proteins in response to

atypical bacteria may not be directly comparable to putative NF-κB proteins during infection of hard ticks and their typical SFG *Rickettsia*.

With the goal of better characterizing the mechanisms of immune signaling occurring during SFG *Rickettsia* infection of *D. variabilis* with SFG *Rickettsia,* the previous chapter of this dissertation research identified *dvrelish,* a transcript encoding a putative Relish-type NF-κB protein (Chapter 2). The current chapter of this dissertation was designed to expand upon those findings by identifying DvRelish protein in *D. variabilis*. The experiments described herein were designed to test the hypothesis that *D. variabilis* express DvRelish and its activated N-terminal truncated form. This identification occurred through: 1) the expression of recombinant DvRelish (rDvRelish) in SF9 cells to confirm the predicted size of DvRelish; 2) the identification of proteins specifically recognized by an anti-DvRelish antibody; and, 3) the determination of DvRelish expression in *D. variabilis* hemocytes exposed to Gram-negative bacteria and Gram-negative bacteria-derived peptidoglycan (PGN).

3.2 Methods and Materials

In order to determine the expression of DvRelish, experiments were designed using recombinant protein expression systems, Western blotting, and immunofluorescence assays (IFA) in uninfected infected tick tissues (Figure 3.1). First, a recombinant DvRelish (rDvRelish) protein was expressed using a baculovirus expression system for the purpose of identifying the molecular weight of rDvRelish and confirming anti-DvRelish antibody binding. Next, utilizing Western blotting and mass-spectrometry, DvRelish and activated N-terminal DvRelish were identified in uninfected *D. variabilis* tissues. Finally, DvRelish expression in hemocytes was

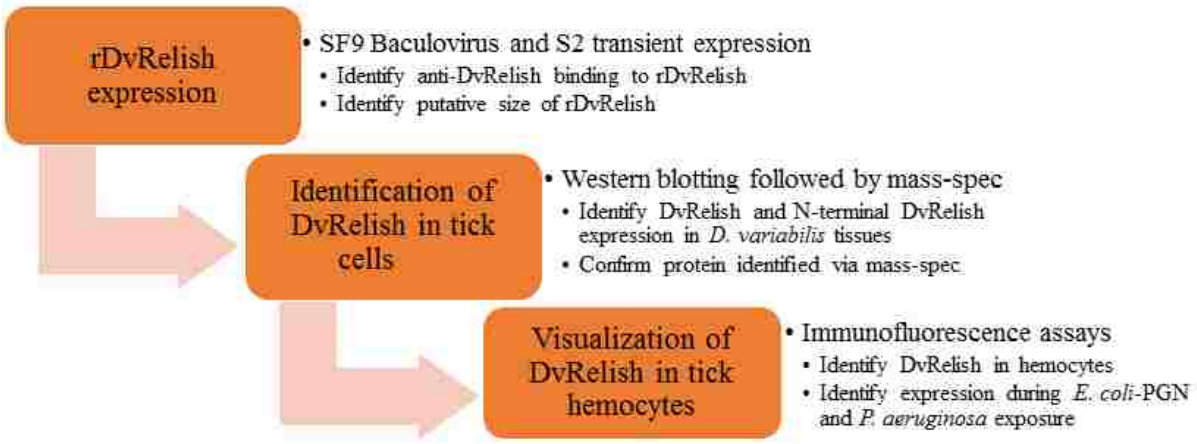

Figure 3.1 Experimental design outlining the identification of a Relish-type NF-κB protein in *D. variabilis* through recombinant protein expression, Western blotting, and immunofluorescence assays.

assessed via IFA in Gram-negative PGN and *Pseudomonas aeruginosa* stimulated *D. variabilis* hemocytes.

3.2.1 Expression of recombinant DvRelish in SF9 cells

Recombinant DvRelish (rDvRelish) was expressed using the Bac-to-Bac baculovirus expression system (Invitrogen) in SF9 cells according to manufacturer's instructions. SF9 cells were maintained in SF900 II serum free medium (Invitrogen) in a 28°C incubator without CO_2. Two constructs were used for expression: 1) the *dvrelish* transcript ORF without a stop codon; and, 2) the N-terminal region of *dvrelish* which corresponds to the Rel-homology domain IPT domain and NLS sequence. Full *dvrelish* and N-terminal *dvrelish* were amplified with FastStart HiFidelity polymerase mix (Roche), 400nM of primers listed in Table 3.1. DvRelish was amplified with primers CACC-FullRelish409F and FullRelish3030Rev-NoStop with the addition of a CACC-overhang for directional cloning and no stop codon for His-inclusion. N-terminal DvRelish was constructed with CACC-FullRelish409F and FullRelishqPCR1725Rev. The PCR

Table 3.1 Primers used for DvRelish and N-terminal DvRelish pENTR-D-TOPO expression plasmid construction.

Primer Name	Primer Sequence (5'-3')
FullRelish409F	ATGCCTATCTGCACTAACTATGAAG
CACC-FullRelish409F	CACCATGCCTATCTGCACTAACTATGAAG
FullRelish3030Rev	TTAGTTGAGAGGGATTTCCAGGAC
3030Rev-NoStop	GTTGAGAGGGATTTCCAGGAC
FullRelishqPCR1725Rev	CGATTGATTCCAGGGTAGGA
CACC-Myc-DvRelish409F	CACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGC CTATCTGCACTAACTATG

cycling parameters were as follows: denaturing at 95°C for 10 minutes, followed by amplification for 40 cycles with denaturing at 95°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 3 minutes. A final extension was performed for 10 minutes at 72°C. PCR products were separated by agarose electrophoresis, stained with SybrSafe DNA Gel stain (Thermo), isolated via Wizard PCR Clean up Kit (Promega) and cloned into the donor pFastBac/HMB-Topo plasmid according to manufacturer's instructions. After transfection into chemically competent *Escherichia coli* and ampicillin selection, the donor plasmid insert was verified via sequencing. The donor plasmid was subsequently isolated and transformed into DH10bac *E. coli* containing the baculovirus shuttle vector. Transposition of the donor vector insert into the baculovirus shuttle vector was verified by PCR after selection of baculovirus DNA-containing colonies via antibiotic selection. After verification of transposition, recombinant baculovirus DNA was isolated and transfected into SF9 cells with Cellfectin II reagent (Invitrogen). To identify expression, cell lysates were analyzed by SDS-PAGE followed by Western blotting with either anti-His or anti-DvRelish antibody. Infected SF9 cells were passed five times to increase viral titer and protein expression. rDvRelish expression was analyzed after each passage via SDS-PAGE and Western blot. SDS-PAGE was performed using Mini-Protean Tris/Glycine 4-15% precast protein gels (Bio-Rad). Separated protein was

transferred to 0.45 µM pore nitrocellulose (Bio-Rad) using a Trans-Blot SD semi-dry transfer machine (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) in TBST and probed with primary 6x anti-His 1:5000 antibody (Clontech) followed by secondary donkey anti-mouse Li-Cor 680CW 1:20,000 antibody (Li-Cor). Western blots were imaged using a Li-cor Odyssey imager (Li-Cor).

3.2.2 Transient expression of recombinant DvRelish in S2 cells

Plasmids were constructed for transfection and transient expression of rDvRelish using the pENTR/D-TOPO entry vector and pMT-DEST48 destination vector. The *dvrelish* ORF was cloned into the pENTR/D-TOPO after PCR using primers CACC-Myc-FullRelish409F and 3030Rev-NoStop (Table 3.1). PCR was performed using 400 nM of each primer, Roche FastStart High Fidelity Polymerase, and 150 ng of pCR4-TOPO plasmid containing *dvrelish* as template. Cycling parameters were as follows: denaturing at 95°C for 10 minutes, amplification for 40 cycles with 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 3 minutes. A final extension was performed for 10 minutes at 72°C. The PCR product was cloned into pENTR/D-TOPO entry vector and insertion was validated by sequencing. The purpose of the entry vector PCR was to add a 5' CACC for directional cloning, a MYC tag, and to remove the stop codon from the ORF prior to cloning into the expression vector. The entry vector insert was recombined into the destination vector using LR Clonase II (Invitrogen). Briefly, 150 ng of entry vector, 150 ng of destination, and 2ul 5x LR Clonase II in TE Buffer (pH 8.0) was incubated at 25°C for 1 hour and stopped with the additional of proteinase K incubated at 37°C for 10 minutes. The recombination reaction was then chemically transformed into One Shot ccdB T1 phage resistant *E. coli* (Invitrogen). Bacteria harboring the recombined destination vector were identified by ampicillin resistance and chloramphenicol sensitivity. The destination vector insert was

sequenced to confirm recombination. *D. melanogaster* S2 cells were cultured in a 28°C incubator without CO_2. Schneider's *Drosophila* medium (Invitrogen) containing 10% heat-inactivated FBS (HyClone) was used for cell maintenance. Cellfectin II was used per manufacturer's instructions for transfection with 2 µg of purified destination vector. After transfection, 500 µM $CuSO_4$ was added to the media to induce expression of Myc-DvRelish-His. To identify expression, cells were lifted, washed with PBS, reconstituted with 200 µl RIPA buffer, and sonicated for 15 sec at 35% amplitude. Lysates were analyzed by Western blotting with anti-Myc and anti-His antibody to confirm the expression of tagged protein. Transfection conditions were optimized by day of induction of cells (1-4 days) and day of harvest of cells post transfection (1-3).

3.2.3 anti-DvRelish peptide antibody production

The production of an anti-DvRelish peptide antibody was commercially produced by Yenzym Antibodies. Briefly, two peptides from the Rel-homology domain of *dvrelish* were chosen and linked to the keyhole limpet hemocyanin (KLH) carrier protein separately. The two peptides used for immunization were as follows: CESSTQQRKTYPT KLENYNTQ-amide (DvRelish amino acids 47-67) and CYRRKIESLQPSQEEQRQLQ-amide (DvRelish amino acids 131-149). These peptides were chosen as candidates because they were predicted to be both hydrophilic and expressed on the surface of the protein. A rabbit was immunized with the combination of the two KLH-conjugated peptides in Freund's complete adjuvant. After two months the rabbit was inoculated with a secondary booster of both peptides. Serum was collected and the specificity of the produced antibodies in serum was determined by enzyme-linked immunosorbent assay. Antibody was then purified by high performance liquid chromatography. Antibodies were delivered from the company at 0.24 mg/ml of purified mono-specific polyclonal IgG.

3.2.4 Detection of DvRelish in tick tissue lysate

Uninfected adult females were dissected, tissues were placed in RIPA buffer with Complete Mini EDTA-free protease inhibitor cocktail (Roche), and homogenized using a sonicating probe (Sonic Dismembranator, Fisher) with 25% amplitude for 5 seconds, 5 times, each on ice. The protein concentration in the tissue lysate was quantified using the Dc Assay (Bio-Rad) per manufacturer's instructions. Tick protein (25 µg per lane) was separated with a Mini-ProteanX 4-15% Tris-Glycine mini-gel (Bio-Rad). Separated proteins were transferred onto a 0.45 µm pore nitrocellulose membrane using a Trans-Blot semi-dry transfer cell at 25V for 25 minutes. The membrane was blocked with 5% BSA in tris-buffered saline with 0.5% Tween 20 (TBST). Primary anti-DvRelish antibody (1:100) in 5% BSA in TBST was followed by secondary donkey anti-rabbit Li-cor 800CW antibody (1:15,000). Peptide competition was performed with 1:100 anti-DvRelish antibody supplemented with 1 µg of each peptide the antibody was raised against. Blots were visualized with a Li-cor Odyssey imager.

3.2.5 Mass-spectrometry analysis

Unfed female *D. variabilis* whole tick tissue was separated by SDS-PAGE. Bands of interest identified concurrently by Western blot at 100 kDa and 70 kDa were excised from a 6% Tris-glycine gel with a clean scalpel blade. Samples were digested prior to mass-spectrometry analysis with porcine pancreas-derived trypsin (Sigma-Aldrich). Digested samples were submitted for MALDI-TOF/TOF mass-spectrometry on a Bruker UltrafleXtreme MALDI-TOF/TOF MS system (Bruker Daltonics) at the LSU Chemistry Department. For identification of submitted samples, reported sample peptide masses were compared to predicted masses for the putative amino acid sequence of DvRelish. *In silico* trypsin digestion analysis of the putative DvRelish amino acid sequence was performed with 1 missed cleavage allowed using the

PeptideMass predictor program from the Swiss Institute of Bioinformatics ExPAsy website (Wilkins et al. 1997, Gasteiger et al. 2005).

3.2.6 Immunofluorescence assay of *D. variabilis* hemocytes

Unfed, adult *D. variabilis* females were injected with either 200 ng of *Escherichia coli* 0111:B4 peptidoglycan (PGN) (InvivoGen) or 10^7 *Pseudomonas aeruginosa* (ATTC 27853); *E. coli*-PGN was resuspended in 1 µl of molecular grade water and *P. aeruginosa* was resuspended in sterile PBS. Prior to injection with *P. aeruginosa*, ticks were surface sterilized with sequential 5 minute incubations of 0.1% bleach, 70% ethanol (3 times), and distilled water. Ticks were immobilized dorsal side down with tape, and injected with *P. aeruginosa* or *E. coli*-PGN into the hemocoel cavity via the coxae of the third left leg. Five unfed, adult females were injected with either 1 µl of PGN solution or *P. aeruginosa* with a 33-gauge needle (Hamilton) and 5 µl glass syringe (Hamilton). Ticks were maintained in a humidified environmental chamber at 27°C for 1 or 6 hours. PGN-injected ticks were incubated for up to 6 hours post-injection (hpi), *P. aeruginosa*-injected ticks were incubated for 1 hpi, and PBS sham injected ticks were incubated for 6 or 1 hours and dissected in sterile PBS. Hemolymph was collected and allowed to dry on glass microscope slides. Hemocytes were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 10 minutes. Hemocytes were washed with 0.01% Triton X-100 in PBS and blocked with 3% BSA in PBS for 30 minutes. Anti-DvRelish antibody (1:50) was applied to the hemocytes in 1% BSA in PBS for 2 hours. No primary antibody controls were also incubated with PBS to determine non-specific binding. Cells were washed 3 times with 0.01% Triton X-100 in PBS for 5 minutes each. Secondary goat anti-rabbit FITC labeled antibody was applied at 1:100 in 1% BSA in PBS for 1 hour. Cells were again washed 3 times with 0.01% Triton-X in PBS for minutes each. Coverslips were mounted

with VectaShield mounting medium containing the DNA stain 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Hemocytes were visualized with an Olympus Fluoview confocal microscope.

3.3 Results

3.3.1 rDvRelish expression in SF9 cells

In order to express a His-tagged DvRelish protein, *dvrelish*-His encoding baculovirus DNA was transfected into SF9 cells. Virus production was allowed to occur for 2 passages before DvRelish Western blotting. This Western blotting produced a band at 100kDa which closely corresponds to the predicted DvRelish-His **(**Figure 3.2). The expression was visualized after two passages of recombinant baculovirus via Western blot with both anti-His and anti-DvRelish antibodies. Both blots resulted in the recognition of a protein of the same size, supporting the idea that both antibodies are recognizing the same protein. The corresponding coomassie stained gel did not reveal a 100 kDa protein, indicating the protein was not highly expressed. The rDvRelish baculovirus was passed 3 more times for propagation of high titer viral

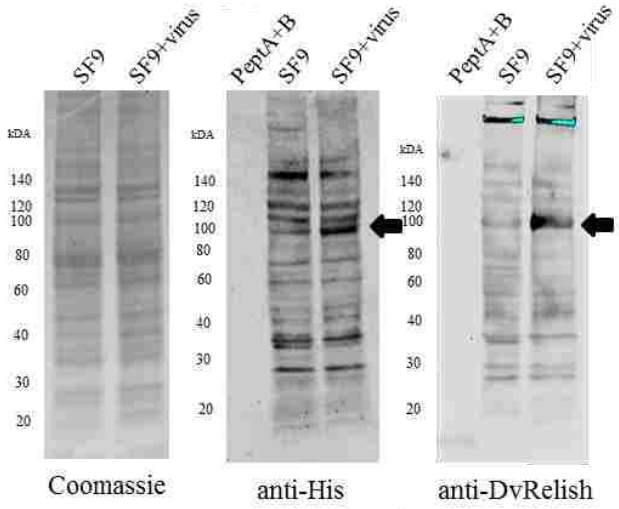

Figure 3.2 Expression of rDvRelish by recombinant baculovirus infection in SF9 cells after 2 viral passages. Arrow indicates recombinant protein expression as recognized by anti-His antibody and anti-DvRelish antibody. SF9 represents an uninfected control, and PeptA+B indicates 10 ng of each peptide the anti-DvRelish antibody were raised against.

stocks. At passage 4 (Figure 3.3) an additional 75 kDa protein was recognized by anti-His antibody Western blot. At passage 5, however, the 100kDa protein is no longer recognized (Figure 3.4). Smaller proteins are recognized by both anti-His, and anti-DvRelish antibody, indicating degradation of rDvRelish. Passage of baculovirus was discontinued after 5 passages due to the loss of rDvRelish expression.

A second baculovirus was constructed to express the N-terminal region of DvRelish containing the Rel-homology domain, immunoglobulin/plexin/transcription factor domain, and a nuclear localization sequence. After 5 passages of recombinant virus in SF9 cells, no expression of the predicted 65 kDa protein was identified via anti-His or anti-DvRelish Western blot (Figure 3.4). Passage of baculovirus was discontinued after passage 5.

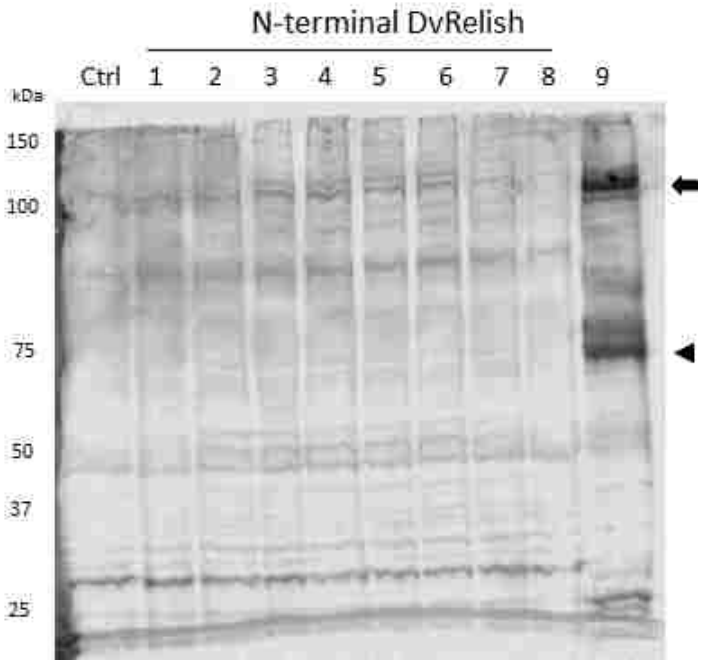

Figure 3.3 Expression of two constructs of DvRelish after 4 passages. Recombinant protein expression was visualized via Western blot with anti-His antibody. SF9 represents control (Ctrl) uninfected SF9 cells, lanes 1 through 8 represent individual populations of N-terminal DvRelish baculovirus infected SF9 cells, and lane 9 represents rDvRelish expressing SF9 cells. The arrow indicates the 100 kDa rDvRelish, and the arrow head indicates the recognition of a 75 kDa protein.

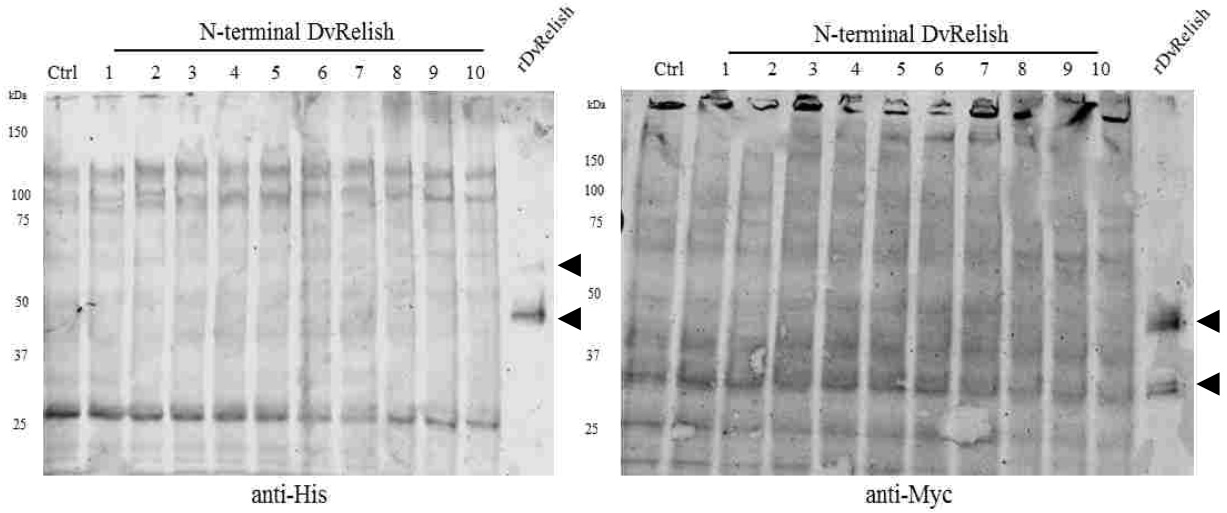

Figure 3.4 Expression of two constructs of DvRelish after 5 passages. Recombinant protein expression was visualized via Western blot with anti-His antibody (left panel) and with anti-DvRelish antibody. SF9 represents uninfected SF9 cells, lanes 1 through 10 represent individual populations of baculovirus infected SF9 cell, and rDvRelish represents the rDvRelish expressing SF9 cells after 5 passages. Arrow heads indicate recognition of rDvRelish cleavage products.

3.3.2 Transient expression of rDvRelish in S2 cells

In order to express rDvRelish for functional characterization, transient expression of rDvRelish in S2 cells via transfection of the pMT-DEST40 destination vector containing *dvrelish* was attempted with varying conditions. After addition of CuSO4 to induce expression of rDvRelish, expression of recombinant protein was determined via Western blot. S2 cells were collected after induction and analyzed for the expression of rDvRelish via SDS-PAGE and Western blot with anti-Myc and anti-His antibodies (Figure 3.5). Cells transfected with 2 µg of plasmid DNA, induced 2 hours post transfection with 500uM $CuS0_4$, and harvested 24 hours later yielded no detectable expressed rDvRelish protein as compared to S2 controls. The conditions of transfection were subsequently optimized by varying the time period prior to induction (days 1-4) and prior to harvest post induction. Under all optimized conditions a 40 kDa anti-Myc reactive protein was identified via Western blot (Figure 3.6). Further analysis of cell lysates revealed the

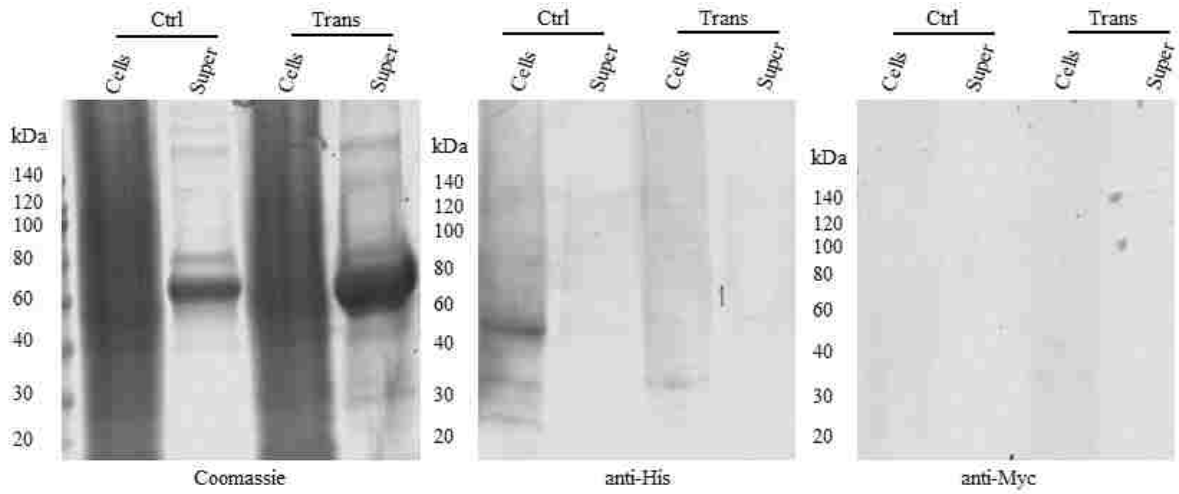

Figure 3.5 Expression of rDvRelish in S2 cells 1 day post transfection. S2 cells were transfected with 2 µg plasmid, induced with 500 µM $CuS0_4$ and harvested 24 hours later. Both cells, and media supernatant were analyzed for recombinant protein expression. Expression was analyzed via Western blot with anti-His and anti-Myc antibodies. Ctrl represents control untransfected S2 cells, and Trans represents transfected cells.

expression of an approximately 70 kDa anti-His reactive protein after 3 days of induction and cell harvest. As the *dvrelish* ORF cloned and sequenced in the transfected pMT-DEST48 encodes a putative 100 kDa product, S2 cell expression was discontinued.

3.3.3 Detection of DvRelish in tick tissue lysate

After identification of specific rDvRelish reactivity via Western blot, DvRelish expression was queried in *D. variabilis* tissues. All tissues of the tick were homogenized and centrifuged to remove insoluble tick materials. Tick protein (25 µg) was analyzed by Western blot with anti-DvRelish antibody, resulting in the recognition of many protein bands (Figure 3.7). A peptide competition assay was performed to determine specificity of anti-DvRelish binding. The addition of 1 µg of each peptide to the primary DvRelish incubation resulted in the loss of signal at 100 kDa and 70 kDa. The secondary antibody only control was performed

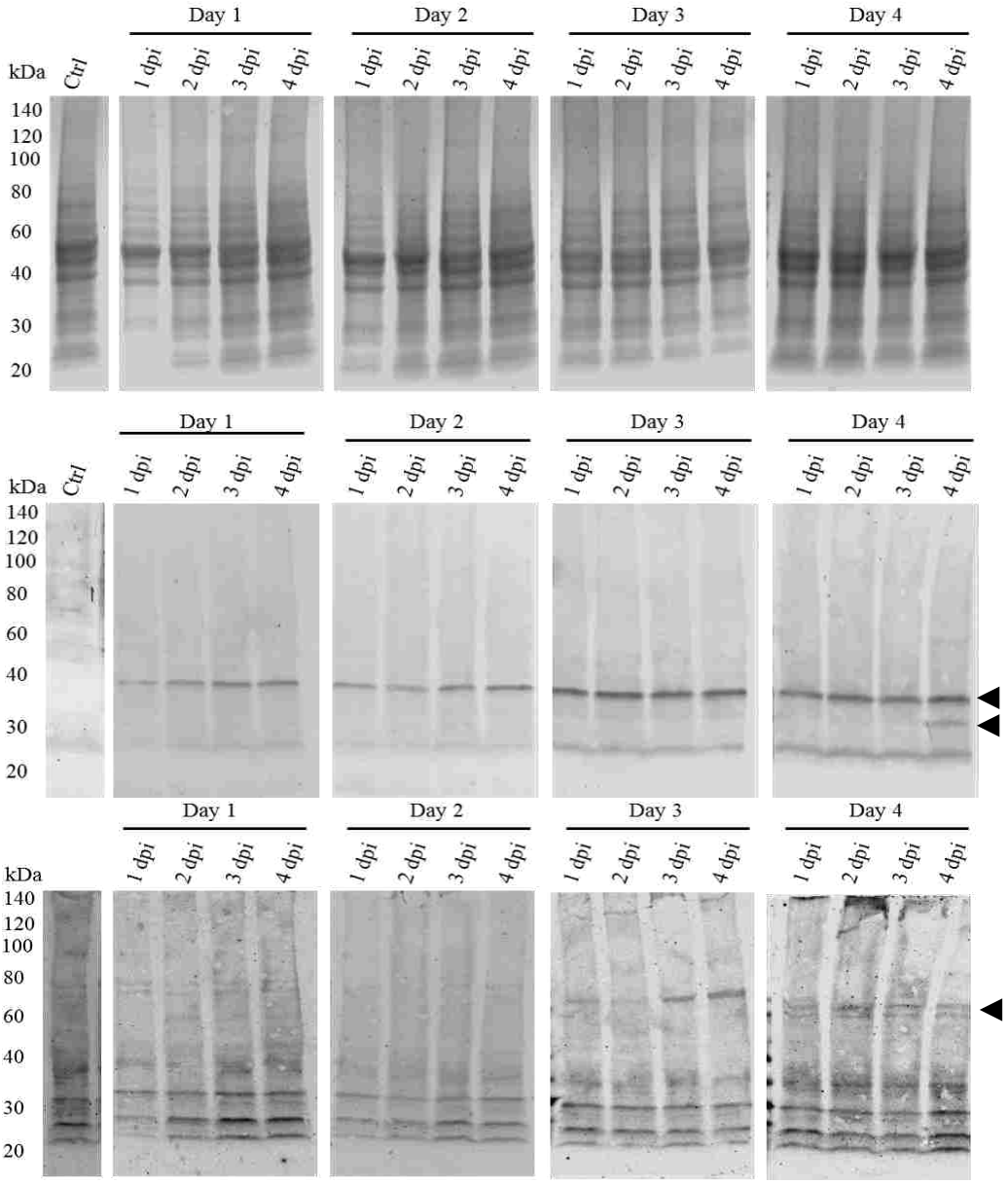

Figure 3.6 Expression of rDvRelish in S2 cells under various conditions. S2 cells were transfected with 2 µg plasmid and induced with 500uM $CuSO_4$. Transfected S2 cells were induced at days 1-4 (labeled with bar) and were harvested at 1-4 days post induction (dpi). Transfected cells and untouched control S2 cells (Ctrl) were analyzed for recombinant protein expression via SDS-PAGE (top row), Western blot with anti-His (middle row) and anti-Myc (bottom row) antibodies.

yielding non-specific binding of the donkey anti-rabbit 800CW antibody to tick tissues. Considerable non-specific binding to *D. variabilis* proteins occurs, but in conjunction with the peptide competition assay, it was concluded that the anti-DvRelish antibody specifically recognized two proteins at 100 kDa and 70 kDa.

3.3.4 Mass-spectrometry analysis

To confirm specific recognition by the anti-DvRelish Western blot, mass-spectrometry was employed on *D. variabilis* tissue lysates. For the purpose of better resolving the previously recognized 100 kDa and 70 kDa proteins, tick tissue samples were separated via SDS-PAGE using a 6% Tris-glycine gel. Protein bands previously visualized by Western blot at 100 kDa and 70 kDa were excised, trypsin digested, and submitted for mass-spectrometry (Figure 3.7). The resultant peptide masses were compared to the masses predicted for DvRelish after *in silico* trypsin analysis. The predicted DvRelish peptide masses corresponded with 18 identified peptide masses and with 9 peptide masses identified within the 70 kDa protein. Additionally, the 70 kDa

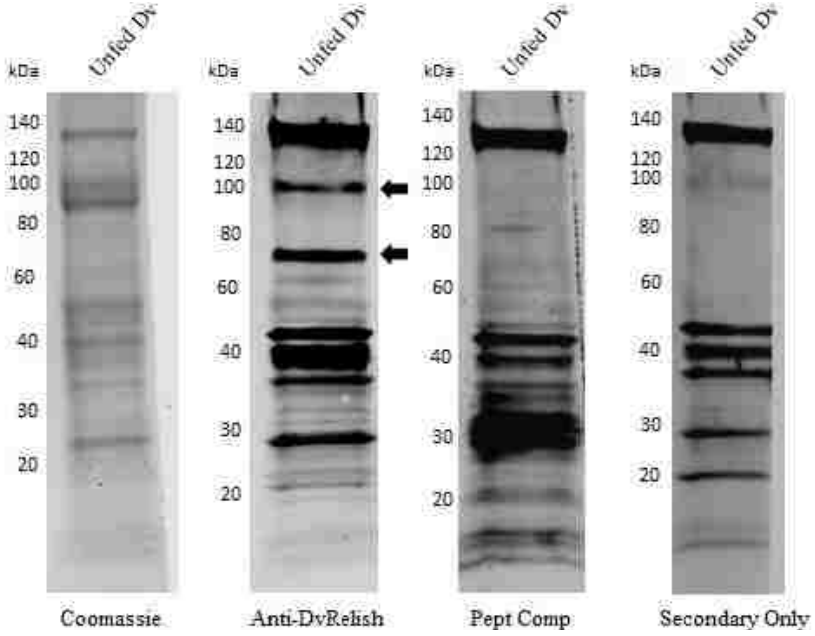

Figure 3.7 Expression of DvRelish in *D. variabilis* tissues. Expression of DvRelish in 25 µg of protein from unfed adult female *D. variabilis* tissue lysate. DvRelish was recognized via Western blot with anti-DvRelish antibody. A peptide competition assay (Pept Comp) decreased recognition of proteins at 100 kDa and 70 kDa.

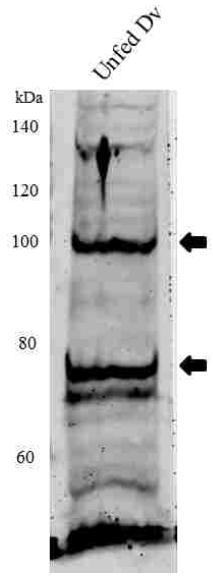

Figure 3.8 Visualization of 100 kDa and 70 kDa protein bands in *D. variabilis* tick tissues. Arrows indicate protein bands visualized by Western blot, excised from an identical 6% Tris-glycine SDS-PAGE gel and submitted for mass-spectrometry.

protein masses identified by mass-spectrometry corresponded with only 3 predicted masses with a translated partial putative *dvdorsal* transcript corresponding to a Dorsal type NF-κB protein previously isolated. Together, these data confirm the recognition of DvRelish via anti-DvRelish antibody at 100 kDa and the N-terminal DvRelish at 70 kDa.

3.3.5 Immunofluorescence assay of *D. variabilis* hemocytes

As hemocytes are an important site of AMP production, the hemocytes of unfed *D. variabilis* females were collected and spotted onto slides for IFA to detect DvRelish expression and nuclear localization in the presence of *E. coli*-PGN or *P. aeruginosa*. Hemocytes from *E. coli*-PGN injected ticks were stained and visualized 3 and 6 hpi (Figure 3.9). DvRelish was present within the cytoplasm of the hemocytes of sham-infected ticks. At 3 and 6 hpi with *E. coli*-PGN, DvRelish was also present in the cytoplasm. Additionally, nuclear DvRelish staining occurred at 6 hpi. In *P. aeruginosa*-injected hemocytes as compared to control hemocytes at 1 hpi

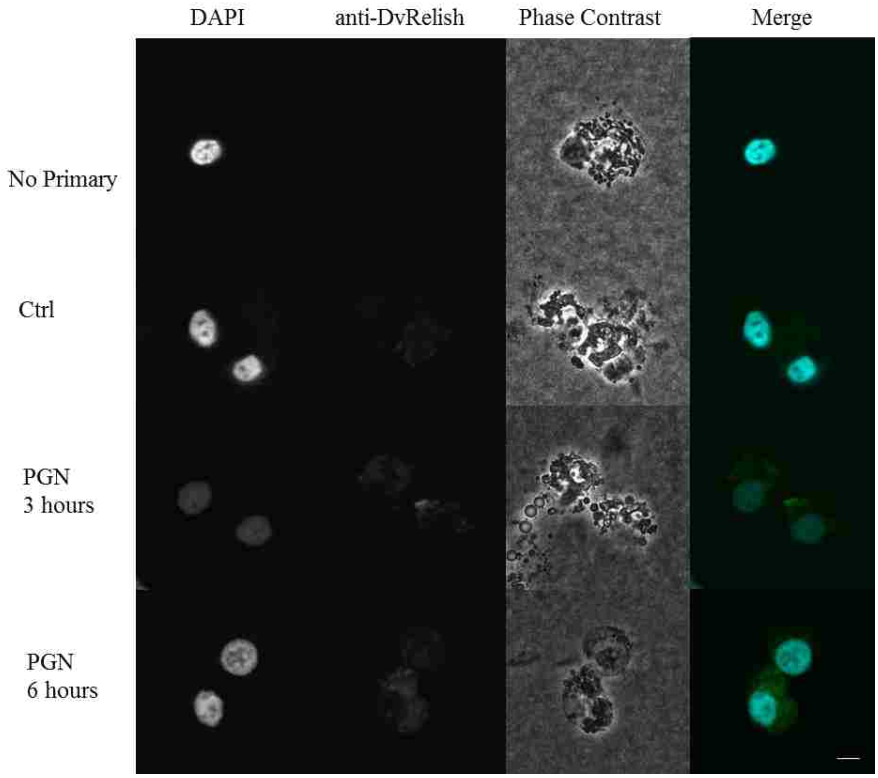

Figure 3.9 Expression of DvRelish in *D. variabilis* hemocytes exposed to *E.coli*-derived PGN. Nuclei were stained with DAPI, DvRelish was visualized with anti-Rabbit-FITC secondary antibody. In the bottom panel, PGN injection resulted in increased DvRelish staining in the cytoplasm of hemocytes after 6 hpi, as compared to both hemocytes at 3 hpi, and basal DvRelish staining in control sham injected tick hemocytes. Bar represents 5 µm.

there was increased DvRelish staining in both the nucleus and cytoplasm (Figure 3.10). Of note, there was also staining of DvRelish within the nucleus and cytoplasm of the control tick hemocytes.

3.4 Discussion

The expression of Relish-type NF-κB transcription factors is a key mediator in the differential immune effector responses in many arthropods, including *D. melanogaster* (Hetru and Hoffmann 2009). While NF-κB proteins are highly conserved across arthropods, the patterns of induction of effector responses has been best characterized in model insects infected with

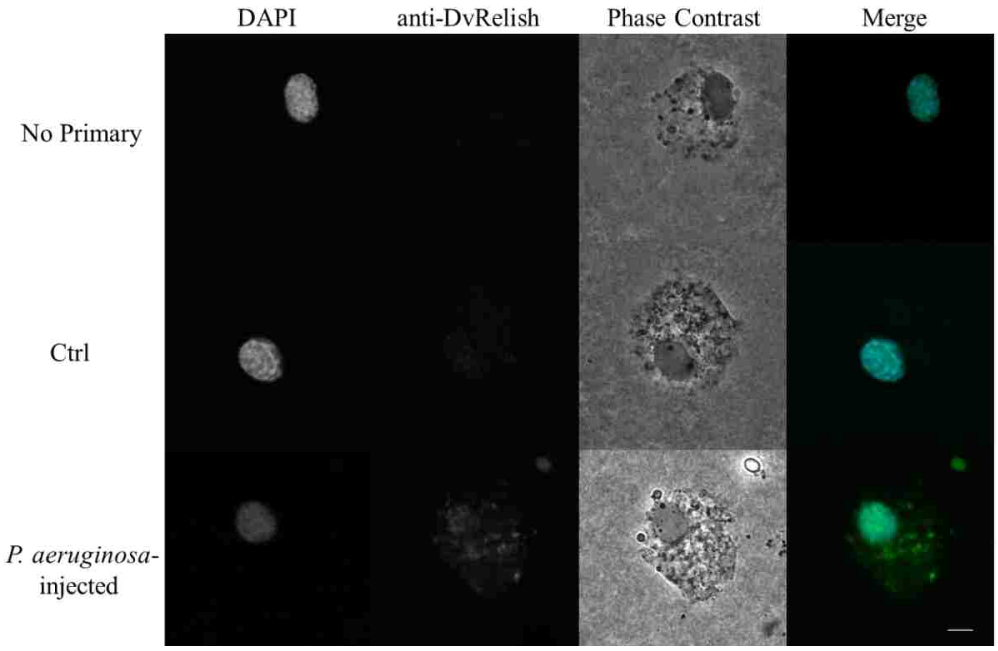

Figure 3.10 Expression of DvRelish in *D. variabilis* hemocytes exposed to *P. aeruginosa*. Nuclei were stained with DAPI, DvRelish was visualized with anti-Rabbit-FITC secondary antibody. Increased cytoplasmic and nuclear anti-DvRelish staining was identified in hemocytes from *P. aeruginosa*-injected *D. variabilis* after 1 hour of exposure (bottom row), as compared to sham inoculated control (Ctrl) hemocytes (middle row). Hemocytes without primary antibody (top row) were visualized to identify and tick cell autofluorescence. Bar represents 5 µm.

atypical bacteria not associated with the specific arthropod (Lemaitre and Hoffmann 2007). Such studies identified that NF-κB proteins are sequestered in the cytoplasm of eukaryotic cells until activated via removal of inhibitory subunits or domains, allowing nuclear translocation and induction of transcription of NF-κB responsive genes (Reichhart et al. 1993, Dushay et al. 1996). Previously identified patterns of NF-κB induction are most likely not applicable to bacteria that are typically associated with arthropods, such as SFG *Rickettsia* and hard ticks.

The immune response to Gram-negative pathogens in many arthropods is controlled through Relish-type NF-κB proteins. Homologous NF-κB proteins have been identified in arthropod vectors of disease. Relish-type NF-κB proteins have been identified in the mosquitoes *Aedes aegypti* (Shin et al. 2003) and *Anopheles gambiae* (Meister et al. 2005); the tsetse fly

Glossina morsitans (Hu and Aksoy 2006); and the hard tick *I. scapularis* (Naranjo et al. 2013). Seminal research describing the response of vectors to typical pathogen pairings has focused on viral and protozoan infections in mosquitoes, as well as protozoan infections in tsetse flies (Hu and Aksoy 2006, Xi et al. 2008, Garver et al. 2012). Infection of *I. scapularis* cells with *Anaplasma phagocytophilum*, a Gram-negative Rickettsiales, was determined to increase binding of Relish proteins to a regulator Subolesin (Naranjo et al. 2013). Studies of NF-κB proteins in vectors described mechanisms of immune defense in mosquitoes, tsetse flies, and prostriate ticks, the mechanisms of immune signaling in *Dermacentor* ticks has been largely unexplored. Mechanisms identified in the prostriate *I. scapularis* may not be applicable to gaining insight to the ecology of *Dermacentor* ticks and their typical SFG *Rickettsia*. This emphasizes the necessity for determining the mechanisms of immune signaling in response to typical Gram-negative bacteria in *D. variabilis.* The studies described in this chapter characterized the expression of the Relish-type NF-κB protein, DvRelish, in *D. variabilis*. To this end DvRelish expression was characterized through the 1) SF9 baculovirus expression of rDvRelish; 2) mass-spectrometry of protein bands identified with an anti-DvRelish antibody; and, 3) visualization of DvRelish expression *D. variabilis* hemocytes.

Arthropod expression systems are an important tool utilized in non-model arthropods in an effort to determine the function of homologous protein expression. Better characterized arthropod model culture systems can be used for protein expression and isolation, or the determination of putative protein functions in cells. An SF9 baculovirus protein expression system was utilized in the present studies for the expression of rDvRelish protein via propagation of recombinant baculovirus that contain full-length DvRelish and truncated N-terminal DvRelish encoding constructs. After 2 passages of recombinant virus, this system allowed for the

identification of the size of the rDvRelish protein and confirmation of anti-DvRelish antibody recognition (Figure 3.1). Both anti-His and anti-DvRelish antibodies recognized protein bands at 100 kDa which were increased in expression as compared to untouched control SF9 cells. In addition to rDvRelish expression, the expression of a shortened N-terminal DvRelish was also attempted. The N-terminal DvRelish construct did not encode the ankyrin repeats which have been identified as inhibiting the translocation of Relish protein into to the nucleus in *D. melanogaster* (Stöven et al. 2000). However, over five passages of virus no recombinant N-terminal DvRelish protein was identified by Western blot with anti-His or anti-DvRelish antibodies. Additionally, after 4 passages of virus, the 100 kDa rDvRelish began to be cleaved, resulting in the recognition of a smaller 75 kDa product. With a subsequent passage multiple cleaved products were recognized via anti-His and anti-DvRelish Western blot. The lack of expression by the N-terminal rDvRelish construct may have been due to unforeseen technical issues and requires further optimization of infection regiment for stable expression. As Relish proteins are endoproteolytically cleaved during signal transduction (Stoven et al. 2003), the cleavage of rDvRelish after 4 passages of may be occurring as the result of cleavage by a currently unknown protease. As neither recombinant protein was expressed at a high level, alternative methods were explored. The expression of full-length and shorter constructs of DvRelish would allow for the determination of binding of the protein to canonical κB promoter elements as identification of novel DvRelish binding promoters through chromatin immunoprecipitation reactions coupled with sequencing of captured promoter DNA. These assays would give a better understanding of the genes induced by DvRelish activation during SFG *Rickettsia* infection.

An alternative method of protein expression utilized in this study was the transient expression of DvRelish in S2 cells. Neither optimization of time of induction or harvest of transfected cell resulted in 100 kDa rDvRelish. Expressed proteins were identified by Western blot with primary antibodies specific for N-terminal Myc and C-terminal His. The anti-Myc Western blot revealed expression of a 35 kDa protein at all time points of induction and harvest days post induction, as well as expression of a His-tagged C-terminal 70 kDa product at later time points. While the expression plasmids were sequenced prior to transfections, aberrant cleavage of the recombinant protein may be occurring as was demonstrated during SF9 cell expression. Interestingly, expression of a recombinant Relish-type NF-κB protein from the horseshoe crab, *Carcinoscorpius rotundicauda*, was determined to be insufficient such that mammalian expression systems were substituted (Fan et al. 2008). As the authors suggested, while both *C. rotundicauda* and *D. melanogaster* are arthropods, this does not assure high levels of recombinant protein expression.

Following anti-DvRelish identification of rDvRelish, DvRelish expression was determined in uninfected, unfed adult female *D. variabilis*. Two protein bands of interest at 100 kDa and 70 kDa were specifically recognized and both were analyzed by mass-spectrometry. The identification of both the full-length DvRelish protein, as well as the cleaved N-terminal DvRelish demonstrated basal levels of expression of DvRelish in tick tissues. Relish-type NF-κB proteins are known to be expressed at basal levels in model arthropods. After immune activation, both mRNA transcription and protein translation are increased (Meister et al. 2005, Fan et al. 2008, Tanji et al. 2010). Ticks are also known to increase mRNA expression of immune responsive genes in response to infection with typical SFG *Rickettsia* (Macaluso et al. 2003, Mulenga et al. 2003, Sunyakumthorn et al. 2012) suggesting the presence of immune responsive

transcription factors. The present study identified basal DvRelish expression through the Western blot analysis of whole tick protein lysate.

As the hemocytes of *Dermacentor* ticks are recognized as an important site of AMP production (Johns et al. 1998, Simser et al. 2004, Hynes et al. 2008), the expression of DvRelish in hemocytes was investigated in ticks injected with *E. coli*-PGN or ticks injected with the atypical bacterium *P. aeruginosa*. After 6 hours of *E. coli*-PGN stimulation, DvRelish staining in both the nucleus and cytoplasm of the hemocytes, with increased expression at all time points in comparison to controls was observed. The expression of Relish-type NF-κB proteins has been previously visualized in cultured *Drosophila* cells indicating nuclear translocation in as little as 10 minutes post-stimulation with E. coli-PGN (Stoven et al. 2003). Interestingly, after injection with *P. aeruginosa*, we observed increased DvRelish staining after 1 hour of incubation in both the nucleus and cytoplasm of hemocytes as compared to controls. Purified PGN of Gram-positive and Gram-negative cells has been shown to elicit AMP production via NF-κB signaling (Hedengren-Olcott et al. 2004); however, the low level of nuclear translocation of DvRelish in *D. variabilis* hemocytes may be due to differences in the dose of PGN utilized or inoculation directly to the arthropod versus in culture conditions. There may be a differential patterns of induction of NF-κB proteins in ticks as compared to other arthropods. These difference may affect the rate of immune stimulation and account for the identified differences in temporal DvRelish nuclear translocation.

This study reports the first identification of Relish-type NF-κB proteins in *D. variabilis* tissues. This was demonstrated by Western blot and mass-spectrometry. Identification of basal DvRelish expression supported the detection of DvRelish expression and activation via hemocyte IFA. Together, these assays will allow for a more complete understanding of the role

of DvRelish expression in during SFG *Rickettsia* infection of *D. variabilis*. Quantitative transcriptional assays in conjunction with protein expression will allow for the identification of potential mechanisms underlying the differences in immune response. Comparison of expression during typical and atypical SFG *Rickettsia* infection, and expression during constitutive infection may give insight into the differential immune signaling mechanisms determining the clearance or establishment of infection in hard ticks.

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CHAPTER 4
EXPRESSION OF DVRELISH, A PUTATIVE RELISH-TYPE NF-KB PROTEIN IN *DERMACENTOR VARIABILIS* IN RESPONSE TO SFG *RICKETTSIA* INFECTION

4.1 Introduction

Hard ticks have been shown to respond differentially to pathogens and endosymbionts (Sonenshine and Hynes 2008). As vectors of spotted fever group (SFG) *Rickettsia*, the differential immune response to host-specific bacteria likely plays a role in the establishment and dissemination of these bacteria in ticks. Specific *Rickettsia*-tick pairings predominate in nature; however, the underlying mechanisms controlling the immune response of hard ticks to SFG *Rickettsia*, and their potential in dictating the specificity of relationships, have not been fully characterized.

The tick response to certain bacterial infections has been characterized, describing the swift AMP production and hemocyte proliferation. For example, in response to infection with the *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, *Dermacentor variabilis* hemocytes populations increased 6.4 times that of uninfected controls, with a peak hemocyte proliferation at 48 hours (Johns et al. 1998). Moreover, in response to an infection with *Borrelia burgdorferi*, *D. variabilis* hemocytes increased the same amount but peaked earlier by 24 hours. In both infection assays, the ticks are able to effectively clear the bacteria, but with differential patterns of immune activation.

Immune responsive genes have been previously identified in *D. variabilis*, demonstrating differential mRNA expression in response bacterial and rickettsial challenge (Macaluso et al. 2003, Mulenga et al. 2003, Jaworski et al. 2010). mRNA expression of immune responsive genes of *D. variabilis* when challenged with *R. montanensis* as compared to *R. amblyommii* have been identified to have tissue-specific expression profiles (Sunyakumthorn et al. 2013). While the mechanisms controlling generalized immune responses in arthropods have been determined

(Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009), the immune response elicited to atypical bacterial infections are most likely not comparable to those elicited against typical bacteria, such as the response of hard ticks to the SFG *Rickettsia* they predominantly harbor. For example, tick cell infection of endosymbiont *R. peacockii* did not upregulate the expression of a lysozyme-like protein, whereas infection with the non-associated bacteria *E. coli* and *Micrococcus luteus* resulted in increased lysozyme expression (Mattila et al. 2007), Conversely, the pathogen *R. rickettsii* is known to affect *Dermacentor* ticks adversely through reduced feeding success, fecundity, and molting success (Niebylski et al. 1999, Schumacher et al. 2016). While the lack of response to *R. peacockii* suggests mechanisms of immune avoidance or senescence, the response of the tick to *R. rickettsii* may be more complex.

Comparing the tick immune response affecting the establishment of SFG *Rickettsia* infection and the response to endosymbiotic *Rickettsia* is necessary to fully understand the ecology of SFG *Rickettsia* in tick vectors. The mechanisms controlling effector response induction are uncharacterized in *D. variabilis,* and the previous chapters of this dissertation have described the identification of a Relish-type NF-κB protein in *D. variabilis*, DvRelish. With similarity to Relish NF-κB transcription factors determined to be crucial to controlling the response to Gram-negative bacterial infections to atypical bacterial infections of arthropods, the expression and activation of DvRelish during hard tick infection with associated SFG *Rickettsia* may give insight into the mechanisms determining tick-*Rickettsia* specificity. In an effort to identify DvRelish expression and activation in *D. variabilis*, the temporal expression of *dvrelish* was characterized in three infection bioassays. The hypothesis tested is if NF-κB induction plays a role in host-specificity and vector competence, then *R. rickettsii* would elicit a greater *dvrelish* response as compared to *R. parkeri*, which is a less pathogenic infection of *D. variabilis*. These

experiments were designed to determine temporal and tissue specific expression of *dvrelish* during SFG *Rickettsia* infection utilizing direct microinjection and natural oral infection (capillary feeding) techniques. Microinjection of SFG *Rickettsia* into the hemocoel of ticks facilitated the direct assessment of expression of *dvrelish* in the hemocytes, which was identified in the previous chapter. Alternately, infection via capillary feeding technique allowed for the identification of *dvrelish* expression in tick tissues in response to a natural route of exposure.

4.2 Methods and Materials

4.2.1 *D. variabilis* colony maintenance and rickettsial exposure

The LSU *D. variabilis* colony was maintained as previously described (Macaluso et al. 2001). Life stages were regularly maintained on laboratory animals at the Louisiana State University School of Veterinary Medicine with all procedures approved by the Institutional Animal Care and Use Committee. Tick life stages were maintained on the following animals: larvae on mice; nymphs on rats, guinea pigs, or rabbits; and adults on rats or guinea pigs. Between feedings, all ticks were surface sterilized with 70% ethanol followed by distilled water before storage in the environmental chamber. All ticks were housed in a 27°C environmental chamber with greater than 90% relative humidity.

4.2.2 Bacteria propagation and purification

Rickettsia rickettsii (str. Sheila Smith) and *Rickettsia parkeri* (str. Portsmouth) were propagated in Vero E6 African green monkey cells. Cells were grown by tissue culture in Dulbecco's modified medium (DMEM) (Invitrogen) supplemented with 5% FBS (Hyclone) in a humidified 34°C incubator with 5% CO_2. *Rickettsia* infection was monitored via cytospin (Wescor) followed by Diff-Quik staining (Seimens) and highly infected cultures were used for

infection assays. All *R. rickettsii* propagation, purification, and infection bioassays were performed in a BSL-3 high-containment laboratory.

For capillary feeding, infected cells were scraped from the flask and 1 ml of *Rickettsia* culture was lysed with 10 passes of a 27 gauge needle followed by low speed centrifugation at 4°C for 10 minutes at 275 x g. The supernatant-containing *Rickettsia* was then passed through a 2 µM pore filter (Whatman) to remove host cell debris. High speed centrifugation at 4°C for 10 minutes at 16,000 x g pelleted the *Rickettsia*. For enumeration and determination of viability, *Rickettsia* were stained using the Baclight viability staining kit (Invitrogen) and counted using a Petroff-Hausser bacterial counting chamber under a Leica fluorescent microscope. For capillary feeding, $2.5x10^8$ *Rickettsia*/ml in whole *Rickettsia*-infected Vero cells was diluted in 0.1% (W/V) Rhodamine B (RhoB) in 0.85% salt solution. In the case of microinjection technique, purified *Rickettsia* was resuspended into PBS at the prescribed dosages. Overnight cultures of *P. aeruginosa* (ATCC 25873) were grown in a 37°C shaking incubator in trypic soy broth (Invitrogen) and assessed for viability and enumerated using the Baclight viability staining kit. Bacteria were pelleted via centrifugation at 4°C for 10 minutes at 16,000 x g and resuspended in sterile PBS as the prescribed dosages.

4.2.3 Microinjection technique

For microinjection of ticks, bacteria were isolated and diluted to 10^5 and 10^7 bacteria/µl of sterile PBS. Ticks were surface sterilized and immobilized as described for capillary feeding. Each tick was injected with 1 µl of bacterial suspension using a 33 gauge Hamilton needle and 5 µl glass syringe into the coxae of the third left leg (Figure 4.5). Ticks were incubated in a 27°C humidified environmental chamber until time of dissection. In the microinjection assays, *D.*

variabilis were incubated for 1 hour, or up to 24 hours before collection of hemolymph via microdissection using

a dissecting microscope. Ticks were dissected in 30 µl of PBS with a scalpel blade without rupturing the tick organs and the hemolymph was placed into 300 µl of Trizol for RNA isolation. Ticks were incubated in a 27°C humidified environmental chamber until time of dissection. Hemolymph, salivary glands, ovaries, and gut were collected and divided for RNA isolation in 300 µl Trizol or 50 µl RIPA buffer for protein lysis. All samples were stored at -80°C until processed.

4.2.4 Capillary feeding technique

Adult *D. variabilis* were fed on a guinea pig for 4 days before being forcibly removed with curved forceps. Ticks were surfaced sterilized with 5 minute incubations of 0.1 bleach, 70% ethanol (3 times), and distilled water before immobilization dorsal-side down. Capillary feeding was performed as previously described (Macaluso et al. 2001). Briefly, $2.5x10^8$ *R. rickettsii*/ ml

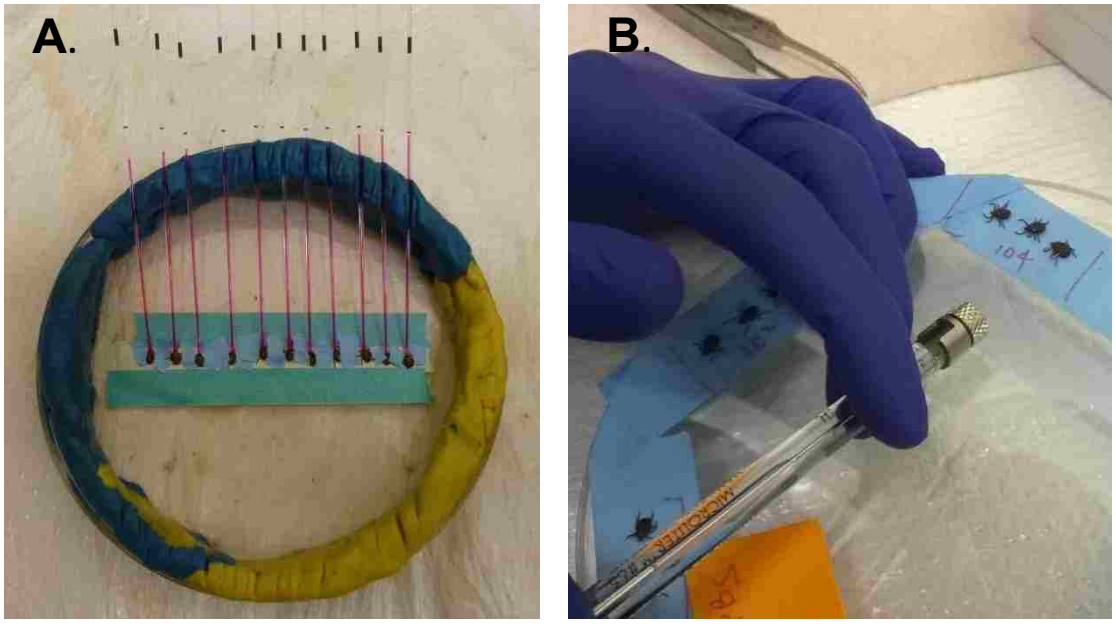


Figure 4.1 Bacterial exposure techniques utilized in *dvrelish* expression assays. (A) Capillary feeding technique. (B) Microinjection technique.

of RhoB solution was fed to immobilized *D. variabilis* for 1 or 3 hours by placing a filled 50 µl capillary tube (Kimble) over the mouthparts of the tick (Figure 4.1). Post capillary feeding, ticks were surface sterilized and feeding was confirmed with the visualization of the feeding biomarker RhoB via fluorescent microscopy with a MVX10 research macro zoom system microscope (Olympus) (Mascari and Foil 2009). After 1 hour of *Rickettsia* exposure via capillary feeding, ticks were collected at 0, 1, 3, 5, and 10 hours post capillary removal. After 3 hours of exposure (hpe), ticks were collected at 1, 3, 6, and 12 hpe. All ticks were surface sterilized with 5 minute incubations of 0.1% bleach, 70% ethanol (3 times), and distilled water before feeding assessment. Ticks were then dissected and salivary glands, ovaries, gut, and hemolymph was collected into 200 µl of Trizol (Invitrogen), homogenized and stored at -80°C until processed.

4.2.5 Tick microdissection

Tick were dissected using a depression slide, fine forceps, and scalpel blades. The instruments were sterilized with 70% ethanol between tick dissections and separate, sterile instruments were used per experimental group. Ticks were dissected in sterile PBS dorsal side down with a dissecting microscope. Micro-cuts were made at the outermost cuticle with special attention paid to cut without rupturing the gut. After cutting around the entire tick, the ventral cuticle was removed and the hemolymph collected. Organs were then transferred to sterile PBS for separation and identification of tissues for processing.

4.2.6 RNA isolation and cDNA synthesis

RNA was isolated using Trizol (Invitrogen). Tissue samples were homogenized with a TissueLyzer (Qiagen) and 3-mm borosilicate glass beads (Sigma) in a 1.7 ml microcentrifuge tube for 4 minutes at 25hz/sec. RNA extractions were performed as per manufacturer's instructions. Briefly, samples were incubated at room temperature for 5 minutes before adding

0.2 ml of chloroform (Sigma) per ml of Trizol. After shaking for 30 seconds by hand and incubating for 2 minutes at room temperature, samples were centrifuged at 4°C for 15 minutes at 12,000 x g. The colorless aqueous phase was transferred to a new tube for RNA precipitation, paying special attention not to disturb the phenol-chloroform phase. RNA was precipitated with 0.5 ml of isopropanol per ml of Trizol, followed by gentle inversion and centrifugation at 4°C for 10 minutes at 12,000 x g. Precipitated RNA was subsequently washed once with 1 ml of 75% ethanol per ml of Trizol and centrifuged at 4°C for 10 minutes at 8,000 x g. The RNA pellet was air-dried for 5 minutes before resuspending in 20 µl of PCR-grade water. Following quantification of RNA via Nanodrop spectrophotometer (Thermo), 2 units of TurboDNase (Ambion) was added and incubated in a 37°C water bath for 30 minutes. RNA was purified and concentrated using the Clean and Concentrator-5 kit (Zymo) and eluted in 15 µl of molecular grade water. RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-rad). No reverse transcriptase reactions were performed for determination of residual DNA by qPCR.

4.2.7 qPCR analysis

A qPCR assay was performed on a LightCycler (Roche) with 10 µl reactions plated in triplicate. Specifically, 2 µl of cDNA, 17.5 µl of iTaq Universal Probes master mix (BioRad), 0.2 µM final concentration of each primer and 0.3 µM final concentration of probe were combined in a 96 well plate and aliquoted in triplicate. The PCR cycling parameters were 95°C for 5 min, 45 cycles of 95°C for 15 sec, 60°C for 60 sec. Primer and probe sets are listed in Table 4.1 and were tested and sequenced for specificity prior to use. Universal Probe Library (Roche) probes were identified for *dvrelish* and *dvactin* using the Universal Probe Library assay design center (Roche). To determine PCR efficiency, standard curves consisting of each amplicon in pCR4-TOPO were diluted with concentrations ranging from 10^8 to 1 copy of linearized plasmid

Table 4.1 Primers and probes for qPCR.

Primer	Sequence 5'-3'	Reference
DvRelish		
DvRHD332F	AATGGCTTTGCCCACAA	This study
DvRHD405Rev	GGAACACTTGGAAGCAGAGG	This study
UPL 71		Roche
DvActin		
DvActin-1424For	CTTTGTTTTCCCGAGCAGAG	(Sunyakumthorn et al. 2012)
DvActin-1572Rev	CCAGGGCAGTAGAAGACGAG	(Sunyakumthorn et al. 2012)
UPL 87		Roche

per 10 µl reaction. Expression of *dvrelish* was determined using the second derivative max calculation methodology and normalized with *dvactin* expression. Expression was normalized using the efficiency modified ΔΔCt method (Roche), which takes into account the efficiency of each PCR reaction. Fold changes of expression were determined in relation to the normalized *dvrelish* expression in control ticks at the first time point of each experiment.

4.2.8 Protein isolation and Western blot analysis

Protein samples in RIPA buffer with Complete mini EDTA-free protease inhibitor (Roche) were homogenized with a TissueLyzer (Qiagen) and 3-mm borosilicate glass beads (Sigma) in a 1.7ml microcentrifuge tube for 4 minutes at 25hz/sec. Lysates were centrifuged at 4°C for 15 minutes at 12,000 x g to remove any insoluble materials. For Western blotting, 25 µg of protein per sample were separating using Mini-Protean Tris/Glycine 4-15% precast protein gels (Bio-Rad), as described in Chapter 3 section 3.2.4. Separated protein was transferred to 0.45 µm pore nitrocellulose (Bio-Rad) using a Trans-Blot SD semi-dry transfer machine (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) in tris-buffered saline with 0.05% Tween-20 (TBST) (Sigma). Membranes were probed with 1:100 anti-DvRelish followed by secondary donkey anti-rabbit 800CW 1:20,000 antibody (Li-Cor).

DvRelish expression was normalized to DvActin determined with primary 1:2,000 mouse anti-Actin followed by secondary 1:20,000 anti-mouse 680CW antibody. Western blots were imaged using a Li-cor Odyssey machine (Li-Cor) and protein band intensities were determined with Image Studio (v4.0) software (Li-Cor).

4.2.9 Experimental design

Three tick infection experiments were designed to determine the effect of SFG *Rickettsia* infection on *dvrelish* expression during the exposure of *D. variabilis* to SFG *Rickettsia* by microinjection and capillary feeding. An overall experimental design is outlined in Figure 4.1.

4.2.9a Microinjection technique assays: hemocyte and combined tissues *dvrelish* expression experimental design

Two microinjection technique assays were designed to determine the response of *D. variabilis* to infection with SFG *Rickettsia*: 1) expression of *dvrelish* in hemocytes after 1

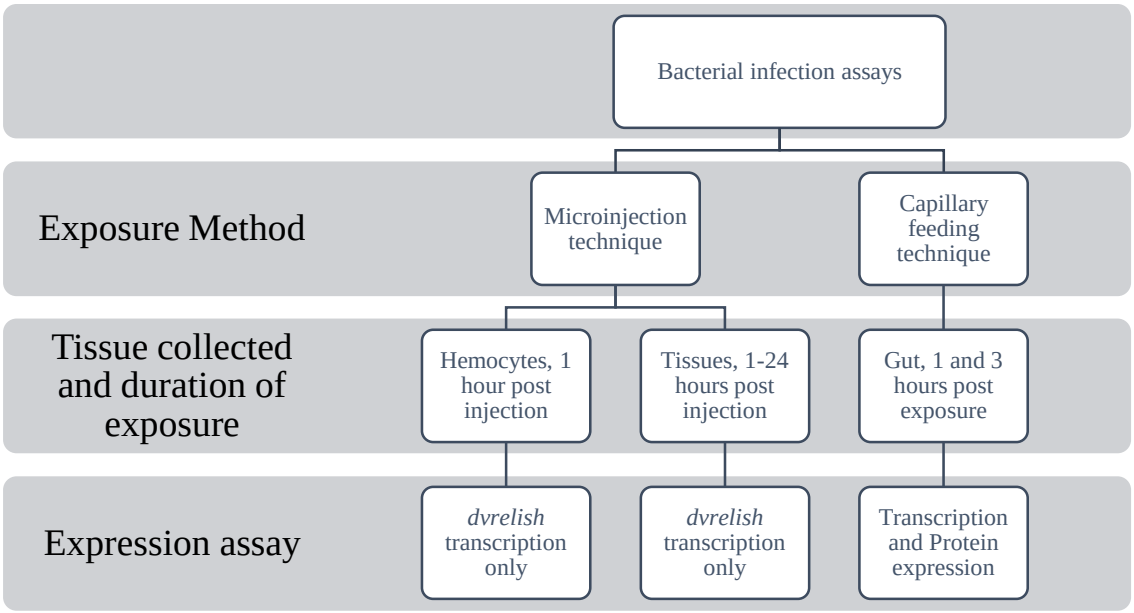
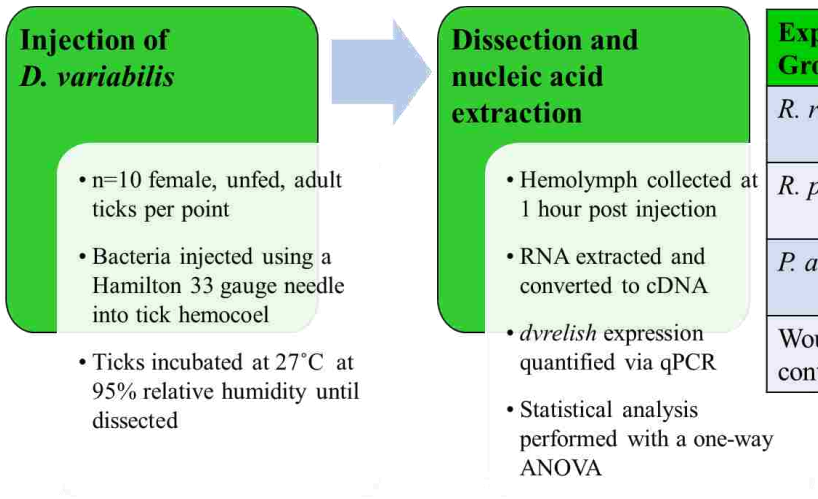


Figure 4.2 Experimental design outlining the three infection assays performed.

hour post-injection (hpi), and 2) expression of in combined tick tissues after injection of SFG *Rickettsia* for 24 hpi. The experimental design for determining the expression of *dvrelish* in hemocytes after one hour of infection is outlined in Figure 4.3.

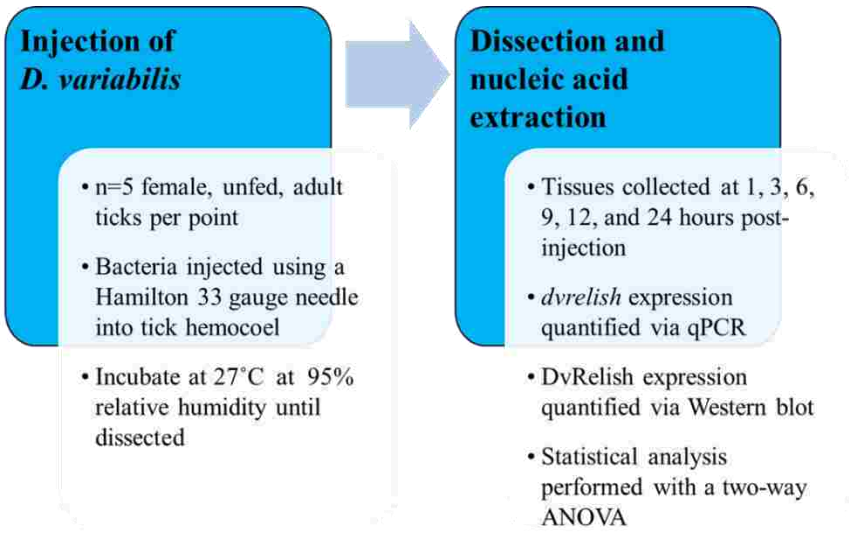
Briefly, the ticks were surface sterilized, immobilized, and injected with 10^5 or 10^7 *P. aeruginosa R. parkeri* or *R. rickettsii*, or sham inoculated with PBS. Each experimental group consisted of 10 *D. variabilis* unfed females. Ticks were dissected 1 hpi and hemolymph was collected separately for each tick into 300 µl of Trizol regent (Invitrogen). RNA was extracted, DNase treated, and reverse transcribed. Expression of *dvrelish* and *dvactin*, was determined via probe-based quantitative real-time reverse transcriptase-PCR (qPCR). Expression of *dvrelish* was normalized with *dvactin* and expressed as relative to the sham inoculated experimental group. Significant changes in *dvrelish* expression were determined using a one-way ANOVA with Tukey's post hoc test with p-value less than 0.05 considered significant.

The experimental design comparing expression in combined tick tissues up to 24 hpi is outlined in Figure 4.4. Briefly, the ticks were surface sterilized, immobilized, and sham



Experimental Groups	Dose
R. rickettsii	10^5 or 10^7 in PBS
R. parkeri	10^5 or 10^7 in PBS
P. aeruginosa	10^5 or 10^7 in PBS
Wounding control	PBS alone

Figure 4.3 Bioassay 1: microinjection and hemolymph stimulation assay.



Experimental Groups	Dosage
R. rickettsii	10^7 in PBS
R. parkeri	10^7 in PBS
E.coli- derived PGN	200 ng
Sham	PBS alone

Figure 4.4 Bioassay 2: experimental design of the microinjection whole tick bioassay of *D. variabilis* with SFG *Rickettsia* with dissection at selected intervals 24 hours post injection (hpi).

inoculated with PBS, with 200 ng *E.coli*-PGN, 10^7 *R. parkeri* or 10^7 *R. rickettsii*. Each experimental group consisted of 5 *D. variabilis* unfed females each at 1, 3, 6, 9, 12, and 24 hpi. Ticks were dissected and in sterile PBS and hemocytes with salivary glands, ovaries, and gut were combined per tick and divided for RNA extraction in 300 µl Trizol, or RIPA buffer for protein. RNA was extracted, DNase treated, and reverse transcribed. No reverse transcriptase (RT) reactions were also reverse transcribed with DNase treated RNA to determine residual DNA contamination. Expression of *dvrelish* was normalized with *dvactin* and expressed as the relative expression compared to sham inoculated experimental group. Significant *dvrelish* expression was determined using a two-way ANOVA with Tukey's post hoc test with p-value less than 0.05 considered significant. For protein analysis by Western blot as described in Chapter 3, DvRelish and N-terminal DvRelish expression was normalized to DvActin expression.

4.2.9b Capillary feeding technique assay: combined tissues *dvrelish* expression experimental design

An experimental design for the determination of *dvrelish* expression in adult, female *D. variabilis* after challenge with SFG *Rickettsia* via capillary feeding technique is outlined in Figure 4.5. Briefly, *D. variabilis* were allowed to feed on a Guinea pig for 4 days before being forcibly removed with curved forceps. Ticks were surface sterilized, immobilized, and capillary fed with *R. rickettsii*-infected Vero cells or uninfected Vero cells suspended in a solution containing the biomarker RhoB for feeding for 1 or 3 hours (Macaluso et al. 2001, Mascari and Foil 2009). Each time point consisted of 5 ticks per treatment group. Once the capillary tubes were removed, RhoB uptake from feeding was identified via florescent microscope. After determining feeding success, ticks were incubated in a 27°C environmental chamber until dissected. Ticks exposed for 1 hour were dissected at 0, 1, 3, 5, and 10 hours post capillary removal, and ticks exposed for 3 hours were dissected at 1, 3, 6, and 12 hours post capillary removal. The combined hemolymph, salivary glands, ovaries and gut were placed into 300 µl of Trizol for RNA extraction. Expression of *dvrelish* was determined with qPCR and

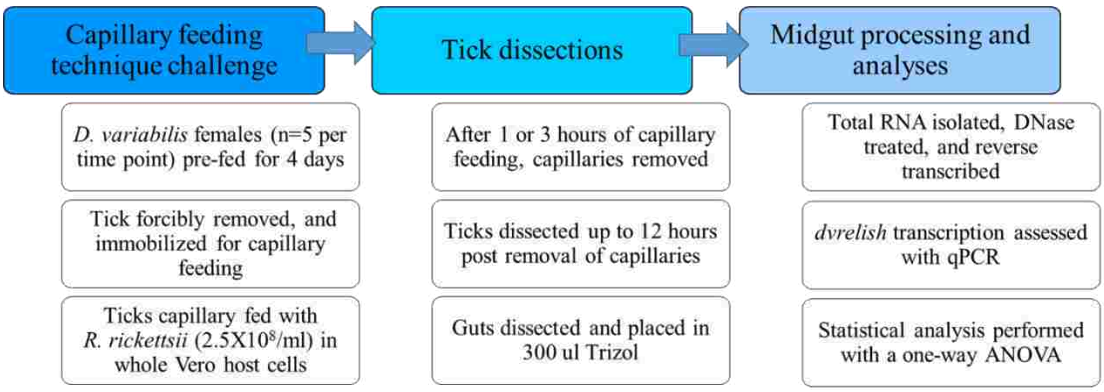


Figure 4.5 Bioassay 3: oral infection bioassay experimental design outlining assessment of *dvrelish* transcription subsequent to *R. rickettsii* infection via capillary feeding technique.

normalized to *dvactin*. Normalized *dvrelish* expression at all time points was compared to Vero cell-exposed ticks at the first time point collected. Significant expression was determined with a one-way ANOVA and Tukey's post hoc test with a p-value of less than 0.05 considered significant.

4.3 Results

4.3.1 Bioassay 1: response of *D. variabilis* hemocytes to microinjection of bacteria at two doses

Adult, unfed *D. variabilis* females were injected with 10^5 or 10^7 *R. parkeri*, *R. rickettsii*, and *P. aeruginosa* (Figure 4.6). Following exposure for 1 hour, ticks were dissected and hemolymph collected to determine expression of *dvrelish* immediately after infection in the hemocytes. 10^5 bacterial injection did not significantly increase *dvrelish* expression as compared to sham injection. There was also no significant difference between any bacteria-injected group at a dose of 10^5 injected bacteria. After injection with 10^7 bacteria, however, expression of

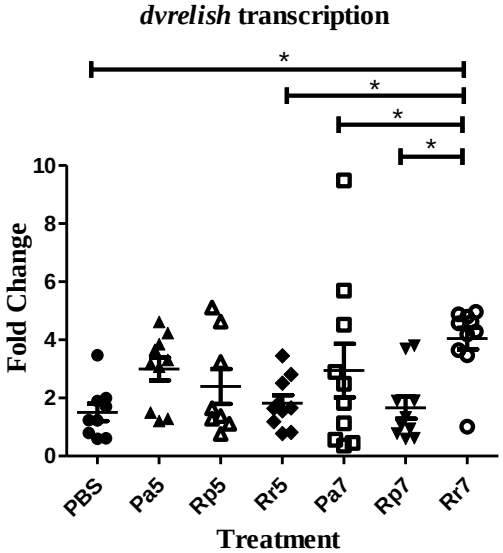


Figure 4.6 Bioassay 1: transcription of *dvrelish* in tick hemocytes after injection of *D. variabilis* with varying amounts of bacteria. *dvrelish* transcription was normalized to transcription of *dvactin* and fold change was determined by comparison to PBS treatment group. Significance was determined using a one-way ANOVA with Tukey's post hoc test with a p-value less than 0.05 considered significant. Error bars represent the standard error of the mean and asterisks denote significance as compared to 10^7 *R. rickettsii* injected group.

dvrelish increased significantly in *R. rickettsii*-injected ticks as compared to *P. aeruginosa* and *R. parkeri* at the same dose. Interestingly, *R. rickettsii* infection had a dose-dependent effect on *dvrelish*, with 4-fold increased expression with after injection with 10^7 bacteria as compared to 10^5 bacteria.

4.3.2 Bioassay 2: response of tissues to infection after microinjection of bacteria

Injection of 10^7 *SFG Rickettsia* into the hemocoel of unfed, *D. variabilis* female ticks were allowed to progress for up to 24 hours. Individual ticks were dissected and salivary glands, ovaries, gut, and hemolymph were divided and pooled for *dvrelish* expression and DvRelish protein expression analysis. *Rickettsia* infections were compared with two control groups, sham PBS injections and inoculations with 200 ng of *E. coli*-PGN. In *R. rickettsii*-injected ticks, expression of *dvrelish* increased 12-fold after 6 hours of incubation as compared to sham inoculated ticks 1 hpi (Figure 4.7). An 8-fold increase in expression in PGN-injected ticks was

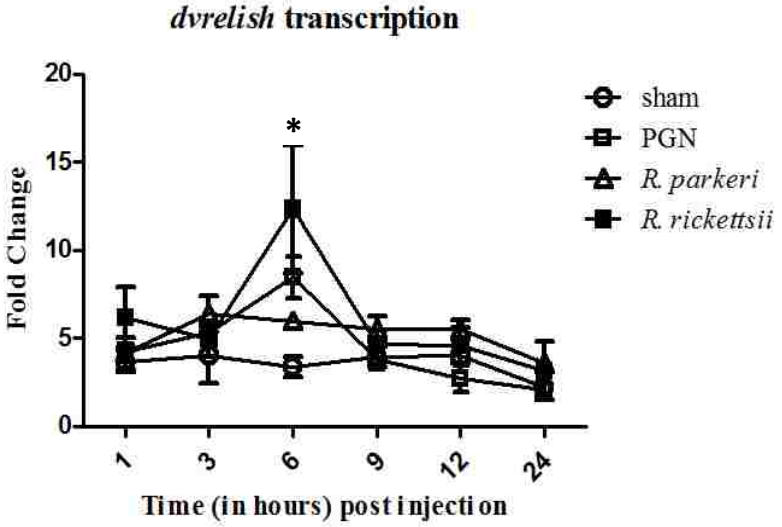


Figure 4.7 Bioassay 2: transcription of *dvrelish* over 24 hours in combined tick tissues (salivary glands, ovaries, gut, and hemolymph) after injection of *D. variabilis* with varying amounts of SFG *Rickettsia*. *dvrelish* transcription was normalized to transcription of *dvactin*. Fold change was determined by comparison to PBS treatment group. Significance was determined using a two-way ANOVA with Tukey's post hoc test with a p-value less than 0.05 considered significant. Error bars represent the standard error of the mean and asterisks denote significance.

observed, but this change was not statistically different from sham inoculated ticks. By 9 hpi, the expression of *dvrelish* returned to expression levels comparable to the sham inoculated ticks. As shown in Figure 4.8, expression of DvRelish protein was determined via Western blot and normalized with the expression of DvActin. The level of DvRelish did not increase significantly in at any time point in response to SFG *Rickettsia* infection, PGN, or sham inoculations. N-terminal DvRelish expression was also analyzed and protein expression did not increase significantly in response to *Rickettsia* infection, PGN or sham inoculation (Figure 4.8). However, expression of N-terminal DvRelish was not detectable in all ticks.

4.3.3 Bioassay 3: response of tick midguts to *R. rickettsii* challenge via capillary feeding

Adult female *D. variabilis* were pre-fed on Guinea pigs before being forcibly removed for capillary feeding with $2.5x10^8$/ml *R. rickettsii* in Vero cells. Ticks were allowed to feed for 1 or 3 hours and guts were dissected from 1 to 12 hours post capillary removal for identification of

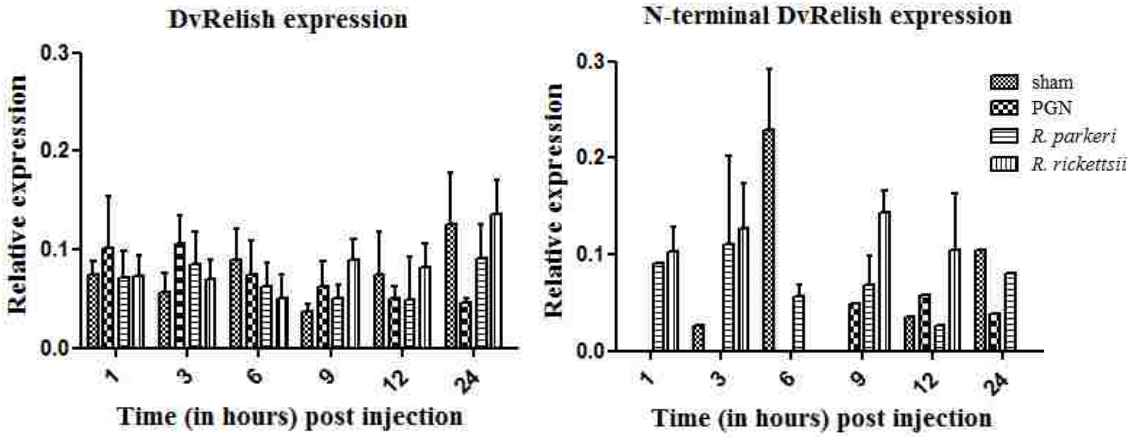


Figure 4.8 Bioassay 2: expression of DvRelish and N-terminal DvRelish over 24 hours in combined tick tissues (salivary glands, ovaries, gut, and hemolymph) after injection of *D. variabilis* with varying amounts of SFG *Rickettsia.* DvRelish was normalized to expression of DvActin. Fold change was determined by comparison to the PBS treatment group. Significance was determined using a two-way ANOVA with Tukey's post hoc test with a p-value less than 0.05 considered significant. Error bars represent the standard error of the mean and asterisks denote significance.

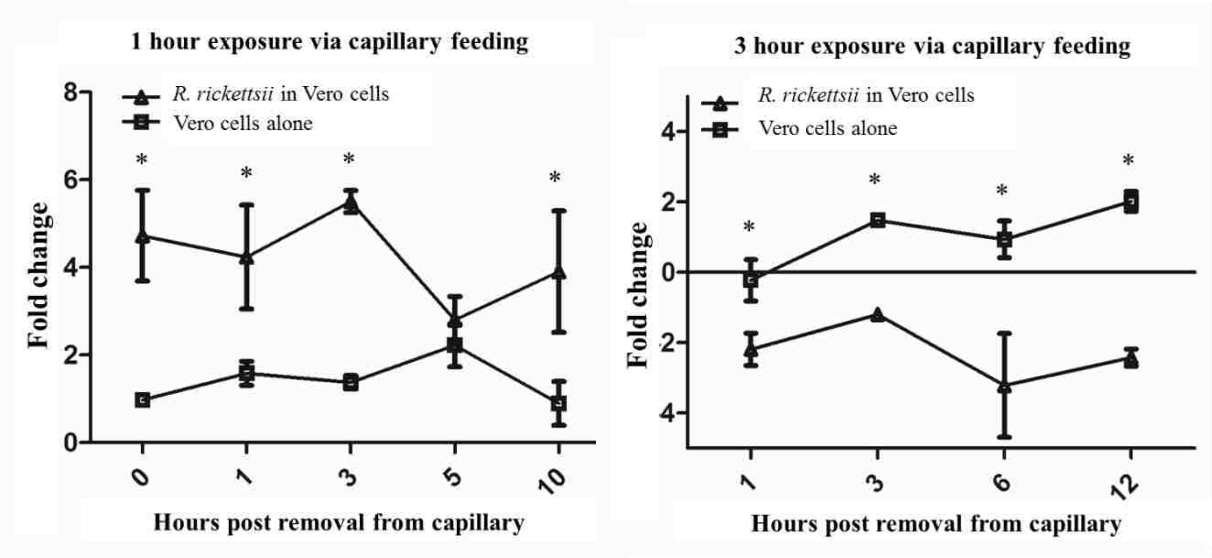


Figure 4.9 Expression of *dvrelish* after exposure to *R. rickettsii* for 1 or 3 hours via capillary feeding technique. *dvrelish* transcription was normalized to transcription of *dvactin*. Significance was determined with a one-way ANOVA and Tukey's post hoc test with a p-value less than 0.05 considered significant. Error bars represent the standard error of the mean and asterisks denote significance between groups at single time points. Fold change for 1 hour exposure was determined by comparison to Vero cell only at 0 hours post capillary removal, and fold change for 3 hour was determined by comparison to Vero cells alone at 1 hour post capillary removal.

dvrelish expression normalized with *dvactin* expression (Figure 4.9). After 1 hour of exposure, RhoB labeling of ticks could not be visualized. Whereas after 3 hours of exposure RhoB labeling could be visualized in all ticks. After exposure with *Rickettsia* for 1 hour, *dvrelish* expression was significantly increased at 0, 1, 3, and 10 hours post capillary removal as compared to ticks capillary fed whole Vero cells. Interestingly, after 3 hour exposure to *R. rickettsii*, *dvrelish* expression in the gut was significantly decreased at 1, 3, 6, and 12 hours post capillary removal as compared to Vero cell only controls.

4.4 Discussion

Infection assays comparing the temporal and tissue specific induction of *D. variabilis dvrelish* in response to SFG *Rickettsia* challenge were performed. Microinjections of specific doses of both vector-associated and non-associated SFG *Rickettsia* into the hemocoel of

D. variabilis identified an *R. rickettsii* dose-specific *dvrelish* response in hemocytes. Over 24 hours, the microinjection of SFG *Rickettsia* into *D. variabilis* elicited an increase in *dvrelish* expression in response to *R. rickettsii* infection. Transcription of *dvrelish* peaked at 6 hpi, but without a corresponding increase in DvRelish expression or activation. In a capillary feeding model, 1 hour exposure of *R. rickettsii* increased *dvrelish* expression, whereas a 3 hour exposure resulted in decreased expression.

As no one methodology captures all aspects of tick infection which may affect immune response induction, multiple methodologies are necessary to determine the spectrum of induced responses. Microinjection and capillary feeding were utilized in this study in order to examine *dvrelish* transcription under many conditions. With microinjection, a dose-dependent increase in *dvrelish* expression was identified. On the other hand, the capillary feeding technique revealed a dose-dependent response conversely related to the intensity of infection. The patterns of expression of DvRelish and molecular characterization of its activation need to be further characterized, as an aspect of vector competence. Therefore, understanding the immune response of hard ticks to their specific SFG *Rickettsia* will allow for a better appreciation of rickettsial epidemiology and its overall impact on the ecology of tick-borne rickettsioses.

A microinjection technique infection of *D. variabilis* with SFG *Rickettsia* enabled the determination of dose-dependent *dvrelish* transcription at 1 hpi. Hemocytes exposed to *R. rickettsii* were collected and *dvrelish* transcription identified. Hemocytes are recognized as vital in the tick response to infections, as evidenced by increased hemocyte proliferation, increased AMP production, and suggestions that the infection of tick hemocytes may facilitate pathogen dissemination in the tick vector (Liu et al. 2011). The characterization of the signaling events occurring in hemocytes during early immune response induction may give insight into the

establishment of pathogenic and non-pathogenic *Rickettsia*. Only after injection with 10^7 *R. rickettsii* was there a significant increase in *dvrelish* transcription in hemocytes. Pathogen infectious dose has been shown to affect the immune response of the host, and doses below the threshold required for disease development may result in lower than detectable immune responses (Ben-Ami et al. 2010, Leggett et al. 2012). However, changes in immune signaling may still be quantifiable. Dose-dependent *P. falciparum* infections in *A. gambiae* induce clear, differential global transcriptional patterns (Mendes et al. 2011). As a transcriptional response may not correlate with increases in protein activation and function, determining both NF-κB gene transcription, NF-κB transcription factor activation and nuclear localization will help to identify the potentially nuanced response of hard ticks to pathogenic, non-pathogenic, and endosymbiotic SFG *Rickettsia*.

A secondary microinjection infection bioassay was designed to examine temporal induction of *dvrelish* transcription over 24 hours. Infecting *D. variabilis* with a dose of 10^7 *R. rickettsii* induced a significant increase in *dvrelish* transcription at 6 hpi as compared to sham, PGN and *R. parkeri*-injected tick tissues. The increased transcription, however, is reduced by 9 hpi. The response of *D. variabilis* to Gram-negative bacterium, such as *P. aeruginosa*, resulted in the induction of immune effort responses with a peak expression by 48 hpi (Johns et al. 1998). Conversely, infection with *B. burgdorferi*, a pathogen associated with other species of hard ticks, occurred quicker, with a peak at 24 hours (Johns et al. 2001). As differing temporal patterns of immune induction do occur, a potential increase in *dvrelish* transcription may not be identified with a single time point and the level of *dvrelish* transcription may not be required for short term effector response. Upregulation of *D. variabilis* immune genes including AMPs in response to rickettsial infection has been previously identified as tissue-specific (Sunyakumthorn et al.

2012). Further identification of *dvrelish* in separate tissues may reveal tissue specific *dvrelish* induction patterns missed in the combined tissue approach taken in the present study.

Whereas microinjection allows for quantifiable infection conditions, the capillary feeding technique best approximates the normal route of SFG *Rickettsia* infection through feeding (Macaluso et al. 2001). In order to determine the expression of *dvrelish* during capillary feeding, ticks were exposed for 1 and 3 hours to *R. rickettsii*-RhoB solution. After fluorescent microscopy, RhoB could not be identified in ticks feeding for only 1 hour; however, after 1 hour exposure a 5 fold increase of *dvrelish* expression was observed as compared to ticks capillary fed Vero cells alone. Thus, the amount of *Rickettsia* imbibed in 1 hour induced an immune response, even while the amount of solution imbibed was below the limit of visualization of the feeding biomarker RhoB. After 3 hours of capillary feeding, RhoB was visualized in most ticks, but the expression of *dvrelish* decreased. Differential RhoB visualization suggests the increased length of exposure time resulted in an increased *R. rickettsii* dose. These results demonstrated an infection intensity-dependent response in *D. variabilis* to *R. rickettsii* infection. Indeed, the intensity of infection has been identified as a mechanism influencing the induction of immune responses in *Plasmodium falciparum* infected *A. gambiae* (Mendes et al. 2011, Garver et al. 2012). During *P. falciparum* infection, immune responsive transcription of genes was altered in a dose-dependent manner with an increase in transcription during high-intensity infections and decreased transcription during-low-intensity infection. This transcriptional pattern differs from the described capillary feeding results, however, intensity-dependent transcriptional induction may be specific to the pathogen-vector pairing. In order to completely identify the spectrum of possible immune responses, infection assays should include different intensities of infection.

Experiments utilizing specific tick-SFG *Rickettsia* pairings are necessary to determine the link between immune signaling, and the transcription and translation of immune effectors such as AMPs. Interestingly, there was no significant increase in *dvrelish* transcription in response to increasing doses of the atypical bacteria *P. aeruginosa* or *R. parkeri* after 1 hour or 24 hours post-microinjection. Effector responses in hard ticks including AMP expression and hemocyte induction occur within 48 hours of infection with non-associated bacteria, such as *P. aeruginosa* (Johns et al. 1998). Compared to increased defensin mRNA transcription in response to the non-pathogen *Rickettsia montanensis, D. variabilis* defensin induction is variable across bacterial infections, *B. subtilis*, *E. coli*, and *R. montanensis* (Johns et al. 1998, Sonenshine et al. 2005, Ceraul et al. 2007). Differential immune signaling mechanisms, such as the differential expression of *dvrelish*, may affect the differential effector responses to invading bacteria. As such, the response of hard ticks to typical and atypical bacteria should not be extrapolated, and must be determined using specific tick-SFG *Rickettsia* pairings.

Of note, the PGN-injected ticks displayed a greater increase in *dvrelish* expression than the non-associated *R. parkeri*, while not statistically different from sham injected ticks. The moderate increase in *R. parkeri*-dependent dvrelish induction as compared to *R. rickettsii*-dependent induction may be due to differing pathogenicity to the tick host. A recent report demonstrated a link between increased immune induction and decreased arthropod fecundity (Nystrand and Dowling 2014). Host fitness costs associated with *R. rickettsii* infection have been identified in *Dermacentor* ticks, potentially influencing immune signaling induction (Niebylski et al. 1999, Schumacher et al. 2016). Furthermore, *Amblyomma maculatum* infected with *R. parkeri* did not result in host fitness costs or lessened transovarial or transstadial transmission in previous studies (Wright et al. 2015). In comparison, *R. parkeri*-free tick colonies result in

lower molt rates, suggesting potential benefits of *R. parkeri* infection in *A. maculatum*. Together, transcription of *dvrelish* in response to SFG *Rickettsia* infection may not be defined in terms of pathogenicity alone, requiring other influencing factors such as the intensity and duration of infection.

Coupled with transcriptional analyses, DvRelish protein translation and activation were identified via Western blot in the same ticks microinjected with 10^7 *R. rickettsii* and *R. parkeri*. During the 24 hours of infection, the expression of DvRelish did not significantly change in response to infection. The expression of the activated form of DvRelish, N-terminal DvRelish, was either undetectable or when detectable, not significantly different from sham inoculated and PGN inoculated ticks. Introduction of Gram-negative environmental pathogens results in an increased protein translation and activation of Relish-type transcription factors in model arthropods (Boutros et al. 2002, Fan et al. 2008). As the tick tissues were divided for qPCR and Western blot analysis, the expression of the N-terminal DvRelish may be below the limit of detection because of limited starting protein sample. In order to better describe the relationship between translation and activation of DvRelish during periods of increased *dvrelish* transcription, additional technical and experimental design changes may be necessary.

The induction of immune responses to bacterial infection has been well characterized in model arthropods, such as *D. melanogaster* (Lemaitre and Hoffmann 2007). Ticks possess an immune system that responds to environmental bacteria in the hemocoel, clearing infections swiftly (Johns et al. 1998, Johns et al. 2001). In non-model arthropods, such as the hard tick *D. variabilis*, the mechanisms controlling immune effector response are largely uncharacterized. In this study, expression of *dvrelish*, a putative Relish-type NF-κB transcription factor was identified in response to bacterial microinjection and capillary feeding. Microinjected ticks

expressed *dvrelish* only at high concentrations of SFG *Rickettsia* in tick tissues, with temporal expression in the tick tissues, and without a corresponding increase in DvRelish protein expression. In response to capillary feeding, *dvrelish* expression was increased or decreased after 1 or 3 hour exposure, respectively. Because specific tick-SFG *Rickettsia* pairings emerge in nature (Macaluso and Paddock 2014), discerning how ticks control differential immune responses and potentially clear or carry an infection with pathogens and endosymbionts will be necessary to better understand SFG *Rickettsia* ecology and epidemiology.

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CHAPTER 5
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

5.1 Discussion of results and future directions

Rickettsioses are zoonotic infectious diseases transmitted by arthropods, specifically ticks, fleas, lice and mites. Tick-borne rickettsioses are caused by the biological transmission of spotted fever group (SFG) *Rickettsia*. Members of this group are recognized pathogens of humans with great variation in infection ranging from self-limiting to severe, including death. The incidence of tick-borne rickettsial diseases (TBRD) in the United States has recently increased, exemplified by the more than 300% increase in reported Rocky Mountain spotted fever (RMSF) cases since 2000 (Openshaw et al. 2010, Drexler et al. 2016). RMSF is caused by an infection with *Rickettsia rickettsii* and is transmitted by its tick vectors *Dermacentor variabilis*, *Dermacentor andersoni*, and most recently described *Rhipicephalus sanguineus* (Demma et al. 2005). SFG *Rickettsia* transmission occurs through transstadial transmission to subsequent tick life cycle stages and through feeding on rickettsemic vertebrate hosts. Specific SFG *Rickettsia*-tick pairings are evident in nature, with particular *Rickettsia* species typically infecting specific tick species (Macaluso and Paddock 2014). Tick-borne rickettsioses have limited distributions which are dependent on the range of their tick vector. In spite of overlapping vector distributions, atypical infections in hard ticks are not sustained. Thus, the identification of the determinants of tick infection and vector competence is important.

The maintenance of SFG *Rickettsia* in ticks is not successful if infection results in negative fitness costs; rather, infection must be above the threshold sufficient for vertical maintenance without exceeding the resources available in the vector host. As observed in other vector host-microbe interactions (Mendes et al. 2011, Garver et al. 2012), the SFG *Rickettsia*-tick interaction is dependent on the checkpoint distinguishing clearance or microbial survival and

is driven by the level of host immune response. Ticks actively respond to infection with SFG *Rickettsia* by modulating transcription of immune-responsive genes (Macaluso et al. 2003, Mulenga et al. 2003, Sunyakumthorn et al. 2013), but it is known that transcription does not always correspond especially in ticks with protein expression (Thepparit et al. 2010). However, it is unknown how the modulation of immune genes factor into the balance of clearance or establishment of infection of vectors of TBRDs.

Identification of the molecular mechanisms central to rickettsial infection and transmission by tick vectors is of paramount importance for the development of novel intervention strategies for control. The tick-derived factors which favor successful tick infection and SFG *Rickettsia* transmission are likely critical in vector competence. Immune responses in ticks include the cell-mediated, hemocyte-driven response and the soluble response derived from the expression of antimicrobial peptides (AMPs) (Sonenshine and Hynes 2008). Tick derived-lysozyme, defensins, and α2-macroglobulins are induced during infection, and have bactericidal effects (Johns et al. 2001b, Buresova et al. 2009). Likewise, SFG *Rickettsia* infection of *Dermacentor* ticks results in the differential mRNA expression of immune genes (Macaluso et al. 2003, Mulenga et al. 2003, Sunyakumthorn et al. 2013). Hosts respond to all microorganisms encountered, but the level of immune response and bacterial evasion of this response determines microbial clearance or persistent infection. The overarching hypothesis of this dissertation work is that the level of response by *D. variabilis* to SFG *Rickettsia* infection occurs in a species specific manner.

AMP induction is a major component of the tick immune response to rickettsial infection (Johns et al. 1998, Johns et al. 2000, Johns et al. 2001a). However, the immune signaling mechanisms controlling this response are unknown. The focus of this dissertation was to

characterize the role of immune regulators during SFG *Rickettsia* infection of *D. variabilis* ticks. In an effort to better describe the signaling mechanisms distinguishing differential tick effector responses, a Relish-type NF-κB gene was identified in *D. variabilis* (Chapter 2) followed by analysis of protein expression during Gram-negative bacterial infection (Chapter 3). Further infection assays were performed utilizing relevant tick-SFG *Rickettsia* pairings, to elucidate the tissue- and dose-specific responses to persistent infection (Chapter 4). Combined, the results of these studies advance the field by moving towards the identification of the mechanisms of tick immune response regulation as potential determinants of infection that drive the occurrence of specific tick-SFG *Rickettsia* pairings.

In Chapter 2, a homologue cloning strategy resulted in the identification of a full-length *dvrelish* transcript. It was hypothesized that *D. variabilis* would encode a Relish-type NF-κB. Based upon the presence of a canonical Rel-homology domain and encoded ankyrin repeats, this transcript was putatively identified as a Relish-type NF-κB. Homologous Relish-type NF-κB proteins have been characterized in the model organism, *Drosophila melanogaster*, and have been demonstrated to act as transcription factors controlling the induction of the response to Gram-negative bacterial infection (Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009). This dissertation focused on identifying a transcript putatively encoding a protein in this class, as SFG *Rickettsia* are Gram-negative bacterium (Azad and Beard 1998). To ascertain the mechanisms of vector competence, these studies determined the expression of the putative Gram-negative NF-κB in response to pathogenic typical and atypical SFG *Rickettsia* infection in *D. variabilis* during a direct infection of the tick hemocytes and a natural oral infection in tick tissues. It is of great interest to describe the discriminatory tick immune processes that allow for the establishment of infection by SFG *Rickettsia* associated with their specific vector, while

quickly clearing tick-borne bacterial species not typically associated with a particular vector. The identification of immune responsive genes, however, is greatly inhibited by the paucity of genomic sequence available for most hard ticks, and specifically for *D. variabilis*, a vector of *R. rickettsii*.

Following identification of the *dvrelish* transcript, in Chapter 3 it was hypothesized that a full-length DvRelish protein, as well as an activated N-terminal truncated form, would be expressed. Western blotting and mass-spectrometry was employed to identify a 100 kDa protein corresponding to DvRelish, and a 70 kDa protein corresponding to the putative activated N-terminal domain of DvRelish without inhibitory ankyrin repeats. DvRelish, and the N-terminal DvRelish were identified in uninfected *D. variabilis* tissues, demonstrating basal expression of NF-κB proteins and mRNA transcription in the tick irrespective of infection status. Similar Relish-type NF-κB expression has been demonstrated in *D. melanogaster*, as these transcription factors have defined additional functions required for host fitness (Lee 2008). Evidence suggests basal NF-κB induction in response to normal gut flora is regulated via multiple points of inhibition, to promote immune tolerance to non-pathogenic infections (Bischoff et al. 2006, Lhocine et al. 2008, Ryu et al. 2008).

Since NF-κB proteins were not described in ticks prior to these studies, the patterns of DvRelish expression in *D. variabilis* were undefined. It was hypothesized that exposure to Gram-negative infection would result in increased DvRelish expression. DvRelish protein expression in hemocytes was identified in response to the Gram-negative bacterium *Pseudomonas aeruginosa* and to *Escherichia coli*-derived PGN exposure by immunofluorescence assay (IFA). While both conditions resulted in anti-DvRelish staining, IFA demonstrated increased staining in the nuclei of *P. aeruginosa* exposed hemocytes. The strength

of these findings was the ability to identify and track the expression and activation in tick hemocytes. As hemocytes are vital in the soluble and cell-mediated responses, characterizing the response of hemocytes directly will expand the understanding of the mechanisms determining SFG *Rickettsia* survival and dissemination in hard ticks (Sonenshine and Hynes 2008, Sunyakumthorn et al. 2013, Hynes 2014).

Hemocytes are critical in the response of ticks to bacterial infection. As such, identifying the expression of immune signaling mechanisms may provide insight into mechanisms of rickettsial persistence. To this end, changes in expression of *dvrelish* was queried in tick hemocytes in response to SFG *Rickettsia* infection via microinjection. Expression of *dvrelish* was significantly increased in *D. variabilis* hemocytes 1 hour after injection with 10^7 *R. rickettsii*. Conversely, expression did not increased with equal or lower doses of the atypical *Rickettsia parkeri* or *P. aeruginosa*. Injections with only 10^7 *R. rickettsii* resulted in the significantly increased expression of *dvrelish* in combined tick tissues only occurring at 6 hours post injection, suggesting a dose-dependent response. When comparing the results for these microinjection bioassays, the increase in *dvrelish* expression occurs after 1 hour in hemocytes, but in combined tissues expression peaks later, at 6 hours. In comparison to expression in the hemocytes, initial induction of *dvrelish* transcription after microinjection with *R. rickettsii* in the hemocytes may have been obscured in the second bioassay with combined tick tissues. Future studies to identify immune signaling induction in the hemolymph, gut, ovaries, and salivary glands individually are needed to identify the temporal and tissue-specific expression as SFG *Rickettsia* infection progress.

Based on other studies in *Drosophila*, it was not presumed that an increase in *dvrelish* transcription will result in increased protein translation or activation. (Dushay et al. 1996, Fan et

al. 2008, Lhocine et al. 2008, Antonova et al. 2009). During microinjection studies, there was no corresponding increase in DvRelish expression in *D. variabilis* tissues over 24 hours of SFG *Rickettsia* infection. While expression of Relish-type NF-κB proteins has been described in arthropods infected with atypical bacterial infections, the level of response may not be directly comparable to DvRelish expression in the tick Studies utilizing specific tick-SFG *Rickettsia* pairings are necessary to identify the immune response to typical bacterial infections. One limitation of the current study was the limited tick tissue available for protein analyses. In subsequent studies, if the initial tick protein sample is a limiting factor obscuring the identification DvRelish expression or activation, pooling ticks may be necessary. Additionally, DvRelish expression varied greatly between samples; thus, increasing the tick sample size may be required for future studies to more accurately quantify DvRelish expression. Also, studies are need to examine protein expression in a temporal fashion. Despite these limitations, the direct assessment of tick tissues infected with both typical *R. rickettsii* and the atypical *R. parkeri* via microinjection identified tissue and *Rickettsia*-specific *dvrelish* transcription over time, while differential DvRelish expression was not observed. Further studies are needed to compare the response of *D. variabilis* to typical infections with varying pathogenicity, including the non-pathogenic *Rickettsia montanensis*. These experiments would more directly assess the immune signaling events occurring during SFG *Rickettsia* dissemination in the vector host. Defining the differential immune induction patterns in response to the pathogenic *R. rickettsii* which induces negative fitness effects and the non-pathogen *R. montanensis* will give insight into putative mechanisms driving the establishment of non-pathogenic SFG *Rickettsia* infection over low levels of identified *R. rickettsii* infected *D. variabilis* in nature (Stromdahl et al. 2010).

Ultimately, understanding the balance may help elucidate the biology behind rickettsial distribution in nature.

Ticks are able to acquire *Rickettsia* through multiple routes, including feeding on a rickettsemic animal. In order for ticks to transmit the infection during subsequent host bloodmeal acquisition, disseminated infections must develop in the tick, resulting from the spread of *Rickettsia* from the gut of the tick to distal salivary glands. During feeding, the first point of *Rickettsia* infection is the gut, where the interactions between the infected blood meal and the tick immune response occur. Therefore, the interaction of the *Rickettsia* in the gut is key to inducing the appropriate immune response. Nevertheless, *Rickettsia* are able to disseminate from the site of infection. However, the pathogenic mechanisms governing the dissemination of *Rickettsia* from the gut are unknown. Infection of *D. variabilis* with SFG *Rickettsia* via a natural oral route complements the microinjection bioassays described in Chapter 4. The expression of *dvrelish* in the gut of *D. variabilis* after exposure via capillary feeding technique with *R. rickettsii* either significantly increased or decreased depending on the rickettsial dose and duration of exposure. Tissue-specific responses of effector genes have been previously identified in *D. variabilis* ticks infected with SFG *Rickettsia,* but the distinguishing signaling pathways are unknown (Macaluso et al. 2003, Mulenga et al. 2003, Sunyakumthorn et al. 2013). Previous studies have reported conflicting immune gene transcription in a tissue-specific manner in response to hard tick infection with typical and atypical SFG *Rickettsia*. These results suggest that mechanisms of immune response induction are likely tissue and SFG *Rickettsia* specific (Mulenga et al. 2003, Ceraul et al. 2008, Sunyakumthorn et al. 2013, Rosa et al. 2015).

In addition to the site of infection, the level of infection may also influence the immune response. During the capillary feeding bioassay, a shorter exposure time to *R. rickettsii* resulted

in the increase in transcription over 10 hours while a longer exposure with the same concentration of *R. rickettsii,* resulting in an increased number of *R. rickettsii,* induced the downregulation of *dvrelish* over 12 hours. This suggests that the number of organisms effects the expression of immune inducible genes, such as *dvrelish*. Moreover, the potential of SFG *Rickettsia* to regulate the immune response should also be examined. Further studies are necessary to identify the precise mechanisms inducing differential immune signaling, including receptors required for the differential recognition. Such receptors may give additional insight into the tick molecules driving microbial clearance or infection. The capillary feeding technique bioassay identified expression in the gut alone; however, the immune response in specific tissues is likely to vary and should be considered. Future studies should also identify immune signaling gene expression, such as *dvrelish,* in all tissues. This would allow for the characterization of immune induction as SFG *Rickettsia* infection progresses and disseminates in the tick host.

Assays determining differential immune responses in tick vectors will benefit from the use of artificial tick feeding systems. In the present study, keeping the concentration of *R. rickettsii* constant during capillary feeding, while varying the exposure time is a limitation. The use of capillary feeding technique in the experimental design in Chapter 4 removes the possibility of comparing responses between treatment groups over time, which is defined after removal of capillary tubes. The utilization of an artificial feeding system would allow for the varying of exposure dose, while keeping the time post exposure constant, allowing for the more accurate assessment of the temporal induction of immune responses as a result of dose-dependent infection models. The temporal induction of immune activation and responses may differ between typical and atypical infections, giving insight into the progression of rickettsial infection and tick vector competence for SFG *Rickettsia*.

Despite the work in this dissertation identifying a putative master regulator of the immune response to Gram-negative bacterial infection in *D. variabilis*, further characterization of DvRelish function is necessary to fully understand the patterns of gene binding, and its overall importance in the tick response to infection. Relish-type NF-κB proteins are known to be endoproteolytically cleaved, revealing a nuclear localization sequence (Stöven et al. 2000, Stoven et al. 2003). The truncated proteins then move into the nucleus, where they can bind to DNA promoter sequences upstream of immune responsive genes (Lemaitre and Hoffmann 2007). It is likely that previously described SFG *Rickettsia* infection responsive gene promoters contain NF-κB binding κB sites. Determining the κB promoter sequence will allow for the global identification of genes that are DvRelish inducible through promoter sequence analysis, using bioinformatic programs such as the motif finder MEME (Bailey et al. 2009). The binding of DvRelish to *D. variabilis* promoter regions could be identified using multiple methodologies, including chromatin immunoprecipitation-sequencing (ChIP-seq), which would allow for the identification of DvRelish induced genes under differing conditions including pathogenic and endosymbiotic SFG *Rickettsia* infection. Differential patterns of DvRelish binding to effector molecule promoter sequences, combined with gene expression, will better define the differential immune patterns which govern SFG *Rickettsia* establishment or clearance.

In order to assess the importance of DvRelish in the tick immune system in response to SFG *Rickettsia* infection, RNAi-mediated knockdown of DvRelish should be performed. Relish-type NF-κB transcription factors are known to be responsive to Gram-negative bacteria, but owing to the redundant nature of immune systems, it is most likely not acting alone (Lemaitre and Hoffmann 2007, Hetru and Hoffmann 2009). Several studies have explored the feasibility of RNAi in *D. variabilis* and RNAi has been a successful technique in the study of immune-

responsive proteins in hard ticks (Kocan et al. 2009, Zivkovic et al. 2010). The silencing of immune proteins resulted in pathogen-specific increases or decreases in infection load, depending on the immune protein silenced. This suggests increased tick immune gene expression in ticks may confer beneficial effects to some bacteria thereby supporting infection, or alternatively induce bactericidal responses decreasing infection load. RNAi could be a valuable tool in understanding the relative importance of DvRelish in the immune response during SFG *Rickettsia* infection.

One of the most pressing needs in vector biology is an expansion in the number of fully sequenced genomes. While the genome of *D. variabilis* has been prioritized for sequencing, the complexity and size of tick genomes make this a challenging task (Pagel Van Zee et al. 2007). As described in Chapter 2, transcriptomes can facilitate the identification of homologous genes in tick vectors, including immune signaling genes. However, the process of homologue cloning is time consuming and will limit the rapid progress towards a global understanding of tick immune responses. The sequencing of the *D. variabilis* genome could greatly increase the number of immune responsive genes that can be characterized, as was realized after the release of the *I. scapularis* genome (Smith and Pal 2014). With increased genomic sequence, the elucidation of the molecular mechanisms influencing the response of *D. variabilis* to SFG *Rickettsia* will be examined at the systems level. Therefore, the genomic sequencing could accelerate the identification of immune signaling mechanisms facilitating the establishment or clearing of SFG *Rickettsia*.

The tick-derived and *Rickettsia*-derived factors contributing to tick vector competence are poorly defined. Infection assays, coupled with the identification of the mechanisms of immune signaling aim to better define the molecular determinants and processes

which govern specific tick-SFG *Rickettsia* relationships. Any one exposure method may not describe the full range of immune responses possible during SFG *Rickettsia* infection. As such, further studies should be cautious to assign biological significance to specific *dvrelish* expression profiles induced in response to SFG *Rickettsia* infection without taking into account differing exposure methods, infection doses, and specific tick-*Rickettsia* pairings. The identification of a Gram-negative Relish-type NF-κB molecule in *D. variabilis* is an essential step forward in the identification of differential immune signaling and understanding of tick host response to infection with SFG *Rickettsia* (Figure 5.1) Further immune characterization may lead to the identification of immune mechanisms required for infection and transmission. Such mechanisms may facilitate the development of novel approaches to control, and better explain the epidemiology of SFG *Rickettsia*. The identification of the differential mechanisms of immune induction in ticks is critical to developing an understanding of the molecular determinants of vector competence.

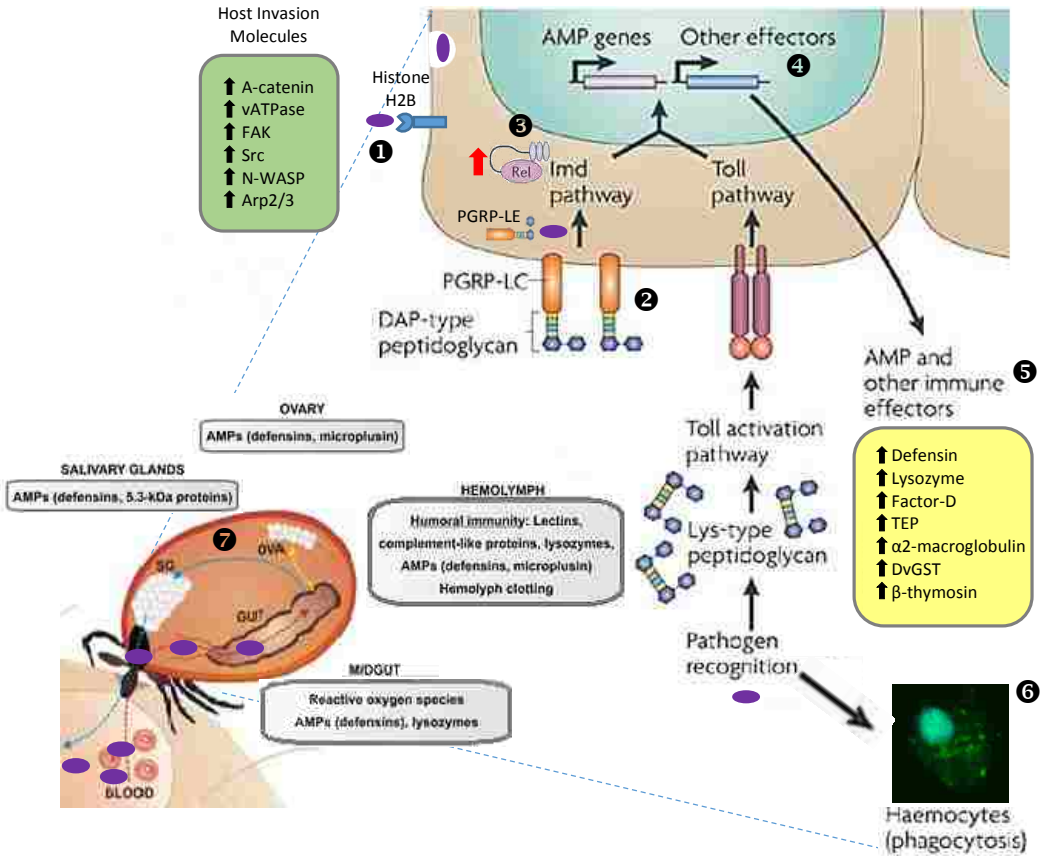

Figure 5.1 SFG *Rickettsia* infection and the tick host immune response. After infectious bloodmeal ingestion, the first site of immune reaction occurs in the tick gut. SFG *Rickettsia* (purple rods) bind to histone H2B and other receptors present on tick cells inducing actin polymerization and *Rickettsia* invasion ❶. Studies in arthropods suggest Gram-negative PGN is recognized by PGRPs located in the cell membrane or by intracellular PGRPs ❷. The induction of the IMD signaling pathway likely activates NF-kB transcription factors, for example DvRelish identified in the present study ❸. Differential transcription of *dvrelish* in response to SFG *Rickettsia* infection was identified; however, the mechanisms of DvRelish protein activation and nuclear localization which induce immune response are not clearly defined ❹. SFG *Rickettsia* infection induces immune effector and AMP upregulation including increased levels of defensin, DvGST, and β-thymosin, α2-macroglobulins, TEPs and Factor D, suggesting an important role in tick-*Rickettsia* interactions ❺. SFG *Rickettsia* that are not cleared by the immune response disseminate to the hemolymph where hemocytes phagocytose SFG *Rickettsia* while continuing to produce effector molecules ❻. Disseminated infections in the salivary glands and ovaries facilitate transmission and stimulate the production of immune effectors ❼. The mechanisms determining the clearance or the establishment of disseminated infection may be controlled through differential immune signaling and effector response, allowing for specific tick-SFG *Rickettsia* pairings to predominate.

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APPENDIX
COMMONLY USED ABBREVIATIONS

AMP – Antimicrobial peptide

BSA – Bovine serum albumin

CDD – Conserved Domain Database

DAP – Diaminopimelic acid

DPI – Days post inoculation

GNBP – Gram-negative binding protein

GST – Glutathione S-transferase

HPI – Hours post inoculation

IMD – Immune deficiency

IPT - Immunoglobulin/plexin/transcription factor

LPS - Lipopolysaccharide

NLS – Nuclear localization sequence

PCR – Polymerase chain reaction

PGN - Peptidoglycan

PGRP – Peptidoglycan recognition protein

qRT-PCR – Quantitative reverse transcriptase polymerase chain reaction

RACE-PCR – Rapid amplification of cDNA ends polymerase chain reaction

RHD – Rel-homology domain

RHOB – Rhodamine B

RMSF – Rocky Mountain spotted fever

RNAi – RNA interference

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFG – Spotted fever group

SRA – Sequence read archive

VITA

Victoria Irene Verhoeve, a native of Saint Petersburg, Florida, is the daughter of William and Janet Verhoeve. She received a Bachelor of Science in microbiology at the University of Florida in 2010. During her undergraduate work, opportunities to volunteer in a vector-borne disease laboratory expanded her interests in parasitology. As her interest in vector-borne disease research grew, she made the decision to enter graduate school in the Department of Pathobiological Sciences at Louisiana State University under the guidance of Dr. Kevin R. Macaluso. She will receive her Doctor of Philosophy degree in May 2016. Upon graduation, Victoria will be taking part in further post-doctoral training opportunities focused in microbial metagenomics of ticks using bioinformatic tools.