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***Anaplasma Phagocytophilum* Modulates Mammalian and Arthropod Signaling for Its Survival and Transmission**

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***ANAPLASMA PHAGOCYTOPHILUM* MODULATES MAMMALIAN AND
ARTHROPOD SIGNALING FOR ITS SURVIVAL AND TRANSMISSION**

by

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ABSTRACT

ANAPLASMA PHAGOCYTOPHILUM MODULATES MAMMALIAN AND ARTHROPOD SIGNALING FOR ITS SURVIVAL AND TRANSMISSION

Supreet Khanal
Old Dominion University, 2021
Director: Dr. Girish Neelakanta

Vector-borne diseases (VBDs) are illnesses transmitted to humans and other animals by arthropods such as ticks, mosquitoes, and fleas. These arthropod vectors transmit infectious pathogens such as viruses, bacteria, and protozoa, to humans during blood-feeding. We have very few control strategies to treat or control these diseases. Human anaplasmosis, caused by the bacterium *Anaplasma phagocytophilum*, is the second most common tick-borne disease in the United States. This work defines three studies elucidating *Anaplasma phagocytophilum*-mediated modulation of cell signaling in mammalian cells and arthropod vector *Ixodes scapularis* ticks.

The first study focused on mammalian PI3 kinases signaling in regulating cell cycle gene expression during *A. phagocytophilum* infection. Using the human megakaryocytic cell line, MEG-01, we observed a differential expression of cell cycle genes in these cells upon *A. phagocytophilum* infection. Both PI3KCA (p110 alpha, catalytic subunit) and PI3KR1 (p85, regulatory subunit) of Class I PI3 kinases and phosphorylated protein kinase B (Akt/PKB) and I κ B were higher at early and late stages of *A. phagocytophilum* infection. Inhibition of PI3 kinases with LY294002 treatment resulted in a significant reduction in the bacterial load and the expression of cell cycle gene expression. These results suggest a role for PI3K-Akt-NF- κ B signaling in the modulation of megakaryocyte cell cycle genes upon *A. phagocytophilum* infection.

The second study showed that *A. phagocytophilum* uses tick transcriptional activator protein-1 (AP-1) as a molecular switch in the regulation of the arthropod antifreeze gene, *iaf*gp. RNAi-mediated silencing of *ap-1* significantly affected *iaf*gp gene expression and bacterial burden in ticks during acquisition from the murine host. The

electrophoretic mobility shift assays (EMSAs) revealed that both the bacterium and AP-1 protein influence *iafgp* promoter and expression. The luciferase assays demonstrated that a 700 bp upstream region of the antifreeze gene is sufficient for AP-1 binding to drive *iafgp* gene expression. Furthermore, survival assays revealed that *ap-1* deficient ticks were more susceptible to cold than the mock control ticks. These data show that AP-1 acts as an upstream transcriptional activator to drive the *iafgp* expression that is critical for *A. phagocytophilum* survival in *I. scapularis* ticks.

The third study identified and characterized the circadian components in *I. scapularis*. The identification of the core clock genes in ticks was made using bioinformatic analysis from the *Ixodes scapularis* genome. Core clock genes like *clock1* and *bmal1* were upregulated upon tick feeding on the murine host. RNAi-mediated knockdown of the arthropod *clock1* gene resulted in an increased bacterial transmission from ticks to the murine host. These results indicate that arthropod clock-mediated signaling is essential for transmitting *A. phagocytophilum* from tick to the vertebrate host. Taken together, these studies highlight several undefined mechanisms that *A. phagocytophilum* modulates for its survival in mammalian cells and ticks.

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This manuscript is dedicated to my parents; Dhurba Prasad Khanal and Gyanu Khanal. To my amazing wife, Bindu Bhandari.

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NOMENCLATURE

AKT/PKB	Serine threonine kinase or protein kinase B
AP-1	Activator Protein 1
ATCC	American Type Culture Collection
bHLH	basic Helix Loop Helix
BLASTp	protein Basic Local Alignment Search Tool
bZIP	basic Leucine Zipper Domain
CDC	Centers for Disease Control and Prevention
CLK	Clock
CRY	Cryptochrome
CYC	Cycle
DC	Dense Core
DNA	Deoxyribonucleic Acid
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic Reticulum
FAD	Flavin Adenine Dinucleotide
GST	Glutathione S-transferase
HCV	Hepatitis C virus
HGA	Human Granulocytic Anaplasmosis
HGE	Human Granulocytic Ehrlichiosis
HSF	Heat Shock Factor
IAFGP	<i>Ixodes scapularis</i> Antifreeze Glycoprotein
IFA	Immuno Fluorescence Assay
IMD	Immune Deficiency pathway
JAK-STAT	Janus Kinase Signaling Transducer Activator of Transcription
LDH	Lactate Dehydrogenase
LGTV	Langat Virus
MEG-01	Megakaryoblast cell line
NAD	Nicotinamide Adenine Dinucleotide
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells

ORF	Open Reading Frame
p.i.	Post-infection
pAKT	phospho AKT
PAMPs	Pathogen Associated Molecular Patterns
PAS	Per-Arnt-Sim domain
PCR	Polymerase Chain Reaction
PER	Period
PI3K	Phosphatidylinositol-3-kinase
PI3KCA	PI3 kinase p110 alpha catalytic subunit
PI3KR1	PI3 kinase p85 regulatory subunit
PSGL-1	P-Selectin Glycoprotein Ligand 1
QRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RC	Reticulate Form
SIT	Sterile Insect Technique
T4SS	Type 4 Secretion System
TBF	Tick Borne Fever
TIM	Timeless
VBDs	Vector Borne diseases
WHO	World Health Organization
ZT	Zeitgeber

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 VECTOR-BORNE DISEASES

Vector-borne diseases (VBDs) are human illnesses caused by pathogens such as bacteria, viruses, and protozoa that are transmitted by an arthropod vector such as ticks, mosquitoes, lice, mites, fleas, and flies. According to the World Health Organization (WHO), annually, more than one billion people are at risk for infection. VBDs are responsible for more than 700,000 deaths worldwide in 2017, which accounts for 17% of all infectious diseases in human beings (WHO, 2019). These VBDs also constitute zoonotic diseases that infect animals other than humans and significantly impact agriculture and the economy (Wikel, 2018). The common VBDs with the highest mortality rates include malaria, leishmaniasis, trypanosomiasis, yellow fever, dengue, Chagas disease, and Japanese encephalitis fever (WHO, 2019). About 19 million arthropod species can act as vectors of several fatal diseases in humans (Neelakanta and Sultana, 2015). An increase in climate change and other environmental factors has led to a rise in many VBDs, especially tick-borne diseases such as Lyme disease and human anaplasmosis (Neelakanta and Sultana, 2015). As per the report from the Center for Disease Control and Prevention (CDC), 275,589 cases of Lyme disease (208,834 confirmed and 66,755 probable) were reported between 2008 to 2014. About 30,000 Lyme disease cases are reported annually that are carried by vectors such as ticks. CDC suggests that more than 300,000 people may be affected annually in the United States. Since 2000, the number of human anaplasmosis cases has been increasing (CDC, 2014).

An increase in global travel and urbanization is the major contributing factor for the growth of VBDs in new regions and countries. This places more people at risk because of the commerce or movement of infected travelers worldwide (CDC, 2014; WHO, 2019). These diseases can be challenging to control and prevent because vaccines are available for only a few of these illnesses. According to the CDC Vital

Signs Report, the disease cases from vectors such as mosquito, tick, and flea have tripled in the US from 2004 to 2016. Since 2004, Nine novel new pathogens have been discovered that are transmitted by mosquitoes and ticks. About 80% of the vector control organizations lack serious prevention and control strategies (CDC, 2014).

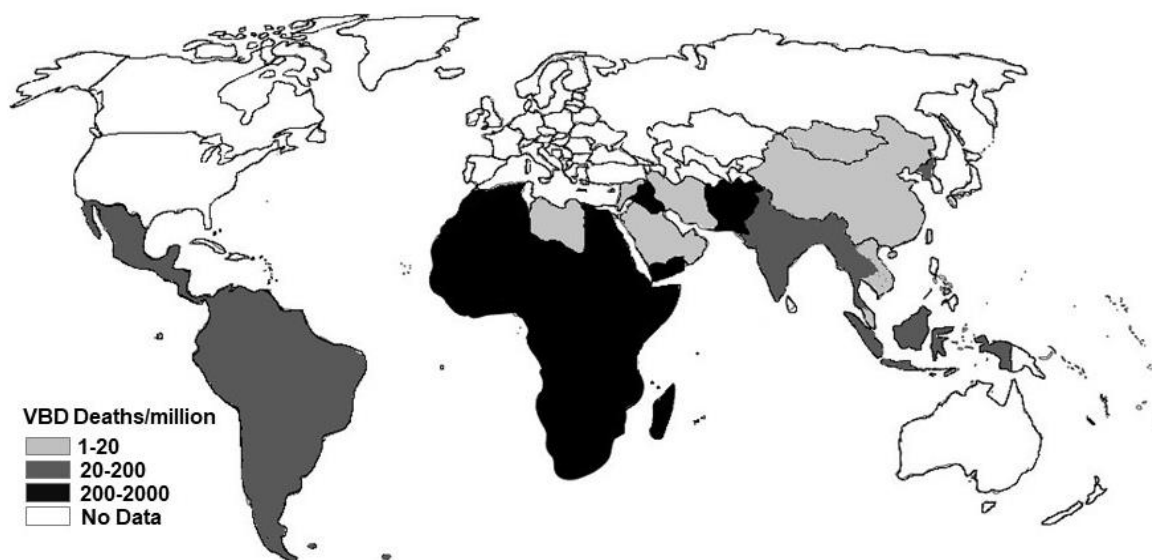


Figure 1. World Map Showing the Number of Deaths from Vector-Borne Diseases

The image demonstrates the distribution of fatalities from VBDs around the world. Africa and Southeast/West Asia, and South America suggest a higher burden of deaths caused by VBDs. The figure was adapted from “Malaria control: the power of integrated action,” a part of the WHO. (<https://www.who.int/heli/risks/vectors/malariacontrol/en/>).

Vectors

Zoonotic diseases are the infections shared between humans and other vertebrate species. It forms a significant group of diseases that impact global public health that is ever increasing. More than 60% of human infectious diseases that emerged between 1940 and 2004 were zoonotic, resulting in significant global morbidity, mortality, and financial loss (Jones et al., 2008). Around 71.8% of them come from wildlife, and the remaining 22.8% are arthropod-borne infections. Since the last two decades, the incidence of emerging vector-borne zoonoses has augmented. Among various

arthropod vectors, ticks transmit the most diverse infectious agents, making them the most important vectors for pathogens transmitted to humans and animals (Jones et al., 2008). Tick-borne illnesses are zoonotic diseases transmitted by interactions between vector hosts (ticks) and vertebrate hosts including humans at various strata of interactions. The global burden of tick-borne diseases is attributed to global warming and increasing incidence of ticks (Wikel, 2018).

To address the role of pathogen distribution and infections from ticks, defining tick-host-pathogen interactions is essential. This requires extensive studies in characterizing pathogen transmission, elucidating pathogenesis, and identifying novel checkpoints to control both vectors and pathogens. Arthropod vectors and their transmission of pathogens carrying disease-causing agents are significantly influenced by weather and climate, so the chances of coinfections are more significant if a single vector harbors multiple pathogens (Schmidt et al., 2013).

Vector Control

Vector control is an essential approach for controlling specific pathogens, thereby preventing their transmission. It is a technique to restrict or eliminate vectors that transmit pathogens to humans and animals. Many methods have been used that fall into four categories: reducing contact, chemical control, habitat and environment control, and biological control (Golding et al., 2015). Habitat and its environment control reduce the habitats or potential breeding areas for vectors. This approach limits the opportunity for mosquitoes and ticks to spread diseases to humans and animals via saliva and feces. Reducing contact means lessening chances for exposure to infectious vectors. This can be achieved by using bed nets, window screens, or wearing protective clothing to avoid mosquito and tick bites. For chemical control, the use of repellents, insecticides, larvicides, and rodenticides is commonly applied (Golding et al., 2015). Using insecticides to bed nets and walls, spraying repellents, and treating wastewater with larvicides has been boosted by the WHO to control vectors effectively. Biological control uses vector predators such as introducing mosquito larvae-eating fishes or using bacterial toxins to kill vectors. This methodology substantially diminishes the risks of VBDs.

Recently, a new pest control method called the sterile insect technique (SIT) has been established to effectively reduce the infection of mosquito-borne diseases (Golding et al., 2015). This environmentally friendly insect pest control method involves breeding in mass-rearing and sterilization, followed by systematically releasing the sterile males into nature over a defined area. SIT-engineered males mate with females producing no offspring. This natural method does not introduce any invading species into the local ecosystem (Vreysen et al., 2000). Nevertheless, these approaches are compelling; the elevated cost for these vector control methods proves costly. Infectious vectors transported globally via international travel and transportation are challenging to control. This may also impact the vector control measures in the developed areas.

1.2 *IXODES SCAPULARIS*

The black-legged tick, *Ixodes scapularis*, is the primary vector that carries and transmits several pathogens to humans (Sonenshine and Roe, 2014). These ticks are three-host, non-nidicolous ticks with larvae and nymphs that feed on small rodents like white-footed mice and birds, and adults that feed on large mammals such as white-tailed deer and humans. Its habitat has been expanding southward from its endemic habitats in the Northeast and Upper Midwest US over the last several decades (Jones et al., 2008; Spielman, 1994). Until the mid-1990s, *Borrelia burgdorferi* and *Borrelia microti* were the only pathogens transmitted by black-legged ticks (Pancholi et al., 1995; Steere et al., 1983). As of 2017, seven pathogens that *I. scapularis* transmit are known to cause illness in humans, including five bacteria (*Anaplasma phagocytophilum*, *B. burgdorferi*, *B. mayonii*, *B. miyamotoi*, and *Ehrlichia muris euclarensis*), one protozoon (*Babesia microti*), and one virus (Powassan virus) (Telford et al., 1997). The last two decades marks a significant shift in the perceived medical importance of the Ixodidae ticks. *I. scapularis* ticks that are naturally infected are shown to transmit these pathogens to the animal host during blood feeding. Moreover, *I. scapularis* also plays a minor role as a vector for the tularemia agent, *Francisella tularensis* (Eisen, 2018; Nelder et al., 2016). Humans are incidental hosts for *I. scapularis* and its associated pathogens and are not essential for either the survival of ticks or pathogen preservation (Eisen and Eisen, 2018; Merten and Durden, 2000). Immature ticks (larvae and

nymphs) have a broad host range, including rodents, insectivores, and birds. In contrast, adult ticks are restricted to medium and large-sized mammals, primarily white-tailed deer (Eisen and Eisen, 2018).

I. scapularis ticks have four stages in their life cycle; egg, larva, nymph, and adult (male and female), which lasts for nearly two years. Ixodid ticks need to have a new host at each stage of their life cycle. After the eggs hatch, these ticks require a blood meal at every stage to survive. Engorged females lay eggs on the ground beginning in late spring, usually near the site where they detach from their hosts. These ticks can feed on mammals, reptiles, birds, and amphibians (Keirans et al., 1996). Eggs deposited in late spring hatch into six-legged larvae, which feed once on small mammals or birds; engorged larvae after feeding drop from the host to the ground and molt into eight-legged nymphs. Nymphs overwinter, and following spring, nymphs begin to feed during the summer months. During this time, the engorged nymph may transmit disease-causing organisms to humans and animals. Both fed larvae and nymphs can become infected with tick-borne pathogens when they feed on infected white-footed mice. These mice are the principal reservoir host for *B. burgdorferi*, *B. microti*, and *A. phagocytophilum*. During the fall, nymphs molt into adult male and female ticks. They seek medium to large mammalian hosts such as white-tailed deer in the next spring. The females feed on large mammals, mate, and lay eggs, thus continuing another cycle (Garnett et al., 2011; Keirans et al., 1996).

As the ticks cannot fly or jump, they wait for a host on the tips of grasses and shrubs, a phenomenon called "questing" to seek the host. The blood-feeding process between its life cycle stages facilitates ticks to acquire and transmit pathogens. *I. scapularis* ticks feed on the host for several days (Sonenshine and Roe, 2014; Sonenshine and Roe, 2013). If the host animal is infected with a bloodborne pathogen, the tick may ingest the pathogen and become infected. If the infected tick later feeds on a human host or an animal, it transmits the ingested pathogen. Once infected, the ticks can transmit the infection throughout their lifetime (Garnett et al., 2011).

1.3 HUMAN GRANULOCYTIC ANAPLASMOSIS (HGA)

Human granulocytic anaplasmosis (HGA), previously known as human granulocytic ehrlichiosis (HGE), is a tick-borne disease caused by *A. phagocytophilum*. These obligate intracellular bacteria are spread to humans by tick bites, primarily from the black-legged tick (*I. scapularis*) and the western black-legged tick (*I. pacificus*). Most cases occur during the spring and summer months. Nymphal deer ticks are most active during this time and are likely to contact humans, and adult deer ticks transmit the infection in the fall. People exposed to HGA agents often have difficulty being diagnosed due to the nonspecific nature of the symptoms. Most patients experience fever, headaches, chills, myalgia, and malaise that can easily be confused with other infectious diseases. Appearances of rashes are rare from people exposed to HGA (Dumler, 2005; Dumler et al., 2005; Stuen et al., 2013).

A. phagocytophilum is a small bacterium infecting typically neutrophils transmitted by *Ixodes* ticks. Granulocytic anaplasmosis is also one of the most widespread tick-borne infections among animals in Europe. The geographical distribution of its tick vector, *I. ricinus* complex (mainly *I. ricinus* and *I. persulcatus*), is increasing (Stuen et al., 2013). More individuals are at greater risk when ticks are active during the spring and summer seasons. Despite the swelling prevalence of *A. phagocytophilum* in the animal hosts, human cases are not frequent. The human cases are underestimated because of the nonspecific clinical signs, for example, flu-like symptoms (Dumler, 2005; Stuen et al., 2013). The strains of *A. phagocytophilum* from the US have shown higher morbidity (< 1%) compared to those from Europe primarily because of the strain heterogeneity that correlates with host preferences and resulting pathogenesis (Bakken and Dumler, 2015).

Clinical Features

The incubation times in humans are varied, ranging from 5 to 21 days, with most cases occurring between April and October. Clinical manifestations of HGA can vary from mild to life-threatening, depending on the patient's age and general health. The clinical presentation starts like an acute nonspecific febrile infection (duration 2-11 days). Of those infected cases, 70-95% of patients are presented with pyrexia, malaise, myalgia,

and headaches. Some also present with arthralgia and elevated hepatic transaminases, neurological problems, gastrointestinal (nausea and vomiting), or respiratory signs (difficulty breathing). The onset of HGA typically begins within 7-8 days of a tick bite and often includes fever, severe headache, malaise, chills, and muscle pain. Other uncommon symptoms may consist of confusion, hemorrhages, and renal failure. Fatal infections are rare, but coinfections can accumulate in multi-system failure. Some patients with tick exposure also present with thrombocytopenia and leukopenia. Coinfection with other tick-borne pathogens is possible, mainly when a rash appears (Bakken and Dumler, 2015; Dumler, 2005).

Diagnosis

The diagnosis for HGA varies, but the common diagnosis is based on the patient's symptoms and the laboratory tests. Patients generally show a low WBC and platelet count, and elevated liver enzyme levels (Dumler, 2005). Microscopy with blood smears is used to observe characteristic morulae (microcolonies) of *A. phagocytophilum* in affected blood cells (usually neutrophils). The gold standard diagnosis of anaplasmosis is the immunofluorescence assay (IFA) using *A. phagocytophilum* antigen to determine a significant rise in antibody titers. During the acute phase of illness, the patient's blood can be tested using PCR (polymerase chain reaction). This method is extremely sensitive early on but quickly loses sensitivity following antibiotics use. Likewise, a negative result does not rule out the diagnosis, as intermittent bacteremia can produce false-negative results. Identification using culture isolation is not routinely available as *A. phagocytophilum* is intracellular, and routine hospital blood cultures cannot detect *A. phagocytophilum* (Bakken and Dumler, 2015; Dumler, 2005; Dumler et al., 2005).

Treatment

Prophylaxis is usually not recommended after a tick bite, even in endemic regions. According to the Center for Disease Control (CDC), the drug of choice for HGA in adults and children (including those younger than eight years of age) is tetracycline (usually doxycycline) for two weeks (Dumler, 2005). An extended treatment period may be required for severe symptoms or coinfection with Lyme disease. Generally, quick

response to treatment with a marked clinical improvement is seen within 24 to 72 hours. But for patients with doxycycline allergies or with mild disease during pregnancy, rifampicin is prescribed. However, other antibiotics like cephalosporins, quinolones, macrolides, and penicillin are ineffective (Dumler, 2005), and using sulfa drugs during acute illness may worsen the severity of the infection.

1.4 ANAPLASMA PHAGOCYTOPHILUM

Anaplasma phagocytophilum is a tick-borne intra-granulocytic alpha-proteobacterium that is transmitted by *I. persulcatus* complex. These are gram-negative and obligate intracellular bacteria that infect a vast range of hosts worldwide (Dumler, 2005). They are the causative agent of tick-borne fever (TBF) in ruminants (ovine granulocytic anaplasmosis) and human granulocytic anaplasmosis (HGA) in humans. As ticks feed on a large variety of vertebrates, *A. phagocytophilum* epidemiological cycles are intricate and involve different ecotypes circulating in host species. The epidemiological cycle of this bacterium is poorly understood (Ismail et al., 2010; Rikihisa, 2010).

Genomic Features of *A. phagocytophilum*

The genome size of *A. phagocytophilum* strain HZ is 1.47 Mb, one-quarter of the size of the model bacteria, *Escherichia coli*. There are no plasmids, intact prophages, or transposable elements. The genome of *A. phagocytophilum* has several repeats (12.7% of the genome), for example, over 100 *p44* (*msh2*) genes, type IV secretion system (T4SS) genes, and tandem repeat genes. The genes required for the lipopolysaccharide and peptidoglycan biosynthesis are absent (Hotopp et al., 2006). Approximately 747 ORFs (55%) from this bacterium have an assigned function and 82 ORFs encode conserved hypothetical proteins. Roughly 458 ORFs encode exclusive hypothetical proteins (Hotopp et al., 2006). In a published study (Lin et al., 2011), the authors performed a global proteomic analysis reporting 1,212 *A. phagocytophilum* proteins in infected human leukocytes, 89.3% of the predicted bacterial proteome. Virtually all bacterial proteins ($\geq 99\%$) with their known functions are expressed, but only 80% of the "hypothetical" proteins were observed in infected human cells. Most hypothetical proteins in *A. phagocytophilum* are surface-exposed, inclusion membrane proteins, and

T4SS molecules that confer unique nature to this bacterium (Ge and Rikihisa, 2006; Rikihisa, 2010). *A. phagocytophilum* also contains genes for the biosynthesis of necessary nucleotides, vitamins, and cofactors, including folate, biotin, FAD, NAD, thiamine, coenzyme A, and protoheme, essential for colonization in tick vectors (Figure 2). It can only synthesize four amino acids, glycine, glutamine, glutamate, and aspartate; therefore, it must obtain the remaining amino acids and other compounds from the host. *A. phagocytophilum* has genes for metabolism and protein processing while replicating and surviving in several host cells (Figure 2). Also, *A. phagocytophilum* has 41 genes for transport and binding proteins that are critical for its survival (Hotopp et al., 2006; Rikihisa, 2010).

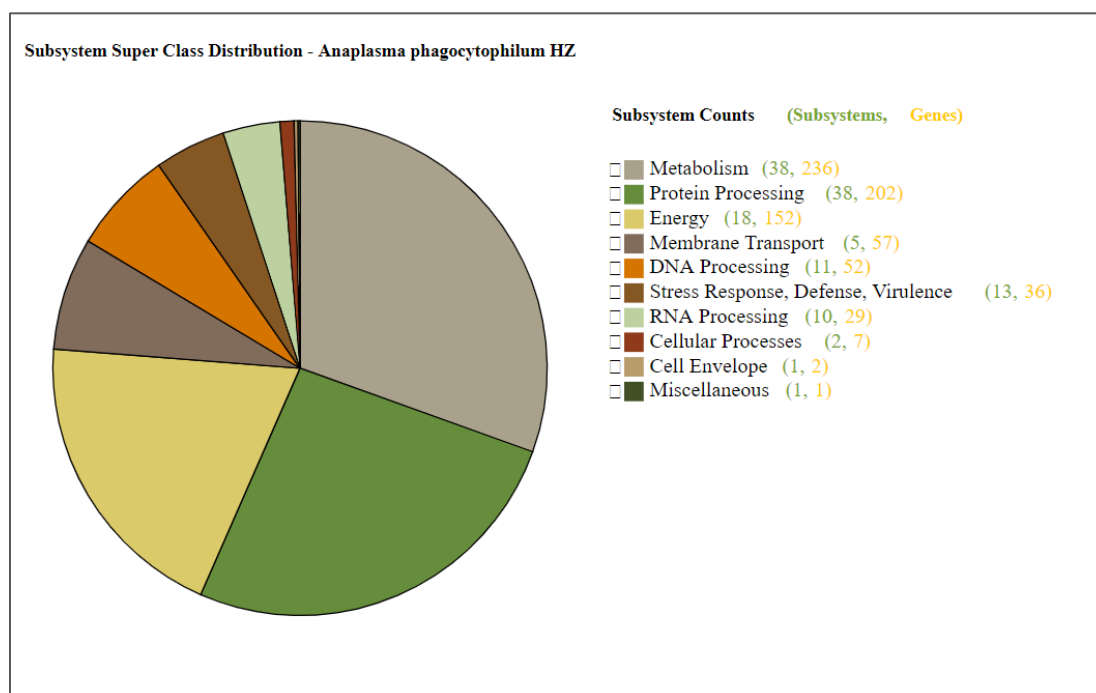


Figure 2. Representative Genome Information for *A. phagocytophilum* HZ Strain

The image shows the distribution of subsystem functions inside the *A. phagocytophilum* genome. The schematic was drawn from Patric.org using the complete reference genome of the *A. phagocytophilum* HZ strain. The image was drawn using the web version of the Patric software.

Surface Proteins/Msp2/P44

One of the most abundant repeats is the genes for surface proteins, P44/Msp2 in *A. phagocytophilum*. These are the major surface antigens used for serodiagnosis (Bakken and Dumler, 2015). The genome of *A. phagocytophilum* contains 113 *p44 (msp2)* paralogous genes encoding P44/Msp2 proteins in its genome (Hotopp et al., 2006). The *p44 (msp2)* genes have a central hypervariable region flanked with 5' and 3' conserved sequences. Most of the *p44* genes are shorter pseudogenes that are truncated partially at both ends. A single full-length *p44 (msp2)* locus is interesting because it is highly polymorphic (Lin and Rikihisa, 2003). This polymorphism allows P44 antigens to be rapidly exchanged while maintaining similar transcriptional regulation from the same promoter. More than 110 P44/Msp2 proteins are expressed in infected HL-60 cells (human promyelocytic cells), and that 88 of them are encoded by the pseudogenes (Lin et al., 2011). The composition of the *p44* allelic population in *A. phagocytophilum* is dependent on the tissue environments and governed by immune pressure and bacterial physiological conditions. This antigenic variation of P44 allows *A. phagocytophilum* to evade the host immune system and replicate and persist in hosts (Hotopp et al., 2006; Lin et al., 2011; Lin and Rikihisa, 2003).

Replication of Bacteria and Intracellular Developmental Cycle Inside Host Cells

There are two morphological and developmental types of this bacteria in both mammalian cells and tick cell cultures; a larger reticulate form (RC) and a smaller dense-core form (DC) that contains condensed protoplasm (Rikihisa, 2010). Electron microscopy imaging shows that only DC form binds to HL-60 cells, but both DC and RC forms bind to and enter in tick cells (Munderloh et al., 2004). *A. phagocytophilum* primarily infects mature neutrophils in the vertebrate hosts. The infection of *A. phagocytophilum* involves major surface proteins Msp2 (P44), Asp55, and Asp62 and other potential receptors for ligand-receptor interactions (Rikihisa, 2010; Troese and Carlyon, 2009). The replication of *A. phagocytophilum* occurs within-host cell-derived vacuoles from mammalian and tick cells, including HL-60 cells, embryonic-derived tick cells, and primate RF/6A endothelial cells (Carlyon and Fikrig, 2006; Munderloh et al., 2004). *A. phagocytophilum* also replicates in endothelial cells of various tissue sections

recovered from infected animals. During its growth in cultured cells, *Anaplasma* cycles between a dense-cored (DC) phenotype that binds and invades host cells and a reticulate cell (RC) morphotype that replicates inside the pathogen-occupied vacuoles (Munderloh et al., 2004; Troese and Carlyon, 2009). Immunofluorescence data showed that only small-sized bacteria (<1- μ m), equivalent to the DC form, enter human peripheral blood neutrophils. But in HL-60 cells, both small or larger (>1- μ m) bacteria are taken up and are delivered to lysosome-associated membrane protein 1 (LAMP1) positive regions from the host cells. These changes in the developmental stages of *A. phagocytophilum* have discrete biological properties that allow them to enter host cells and avoid host immune responses through different receptors and internalization pathways (Niu et al., 2008). The DC and RC forms of *A. phagocytophilum* have been reported in the tick tissues of experimentally infected and field-derived adult deer (Telford et al., 1997).

As the bacteria infect a different host (Figure 3), it shows a differential expression of its genes, maintaining the secretive nature and evading immune response from the host cells. The host environment in which the bacterium replicates and the bacterial concentration regulate the transcription of *A. phagocytophilum* genes. The gene expression profiles of *A. phagocytophilum* HGE1 isolate during the invasion and replication from its vector and human host show a differential expression reflecting its ability to adapt to these biologically different hosts (de la Fuente et al., 2017; de la Fuente et al., 2016a). For example, the analysis of gene activation in *A. phagocytophilum* from infected HL60 cells infected showed the bacteria to be pre-programming an invasion cascade in the same mammalian host cell (Carlyon and Fikrig, 2003). However, the expression profiles of HL-60- or tick cell (ISE6) derived *Anaplasma* when incubated with uninfected tick cells demonstrated significant upregulation of new genes compared to mammalian cells, suggesting the bacteria was primed for multiple rounds of infection and invasion of mammalian cells, but not tick cells (Nelson et al., 2008). These results show that the bacteria recognize various host cells and adapt quickly, displaying a differential upregulation of host-cell-specific genes (Nelson et al., 2008).

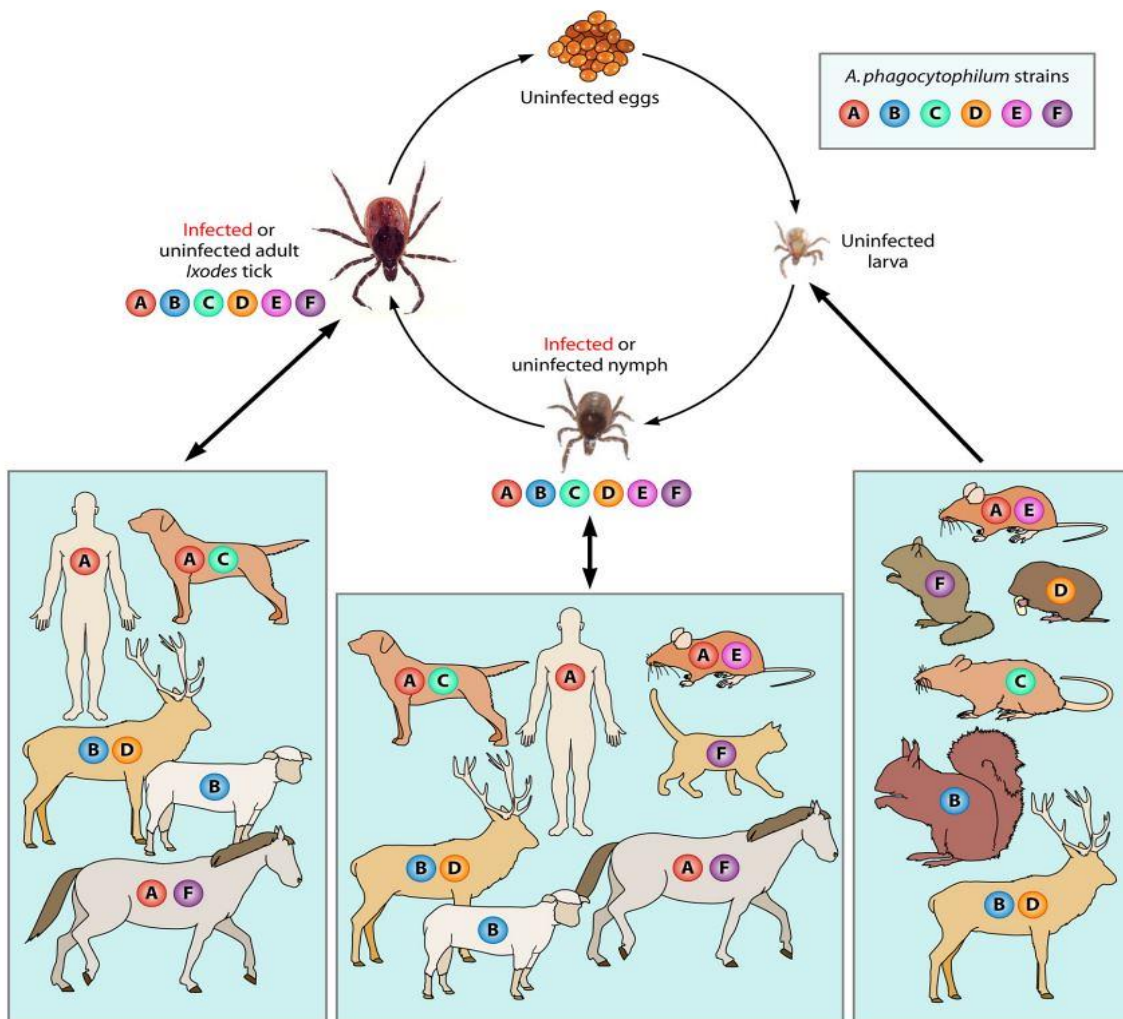


Figure 3. The Life Cycle of *A. phagocytophilum* in Various Hosts

The image above shows different strains of *A. phagocytophilum* with varying host susceptibilities. The female lays uninfected eggs and larvae. Developing-stage ticks such as larvae, nymphs, or adults can acquire *A. phagocytophilum* during feeding from their infected host counterparts. Ticks maintain *A. phagocytophilum* through molting stages and transmit the pathogen to a susceptible host during their next blood-meal. This image is reproduced from a published journal article (Rikihisa, 2011).

A. phagocytophilum infection is maintained primarily on circulating neutrophils rather than other peripheral tissues or hematopoietic tissues. However, it can infect cells from bone marrow progenitors, megakaryocytes, and endothelial cells (Herron et al., 2005; Hodzic et al., 1998). It can attach and enter susceptible host cells and internalize

inside them. It has been shown that host P-selectin glycoprotein ligand 1 (PSGL-1) is required for *A. phagocytophilum* binding and infection in HL-60 cells (Herron et al., 2005). This binding of *A. phagocytophilum* to HL-60 cells initiates the PSGL-1 signaling pathway, which results in the tyrosine phosphorylation of ROCK1 (Thomas and Fikrig, 2007).

1.5 *A. PHAGOCYTOPHILUM* SIGNALING IN *I. SCAPULARIS*

Vectorbase and GenBank remain the major databases to extract vector information, especially for tick genome information. It has been reported that 234 genes in tick immunity are categorized into one of the following nine major immune pathways: proteases (33), leucine-rich repeat (LRR) proteins (21), gut-microbe homeostasis (17), free radical defense (13), agglutination (37), coagulation (11), phagocytosis (33), signal transduction via Toll, IMD, and JAK-STAT pathways (55), and anti-microbial peptides (14). The *I. scapularis* genome encodes representative genes from all the major three pathways; immune deficiency (IMD), Toll, and Janus kinase (JAK)- signaling transducer and activator of transcription (STAT) pathway that activates the immune response within arthropods (Smith and Pal, 2014).

The transmission of pathogens mainly occurs during the blood-feeding stages during the tick life cycle (Figure 3). During a blood meal, tick saliva contains numerous pharmacologically active molecules that affect the host immune responses to facilitate tick feeding (Sonenshine and Roe, 2014). This salivary secretion from feeding ticks modulates vertebrate innate and adaptive immunity (Kotál et al., 2015). Tick salivary secretion is essential in the interaction of ticks with the vertebrate hosts and the pathogens they transmit (Kotál et al., 2015). Many pathogens that persist and colonize and transmit through ticks during their life cycle are distinct evolutionarily and own unique structures (Hajdusek et al., 2013). In humans, the rickettsial pathogen *A. phagocytophilum* has developed various strategies to infect and survive in neutrophils and other hematopoietic cells, including megakaryocytes. This pathogen regulates different tick molecules for its survival and replication and the colonization of *A. phagocytophilum* in *I. scapularis* ticks (Dahmani et al., 2020; Khanal et al., 2017;

Neelakanta and Sultana, 2015; Neelakanta et al., 2010; Ramasamy et al., 2020; Taank et al., 2017; Turck et al., 2019).

Tick-borne pathogens have evolved to persevere and be transmitted by a specific tick species, showing that they coevolved and developed a significant intimate relationship with the hosts. Additionally, to successfully colonize and replicate in various hosts, these tick-borne pathogens have also evolved particular mechanisms for persistence in the vector and evade innate immune insults (de la Fuente et al., 2016a). These interactions were well studied, where the authors showed that the presence of *A. phagocytophilum* in *I. scapularis* ticks increases their physiological fitness (Khanal et al., 2018; Neelakanta et al., 2010). The *I. scapularis* antifreeze glycoprotein, IAFGP, was induced by the pathogen inside ticks, thereby increasing the cold tolerance of vector hosts.

Signaling in Tick-Pathogen Interactions

The pathogens harboring inside ticks induce the expression of various tick molecules. These tick-pathogen protein-protein interactions play a vital role during pathogen infection, transmission, and persistence. The analysis of *A. phagocytophilum* proteins differentially represented during infection in ticks demonstrated that tick AP-1 interacts with *iafgp* promoter that induces this gene critical for ticks to survive better at cold and freezing temperatures (Khanal et al., 2018; Neelakanta et al., 2010). Various type IV secretion system (T4SS) proteins in *A. phagocytophilum* are reported to be involved in the secretion of HSP70 and the surface proteins, MSP4. Several tick molecules, used by *A. phagocytophilum*, assist in the colonization of the pathogen in the tick tissues, such as the midgut and salivary glands (de la Fuente et al., 2005; Gulia-Nuss et al., 2016).

A. phagocytophilum is naturally maintained in a tick-rodent cycle. Upon tick feeding, the bacteria replicate and migrate from the salivary glands to the mammalian host (to invade granulocytes). The transmission of *A. phagocytophilum* occurs between 24 and 48 hours after tick engorgement (Hodzic et al., 1998). *A. phagocytophilum* uses the vector host molecules for its survival and thus have evolved developed intimate and symbiotic relationships between pathogen and arthropod (de la Fuente et al.,

2016a). When the *A. phagocytophilum* is transmitted into a naïve tick, the infection of tick salivary glands results in the inhibition of the intrinsic apoptosis pathway. Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway are activated in tick's midgut during *A. phagocytophilum* infection to inhibit apoptosis and promote pathogen survival. In ISE6 cultured tick cells, *A. phagocytophilum* infection promotes protein misfolding in the endoplasmic reticulum (ER), reducing the tick cell response to bacterial infection (Alberdi et al., 2016). But tick cells activate protein targeting and degradation, reducing ER stress and apoptosis, eventually progressing *A. phagocytophilum* infection. These studies show that inhibition of tick cell apoptosis is a standard mechanism used by *A. phagocytophilum* to survive and multiply in both tick and vertebrate host cells (de la Fuente et al., 2017; de la Fuente et al., 2016a; Sarkar et al., 2012).

Effect of Pathogen Infection on Vector Fitness

Understanding the role of vector-host-pathogen triad interactions is essential to highlight the relationship between *I. scapularis* and *A. phagocytophilum*. Modulation of arthropod cell signaling benefits tick health and allow pathogen persistence (de la Fuente et al., 2017; de la Fuente et al., 2016a; Dumler, 2005; Gulia-Nuss et al., 2016; Rikihisa, 2010). At the tick-pathogen interface, the pathogen *A. phagocytophilum* induces the expression of IAFGP, AP-1, OAPTs, src kinase, tick specific miRNA-133, and heat shock proteins (HSPs) to increase the survival and feeding fitness of its vector host (Dahmani et al., 2020; Khanal et al., 2018; Neelakanta et al., 2010; Ramasamy et al., 2020; Taank et al., 2017; Turck et al., 2019). Besides, induced phosphorylation of actin upon *A. phagocytophilum* selectively regulates the salp16 expression in both ticks and tick cells (Sultana et al., 2010). For example, *A. phagocytophilum* induces tick *iafgp* and AP-1 to facilitate tick survival in the cold, thus enhancing physiological fitness against freezing temperatures. All these studies in understanding vector-host-pathogen triad interactions show that pathogens have established strategies, such as exploiting the host immune system for their infection and transmission. Most of these strategies are used by tick-borne pathogens to infect both ticks and mammalian hosts (de la Fuente et al., 2016a; de la Fuente et al., 2016b). These studies have illustrated that pathogens modulate the

arthropod signaling mechanisms not only to improve the fitness of their vector host but also for their replication and survival in the vectors.

1.6 CIRCADIAN RHYTHMS IN INVERTEBRATES

The axial rotation of the Earth produces a 24-hour cycle of environmental variations that provides a selective force that eventually has evolved into an endogenous circadian clock for daily processes in all living beings including insects. For example, *Anopheles gambiae*, the malaria mosquito vector, displays circadian rhythms in antennal olfactory sensitivity to host odorants that correlate with blood-feeding (Maliti et al., 2016; Rund et al., 2013). These approximately 24-hour cycles of physiology are so engrained in behavior that even *Drosophila melanogaster* that was housed in the dark for 1,300 generations still showed robust daily rhythms in locomotor activity upon entrainment in light-dark cycles (Imafuku and Haramura, 2011). Other experimental data shows that the circadian clocks deliver organisms with robust fitness advantage in their environment (Dibner et al., 2010; Woelfle et al., 2004). The circadian clocks are thought to exist and have been continuously studied in organisms from bacteria to higher eukaryotes. The evolution in the circadian system remains unclear. Several hypotheses have been proposed to explain the evolution of the circadian system. Possibly the most accepted theory is that the circadian clock components restrict cell division and DNA replication to night-time to reduce UV-induced DNA damage and mutations (Meireles-Filho and Kyriacou, 2013; Ozturk et al., 2011).

The core oscillator proteins such as PER (period), TIM (timeless), CLK (clock), CYC (cycle), and CRY (cryptochrome) represent key protein elements in invertebrates that assimilate metabolic and environmental signals to regulate cellular biology and physiology. This set of proteins are predicted to be highly conserved throughout invertebrate lineage and possibly even throughout the metazoan evolution line. Amazingly, comparative analysis of the clock oscillator proteins from the pre-metazoans to numerous metazoan lineages showed significant divergence underscoring an unexpected level of plasticity in oscillator design. It has been demonstrated that zeitgebers, like, light-dark cycles and temperature, interact to affect entrainment (Gentile et al., 2013; Ozturk et al., 2011). However, future studies are essential to

analyze interactions between different zeitgeber at neuronal levels to shape the clock output and observe when these interactions are ecologically and physiologically relevant.

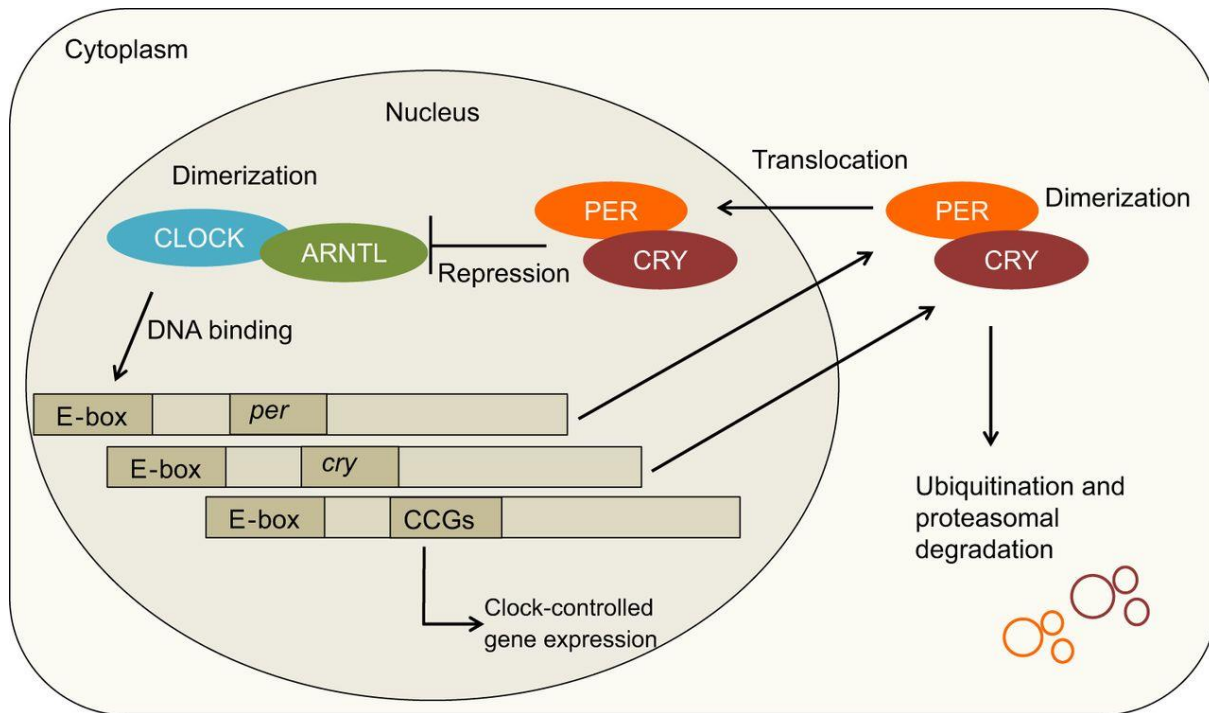


Figure 4. The Regulatory Feedback Loops in Circadian Rhythms

CLOCK dimerizes with ARNTL (also called BMAL1) in the nucleus and then binds to E-box elements in the promoter regions of *per*, *cry*, and other clock-controlled genes. PER and CRY proteins dimerize in the cytoplasm and then translocate to the nucleus. Post-translational modifications of proteins control the accumulation of PER–CRY dimers. PER and CRY inhibit the CLOCK activity–ARNTL dimers in the nucleus, eventually allowing the transcriptional cycle to start again. This image was modified from (McIntosh et al., 2010).

CLOCK/CYCLE/BMAL1

These are bHLH-PAS transcription activators that have long been established as the main driving force for the circadian transcription inside cells (Darlington et al., 1998; Zhao et al., 2003). Depending on the species type, either CYC/BMAL1 or CLK binds to

E-box elements (CACGTG) and activates the transcription of clock-regulated genes. Both CLK and CYC appear early in metazoan evolution, but CLK seems to have evolved earlier (Hardin and Panda, 2013). Predominantly CLK activates the primary circadian transcription without CYC as a heterodimeric partner. But other unknown proteins could reinstate their function. This pattern appears to be coherent in bilaterian lineages; either CLK and CYC are lost, or only CYC is lost, suggesting that CLK is likely the dominant circadian transcriptional activator (Hao et al., 1997). Studies in understanding the clock-mediated regulation of tick genes are not explored. Therefore, this study aimed to characterize the role of circadian genes in tick- *A. phagocytophilum* interactions.

CHAPTER 2

***ANAPLASMA PHAGOCYTOPHILUM* MODULATES EXPRESSION OF CELL CYCLE GENES IN MEGAKARYOCYTES THROUGH PHOSPHATIDYLINOSITOL-3-KINASE SIGNALING**

2.1 INTRODUCTION

Anaplasma phagocytophilum is a tick-transmitted intracellular bacterium that infects granulocytes that cause human granulocytic anaplasmosis (HGA). HGA is one of the most common tick-borne diseases, and *A. phagocytophilum* is one of the common pathogens harbored in various vectors (Anderson and Magnarelli, 2008; Dumler et al., 2005). This intracellular bacterium has developed strategies to survive in several hosts, including humans, horses, cattle, deer, sheep, mice, and ticks, among other species (Carlyon and Fikrig, 2003; Foley et al., 1999; Madigan et al., 1995; Neelakanta et al., 2010; Stuen et al., 1998; Sultana, et al., 2010). Previous studies have shown that nearly 30% of the human population in the endemic areas may have been exposed to *A. phagocytophilum* infections (Aguero-Rosenfeld et al., 2002; Bakken et al., 1998). Anaplasmosis is an emerging infectious disease that has been reported since 1995, with a 12-fold increased rate in 2001-2011 (Bakken and Dumler, 2015). Usually, infections, in many cases, are asymptomatic and mild (Aguero-Rosenfeld et al., 2002; Bakken and Dumler, 2015; Dumler, 2005; Dumler et al., 2005). Sometimes, HGA infections could lead to severe illness and fatality in many individuals (Bakken and Dumler, 2015).

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The typical clinical manifestation of HGA includes fever, malaise, headache, and myalgia, but nausea, vomiting, or cough may occur in some infections.

Thrombocytopenia (reduced platelet condition), leucopenia, anemia, and elevated liver enzyme levels are often evident in HGA cases (Bakken and Dumler, 2015; Dumler, 2005; Dumler et al., 2005). *A. phagocytophilum* successfully creates a vacuolar protective layer within neutrophils as well as mammalian myeloid and endothelial cells. It survives primarily in the neutrophils, where it enters vacuoles that do not fuse with a lysosome, thereby surviving from the host immune system (Mott et al., 1999; Webster et al., 1998). *Anaplasma phagocytophilum* delays apoptosis in neutrophils by modulating multiple apoptotic pathways (Ge and Rikihisa, 2006; Yoshiie et al., 2000). Several studies have shown that *A. phagocytophilum* modulates host gene expression for its survival and replication (de la Fuente et al., 2005; Garyu and Dumler, 2005; Ge et al., 2005; Lee and Goodman, 2006; Thomas and Fikrig, 2007; Yoshiie et al., 2000). *Anaplasma phagocytophilum* is also known to infect other hematopoietic cells, as demonstrated (Bakken and Dumler, 2015; Munderloh et al., 2004; Rikihisa, 2011). Multiple studies have shown the interactions of *A. phagocytophilum* with neutrophils. However, very little is known about how this bacterium survives and persists within other hematopoietic cells.

Megakaryocytes are hematopoietic cells that are the precursor cells for the production of platelets. The maturation and differentiation of megakaryocytes occur in bone marrow (Machlus et al., 2014). After differentiation, they form extended cytoplasmic structures, termed proplatelets, which lead to platelet formation (Machlus et al., 2014). These studies provide a strong foundation for using MEG-01 cells to study infection-induced changes in megakaryocytes (Granick et al., 2008). A previous study has reported that *A. phagocytophilum* readily infects MEG-01 cells by using sialylated ligands, such as PSGL-1, to enter these cells and the absence of sialylation, or blocking of PSGL-1, limited infection susceptibility (Troese et al., 2009). *A. phagocytophilum* failed to alter the formation of platelets in MEG-01 cells (Granick et al., 2008). Infected MEG-01 cells produced proplatelets and platelet-like particles comparable to uninfected control. The pathogen also decreases MEG-01 proliferation using PSGL-1 (Granick et al., 2008). The mechanism why *A. phagocytophilum* decreases MEG-01 cell

proliferation is currently not understood.

HGA-induced thrombocytopenia in many of the cases is not entirely understood. However, some evidence suggests that immunosuppression in the infected host cells could lead to thrombocytopenia (Ismail et al., 2010; Walker and Dumler, 1997). Due to the regular incidence of thrombocytopenia in HGA cases, studies in understanding *A. phagocytophilum* within the cells of hematopoietic origin are necessary. This study addressed whether decreased cell proliferation of MEG-01 cells upon *A. phagocytophilum* infection, as observed (Granick et al., 2008), is due to the changes in the cell cycle of these cells. Our data show that *A. phagocytophilum* modulates the expression of cell cycle genes differentially at early and late time points of infection in MEG-01 cells via PI3-kinase signaling for its survival in these cells.

2.2 RESULTS

***A. phagocytophilum* Infection Kinetics in Megakaryocytic Cell Line MEG-01**

A. phagocytophilum NCH-1 strain readily infects MEG-01 cells (Granick et al., 2008). Similar infection kinetics with *A. phagocytophilum* HZ-1 strain in MEG01 cells during the course of infection (Figure 5) was observed. QRT-PCR analysis showed increasing loads of the bacteria with increasing time points. *A. phagocytophilum* loads were significantly ($P < 0.05$) higher at day 7 p.i. in comparison to day 1 p.i. of MEG-01 cells (Figure 5A). Day 1 p.i. was considered as earlier time point, and day 7 p.i. was regarded as the late point of *A. phagocytophilum* infection in these cells. Cytotoxicity assays via measuring LDH release from the cells into the supernatant were performed. Decreased cytotoxicity ($P < 0.05$) was observed in *A. phagocytophilum* infected cells when compared to the uninfected controls (Figure 5B).

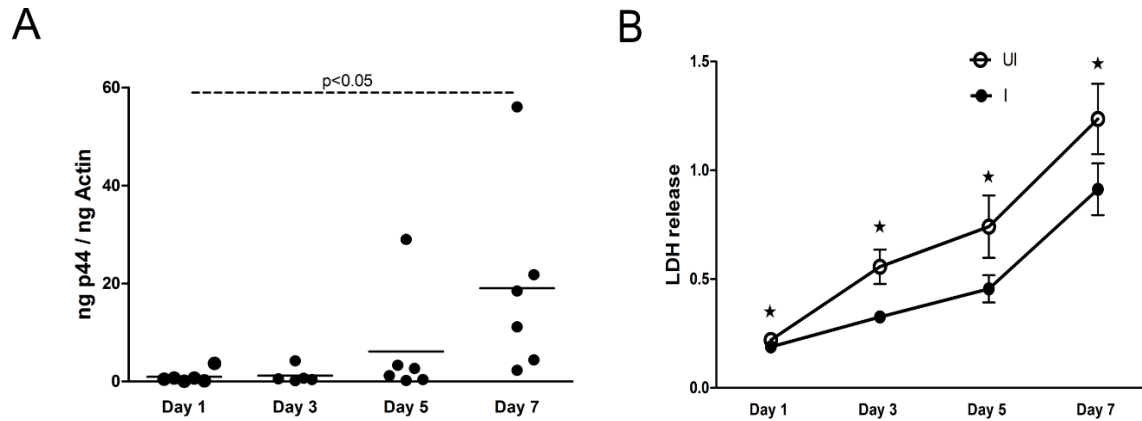


Figure 5. *A. phagocytophilum* Infection Decreases Cytotoxicity in MEG-01 Cells

(A) QRT-PCR showing bacterial load in infected-MEG-01 cells at different days (1, 3, 5, 7) p.i.. Each circle represents an individual experimental sample taken from a single culture well performed in duplicates.

The *p44* DNA levels from *A. phagocytophilum* were normalized to human beta-actin levels.

(B) LDH release assay performed with cell culture supernatants collected from uninfected (UI) or *A. phagocytophilum*-infected (I) at different time points (day 1, 3, 5, 7) p.i.

The values on the Y-axis show the absorbance for LDH release (samples minus background absorbance) in the cell culture supernatants. The culture medium was not changed daily. The mean values plotted on the graph represent the average of 6 replicates performed in duplicates. The asterisk above the error bar indicates statistical significance ($P < 0.05$) between UI and I sample from the same time point.

Infection of MEG01 Cells with *A. phagocytophilum* Modulate the Expression of Cell Cycle Genes

To observe the cellular proliferation of MEG-01 cells, the expression of cell cycle genes from various phases, notably G1 and S phases, was analyzed. Previously, *A. phagocytophilum* was observed to decrease cell proliferation of MEG-01 cells (Granick et al., 2008). Therefore, this study tested whether *A. phagocytophilum* modulates cell cycle genes for its survival in these cells. QRT-PCR analysis revealed that at the earlier time point of infection (day 1 p.i.), mRNA levels for cell cycle genes such as CDC2 (Figure 6A), CDC25A (Figure 6B), Cyclin E (Figure 6C), CDK5 (Figure 6D), CDK8 (Figure 6E) and Cyclin G1 (Figure 6F) were significantly upregulated in *A. phagocytophilum*-infected cells in comparison to uninfected cells. However, at the late time point of infection (day 7 p.i.), the mRNA transcript levels for the cell cycle genes

were significantly ($P<0.05$) down-regulated in *A. phagocytophilum*-infected cells in comparison to uninfected MEG-01 cells (Figure 6A-6F). These results show that *A. phagocytophilum* differentially modulates the expression of cell cycle genes at various stages of infection in megakaryocytic cells.

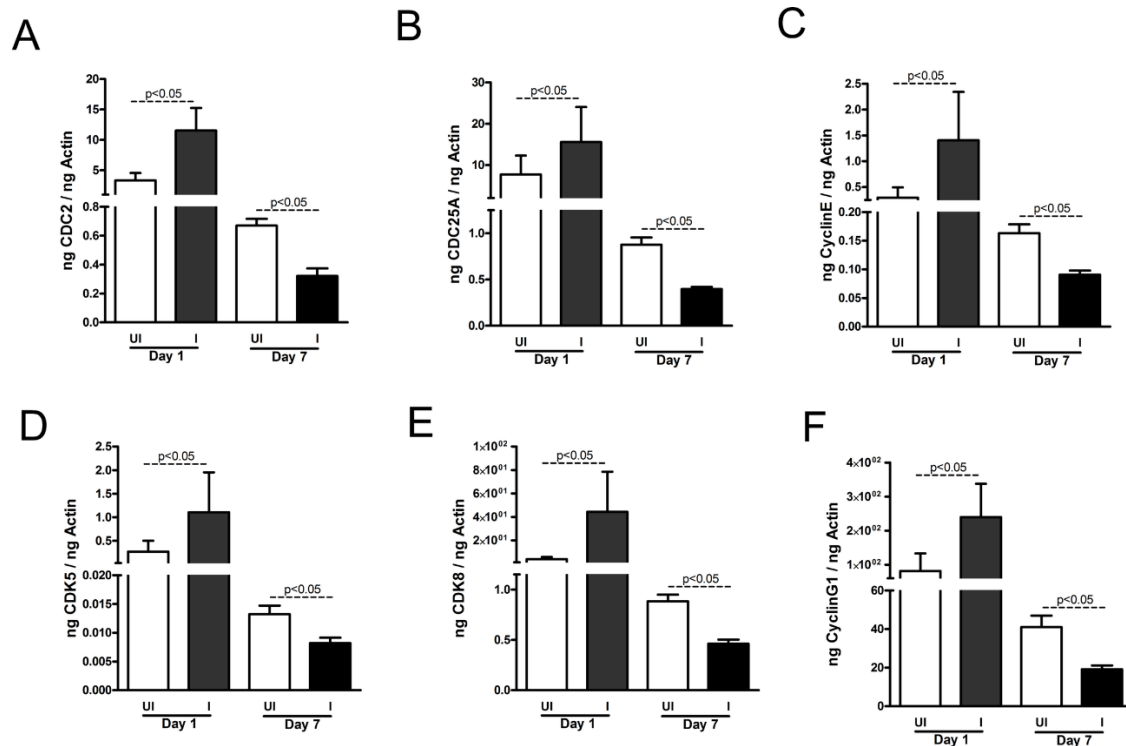


Figure 6. *A. phagocytophilum* Infection Alters the Gene Expression of Cell Cycle Genes in MEG-01 Cells

(A-F) Expression of CDC2, CD25A, Cyclin E, CDK5, CDK8, and Cyclin G1.

QRT-PCR analysis showing the expression levels in uninfected (UI) and *A. phagocytophilum*-infected (I) MEG-01 cells at two different time points of infection. The normalization of mRNA levels of cell cycle genes was done with human beta-actin. The qPCR data shown above was performed on six biological samples with duplicates generated from three independent experiments.

To observe the viability of the MEG-01 cells during infection, viability assays with trypan blue staining along with cytotoxicity were performed. Live (viable) cells exclude the dye, but dead (non-viable) cells or cells with a compromised membrane are stained with intense blue color. This viability assay also revealed significantly ($P < 0.05$) increased number of live *A. phagocytophilum*-infected MEG-01 cells when compared to uninfected controls (Figure 7).

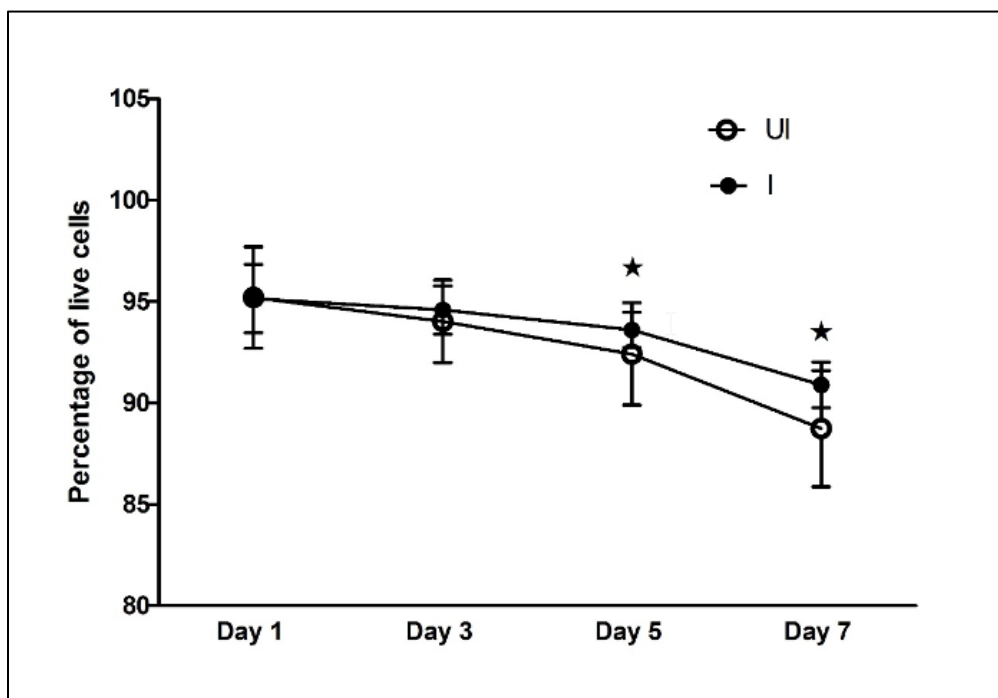


Figure 7. Viability Assay of MEG-01 Cells Upon *A. phagocytophilum* Infection

Trypan blue staining results performed with uninfected or *A. phagocytophilum* infected MEG-01 cells at different time points of infection (day 1, 3, 5, 7) p.i is shown. Values on the Y-axis show the percentage of live cells. The percentage data is the average reading of 24 microscopic fields obtained from 3 wells per group performed in duplicates.

***A. phagocytophilum* Modulate the Expression of Class I PI3 Kinases**

A. phagocytophilum activates the PI3 kinase/AKT signaling pathway in neutrophils (Sarkar et al., 2012). Therefore, this study tested whether *A. phagocytophilum* affects the expression of PI3KCA (p110 alpha catalytic subunit) and PI3KR1 (p85 regulatory subunit) subunits of class I PI3 kinases. QRT-PCR analysis revealed that *A. phagocytophilum* significantly ($P < 0.05$) upregulated mRNA levels of both PI3KCA and PI3KR1 (Figure 8A) at the earlier time point (day 1 p.i.) of infection in comparison to uninfected MEG-01 cells. Immunoblotting assays revealed increased (~ 2-fold) PI3KCA and PI3KR1 at both time points (early and late time points p.i.) upon infection of *A. phagocytophilum* in MEG-01 cells in comparison to uninfected controls (Figure 8E).

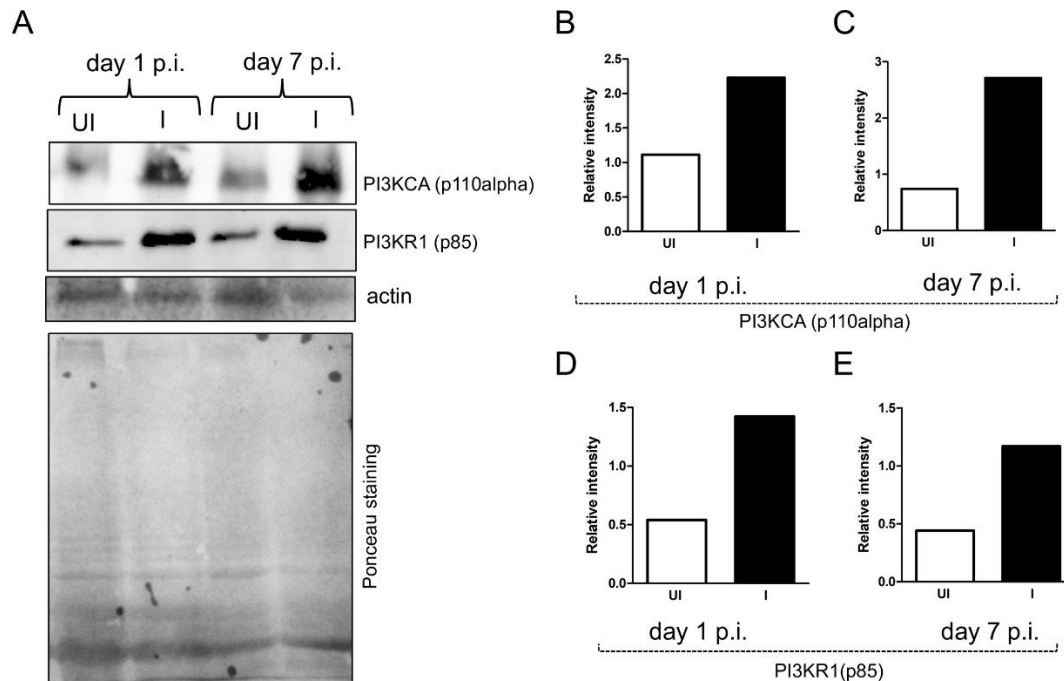


Figure 8. Expression of PI3KCA and PI3KR1 in *A. phagocytophilum* Infection

(A) Immunoblot showing levels of PI3KCA, PI3KR1 in uninfected (UI), or *A. phagocytophilum*-infected (I) MEG-01 cells at days 1 and 7 p.i. The actin (detected by immunoblotting) and Ponceau stained gel image for total protein served as a loading control.

(B and C) The densitometry analysis depicting the levels of PI3KCA at day 1 and day 7.

(D and E) The densitometry analysis depicting the levels of PI3KR1 at day 1 and day 7.

Inhibition of PI3 Kinase Affects Bacterial Loads and *A. phagocytophilum*-Mediated Modulation of Cell Cycle Gene Expression

To test whether *A. phagocytophilum* modulates cell cycle gene expression in the megakaryocytic cell line through PI3 kinase signaling, we used LY294002, a potent inhibitor of phosphoinositide 3-kinases. *A. phagocytophilum*-infected MEG-01 cells were treated with LY294002. With phase-contrast microscopy, no significant changes in cellular morphology were observed when treated with the inhibitor at both early and late time points (Figure 9).

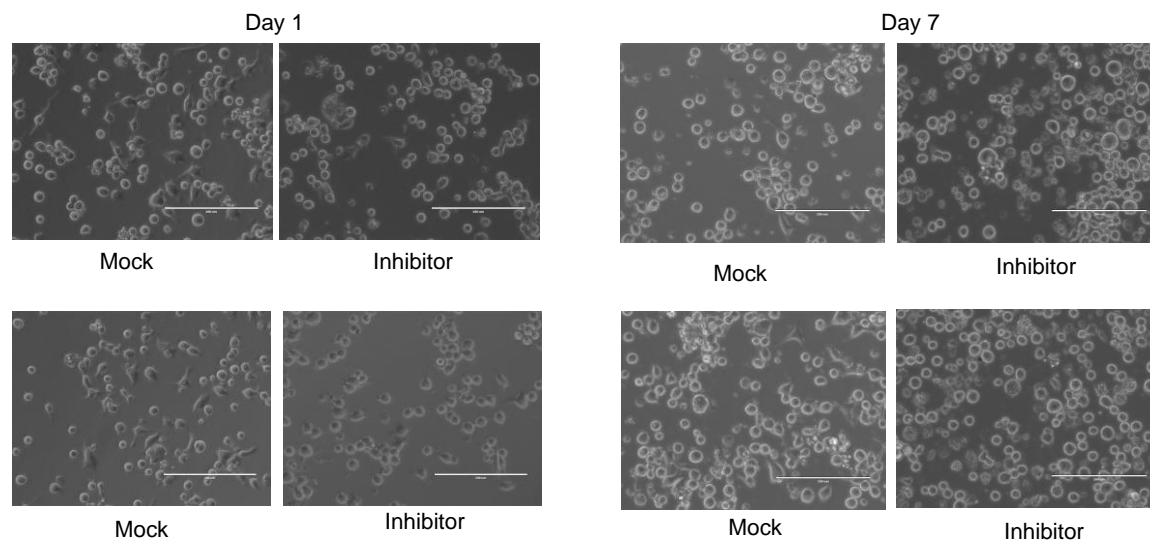


Figure 9. LY294002 Treatment Had No or Reduced Cytotoxicity on MEG-01 Cells

Phase-contrast microscopic images of *A. phagocytophilum*-infected mock-treated (DMSO) or LY294002-treated (PI3K inhibitor; at 100 μ M concentration) MEG-01 cells on different days (1 and 7 p.i.) are shown. Two representative images from each group and at each time point are shown. The scale bar represents 200 μ m in each image.

The *A. phagocytophilum*-infected MEG-01 cells treated with the mock solution were used as controls. QRT-PCR analysis revealed that *A. phagocytophilum*-infected LY294002-treated cells had a significant ($P<0.05$) reduction in the expression of CDC2 (Figure 10B), CDC25A (Figure 10C), Cyclin E (Figure 10D), CDK5 (Figure 10E), CDK8 (Figure 10F) and Cyclin G1 (Figure 10G) at an earlier time point of infection (day 1 p.i.). However, no differences in the *A. phagocytophilum* burden were evident at day 1 p.i. in LY294002-treated MEG-01 cells when compared to the mock-treated controls (Figure 10A). Significant ($P<0.05$) reduction in the mRNA levels for all tested cell cycle genes was also evident in the *A. phagocytophilum*-infected LY294002-treated cells in comparison to mock-treated controls at a later time point (day 7 p.i.) of infection.

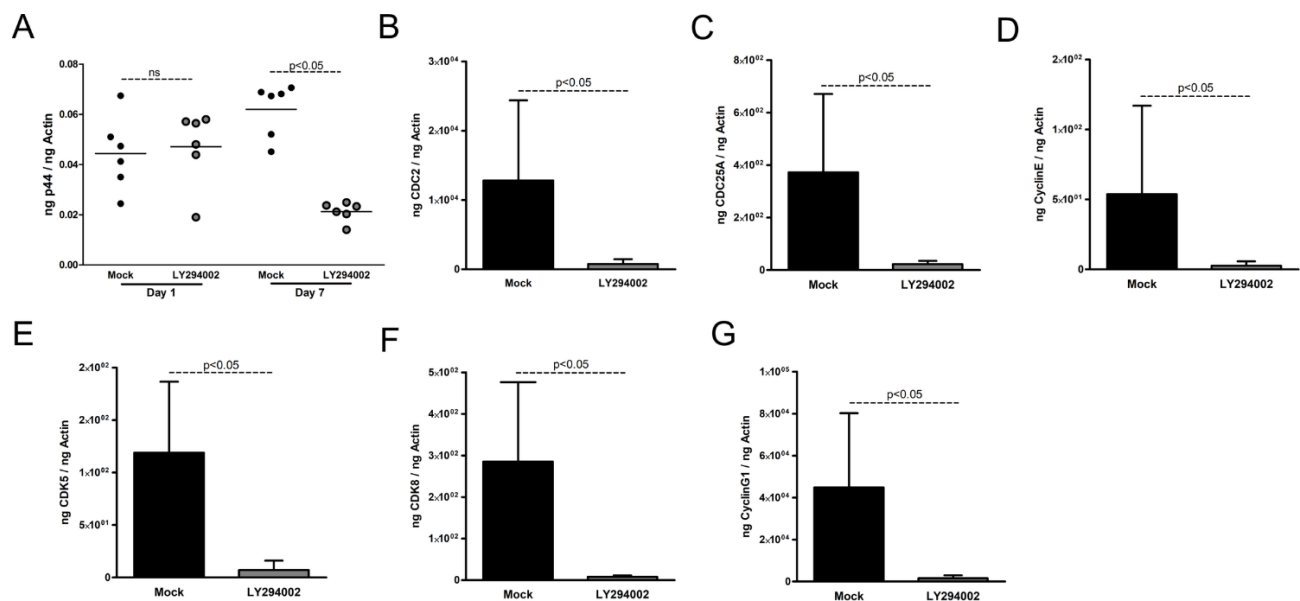


Figure 10. Inhibition of PI3 Kinases Reduces the Expression of Cell Cycle Genes and *A. phagocytophilum* Replication in MEG-01 Cells

(A) QRT-PCR analysis showing bacterial loads in *A. phagocytophilum*-infected MEG-01 cells treated with mock (DMSO-treated) or LY294002 (PI3K inhibitor) at days 1 and 7 p.i. *A. phagocytophilum* p44 DNA loads were normalized to human beta-actin. Each circle represents one individual sample.

(B-G) Expression of CDC2, CD25A, Cyclin E, CDK5, CDK8, and Cyclin G1.

QRT-PCR analysis showing expression levels in *A. phagocytophilum*-infected mock or LY294002 (PI3K inhibitor)-treated MEG-01 cells at day 7 p.i. The qPCR data shown above was performed on six biological samples with duplicates generated from three independent experiments. The P-value ($P<0.05$) calculated from the Student's t-test was considered significant.

Inhibition of PI3 Kinase Reduces Infection-Associated Cytotoxicity

A. phagocytophilum is reported to extend the life of neutrophils by modulating gene expression in these short-lived cells, thereby maximizing its survival and replication time in these cells (Carlyon and Fikrig 2006; Rikihisa, 2010). Therefore, this study tested whether *A. phagocytophilum* has a similar effect in megakaryocytes. Supernatants were collected from *A. phagocytophilum*-infected MEG-01 cells and uninfected cells at various time points p.i. and tested for lactate dehydrogenase activity (LDH cytotoxic assay). An increase in the LDH release indicates more release of LDH in the supernatant from dead cells. Assays performed with supernatants collected from *A. phagocytophilum*-infected MEG-01 cells treated with LY294002 on day 1 showed reduced LDH release compared to the mock-treated cells (Figure 11A). Though, assays performed with supernatants collected from *A. phagocytophilum*-infected MEG-01 cells treated with LY294002 at a late time point (day 7) showed increased LDH release in comparison to the mock-treated cells (Figure 11A). These results suggest that *A. phagocytophilum* modulates the expression of cell cycle genes and reduces the cellular cytotoxicity through PI3 kinase signaling to arrest the growth of megakaryocytes for its survival in these cells.

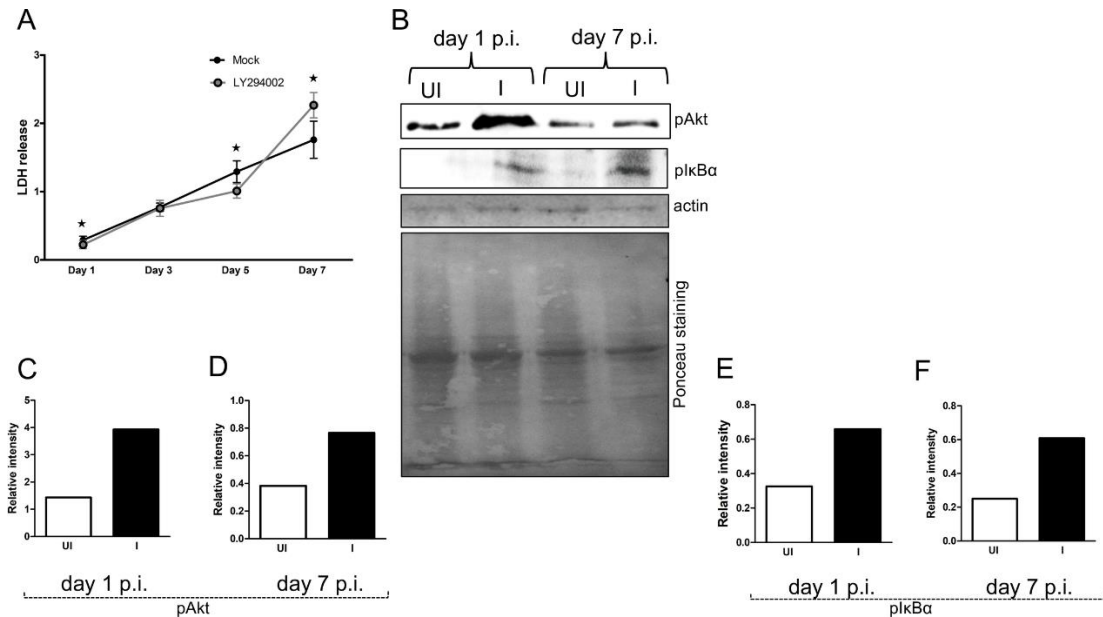


Figure 11. *A. phagocytophilum* Infection Increases Activation of Akt and NF-κB

(A) LDH assay performed with *A. phagocytophilum*-infected cell culture supernatants collected from mock-treated (mock) or LY294002 (PI3K inhibitor) treated at different days (1, 3, 5, 7). Y-axis shows the absorbance for the release of LDH in the cell culture supernatants. The data displayed above was performed on three biological samples with duplicates generated from three independent experiments. (B) Immunoblot showing the levels of phosphorylated Akt and IκB alpha in uninfected (UI) or *A. phagocytophilum*-infected (I) MEG-01 cells. (C and D) The densitometry analysis depicting the levels of pAkt at day 1 and day 7. (E and F) The densitometry analysis showing levels of plkBα at day 1 and day 7.

***A. phagocytophilum* Infection Tempers PI3K-Akt-NF-κB Signaling in MEG-01 Cells**

The intracellular bacterium, *A. phagocytophilum* activates PI3K/Akt and NF-κB survival pathways in neutrophils (Sarkar et al., 2012). NF-κB is an essential transcriptional regulator that regulates the metabolism of cell cycle genes (Joyce et al., 2001). Therefore, this study tested whether the upregulation of PI3 kinase subunits (PI3KCA and PI3KR1) upon *A. phagocytophilum* infection (Figure 5A) activates Akt and NF-κB levels. Immunoblot showed that upon *A. phagocytophilum* infection of MEG-01 cells, approximately 2-3-fold increased level of phosphorylated Akt (Figure 11B-11D), and IκB (Figure 11B, 11E and 11F) was evident at both days 1 and day 7 p.i. However, upon inhibition of PI3K kinases, a reduced level of phosphorylated Akt (~ 2-fold) was

observed in *A. phagocytophilum* infected LY294002-treated cells in comparison to mock controls at both time points (Figure 12A–12C). These results collectively suggest that there is a differential modulation of cell cycle gene expression, and also, their reduction in their cellular toxicity in megakaryocytes is mediated through PI3K-Akt-NF- κ B signaling.

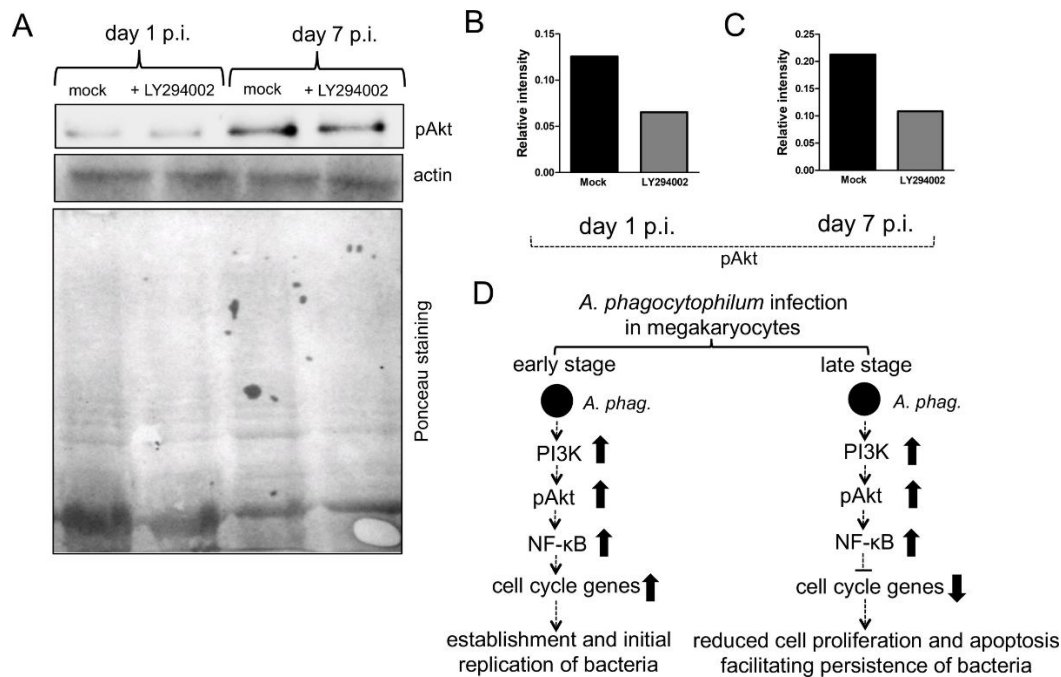


Figure 12. *A. phagocytophilum* Infection Modulates PI3K-Akt-NF- κ B Signaling in MEG-01 Cells

(A) Immunoblot images showing levels of phosphorylated Akt in *A. phagocytophilum*-infected mock or LY294002-treated MEG-01 cells at both time points of infection (days 1 and 7 p.i.). Actin levels and Ponceau stained gel image for total protein serve as a loading control.

(B) Densitometry showing the levels of phosphorylated Akt at day 1 p.i.

(C) Densitometry showing the levels of phosphorylated Akt at day 7 p.i.

(D) Model showing the role of *A. phagocytophilum*-infection on the expression of megakaryocyte cell cycle genes at early and later stages of infection.

2.3 DISCUSSION

Human pathogens have developed various strategies to manipulate host-cell function, including influence on cell cycle, presumably for their benefit. The observation that *A. phagocytophilum* initially upregulates and then down-regulates cell cycle genes in the later stages of infection in MEG-01 cells suggests an interesting model to understand host-pathogen interactions in the cells of hematopoietic origin.

Rickettsial pathogen *Ehrlichia chaffeensis* modulates cell cycle genes to survive in the human monocytic cell line (Zhang et al., 2004). Variable expression of cell cycle genes was evident upon *E. chaffeensis* infection in a human monocytic cell line with CDC2, CDK5, CDK8, and cyclin G1 down-regulated at 1h p.i and cyclin E1, cyclin E2, and CDC25 upregulated between 7-24 h p.i. *A. phagocytophilum* is very closely related to *Ehrlichia species* (Dumler, 2005; Ismail et al., 2010; Rikihisa, 2010). The significance of the variable regulation of cell cycle genes upon *A. phagocytophilum* infection in MEG-01 cells at different time points of infection is currently not understood. This study hypothesized that *A. phagocytophilum* upregulates cell cycle genes and reduces cellular toxicity during the initial phase of infection. The downregulation of the cell cycle genes during the later phases suggests that *A. phagocytophilum* reduces megakaryocytes' cell proliferation as the infection progresses. This notion supports the previous observation of the reduced proliferation of MEG-01 cells upon *A. phagocytophilum* infection (Granick et al., 2008). The decrease in the cellular toxicity in MEG-01 cells at later time points of *A. phagocytophilum* infection suggests that the bacterium may hijack these cells presumably to extend the life of megakaryocytes without allowing increased proliferation. This "arrest mechanism" by *A. phagocytophilum* may result in controlling megakaryocytes in a stagnant stage that could facilitate the persistence of this bacterium for a longer time in these cells before escaping to infect other cells. With any of these hypotheses, this study provides an interesting model to understand the biology of the modulation of cell cycle genes in the persistence of a human pathogen in megakaryocytes.

The data in this study shows that PI3KCA and PI3KR1 mRNA levels are upregulated at an earlier time point (day 1 p.i.) and down-regulated at a later time point (day 7 p.i.) of *A. phagocytophilum* infection in MEG-01 cells. Decreased PIK3CA and

PI3KR1 transcripts at day 7 p.i. could impact the reduced production of these proteins at later time points. However, the observation of reduced expression of cell cycle genes, decreased *A. phagocytophilum* burden, and increased cellular toxicity upon inhibition of PI3 kinases at a later time point of infection suggests a role for PI3 kinase-mediated signaling pathways in facilitating the survival of this bacterium and cell cycle regulation in megakaryocytes.

Multiple studies have highlighted the role of PI3 kinases in the progression of the cell cycle (Chang et al., 2003; Liang and Slingerland, 2003; van Opstal and Boonstra, 2006). The upregulation of Class I PI3 kinases subunits PIK3CA and PI3KR1 in this study suggests an increased PI3 kinase activity (at early time points) in MEG-01 cells upon *A. phagocytophilum* infection. The observation of increased pAKT levels (Figure 11B) at an earlier time point (day 1 p.i.) further supports this notion. PI3K/AKT pathway regulates NF- κ B activity (Ozes et al., 1999). NF- κ B is one of the significant inducible transcription factors that regulate cellular metabolism, including genes involved in apoptosis, proliferation, differentiation, and host immune responses (Aggarwal, 2000). The increase in PI3K/AKT activity may subsequently influence NF- κ B activation. The activation of NF- κ B has been reported to be involved in cell cycle progression and regulation (Joyce et al., 2001). The data in this study suggest that *A. phagocytophilum* activates Class I PI3 kinases and increases AKT activity at an early point of infection of MEG-01 cells. The observation of no differences in the pAKT levels between uninfected and *A. phagocytophilum*-infected MEG-01 cells at day 7 p.i. suggests that early AKT activation may be enough to influence signaling to NF- κ B for the downregulation of cell cycle genes at the later time point of infection. Thus, it would be interesting to determine whether *A. phagocytophilum* differentially activates PI3K/AKT signaling that regulate NF- κ B-mediated cell cycle gene regulation in megakaryocytes.

The intracellular bacterium, *A. phagocytophilum*, secretes several effector proteins such as Ankyrin repeat protein, AnkA, into mammalian cells (Rikihisa, 2010). AnkA plays an essential role in the modulation of host cell gene expression, so the role of AnkA in *A. phagocytophilum*-mediated modulation of cell cycle genes in MEG-01 cells needs to be explored (Rikihisa, 2010). The proposed role for *A. phagocytophilum* effector proteins in cell cycle gene regulation is not unique to this bacterium. Several

studies have shown that some bacterial pathogens secrete cyclomodulins that could function as inhibitory or stimulatory molecules in cell cycle regulation (Oswald et al., 2005). Upon *A. phagocytophilum* infection, the increase in PI3K activity levels leads to enhanced Akt activation, which subsequently increases NF- κ B activation. The activated NF- κ B translocates to the nucleus and may directly or indirectly regulate cell cycle gene progression. The upregulation of cell cycle genes at an early stage of infection could facilitate *A. phagocytophilum* to establish and replicate initially. The downregulation of cell cycle genes at a later stage of infection could protect cells from apoptosis and allow the bacterium to persist for extended period in these cells. In summary, this study shows that *A. phagocytophilum* persists in megakaryocytes by modulating cell cycle gene expression via class I PI3 kinases, and inhibition of PI3 kinases affects bacterial replication in these cells. This study not only provides evidence for greater understanding of *A. phagocytophilum* infection in megakaryocytes but might lead to the development of newer strategies to interrupt its survival in the cells of hematopoietic origin.

2.4 EXPERIMENTAL PROCEDURES

Bacterial Isolates and Megakaryocytic Cell Line

HZ-1 strain of *A. phagocytophilum* was used in this study. This isolate was maintained in HL60 cells in RPMI media with 10% FBS as described (Severo et al., 2013). The human bone marrow-derived megakaryoblast cells (MEG-01 cells) were purchased from ATCC and cultured in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS). The megakaryocytic cell line, MEG-01 cells were maintained inside 37°C incubator with a 5% CO₂ supply.

***In vitro* MEG-01 Cell Line Infection Experiments**

Anaplasma phagocytophilum HZ-1 was maintained in the human promyelocytic cell line (HL-60). Host cell-free bacteria were isolated from infected HL-60 cells and used for *in vitro* infection as described (Sultana et al., 2010; Thomas and Fikrig, 2007). Briefly, *A. phagocytophilum*-infected HL-60 cells were centrifuged for 10 mins at 4,000 rpm, and the cell pellet was re-suspended in IMDM, lysed by 6-8 passages through 25-gauge,

followed by 6-8 more passages through 27-gauge syringes. The lysed cells were centrifuged at 1,200 rpm for 3 mins to obtain host cell-free bacteria in supernatants. The host-cell free supernatant was used for infection of MEG-01 cells. Inhibitor studies were carried using LY294002, a common inhibitor of PI3 kinases. MEG-01 cells were pre-treated with 100 μ M of LY294002 for 4 hours, then were infected with host-cell-free *A. phagocytophilum*. The infected cells were treated with similar amounts of DMSO as the mock controls. The concentration of LY294002 (EMD Millipore Sigma) used in this study had no or marginal effects, compared with mock controls, on cellular viability during infection. However, ~ 50-60 % of LY2849002-treated cells were still viable at day 7 p.i..

Total RNA/DNA Extractions and Gene Expression Analysis

Total RNA from MEG-01 cells was generated using the Aurum Total RNA Mini kit (BioRad, USA) following the manufacturer's instructions. RNA was converted to cDNA using the iSCRIPT cDNA synthesis kit (BioRAD, USA), which was used for the qPCR template for quantifying transcripts of all the cell cycle genes, PIK3CA (*pi3k* 110 α) and PIK3R1 (*p85*) subunits. The housekeeping gene, beta-actin, was used as a normalizing control for quantifying transcripts. QRT-PCR was performed using the CFX96 QPCR system (BioRad) and iQ-SYBR Green Supermix (BioRad, USA). To quantify the *Anaplasma* load, genomic DNA from *A. phagocytophilum*-infected MEG-01 cells was extracted using the DNeasy kit (QIAGEN) and processed for PCR primers specific for the *A. phagocytophilum* *p44* gene. A standard curve was then generated using 10-fold serial dilutions of the quantifying genes for qPCR analysis.

Cytotoxicity Assays

The cytotoxicity assays were conducted for uninfected or *A. phagocytophilum*-infected MEG-01 cells and measured using a lactate dehydrogenase (LDH) assay kit (Pierce, USA) following the manufacturer's protocol. The final absorbance was calculated after subtracting the respective background from blank controls. A similar procedure was adapted to measure LDH release from uninfected or *A. phagocytophilum*-infected cells (treated with LY2849002 or mock control) collected at different time points (day 3, 5,

and 7 p.i.). All of the cultures were started simultaneously at the same time and without the change in the culture medium.

Trypan Blue Staining

Trypan blue staining (Trypan blue 0.4% solution, Gibco, USA) was performed to estimate the percentage of live cells in each experimental group. Briefly, 1×10^5 MEG-01 cells were plated in triplicates and infected with *A. phagocytophilum*, as described in the earlier section. Cells were then washed with 1X phosphate buffer saline (PBS), 400 μ l of trypan blue solution was added and incubated for 10 minutes. The PBS and trypan blue stain were removed, and 200 μ l of fresh 1X PBS was added to the wells. The wells were imaged with the EVOS imaging system (Thermofischer, USA). Representative images (24 images from 8 fields/well) were captured and analyzed for the number of live and dead cells. The total percentage of live cells was quantified.

Immunoblotting

Total cell lysates from uninfected or *A. phagocytophilum*-infected MEG-01 cells at various time points were prepared in modified RIPA buffer (BioExpress) supplemented with an EDTA-free protease inhibitor cocktail (Sigma, USA). Protein concentrations were determined by the BCA protein assay kit (Pierce, USA). A similar concentration of total lysates from each group was loaded onto a 12% non-reducing SDS-PAGE gel and processed for immunoblotting. Antibodies against PIK3CA and PIK3R1 were purchased from Cell Signaling Technologies (USA). The pAKT and the rabbit polyclonal anti-IgG HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). ECL reactions were performed using the Advanced WesternBright ECL substrate kit (Advansta, USA), and chemiluminescence reaction was imaged using Chemidoc MP imager (BioRad). The total protein profile measured by Coomassie-stained gels or beta-actin levels. The densitometry analysis was achieved using Image Lab software by measuring their intensities against the loading control (actin band).

Statistics

The statistical analysis was performed using Student's *t*-test using GraphPad Prism6 software and Microsoft Excel 2016. For the comparison of two means, the non-paired Student *t*-test was performed. The P-value of <0.05 was considered significant in all tests. Wherever necessary, statistical tests and P values are reported.

CHAPTER 3

ARTHROPOD TRANSCRIPTIONAL ACTIVATOR PROTEIN-1 (AP-1)

AIDS TICK-RICKETTSIAL PATHOGEN SURVIVAL IN THE COLD

3.1 INTRODUCTION

Human anaplasmosis, caused by *Anaplasma phagocytophilum*, is one of the most common arthropod-borne diseases in the United States. It is caused by a rickettsial bacteria, *A. phagocytophilum*, that resides in *I. scapularis* (Anderson and Magnarelli, 2008; Dumler et al., 2005; Sonenshine and Roe, 2014). *A. phagocytophilum* is transmitted to humans from an infected black-legged tick. Ticks spend most of their life off the hosts in a natural microenvironment, including extreme cold and freezing temperatures (Anderson and Magnarelli, 2008; Needham and Teel, 1991; Sonenshine, 1993). When *A. phagocytophilum* enters a tick body, it enters the gut and colonizes the salivary glands (Anderson and Magnarelli, 2008; Hodzic et al., 1998). This bacterium is maintained transstadially (Bakken and Dumler, 2015; Brownstein et al., 2003; de la Fuente et al., 2016a; Hodzic et al., 1998; Noda et al., 1997; Yuval and Spielman, 1990). The signaling mechanisms that *A. phagocytophilum* uses to survive in its vector host during overwintering stages are not clearly understood. A tick antifreeze molecule, IAFGP (*I. scapularis* antifreeze glycoprotein) induced by *A. phagocytophilum* helps these ticks survive better in the cold. Also, transgenic expression of IAFGP rendered cold tolerance in mice and flies (Heisig et al., 2015; Neelakanta et al., 2012).

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Several studies reported that *A. phagocytophilum* modulates the expression of several genes in vertebrate and invertebrate hosts for its survival (Taank et al., 2017). Several other studies focused on understanding the modulation of signaling pathways during *A. phagocytophilum*-tick interactions (Cabezas-Cruz et al., 2016; Cabezas-Cruz et al., 2017; de la Fuente et al., 2017; Mansfield et al., 2017; Chavez et al., 2015; Shaw et al., 2017). However, studies delineating the molecular gene regulation of *iafcp* upon *A. phagocytophilum* infection and survival in ticks have not been studied.

There are several transcriptional factors encoded in the genome of *I. scapularis* ticks (Gulia-Nuss et al., 2016). Activator protein-1 (AP-1) family of proteins are sequence-specific DNA binding transcription factors. They maintain the basal expression of several genes and are the crucial molecules that drive cellular processes, differentiation, proliferation, immune regulation, and apoptosis (Angel and Karin, 1991; Karin et al., 1997; Kouzarides and Ziff, 1988; Kramer et al., 2015; Wagner and Eferl, 2005; Ye et al., 2014; Zarubin and Han, 2005). In humans, the AP-1 family comprises homodimer and heterodimer of Jun and Fos, including c-Jun, JunB, and JunD; and c-Fos, FosB, Fra-1, and Fra-2. Other molecules in the AP-1 family include ATF and MAF (Karin et al., 1997; Kouzarides and Ziff, 1988; Wagner and Eferl, 2005; Ye et al., 2014; Zarubin and Han, 2005). They contain a highly conserved dimeric basic leucine zipper DNA-binding domain (Karin et al., 1997; Kouzarides and Ziff, 1988). These are cellular regulators frequently involved in dimerization, DNA-binding, transactivation, and ubiquitination. The AP-1 protein complex binds to specific promoters of several genes, activates or represses them (Karin et al., 1997; Kouzarides and Ziff, 1988; Ye et al., 2014). AP-1 protein is a highly conserved transcription factor across various organisms with varied functions (Britton et al., 2017; Kockel et al., 2001; Xiang et al., 2014). Recent studies have reported vertebrate AP-1 in host-pathogen interactions, including a role in *Chlamydia pneumoniae* persistence in human epithelial cells (Hess et al., 2004; Kramer et al., 2015; Xiang et al., 2014). The precise role of AP-1 in tick biology and the host-pathogen interactions in ticks remains to be studied. Here, this study aimed to examine the regulatory role of AP-1 in *Ixodes scapularis*. *A. phagocytophilum* uses this arthropod AP-1 molecule as a molecular switch to regulate its vector antifreeze gene and help the ticks survive better in the cold.

3.2 RESULTS

Genomic Organization of *iafgp* Gene

Bioinformatics analysis of the genomic region (1108462552178 genomic scaffold) in *I. scapularis* derived from the whole-genome shotgun sequence (GenBank accession number DS704943, 61161 bp) revealed two putative (CDS-1 and CDS-2) exons in the coding sequences of *iafgp* gene (Figure 13A). A strong TATA-binding region (TATATAAT, 18360-18367 bp) in the putative *iafgp* promoter region was identified using bioinformatics tools. More than 100 different sites for various transcription factors were predicted using TFBIND (Tsunoda and Takagi, 1999). These include, but are not limited to, two AP-1 binding sites (TGACGCT and TGTCGCA) with positions 18054-18060 bp and 18167-18173 bp, respectively. The heat shock factor-1 (HSF-1) binding site (TGTGCCTTCC) corresponds to the genomic position 18113-18122 bp (Figure 13A). The eukaryotic promoter for the TATA consensus sequence is TATA(A/T)A(A/T)(A/G) (Basehoar et al., 2004). The consensus sequence for the AP-1 binding region is TGAC/GTCA (Shaulian and Karin, 2001). Of the many putative sites, the TATA box and AP-1 sites matched the upstream sequence of *iafgp* gene very closely. AP-1 is a global regulator (Hess et al., 2004; Kramer et al., 2015; Xiang et al., 2014). Due to the presence of the AP-1-binding site that is upstream to the putative *iafgp* TATA box, this study aimed to study the role of AP-1 in vector-pathogen interactions.

Expression of *ap-1* Transcripts in *Ixodes scapularis* Life Stages

To check the *ap-1* expression at various developmental stages of tick life cycle, this study used unfed uninfected ticks from different developmental stages. Ticks encode *ap-1* gene in their genome (GenBank acc. no. XM_002404527). As shown by QRT-PCR analysis, larvae and nymphs expressed higher levels of *ap-1* mRNA ($P < 0.05$) when compared to both adult males and females (Figure 13B). This differential expression indicates differential regulation of *ap-1* mRNA at various developmental stages of ticks. Furthermore, analysis in whole unfed nymphs (Figure 13C) or salivary glands (Figure 13D) isolated from unfed nymphs revealed significantly higher levels ($P < 0.05$) of *ap-1* transcripts upon *A. phagocytophilum* infection of ticks compared to uninfected controls.

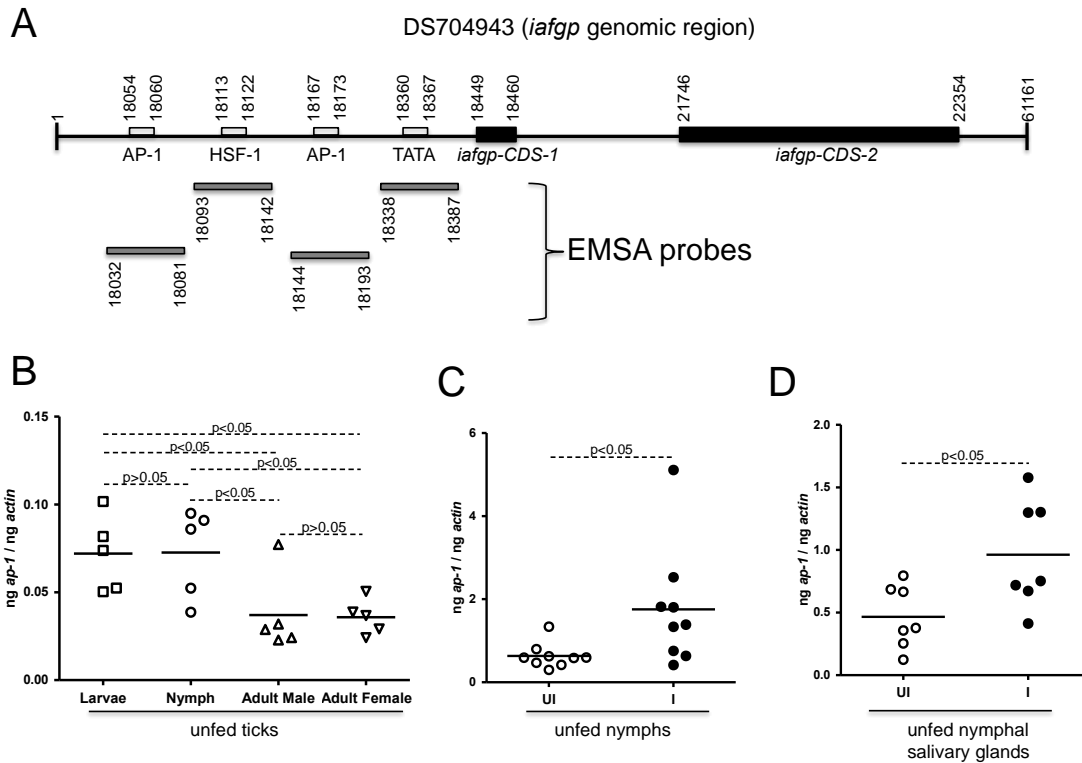


Figure 13. *ap-1* mRNA is Upregulated in *A. phagocytophilum*-Infected Unfed Nymphs

(A) Genomic region in *I. scapularis* 1108462552178 genomic scaffolds derived from WGS (not to scale). The rectangular boxes on the top of the bold line indicate AP-1, HSF-1 binding sites, or TATA region. The location and position of sequences used to generate EMSA probes are indicated. Two putative exons for the *iafgp* gene sequences are shown. All position numbers correspond to GenBank accession number DS704943.

(B) QRT-PCR analysis showing levels of *ap-1* transcripts in tick developmental stages (naïve unfed larvae, nymphs, adult male, and adult female ticks).

(C) Gene expression in naïve (UI) and *A. phagocytophilum*-infected (I) whole nymphs.

(D) Gene expression of salivary glands obtained from unfed naïve and *A. phagocytophilum*-infected whole nymphs.

The larvae data in panel B was generated from pooled tick samples (5-7 ticks/pool). Each square, triangle, circle, or inverted triangle represents one tick sample performed in duplicates.

Sequence Analysis of *I. scapularis* AP-1 with Other Orthologs and AP-1-Like Proteins

The tick AP-1 annotated amino acids sequence (XP_002404571) was used to analyze the AP-1 protein domain(s) structure. Domain analysis showed that the putative AP-1 protein contained a Jun domain (1-143 aa) at the N-terminal and a basic leucine zipper (bZip) domain (156-216 aa) at the C-terminal (Figure 14A). Within the bZip domain, the DNA-binding domain (161-176 aa) and polypeptide-binding domains (175-216 aa) were observed. A BLAST search in NCBI followed by ClustalW alignment (using DNASTAR) revealed the presence of AP-1 like orthologs in the closer family of other ticks (Figure 14B); for example, *Ixodes ricinus* (JAP70916), *Amblyomma triste* (JAC33128), *Amblyomma cajennense* (JAC21012), and *Rhipicephalus microplus* (AIT40211).

A higher degree of conservation across the entire amino acid sequences was seen in *I. scapularis* AP-1 while comparing it with other ticks. AP-1 from *I. scapularis* was closest to *Ixodes ricinus* (99% identity), among others. Most of the bZIP region (both DNA and polypeptide binding regions) was found to be conserved (Figure 14B). Furthermore, we noted that *I. scapularis* AP-1 shares 43% identity with *Rattus norvegicus* (rat) AP-1, 43% identity with *Mus musculus* AP-1, 44% identity with *Macaca mulatta* AP-1, and 46% identity with *Homo sapiens* AP-1 (Figure 14B). The phylogenetic tree-derived from the ClustalW alignment revealed that *I. scapularis* AP-1 and other tick AP-1 orthologs fall within the same clade. In contrast, the AP-1 family of proteins from mice, rats, monkeys, and humans forms a different clade (Figure 14C).

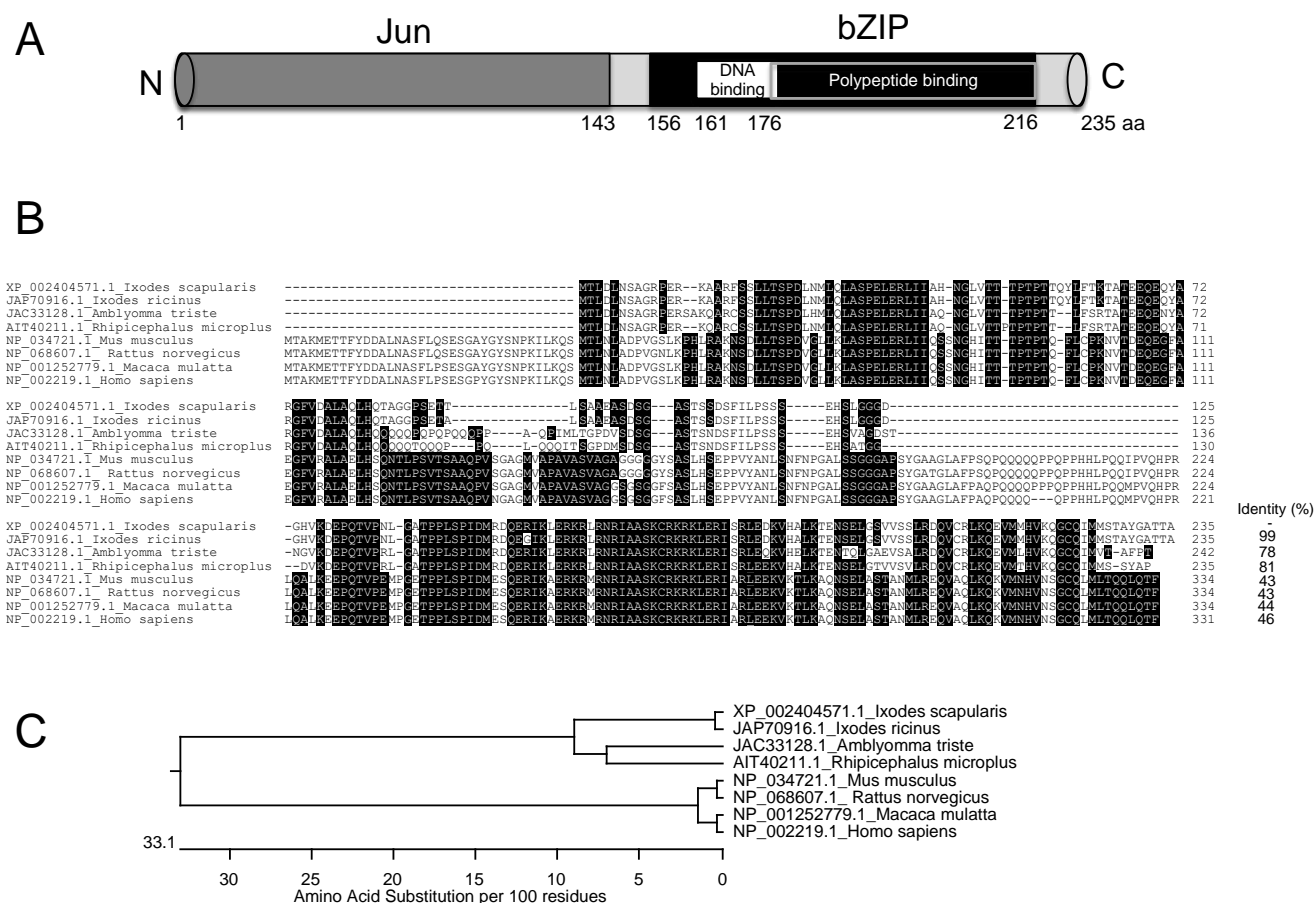


Figure 14. Alignment and Phylogenetic Analysis of *Ixodes scapularis* AP-1 with Other Orthologs

(A) Domain analysis of *I. scapularis* AP-1 showing Jun domain (1-143 aa) at N-terminus and bZIP domain at C-terminus (156-216 aa).

(B) The *I. scapularis* AP-1 amino acid sequence alignment (with other orthologs) using the ClustalW algorithm. The residues that match are shaded in black color. The total length and percent identities of the amino acid sequences are shown at the end.

(C) Phylogenetic tree shown was generated by ClustalW slow/accurate alignment method using the Gonnet algorithm as the default value for protein weight matrix using 1000 bootstraps. The scale at the bottom denotes amino acid substitutions per 100 amino acid residues.

***A. phagocytophilum* Influences *iafgp* Promoter via *I. scapularis* AP-1**

A previous study showed that *A. phagocytophilum* induces the expression of *iafgp* in ticks (Neelakanta et al., 2010). But the molecular mechanism of this gene regulation has not been elucidated. To provide evidence for the *iafgp* gene regulation, Electrophoretic mobility shift assays (EMSAs) were performed. Total nuclear extracts from naïve or *A. phagocytophilum*-infected unfed nymphs were prepared, and we tested its binding on the biotin-labeled DNA probe (the position corresponding to 18338-18387 in GenBank acc. no. DS704943) containing putative *iafgp* TATA promoter region (Figure 14A). The shift of the observed bands in the biotin-labeled DNA probe indicates the binding of nuclear proteins to the probe. EMSAs showed ~ 3-4-fold increase in the shift of *iafgp* TATA probe with incubation with nuclear proteins from *A. phagocytophilum*-infected ticks when compared to the incubation with nuclear proteins isolated from naïve ticks (Figure 15A). The incubation of the biotin-labeled *iafgp* promoter region DNA probe with putative AP-1 binding site (Figure 13A) revealed a 2-3-fold increased binding of the probe upon incubation with the total nuclear proteins isolated from *A. phagocytophilum*-infected ticks when compared to incubation with nuclear proteins isolated from naïve ticks (Figure 15B). However, biotin-labeled *iafgp* promoter region DNA probe (the position corresponding to 18032-18081 in GenBank acc. no. DS704943) containing another putative AP-1 binding site or heat shock factor-1 (HSF-1) binding site (data not shown) did not show any binding upon incubation with nuclear lysates prepared from either group. These results derived from gel shift assays indicated that *A. phagocytophilum*-mediated influence on the *iafgp* promoter is dependent on the TATA region (the position corresponding to 18360-18367) and a specific AP-1 binding region (18167-18173 in GenBank acc. no. DS704943).

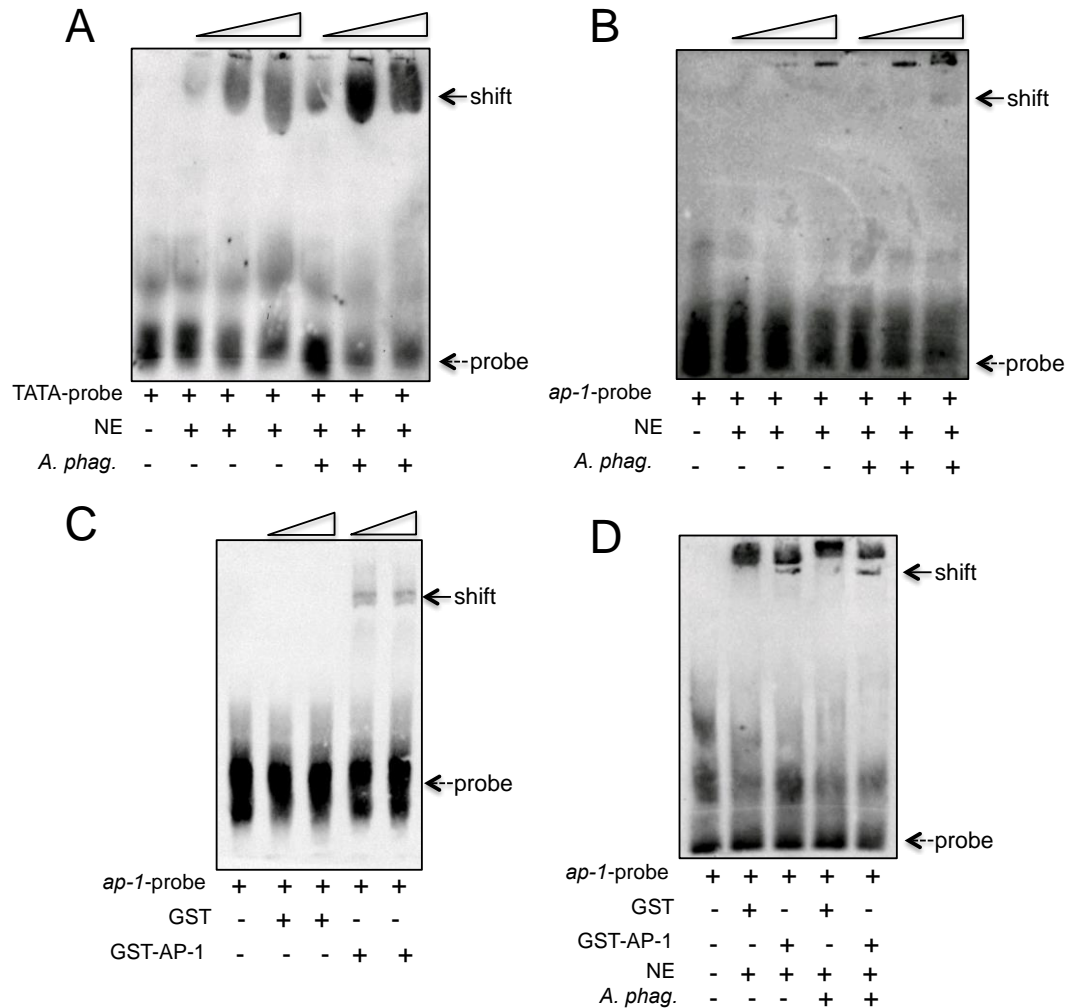


Figure 15. *A. phagocytophilum* and AP-1 Influence *iafgp* Promoter

(A) Gel shift assay results showing enhanced binding of *iafgp* TATA-probe upon incubation with nuclear proteins isolated from *A. phagocytophilum*-infected nymphs compared to the naïve nymphs. The biotinylated probe from the *iafgp* TATA-binding promoter region (DS704943, 18338-18387 bp) and nuclear proteins from naïve or *A. phagocytophilum*-infected nymphs were used in EMSAs. The wedges represent increasing amounts of nuclear protein lysate (1, 3, and 5 µg).

(B) EMSAs with biotinylated *iafgp* promoter probe containing AP-1 binding site (DS704943, 18144-18193 bp) and nuclear proteins (1, 2, and 3 µg) from naïve or *A. phagocytophilum*-infected nymphs are shown.

(C) EMSAs with biotinylated *iafgp* promoter probe containing AP-1 binding site (DS704943, 18144-18193 bp) and recombinant GST or GST-AP-1 protein (1 and 1.5 µg) is shown.

(D) EMSAs with biotinylated *iafgp* promoter probe containing AP-1 binding site (DS704943, 18144-18193 bp), recombinant GST or GST-AP-1 protein (1.5 µg), and nuclear proteins (3 µg) prepared from naïve or *A. phagocytophilum*-infected nymphs are shown.

The dotted arrow indicates a free probe, and a solid arrow indicates a shift. NE indicates nuclear extracts.

After performing EMSAs with nuclear extracts from ticks, this study tested if purified tick recombinant AP-1 protein (rAP-1) directly binds to the *iafgp* promoter region. For this, full-length AP-1 was cloned and expressed in *E. coli* cells. The purified recombinant protein was designated as GST-AP-1 (~ 54 kDa). Recombinant GST (26 kDa) protein was also purified (Figure 16). Gel shift assays revealed a clear binding of the AP-1 probe upon incubation with GST-AP1. However, no shift was observed upon incubation of the AP-1 probe with GST alone (Figure 15C). However, enhanced binding in the AP-1 probe was noted upon incubation with GST-AP-1 and nuclear proteins isolated from naïve or *A. phagocytophilum*-infected ticks (Figure 15D) indicating a direct binding of rAP-1 binding on the *iafgp* promoter region.

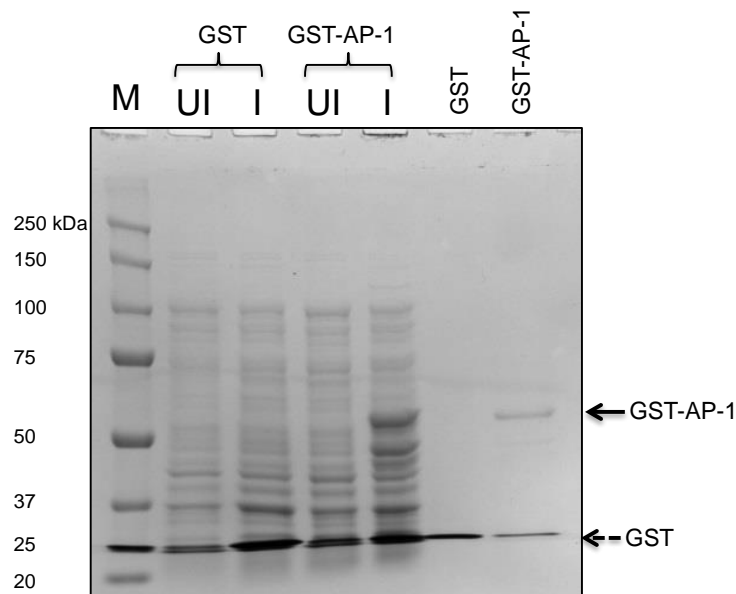


Figure 16. Coomassie Blue Staining of SDS-PAGE Gel Showing Induction of GST-AP-1 Protein

The image shows the induction of rAP-1-GST or GST proteins in induced (I) *E. coli* BL21 cell lysates. Uninduced (UI) GST-AP-1 or GST *E. coli* BL21 cell lysates are shown as control. Purified recombinant proteins from *E. coli* BL21 cell lysates are shown and indicated with a solid arrow (rAP-1-GST) or dotted arrow (GST). A band at the size similar to GST in rAP-1-GST lane indicates possible degradation of later protein. M indicates protein marker, shown on the left of the gel with respective molecular sizes in kDa.

Upstream Region of ~700 bp of the *iafgp* Coding Sequence is Sufficient for *A. phagocytophilum*-Mediated Influence on Tick Antifreeze Gene Expression

Gel shift assays revealed a strong TATA and AP-1 binding site regions upstream of the *iafgp* CDS-1, so ~700 bp upstream region from *I. scapularis* genomic DNA (the position corresponding to 17757-18463 in GenBank acc. no. DS704943) was PCR amplified (Figure 17A), sequenced, and cloned in pGLuc promoterless vector (Figure 18A). Once the sequencing was confirmed (Figure 17B) for the corresponding genomic region, the construct was processed for luciferase assays.

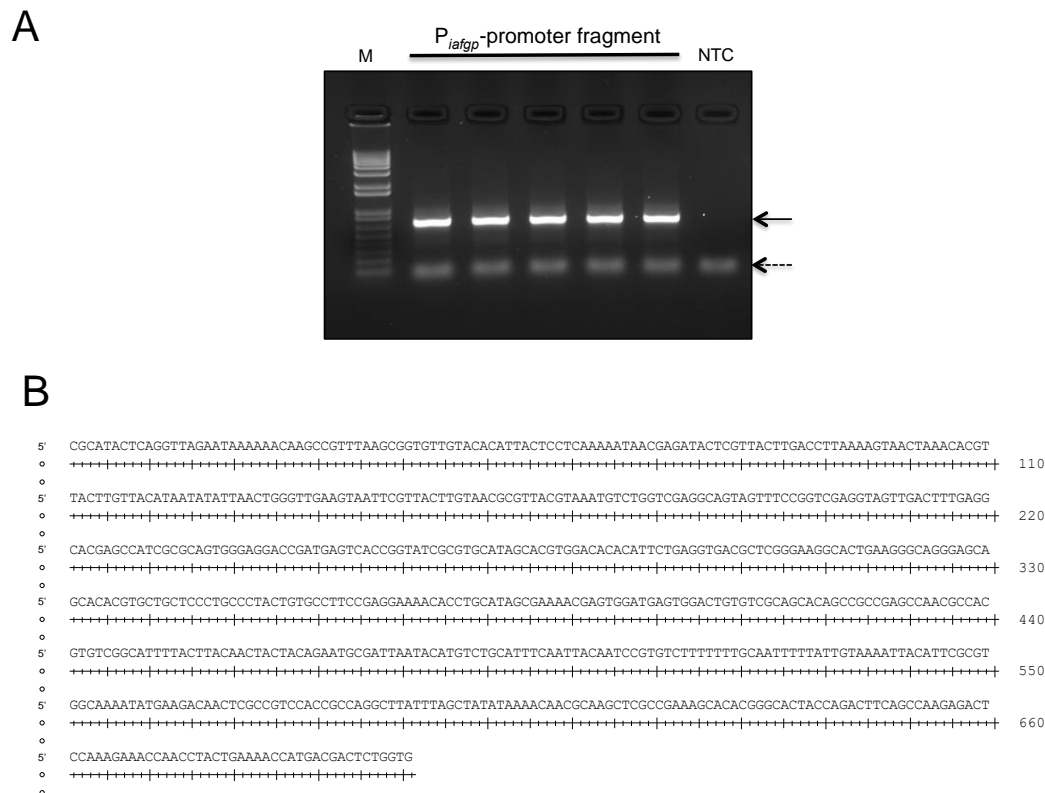


Figure 17. PCR Amplification and Sequencing Confirmation of 700 bp Upstream Region of *iafgp* Promoter

(A) Agarose gel electrophoresis image showing PCR amplification of ~ 700 bp DNA fragment containing *iafgp* promoter from *I. scapularis* genomic DNA.

(B) Sequence confirmation of the nucleotide sequence of the ~ 700 bp DNA fragment cloned in the pGLUC vector is shown. The sequence is shown from 5'-3' direction. The length of the sequence is shown on one side of the sequence.

The transfection for the luciferase assays was performed, and luciferase activity was measured in tick cells. The naïve or *A. phagocytophilum*-infected tick cells showed no or very low morphological differences when transfected with pGLuc vector carrying *iafgp* promoter (pGLuc+P_{*iafgp*}) or empty pGLuc vector even after 48 hours of transfection. The supernatant was collected 48 h post-transfection to measure luciferase activity. Enhanced ($P < 0.05$) luciferase activity (via luminescence) was observed in both naïve and *A. phagocytophilum*-infected tick cells that were transfected with pGLuc+P_{*iafgp*} in comparison to the respective control cells transfected with an empty vector (Figure 18B). Luciferase activity was observed to be approximately two-fold more in *A. phagocytophilum*-infected tick cells upon transfection with the putative *iafgp* promoter region, pGLuc+P_{*iafgp*} in comparison to the naïve tick cells transfected with pGLuc only (Figure 18B). Using QRT-PCR analysis, luciferase mRNA in tick cells transfected with either of the constructs was also detected. Detection of luciferase mRNA (Figure 18C) in each transfected group further supported the results from luciferase promoter assays measuring luminescence in the supernatant. Also, a significant decrease in luciferase activity was observed upon transfection of pGLuc+P_{*iafgp*} construct in *A. phagocytophilum*-infected *ap-1*-silenced tick cells compared to the activity observed in mock-dsRNA treated tick cells (Figure 18D). These results collectively show that a region of ~700 bp upstream of the *iafgp* coding sequence contains the promoter region for *iafgp*, and it is sufficient for *A. phagocytophilum*-mediated AP-1-dependent regulation of tick antifreeze gene.

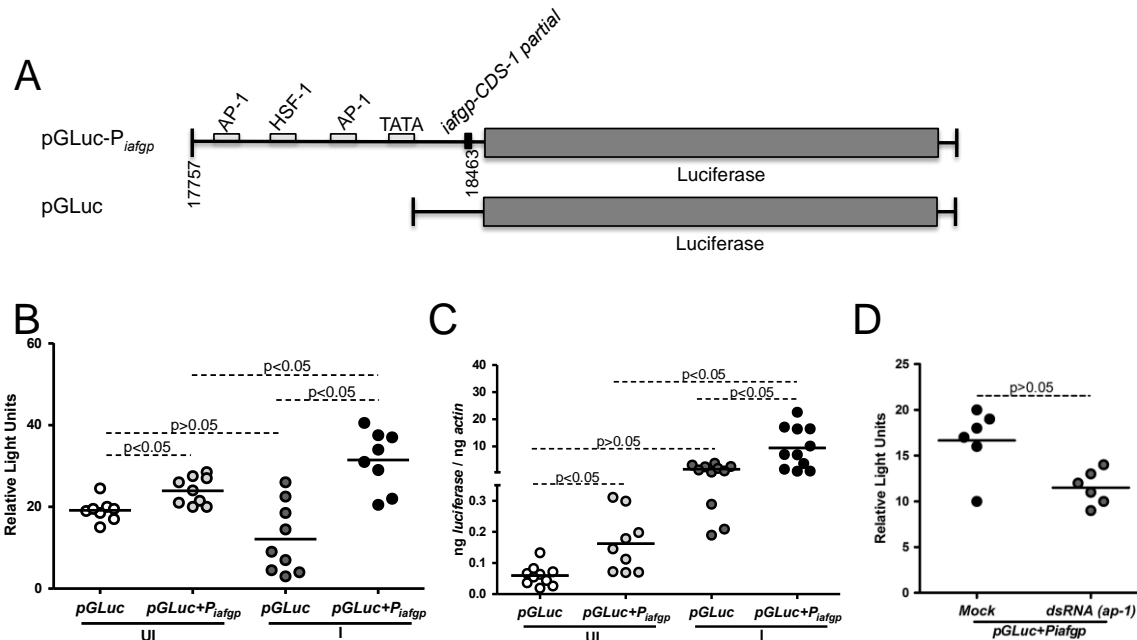


Figure 18. Approximately 700 bp DNA Sequence Upstream of *iaifgp* is Sufficient to Drive its Gene Expression

(A) A schematic representing the *iaifgp* promoter construct used for promoter activity in tick cells is shown. The picture is not drawn to scale. The ~700 bp DNA sequence upstream of the *iaifgp* gene containing all the analyzed binding sites (AP-1, HSF-1, TATA-region) was cloned into a promoterless pGLuc (pGLuc-P_{iaifgp}) vector using an empty pGLuc vector as control.

(B) Luciferase activity determined from culture supernatants from naïve (UI) or *A. phagocytophilum*-infected (I) ISE6 cells transfected with pGLuc-P_{iaifgp} or pGLuc constructs is shown.

(C) Expression levels of luciferase transcripts in naïve (UI) or *A. phagocytophilum*-infected (I) ISE6 cells transfected with pGLuc-P_{iaifgp} or pGLuc constructs is shown. The expression levels of luciferase transcripts were normalized to the levels of beta-actin transcripts.

(D) Luciferase activity determined from culture supernatants from *A. phagocytophilum*-infected ISE6 cells treated with mock-dsRNA or *ap-1*-dsRNA and transfected with pGLuc-P_{iaifgp} constructs is shown.

In each figure panel, each circle represents luciferase assay measurement from one culture well performed in duplicates.

RNAi Silencing of *ap-1* Affects *iafgp* Expression and *A. phagocytophilum* Burden in Nymphs During Acquisition

Then, *ap-1* mRNA levels were measured in nymphs upon feeding on either naïve or *A. phagocytophilum*-infected murine hosts. A significant increase ($P < 0.05$) in *ap-1* mRNA levels in the ticks that were engorged on mice infected with *A. phagocytophilum* was noted when compared to the levels noted in ticks that were fed on naïve mice (Figure 19A). Microinjection with *ap1-dsRNA* to generate *ap-1*-knockdown ticks was performed as described (Neelakanta et al., 2010; Sultana et al., 2010; Taank et al., 2017). QRT-PCR assays showed ~ 2-3-fold reduced ($P < 0.05$) levels of *ap-1*- transcripts in the *ap-1*-knockdown ticks (Figure 19B). Approximately 3-4-fold reduced ($P < 0.05$) levels of *iafgp* transcripts (Figure 19C) and *A. phagocytophilum* burden (Figure 19D) was noted in *ap-1*-knockdown ticks when compared to the mock controls. Gel shift assays performed with nuclear proteins isolated from *ap-1* knockdown fed nymphal ticks revealed a decrease in the *ap-1* probe shift compared to the shift observed with nuclear proteins isolated from mock control ticks (Figure 19E). These results further substantiate the role of AP-1 as the upstream transcriptional activator in *iafgp* gene regulation during *A. phagocytophilum* acquisition by ticks.

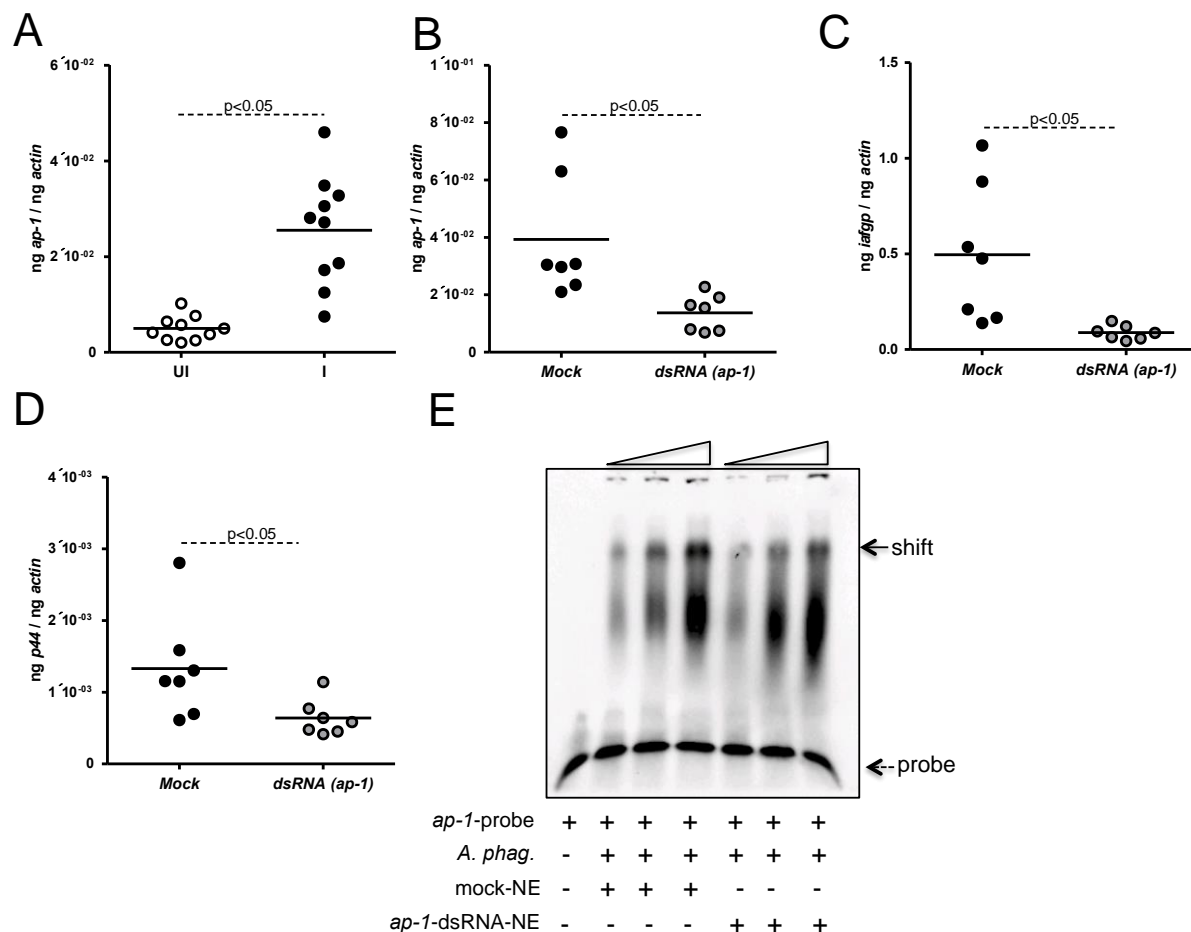


Figure 19. Silencing of *ap-1* Affects Bacterial Acquisition and *iafgp* Gene Expression in Ticks

(A) QRT-PCR analysis showing levels of *ap-1* transcripts in nymphs fed on naïve or *A. phagocytophilum*-infected mice.

(B) *ap-1* expression in mock or *ap-1*-deficient ticks upon bacterial acquisition from the murine host.

(C) *iafgp* expression in mock or *ap-1*-deficient ticks upon bacterial acquisition from the murine host.

(D) Bacterial load in 48 hours post-repleted ticks, either from mock or *ap-1*-deficient nymphs fed on *A. phagocytophilum*-infected mice.

(E) EMSAs with biotinylated *iafgp* promoter probe containing AP-1 binding site (DS704943, 18144-18193 bp) and nuclear proteins prepared from *A. phagocytophilum*-infected mock or *ap-1*-deficient ticks.

The dotted arrow indicates a free probe, and a solid arrow indicates binding to the promoter region. NE means nuclear extracts.

***ap-1* Silencing Impacts *iafgp* Expression and *A. phagocytophilum* Burden in Tick Cells**

The levels of *ap-1* and *iafgp* mRNA were analyzed in ISE6 *in vitro* tick cells. The transcripts of *ap-1* were significantly upregulated in *A. phagocytophilum*-infected tick cells when compared to the naïve cells (Figure 20A). RNAi-mediated silencing showed reduced ($P<0.05$) levels of *ap-1* transcripts in *ap-1* knockdown tick cells when compared to mock controls (Figure 20B). Reduced *iafgp* mRNA levels were also observed in *ap-1* knockdown tick cells when compared to the levels noted in mock control (Figure 20C). A reduced bacterial load in *ap-1* knockdown tick cells was observed compared to mock controls (Figure 20D). Gel shift assays performed with nuclear proteins isolated from *ap-1* knockdown tick cells revealed a ~ 2-3-fold decrease in *ap-1* binding when compared to the nuclear proteins isolated from mock-treated tick cells (Figure 20E). These results collectively indicate an essential role for AP-1 in *iafgp* gene regulation and survival of *A. phagocytophilum* in tick cells.

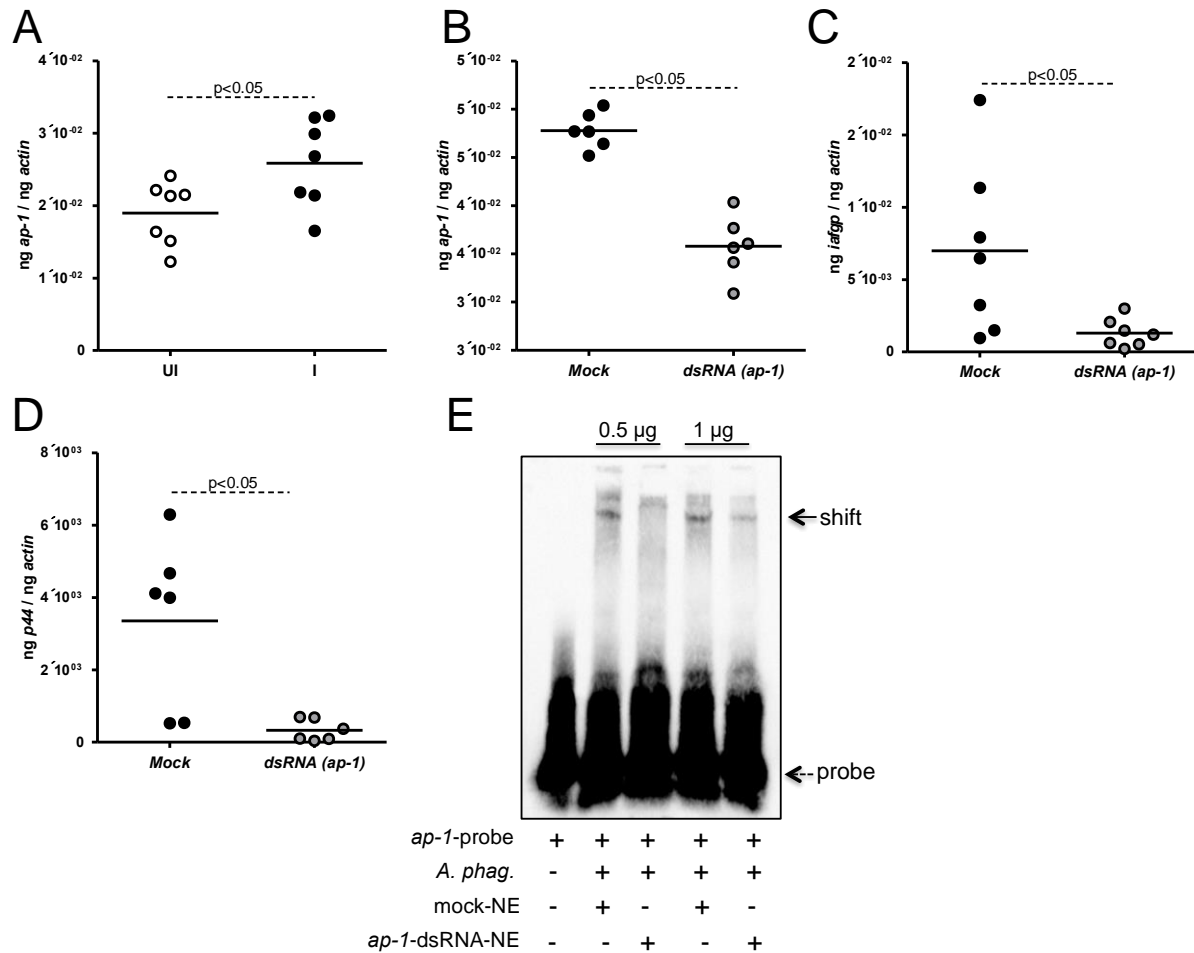


Figure 20. Silencing of *ap-1* in Tick Cells Affects Expression of *iafgp* and Bacterial Burden

(A) QRT-PCR results showing *ap-1* transcripts in naïve or *A. phagocytophilum*-infected ISE6 cells at 48 h post-infection.

(B) *ap-1* expression in tick cells with mock or *ap-1*-dsRNA treatment is shown.

(C) *iafgp* expression in tick cells with mock or *ap-1*-dsRNA treatment is shown.

(D) Bacterial load in mock or *ap-1*-silenced ISE6 cells at 24 hours post-infection is shown.

(E) Gel shift assays with a biotinylated *iafgp*-promoter probe containing AP-1 binding site (DS704943, 18144-18193 bp) and nuclear proteins (0.5 and 1 µg) prepared from *A. phagocytophilum*-infected mock or *ap-1*-silenced ISE6 cells is shown.

The dotted arrow indicates a free probe, and a solid arrow indicates a shift. Each circle in the panels A-D indicates RNA/DNA samples generated from one tick cell culture well performed in duplicates.

AP-1 Induces *iafgp* Gene Expression in Unfed Nymphs at Cold Temperature

The effect of cold temperature on *ap-1* mRNA levels was tested in *A. phagocytophilum*-infected ticks. A significantly higher level ($P < 0.05$) of *ap-1* transcripts was observed in *A. phagocytophilum*-infected ticks when compared to the uninfected controls at cold ($10 \pm 1^\circ\text{C}$) temperature (Figure 21A). Besides, *ap-1* transcripts were also upregulated upon ticks' incubation at cold ($4 \pm 1^\circ\text{C}$) temperature. Gel shift assays performed with total nuclear proteins isolated from *A. phagocytophilum*-infected ticks (incubated at cold temperature) revealed increased binding of nuclear proteins to *iafgp* TATA (Figure 21B) and *ap-1* probes (Figure 21C) in comparison to the shifts observed upon incubation with extracts prepared from the uninfected controls. These results further support the vital role of AP-1 in *A. phagocytophilum* induced *iafgp* gene expression at cold temperatures.

Silencing of *ap-1* Affects Tick Survival in the Cold

A previous study showed that knockdown of *iafgp* expression affects the survival of nymphs at a cold temperature (Neelakanta et al., 2010). As *ap-1* is crucial for *iafgp* expression, this study tested whether knockdown of *ap-1* influences ticks' ability to survive at cold temperatures. Cold tolerance assays were performed to observe the survival of ticks at cold temperatures as described (Neelakanta et al., 2010). RNAi-mediated gene silencing showed a significant reduction in *ap-1* mRNA (Figure 21D) and *iafgp* mRNA (Figure 21E) levels in *ap-1* knockdown ticks when compared to the mock controls. Cold-tolerant assays with *ap-1* knockdown or mock control ticks were performed. Cold tolerance assays revealed a significant reduction in the survival of *ap-1* knockdown ticks than mock control ticks (Figure 21F). The ticks that were *ap-1* deficient weren't able to survive cold temperatures. Also, around two-three-fold reduced mobility was observed in *ap-1* knockdown ticks than the mock control ticks (Figure 21G). Together, these results suggest that arthropod transcriptional activator AP-1-mediated regulation of *iafgp* expression is vital for the survival of ticks at cold temperatures.

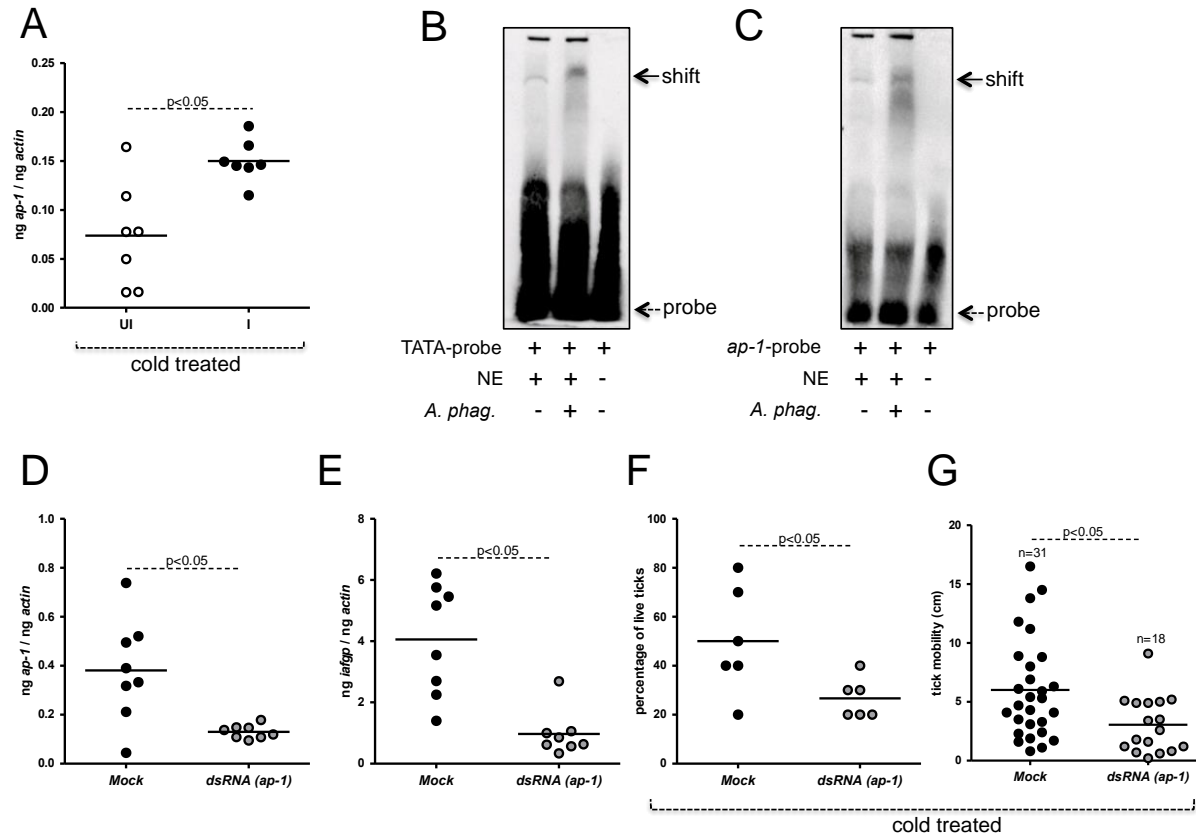


Figure 21. Silencing of *ap-1* Reduces *iaftp* Expression and Survival of Ticks at Cold Temperature

(A) QRT-PCR results showing *ap-1* transcripts in naïve or *A. phagocytophilum*-infected ticks incubated at 10°C for 8 hours.

(B) EMSA with biotinylated *iaftp* TATA probe (DS704943, 18338-18387 bp) is shown.

(C) EMSA with biotinylated *iaftp* AP-1 probe (DS704943, 18144-18193 bp). Nuclear proteins (2.5 µg) were prepared from naïve (UI) or *A. phagocytophilum*-infected (I) nymphs incubated at 10 °C for 8 hours is shown.

(D) *ap-1* expression in *ap-1*-dsRNA-injected uninfected nymphs compared with the mock controls.

(E) *iaftp* expression in *ap-1*-dsRNA-injected uninfected nymphs compared with the mock controls.

(F) Percentage survival of mock- or *ap-1*-dsRNA-injected nymphs at the LT₅₀ time point.

(G) Mobility (in cm) by mock- or *ap-1*-dsRNA-injected nymphs at LT₅₀ time point (–20°C, 25 min).

In panels A, D, E, and G, each circle represents one individual tick performed in duplicates, whereas, in panel F, each circle represents one experiment performed with ten ticks/group.

3.3 DISCUSSION

Numerous studies have focused on understanding the molecular and cellular signaling in blood-sucking arthropods upon infection with various pathogens (de la Fuente et al., 2016b; Dutra et al., 2016; Neelakanta and Sultana, 2015, 2016; Nelson et al., 2008; Oliva Chavez et al., 2015; Sandiford et al., 2015; Saraiva et al., 2016). A previous study showed an essential role for tick antifreeze glycoprotein, IAFGP, that allows the survival of *A. phagocytophilum* in its vector host in the cold (Neelakanta et al., 2010). This study provides evidence that *A. phagocytophilum* modulates a transcription factor, AP-1, as an upstream activator for regulating tick *iafgp* gene expression that is critical for their survival in the cold (Figure 22). The domain analysis revealed the presence of bZIP domain at the C-terminus region of *I. scapularis* AP-1. The bZIP domain-containing proteins are present in a wide range of eukaryotic proteins (including AP-1 proteins) recognized as DNA-binding transcription factors (Hurst, 1995). Ticks belonging to the Ixodidae family are medically important vectors that transmit various pathogens to humans (Anderson and Magnarelli, 2008). A relatively higher degree of conservation across the entire *I. scapularis* AP-1, in particular at the bZIP domain region, was observed with AP-1 orthologs from other Ixodidae ticks. This suggests a possible conserved role for this arthropod AP-1 in tick-pathogen interactions from other species as well.

Upon entry into ticks, *A. phagocytophilum* colonizes the salivary glands (Rikihisa, 2010). The upregulation of AP-1 in the unfed nymphal tick salivary gland suggests a role for this molecule in *A. phagocytophilum* colonization in ticks. Also, the RNAi analysis results indicate that AP-1-mediated upregulation of *iafgp* gene expression is essential in acquiring *A. phagocytophilum* by uninfected naïve ticks. This observation is also supported by our previous finding of reduced *A. phagocytophilum* loads in *iafgp*-knockdown ticks upon feeding on an infected murine host (Neelakanta et al., 2010). Numerous experiments, including EMSAs with TATA and AP-1 probes, recombinant AP-1 protein, with nuclear extracts, support the transcriptional role of AP-1 as an essential transcriptional activator in the regulation of *iafgp* promoter activity. A ~ 700 bp upstream region was considered as a putative promoter for the *iafgp* gene because the genomic region contained both TATA and AP-1 binding sites. The observation of no

shift with the other tested AP-1 probe (a region 18032-18081 in GenBank acc. no. DS704943) and HSF-1 probe (a region 18093-18142 in GenBank acc. no. DS704943) upon incubation with nuclear proteins isolated from naïve or *A. phagocytophilum*-infected nymphs suggests a non-definitive site for AP-1 or HSF-1 binding, respectively. The transfection of pGLuc construct containing the promoter region (P_{iafgp}) with all the tested sites (two AP-1 sites, one TATA site, and one HSF-1 site) showed increased promoter activity when compared to the control. This finding supports that approximately 700 bp is the putative *iafgp* promoter. It is easier to hypothesize that ~ 320 bp region (18144-18463 bp in GenBank acc. no. DS704943) containing one each of confirmed AP-1 and TATA site is sufficient for *iafgp* expression. Future studies would reveal whether the ~ 320 bp promoter region is adequate to direct *iafgp* gene expression.

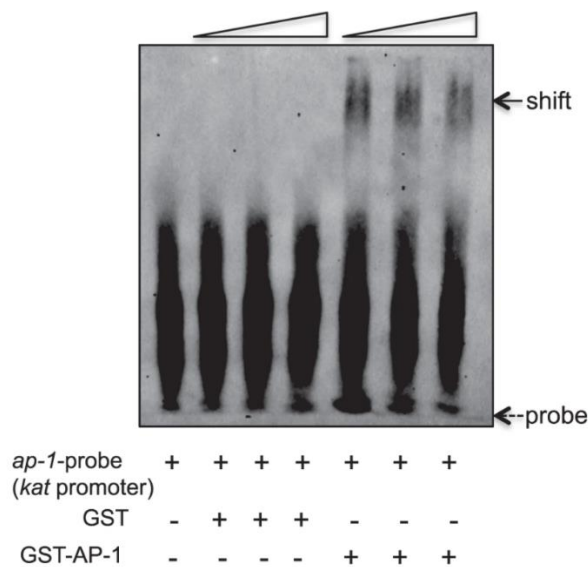


Figure 22. AP-1 Binds *kat* Gene Promoter

EMSA was performed with the biotinylated AP-1 region probe (DS929842, 124879–124830 bp) from putative *kat* promoter containing AP-1 binding site (DS929842, 124858–124852 bp) and rGST alone or rGST-AP-1 protein (wedges indicates increasing amounts of nuclear extracts). The dotted arrow indicates free probe, and the solid arrow indicates band shift.

Bioinformatics analysis revealed the presence of more than 100 transcription factors on the *iafgp* promoter. Other transcription factors, as shown by bioinformatics analysis, include but are not limited to HSF-2. The HSF-1 probe that was used in this study also contained a putative HSF-2 binding site, thus excluding the later protein's possibility to bind to the *iafgp* promoter region at that predicted sites. Multiple studies have shown that genes containing TATA-box promoters are associated with stress and are highly regulated compared to the genes that are TATA-less promoters (Basehoar et al., 2004; Otterbein and Choi, 2002). The *iafgp* is a stress-induced (infection and cold) gene with a strong TATA-box, so it is entirely reasonable to hypothesize that multiple transcription factors could bind to different *iafgp* promoter regions. However, the presence of a strong binding site in the *iafgp* promoter (Figure 13), a role in stress (Hess et al., 2004), and interactions with pathogens (Hess et al., 2004; Kramer et al., 2015; Xiang et al., 2014), strongly supports that AP-1 is an essential transcriptional activator of tick antifreeze gene expression. A previous study reported the OATP-KAT pathway in the survival of *A. phagocytophilum* in ticks (Taank et al., 2017). The binding of AP-1 to the KAT promoter (Figure 22) further suggests that *A. phagocytophilum* modulate AP-1 as a dominant player to control downstream tick genes critical for its survival. Future studies would reveal exciting aspects of any other possible *I. scapularis* transcription factors in the regulation of the *iafgp* gene or the interactions of AP-1 with the *iafgp* or other tick promoters.

The silencing of *ap-1* using *ap-1*-dsRNA in ticks reduced their survival as well as mobility at cold temperatures and the survival of *A. phagocytophilum* in ticks. This observation suggests that a lack of AP-1 affects *iafgp* gene expression. Being a global transcriptional regulator, it is possible to reason that the silencing of the *ap-1* gene could influence other tick cold tolerance strategies and pathogen acquisition. Also, the reduced *iafgp* expression at cold temperatures due to *ap-1* silencing and lower ability of the *ap-1* knockdown ticks to survive cold strongly supports the role of AP-1-IAFGP signaling in the survival of ticks at cold temperatures. A previous study reported reduced ability of *iafgp*-deficient ticks to survive at a cold temperature (Neelakanta et al., 2010). In summary, this study shows that *A. phagocytophilum* modulates arthropod AP-1 molecule to activate tick antifreeze gene expression thereby facilitating the survival of

both the rickettsial pathogen and its vector host in cold temperatures. Understanding the molecular signaling, such as this, in vector-pathogen interactions, would serve as essential means to develop newer strategies to combat several rickettsial vector-borne diseases.

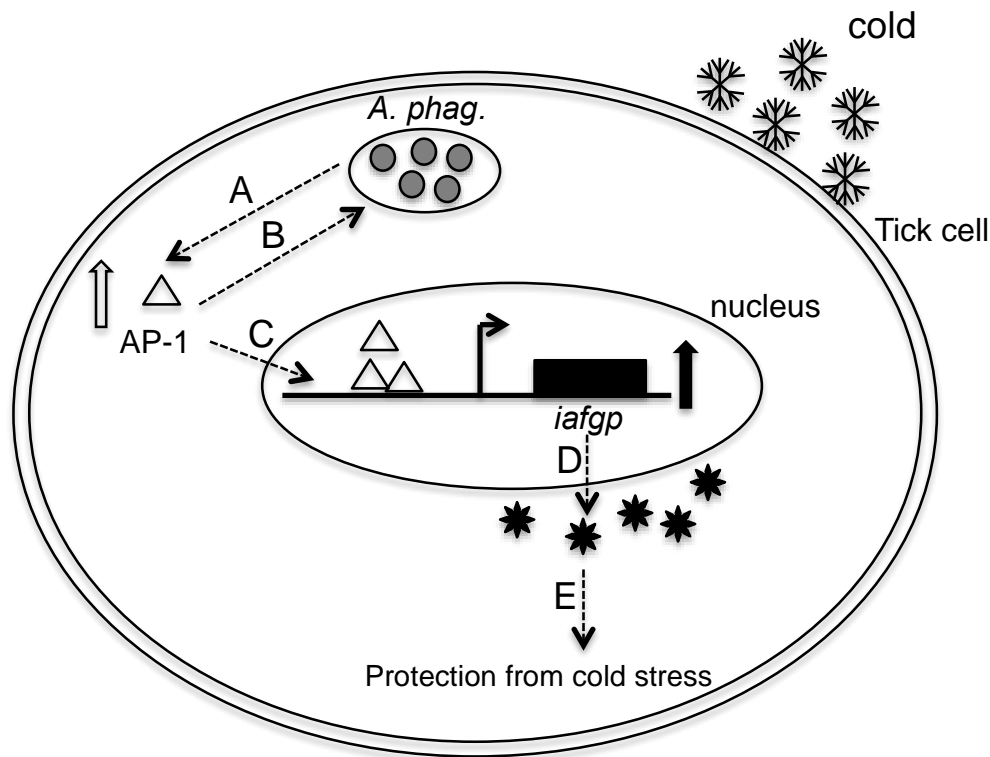


Figure 23. A Schematic Model Showing *A. phagocytophilum* Mediated AP-1-IAFGP Signaling in Tick Cold Tolerance

- (A) Upon *A. phagocytophilum* (shown as morulae within a tick cell) infection, and cold shock upregulate *ap-1* gene expression, leading to enhanced AP-1 protein production.
- (B) The increased production of AP-1 proteins promotes *A. phagocytophilum* survival in ticks. Also, AP-1 translocate into the nucleus and binds to the *iafgp* promoter.
- (C) Increased tick antifreeze gene expression leads to increased production of IAFGP.
- (D) Increased tick antifreeze mRNA, therefore, results in higher *iafgp* protein in ticks.
- (E) Increased production of IAFGP protein protects *A. phagocytophilum* and tick cells from cold.
- The schematic representation is not drawn to scale.

3.4 EXPERIMENTAL PROCEDURES

Bacterial Isolates and ISE6 Cells

Two isolates of *A. phagocytophilum* were used during this study. NCH-1 isolate was used in generating infected ticks as described (Taank et al., 2017), and the HZ isolate was used in cell line experiments. Both isolates are mentioned as *A. phagocytophilum*. The pGLuc constructs were transformed and maintained into *Escherichia coli* DH5 α cells. The *E. coli* BL21 strain was used for recombinant AP-1 protein expression and purification. Naïve uninfected *I. scapularis* ticks (larvae and nymphs) were obtained from the Connecticut Agricultural Experiment Station (New Haven, CT). *ap-1* knockdown experiments using RNAi were performed in ticks obtained from BEI Resources, NIAID, NIH. The *in vitro* ISE6 cell line was obtained from Dr. Ulrike Munderloh and was propagated in our laboratory (Felsheim et al., 2006). *A. phagocytophilum* isolate HZ was maintained in HL-60 cells (human promyelocytic cell line). Different stages of ticks were housed in the controlled environment chamber (Parameter Generation and Control, Black Mountain, NC) with settings for the temperature at 23 °C, humidity at 90-93 %, and photoperiod time set for 14h light and 10h dark exposure.

Ticks and Mice

All the animal experiments in this study were performed with 4-6 weeks old female C3H/HeN mice (Charles River Laboratories, USA). The unfed nymphs were generated by feeding larvae on either naïve or *A. phagocytophilum*-infected mice. Fed larvae were housed in a controlled environment chamber for molting into nymphs. In knockdown assays, *ap-1*-dsRNA was microinjected into naïve nymphs and fed on *A. phagocytophilum* infected mice. Mock injections were performed similarly in naïve ticks with dsRNA fragment generated from multiple cloning sites of pL4440 vector and empty pL4440 vector were used as controls. The repleted and engorged ticks were collected, and RNA or DNA extractions were performed. Real-time PCR analysis was carried out on these tick samples to evaluate knockdown efficiency, bacterial burden, and gene expression. Institutional biosafety committee approval (number 15-012) was used to perform experiments in this study. The mice studies were performed based on the

animal protocol (16-017) approved by Old Dominion University Institutional Animal Care and Use Committee (IACUC).

Prediction of *iafgp* Promoter Region and Phylogenetic Analysis

The nucleotide sequence with GenBank accession number DS704943 was downloaded from the National Center for Biotechnology Information (NCBI). The region corresponding to -692 to +15 bp from the start of *iafgp* CDS was analyzed to predict the TATA box and other putative transcription factors. We used the Neural network promoter prediction tools, H Ctata and TSSW servers to predict the TATA box regions as described (Sultana et al., 2010). Three predictions were compared, and the TATA box site mapped by all three software were selected for designing the EMSA probes. We used TFBIND to predict the putative transcription factor binding sites (Tsunoda and Takagi, 1999), and the results were compared with their consensus sequences. The highest match between TFBIND prediction and known consensus sequences was considered for designing the EMSA probes and predicting putative transcription factor binding sites on the *iafgp* promoters. Phylogenetic analysis for AP-1 sequences was done using a rooted tree generated based on CLUSTALW alignment of amino acid sequences in DNASTAR using 1000 bootstraps. The Kimura distance method was used to calculate distance values.

Gene Expression Analysis and Determining Bacterial Burden

Quantitative real-time PCR analysis was conducted as reported (Neelakanta et al., 2017b; Vora et al., 2017). Total RNA from different developmental stages of ticks (larvae, nymphs, adult ticks), fed naïve or *A. phagocytophilum*-infected nymphs, and from ISE6 cells was extracted and processed for cDNA synthesis and QRT-PCR as reported (Neelakanta et al., 2017b; Taank et al., 2017; Vora et al., 2017). Table 1 in the Appendix contains sequences for the oligonucleotides used in this study. The *ap-1* or *iafgp* mRNA levels in samples were normalized to tick *beta-actin* mRNA levels in the same sample. *A. phagocytophilum* load was quantified from the genomic DNA isolated from infected unfed/fed nymphal ticks or tick cells. Total genomic DNA extractions were performed as described (Taank et al., 2017) and processed to detect *A.*

phagocytophilum p44. A standard curve from six 10-fold dilutions (1 ng to 0.00001 ng) of *ap-1*, *iafgp*, *p44*, or actin was used for gene expression analysis.

RNAi Experiments in Ticks

The *ap-1*-dsRNA was generated as reported previously (Neelakanta et al., 2010; Sultana et al., 2010; Taank et al., 2017). The *ap-1* fragment was PCR amplified with oligonucleotides containing two restriction enzyme sites (BglII and KpnI). The PCR fragment containing the *ap-1* gene sequence was later gel purified using a gel extraction kit (Qiagen, USA) and then digested with BglII-KpnI restriction enzymes. Ligations were performed at BglII-KpnI digested sites of L4440 vector as reported previously (Neelakanta et al., 2010; Sultana et al., 2010; Taank et al., 2017). Transformations were performed in *E. coli* DH5 alpha cells, and the clones were sequenced to confirm the presence of the promoter region. Then, the construct was used for synthesizing dsRNA using MEGAscript RNAi Kit (Ambion Inc. USA). This dsRNA was used for in vitro transfection experiments as well as microinjecting into the ticks. Microinjections with *ap-1* dsRNA or empty L4440 vector were performed as described (Neelakanta et al., 2010; Sultana et al., 2010; Taank et al., 2017). After microinjection, ticks were incubated for 24 hours in a desiccator housed in a walk-in-incubator with temperature settings at 23±2°C, humidity at 88-92%, and a photoperiod time set at 14 h light and 10 h dark exposure. Microinjected ticks were later fed on naïve or *A. phagocytophilum*-infected mice after 24 hours of adaptation inside the incubator. The engorged ticks were collected after repletion, and RNA/DNA was extracted. QRT-PCR was done to determine gene expression, silencing efficiency, and bacterial load. However, for some experiments, unfed ticks injected with *ap-1*-dsRNA or mock were directly used for RNA/DNA extractions after 24 hours of incubation in the desiccator.

***ap-1* Silencing Experiments, Transfection in Tick Cells and Luciferase Assays**

Silencing was achieved using the *ap-1* dsRNA using transfection in tick cells (Taank et al., 2017). Briefly, 1 x 10⁵ tick cells were seeded onto a 12 well plate in L-15B300 medium and incubated for 20-24 hours. After incubation of 24h, 750 ng of *ap-1* dsRNA or an equal volume of mock solution or pGLuc/pGLuc-P_{*iafgp*} constructs were transfected

using Lipofectamine 2000 reagent. The plates were incubated for additional 6 hrs. Then, a 2X L15-B300 medium was added after 6 h incubation to recover the cells from the lipofectamine treatment. Plates were then incubated for 18 h. *A. phagocytophilum* HZ strain was used to infect these cells. Host cell-free bacteria isolated from *A. phagocytophilum*-infected HL-60 cells were added to tick cells, and plates were incubated for an additional 24 h. After 24 h post-infection (p.i.), tick cells were collected, and RNA or DNA extractions were performed. The pGLuc-P_{iafgp} construct was prepared by cloning ~700 bp fragment (17757-18463 region in GenBank acc. no. DS704943) into the pGLuc vector. Shortly, ~700 bp fragment was PCR amplified using primers containing EcoRI and HindIII restriction sites. The PCR fragment was then digested and ligated into EcoRI and HindIII digested pGLuc vector. The ligation mix was then transformed into *E. coli* DH5 α strain and clones were selected on Luria-Bertani agar plates containing Ampicillin (50 μ g/ml). Plasmids from clones were isolated using the Qiagen Plasmid Maxi kit (Qiagen, USA), sequenced, and used for transfection. Luciferase assays were performed with cell culture supernatants collected after 24 h post-infection and 48h post-transfection using the BioLux Gaussia Luciferase assay kit (New England Laboratories, USA). Luminescence from the cell culture supernatants was measured using a TECAN Plate reader (Tecan, USA).

Electrophoretic Mobility Shift or Gel Shift Assays (EMSAs)

EMSAs were performed on the nuclear extracts from ticks and tick cells. Nuclear proteins from unfed or fed, naïve, or *A. phagocytophilum*-infected ticks or tick cells were extracted as described (Sultana et al., 2010; Taank et al., 2017). For cold treatment experiments, unfed nymphal ticks were incubated at $10 \pm 1^\circ$ C for 8 hours and then processed to prepare nuclear extracts. We used BCA kit (Pierce, USA) to quantify nuclear extracts. The sequences for the probes were designed based on the bioinformatics prediction using tools, as described (Sultana et al., 2010). Probes for EMSAs were prepared by annealing complementary oligonucleotides containing *iafgp* TATA region or AP-1 or HSF-1 binding sites. Biotin labeling of oligonucleotides was performed as described. The EMSAs were performed on 6% polyacrylamide gels, as reported previously (Sultana et al., 2010; Taank et al., 2017). After the run, samples

from gels were transferred and processed to detect gel shifts using the chemiluminescent detection method reported previously (Taank et al., 2017). The ChemiDoc MP imager system (BioRad, USA) was used for imaging.

Tick Cold Tolerance and Mobility Assays

Cold tolerance assays and the mobility determination in ticks were performed as described (Neelakanta et al., 2010). Briefly, *ap-1*-dsRNA or mock-treated unfed naïve or *A. phagocytophilum*-infected nymphs were incubated at $23 \pm 1^\circ \text{C}$ for 24h in a desiccator. They were later split into ten ticks/groups in plastic vials with ventilated mesh clothes for aeration. These vials were then immediately incubated at -20°C on a flat surface for 25 min. After 25 min, vials were immediately shifted to 4°C for 1 hour and then later incubated at room temperature for 60 minutes to reacclimatize the ticks. These reacclimatized ticks were given a breath test and then immediately placed side-by-side (1 cm apart) in a line on the Whatman filter paper. Any mobile tick moving out of the line within 10 minutes was considered to have survived the cold temperature. A total of six independent experiments (containing 10 ticks/group/experiment) for uninfected and three independent experiments (containing 10 ticks/group/experiment) for *A. phagocytophilum* infected ticks were performed. The percentage of tick survival at -20°C was determined for each experiment. The mobility of ticks after the rapid cold shock was determined by measuring each alive tick's distance traveled. Distance from the start point and endpoint was measured in centimeters for each tick.

Recombinant AP-1 Protein Expression and Purification

Full-length AP-1 protein purification was performed as described (Neelakanta et al., 2017a). The full-length *ap-1* sequence was amplified using oligonucleotides containing BamHI and Not I sites. The full-length PCR product was then digested with BamHI-NotI restriction enzymes and ligated in-frame at BamHI-NotI digested pGEX vector (Amersham, USA). An empty vector without an *ap-1* sequence was used as control. The ligation mix was transformed into competent *E. coli* BL21 cells, and the clones were selected on LB agar plates containing ampicillin antibiotic (50 $\mu\text{g/ml}$). The rAP1-GST and GST alone were purified from BL21 cells following Hook GST protein Spin

purification kit (G-Biosciences Inc. USA) as described (Neelakanta et al., 2017). The recombinant protein concentration was measured using the BCA Assay Kit.

Isolation of Tick Tissues

Different tick tissues such as salivary glands and guts from each tick were isolated as reported previously (Sultana et al., 2010; Taank et al., 2017). These tissues were homogenized in lysis buffer provided in the Aurum Total RNA Isolation kit (Biorad, USA) and processed for total RNA extractions. The cDNA was prepared as described in the earlier sections and was used for QRT-PCR analysis.

Nucleotide and Amino Acid Accession Numbers

The following GenBank accession numbers used in this study: DS704943 (the genomic region of *iafgp*), XP_002404571 (*Ixodes scapularis* AP-1), XM_002404527 (*Ixodes scapularis ap-1* mRNA), JAP70916 (*Ixodes ricinus* AP-1), JAC33128 (*Amblyoma triste* AP-1), JAC21012 (*A. cajennense* AP-1), AIT40211 (*Rhipicephalus microplus* c-Jun AP-1), JAP80402 (*R. appendiculatus* AP-1), NP_068607 (*Rattus norvegicus* AP-1), NP_034721 (*Mus musculus* AP-1), NP_001252779 (*Maca mulatta* AP-1) and NP_002219 (*Homo sapiens* AP-1).

Statistics

The statistics were performed using GraphPad Prism6 software and Microsoft Excel 2016. A non-paired Student t-test compared the average means from the data analysis from two different samples. The P-value lesser than 0.05 was considered significant. The experimental analysis was performed with at least five ticks in duplicates to minimize the variation between the data among ticks in a group. The horizontal lines in the scatter plot represent the average value of the readings. P values are indicated wherever necessary.

CHAPTER 4

***ANAPLASMA PHAGOCYTOPHILUM* MODULATES TICK CLOCK**

GENE EXPRESSION DURING TICK FEEDING

4.1 INTRODUCTION

Ixodes scapularis is one of the primary vectors for several diseases, transmitting pathogens to animals, including humans. *A. phagocytophilum* replicates and persists through different tick molting stages. This bacterium increases the ticks' ability to survive various stress conditions and establishes a 'secondary facultative symbiont' relationship with ticks that enhances vector competence (de la Fuente et al., 2016b; Khanal et al., 2018; Neelakanta et al., 2010). Circadian rhythms are often related to multiple stress-related responses in different living systems and insects (Reitzel et al., 2013; Sandrelli et al., 2008).

A circadian rhythm is a nearly 24-hour cycle in living beings regulating many biologic functions and physiological processes. The global clock proteins such as clock (CLK), Period (PER), timeless (TIM), and cycle (CYC), are the master timekeepers regulating the core components of the circadian system affecting locomotion, mating, and feeding activity (Meireles-Filho et al., 2006; Meireles-Filho and Kyriacou, 2013). These biological clocks can be modulated by external cues such as light, temperature, PAMPs (Pathogen Associated Molecular Patterns), and even bacteria and viruses (Meireles-Filho and Kyriacou, 2013). Although circadian rhythms are cell-autonomous, there have been fewer studies regarding a pathogen's role in altering their hosts' endogenous clocks. Hepatitis C virus and Influenza virus modulates the host circadian rhythms (Zhuang et al., 2019). Clock disruption leads to increased virus replication and dissemination, indicating that circadian timekeeping influences the severity of acute infections (Mazzocchi et al., 2020). This study aimed not only to study whether *A. phagocytophilum*, being an obligate intracellular bacterium in its vector host *I. scapularis*, regulates the clock genes in ticks but also to explore whether modulation of these circadian genes influences their immune system and blood-feeding behavior.

Circadian Clock During Pathogen Entry

Unicellular eukaryotes and prokaryotes comprise several pathogens to metazoans, but their role in chronobiology has not been described much—except for photoreceptive cyanobacteria and the purple bacteria. Some evidence on circadian rhythms has been reported in non-photosynthetic bacteria for well-known species like *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas putida*, and *Bacillus subtilis*, based on growth kinetics, physiological swarming response, and expression of reporter genes (Costantini et al., 2020). Comparative genomics of all prokaryotes has found homologs of the cyanobacterial circadian gene *kaiC* in numerous other bacteria, including *Rhizobacteria* and *P. putida*. Several pathogens stated above cause various acute human infections, where circadian rhythms play an important role. *Escherichia coli* and *Klebsiella sp.* are the typical gut microbiota and part of the natural mammalian microbiome (Costantini et al., 2020; Hardin and Panda, 2013). The indispensable role of viruses such as HCV has been implicated in modulating host circadian behavior, thereby allowing its replication and survival. The intricate relationship between a healthy host and its gastrointestinal flora is mutualistic (Zhuang et al., 2019). But the entry of pathogens modifies the circadian behavior causing disturbance of the gut flora resulting in nutritional deficiencies and inflammatory conditions (Panda, 2016; Parkar et al., 2019). The disruption of the sleep-wake cycle in infected patients during travel has been shown to rapidly affect the human gut microbiome in larger groups of population samples in multiple studies (O'Donnell et al., 2011; Scheer et al., 2009).

Clock genes are commonly known to control circadian behavior in several feeding insects (Reitzel et al., 2013). A previous study (Gentile et al., 2013) has shown the impact of mating and blood-feeding on the expression of clock genes in *Aedes aegypti*. They reported that blood intake downregulates the clock genes, and the peak activity of the mosquitoes was reduced after silencing the timeless gene using RNAi. Compared to many studies that have focused on understanding the role of circadian genes in mammalian cells, very few studies have focused on arthropod vectors. Due to the critical function of central and peripheral clocks in physiology and metabolism, more studies need to focus on understanding the circadian oscillation in medically important

arthropod vectors like ticks. This study is focused on understanding the role of circadian genes in tick-*A. phagocytophilum* interactions.

4.2 RESULTS

Identification of Circadian Genes in *Ixodes scapularis*

Many of the genes involved in circadian clock organization are similar across several taxonomic groups (Dunlap and Loros, 2004). However, there appear to be differences among insect orders (Sandrelli et al., 2008; Yuan et al., 2007). Four core clock orthologs were identified in *I. scapularis* (CLOCK, BMAL1, PERIOD, TIMELESS). BLASTp analyses provided evidence supporting the similarity of these sequences to other nearby clock-related proteins and a closer affinity to mammalian orthologs than those of *Drosophila* spp. The more rigorous phylogenetic analyses further supported these results (Figure 24).

The tick CLOCK protein contained conserved domains matching to *Mus musculus* and *Homo sapiens*. Analyses performed with BLASTp in GenBank and Vectorbase databases confirmed the presence of the helix-loop-helix domains and Per-Arnt-Sim (PAS) domains. These analyses revealed functional similarity in amino acid residues among the CLOCK protein orthologs in the analyzed taxa. The evolutionary relationships of BMAL1/CYCLE orthologs were studied based on the Kimura distance method. The study noted that the complete sequence for BMAL1 in the NCBI was smaller than BMAL1 from higher vertebrates like mice and humans. The PDB structure analysis revealed BMAL1 was found in the CLOCK/ARNT/BMAL/PAS group. BMAL1 was identified in all species, but most of the insects contained CYCLE in addition to BMAL1 circadian protein. The tick PERIOD matched with *Drosophila melanogaster* PERIOD circadian protein with 42% identity. It contained the PAS domain and period-circadian-like the C-terminal domain. A complete RNA transcript for the *timeless* gene was identified. *I. scapularis* TIMELESS contained the conserved TIMELESS domain similar to the one found in other orthologs.

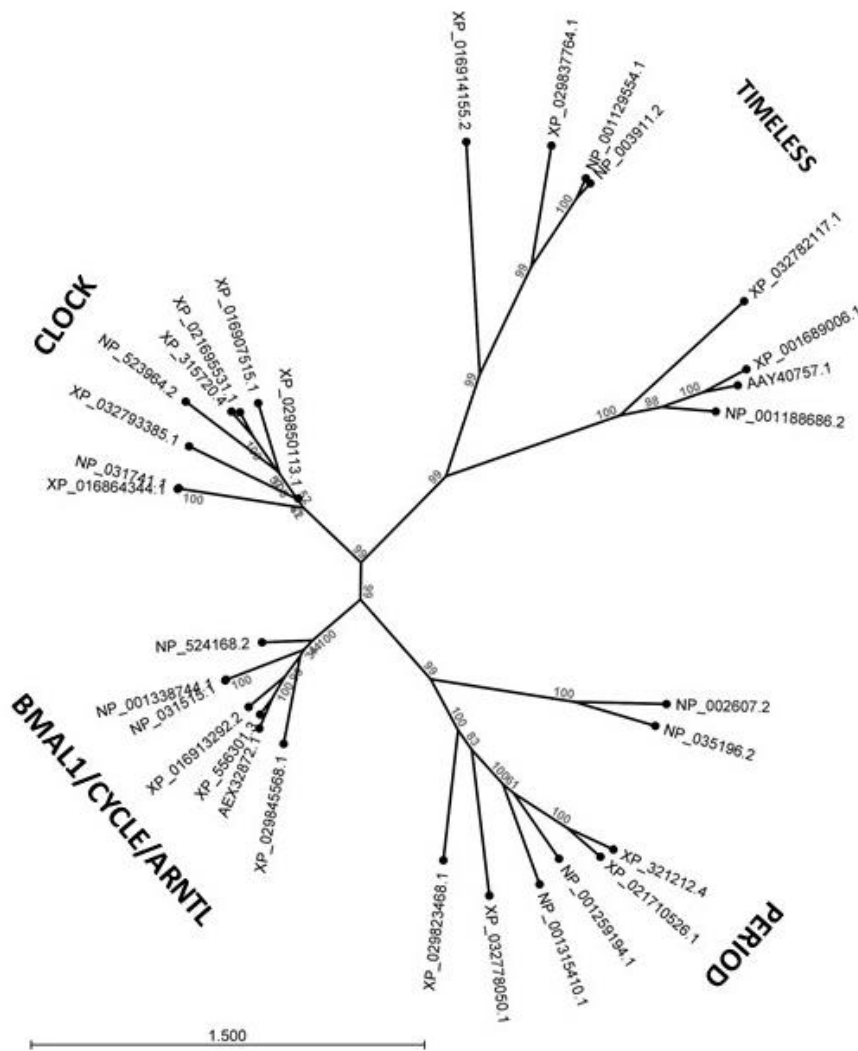


Figure 24. Phylogenetic Analysis of *I. scapularis* Circadian Clock Genes

CLOCK, BMAL1, PERIOD, TIMELESS PROTEINS are represented in the phylogenetic tree. The phylogenetic tree was constructed using the Kimura distance method with CLC Bio software, and the numbers on the nodes represent percent bootstrap values based on 1000 replicates.

***I. scapularis* Shows Circadian Oscillation in Unfed Naïve Nymphs**

Ticks encode circadian genes in their genome that show high identity to *M. musculus* and *D. melanogaster* circadian system. To check the oscillation pattern of the circadian genes in *I. scapularis*, naïve unfed nymphs were used. These ticks were subjected to 12

hours of light and dark cycles (LD). Figure 24 shows the relative expression of *I. scapularis* nymphs monitored for 48 hours under LD cycles. The expression profile of core clock genes such as *clock1*, *bmal1*, *period*, and *timeless1* shows an oscillation pattern in response to the constant light and dark stimuli.

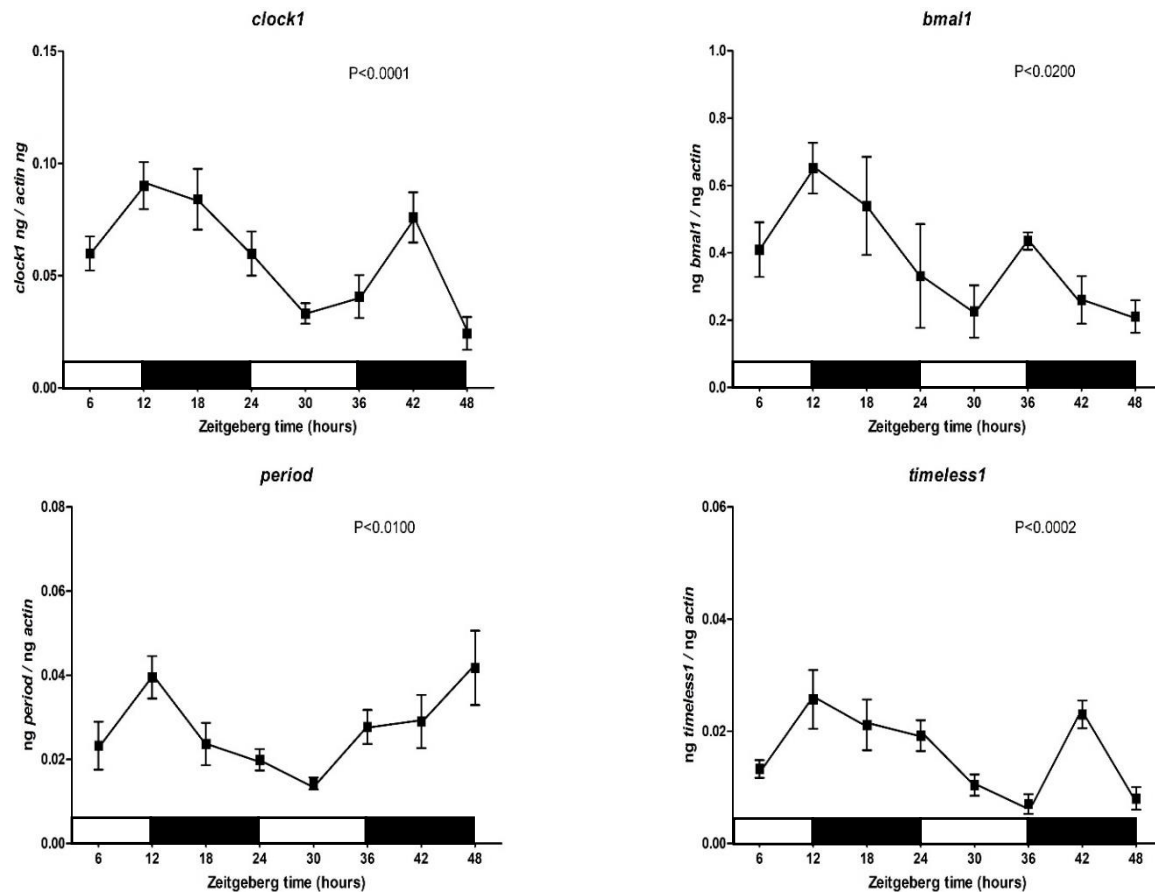


Figure 25. Circadian Expression of Clock Genes in *I. scapularis*

The graphs show the expression of *clock1*, *bmal1*, *period*, and *timeless1* in *I. scapularis* ticks for 12 h of light and 12 h of dark cycles. The y-axis indicates relative mRNA transcripts normalized with tick beta-actin and the x-axis represents time points in zeitgeber units. Each zeitgeber time point was obtained by averaging the data from 5 individual ticks. Error bars represent the standard deviation. One-way ANOVA was used for the statistical analysis mentioned in each graph.

Using gene-specific primers and quantitative real-time PCR, the expression of core circadian genes was monitored in ticks that were exposed to light-dark (LD) and constant darkness (DD) cycles (Figure 24). The two main regulatory loops of the circadian clock involving the genes *clock1* and *bmal1* were noted to be oscillating in a similar fashion. Expression of both genes was noted to be peaked at 12 hours and a dipping pattern in the expression was noted at 30 hours of LD stimulus. The *period* and *timeless1* gene oscillation peaked at 12 hours of the light stimulus and dipped at 30 and 36 hours after light and dark stimulation, respectively. Overall, these results show oscillation patterns in the gene expression for core circadian genes in *I. scapularis* unfed nymphs.

***A. phagocytophilum* Perturbs Circadian Gene Expression in Unfed Nymphs**

A. phagocytophilum modulates several genes in ticks (Dahmani et al., 2020; Granick, Jennifer L et al., 2008; Khanal et al., 2018; Neelakanta et al., 2010; Ramasamy et al., 2020; Sultana et al., 2010; Taank et al., 2017; Turck et al., 2019). The effect of *A. phagocytophilum* on the expression of tick circadian genes was analyzed. The expression pattern of the core clock genes; *clock1*, *bmal1*, *period*, and *timeless1* was analyzed by quantitative RT-PCR. The pattern of gene expression for circadian genes in uninfected nymphs was noted to be similar to the previous studies (Figure 26). The expression of all the clock genes was induced in *A. phagocytophilum* infected ticks at various Zeitgeber times. There was a significant upregulation ($P < 0.001$) in all the four analyzed circadian genes (*clock1*, *bmal1*, *period*, and *timeless1*) at ZT30, in the light cycle (Figure 26). After peaking expression at ZT30 in *A. phagocytophilum*-infected nymphs, the expression pattern of all the circadian genes dipped down and was comparable to the uninfected controls (Figure 26). However, it was noted that *clock* gene expression was also reported to be upregulated in the dark cycle (ZT12-24 h) in *A. phagocytophilum*-infected ticks when compared to uninfected controls. Collectively, these results suggest that *A. phagocytophilum* modulates circadian gene expression in ticks.

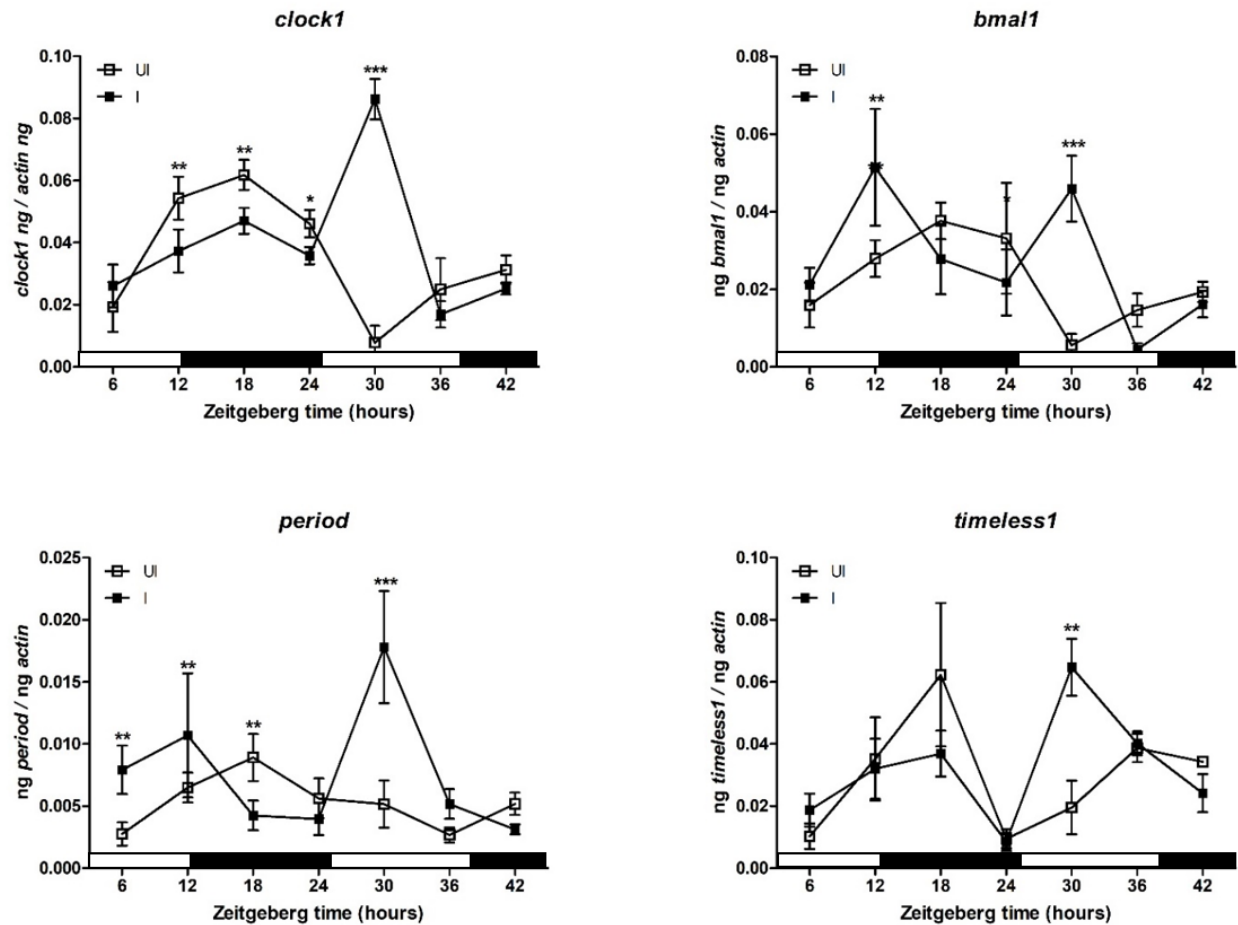


Figure 26. *A. phagocytophilum* Alters Circadian Oscillation in Unfed *I. scapularis*

The graphs show clock genes' expression in uninfected (UI) and *A. phagocytophilum* infected (I) unfed whole nymphs in cycles of 12 h of light and 12 h of dark. The Y-axis indicates relative mRNA abundance normalized with tick beta-actin, and the X-axis represents Zeitgeber time points. Each average in Zeitgeber time was obtained through the average of 5 individual ticks. Error bars represent the standard deviation. Two-way ANOVA was performed for the statistical analysis, and statistical significance is indicated on each graph.

***clock1* and *bmal1* are Upregulated in Fed Nymphs**

The mRNA levels of circadian genes were analyzed in uninfected and *A. phagocytophilum* nymphal ticks upon feeding on the naïve murine host. A significant upregulation of *clock1* and *bmal1* in *A. phagocytophilum* infected fed nymphs was observed when compared to the levels noted in uninfected control fed ticks (Figure 27).

The mRNA levels of *timeless1* and *period* were unaltered between the fed tick groups. No significant differences in the analyzed circadian genes were observed between unfed uninfected and *A. phagocytophilum* infected ticks (Figure 27).

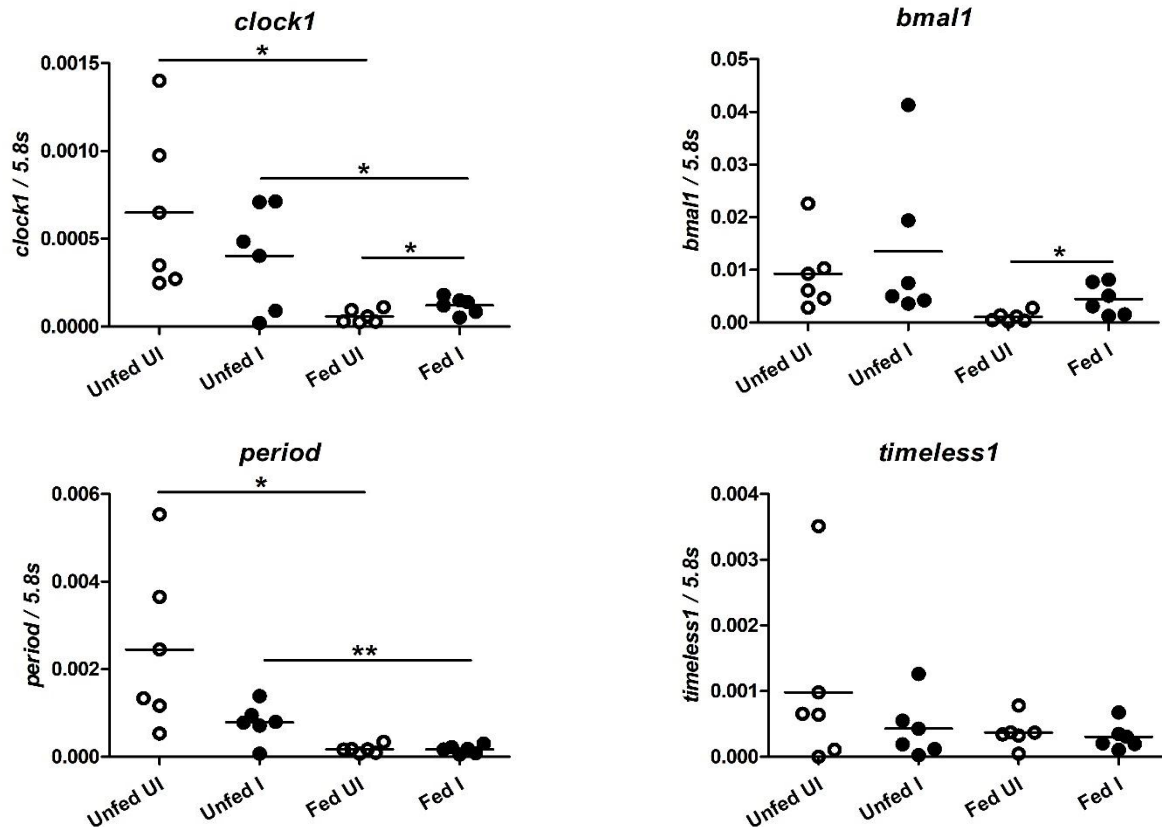


Figure 27. *clock1* and *bmal1* are Upregulated Upon Feeding in *A. phagocytophilum* Infected Ticks
QRT-PCR results showing levels of circadian gene transcripts (*clock1*, *bmal1*, *period*, *timeless1*) in nymphs fed on uninfected or *A. phagocytophilum* infected mice. Both unfed and fed ticks were generated from the same batch for consistency. Statistical analysis was performed using Student's t-test comparing uninfected (UI) and *A. phagocytophilum* infected (I) ticks in each group. A P-value of less than 0.05 was considered significant.

***clock1* Deficient Ticks Feed Late and Have Lower Engorgement Weight**

Ticks deficient for *clock1* were generated by microinjecting *clock1* dsRNA into *A. phagocytophilum* infected ticks. Microinjected ticks were fed on naïve C3HeN mice after

24 hours of incubation and acclimatization. Microinjections were done as described in our previous studies (Khanal et al., 2018; Neelakanta et al., 2010). The repleted and engorged ticks were collected, and repletion time and tick body weights were measured. During transmission, a significant decrease ($P < 0.05$) in the *clock1* mRNA levels was observed in *clock1*-dsRNA (*dsClock1*) injected ticks when compared to mock controls, showing that the expression of the *clock1* gene was silenced (Figure 27A). The repletion time (time after feeding and repletion) was also delayed in the knockdown group when compared to the control group (Figure 27B). The engorged nymphs collected from the *clock1* knockdown group showed significantly less bodyweight than its mock control (Figure 28C). Together, these results indicate an essential role for *clock1* in tick feeding during pathogen transmission.

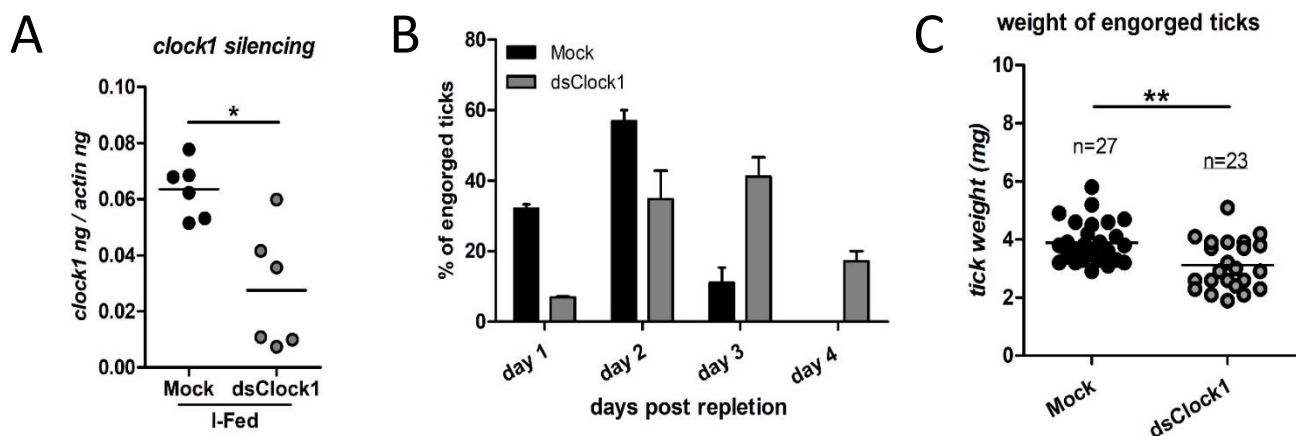


Figure 28. Silencing of Arthropod *clock1* Gene Decreases Tick Weight and Delays Feeding

(A) QRT-PCR results showing silencing efficiency of *clock1* knockdown in *A. phagocytophilum* infected ticks fed on naïve uninfected mice.

(B) Percentage of ticks collected post repletion in mock and dsClock1 knockdown ticks.

(C) Weight of repleted ticks in mock and *clock1*-deficient ticks. All ticks were collected for their body weight measurement.

The data shown above represents a single transmission experiment. Statistical analysis was performed using Student's t-test, and a P-value of less than 0.05 was considered significant in panels A and B.

Tick *clock1* Silencing Increases Bacterial Load During Transmission

Studies were performed to analyze whether *A. phagocytophilum* infection modulates *clock1* gene levels during its transmission from ticks to the murine host. QRT-PCR analysis revealed that silencing of the *clock1* gene expression in nymphal ticks before (Figure 27A). Since *clock1* played a vital role during tick feeding on mice (Figure 27), this experiment aimed to see the effect of *clock1*-knockdown on the transmission of the pathogen from the tick vector to the murine host.

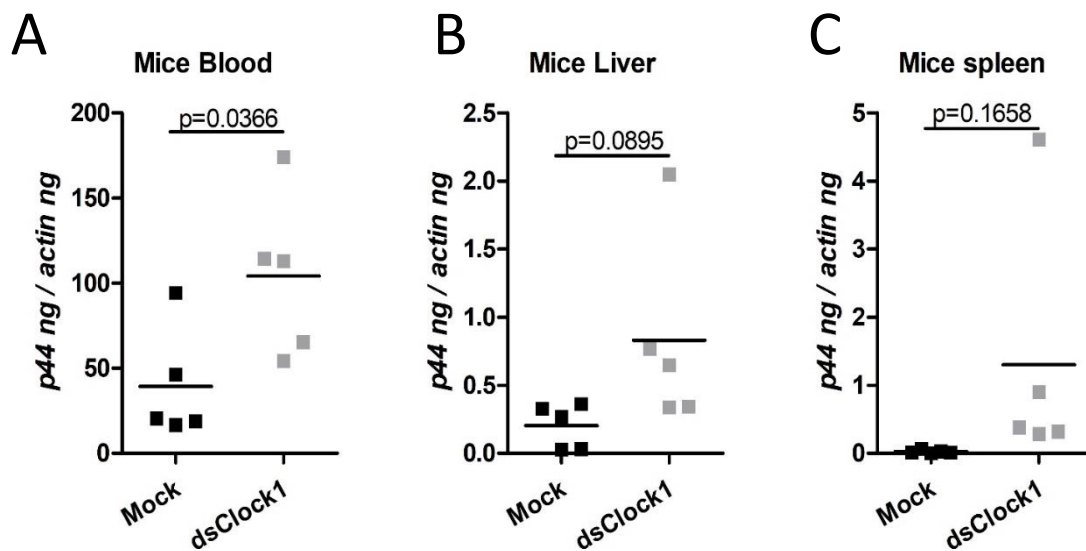


Figure 29. *A. phagocytophilum* Bacterial Load in Mice Tissues after Transmission

(A-C) Bacterial burden in mice tissues (blood, liver, and spleen).

DNA was extracted from the mice tissues from each group, and *A. phagocytophilum* p44 DNA levels were normalized to mice beta-actin levels. The data was generated from a transmission experiment with five mice in each group. The bacterial load from the mice tissues was determined from the mice infested with *A. phagocytophilum*-infected nymphal ticks injected with *clock1*-dsRNA, or mock is shown. Statistical analysis was performed using Student's t-test, and P-value is shown.

To check whether the *clock1* gene regulation affects bacterial transmission from ticks to vertebrate host, *A. phagocytophilum*-infected unfed nymphal ticks were microinjected with *clock1* dsRNA or mock control and then fed on uninfected mice.

QRT-PCR analysis showed increased bacterial load in mice tissues infested with *clock1*- dsRNA injected ticks compared to mock control-injected ticks. A significant increase in bacterial burden ($P < 0.05$) was observed in mice blood (Figure 29A), and an increasing trend was also seen in mice tissues such as liver and spleen (Figure 28B & C). These results show that downregulation of the *clock1* gene allowed increased transmission of *A. phagocytophilum* to the vertebrate host. Collectively, these results elucidate that *A. phagocytophilum* modulates the tick *clock1* gene for its transmission from tick to the murine host.

4.3 DISCUSSION

Animals have developed diurnal circadian rhythms that are often entrained by day and night cycles (Kreitzman and Foster, 2011). The presence of one or other circadian systems (diurnal and nocturnal) has been previously observed from lower invertebrates to higher mammals (Lam and Chiu, 2017). Despite the lack of a nervous system, animals like sponges and protozoa can detect light and display diurnal physiological and reproductive patterns, resembling a circadian clock (Rivera et al., 2012). In this study, circadian genes in *I. scapularis* were screened from the tick genome and characterized during tick feeding and pathogen transmission.

Domains like bHLH-PAS are the core transcriptionally functional domains found in *clock/bmal/cycle* circadian genes. Clock and ARNTL/Cycle are the central positive regulatory elements of the metazoan circadian network system (Simionato et al., 2007). *I. scapualris* genome contains a *clock* gene, but no ortholog for *cycle* was noted, although a closely related *ARNT* gene is present. Previous studies from (Egekwu et al., 2014; Gulia-Nuss et al., 2016) have reported circadian genes in ticks, but their physiological roles have not been extensively studied. Circadian genes play an important role in physiological responses of an organism, development, feeding behavior, symbiosis, and metabolism (Kreitzman and Foster, 2011; Lam and Chiu, 2017; Meireles-Filho and Kyriacou, 2013). All of the studied circadian genes showed an oscillating expression with 12 hours of light and dark incubation. The laboratory conditions do not mimic the natural habitats where the ticks are found, but it shows diurnal expression using light as a stimulus. However, this study provides preliminary

observations on the oscillation of circadian genes in ticks in response to the day-light cycle.

The oscillations of the circadian genes like *clock1*, *bmal1*, *period*, *timeless1* indicate the activity of the circadian system in ticks. Previous studies suggest that organismic physiological behavior, humoral and neural factors control myriad aspects of metabolism affected by the circadian system (Edery, 2000; Kreitzman and Foster, 2011; Xie et al., 2019). In *A. phagocytophilum* infected ticks, the circadian rhythms were perturbed at different zeitgeber times. This shows the manipulation of the host circadian system by the pathogen for its survival and growth. To further have a broader view of this multisystem network, "Circadian–Metabolic–Microbial" Supersystem was also suggested, where the component systems can influence each other (Barik, 2019). The capacity to maintain the cyclic output for a more extended period after removing the entraining cue is one of the prominent defining features of the classical circadian network regulating varied metabolic functions within an organism (Meireles-Filho and Kyriacou, 2013; Xie et al., 2019). The results from this study provide evidence that circadian factors can all regulate the host's interaction with intracellular pathogens like *A. phagocytophilum* in *I. scapularis* ticks. The complexity of the circadian network makes it difficult to decipher if the modulation of the host's circadian rhythms is a direct or an indirect phenomenon by the pathogen.

The daily metabolic requirements vary drastically between rest and feeding behavior in arthropods like mosquitoes (Meireles-Filho and Kyriacou, 2013; Panda, 2016; Sandrelli et al., 2008). Previous reports suggest that the mosquitoes' sugar and blood-feeding are restricted to the night phase (Clements, 1992). These temporal changes and nutritional behavior are attributed to circadian control (Meireles-Filho and Kyriacou, 2013; Panda, 2016). Internal metabolic pathways such as oxidative phosphorylation, lipogenesis, and gluconeogenesis are rhythmically expressed in many organisms. Periodic gene expression of the clock genes is interesting as they are upregulated by sugar starvation, light sensitivity, and blood-feeding (Panda, 2016). Ingestion of blood meal in mosquitoes is under the circadian control and modulated by a light stimulus, both in a clock-dependent and in an independent manner (Das and Dimopoulos, 2008). The authors used RNAi to demonstrate the essential function of

clock genes during mosquito feeding. In this study, the circadian components, *clock1* and *bmal1* were upregulated in *A. phagocytophilum* infected ticks upon feeding on the murine host. The other circadian genes, *period* and *timeless1*, were unaffected by the pathogen during transmission, suggesting a specific role for *clock1* in *A. phagocytophilum* transmission during blood feeding. The observation of reduced transcripts of circadian genes in fed ticks compared to the levels noted in unfed ticks suggests modulation of signaling by circadian factors during tick feeding.

The observation of reduced engorgement weights in *clock-dsRNA*-injected ticks compared to the weights noted in mock-treated control ticks suggests a role for *clock1* in tick blood-feeding. The observation of delayed feeding time also supports the role of *clock1* in successful feeding and complete engorgement during tick blood-feeding. The circadian genes are rhythmically expressed in the ticks, and deficiency of this metabolic gene affects the feeding behavior in the ticks. The absence of *clock1* could affect other circadian genes important for blood-feeding or could affect other factors important in tick blood-feeding. It is also plausible to state that the circadian transcriptional regulation in the presence of a pathogen is reprogrammed and allows the pathogen to multiply and modulate the host's circadian pattern. The vital role of *Anopheles gambiae* clock genes during blood feeding is light-dependent and was shown by RNAi-mediated gene silencing and DNA microarray analysis (McDonald and Rosbash, 2001). During *A. phagocytophilum* transmission, the load of bacteria in mice blood, liver, and spleen with *clock1* knockdown was found to be increased. This observation of higher bacterial load in mice infested with *clock1* dsRNA compared to mock controls suggests the symbiotic role of *A. phagocytophilum* in its vector hosts, thereby modulating their circadian behavior during a blood meal (Rund et al., 2013; Rund et al., 2011). The combined observation of increased *clock1* expression and increased bacterial transmission from *clock-1* deficient ticks suggest a host (vector) response limiting the bacterial dissemination. A putative beneficial relationship between ticks and *A. phagocytophilum* could encourage ticks to limit this bacterial transmission from vector to the vertebrate host. This study provides a fascinating model on the pathogen-associated modulation of circadian genes during blood feeding and pathogen transmission.

4.4 EXPERIMENTAL PROCEDURES

Identification of Circadian Genes from *I. scapularis* Genome

To identify tick circadian clock genes, the amino acid sequences were downloaded from the *Ixodes scapularis* genome. BLAST searches of the *I. scapularis* genome were performed within Vectorbase (www.vectorbase.org; www.ncbi.nlm.nih.gov) and NCBI (NCBI, <http://www.ncbi.nlm.nih.gov/>) databases and used them as query sequences to conduct BLASTp searches in the *I. scapularis* genome. The top hit sequences were selected based on their lowest E-value using a cutoff E-value of 1e-30. SMART, InterPro, and CDD algorithms were used to predict their domains and motifs. Motifs with a higher than 40% identity were considered conserved. The circadian clock protein sequences from other nearby orthologous groups were searched, and their orthology was accessed comparing the domains and motifs present in the common orthologs.

Phylogenetic Analysis

Phylogenetic investigation of circadian clock proteins was done using CLC Bio software from the Old Dominion University High-Performance Computing Cluster. The neighbor-joining method was applied to construct circadian clock trees with the p-distance model, pairwise deletion of gaps, and bootstrapping (1000 replicates: random seed). The whole genomic sequence of tick circadian clock proteins was retrieved from the National Center for Biotechnology Information or Vectorbase. The genomic location was searched, and the domains were identified for orthology.

Ethics Statement

All experiments in this study were performed following relevant guidelines and regulations. All the animal work was ethically carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. Old Dominion University Animal and University Institutional Animal Care and Use Committee (IACUC) approved the current experimental protocol (permit number 16–017 and 19–009). Institutional biosafety committee approval (number 15-012) was used to perform experiments in this study.

Ticks and Mice

I. scapularis nymphal ticks were used throughout unless specified. Laboratory-reared specimens of black-legged *I. scapularis* ticks were used. Various developmental stages of ticks (larvae, nymphs, adult male, and female) used in this study were either obtained from BEI Resources (NIAID, NIH) or reared inside the laboratory facility. Ticks used in this study were maintained at the Department of Biological Sciences (ODU) or from a continuously maintained tick colony at the Connecticut Agricultural Experiment Station (New Haven, CT). Ticks were fed on uninfected or *A. phagocytophilum* infected mice with proper incubator conditions. The fed larvae were then allowed to molt into nymphs. The engorged ticks were stored in a Parameter Generation and Control incubator (Black Mountain, NC) at $26\pm1^{\circ}\text{C}$, $92\pm1\%$ relative humidity, and 14:10 (L: D) molting or further tick experiments. The molted *A. phagocytophilum* infected unfed nymphs were used in circadian oscillation experiments, gene expression analysis, and transmission experiments in mice. Fed nymphs were used in the expression analysis of circadian genes.

Bacterial Isolate

NCH-1 strain of *Anaplasma phagocytophilum* was used throughout the study. Uninfected and *A. phagocytophilum* NCH-1 strain–infected human promyelocytic HL-60 cells (ATCC) were cultured. *A. phagocytophilum* NCH-1 strain was maintained in the human promyelocytic cell line (HL-60) as described (Ramasamy et al., 2020). DC form of cell-free bacteria isolated from these cells were used to generate *A. phagocytophilum* infected mice.

RNA/DNA Extractions and Gene Expression Analysis

Total RNA was extracted from various tick samples and tick cells. Total RNA was reverse transcribed into cDNA using a cDNA synthesis kit. Realtime QRT-PCR was performed with SYBR Green Premix using the CFX96 Real-time system (Biorad, USA) as described (Dahmani et al., 2020; Ramasamy et al., 2020). The real-time qPCR results are shown as the ratio of transcripts from individual genes against the housekeeping genes using relative and absolute quantification methods. Normalization

was done using tick beta-actin or tick 5.8s and mentioned wherever necessary. The real-time expression data were analyzed using Prism 7.0. A student's t-test was used to compare statistical significance between the groups, or ANOVA was used to compare the group's variations. The primers that were used in this study are listed in Supplementary Table 2 in the Appendix. At least six biological replicates were used to compare means and indicated wherever necessary. Each biological replicate was performed in duplicates.

dsRNA Synthesis and Transmission Experiments in Mice

The *clock1* gene sequence was amplified, confirmed by sequencing, and T7 promoter sequences were added at 5' end. The entire genomic sequence was synthesized and was used as a DNA template for amplification. The dsRNA for *clock1* synthesis was performed using MEGAscript RNAi Kit (Ambion Inc. USA) following manufacturer instructions and as described (Dahmani et al., 2020; Khanal et al., 2018; Ramasamy et al., 2020; Taank et al., 2017). Microinjections (~4.2 nl/tick) were performed with *A. phagocytophilum* infected nymphs with dsRNA for *clock1* (dsClock1) or its empty L4440 vector controls. The microinjected ticks were incubated for 48 hours in a desiccator (for recovery) inside an incubator set at $23 \pm 2^{\circ}\text{C}$ with 95% relative humidity and a 14/10-hour light/dark photoperiod. After 18-24 hours, the microinjected ticks were fed on five uninfected mice in each group. The engorged ticks were collected after repletion, and their tick measurements were taken as soon as possible for each round of collection. The engorgement time, feeding time, and the mortality of the ticks were carefully monitored. After five days post repletion of all engorged ticks, the mice were dissected, and specific tissues (blood, spleen, liver) were collected separately from each group. Total RNA was extracted from the repleted ticks, and the total genomic DNA was extracted from the mice tissues using the Qiagen DNA extraction kit (Qiagen, USA). QRT-PCR analysis was performed to determine the expression of clock and bacterial load in the mice tissues.

Tick Body Weight Measurements

Engorged fed ticks were collected immediately after repletion, cleaned with brushes to remove any mice hair or feces, and weighed using a microbalance in duplicates to minimize errors. Total body weight measurements were obtained by directly placing each tick on a balance pan. Data in the figures show the number of repleted ticks in each group for analyzing tick body weights.

Statistics

All the data set were statistically analyzed using GraphPad Prism 6 software. The non-paired Student t-test was considered to compare the experimental data sets with two variables. One or two-way ANOVA was performed for comparing data obtained from more than two variables. P values of <0.05 were deemed to be significant for the data analyzed. The statistical method used for the experiments and the P values obtained in the data analyses are mentioned in the figures and in the legends wherever necessary.

CHAPTER 5

CONCLUSION

The black-legged tick, *I. scapularis*, is a medically important vector that transmits several human pathogens, including *A. phagocytophilum*. The development of newer strategies to prevent/target tick-borne diseases is highly warranted, as the incidence of tick-borne illness is on the rise (WHO, 2014). Current studies provide insights on the mechanistic signaling of *A. phagocytophilum* inside vertebrate and invertebrate hosts during host-vector-pathogen interactions. These studies reveal how the bacterium modulates tick and mammalian host cellular signaling for its growth and survival and overall transmission between its vectors.

The first study showed replication of *A. phagocytophilum* in human cells of hematopoietic origin. Differential modulation of cell cycle genes allows the efficient survival and persistence of the pathogen inside the host cells. As *A. phagocytophilum* is intracellular, it causes less cytotoxicity in the dividing host cells for its benefit, both during the early and late stages of infection. The increased PI3 kinases in infected cells show the regulation of PI3 kinases in cell cycle progression. These results might indicate a common signaling pathway used by intracellular pathogens such as *A. phagocytophilum*, using Akt- NF- κ B signaling pathway for cell cycle regulation. NF- κ B activation induces the expression of anti-apoptotic genes in various cells. Especially in megakaryocytes, which undergo endomitosis for platelet formation, it perhaps allows polyploidy via PI3K-NF- κ B pathway during early intracellular infection but after 7 days p.i. inhibits polyploidy, thereby resulting in fewer platelet formation. This study describes a possible cause for thrombocytopenia due to *A. phagocytophilum*-infection. This study provides a fascinating model to study cell cycle gene modulation and provide strategies to combat human intracellular pathogens in megakaryocytes. But the precise role for PI3 kinases in the endomitosis of megakaryocytes during *A. phagocytophilum* infections remain to be studied.

The second study explores the role of AP-1 transcriptional factor in the survival of *A. phagocytophilum* in ticks. Neelakanta et al. 2010 identified a tick antifreeze glycoprotein, IAFGP, that allows ticks to survive colder and freezing temperatures better. However, the transcriptional regulation of *iafgp* was not known. Here, in this study, the essential role of *A. phagocytophilum* modulating AP-1 as an upstream activator for regulating tick *iafgp* gene expression is shown. Bioinformatics analysis showed the presence of the bZIP domain at the C-terminus region of *I. scapularis* AP-1. The phylogeny revealed considerable conservation of AP-1 among the members of the Ixodidae family and vertebrates. *ap-1* was found to be upregulated in unfed nymphal tick salivary glands and during feeding. EMSAs and promoter activity assays showed the upstream region of 700 bp from the *iafgp* gene is enough to drive its expression in ticks. *A. phagocytophilum* was found to induce the expression of *ap-1* and *iafgp*, thus showing a more beneficial role of this pathogen in tick physiology. The ~700 bp region contains the TATA and AP-1 binding sites, and the binding of the probes from those sites proved that the regulatory function of AP-1 induced by *A. phagocytophilum*. This study is in accordance with earlier studies showing that genes containing TATA-box promoters are correlated with stress and are highly regulated compared to TATA-less promoter genes. As *iafgp* is a stress-induced (infection and cold) gene with a strong TATA-box, several transcription factors could bind to different regions of the *iafgp* promoter and induce its expression. RNAi was used to show the survival of the ticks under cold stress. Ticks with *ap-1* expression showed better survivability and fitness compared to *ap-1* knockdown ticks. In summary, *A. phagocytophilum* alters arthropod AP-1 to activate tick *iafgp*, assisting the survival of both the rickettsial pathogen and its vector host in the cold. This study shows an exciting role for this bacterium in tick physiology. Still, the role of AP-1 in the tick colonization of the rickettsial pathogen inside their tick salivary glands and its specific role during transmission needs to be explored.

In the third study, identification, and characterization of circadian genes from *Ixodes scapularis* were reported. The study elucidated a vital role for *clock1* gene in tick feeding. This study uncovers an additional layer of physiological functions of the *clock* gene in tick behavior and physiology. Animals, from lower invertebrates to higher

mammals, have developed a circadian system for their physiological functions and responses. Circadian rhythms are the inherent and natural biological processes that display periodic oscillations regulated by a molecular circadian clock. Domains like bHLH-PAS are the functional domains found in *clock/bmal/cycle* proteins. Among them, Clock and ARNTL/Cycle are the central positive regulatory elements that induce the expression of other circadian genes like *period* and *timeless*. Collectively they assist in the organism's physiological responses, including development, metabolism, and behavior. The presence of *A. phagocytophilum* perturbs the vector host circadian rhythms and manipulates for its survival and transmission. Previous studies show the intrinsic role of circadian clock genes in behavior, metabolism, and physiology. The knockdown of *clock1* in ticks delayed its repletion time and lowered the body weights of engorged ticks. Earlier studies have stressed the significance of the *clock* gene in mosquito feeding and nutritional processes. This study also showed that *clock1* from ticks is crucial in tick feeding and the transmission of the rickettsial pathogen from ticks to the murine hosts.

Together, these studies provide significant evidence on the pathogen-associated modulation of host genes for its replication and survival. These studies would not only provide insights on the pathogen-associated modulation of molecular signaling in host-vector-pathogen interactions but also would provide information for the development of strategies to target rickettsial pathogens transmitted by the vectors.

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Title: *Anaplasma phagocytophilum* infection modulates expression of megakaryocyte cell cycle genes through phosphatidylinositol-3-kinase signaling

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PEER-REVIEWED PUBLICATIONS

- Regmi P, Khanal S, Neelakanta G, Sultana H, (2020) Tick-borne flavivirus inhibits sphingomyelinase (IsSMase), a venomous spider ortholog to increase sphingomyelin lipid levels for its Survival in *Ixodes scapularis* ticks. Front. Cell. Infect. Microbiol. 10, 244
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