

DROSOPHILA MELANOGASTER AS A MODEL FOR STUDYING *EHRlichia*
CHAFFEENSIS INFECTIONS

by

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B.S., University of Pittsburgh, 2001
M.S. Shippensburg University, 2004

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Abstract

Ehrlichia chaffeensis is an obligate, intracellular bacterium that causes human monocytic ehrlichiosis (HME). The bacteria are vectored by the Lone Star tick (*Amblyomma americanum*), which is found primarily in the Midwestern and Southeastern United States. *E. chaffeensis* was first reported in 1986 and HME was designated a nationally reportable disease by the United States Centers for Disease Control in 1999. *Ehrlichia* grows in several mammalian cell lines, but NO consensus model for pathogenesis exists for arthropods or vertebrates. Moreover, the host genes required for intracellular growth of this bacteria are unknown. We first established that the bacteria could infect and replicate both *in vitro* and *in vivo* in *Drosophila melanogaster* S2 cells and adult flies, respectively. We performed microarrays on S2 cells, comparing host gene expression between permissive or non-permissive conditions for *E. chaffeensis* growth. A total of 210 permissive, exclusive and 83 non-permissive, exclusive genes were up-regulated greater than 1.5-fold above uninfected cells. We screened flies mutant for genes identified in our microarrays for their ability to support *Ehrlichia* replication. Five mutant stocks were resistant to infection with *Ehrlichia* (genes *CG6479*, *separation anxiety*, *CG3044*, *CG6364*, and *CG6543*). qRT-PCR confirmed that bacterial load was decreased in mutant flies compared to wild-type controls. In particular, gene *CG6364* is predicted to have uridine kinase activity. Thus, the *in vivo* mutation of this gene putatively disrupts the nucleotide salvage pathway, causing a decrease in bacterial replication. To further test the function of gene *CG6364* in bacterial replication, we obtained cyclopentenyl cytosine (CPEC) from the National Cancer Institute. CPEC is a cytidine triphosphate (CTP) inhibitor known to deplete CTP pools in various cancers and to exhibit antiviral activity. Consequently, it inhibits de novo nucleotide synthesis, but doesn't affect the nucleotide salvage pathway. When S2 cells were treated with CPEC and infected with *Ehrlichia*, an increase in bacterial replication was confirmed by qRT-PCR. Furthermore, addition of cytosine to S2 cells also resulted in increased bacterial replication. Therefore the nucleotide salvage pathway through cytidine appears necessary for bacterial replication. Our approach has successfully identified host genes that contribute to the pathogenicity of *E. chaffeensis* in *Drosophila*.

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Approved by:

Major Professor
Dr. Stephen Keith Chapes

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To all of my family and friends – Thank you for being so good to me always. Our times together are the heartbeat of my existence.

Dedication

Dedicated to my loved ones who have long passed on, but would have undoubtedly celebrated with me if they were still here --- my grandparents Bunny & Evelyn Luce and Steinie & Ella Fedrow; my nanny Martha Miller; and my cousins “Uncle” Jeff and Rachel Ann Abbott. I think of you often....

CHAPTER 1 - Literature Review

Rickettsia

The order *Rickettsiales* includes the families of *Anaplasmataceae* and *Rickettsiaceae* (48). The organisms included in these families are Gram-negative, obligate, intracellular bacteria that grow in the cytoplasm or vacuoles within the host cell, and are vectored by arthropods and trematodes (125, 139). Among the diseases caused by bacteria in the order *Rickettsiales* are: (1) rickettsioses caused by bacteria of the genus *Rickettsia*; (2) ehrlichioses and anaplamoses caused by the members of the *Anaplasmataceae* family; and (3) scrub typhus resulting from infection with *Orientia tsutsugamushi* (125). The first rickettsiosis reported in the United States was *Rickettsia rickettsii* in 1906, which is the causative agent of Rocky Mountain Spotted Fever (145, 184). Since then, diseases resulting from infection with bacteria in the order *Rickettsiales* have been reported in Europe, Asia, Africa, and Australia.

Bacteria of the genus *Rickettsia* are responsible for diseases classified as spotted fever and typhus group rickettsioses. Several members of the *Rickettsia* genus are considered to be emerging pathogens across the world and include bacteria such as *R. japonica* (6), *R. africae* (91), *R. honei* (9), and *R. slovaca* (28). The spotted fever rickettsia are transmitted by hard ticks (*Ixodids*), via the biting of humans/mammals. Upon transmission to humans, the bacteria grow and multiply in the cytoplasm of endothelial cells (125). Symptoms associated with rickettsial infection are flu-like in nature and include the hallmark inoculation eschar at the site of the tick bite (125). Disease resulting from infection with spotted fever group rickettsia can range from mild to lethal.

Members of *Rickettsiaceae* can also cause typhus in humans. *R. felis* is the causative agent of cat-flea typhus and *O. tsutsugamushi* causes scrub typhus. The details concerning infection with *R. felis* are not well described. Suspected infections in humans have been detected in patients from several countries (140, 144, 192). In addition, *R. felis* has also been isolated from fleas in several countries (95, 96, 110, 121, 128, 140).

Among the ehrlichioses and anaplasmoses that are found in the family Anaplasmataceae, four have been shown to cause disease in humans: (1) *Ehrlichia chaffeensis*; (2) *Anaplasma phagocytophilum*; (3) *Neorickettsia sennetsu*; and (4) *E. ewingii* (47). All of these bacteria are transmitted to humans by the bite of an infected tick or through the consumption of contaminated snails/fish. The bacteria are maintained in the tick by transtadial transmission (from one life stage to another) and are maintained in nature by transmission from the tick(s) to mammals, or flukes to mammals via snails or fish, which serve as reservoirs for the bacteria. Humans are an accidental host for the bacteria, which is transmitted through the bite of an infected tick.

E. chaffeensis is the causative agent of human monocytic ehrlichiosis (HME) and was first identified in 1986 in a man bitten by ticks at Fort Chaffee, Arkansas (47). Earlier reports of illness resulting from the bite of Lone Star ticks were reported in troops in 1943 at Camp Bullis (71, 185). It is suspected that *E. chaffeensis* may have been the causative bacteria in these early reports. From 1986-2006, approximately 3600 cases of HME were reported to the CDC; among which 338 were confirmed by a United States national surveillance program (64, 123, 187). At commercial labs in the United States, approximately 1200 cases of HME were reported between

1992-1998 (123). 828 cases were reported for 2007, which represent a 44% increase in over those reported for 2006, and an overall 159% increase since 2003 (76). In addition to being reported in the United States, the occurrence of *E. chaffeensis* has also been documented in Africa, Europe, China, and Brazil (23, 36, 108, 180).

A. phagocytophilum causes human granulocytic ehrlichiosis (which is now regarded as human granulocytic anaplasmosis) and was first reported in the United States in 1994 (30). Like *E. chaffeensis*, cases of *A. phagocytophilum* have also been recorded in the United States. Since 1994, approximately 2963 cases have been recognized (29). Moreover, *A. phagocytophilum* has been documented in humans and animals across Europe (19, 125, 130).

N. sennetsu is an obligate, intracellular bacterium believed to be harbored by trematodes found in fish, and is transferred to humans by ingestion of raw or undercooked fish (118, 177). The bacteria was first reported in Japan in 1954 and then seemed to disappear until 1985, when antibodies were isolated from patients in Malaysia (118). The body of knowledge surrounding *N. sennetsu* is limited and information concerning its incidence and geographic associations require additional research. Some concern over its prevalence has recently emerged due to the spreading trends of consuming raw fish, particularly in the United States and East Asia (118).

E. ewingii is vectored by the same tick species as *E. chaffeensis*, *Amblyomma americanum*. While the first infections of *E. ewingii* were reported in dogs in the United States in 1971, the first cases in human were not reported until 1999 (47, 56). An additional four human infections in HIV patients were reported in 2001, which reflects the opportunistic nature

of these organisms (124). Diagnosis of *E. ewingii* is difficult because the bacteria are uncultivable in the lab and have high antibody cross-reactivity with *E. chaffeensis*. The most confirmatory and recently used technique for verifying infections is polymerase chain reaction (PCR) (7, 105, 167). Hence, statistics concerning the prevalence of the bacteria are lacking.

Other old and new members of Anaplasmataceae have recently been suspected of having the potential to cause infection in humans. *E. canis* was isolated from a man in Venezuela and *E. ruminantium* from patients in South Africa (107, 129). Two new members of the *E. canis* group were identified from ticks in Mali and Niger; and *Ixodes ovatus ehrlichiae* (thought to be closely related to *E. chaffeensis*) was isolated from ticks in Japan. It is not known if this newly isolated species is capable of infecting humans (126, 159).

Ehrlichia chaffeensis

Ehrlichia chaffeensis is an obligate, intracellular bacterium, and the causative agent of human monocytic ehrlichiosis (HME). Its genome sequence was completed in 2006 and contains 1,176,248 base pairs (81). *E. chaffeensis* belongs to the family *Anaplasmataceae* and the Ehrlichiosis group of diseases which includes *Neorickettsia sennetsu* (causative agent of Sennetsu fever), *Anaplasma phagocytophilum* (causative agent of human granulocytic ehrlichiosis), *E. ewingii* (causative agent of granulocytic ehrlichiosis in dogs), and *E. chaffeensis*. Symptoms of HME usually begin 1-21 days post infection and include fever, headache, chills, muscle aches, fatigue, nausea/vomiting, swollen glands, diarrhea, delirium, and coma (174). In addition, a rash also occurs in approximately 40% of HME cases, and central nervous system

involvement and gastrointestinal disorders may also be observed (174). The drug of choice for treatment of HME is tetracycline or doxycycline, which inhibits protein synthesis of bacteria by reversible binding to the 30S ribosomal subunit, thereby preventing formation of peptide chains (10, 123). Case fatality rates are approximately 3% for HME, but without appropriate treatment(s) of the disease, irreversible neurological damage may result due to acute inflammatory responses (10, 123, 174). Diagnostic procedures for detection of the bacteria in patient samples include PCR amplification, antibody-based immunofluorescence assay or Western blot, and/or cultivation of the bacteria in cell monolayers (125).

Ehrlichiae are gram-negative, cocci (round) shaped bacteria, measuring 0.5-2 μm in diameter (133, 134) that, upon infection, form vacuole bound colonies (called morulae) mostly in leukocytes (123). Intracellularly, the bacteria are observed as either an infectious dense-cored form or a dividing reticulate form within intracellular vacuoles (193). Although *E. chaffeensis* is classified as a Gram-negative organism, this organism does not have genes for the synthesis of lipopolysaccharide (LPS) or peptidoglycan (106), which may ultimately affect the host immune response to infection with this organism.

E. chaffeensis is primarily vectored by the *Ixodes* tick *Amblyomma americanum* (also known as the lone star tick) and white-tailed deer (*Odocoileus virginianus*) are considered to be the major reservoir for the organism. More recently, *E. chaffeensis* was found to also be present in *Dermacentor variabilis* and *Ixodes pacificus* ticks (178). The bacteria are transmitted transtadially (from one life form to the next), but not transovarially among the ticks. The distribution of *A. americanum* occurs from western Texas across the southeastern and

midwestern United States. Correspondingly, the majority of cases of HME are reported from these regions of the United States. However, cases of HME have been reported in the northeastern United States and *A. americanum* ticks from Connecticut and Rhode Island have tested PCR-positive for *E. chaffeensis* (123). *A. americanum* ticks follow a three-host life cycle that takes 2-4 years for completion (127) and all stages of *A. americanum* may bite humans. In order to molt from one life stage to the next (e.g. egg to larvae to nymph to adult), the tick must take a blood meal from a vertebrate host. Immature feeding states (larvae and nymphs) can usually be found taking a blood meal on smaller mammalian hosts such as *Peromyscus leucopus* (white-footed mouse), *Tamias striatus* (chipmunk), *Microtus pennsylvanicus* (short-tailed shrews), *Sylvilagus floridanus* (cottontail rabbits), and *Turdus migratorius* (American robin) (79). Feeding adults are most often found on medium/larger-sized mammals. The white-tailed deer is the most commonly parasitized species, but the ticks have also been found on *Ursus americanus* (black bears), *Didelphis virginiana* (opossums), *Procyon lotor* (raccoons), *Sciurus carolinensis* (gray squirrels), and *Vulpes vulpes* (red fox) (79). Each feeding stage takes its blood meal, detaches from the host, drops from the host and molts to the next stage. Adults feed to repletion on their final host, the males die on the host, females fall to the ground, lay their eggs, and die. The three-host lifecycle is in contrast to the one-host lifecycle followed by other *Ixodes* ticks, such as *D. albipictus* (winter tick) (4). In the case of a one-host lifecycle, all life stages and mating take place on one vertebrate host. Thus transmission of bacteria occurs only between the tick and its one host. During the three-host life cycle, the tick has the potential to infect or be infected by multiple different hosts.

E. chaffeensis has been defined as containing immunodominant 29-kilodalton outer membrane proteins (119, 142, 161). The genes encoding the p28-Omp are contained in a

multigene locus containing 22 tandem genes (119, 142, 161). Other members of *Rickettsiales* such as *E. canis*, *E. ruminantium*, and *Anaplasma* species also have homologous multigene loci (11, 27, 31). It has been hypothesized that differential expression of the proteins from these genetic loci may contribute to the lifecycle and persistence of *E. chaffeensis* in invertebrate and vertebrate hosts. To better define protein expression from the p28-Omp locus, Singu et al. (161, 162) performed a series of proteomic studies to determine the expression of these proteins in vertebrate (canine DH82 macrophage cell line) and invertebrate (*Ixodes scapularis* tick cells) hosts. In addition, the group also demonstrated the presence of post-translations modifications of p28-Omp locus proteins. It was found that 50% of the proteins were expressed differentially between the *E. chaffeensis* grown in canine macrophage cells and the bacteria grown in the *Ixodes* tick cells. Accordingly, the immunodominant proteins expressed by *E. chaffeensis* grown in the canine macrophages were p28-Omp19 and -20; and p28-Omp14 for the *E. chaffeensis* grown in the tick cells.

Singu et al. (2006) also detailed the gene expression from the p28-Omp locus of *E. chaffeensis* isolates of Group I (Arkansas), II (St. Vincent), and III (Jax) (31). In addition, they monitored gene expression of *E. chaffeensis* grown in an *Amblyomma americanum* cell line, which was derived from the natural tick vector for the bacteria. They confirmed that the expressed 28-30 kilodalton proteins were orthologues of the p28-Omp19 and -20 genes. They also found that protein expression in the *A. americanum* cells was similar to that in the *I. scapularis* cells – p28-Omp gene 14 expression.

The host response to *Ehrlichia chaffeensis*

The differential gene expression from the p28-Omp locus in vertebrate and invertebrate systems may be an important factor for bacterial survival/persistence in its tick and mammalian hosts. There were significant differences in clearance of bacteria, antibody responses, macrophage activation, generation of memory cells, and cytokine production in mice that were infected with the bacteria that had been grown in DH82 compared to bacteria grown in tick cells (61). In particular, mice infected with bacteria grown in the tick cells took approximately 1.5 weeks longer to completely clear the infection, and bacterial loads in the spleen, peritoneum, and liver were higher than in the mice that were challenged with DH82-grown *Ehrlichia chaffeensis* (61). Similarly, the IgG levels in mice infected with tick cell-derived bacteria were also higher for a longer period of time, which reflected the prolonged infection (61). While nitric oxide production and effector cell memory were similar between the two groups of mice, IL-6 and spleen cytokine production was higher in mice infected with the *E. chaffeensis* that was grown in DH82 cells (61). This suggested that the innate responses may be critical to the success of the bacterial infection.

Various types of mice have been used extensively to study the host immune response to *Ehrlichia* species. *E. chaffeensis*, *E. muris*, and *Ixodes ovatus ehrlichiae* (IOE) all infect mice, and each pathogen presents differently in the host. *E. chaffeensis* is cleared by immunocompetent mice; *E. muris* causes persistent infection; and IOE causes fatal infection. Among the three models of HME, *E. muris* and IOE most closely resemble the symptoms/pathogenesis observed during acute human monocytic ehrlichiosis (120). The role(s) of antibodies and T-cells have been investigated for each type of bacterial infection.

One of the first studies to detail the murine immune response to *E. chaffeensis* was performed by Telford and Dawson (1996) (171). In their study, C3H/HeJ mice were infected with *E. chaffeensis* and monitored for their antibody response and ehrlichial invasion of the blood, cells, and organs. C3H/HeJ mice have macrophages that do not respond to endotoxin and display reduced Fc-binding (175). In the C3H/HeJ mice, seroconversion was observed in 92% of the mice by 15 days following infection and the mice developed persistent infections for at least one month. The persistent infection was marked by a high IgG antibody titer specific for *E. chaffeensis*. Blood and spleen samples were also monitored for the presence of ehrlichiae. Visual inspections of blood smears did not show the presence of morulae within the cells, but polymerase chain reaction (PCR) demonstrated the presence of ehrlichial DNA for up to one month following infection. Furthermore, lesions were not detected in organs taken from the C3H/HeJ mice at 14 days following infection. They concluded that the C3H/HeJ mice may not serve as an appropriate model for studying the pathogenesis of human HME, but they were a good model for studying the development of protective immunity against *E. chaffeensis*.

Another animal model for HME was introduced by Winslow et al. (1998) (182). These investigators compared immunocompetent and immunocompromised (SCID) mice to demonstrate that T- and B-cells were necessary for adaptive immunity during *E. chaffeensis* infections. C.B-17, C.B-17-*scid*, C.B-17-*scid/bg*, C57BL/6, and C57BL/6-*scid* mice were monitored for infection, morbidity, and pathology. Bacteria were detected in immunocompetent mice, but the infection was cleared by 17 days post infection. In contrast, immunodeficient mice were unable to resolve the infection and by 17 days post infection, bacteria were detected in the liver, spleen, lymph nodes, peritoneal exudate cells, lung, brain, bone marrow, and blood

samples. By 24 days post infection, the immunodeficient mice were moribund. Splenomegaly was observed in both immunocompetent and immunodeficient mice. This condition was resolved in immunocompetent mice, but continued to worsen in the immunodeficient mice. Tissue damage was also more severe in the SCID mice. Extensive liver necrosis, lymphadenopathy, pericarditis, bone marrow hypercellularity, and granulomatous infiltration of the brain were observed. The immunocompetent mice displayed some granulomatous infiltration of the liver during early infection, but it did not progress into the later stages of infection. Consequently, SCID mice were unable to clear infections with *E. chaffeensis*. The lack of B-cells and T-cells in these mice effectively demonstrates the necessity of the adaptive immune response for modulation of *E. chaffeensis* infections in mice.

The humoral immune response contributes to the host resistance to *Ehrlichia* (104, 183). SCID mice were protected from *E. chaffeensis* by immune serum adoptively transferred from wildtype mice. There was a reduction in bacteremia in the tissues and a lack of morbidity and pathology. Monoclonal antibodies specific for *E. chaffeensis* also protected SCID mice from infection (104). The protective antibodies were IgG2a or IgG3 isotypes and specifically recognized the outer membrane protein (OMP)-1g. A similar antibody response was also reported by Ganta et al. (62, 63), with IgG2a, IgG2b, and IgG3 being the predominant isotypes following infection. Comparatively, studies on *E. muris* infections showed that passive transfer of *E. muris*-specific antibodies protected SCID mice (59); and that priming of mice with *E. muris* followed by challenge with IOE produced IgG2a isotypes (88). Moreover, adoptive transfer of the *E. muris*-specific antibodies increased the survival of naïve mice that received a high dose challenge of IOE (88). The importance of the B-cell to host resistance was confirmed by studies

that demonstrated that B-cell-deficient mice could not be protected from IOE challenge with or without priming by *E. muris* (18, 189). In fact, antibody alone may provide host resistance. Antibodies specific for OMPs of *E. muris* could be detected in CD4-deficient and MHC II-deficient mice following infection (18). Likewise, serum from IOE-infected mice contained antibodies specific for OMPs and upon adoptive transfer it decreased bacterial load in infected mice (189). It should not be a surprise that antibodies are protective. The induced OMP-specific isotypes are known to be involved in complement fixation and FcR binding (68, 141, 163, 164). These observations are important for better understanding the relevance of humoral immunity during infection and translating the results to cases of human *Ehrlichiosis*.

Although humoral immunity can provide host resistance to *Ehrlichia* infection, both innate immunity and T-cell immunity contribute to optimal host responses. Ganta et al (2002, 2004) found that gene disruptions in mouse T-cell genes or in *tlr4* could alter the course of an *E. chaffeensis* infection (62, 63). In mice that were mutant for *tlr4*, clearance of the bacteria was delayed approximately two weeks compared to wildtype mice (63). This was accompanied by decreased macrophage secretion of nitric oxide and IL-6 (63). Decreases in these cytokines may have impacted macrophage stimulation, reflected by the impaired response.

The impact of helper T-cells on *Ehrlichia* infections has also been monitored using mice. MHC II-deficient mice were unable to clear *E. chaffeensis* infections, but did not exhibit differences in nitric oxide levels compared to wildtype animals (62, 63). The unchanged nitric oxide levels along with decreased liver inflammation in these persistently infected mice indicated that other immune responses are active even when CD4⁺ T-cells are absent. The T-cell

contribution to *Ehrlichia* host defense is exemplified by the fact that mice lacking CD4⁺ T-cells cleared *Ehrlichia* infections approximately two weeks later than wildtype animals (62). This is an interesting contrast to MHCII-deficient mice which also lack CD4⁺ T-cells. It is known that CD4-deficient mice also have a population of CD4-CD8⁻ T-cells (135), so help from those cells or activation of those helper cells may contribute to clearance of the infection. The T-cell requirement was also demonstrated by secondary challenge experiments. Clearance of the bacteria was not enhanced in CD4-deficient mice by a second challenge of *E. chaffeensis* as was observed in wildtype mice. The participation of CD4⁺ T-cells was confirmed in other model systems (24, 26, 115). There was an increase in IFN γ -secreting CD4⁺ cells during *E. muris* and low dose IOE infections but not in lethal/high dose IOE infections (17, 88). Decreased survival to *E. muris* infections occurs in CD4-depleted, MHCII-deficient, and IFN γ /TNF α -depleted mice (59). These data suggest that one of the primary roles of CD4⁺ TCs during *E. muris* and low dose IOE is the secretion of IFN γ which would lead to the development of a strong Th1 response (17, 88). This hypothesis is supported by the fact that priming of mice with *E. muris* can protect against high dose IOE challenge (88), suggesting that the generation of *Ehrlichia*-specific T-cells protects against a lethal IOE challenge only if the appropriate idioType(s) have had ample time to increase in frequency. The decrease in CD4⁺ T-cells observed during lethal IOE challenge has been attributed to increased apoptosis, because an increased percentage of apoptotic CD4⁺ T-cells was observed in the spleen following IOE infection (87). Hence, CD4-dependent immunity also contributes to the host defense against different *Ehrlichia* species.

Although IFN γ -producing CD4⁺ T-cells may contribute to host defense against *Ehrlichia*, cytotoxic T-cells were not critical to host resistance (62). Cytolytic activity was

detected in MHCII-deficient mice after a second challenge of bacteria, yet the mice were unable to clear the bacteria. Wildtype mice cleared the bacteria after a single challenge without generation of a strong cytotoxic T-lymphocyte (CTL) response. Although, the cytotoxic response to *E. chaffeensis* does not appear to be required for clearance, CD8⁺ T-cells may still have other roles in the immune response. When MHC I-deficient mice were infected with *E. muris*, 81% fatalities were reported, indicating that CD8⁺ T-cells were necessary (59). Interestingly, infection of β_2m -deficient or TNFR-deficient mice with IOE resulted in decreased bacterial burden and liver injury (87, 89). TNFR-deficient mice developed increased bacterial burdens during early infection, suggesting that the lack of TNF α production led to impaired bacterial clearance. Since IL-10 and TNF α levels decreased in infected β_2m -deficient mice, but IL-10 increased in TNFR-deficient and wildtype mice during IOE infection (87, 89), the data suggested that CD8⁺ T-cells are responsible for the overproduction of TNF α in some types of infections. The current hypothesis is that the dysregulation of these cytokines influenced processes such as macrophage activation, apoptosis/necrosis, and inflammation. The toxic-shock like syndrome that is associated with fatal IOE infections is dependent on antigen specific CD8⁺ T-cells and the production of TNF α .

The route of administration of *Ehrlichia* can also affect the immune response(s) (165). Whereas intradermal (ID) IOE infection caused mild disease, intraperitoneal (IP) infection caused fatalities with as little as 100 bacteria. Compared to IP infection, ID administration of IOE caused minimal liver damage, lower bacterial burden and an increase in CD4⁺CD8⁺ T-cells and a Th1 response. The Th1 response was defined by increased production of IFN γ and TNF α in the spleen. Consequently, the survival of ID-infected mice may be attributed to better initial

containment of the bacteria and/or an accelerated priming of T-cells, resulting in better initial control of the infection. A notable difference in mice that were inoculated IP was an increase in the numbers of apoptotic cells, which correlated with increased levels of TNF α and IL10 in the serum and decreased numbers of CD4 $^{+}$ and CD8 $^{+}$ splenic T-cells. Therefore, increased levels of pro-inflammatory/apoptotic cytokines and decreased levels of T-cells most likely contributed to the fatal responses of the IP-infected mice. These differences are important observations because the natural transmission of *Ehrlichioses* is via tick bites and therefore better compares to ID inoculation of bacteria.

To understand the impact of *E. chaffeensis* at the cellular level, microarrays were done on THP-1 monocyte cells up to 24 hours following infection (194). THP-1 cells were chosen because of the macrophage tropism of *E. chaffeensis*. Gene expression levels were monitored following infection of the cells with *E. chaffeensis* at 1, 7, 11, and 24 hours post infection. Beside down-regulating the innate immune response and alternately regulating cell cycle genes, *E. chaffeensis* altered transcriptional activity of genes that were involved in biosynthesis/metabolism, ion channel transport, cell differentiation, signal transduction/transcription, inflammation, and membrane trafficking. In particular, the authors concentrated on genes that affected innate immunity and cell cycling that were down-regulated during infection(s). Innate immune response genes responsible for cytokine production, apoptosis, and phagosome-lysosome fusion were all down-regulated in THP-1 cells in response to infection with *E. chaffeensis* (194). It was hypothesized that down-regulation of IL-12, 15, and 18 may decrease IFN- γ production and the subsequent activation of macrophages, natural killer cells and CTLs. This was thought to lead to impaired killing of infected cells - a condition

that favored bacterial replication. In addition to down-regulating the expression of distinct cytokine genes, *E. chaffeensis* infections also down-regulated the JAK-STAT pathway. This pathway is involved in activation of cytokine signaling and its down-regulation could compromise the host's ability to kill *E. chaffeensis*-infected cells. Regulation of apoptotic genes was also affected by *E. chaffeensis* infection. Expression of apoptosis-related genes was also reduced early in infection and returned to normal levels at later times. During later stages of infection, pro-apoptotic genes were induced. This pattern of gene expression favors the survival/growth of the *E. chaffeensis* in the cell during early infection and release of bacteria out of the cell during late infection. Lastly, *E. chaffeensis* infection down-regulated a number of proteins that are necessary for fusion of bacteria-containing phagosomes with lysosomes (3). This also promotes survival by allowing the *E. chaffeensis* to avoid destruction in phagolysosomes. Therefore, there is a clear pattern that indicates that THP-1 monocytes are hijacked after infection to promote *E. chaffeensis* survival and subsequent replication in the host.

Significance of studying *Ehrlichia chaffeensis*

Much remains to be learned about the bacterial pathogenesis and host-response to *E. chaffeensis*. Since its discovery in 1986, infections have been reported in Africa (22), Europe (113), China (180), and South America (108). Moreover, the expansion of the white-tailed deer population and increased tick populations in the United States may cause increased human exposure to the bacterial vector. The increase in susceptible populations, such as the elderly and immunocompromised people in the United States, may also contribute to the emergence of new HME cases. A better understanding of the bacteria will facilitate optimum development of rapid diagnostics and the possibility of a vaccine(s).

Drosophila melanogaster

Drosophila melanogaster belongs to the order Diptera, the family Drosophilidae, and is commonly known as the fruit fly. It was popularized by Thomas Hunt Morgan (114) and it has been one of the most frequently used model organisms among researchers. The advantages of *D. melanogaster* as a model organism include: ease of rearing and maintenance of stocks (116), high fecundity and short generation time (116), ease of genetic manipulation (117), accessibility to mutants (16), and a completed genome (15). The entire life cycle of *Drosophila* lasts approximately 12-14 days and includes the life forms of egg, first instar larva, second instar larva, third instar larva, pupa, and adult. The egg stage lasts approximately one day and the larvae hatch, which is followed by approximately five days of eating by the larvae (116). The larvae crawl out of the food source and then molt to non-motile pupa for approximately five days before eclosing as adults (116). Adults are fully developed upon hatching and begin mating within twelve hours of eclosure (116).

***Drosophila melanogaster* immunity**

Drosophila melanogaster constantly comes in contact with various pathogens and microbes because of their lifestyle. They congregate on decaying fruit/food and/or yeast. Therefore, *Drosophila* have a well-developed innate immune system (99). Acquired immunity in the *Drosophila* system has not yet been described, although some primordial genes reminiscent of T- or B-cell receptor genes have recently been discovered (82). The innate immune responses of *Drosophila* include epithelial barriers for protection from microbes (99), production of antimicrobial peptides (humoral/systemic response) (99), and phagocytosis of

invading pathogens by cells found in the hemolymph (cellular response) (99). The relationship between the humoral and cellular response has not yet been clearly defined.

Epithelial immunity begins at the cuticle of *Drosophila* and also extends to the reproductive system and the respiratory and digestive tracts (99). These epithelia are exposed to microbes via food, respiration, and mating. It has been shown that they can all serve as routes of infection for microbes (122, 173). Perturbations to the epithelia lead to formation of clots at the site of disturbance that act to surround invading microbes and stop hemolymph from being lost (152). Hemolectin and fondue are two of the proteins involved in clot formation, and both are expressed by plasmatocytes (72, 153). *Drosophila* RNAi mutants for these proteins did not die after bacterial challenge with a needle, but larger scabs/clots were produced than observed in wildtype flies (72, 152, 153). Another innate epithelial response to pathogens involves the production of reactive oxygen species (ROS), particularly in the gut, controlled by the *Duox* and *immune responsive catalase* (IRC) genes (74, 75). *Duox* RNAi flies had no ROS production and were unable to control microorganism growth when infected orally (74). RNAi of IRC in flies caused an increase in ROS and subsequent death of the flies (74, 75). Thus there appears to be a balance between the *Duox* and IRC gene products in the control of the ROS response to microbes. A third mechanism of epithelial immunity in *Drosophila* involves the local production of antimicrobial peptides (AMPs) either in a constitutive or inducible manner. *Drosomycin* is constitutively expressed (regardless of infection) in salivary glands and in the female spermatheca (173). *Cecropin* is produced in the male ejaculatory duct (173). Inducible expression of *Drosomycin* and *Diptercin* has been observed in response to Gram-negative bacteria, but not to Gram-positive bacteria or Fungi (173). The induction is controlled by the Imd pathway and has been observed in the trachea and gut of flies (13, 122). Therefore, the

defenses associated with epithelia are complex and serve as part of the first responses during bacterial challenge(s) to the fly.

The humoral/systemic immune response in *Drosophila* involves the production of AMPs which are controlled by the Toll and Imd pathways. Approximately 20 genes encoding AMPs are present in *Drosophila* (99). The AMPs can be grouped according to their activity for combating either fungi (83), Gram-negative (83), or Gram-positive bacteria (83). Antifungal AMPs include Drosomycin, Attacin, Cecropin and Metchnikowin (8, 25, 53, 58, 98, 103). Anti-Gram-negative AMPs include Diptericin, Drosocin, Cecropin and Attacin (8, 25, 98, 181). An anti-Gram-positive AMP is Defensin (44). AMPs are produced by the fat body of the fly in response to the presence of pathogens within the hemolymph (20) and the activity of the AMPs has been observed to last for several days at a time (20, 99). AMP production is achieved through the binding of transcription factors to promoter sequences, and this transcriptional regulation is the result of activation of either the Toll or Imd immune pathways. The Toll and Imd pathways are the only known immune-regulating pathways in *Drosophila*, and studies using *Toll/imd* double mutants demonstrated that 80% of genes associated with septic injury are regulated by either one or both of the pathways (40). To date, natural deletion mutants for AMPs have not been identified. This probably reflects the essential nature of these genes.

The Toll pathway was first described for its role in dorso-ventral developmental patterning processes in *Drosophila* (5), and was subsequently found to participate in antifungal and anti-Gram-positive responses in the fly (101, 149). The pathway contains components that are homologous to Toll-like receptor, interleukin-1 receptor, and tumor necrosis factor receptor

signaling molecules (78, 147). The main components of the Toll pathway include Spatzle, Toll1 receptor, Tube, MyD88 (homolog of mammalian MyD88), Pelle (homolog of mammalian IRAK), Cactus (homolog of mammalian I κ B), and Dorsal and Dif (homologs of mammalian NF- κ B) (77). Activation of the pathway begins when Gram-positive bacteria are recognized by peptidoglycan recognition proteins (PGRPs) PGRP-SA, PGRP-SD, or GNBP1 (Gram-negative binding protein 1) (70, 111, 132); or when fungi are recognized by glucan binding protein 3 (GNBP3) (73). Binding induces the activation of Spatzle processing enzyme (SPE), which cleaves and activates Spatzle so that it can bind to the Toll1 receptor (179). This leads to recruitment of the intracellular death domain proteins MyD88 and Tube, which in turn recruit Pelle kinase (168, 170, 172). The phosphorylation of Cactus by Pelle results in the degradation of Cactus, releasing Dif and Dorsal to translocate to the nucleus of the cells, leading to the transcriptional activation of the AMP genes *Defensin*, *Drosomycin*, *Cecropin*, and *Metchnikowin* (40, 41, 60, 69, 86). Dif and Dorsal are described as NF- κ B-related proteins that have been shown to bind to κ B sites, thereby activating transcription of AMPs (84, 143). Flies mutant-deficient for components of the Toll pathway such as Spatzle, Toll, and Pelle render flies susceptible to fungal and Gram-positive infections and disrupt the production of AMPs (101, 149).

The *Drosophila* Imd pathway is activated in response to the presence of Gram-negative pathogens. The recognition of the Imd pathway was preceded by the discovery of the *immune deficiency (imd)* mutation that cause decreased survival in flies infected with Gram-negative bacteria and impaired production of the anti-Gram-negative AMPs (100). Components of the Imd pathway include PGRP-LC, TAK1, TAB2, DIAP2, IKK β /ird5, IKK γ /Kenny, dFADD,

Dredd, and Relish (99). The exact roles of all components of the Imd pathway have not been deduced. In general and in contrast to the Toll pathway, the Imd pathway is directly activated by binding of Gram-negative bacteria to the PGRP-LC receptor (94), which results in the recruitment of the Imd protein and interaction with dFADD (191). The Imd protein is a homolog of the mammalian tumor necrosis factor receptor interacting protein (RIP) (67). dFADD associates with Dredd (191), which is believed to cleave the phosphorylated form of the transcription factor Relish (102), thereby releasing the Rel domain to the nucleus of the cell for subsequent activation of immune genes (50). Similar to the Dif and Dorsal proteins of the Toll pathway, Relish has also been described as an NF- κ B-related protein binds to κ B sites (50). It is believed that the phosphorylation of Relish is performed by the IKK β /ird5/ IKK γ /Kenny complex (160). TAK1, TAB2, and DIAP2 have not been ascribed distinct roles in the Imd pathway, but are thought to act as adaptor and/or activator molecules for the IKK complex and possibly each other (99). *Drosophila* that are mutant-deficient for various components of the Imd pathway are viable and fertile, but succumb quickly to infections with Gram-negative bacteria.

The similarity of the Dif, Dorsal and Relish proteins and their NF- κ B-relatedness has led to the question of whether or not crosstalk/cooperation exists between the Toll and Imd pathways, especially at the level of transcription factors. Tanji et al. (2007) (169), demonstrated that the pathways can act in concert to activate AMP genes and that the activation is dependent on Dif, Dorsal, and Relish. They showed that direct stimulation *in vitro* and *in vivo* with ligands of the Toll and Imd pathways induced the greatest expression of AMPs, compared to when the ligands were assayed separately. Moreover, it was shown that the synergy between the pathways

was not the result of Imd ligands binding to extracellular Toll, but resulted from the activation of a separate, intracellular pathway. The proposed mechanism for the synergistic activation of the pathways was determined by mutation of κ B binding sites of the *Drosomycin* promoter. The model proposed that different κ B binding sites are only permissive for the binding of Dorsal or Dif homodimers or Relish homodimers. Once the homodimers have bound to their respective sites, cooperation between them activates transcription of the AMP gene(s). In an alternate situation, Relish may form a heterodimer with Dorsal or Dif, bind to its respective site, and then interact with the Dorsal/Dif homodimer that is bound to a separate site. Overall, the binding of κ B sites at the promoters of AMP genes by the different transcriptional activators of the Toll and Imd pathways may be the determining factor for the synergistic expression of distinct sets of AMP genes.

The cellular immune response in *Drosophila* involves the phagocytosis and encapsulation of microorganisms and foreign invaders. Three types of cells contribute to the *Drosophila* cellular immune response: (1) plasmatocytes; (2) lamellocytes; and (3) crystal cells (99). The major immune functions of these cells are phagocytosis, encapsulation, and melanization, respectively. Plasmatocytes are responsible for phagocytosis of invading pathogens and foreign substances, and are likened to antigen presenting cells (APCs) of mammalian systems (166). These cells account for 90-95% of mature larval hemocytes (99) and are also present in embryos and adult flies. However, the plasmatocytes in the adult are sessile unless an immune stimulus is present; they circulate freely within embryos and larvae (80). Encapsulation is mediated by lamellocytes and occurs when the foreign substance/object is too large to be phagocytosed. Lamellocytes have only been reported in larvae, specifically larvae that are infected with

parasitoid wasp eggs - which necessitate encapsulation (99). A *Drosophila* mutant that lacks lamellocytes was unable to encapsulate parasitic wasp eggs after experimental infection (55). Crystal cells are also found in larvae, and make up approximately 5% of the total cell population (99). These cells store a crystallized form of pro-phenoloxidase (pro-PO), which is released when melanization of wounds or invading organisms is required (146).

***Drosophila* as an experimental system**

There have been over one hundred *Drosophila* cell lines established from embryos and larvae in the past 40 years (14). Among these are the *Drosophila* S2 (148), Kc (34), mbn (malignant blood neoplasm) (66), and S2R+ (190) lines. The S2 and Kc lines were established in the late 1960's and early 1970's and were made from spontaneously immortalized cells taken from embryos (51, 93, 155). The mbn lines were established approximately 10 years later from primary embryo cultures that harbored blood cell tumors (65). The most recently established cell line is the *Drosophila* S2R+, which was isolated from the S2 cell line and has qualities associated with hemocytes taken from larvae (190). All of the above cells lines display functional immune signaling and mirror the characteristics of hemocytes observed *in vivo* and in primary larval cultures (14), including phagocytosis. Additionally, the S2 and mbn lines are known to express AMP genes in response to appropriate stimuli (150). The hemocytic properties and immune responsiveness of these cells lines make them appropriate and attractive tools for studying *Drosophila* host-pathogen relationships.

Isolates of *Ehrlichia chaffeensis* are most often cultured in the canine macrophage cell line DH82. The bacteria have also been shown to grow in various cell lines, including human

microvascular endothelial cells, African green monkey kidney cells, human cervical epithelioid carcinoma cells, human monocytic leukemia cells, mouse embryo cells, buffalo green monkey cells, and murine fibroblasts (123). Although these cells lines are helpful for exploring host-pathogen interactions of *E. chaffeensis*, *Drosophila melanogaster* would be more useful as a model system to study infections. *Drosophila* offer advantages that include ease of manipulation, amenability to large screens, a completed genome, availability of a large number of mutants, and ease of RNAi-mediated silencing of genes *in vitro*. Moreover, the genetic similarities between *Drosophila* and mammalian systems are numerous and easily translated. This is advantageous because many bacteria that are infectious to humans can also be used to infect *Drosophila*. Genes discovered to play a role in *Drosophila*-pathogen interactions can often be used to identify the corresponding mammalian homologs and their function(s). The advantages of *Drosophila* have been successfully manipulated in several *in vitro* and *in vivo* studies of pathogens including *Listeria monocytogenes* (2, 3), *Chlamydia trachomatis* (54), *Mycobacterium marinum*(2, 45, 97, 131), *Francisella tularensis* (151, 176), and the protozoan parasite *Plasmodium gallinaceum* (21, 154). Ultimately, these types of studies contribute to broadening the knowledge base of different host-pathogen relationships in invertebrate and vertebrate systems.

Host-pathogen studies using *Drosophila*

Listeria monocytogenes is a facultative, intracellular pathogen which grows and replicates in *Drosophila* S2 cells, larvae, and adults (32, 109). When an actin-nucleating mutant strain of *Listeria* was used for infections, bacteria spread less efficiently to surrounding cells, both *in vitro* and *in vivo* (109). The bacteria acquired actin tails during infections in the S2 cells

and in larvae (109). These observations implicated actin as a key factor during infections, which is similar to findings in mammalian systems (38, 85).

In addition to identifying bacterial components that contributed to pathogenesis, the host response to *L. monocytogenes* was dissected by infecting *Drosophila Toll* and *Imd* mutant flies. Increased susceptibility to infection occurred in both types of mutants (109), which indicated the participation of both immune pathways in host defense. Traditionally, the *Drosophila Toll* pathway is activated in response to Gram-positive bacteria. Therefore, some undefined component of *Listeria* is eliciting an immune response through the *Imd* pathway.

Studies of the human pathogen *Chlamydia trachomatis* are particularly important because *C. trachomatis* infections are one of the most widespread and commonly reported sexually transmitted diseases in the United States (186). Elwell & Engel (2005) (54) demonstrated that early infection processes of *Chlamydia* are similar between *Drosophila* S2 and mammalian cells. Both S2 and mammalian cells required heparin sulphate-decorated molecules for attachment to host cells and actin rearrangements. Following entry into the S2 cells, the Chlamydia-containing phagosome avoided fusion with lysosomes, and the bacterial elementary bodies differentiated into metabolically active reticulate bodies. The best demonstration that infections in *Drosophila* parallel events in human cells was accomplished using RNAi techniques in the S2 cells. Knockdown of the Rho family GTPase gene *Rac ½* with dsRNA resulted in decreased infection in the cells (54), which similarly occurs in mammalian cells (157, 158).

Dionne et al. (2003) found that *Mycobacterium marinum* caused lethal infection in adult *D. melanogaster* (45). It proliferated in the hemocytes, disseminated as infection progressed, and caused tissue damage. To compare infections in the fly to those in mammals, *M. marinum* carrying the promoter gene *mag24::GFP* was used for infections. *Mag24* encodes a gene from the member of PE-PGRS family (137). The function(s) of *mag24* are not completely defined, but are hypothesized to be specific to pathogenic mycobacteria and to contribute to bacterial/host interactions (42, 137). In mouse macrophages, *mag24* is activated only upon phagocytosis and its deletion caused decreased virulence of the bacteria (137). The same results were observed in *Drosophila* hemocytes when the *mag24::GFP Mycobacteria* was used for *in vivo* infections. Likewise, *M. marinum* could not be found in the acidic vacuoles of the fly hemocytes, suggesting that the bacteria may have a mechanism for blocking vesicle acidification, thereby promoting their own survival. The ability of the bacteria to stop acidification has also been observed in vertebrate macrophages (12). Therefore, the function of fly hemocytes clearly does parallel those of macrophages and can provide valuable insights for better understanding *M. tuberculosis*, which infects approximately one third of the world's population (188).

Multiple studies have utilized *Drosophila* S2 or *mbn-2* cells to detail infections of the facultative, intracellular bacteria *Francisella tularensis* (151, 176). *F. tularensis* is classified as a category A bioterrorism agent (57) and little is known about the pathogenesis of *F. tularensis* in its arthropod vectors. *F. tularensis* caused a lethal infection in adult, wild-type flies, was widely disseminated, and infected larval hemocytes. Adult flies that were mutant-deficient for components of the Imd signaling pathway succumbed to infection faster than wild-type flies, indicating that the Imd pathway serves as one of the primary host defenses. Most importantly,

fly experiments were used to demonstrate that the intracellular growth locus (*igl*) virulence factors of *Francisella* contributed to pathogenesis. Although the *igl* mutant bacteria were lethal to the flies, death occurred at a slower rate than when wild-type bacteria were used for infections. It was hypothesized that the impact of *igl* virulence factors in S2 cells may be involved in fusion/non-fusion of the phagosome(s) with the lysosome(s). Further investigations using *Drosophila* cells will be necessary to ultimately define the role of *igl* virulence factors in the pathogenesis of *F. tularensis*.

Drosophila models of host-pathogen interactions have not only been used to learn more about mammalian systems but have also been used to identify mosquito genes important to *Plasmodium gallinaceum* pathogenesis (21). *P. gallinaceum* developed in adult *D. melanogaster* (154), and the sporozoites isolated from the fly could be used to infect white leghorn chickens (154). In addition, when *Aedes aegypti* mosquitoes fed on the infected chickens, they became infected with *Plasmodium*. This demonstrated that the *Plasmodium* was completing part of its lifecycle in the fly and maintained its virulence throughout. Following this work, Brandt et al. (2008) screened 1,452 mutant *Drosophila* lines and identified 18 mutants that displayed decreased growth of *P. gallinaceum*. Of those, 18 genes were found to have strong homologs in *An. gambiae*. RNAi was subsequently used for *in vivo* silencing of the genes in *An. gambiae*. Specifically, silencing of the mosquito *oxr1* (oxidation resistance) and *argk* (arginine kinase) genes led to decreased *Plasmodium* infection in the mosquitoes. Thus, the homologs identified in *Drosophila* were used to successfully identify novel genes that contribute to *Plasmodium* infection in its natural host and that had not been previously implicated in *Plasmodium* infections in the mosquito.

Screening in *Drosophila*

Large scale RNAi screens in *Drosophila melanogaster* can be based on the use of double-stranded (dsRNA), short-interfering (siRNA), or short-hairpin (shRNA) RNA's to silence target genes via destruction of the gene message/mRNA. The use of RNAi in *Drosophila* is advantageous because: (1) long dsRNAs are taken up by *Drosophila* cells without the use of a transfection reagent (37); and (2) full-genome RNAi screens make it possible to test the function of all genes in the *Drosophila* genome. Several types of reporters are available for RNAi screens depending on the gene, model system, and antibody reagents that are available. Transcriptional reporters are based on fluorescence that is activated in response to gene expression (39). Antibody-based screens use specific antibodies and FACS-based reporting to monitor phenotypes (52). Microscopy-based screens are useful when visual imaging is adequate to confirm phenotypes; however, these screens are labor intensive (136). Nevertheless, RNAi screens have been used to define components of *imd* signaling, JAK/STAT signaling, phagocytosis, actin remodeling, and genes involved in the survival/replication of intracellular bacteria (35). Moreover, RNAi has been used extensively in *Drosophila* cell lines to study host/pathogen interactions of organisms including: *Escherichia coli* (40, 138), *Staphylococcus aureus* (132), various *Mycobacterium* species (131), *Legionella pneumophila* (46), different *Chlamydia* species (43), and *Listeria monocytogenes* (33). The use of RNAi has provided extensive contributions to the underlying body of knowledge concerning host/pathogen interactions in *Drosophila*, and most importantly has been translated into mammalian systems in several instances.

Gene expression profiling via microarray has also been used to better understand host-pathogen relationships of *Drosophila* (40, 86, 92). In general, microarrays have been used to

better understand the genes involved in cellular function(s) and biochemical pathways involved in normal and diseased conditions. Microarrays have become popular tools for large scale gene expression studies because of their cost efficiency, accessibility, and the universality of their standard protocol (90). Affymetrix was the first to develop *Drosophila* oligonucleotide arrays/gene chips (112). The most current Affymetrix *Drosophila* gene chip is the *Drosophila* Genome 2.0 Array, which measures 18,500 different transcripts and variants (1). The design of the 2.0 Array was based off of the Berkeley *Drosophila* Genome Project annotation (release 3.1) and includes fourteen pairs of oligonucleotide probes to measure transcription levels for each sequence on the chip (1). Briefly, the protocol for chip preparation involves the isolation of RNA from cells and/or tissues of interest, converting it to DNA, labeling the product, and hybridizing to the gene chip. Hybridization can be detected by phosphor-imaging and/or fluorescence scanning (156). *Drosophila* genes involved in the recognition, phagocytosis, melanization, and coagulation following pathogen challenge (40); pathogen-specific immune-related protease and apoptotic genes (86); and those genes dependent on hemocyte function (92) have all been defined using microarray analysis.

Philips et al. (2005) (131) used a genomewide RNAi screen to determine host factors required for non-specific and specific phagocytosis of *M. fortuitum*. The screen employed GFP reporter constructs under the control of Map24 and Map49 promoters that were induced by phagocytosis. Visual inspections and automated image analyses were used to confirm the ability of *M. fortuitum* to infect dsRNA-treated S2 cells. 86 genes were identified in this screen. These genes were involved in processes that included vesicle trafficking and actin-mediated rearrangements. These host genes were labeled as non-specific because their disruption also

impacted uptake of fluorescently labeled *E. coli*. However one host gene, *CG7228*, was found to be specifically necessary for mycobacterial uptake. *CG7228* is a scavenger receptor and member of the CD36 family. dsRNA treatment blocked S2 cell phagocytosis of *M. fortuitum*. To confirm that *CG7228* was necessary for *M. fortuitum* infections, it was expressed in human embryonic kidney 293 (HEK293) cells. These cells are normally resistant to *M. fortuitum* infection. Expression of *CG7228* in the HEK293 cells allowed for *M. fortuitum* infection in the cells. In follow-up experiments, human and mouse CD36 family scavenger receptors were expressed in HEK293 cells. They found that the mammalian class B scavenger receptors increased *M. fortuitum* infection in HEK293 cells (131).

This same group used *Drosophila* SL2 cells in an RNAi screen to identify host factors required for *Listeria monocytogenes* infections (2). They identified host genes that contributed to entry, intracellular replication, and vacuole escape. When genes involved in vesicular trafficking, signal transduction, and cytoskeletal organization were silenced, a decrease in infection was observed and believed to be the result of defects in the entry of the bacteria into the cells. Conversely, the genes involved in cell cycle and RNA processing contributed to increased growth of bacteria. This screen also confirmed that *CG7228* decreased the entry/uptake of *L. monocytogenes*, and identified an additional 59 host genes that specifically decreased *L. monocytogenes* infections. Among these were a predicted glucose and lysosomal transporter. The comparative results of these two studies (2, 131) will be valuable in further identifying the mammalian homologs that may contribute to infection by these types of intracellular bacteria.

Cheng et al. (2005) (33) used three different *L. monocytogenes* strains in a high-throughput RNAi screen to identify genes involved in listeriolysin O (LLO)-dependent entry, vacuolar escape, growth, and LLO toxicity. The genes *CG3573*, *myotubularin*, and *sbf* were found to be involved in entry, vacuolar escape, and intracellular growth processes. Knockdown of the genes *MESR4*, *string*, *CG5451*, and *CG5505* all led to increased growth of *Listeria*. Therefore, these genes probably control bacteria replication. In their screen looking at LLO-dependent processes, these authors identified the genes *SNF7*, *Vps4*, *Bro1*, and *Vps16*, which control vesicular trafficking. The final RNAi screen used a *Listeria* mutant that expresses LLO in the cytosol of the host cell, which causes cellular toxicity. The enzyme SPT, which is involved in the biosynthesis of sphingolipids, was crucial to controlling toxicity (33). Pharmacological inhibitors of SPT confirmed its role in pathogenesis in murine bone marrow derived macrophages (33). Therefore, this genomewide RNAi screen revealed a myriad of host genes that contribute to several aspects of *Listeria* infections. The availability of the bacterial mutants was invaluable in this screen for shedding light on the more distinct and specific host processes involved in *Listeria* infections.

RNAi screening has also been used to determine host genes that contribute to *Legionella pneumophila* pathogenesis (46). In contrast to other studies utilizing *Drosophila* for RNAi screening, this study did not employ a whole genome screen approach. Instead, a set of 73 dsRNAs for genes in the categories of secretory pathways, endoplasmic reticulum (ER) dynamics, and endocytic transport were used to determine their impact on *Legionella* infection in *Drosophila* Kc167 cells. Silencing of genes involved in the transport protein particle (TRAPP) complex slightly affected replication of the bacteria (46). The greatest decrease in bacterial

replication was observed only when knockdown of multiple genes involved in the TRAPP complex was performed. The authors concluded that multiple components of the TRAPP complex were involved in the *L. pneumophila* replication cycle. Among the dsRNAs that singly produced a significant phenotype was *Cdc48/p97* (*Drosophila Ter94*). *Cdc48/p97* functions to remove ubiquitinated proteins from the ER before they are destroyed by the proteasome. To confirm *Cdc48/p97*'s role in infection, it was targeted by hairpin siRNA in HEK293 cells. Silencing caused a decrease in bacterial replication in the HEK293 cells. In addition, dsRNA treatment of genes that associate with *Cdc48/p97* was also performed simultaneously with the silencing of *Cdc48/p97*. The dual silencing of *Cdc48/p97* with the *Pac10* gene or *CG32566* caused a significant decrease in *Legionella* replication, indicating a possible role for these genes as cofactors of *Cdc48/p97*. Therefore, this RNAi screen was important because it was the first to demonstrate the involvement of *Cdc48/p97* in the intracellular replication of *L. pneumophila*.

Derre et al. (2007) (43) used an RNAi screen to identify 162 candidate genes that decreased *Chlamydia caviae* infection in *Drosophila* SL2 cells upon silencing. 54 of the genes were confirmed for their ability to control growth of *C. caviae*, but only four genes, classified as members of the *Drosophila* mitochondrial membrane translocase, were found to be specific to *C. caviae* infections. To test if these genes had similar impacts in infection in a mammalian system, *Tom40* and *Tom22* were silenced using RNAi in HeLa229 cells. Derre et al. (2007) found that silencing *Tom40* and *Tom22* reduced the overall size of *C. caviae* inclusions in the cells, interfered with reticulate/elementary body formation in the cells, and reduced the number of infectious cells produced during *C. caviae* infections. The *Tom40* and *Tom22* requirement was specific to *C. caviae* infections because *Tom40* or *Tom22* silencing had no effect on infection

when the same experiments were performed using *C. trachomatis* (43). The results of this screen present another example in which whole genome *Drosophila* RNAi screens were successful in identifying host genes that contribute to the infection cycle of an obligate, intracellular bacterium in both specific and non-specific fashions.

Mycobacterium marinum is an intracellular bacterium that normally infects fish and amphibians (45). Because of its genetic and pathogenic similarities to *M. tuberculosis*, it is considered to be a model for infections of *M. tuberculosis*. In addition, it will complete its growth cycle at temperatures of 25-28°C. Therefore, it can grow at temperatures at which *Drosophila* S2 cells grow. Since *M. tuberculosis* completes its replication cycle at such low temperatures, Koo et al. (2008) (97) used *M. marinum* infection of *Drosophila* S2 cells to identify host genes critical to replication (97). Among approximately 1000 *Drosophila* genes screened, Koo et al. (2008) found that knockdown of the gene *Hexo2* increased bacterial growth. *Hexo2* encodes the *Drosophila* homolog of the β -subunit of mammalian lysosomal enzyme β -hexosaminidase. The discovery of the contribution of *Hexo2* to *M. marinum* infections was translated to a mammalian system using bone marrow derived macrophages from *HexB* (*Hexo2* homolog) knockout mice. Infected cells from the knockout mice had increased bacteria levels compared to infected cells from wildtype mice. It was discovered that β -hexosaminidase is secreted from infected cells and that it is bactericidal to *M. marinum*. The bactericidal activity was measured in the absence of phagocytosis, and had similar cytotoxicity when phagocytosis was not blocked. These data suggest that killing of *M. marinum* by β -hexosaminidase occurred intra- and extra-cellularly. Since *M. marinum* is a model of the pathogenesis of the human

disease tuberculosis, the results of this study will contribute to the knowledge surrounding an important and worldwide human pathogen.

Conclusion

The use of *Drosophila melanogaster* for deciphering host-pathogen interactions and identifying mammalian homologs has been successfully executed for a wide range of bacterial pathogens. Using the knowledge gained from these studies, new and old biochemical pathways have been redefined and previously undefined host genes have been assigned function(s). This has contributed to a refinement and overall better understanding of the pathogenesis by different microorganisms. Therefore, *D. melanogaster* was tested in this project to determine if it could serve as an appropriate model system to better understand the pathogenesis of *E. chaffeensis*. *E. chaffeensis* causes human monocytic ehrlichiosis, which is considered an emerging, infectious disease. Its emergence can be attributed to increases in the population density and geographic distribution of *A. americanum*, in vertebrate host populations, in human contact with natural foci of infection, and in the size of the aging and/or immunocompromised population (123). No vaccine is yet available for controlling *E. chaffeensis* infections. Continued studies of the pathogenesis of the organism will contribute to the development of possible vaccine targets, immunodiagnostics, and to keep the general public well-informed of the risks associated with HME.

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CHAPTER 2 - Use of *Drosophila* S2 cells as a model for studying *Ehrlichia chaffeensis* infections

ABSTRACT

Ehrlichia chaffeensis is an obligate intracellular bacterium and the causative agent of human monocytic ehrlichiosis (HME). Although this pathogen grows in several mammalian cell lines, no general model for eukaryotic cellular requirements for bacterial replication has yet been proposed. We found that *Drosophila* S2 cells are permissive for the growth of *E. chaffeensis*. We saw morulae (aggregates of bacteria) by microscopy, detected *E. chaffeensis* 16S ribosomal RNA gene by RT-PCR, and used immunocytochemistry to detect *E. chaffeensis* in S2 and mammalian cells. Bacteria grown in S2 cells reinfected mammalian macrophages. S2 cells were made non-permissive for *E. chaffeensis* through incubation with lipopolysaccharide. Our results demonstrate that S2 cells are an appropriate system for studying the pathogenesis of *E. chaffeensis*. The use of a *Drosophila* system has the potential to serve as a model system for studying *Ehrlichia* due to its completed genome, ease of genetic manipulation, and availability of mutants.

INTRODUCTION

Ehrlichia chaffeensis, the causative agent of human monocytic ehrlichiosis (HME), was first reported in 1987. It has subsequently been reported in 30 UNITED STATES states, and was designated a nationally reportable disease by the UNITED STATES Centers for Disease Control in 1999 (3). The bacteria are vectored by *Amblyomma americanum* (lone star tick) and white-tailed deer are considered to be the major reservoir for the bacteria. Although some details are

known about the host response(s) of vertebrates to *E. chaffeensis*, little is known about the invertebrate response to the bacteria in the tick (7). Ehrlichiae are Gram-negative, cocci (round) shaped bacteria, measuring 0.5-2 μm in diameter that, upon infection, form vacuole bound colonies (called morulae) in leukocytes (2). In particular, *E. chaffeensis* infects mononuclear leukocytes (monocytes and macrophages). Clinical symptoms of the disease include fever, headache, chills, muscle aches, fatigue, nausea/vomiting, swollen glands, and/or delirium. Drugs such as tetracycline/doxycycline, which inhibit protein synthesis of the bacteria by binding to the 30S ribosomal subunit, are often used during treatment(s) (2, 15). In approximately 40-60% of cases, hospitalization is necessary (20). Although case fatality rates are approximately 3% (15), inappropriate treatment of the disease may lead to irreversible neurological damage due to the result of acute inflammatory responses.

Isolates of *E. chaffeensis* are most often cultured in the canine, macrophage-like cell line DH82. The bacteria also grow in other cell lines, including human microvascular endothelial cells, African green monkey kidney cells, human cervical epithelioid carcinoma cells, human monocytic leukemia cells, mouse embryo cells, buffalo green monkey cells, and murine fibroblasts (15). However, no clear requirements for cell tropism have been defined. The host genes that are required for intracellular growth of *E. chaffeensis* are not known. Some efforts to characterize gene expression in mammalian macrophages have been initiated (21), but those studies have not revealed significant details about host requirements. The *Drosophila melanogaster* system offers several advantages over mammalian macrophages for these studies. For example, the *D. melanogaster* genome is well defined, with many of the innate immune response genes having homologues in mammals. S2 cells were chosen because *E. chaffeensis* is macrophage-tropic and S2 cells have characteristics of hemocytes, the insect equivalent of

macrophages. S2 cells are more easily transfected than mammalian macrophages. This characteristic is advantageous for experiments utilizing siRNA techniques. Moreover, while the tick is the natural host for the bacteria, the tick system is less defined than *D. melanogaster*. Thus, the *D. melanogaster* system offers a closer parallel as an insect system versus a mammalian systems for studying the tick-derived bacteria, *E. chaffeensis*. Most recently, it has been recognized that insect systems are an extremely useful tool for studying the innate immune responses elicited by various pathogens in comparison to the response(s) observed in vertebrate systems (11). The *D. melanogaster* derived cell line, S2 (Schneider line-2 cells), were isolated from primary cultures of 20-24 hour old embryos over 20 years ago (10). These cells are classified as hemocytes, which are known to circulate freely in the hemolymph of *Drosophila*, and are phagocytic in nature. In addition, these cells are responsible for the synthesis/secretion of antimicrobial peptides (19). Indeed, *Drosophila* S2 cells have served as an *in vitro* model to study another obligate intracellular pathogen, *Chlamydia trachomatis* (8). The early steps of *C. trachomatis* infection in S2 cells mirror those seen in mammalian cells. Conservation of infection was observed between steps of entry, inclusion formation, inhibition of phagolysosomal fusion, and acquisition of golgi-derived sphingolipids. Other intracellular bacteria, including *Listeria monocytogenes* and *Mycobacteria marinum* have also been grown in S2 cells (14, 16, 18). Consequently, the use of S2 cells for the study of intracellular bacteria is helping to contribute to the elucidation of bacterial and cellular mechanisms that are important to these infections. To date, there are no reports of successful *E. chaffeensis* growth in *Drosophila* S2 cells. Therefore, we tested the hypothesis that *E. chaffeensis* would grow in *D. melanogaster*-derived S2 cells to determine if it could serve as a model system. The data in this manuscript document conditions that allow *E. chaffeensis* replication in S2 cells.

MATERIALS & METHODS

Maintenance of cell lines and *Ehrlichia chaffeensis* infections.

The canine macrophage cell line, DH82, was maintained at 37°C in Dulbeccos Modified Eagle's Medium with 3.5% fetal bovine serum and 3.5% Nu Serum (DMEM-7). The *E. chaffeensis* Arkansas isolate was continuously cultivated in the DH82 cell line at 37°C, 8% CO₂ in DMEM-7 medium. Bacteria were passaged when infectivity reached 80-90% as visualized using cytopsin prepared slides (stained with Hema3 fixative and Dif-Quik stain) to monitor formation of morulae in the cells. Infected cells were removed by scraping each plate with a cell scraper, transferring the culture to a conical tube, and vortexing the suspension with glass beads. The freed bacteria were purified by centrifuging the suspension at 600 x g for twenty minutes to remove cell debris. The bacteria-containing supernatant was removed, transferred to a sterile conical tube, and was centrifuged at 15,000 x g for twenty minutes to pellet the free bacteria. The final supernatant was removed/discarded and the pellet was re-suspended in an appropriate amount of sterile PBS. Purified bacteria were used to re-infect fresh DH82 cells. *Drosophila* S2 cells were cultivated at 28°C in Schneider's *Drosophila* medium (Gibco, Grand Island, NY, #11720) supplemented with 10% fetal bovine serum (Atlanta Biologicals, #S11150).

Time Course Infections of DH82 and *Drosophila* S2 cells.

Drosophila S2 cells were plated in 6-well tissue culture plates (60-mm, Techno Plastic Products AG, #92406) at a concentration of 1×10^6 cells per ml. The cells were allowed to adhere for at least 30 minutes but no longer than 24 hours. Bacteria purified from infected DH82 (85-100%) cells, approximately 3×10^7 bacteria, were added to S2 cell cultures and then were monitored for infection at 12, 24, 48, 72, 96, and 120 hours post-infection (hpi). At each time point, RNA was

isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, #TR118). S2 cells were removed by pipetting the cells off the dish and pelleted by centrifugation at 300 x g for 5 minutes. The supernatants were discarded and 2 ml of TriReagent was used to lyse the pellet. For the DH82 cells, spent medium was aspirated from the culture dishes and cells were removed from the dish using 2 ml of TriReagent and repeated pipetting. The TriReagent plus cells was then transferred to polypropylene tubes, 200 μ l of chloroform was added, mixtures were incubated on ice for 15 minutes, and centrifuged for 15 minutes at 10,000 rpm. 750 μ l of the aqueous phase was mixed with 750 μ l of isopropanol and incubated overnight at 4°C. Samples were then centrifuged for 15 minutes at 13,000 rpm, 4°C. The pellets were washed with 75% ethanol, centrifuged for 10 minutes at 8,000 rpm (4°C), and resuspended in 50 μ l of nuclease free water. RNA concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE). Infections were quantitated by assessing morulae formation on cytopins of infected cells. Cells were randomly scored for the presence or absence of morulae. Lastly, the bacteria from infected S2 cells were isolated at each time point as described above. Purified bacteria (from each time point of the S2 time course) were used to re-infect fresh DH82 cells to determine if S2-grown bacteria would be infectious. These DH82 cells were set up as a time course: cells were plated in 6-well tissue culture plates at a concentration of 1×10^6 cells per ml and were subsequently infected with a range of purified bacteria concentrations derived from S2 cells that had been infected for various lengths of time. The DH82 cells were assessed for infection 72 hours later as described above. RNA was also isolated from the DH82 cells.

Assessment of bacterial numbers for infection.

The amount of bacteria used for infection experiments of DH82 and *Drosophila* S2 cells was estimated using a TaqMan-based real-time reverse transcription-PCR as previously described (9,

17). This TaqMan-based assay targets the *E. chaffeensis* 16S rRNA gene. Real time PCR was performed on ten-fold serial dilutions of RNA extracted from 80-100% infected DH82 cells (3 different samples), using the Smart Cycler system (Cepheid, Sunnyvale, CA). Standard curves were generated by plotting the log number of bacteria versus corresponding Ct value (average of 3 experiments). The lowest detection limit, or the presence of 1 bacterium, was considered to be the dilution at which the Ct value approaches 40 (zero).

Determination of infection by RT-PCR.

Infections were assessed by using the reverse transcriptase polymerase chain reaction (RT-PCR) using the Promega Access One-Step RT-PCR kit (Madison, WI). 500-1000 ng of RNA were used for each reaction. Each reaction contained the following components: 1X buffer, 0.2 mM dNTPs, 2 μ M forward primer, 2 μ M reverse primer, 1.5mM MgSO₄, 1U per μ l DNA polymerase, 1U per μ l reverse transcriptase, and nuclease free water for a final reaction volume of 25 μ l. RT-PCR reactions were performed in a (Eppendorf Mastercycler Gradient) thermocycler, based on primers specific for the 16S ribosomal RNA gene of *E. chaffeensis*. Primers (Integrated DNA Technologies, Coralville, IA) used for detecting *E. chaffeensis* in both S2 and DH82 cells were RRG27 (5' GTATTACCGCGGCTGCTGGCAC 3') and RRG3 (5' CAATTGCTTATAACCTTTTGGTTATAAAT 3') (accession #M73222) (4).

Cycling conditions for these primers were as follows: 48° C for 5 minutes, 42° C for 5 minutes, 45° C for 5 minutes, 48° C for 30 minutes, 94° C for 4 minutes, then 35 cycles of 94° C for 30 seconds, 52° C for 30 seconds, and 72° C for 1 minute. Total RNA input was assessed using housekeeping genes for ribosomal protein 49 (rp49)(accession #U92431) (*Drosophila*) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH canine) (accession #AB038240).

Sequences for housekeeping primers included rp49 (5' ATCGGTTACGGATCGAACAA 3');

5'GACAATCTCCTTGCGCTTCT 3') for *Drosophila* S2 cells and GAPDH (5'GATTGTCAGCAATGCCTCCT 3'; 5'GGCAGGTCAGATCCACAAC 3') for the DH82 cells. Cycling conditions for rp49 primers were as follows: 48°C for 45 minutes, 94°C for 2 minutes, and then 35 cycles of 94°C for 45 seconds, 50°C for 1 minute, and 72°C for 1.5 minutes. Cycling conditions for GAPDH primers were as follows: 48°C for 45 minutes, 94°C for 2 minutes, and then 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 68°C for 1 minute. RT-PCR reactions were also performed without reverse transcriptase in order to assure that DNA was absent from the sample(s). RT-PCR products were identified on a ChemiImager after electrophoresis in 2% agarose gels and staining with ethidium bromide. The sized amplicons for each primer set were: RRG 27 & 3 (430 bp), rp49 (165 bp), and GAPDH (308 bp).

Immunocytochemistry.

Specific *E. chaffeensis* infections were confirmed by immunocytochemical techniques. Immunocytochemistry was performed on *E. chaffeensis*-infected S2 cells, *E. chaffeensis*-infected DH82 cells, uninfected S2 cells, and uninfected DH82 cells. Cells were prepared using cytopspin slides and dried for 24 hours at room temperature. Cells were fixed with acetone, dried ten minutes, and outlined using a Pap Pen (The Binding Site, Inc., San Diego, CA). Samples were placed in PBS containing 0.01% Tween for five minutes. The samples were blocked in 10% blocking solution (PBS containing 50% normal goat serum) for 30 minutes at 37°C, washed in PBS-Tween for five minutes, and incubated in primary antibody (mouse-anti-*E. chaffeensis*; 1:100 dilution) for 24 hours in the dark at 4°C. Primary antibody was either normal mouse serum or serum taken from mice infected with *E. chaffeensis*. After incubation, the slides were washed with PBS-Tween for five minutes and were incubated for one hour in the dark at room

temperature with a 1:50 dilution of goat-anti-mouse IgG conjugated with Rhodamine (Organon Teknika Corp., West Chester, PA). Samples were washed in PBS-Tween for five minutes, then in distilled water for five minutes before viewing.

Activation of S2 cells with Lipopolysaccharide.

To determine if lipopolysaccharide (LPS) activation would inhibit the growth of *E. chaffeensis*, S2 cells were plated at a concentration of 1×10^6 cells per plate in 6-well plates and were allowed to adhere for at least 30 minutes. LPS (from *Salmonella minnesota*; Sigma) was sonicated for one hour and then added to each well at a concentration of 10 μg per ml. The cells plus LPS were incubated for five hours. S2 cells were infected with *E. chaffeensis* purified from DH82 cells. RNA was extracted (using Tri-reagent) from each sample at 48 hpi, and assessed for the presence of *Ehrlichia* using RT-PCR, as previously described.

Transmission electron microscopy and sample preparation.

Infected S2 (72 hpi (hpi)), uninfected S2, infected DH82 (72 hpi), and uninfected DH82 cells were pelleted (5-10 μl pellet) and immersion fixed in fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 sodium cacodylate buffer (pH 7.4) for 16 hours at room temperature with constant rotation. Each sample was washed 3 times for 5 minutes in 0.1M sodium cacodylate buffer at room temperature (RT) with constant rotation. The samples were post-fixed with 2% Osmium tetroxide in 0.1M sodium cacodylate buffer with constant rotation for 1-2 hours and then washed 3 times for 5 minutes in 0.1M sodium cacodylate buffer at RT with constant rotation. Samples were pre-embedded/stained with 2% uranyl acetate in 0.2M sodium acetate buffer (pH 5.2) for 1 hour at RT, light-protected, with constant rotation, and subsequently washed 3 times for 5 minutes in 0.2M sodium acetate buffer at room temperature with constant rotation. The samples were dehydrated in an ascending acetone series (50, 60, 70,

80, 90, 95, and 100%) with constant rotation at RT and then in 100% propylene oxide for 10 minutes. Infiltration of samples was accomplished using EMBED 812/Araldite 502 resin at RT with constant rotation and consisted of 10 minutes in 1:1 propylene oxide:resin, 20 minutes in 1:2 propylene oxide:resin, 10 minutes in 100% resin. New 100% resin was finally added to the sample, allowed to infiltrate for 16 hours, and then polymerize in a drying oven at 60° C for 24-48 hours. The images were collected on a CM 100 (FEI company) transmission electron microscope at 100kV using a Hamamatsu C8484 digital camera and Advanced Microscopy Techniques Corp. software version 5.4.2.22B.

Statistics.

Statistical values were determined using the Student's *t*-test (two-tailed, general). *P* values of <0.02 (as indicated) were considered highly significant. Data are presented as mean ± SD.

Differences were determined using the StatMost Statistical Package (Data XIOM, Los Angeles, CA, USA).

RESULTS

To test the hypothesis that *E. chaffeensis* can be propagated in macrophage-like S2 cells, we infected the S2 cells and assessed the infection via morphological, molecular, and immunological techniques. In our initial experiments, bacteria isolated from heavily infected DH82 cells (~1.5 x 10⁷ bacteria) was used in an attempt to infect the S2 cells. The S2 cells were tested for the presence of bacteria at time points ranging from 24 hpi to as late as 120 hpi. At 24 hpi, 18.8% of the S2 cells contained one or more morulae. At 120 hpi, 91.7% of the S2 cells contained one or more morulae (Figure 2.1). We observed an increase in the percentage of cells infected with each subsequent time point. At the 120 hour time-point, many of the S2 cells

contained large numbers of morulae. S2 cells had more morula than DH82 cells at each time point beyond 24 hpi ($P<0.02$) (Figure 2.1). Moreover, the cells became progressively more vacuolated and mis-shaped throughout the infection. To confirm the infection in the S2 cells, RNA was assessed for the presence of the *E. chaffeensis* 16S ribosomal subunit transcript. Bacterial message was detected at 12 hpi through 120 hpi, which correlated with the observed morulae formation in the infected S2 cells (Figure 2.2). Additionally, we compared the kinetics of the infection between S2 cells and DH82 cells. At 24 hpi, 12.6% of the DH82 cells contained one or more morulae, compared to almost 20% of the S2 cells. By 120 hpi 57.4% of the DH82 cells contained morulae, compared to >90% of the S2 cells (Figure 2.1). Therefore it was clear that *Ehrlichia* were replicating in S2 cells.

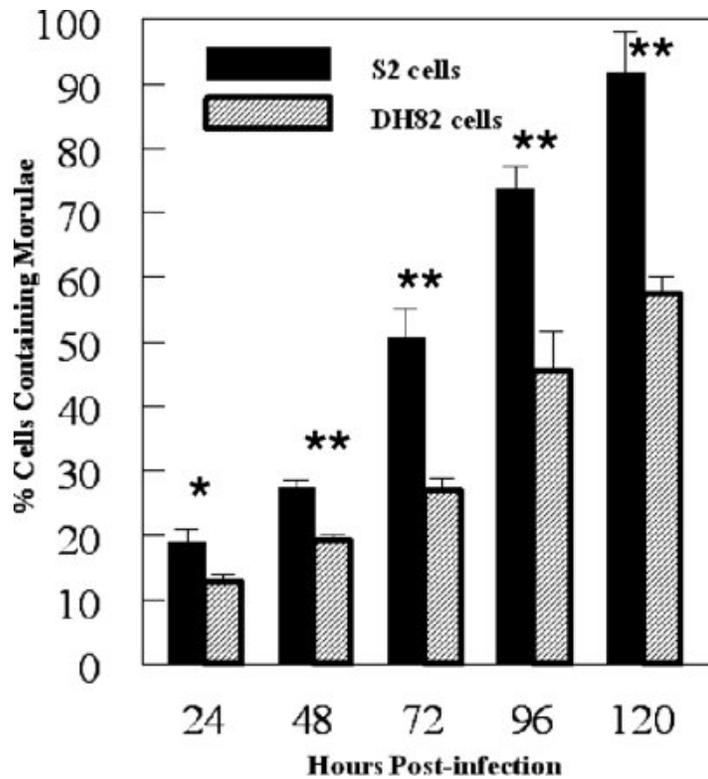


Figure 2.1 Percentage of cells containing morulae in S2 and DH82 cells after infection with *E. chaffeensis*. Cells were considered positive when one or more morulae were present. Results are the averages of three separate infection experiments (mean \pm S.d., n=3). Statistical significance is represented by * ($P<0.06$) or ** ($P<0.02$).

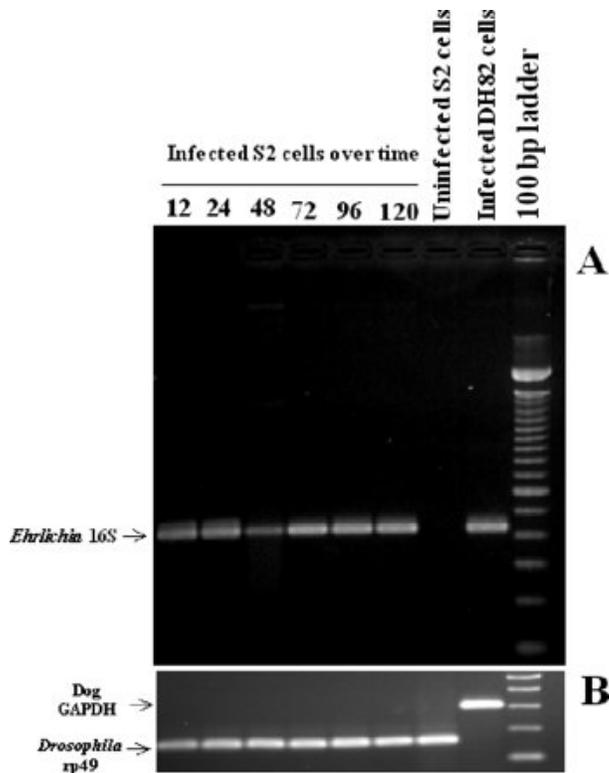


Figure 2.2 A, RT-PCR of *E. chaffeensis* 16S ribosomal RNA gene (band at 430 bp) after infection of S2 cells at 12-120 hpi. Time course infection experiments and RT-PCR of 16S ribosomal RNA gene were repeated greater than 6 times with the same outcome. One representative experiment is shown. B, RT-PCR of *D. melanogaster* rp49 (165 bp) and dog GAPDH (308 bp) housekeeping genes, performed on samples shown in A.

To confirm that the bacteria grown in S2 cells were completing replication and were infectious to vertebrate cells, DH82 cells were infected with bacteria purified from S2 cells at 24, 48, 72, 96, and 120 hpi of the S2 cells. The purified bacteria were used to infect the DH82 cells and the amount of bacteria present in DH82 cells was assessed 72 hours later by quantitation of morulae. When S2 cells were only infected for 24 hours, the bacteria isolated from those cells infected 8% of the DH82 cells (Figure 2.3). 67% of the DH82 cells were infected with bacteria isolated from S2 cells infected for 120 hours (Figure 2.3). We confirmed these infections by RT-PCR of the 16S *E. chaffeensis* rRNA (Figure 2.4). Consequently, *E. chaffeensis* is capable of

completing its lifecycle in S2 cells and also maintains its ability to reinfect the mammalian macrophage cell line, DH82.

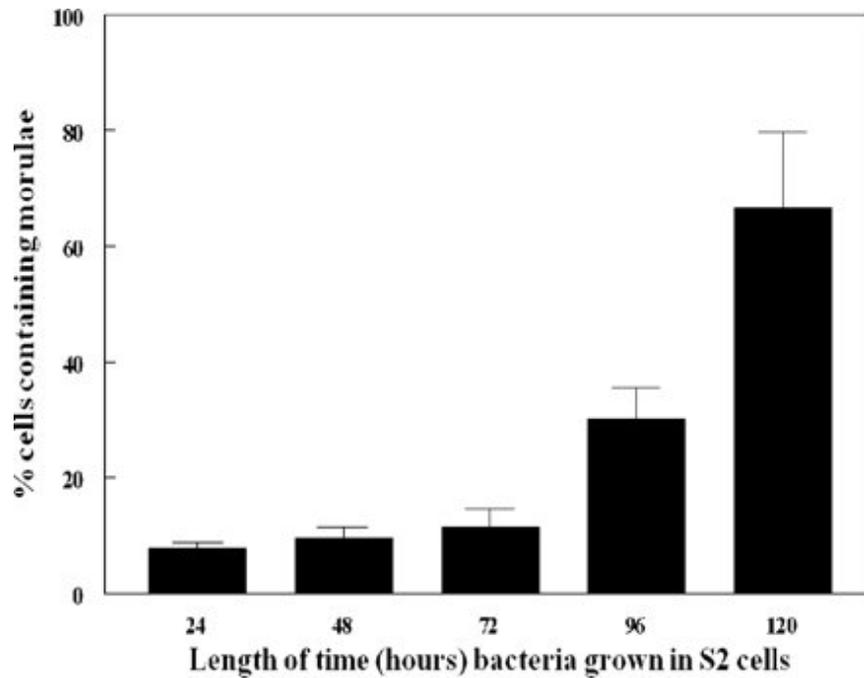


Figure 2.3 Growth of *E. chaffeensis* (originally grown in S2 cells) in DH82 cells. Bacteria grown in S2 cells for the indicated length of time were used to infect DH82 cells. The percentage of DH82 cells containing morulae was assessed 72 hours later. Results are the averages of three separate time course experiments (mean \pm S.d., n=3).

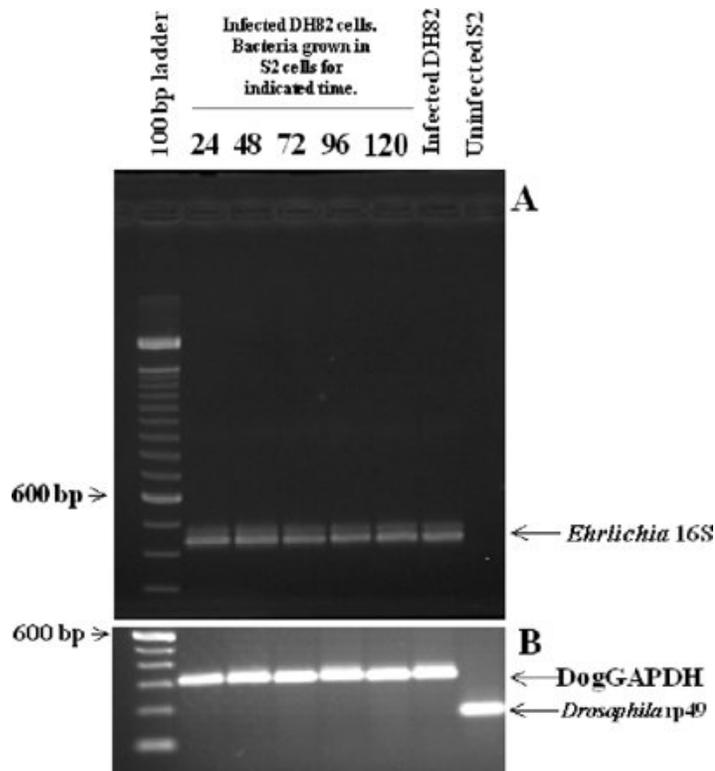


Figure 2.4 Reinfection of DH82 cells with S2 cell-grown bacteria (A). RT-PCR of *E. chaffeensis* 16S ribosomal RNA gene (band at 430 bp) after DH82 cells were infected with bacteria grown in S2 cells for the indicated times. RT-PCR of *D. melanogaster* rp49 (165 bp) and dog GAPDH (308 bp) housekeeping genes, performed on samples shown in A (B).

To further demonstrate that the inclusions seen in the S2 cells after infection with *E. chaffeensis*, we utilized immunocytochemistry to detect bacteria. Slides were made with infected S2 and DH82 cells or with uninfected cells. Sera collected from *E. chaffeensis*-infected mice or from normal mice were used as primary antibodies. Only *E. chaffeensis*-specific antiserum reacted with the infected DH82 (Figure 2.5D) and S2 cells (Figure 2.5A-C). No bacteria were detected with the normal mouse serum incubated with infected or uninfected cells, or by secondary antibody alone (Figure 2.5E-H).

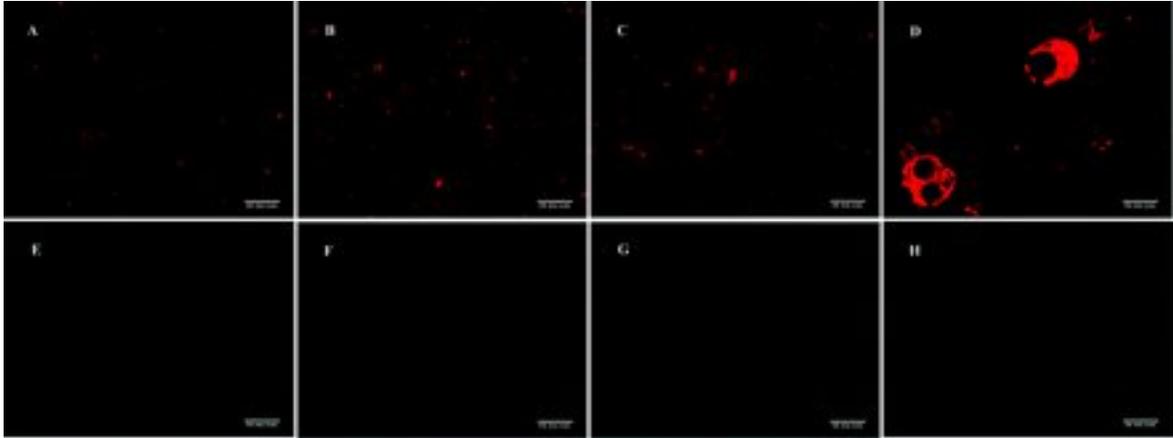


Figure 2.5 Immunocytochemical detection of *Ehrlichia* in S2 and DH82 cells. A,B,C Infected S2 cells incubated with *E. chaffeensis*-specific mouse serum and secondary antibody; D, Infected DH82 cells incubated with *E. chaffeensis*-specific mouse serum and secondary antibody; E, Infected S2 cells incubated with normal mouse serum (NMS) and secondary antibody; F, Infected S2 cells incubated with secondary antibody only; G, Uninfected S2 cells incubated with *E. chaffeensis*-specific mouse serum and secondary antibody; H, Uninfected DH82 cells incubated with *E. chaffeensis*-specific mouse serum and secondary antibody. Each image captured at 20X magnification.

We also demonstrated the presence of morulae in S2 and DH82 cells using transmission electron microscopy (Figure 2.6). The S2 and DH82 cells used in this experiment were infected at the same time with the same batch of *E. chaffeensis*, and were both fixed and imaged at 72 hpi. Morulae were seen in both S2 and DH82 cells. By 72 hpi most of the morulae in DH82 cells contained numerous dense, elongated bacterial forms (Figure 2.6E). Morulae in S2 cells also contained many bacteria with both reticulate and dense forms (Figure 2.6B,C). The morulae in both cell types were confined to vacuoles. Uninfected S2 and DH82 cells contained minimal vacuoles. In instances where vacuoles were seen in the uninfected cells (Figure 2.6A), they contained no bacteria.

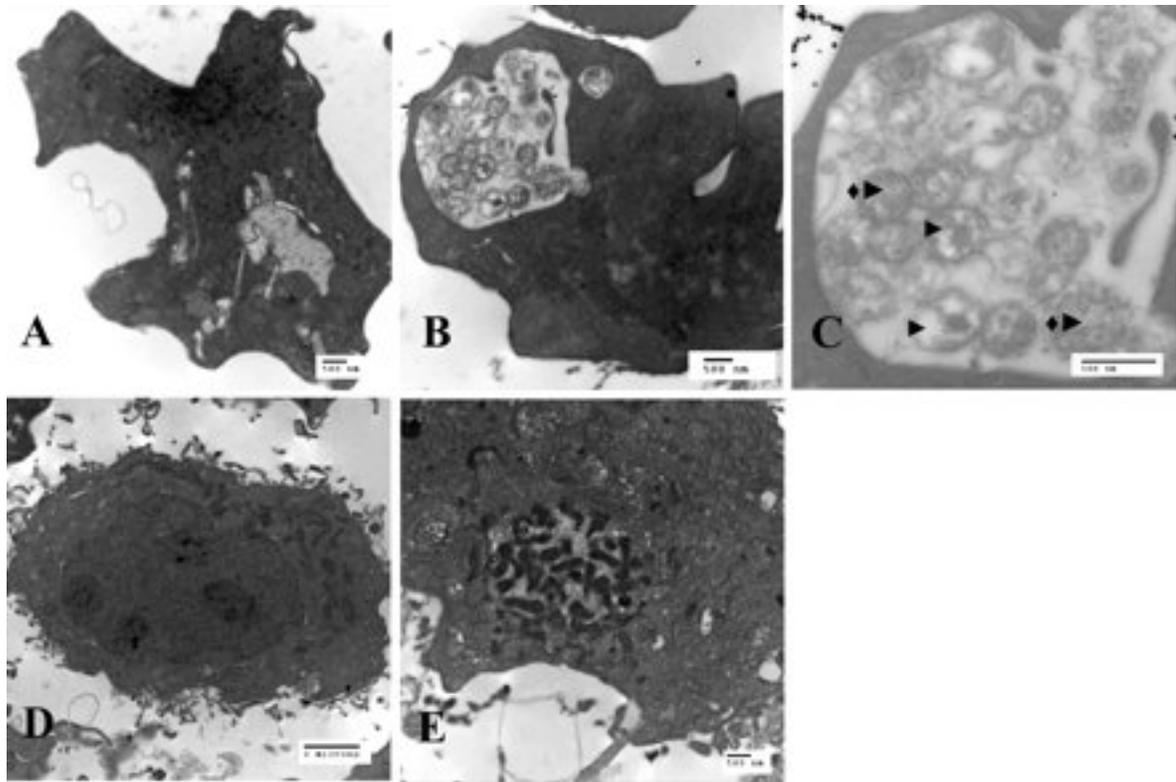


Figure 2.6 Transmission electron micrographs of S2 and DH82 cells infected with *E. chaffeensis* or uninfected. A, uninfected S2; B, infected S2; C, infected S2 (black arrowheads indicate dense core form; diamond with black arrowhead indicates reticulate form); D, uninfected DH82; E, infected DH82.

Lastly, it is known that mammalian macrophages are non-permissive for *E. chaffeensis* growth after LPS activation (13). Therefore, we wanted to determine if S2 cells could be resistant to *E. chaffeensis* infection. To test this hypothesis, we incubated S2 cells with LPS (10 μg per ml) for 4 hours, and then we challenged the cells with increasing doses of purified *E. chaffeensis* bacteria. RNA was extracted from the cells 48 hpi, and the RNA samples were analyzed by RT-PCR for *Ehrlichia* infection. S2 cells activated with LPS (10 μg per ml) were non-permissive for *E. chaffeensis* regardless of the number of bacteria used for infection (Figure 2.7). As a control, unstimulated S2 cells were infected at the same time using the same number

of bacteria (Figure 2.7). Thus, activated S2 cells become non-permissive to infection by *E. chaffeensis*, similar to results seen in activated human monocytes (13).

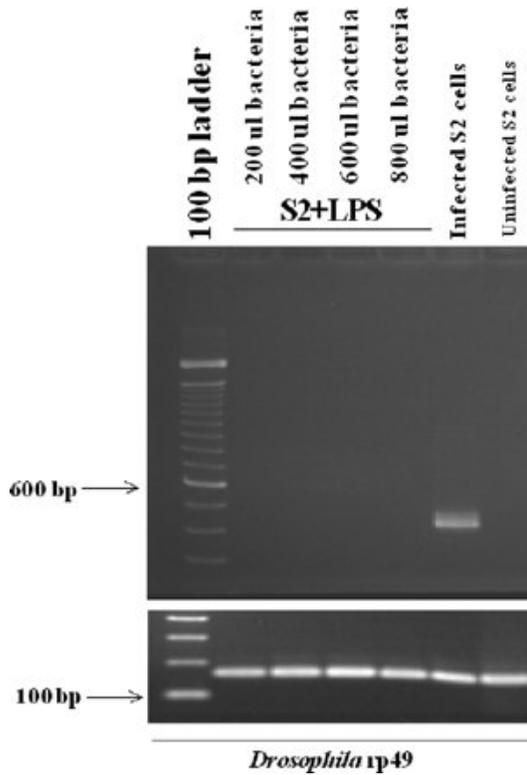


Figure 2.7 RT-PCR results of LPS activation of S2 cells. *E. chaffeensis* 16S ribosomal RNA (band at 430 bp) present only in cells not treated with LPS. S2 cells were infected with increasing numbers of bacteria with 1.4×10^7 bacteria/200 μ l. Housekeeping gene is *Drosophila melanogaster* ribosomal protein 49 (rp49).

DISCUSSION

We have presented novel data showing that *Drosophila* S2 cells can be successfully infected with the obligate, intracellular bacterium, *E. chaffeensis*. Infection was confirmed using three distinct criteria: (1) presence of morulae in the S2 cells; (2) the detection of 16S ribosomal RNA of *E. chaffeensis* in infected cells; and (3) the specific detection of *E. chaffeensis* in S2

cells by bacteria-specific antibody. Morulae in mammalian cells are known to have two forms: dense core and reticulate cells (15). We found the presence of both dense and reticulate forms of bacteria in S2 cells, particularly a preponderance of reticulate forms in infected S2 cells.

Considering the differences observed between bacterial growth in the S2 and DH82 cells (Figure 1), this is not surprising. The reticulate form is the dividing form of the bacteria, and thus an increased number of dividing bacteria coincides with the increase bacterial kinetics observed in the S2 cells when compared to the DH82 cells. Zhang et al. (2007) found that the dense core form of bacteria were present exclusively during early infection (0-1 hpi). Between 24 and 48 hpi, the reticulate form predominated and binary fission was often observed. Subsequent to that, dense core forms reappeared. It is possible that the reticulate forms predominate in insect cells grown at lower temperatures or that the reappearance of dense core forms occurs later.

Alternatively, the presence of reticulate and dense core forms may be cell-line and/or temperature-dependent. Additional electron microscopy work will be necessary to determine this. Nevertheless, these data confirm that *E. chaffeensis* can be propagated in S2 cells and its developmental patterns are similar to those seen in mammalian macrophages. In addition, vacuoles containing appropriately sized inclusions (presumably bacteria) were observed by light microscopy/live video imaging in both S2 cells and DH82 cells that had been infected with *E. chaffeensis* (data not shown). The bacteria were confined to the vacuoles in the infected cells; conversely, no bacteria were observed in the uninfected S2 or DH82 cells.

The kinetics of bacterial infection of S2 cells was comparable to the growth of bacteria in DH82 cells, which are one of the most commonly used cell lines to propagate infections of *E. chaffeensis*. Moreover, bacteria isolated from infected S2 cells were infectious and could be used to infect DH82 cells. Therefore, the growth of *E. chaffeensis* in dipteran insect cells does

not compromise the viability of the bacteria. This is consistent with the natural life cycle of *Ehrlichia* that includes an arachnid host, the tick. The host requirements for *E. chaffeensis* to grow in ticks are not clear. The *D. melanogaster* system offers the advantage of having a well defined genome. Using the S2 cells to identify which host genes are necessary to support *E. chaffeensis* growth will eventually allow us to study homologous genes in both arachnids and vertebrates. Our data are also consistent with observations that S2 cells are capable of supporting other macrophage-tropic intracellular bacteria. For example, *Chlamydia trachomatis*, *Listeria monocytogenes* and *Mycobacterium fortuitum* all infect S2 cells (1, 5, 6, 8, 14, 16). Studies on these bacteria in S2 cells have provided valuable information about early and late infection processes of *C. trachomatis* (8). S2 cells have also been critical to the identification of key components involved in the pathogenesis of *L. monocytogenes* (6), and the involvement of CD36 in *M. fortuitum* infections (16). Nevertheless, our findings open the molecular and genetic tool box of the *Drosophila* genome for the study of *E. chaffeensis*.

In addition to the observation that *E. chaffeensis* is capable of infecting the *Drosophila* S2 cells, we also were able to make the S2 cells non-permissive for infection by *E. chaffeensis*. Although *E. chaffeensis* does not make LPS or peptidoglycan (12), we wanted to determine if LPS could activate the S2 cells. Activation with LPS prevented bacterial replication even at high MOIs. More importantly, the option of a system made non-permissive by the addition of LPS increases the utility of S2 cells and makes them comparable to vertebrate macrophages in this respect (13). In particular, comparing gene expression in activated versus infected S2 cells will allow us to pinpoint genes specific to *E. chaffeensis* infections as well as genes that are simply activation-specific. It is our goal to define the mechanisms that allow for maintenance of *E. chaffeensis* in its invertebrate and vertebrate hosts.

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CHAPTER 3 - *Ehrlichia chaffeensis* infections in *Drosophila melanogaster*

ABSTRACT

Ehrlichia chaffeensis is an obligate, intracellular bacterium, transmitted by the tick *Amblyomma americanum*, and is the causative agent of human monocytic ehrlichiosis infections. We previously demonstrated that *E. chaffeensis* is capable of growing in *Drosophila* S2 cells. Therefore, we tested the hypothesis that *E. chaffeensis* can infect adult *D. melanogaster*. Adult *Drosophila* were experimentally challenged with intra-abdominal injections of bacteria. *Ehrlichia*-infected flies showed decreased survival compared to wild-type flies and bacteria isolated from flies could reinfect mammalian macrophages. *Ehrlichia* infections activated both the cellular and humoral immune responses in the fly. Hemocytes phagocytosed bacteria after injection and antimicrobial peptide pathways were induced following infection. Increased pathogenicity in flies carrying mutation for genes in both the Toll and Imd pathways suggests that both immune defense pathways participate in host defense. Induction of *Drosophila* cellular and humoral responses and the *in vivo* replication of *E. chaffeensis* suggests that *D. melanogaster* is a suitable host for *E. chaffeensis*. In the future, it will be a useful tool to unlock some of the *in vivo* mysteries of this arthropod-borne bacteria.

INTRODUCTION

Drosophila melanogaster is a valuable tool for studies focused on innate immune responses. It is especially attractive because of the ability to study innate host defense without the complicating variables of acquired immunity (28, 58). *Drosophila* innate immunity involves

both cellular and humoral components. The cellular immune response involves phagocytosis, encapsulation, and/or melanization of pathogens via hemocytes, plasmatocytes, or crystal cells, respectively (18, 31, 63). Humoral immunity involves the production of antimicrobial peptides through either the Toll or *immune deficiency* (Imd) pathways (18, 31, 63). The Toll pathway is activated by Gram-positive bacteria or fungi, and elicits production of the antimicrobial peptide Drosomycin (18, 31, 63). The Imd pathway is activated by Gram-negative bacteria and is characterized by the production of antimicrobial peptides such as Attacin and Diptericin (18, 31, 63). Additionally, *D. melanogaster's* completed genome, ease of manipulation, availability of mutants, and homology to vertebrate systems make it an attractive tool as a model system for detailing the innate immune responses to various pathogens. In particular, it has been used to characterize immune reactions elicited in response to *Erwinia carotovora*, *Mycobacterium marinum*, *Plasmodium gallinaceum*, *Francisella tularensis*, *Serratia marcesans*, *Listeria monocytogenes*, and *Salmonella enterica* serovar *typhimurium* (3, 7, 13, 40, 42, 52, 64).

Ehrlichia chaffeensis is an obligate, intracellular bacterium and is vectored by *Amblyomma americanum* (lone star tick). It is the causative agent of human monocytic ehrlichiosis (HME), which can be particularly life-threatening in young, elderly, and/or immunocompromised patients. In 2006, the CDC reported an infection rate of 0.2/100,000 persons in the United States (41). *E. chaffeensis* is classified as a Gram-negative bacterium, but lacks the genes necessary for the synthesis of peptidoglycan (PGN) or lipopolysaccharide (LPS) (37). Therefore, many questions exist about early host resistance to *Ehrlichia* as well as questions about the host genetic requirements for bacterial growth. *Drosophila melanogaster* could be a useful tool to address these questions. We have established that *E. chaffeensis* can infect and replicate in the hemocytic, macrophage-like *Drosophila* S2 cell line (38). We

hypothesized that *E. chaffeensis* would infect adult flies and activate host defenses. We present evidence indicating that *Ehrlichia* can infect and replicate in adult *Drosophila*, that hemocytes respond to the infection, and *Drosophila* humoral immune pathways are activated.

MATERIALS AND METHODS

Maintenance of cell lines and *Ehrlichia chaffeensis* infections.

The canine macrophage cell line DH82 was maintained at 37°C in Dulbecco's modified Eagle's medium with 3.5% fetal bovine serum and 3.5% Nu serum (DMEM-7). The *E. chaffeensis* Arkansas isolate was continuously cultivated in the DH82 cell line at 37°C, 8% CO₂ in DMEM-7 medium. Bacteria were passaged when infectivity reached 80 to 90% as visualized by using cytopsin-prepared slides (stained with Hema3 fixative and Dif-Quik stain) to monitor the formation of morulae in the cells. Infected cells were removed by scraping each plate with a cell scraper, transferring the culture to a conical tube, and shaking the suspension with glass beads. The liberated bacteria were purified by centrifuging the suspension at 600 x g for 20 minutes to remove cell debris. The bacteria-containing supernatant was removed, transferred to a sterile conical tube, and centrifuged at 15,000 x g for 20 minutes to pellet the free bacteria. The final supernatant was removed and discarded, and the pellet was resuspended in sterile phosphate-buffered saline (PBS).

For preparation of dead bacteria cultures, the final pellet was resuspended in sterile PBS and the tube was subsequently placed in a boiling water bath for 15 minutes. The boiled bacteria was then centrifuged at 15,000 x g for 20 minutes to re-pellet the bacteria. The supernatant was removed and the dead bacterial pellet was resuspended in 5 ml sterile PBS.

Infected flies were used to re-infect DH82 cells. Ten flies that had been infected for 168 hours were anesthetized using CO₂ and transferred to a sterile, 1.5 ml conical tube (Kimble Kontes #749510-1500). Flies were homogenized with a disposable pestle (Kimble Kontes #749521-1590) in 1 ml of sterile PBS. The homogenate was spun at 10,000 x g for 10 seconds in a microcentrifuge and the resulting supernatant was added to a 150-mm plate of 40% confluent DH82 cells (treated with 0.13 µg per ml of Fungizone). Cells were observed for the formation of morulae, using cytospin slide preparations. At two weeks after infection, morulae formation was observed in 90-100% of the cells. RNA extraction and RT-PCR analysis for *Ehrlichia* 16S ribosomal gene was performed on the infected and on uninfected DH82 cells as previously described (38).

Assessment of bacterial numbers for infection.

The number of bacteria used for infection experiments of DH82 cells and flies was estimated using TaqMan-based real-time reverse transcriptase PCR (RT-PCR) as previously described (55). This TaqMan-based assay targets the *E. chaffeensis* 16S rRNA gene transcripts. Detection of the 16S rRNA is 100 times more sensitive than rDNA (20, 55). We have confirmed that there is no difference in the relative levels of 16S rRNA and rDNA during the course of an infection using qRT-PCR and qPCR. Real-time quantitative PCR (qRT-PCR) was performed on 10-fold serial dilutions of RNA extracted from 80- to 100%-infected DH82 cells (three different samples) using a Smart Cycler system (Cepheid, Sunnyvale, CA). Standard curves were generated by plotting the log number of bacteria versus the corresponding threshold cycle value (mean of the results of three experiments). The lowest detection limit, or the presence of 1 bacterium (100 rRNA copies = 1 bacterium), was considered to be the dilution at which the threshold cycle value approaches 40 (zero).

Fluorescent labeling of bacteria for injection.

Cell-free *E. chaffeensis* was purified as described above and was fluoresceinated as previously described (10) with modifications described as follows. Fluorescein isothiocyanate (FITC) was dissolved into 0.2M Na₂CO₃ (pH 9.5) buffer to a final concentration of 5 mg per ml. One ml of the FITC solution was added to the cell-free *E. chaffeensis* pellet, mixed gently, and incubated with rocking for 15 minutes at room temperature (protected from light). The FITC-bacteria mixture was then added to 4 ml of 135 mM NaCl/10 mM phosphate buffer (pH 7.4) and incubated for 5 minutes at room temperature. Following incubation, the mixture was centrifuged at 10,000 x g for 5 minutes, the supernatant was removed, the pellet was resuspended in 2.83% Na₂HPO₄ (pH 8.5), and the mixture was centrifuged again. The resulting pellet was resuspended in sterile PBS and subsequently washed three times with sterile PBS. The bacterial pellet was resuspended in sterile PBS for fly injections.

Bacteria were also labeled following the protocol of the pHrodo Phagocytosis Particle Labeling Kit for Flow Cytometry (Invitrogen, Carlsbad, CA; #A10026). Briefly, cell-free *E. chaffeensis* was obtained as previously described. The bacterial pellet was resuspended in 3 ml of 100 mM sodium bicarbonate buffer (Component F) and centrifuged at 16,000 x g in a microcentrifuge for 60 seconds. The pellet was then suspended in 750 µl of Component F, 18.25 µl of the pHrodo dye (Component D) was added, and the bacteria were incubated with rocking for 45 minutes at room temperature (protected from light). 750 µl of wash buffer (Component C) was added to the bacteria mixture, centrifuged at 16,000 x g for 60 seconds, and the supernatant was removed. The bacterial pellet was resuspended in 1.5 ml of 100% methanol, vortexed for 30 seconds, centrifuged at 16,000 x g for 60 seconds, and the supernatant was removed. The bacteria were resuspended in 1.5 ml of Component C, vortexed for 30 seconds, and centrifuged

at 16,000 x g for 60 seconds. The wash step was repeated and the final bacterial pellet was resuspended in sterile PBS for injection.

For fly injection, equal volumes of the FITC-labeled and pHrodo-labeled *E. chaffeensis* were mixed and simultaneously injected into *yellow white* flies. Flies were viewed on an Olympus SZX12 fluorescent, dissecting microscope with emission filters of 535/50 for FITC detection and 620/60 for pHrodo detection.

Salmonella enterica serovar typhimurium .

S. enterica serovar *typhimurium* (strain KSU-7)(15) was grown and streaked for isolation on MacKonkey agar plates. Nutrient broth was inoculated with a single colony and grown overnight at 37°C. The cultures were centrifuged at 10,000 rpm for 10 minutes and rinsed with PBS. Bacterial pellets were resuspended in PBS, absorbance was measured on a NanoDrop Spectrophotometer (Thermo Scientific; Wilmington, DE), and bacterial concentration was estimated as previously described (9).

Flies.

Flies were maintained on standard dextrose/molasses/yeast medium at 18-29°C. For initial *E. chaffeensis* infections and dose response infections, *w;Hemese-Gal4, UASGFP* flies were used as wild-type (WT) in these experiments (from Dr. Michael J. Williams; Umea Centre for Molecular Pathogenesis; Umea University; Umea, Sweden) (66). *yellow white* (*yw*) flies are continuously maintained at Kansas State University for use as a wild-type stock. Transgenic flies expressing green fluorescent protein (GFP) under the control of the Attacin (*Attacin-GFP(II)*)(*attacin-GFP*), Dipteracin (*Pw⁺/Pw⁺ III (Dipt-GFP-Drom)*)(*dipteracin-GFP*), or Drosomycin (*ywP(w⁺,Drom-GFP)D4*)(*drosomycin-GFP*) promoters; and mutants for *dredd* (*ywD44*)(*dredd^{D44}*) and for *relish* (*rel^{E20}, e⁺*) (from Dr. Bruno Lemaitre; Ecole Polytechnique Federale de Lausanne; Lausanne,

France)(35, 62) were used to study the Imd and Toll pathways. Toll-constitutive flies ($Tl^3, ri^+ e^- / TM3, Ser$)(Tl^3) were received from Drs. Claudia Mizutani and Ethan Bier (University of California at San Diego)(1). *pelle* mutant (pll^2) stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University, Bloomington, Indiana, $e^1 pll^2 ca^1 / TM3, Sb^1$ (#3111)(2). *cactus* deficient flies ($cact^{D11}/CyO; cact^{D13}/CyO$)($cact^{D11}$ and $cact^{D13}$) have been previously described (51).

Fly Infections.

Flies were transferred to fresh food at least 24 hours prior to injection/infection. For injection/infection, adult male and female flies were anesthetized with CO₂ (for no longer than 15 minutes at a time). Flies were injected with approximately 50 nl of sterile PBS with or without bacteria, using pulled, glass capillary needles. Injections were made in the abdomen of the fly, close to the junction of the thorax, and ventral to the junction between the dorsal and ventral cuticles. Following injection, flies were maintained in clean bottles with molasses caps that were changed every other day throughout the course of the experiments. Survival was monitored daily.

Bead injection.

In order to inhibit phagocytosis by hemocytes (13, 16, 47, 52), flies were injected with a 2% solution of PBS mixed with FluoSpheres carboxylate-modified microspheres (0.2 μm, red fluorescent – 580/605)(Invitrogen #F8810) or PBS alone. Injections were performed four hours or 24 hours prior to injection with *E. chaffeensis*. Survival was monitored for 96 hours post infection.

Assessment of fluorescence in antimicrobial peptide promoter-EGFP expressing *Drosophila*.

Live, adult flies were assayed for eGFP expression by placing each fly into a 0.5 ml microfuge tube containing 120 μ l of sterile PBS. The flies were pulverized using a Dremel 100 Series rotary tool with a conical shaped grinding bit. The bit was sterilized by flaming the bit after dipping it in 70% ethanol. The bit was cooled between each sample. After pulverization, the tubes were briefly microfuged at 12,000 x g and 50 μ l of each supernatant was transferred to one well of a 96-well plate. The fluorescence was quantitated using a Perkin Elmer Wallac Victor³ Multilabel Counter.

RNA extraction and quantitative real time reverse transcription – PCR (qRT-PCR).

To quantitate transcript levels, flies were anesthetized, placed in 1.5 ml tubes (Kimble Kontes #749510-1500), and homogenized with disposable pestles in 1 ml of TriReagent (Molecular Research Center) (Kimble Kontes #749521-1590). Homogenates were transferred to 2.0 ml, Heavy Phase Lock Gel tubes (5 Prime/Eppendorf; Westbury, New York; #2302830). 300 μ l of chloroform was added and the mixture was shaken (not vortexed) for 15 seconds. The samples were then centrifuged at 12,000 x g for 10 minutes at 4°C and the aqueous phase was transferred to clean 1.5 ml tubes. 500 μ l of isopropanol was added and RNA was precipitated at -20°C for 24 hours. Samples were subsequently centrifuged at 12,000 x g for 10 minutes. The RNA pellet was washed with 1 ml of 70% ethanol and samples were centrifuged at 7.4 x g for 5 minutes. The 70% ethanol was decanted from the pellet, the pellet was allowed to slightly air dry and was resuspended in 50 μ l of nuclease-free water.

qRT-PCR was performed using the Invitrogen's One-Step Platinum qRT-PCR kit (#11732) or Invitrogen's Superscript III Platinum SYBR Green One-Step qRT-PCR kit (#11732) in a Cepheid Smart Cycler. *E. chaffeensis* was detected as described above. *Drosophila*

ribosomal protein 15a (Accession #NM_136772) was detected using left primer TGGACCACGAGGAGGCTAGG, right primer GTTGGTGCATGGTCGGTGA, and Taqman probe TGGGAGGCAAAATTCTCGGCTTC (13). Antimicrobial peptides were detected using the following primer sets: *diptericin* (Accession #NM_057460), (5') ACCGCAGTACCCACTCAATC, (5') CCCAAGTGCTGTCCATATCC; and *drosomycin* (Accession #NM_079177), (5') GTACTTGTTTCGCCCTCTTCG, (5') CTTGCACACACGACGACAG (52).

Transfection of S2 cells.

Plasmids carrying GFP reporter-AMP promoter constructs for Attacin, Diptericin, and Drosomycin were obtained from the lab of Bruno Lemaitre (62). Competent *E. coli* cells (Invitrogen #C404003) were transformed and plated on nutrient agar containing 50 µg per ml of ampicillin. The presence of the *attacin*, *diptericin*, or *drosomycin* plasmid was confirmed using colony PCR. For detection of *diptericin* and *drosomycin*, the previously mentioned primer sets were used. For the detection of *attacin* (Accession #NM_079021), the following primer set was used: left primer-CAATGGCAGACACAATCTGG and right primer-ATTCCTGGGAAGTTGCTGTG (13). PCR reactions were performed using the Platinum Taq Polymerase kit (Invitrogen #10966-034). Upon confirmation of the plasmid of interest, nutrient broth cultures containing 50 µg per ml of carbenicillin were inoculated and grown overnight at 37°C. Plasmid DNA was extracted using the Qiagen Midi-Prep Kit (#12145). For transfection, 5×10^5 S2 cells were plated in 60 mm² tissue culture wells of 6-well plates and incubated overnight. One µg of plasmid DNA was mixed with 15 µl of Cellfectin Reagent (Invitrogen #10362), and subsequently mixed in incomplete S2 medium (no serum) in a total volume of 200 µl. The mixture was incubated at room temperature for 30 minutes. The S2 cells were rinsed

with incomplete S2 medium and a final volume of 800 μ l of incomplete medium was added to the cells. Plasmid/CellFectin complexes were added to the cells, the cells were briefly shaken, and incubated with the complexes for 5.5-6 hours. Cells were then washed with complete S2 medium and resuspended in a final volume of 2 ml. To test for activation of the transgene, viable or heat-killed cell-free *E. chaffeensis* was added to the cells 48 hours post-transfection and fluorescence was measured at 24 hours later using a Perkin Elmer Wallac Victor³ Multilabel Counter.

Statistics.

Data are presented as the mean \pm standard error (SE) of independent experiments. Differences in mean were determined by using Student's *t* test (two-tailed, general) (StatMost statistical package (Data XIOM, Los Angeles, CA)). Survival data were analyzed for significance using the log-rank test of Kaplan Meier plots using Prism Graphpad software (La Jolla, CA). *P* values of <0.05 were considered highly significant.

RESULTS

Growth and pathogenicity of *E. chaffeensis* in adult *Drosophila*.

We previously demonstrated that *E. chaffeensis* replicated in the *Drosophila* hemocyte-like S2 cells (38). These data raised the issue of whether *E. chaffeensis* could grow *in vivo*. To test the hypothesis that adult *D. melanogaster* can be infected with *E. chaffeensis*, we injected cell-free *E. chaffeensis* into the abdomens of WT adult male and female *D. melanogaster*. Negative control male and female flies were injected with sterile PBS. Survival was monitored in a total of 20-25 flies per experiment for 120 hours. There was a significant amount of death in the infected flies compared to those injected with PBS (Figure 3.1). For example, at 24 hpi, a mean

of 86% of flies injected with *Ehrlichia* survived compared to 97% of the PBS-injected controls. By 120 hpi, only 25% of the flies injected with *Ehrlichia* were still alive, compared to 85% of the PBS controls (Figure 3.1; $P < 0.05$). To confirm that our results were not particular to the *w;Hemese-Gal4, UASGFP* flies we chose to use as WT, we compared their survival with that of *yw* flies. We detected no significant differences in survival between the *yw* and WT flies that were challenged with *Ehrlichia* (Figure 3.1). Therefore, *Ehrlichia* pathogenesis was not unique to the *w;Hemese-Gal4, UASGFP* flies.

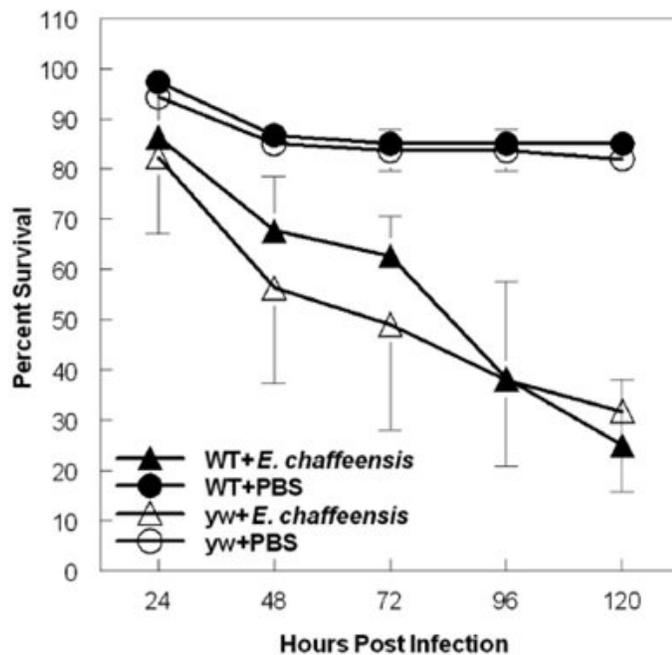


Figure 3.1 Comparison of survival of adult, wild-type (WT)(*w;Hemese-Gal4, UASGFP*) and yellow white (*yw*) flies infected with *E. chaffeensis*. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean \pm SEM of 3 independent experiments. 20-25 flies were injected per treatment group per experiment.

The number of flies that survived was dependent on bacterial dose (Figure 3.2). At 96 hours post infection, a mean of 47% of flies survived at the highest challenge dose, with 68%, 72%, and 77% survival when challenged with decreasing doses (Figure 3.2). Bacteria replicated in the flies over time. RNA was collected from live flies at several time points for subsequent qRT-PCR to measure 16S rRNA *E. chaffeensis* and *Drosophila ribosomal protein 15a*. By 24 hours, there were usually more bacteria detected than were originally injected and the number of bacteria increased and decreased in a cyclical fashion over a period of 120 hours (Figure 3.3). By 96 hours post infection there were higher bacterial loads in the flies than in the original injections in 9 of the 11 measurements made (Figure 3.4). Therefore, these data indicate that *E. chaffeensis* is capable of infecting *D. melanogaster* in a dose-dependent fashion and the bacteria were actively replicating in adult flies. Furthermore, bacteria isolated from adult flies were used to reinfect DH82 cells *in vitro*, as determined by RT-PCR and by the identification of morulae in the infected cell after infection (data not shown). Therefore, the bacteria that replicate in adult flies are capable of re-infecting mammalian cells.

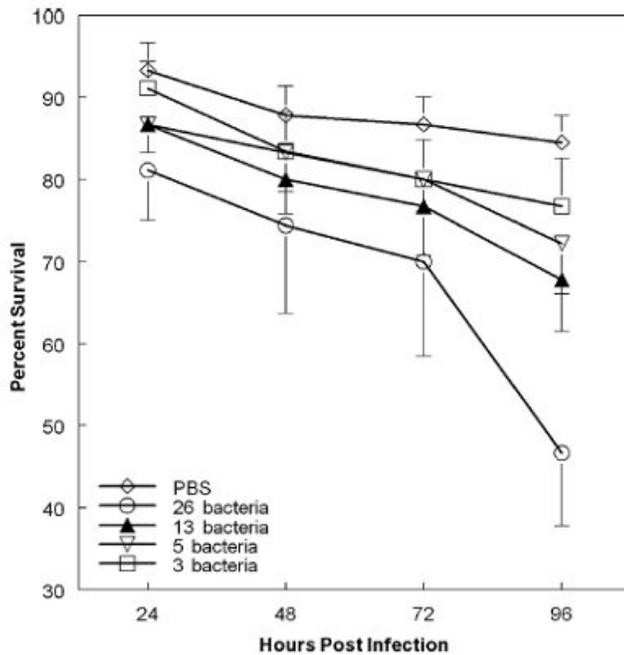


Figure 3.2 Dose-dependent survival after *E. chaffeensis* challenge. Wild-type flies were challenged with an mean, initial dose of ~26 bacteria or 1:2, 1:5, and 1:10 dilutions of the initial dose. Data represent the mean \pm SEM of 3 independent experiments, 30 flies per treatment group challenged per experiment.

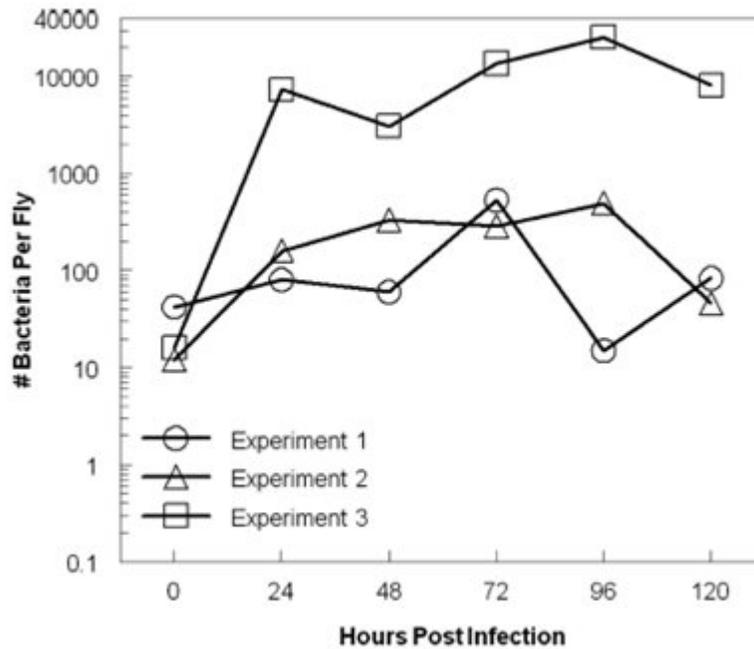


Figure 3.3 Quantitation of bacterial load in flies infected with *E. chaffeensis*. Bacterial load was estimated by qRT-PCR for *Ehrlichial* 16S rRNA as described in the materials and methods. Three independent experiments are illustrated. Each point represents 4-7 flies per RNA preparation.

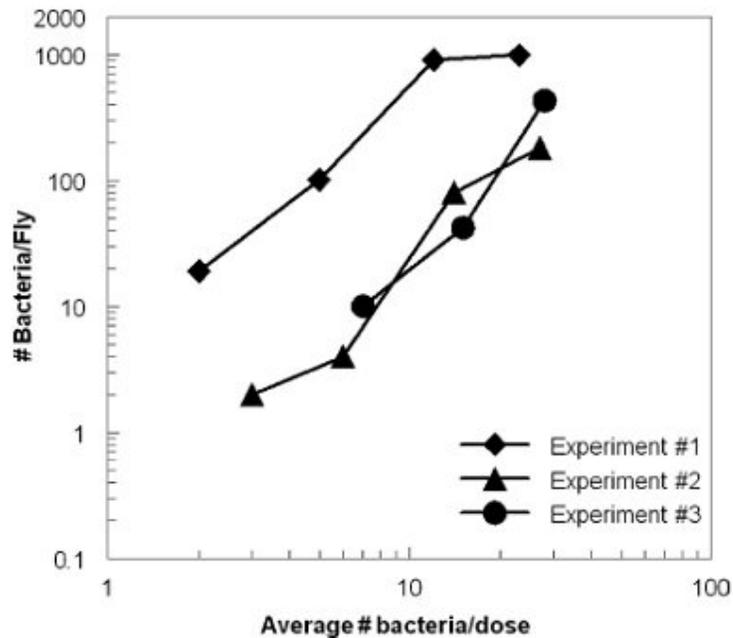


Figure 3.4 Bacterial growth in adult *Drosophila*. Wild-type flies were infected with the dose of *E. chaffeensis* indicated on the abscissa and the number of bacteria present at 96 hpi was quantitated (ordinate). Bacterial load was estimated based on copies of 16S rRNA as described in the materials and methods. Each point represents 13-26 flies per RNA preparation.

Injection of beads affects fly survival.

E. chaffeensis infects monocytes and macrophages of vertebrate hosts (45). *E. chaffeensis* can also infect and replicate in phagocytic *Drosophila* S2 cells (38). Consequently, we hypothesized that interfering with hemocyte/phagocyte function in the adult flies would have an impact on *E. chaffeensis* infections. Injection of polystyrene beads has been shown to disrupt *Drosophila* hemocyte/phagocyte function in other studies (13, 47, 52). Therefore, we used that technique to explore the role of hemocytes in host resistance to an *E. chaffeensis* challenge. We injected WT adult male and female flies with a 2% solution of 0.2 μm polystyrene beads/PBS or with PBS alone. Flies were challenged with *Ehrlichia* either four hours or 24 hours after injection of the beads/PBS or PBS alone. Although bead injection did not affect fly survival more than the PBS

injection (Figure 3.5), the bead injection had an immediate impact on flies challenged with *Ehrlichia* ($P < 0.05$, log rank test). When flies were challenged four hours after bead injection, 60% of the flies died compared to 30% of the flies challenged with *Ehrlichia* alone, at 24 hours post infection. The impact of the beads diminished over time. By 96 hours post infection, there were no differences in survival ($P > 0.05$, t-test) between bead-injected and non-bead-injected flies that were challenged with *Ehrlichia*. Flies challenged with *Ehrlichia* 24 hours after bead injection had a similar pattern compared to the flies that were challenged 4 hours after bead injection (Figures 3.5A, B). In addition, we performed qRT-PCR to determine if bead injection had an impact on the number of bacteria present in surviving flies. We observed an increase in the number of bacteria present per fly in those flies that were injected with the beads prior to *Ehrlichia* challenge (Figures 3.5C, D). For example, flies injected with beads four hours prior to challenge had an average of 40 bacteria per surviving fly compared to 23 bacteria per control flies at 24 hours after *Ehrlichia* challenge. The increased bacterial load was observed when the beads were injected four or twenty-four hours before *Ehrlichia* challenge (Figure 3.5).

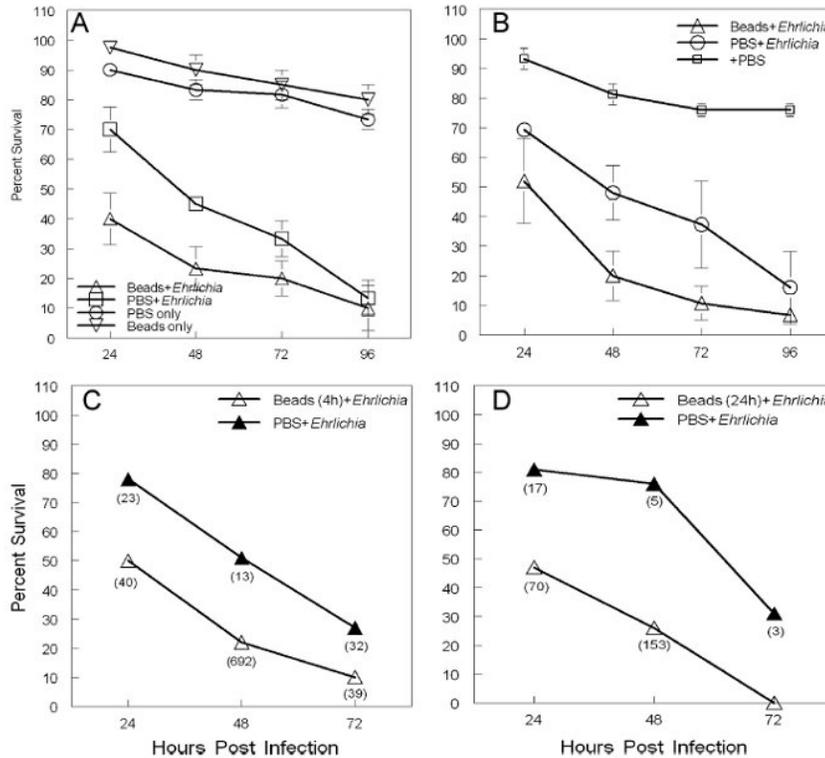


Figure 3.5 Effects of polystyrene bead injection on *E. chaffeensis* challenge. **A**, beads or PBS injected 4 hours prior to *Ehrlichia* challenge; **B**, beads or PBS injected 24 hours prior to *Ehrlichia* challenge; **C**, beads or PBS injected 4 hours prior to *Ehrlichia* challenge. For **C** and **D**, the numbers in parantheses indicate the bacterial load per fly at the corresponding time point. For panels **A** and **B**, the data represent the mean \pm SEM of 3-5 independent experiments (20 flies per treatment group per experiment); absence of error bars indicates error smaller than the size of the marker. For panels **C** and **D**, one representative experiment is shown.

Drosophila hemocytes phagocytose *E. chaffeensis*.

To determine if the *Drosophila* hemocytes were phagocytosing the injected *E. chaffeensis*, we utilized pHrodo dye, an amine-reactive succinimidyl ester, which becomes fluorescent as the local environment becomes more acidic. *Drosophila* hemocyte phagosomes have a lower pH compared to the extracellular environment (26). Therefore, we hypothesized that pHrodo-

labelled *E. chaffeensis* would fluoresce only if it was phagocytosed by hemocytes. Additionally, we FITC-labeled *E. chaffeensis* to visualize bacterial trafficking and localization. FITC-labeled bacteria were identified in 98% of the flies (3 experiments, 20 flies/experiment) examined 4 hours after injection and in 93% of the flies examined at 24 hours after injection. We observed pHrodo-labeled bacteria in 82% of the flies at 4 hours and 88% of the flies at 24 hours after injection. Therefore, it appeared that the lower pH environment of the hemocytes caused the bacteria to fluoresce red. Hemocytes and extracellular bacteria were distinctly observed in the central, dorsal abdomen at four and 24 hours post injection (Figure 3.6), and was the most frequent location where hemocytes and bacteria were observed. We also observed dissemination of the hemocytes and bacteria throughout the fly (Figure 3.6).

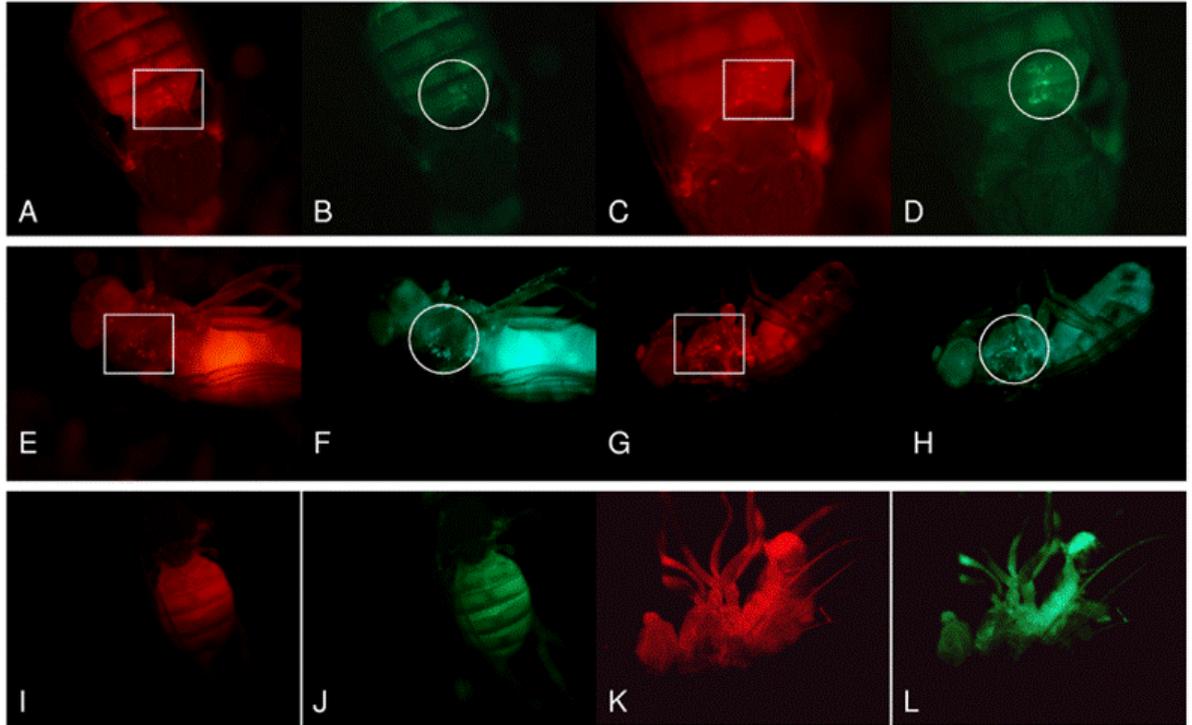


Figure 3.6 Hemocytes participate in *E. chaffeensis* infection. Flies were injected with a mixture of pHrodo-labelled and FITC-labelled *E. chaffeensis*. Panels A-H are flies that received a mixture of pHrodo and FITC-labelled bacteria; panels I-L are flies that did not receive an injection. The same fly was photographed in panel A-B; C-D; E-F; G-H; I-J; and K-L. Hemocytes (pH-rodo) are designated by boxes and FITC-labeled bacteria are designated by circles in panels A-H. Panels A-H are dorsal views of hemocytes and bacteria and panels E-F show presence of hemocytes and bacteria in the thoracic region. The high intensity foci on the image represent the hemocytes. Images in panels A-H were photographed at 4-24 hpi, at 25-30X magnification.

Antimicrobial peptides are induced in response to *E. chaffeensis* infection in *D. melanogaster*.

Our data suggested that hemocytes were mobilized after injection of *E. chaffeensis*. *Drosophila* innate immunity also includes humoral components, principally the production of antimicrobial peptides through either the Toll or Imd pathways. The Toll pathway is most often activated by

Gram-positive bacteria and fungi, which elicits production of the antimicrobial peptide Drosomyacin (18, 31, 63). The Imd pathway is most often activated by Gram-negative bacteria and is characterized by production of antimicrobial peptides such as Attacin and Diptericin (18, 31, 63). Although *E. chaffeensis* is Gram-negative, it lacks the genes for synthesis of both lipopolysaccharide and peptidoglycan (37). Because *E. chaffeensis* has this atypical outer membrane, we were particularly interested in determining if the host defense pathways (Imd and/or Toll) were activated in response to *E. chaffeensis* challenge.

To address this question, we challenged transgenic flies expressing green fluorescent protein (GFP) under the control of the *attacin*, *diptericin*, or *drosomyacin* promoters (62) with *E. chaffeensis*. After injection with bacteria or sterile PBS, GFP-expressing flies were examined under a fluorescent, dissecting microscope at 24 hpi as described in the methods. There were distinct differences in *attacin-GFP* and *diptericin-GFP* expression between the flies that received bacteria compared to those that received PBS or no injection at all (Figure 3.7). Fluorescence was also quantitated in the transgenic flies that were injected with *Salmonella enterica* serovar *typhimurium*, as described in the methods. *Salmonella* is a Gram-negative bacterium that is lethal to flies (47). No significant differences in antimicrobial peptide induction were detected between flies that were challenged with *Ehrlichia* and *Salmonella* (Figure 3.8). For the *Ehrlichia*-injected flies, the *attacin-GFP* flies had a significant increase ($P < 0.05$, $n = 3$ independent experiments) compared to control flies (injected with PBS only) at both 6 and 24 hours post infection (Figure 3.8). For example, at 6 hours post infection, *attacin-GFP* expression in the *Ehrlichia*-injected flies had a mean of 95,009 arbitrary fluorescent units (AFU) compared to 54,894 AFU in the PBS-injected flies. Significant increases in GFP expression were also observed in the *diptericin-GFP* flies that were injected with *Ehrlichia*

compared to controls (Figure 3.7). At 6 hours post infection, the mean *dipteracin* expression was 66,635 AFU in the *Ehrlichia*-injected flies and 48,309 in the PBS-injected flies. The activation of *drosomycin* was distinctly different from the activation of *dipteracin* and *attacin*. There were minimal visual and quantitative (Figures 3.7 and 3.8) differences in GFP expression in the *drosomycin-GFP* flies that received *Ehrlichia* from those that received PBS or no injection. GFP expression was also significantly lower in the *drosomycin-GFP* flies compared to the *attacin-GFP* and *dipteracin-GFP* flies.

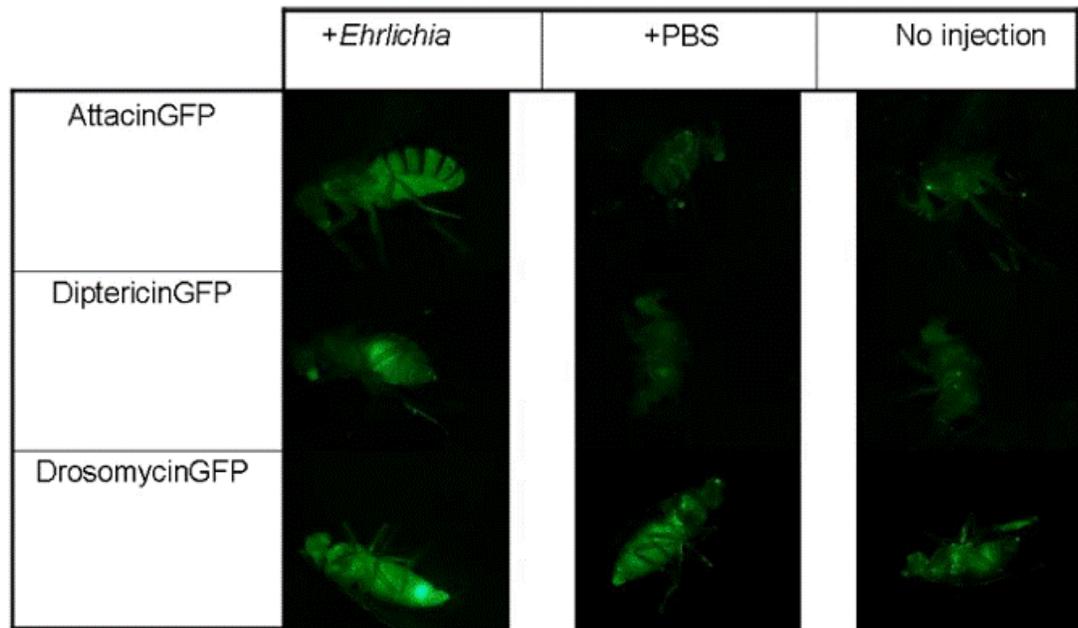


Figure 3.7 *E. chaffeensis* induces antimicrobial peptide transcription. Transgenic flies expressing GFP under the control of the *attacin*, *dipteracin*, or *drosomycin* promoter were injected with *E. chaffeensis*, PBS, or received no injection. At 24 hpi, flies were viewed under a fluorescence, dissecting microscope at 25-30X magnification.

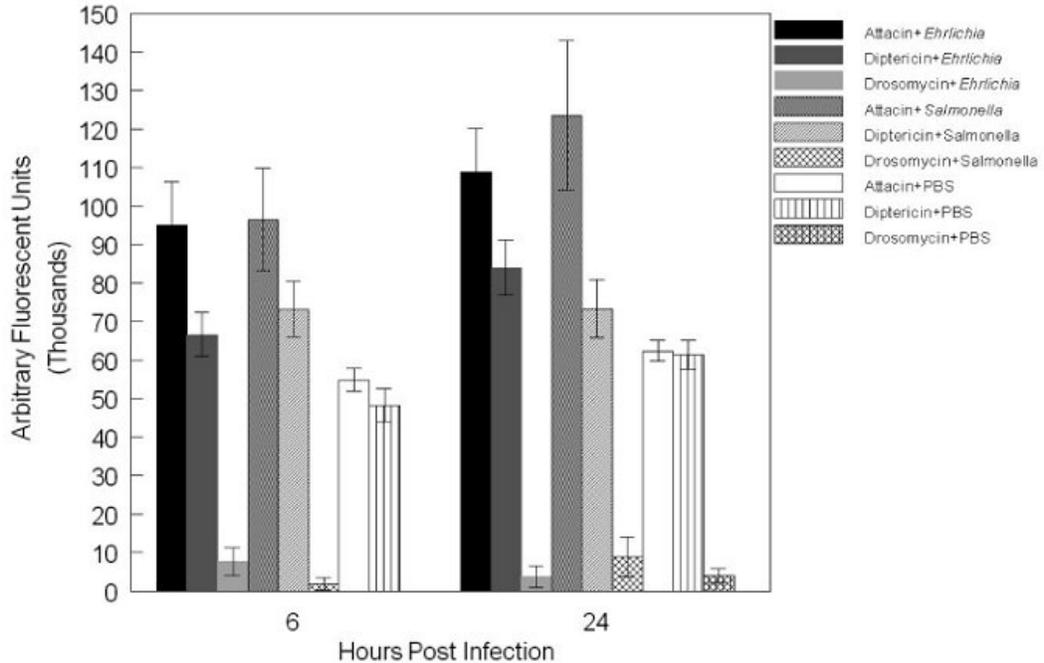


Figure 3.8 *E. chaffeensis* induces antimicrobial peptides. Transgenic flies expressing GFP under the control of the *attacin*, *dipteracin*, or *drosomycin* promoter were injected with *E. chaffeensis*, PBS, *S. enterica* serovar *typhimurium*, or received no injection. At 6 and 24 hpi, individual flies were homogenized and GFP expression was measured on a spectrophotometer. Data shown are the mean of 3 independent experiments \pm SEM, ≥ 10 flies per treatment group per experiment.

***relish* and *dredd* mutants are more susceptible to *E. chaffeensis* infection.**

The apparent up-regulation of *attacin* and *dipteracin* in adult flies challenged with *E. chaffeensis* suggested that the Imd pathway was activated in response to the *E. chaffeensis* and might be important to host resistance. To test this hypothesis, we experimentally challenged flies that carried mutations in the genes that encode for specific proteins of the Imd pathway. These mutations included those that affected the Dredd or Relish proteins. WT flies were concurrently infected as controls in these experiments. Both the *relish* and *dredd* mutants had significantly increased mortality compared to the control flies (Figure 3.9). Statistically significant

differences in survival between *relish* mutants and wild-type flies were observed at 24, 48, 72, 96 and 120 hours post infection ($P < 0.05$, t-test and log-rank test). For the *dredd* mutants, significant differences in survival compared to the wildtype flies were confirmed at 24 and 96 hours post infections (t-test $P < 0.05$); and significant difference in the survival curves was confirmed by the log-rank test. We confirmed that the *dredd* and *relish* mutants maintained their phenotypes. When we used qRT-PCR to measure the AMP transcript levels in the mutants, there were decreased transcript levels for *diptericin* in the *dredd* and *relish* mutants (compared to wild-type flies); *drosomycin* transcript levels were not altered in either of these mutants.

We also used qRT-PCR to measure the bacteriemia in the mutant flies after infection to give us a second measure of pathogenesis. Infection was detected in all mutant flies, and was higher in the *relish* mutants compared to the wild-type flies. These results suggested that Relish was a key component of the Imd pathway necessary for *Drosophila* to combat *E. chaffeensis* infections.

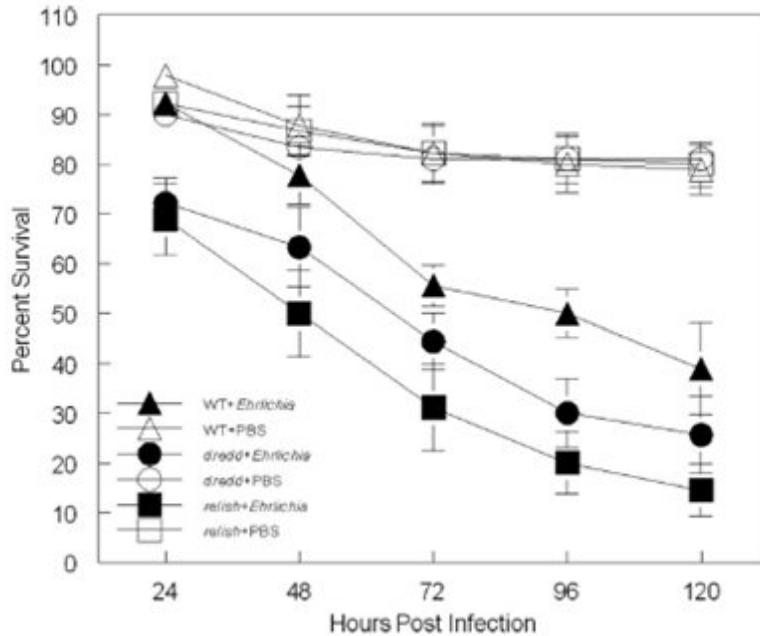


Figure 3.9 Survival of Imd pathway mutants after *E. chaffeensis* challenge. *dredd* and *relish* mutants and wild-type flies were infected with *E. chaffeensis* and monitored for survival for 120 hours. Data presented are the mean of 3 independent experiments \pm SEM, 30 flies per treatment group per experiment. *dredd* and *relish* mutant fly survival was significantly different from wild-type fly survival ($P < 0.05$, log-rank test).

pelle, but not *Toll* and *cactus* mutants are more susceptible to *E. chaffeensis* infections.

The data from the *drosomycin-GFP* transgenic flies suggested that the Toll pathway was activated to a lesser extent after *Ehrlichia* infection compared to *attacin* and *diptercin*. To directly assess the role of the Toll defense pathway in host resistance to *E. chaffeensis*, we also challenged adult, *pll²*, *Tl³*, *cact^{D11}* and *cact^{D13}* flies. *Tl³* mutant flies have constitutively active Toll and enhanced Drosomycin activity. *pll²* flies are mutants lacking the Pelle kinase and have an impaired ability to make Drosomycin. *cact^{D11}* and *cact^{D13}* flies are mutants lacking the Cactus protein, which is a negative inhibitor of the Toll pathway. Therefore, these flies have enhanced Drosomycin activity. We observed decreased mortality ($P < 0.05$, log-rank test) in the *Tl³* flies

compared to wild-type flies. (Figure 3.10). We observed no significant difference in death at any time point in the *cactus* mutants (Figure 3.11). In contrast to the *Toll* and *cactus* mutants, the *pelle* mutants displayed significantly decreased survival ($P < 0.05$) compared to wildtype flies at all time points (Figure 3.12). At 24 hpi, a mean of 75% of the *pelle* mutants were alive compared to 92% of wild-type flies. By 120 hpi, a mean of 25% of *pelle* mutants survived compared to 53% of wild-type flies.

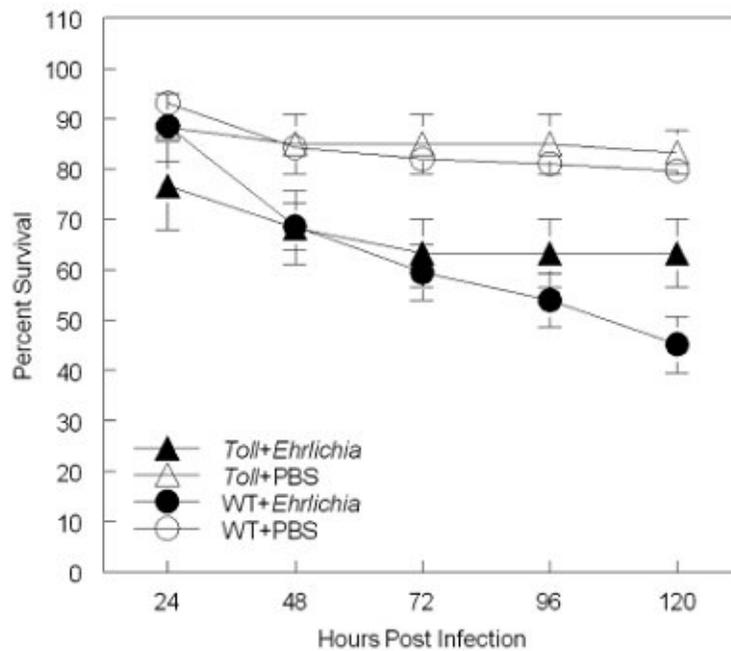


Figure 3.10 The *Toll*-constitutive mutant flies are less susceptible to *E. chaffeensis* infections. Mutants for the Toll protein and wild-type flies were infected with *E. chaffeensis* and monitored for survival for 120 hours. Numbers represent mean \pm SEM of ≥ 3 independent experiments, 20 flies per experiment. Survival of wild-type flies was significantly different from *Toll* mutants ($P < 0.05$, log-rank test).

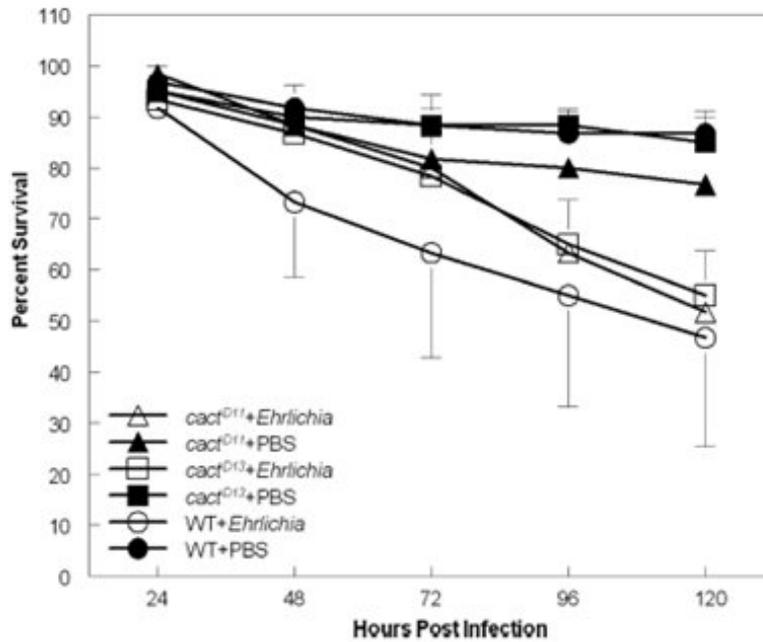


Figure 3.11 Effect of *E. chaffeensis* infection on *cact*^{D11} and *cact*^{D13} mutants. *cactus* mutants and wild-type flies were infected with *E. chaffeensis* and monitored for survival for 120 hours. Data represents the mean of 3 independent experiments \pm SEM, 20 flies per treatment group per experiment. Absence of error bars indicates error smaller than the size of the marker.

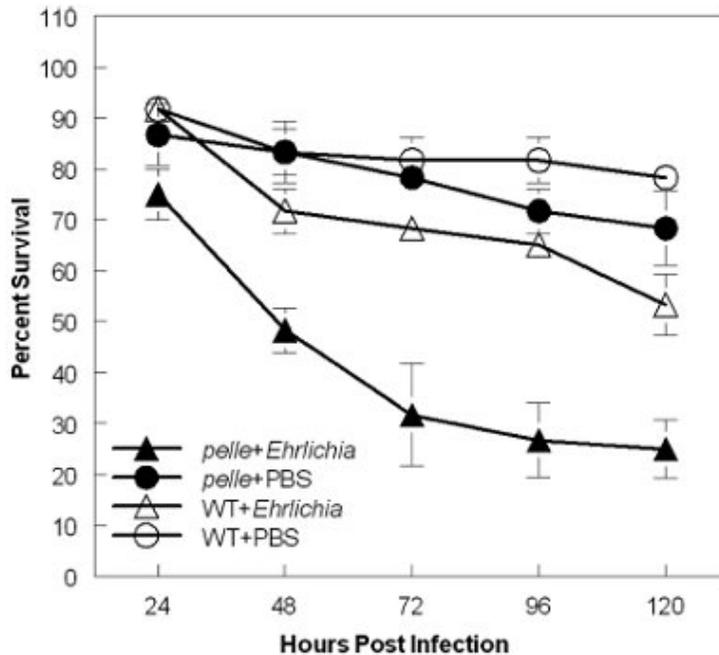


Figure 3.12 Effects of *E. chaffeensis* infection on *pelle* (3111) mutants. *pelle* mutants and wild-type flies were infected with *E. chaffeensis* and monitored for survival for 120 hours. Data represent the mean of 3 independent experiments \pm SEM, 20 flies per treatment group per experiment. Significant decreases in survival were observed between wild-type and mutant flies ($P < 0.05$, log-rank test). Absence of error bars indicates error smaller than the size of the marker.

***E. chaffeensis* membrane components activate the Imd pathway.**

In mammalian systems, *E. chaffeensis* and other *Rickettsia* activate macrophages through the Tlr4 pathway (11, 22, 27), despite the fact that the bacteria do not synthesize LPS. Therefore, the atypical outer membrane of *Ehrlichia* likely contains molecular patterns that can serve as alternative ligands for Tlr4. Since disruption of components of both the Toll and the Imd pathways led to decreased fly survival after an *Ehrlichia* challenge, we wanted to determine if activation was dependent on bacterial replication/infection or if exposure to the bacteria and their respective molecular patterns was sufficient for activation of these pathways. To test this

hypothesis, we used the AMP-GFP reporters used in the fly experiments above as an *in vitro* readout system using *Drosophila* S2 cells as outlined in the methods. Results from a microarray analysis (data not shown) revealed that several antimicrobial peptide genes were up-regulated in S2 cells after infection with *E. chaffeensis*. *attacin*, *diptericin*, and *drosomycin* were among those genes. Therefore, we anticipated that the up-regulation of GFP in S2 cells would occur in response to the appropriate signals either delivered by exposure to bacteria alone, or by infection by live bacteria. *attacin-GFP* and *diptericin-GFP* expression were both up-regulated in the transfected S2 cells in response to *E. chaffeensis* infection by viable bacteria (Figure 3.13). *drosomycin* was also up-regulated in response to infection (Figure 3.13). Interestingly, we also observed induction of the antimicrobial peptides when heat-killed *E. chaffeensis* was added to the cells (Figure 3.13). However, the intensity of the activation was lower compared to viable bacteria. These results suggest that some component of the *Ehrlichia* membrane is activating the AMP pathways and that replication is able to augment that response.

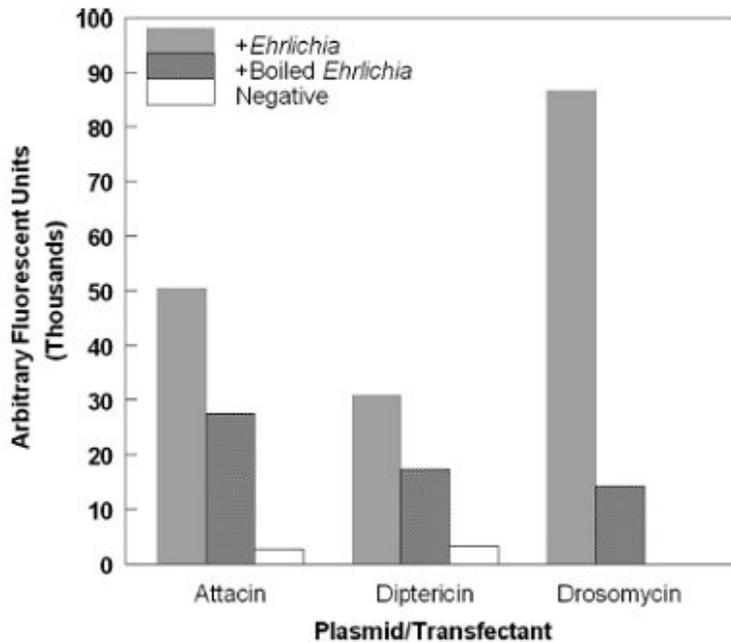


Figure 3.13 Antimicrobial peptides are activated in response to *E. chaffeensis* in S2 cells. S2 cells were transfected with *attacin*, *dipterucin*, or *drosomycin* promoter-GFP constructs. The S2 cells were dosed with live or boiled *E. chaffeensis*, or with sterile S2 medium (negative). GFP output was measured on a spectrophotometer. Numbers represent the mean of two independent experiments.

DISCUSSION

E. chaffeensis can infect and complete its replication cycle in adult *D. melanogaster*.

This is the first demonstration that *Ehrlichia chaffeensis* is able to replicate in an arthropod other than the tick. We previously demonstrated that that *Ehrlichia* could grow in *Drosophila* S2 cells (38). Therefore, establishing infections at the organismic level represents an important step forward in our ability to work in an alternative arthropod system. We concede that the mode of *Drosophila* infection was not similar to the natural route of infection in a tick, which acquires the infection by a blood meal. However, the ability to grow the bacteria in the fly opens the

biological and genetic tool box of *Drosophila melanogaster* to address questions about *Ehrlichia* that cannot be addressed in the tick.

E. chaffeensis infection was pathogenic to adult flies. It is not completely clear how this outcome differs when bacteria infects ticks. Little is known about tick survival after *Rickettsia* infection. Our results are consistent with the observation of a decrease in larval molting and overall survival among *Ixodes scapularis* ticks when allowed to feed on mice infected with various isolates of *Anaplasma phagocytophilum* (50). Increased death during molting to adult stage or before feeding of *Dermacentor andersoni* larvae and nymphs after experimental infection with *Rickettsia rickettsii* has also been reported (44). In the same study, fewer larvae developed from infected ticks. Decreases in molting success and increased death were also observed in a study in which *Rhipicephalus sanguineus* ticks were experimentally infected with *Rickettsia conorii* (36).

Although we observed a dose-dependent survival when flies were injected at any one particular time (Figure 3.2), there was some experiment-to-experiment variability in the number of bacteria that caused death in the challenged flies. This variability probably resulted from a combination of factors including differences in bacterial preparations (ratio of dense core to reticulate forms (67)), variability in the custom made glass needles used to inject the bacteria, the ages and sexes of the flies (14, 19, 46), and the skill of the individual doing the injections. Therefore, the experiments we presented were always reported as the outcome of multiple independent experiments. Controls and direct comparisons were done at the same time.

Hemocytes appear to participate in the *Drosophila* cellular immune response to *E. chaffeensis*. This is supported by three different experiments. There was significantly decreased survival when the flies received an injection of polystyrene beads prior to bacterial challenge.

We also detected phagocytosis of the *Ehrlichia* using the pHrodo dye and an increase in the number of bacteria in flies receiving the beads compared to the bead-free controls. It is possible that the beads inhibited hemocyte phagocytosis, rendering the fly less capable of controlling the initial infection of the bacteria. This hypothesis is supported by previous studies where polystyrene beads inhibited phagocytosis of *Streptococcus pneumoniae*, *Plasmodium gallinaceum*, *Mycobacterium marinum*, and *Escherichia coli* (13, 16, 47, 52). Alternatively, the beads may inhibit *Ehrlichia*-specific receptors on the hemocytes. This could lead to an overgrowth of bacteria in the haemocoel, resulting in tissue damage that may be fatal.

Our attempts to isolate hemocytes from infected or uninfected adult flies were unsuccessful, even after using several different techniques that were successful in other dipterans or *Drosophila* (8, 29). Therefore we visualized hemocyte location within the fly with the pHrodo dye. Since not all the FITC-labelled bacteria were localized to hemocytes (identified with pHrodo-labelled bacteria), it appears that some of the bacteria (~25%) were not phagocytosed (extracellular bacteria). We observed an abundance of hemocytes as well as extracellular bacteria as early as one hour after and as late as 120 hours after injection of bacteria. Hemocytes and extracellular bacteria were most often observed at the dorsal midline at the anterior end of the abdomen of the fly. There were also hemocytes and free bacteria distributed throughout the body of the fly. We suspect that *Ehrlichia* are capable of growing in hemocytes since bacteria can grow in S2 cells (38). However, we have yet to determine whether the pathogenesis we see is due to bacteria growing inside the hemocyte, extracellularly, or by infecting other tissues. It has been reported in *Ixodid* ticks that *Rickettsiae* infect hemocytes and thus disseminate to all tissues and organs and can survive in the tissues and the body cavity for long periods of time (56). Additional experiments will be needed to determine if this dynamic is

also the case in *Drosophila* and were beyond the goals of this investigation. However, based on the survival kinetics after bead injections, it does appear that the early plasmatocyte/hemocyte response does work to control the infection.

Ehrlichia infection in flies appears to activate both the Toll and Imd host defense pathways. This conclusion is supported by several different experiments. For the Toll pathway, we saw increased survival in the *Tl³* flies, and decreased survival in the *pelle* mutant flies. The *Tl³* flies were created with the mutagen ethyl methanesulfonate and have a mutation that renders Toll constitutively active (1, 32, 33, 49, 53). The *pelle* mutant flies were created with the mutagen ethyl methanesulfonate and have a mutation that ablates that function of the *pelle* kinase (54), which is believed to play a role in the degradation of Cactus (31, 61). The continuous expression of *drosomycin* in the *Tl³* flies and the loss of *drosomycin* in the *pelle*-mutant flies would support some role for this pathway after *Ehrlichia* challenge. Spaetzle is the ligand for Toll and the interaction initiates Toll signaling (24, 33, 65), followed by interaction of MyD88/Pelle/Tube, which causes the degradation of Cactus (4, 59). Cactus is an inhibitor of the Toll pathway (43). Therefore, the loss of function Cactus mutants have a constitutively active Toll pathway (5, 51). Toll pathway involvement was also supported by activation of the *drosomycin* promoter when S2 cells were infected *in vitro*. It is not clear why activation of *drosomycin* was lower than that of *attacin* and *diptericin in vivo*. *drosomycin* expression has been shown to be up-regulated later than *diptericin* (6, 34). Therefore, it is possible that our data reflect those kinetics.

The Imd pathway also appears to be activated after an *Ehrlichia* challenge. We observed activation of the promoters of the Imd-dependent antimicrobial peptides *attacin* and *diptericin* both *in vivo* and *in vitro*, and increased transcript levels of *diptericin* in wild-type flies after

infection (data not shown). We also observed significantly diminished survival of *relish* and *dredd* mutant flies after experimental challenge. Relish is a key component of the Imd pathway, which is activated by Gram-negative bacteria (31, 48, 64). It is a homologous component to the p100 and p105 precursors in the NF- κ B family of transcription factors and is essential for the transcription of antimicrobial peptides in *D. melanogaster* (57). The activation of the Imd pathway by the obligate intracellular pathogen *E. chaffeensis* is similar to observations made with another Gram-negative, intracellular bacterium, *Francisella tularensis* (64). We also observed a significant decrease in the survival of *dredd* mutants. Dredd is the upstream caspase activator of Relish (17) (39) and it has been previously demonstrated that *dredd* mutants are more susceptible to Gram-negative bacterial infections (35). Therefore, several different observations demonstrate that Imd plays a role in the host defense of *E. chaffeensis*.

Our data show that bacteria are growing in adult flies and the oscillatory nature of the bacterial load over time suggest that there is an active host response against the bacteria just as there is in mice (21). Interestingly, the activation of AMPs did not appear to require bacterial replication. Exposure to boiled *E. chaffeensis* (dead) was sufficient for activation of AMPs in the S2 cell reporter system. The activation of AMP production is consistent with the control of these innate responses by pattern recognition receptors after they engage their ligands (31). In flies, the Toll and Imd pathways are activated by different forms of peptidoglycan, but not by LPS (31). In rodents, *E. chaffeensis* and other Rickettsia appear to engage Toll-like receptors even in the absence of LPS (22, 27, 60). Therefore, the *Ehrlichia* outer membrane components responsible for activation of antimicrobial peptides may be lipoproteins (25). The isolation of several immunogenic lipoproteins from the outer membrane of *E. chaffeensis* would support this hypothesis (25). Moreover, the activation of both the Imd and Toll pathways would be

consistent with the atypical outer membrane of *E. chaffeensis* compared to the unique activation of Imd by classical, pyogenic Gram-negative, LPS-containing bacteria (12, 23, 30).

In conclusion, we have demonstrated that *E. chaffeensis* is able to grow and replicate in adult *D. melanogaster*. Infection induces innate cellular and humoral responses in the fly that contribute to host resistance. These findings are significant because the *Drosophila* system will allow us to dissect the role of host genes in bacterial replication and elucidate which bacterial components contribute to the generation of innate resistance in arthropods.

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CHAPTER 4 - Identification of host genes that contribute to *Ehrlichia chaffeensis* infections in *Drosophila melanogaster*

ABSTRACT

Ehrlichia chaffeensis is an obligate, intracellular bacterium that causes human monocytic ehrlichiosis (HME) and the bacteria are vectored by the Lone Star tick (*Amblyomma americanum*). *Ehrlichia* grows in several mammalian cell lines, but NO consensus model of pathogenesis exists for arthropods or vertebrates and the host genes required for intracellular growth of the bacteria are unknown. We performed microarrays on S2 cells, comparing host gene expression between permissive or non-permissive conditions for *E. chaffeensis* growth. A total of 517 genes were identified that had increased transcript levels only under permissive growth conditions at 24 hours post infection. We screened adult flies which were mutant for some of the permissive genes identified in our microarrays for their ability to support *Ehrlichia* replication. Five mutant stocks were resistant to infection with *Ehrlichia* (genes *CG6479*, *separation anxiety*, *CG3044 (Cht11)*, *CG6364*, and *CG6543*). qRT-PCR confirmed that the bacterial load was decreased in these mutant flies compared to wildtype controls. Gene *CG6364* is described as potentially having uridine kinase activity and the *in vivo* mutation of this gene is predicted to disrupt the nucleotide salvage pathway, which caused a decrease in bacterial replication *in vivo*. Disruption of cytidine triphosphate synthesis with cyclopentenyl cytosine (CPEC), a CTP synthetase inhibitor, demonstrated that the *de novo* synthesis of CTP from glutamine was unnecessary. S2 cells treated with CPEC and infected with *Ehrlichia* had higher bacterial loads than untreated controls. Furthermore, addition of cytosine to S2 cells also

resulted in increased bacterial replication. Therefore the nucleotide salvage pathway via cytidine may be essential for bacterial replication. We also investigated what role *CG3044 (Cht11)* had in the replication of *Ehrlichia*. We hypothesized that it may be involved in cholesterol regulation or have a regulatory role in cellular activation. Our approach has successfully identified host genes that contribute to *E. chaffeensis* replication in *Drosophila*.

INTRODUCTION

Drosophila melanogaster has been used to study a variety of intracellular pathogens and has been successfully manipulated for successful identification of the genes involved in host-pathogen interactions in several *in vitro* and *in vivo* studies of pathogens. Some of these studies have focused on *Drosophila* interactions with *Listeria monocytogenes* (1, 2), *Chlamydia trachomatis* (10), *Mycobacterium marinum* (1, 9, 30, 43), *Francisella tularensis* (45, 54), and the protozoan parasite *Plasmodium gallinaceum* (5, 47).

We previously demonstrated that the obligate, intracellular bacterium *Ehrlichia chaffeensis* is capable of infecting, completing its lifecycle, and maintaining its pathogenicity in both *Drosophila* S2 cells and in adult flies (34, 35). *E. chaffeensis* is the causative agent of human monocytic ehrlichiosis, of which 828 cases were reported for 2007 (25). This represents a 44% increase in over those reported for 2006, and an overall 159% increase since 2003 (25). In addition to being reported in the United States, the occurrence of *E. chaffeensis* has also been documented in Africa, Europe, China, and Brazil (7, 8, 36, 56). Therefore, establishing a system to determine the host genes that contribute to the replication and pathogenicity of *E. chaffeensis* is important. To date, no such model system is yet available.

We previously identified growth conditions that were “non-permissive” for the growth of *E. chaffeensis* infection in *Drosophila* S2 cells. Incubating S2 cells with lipopolysaccharide

(LPS) effectively stopped bacterial replication (34). We hypothesized that microarray analysis of permissively-infected and non-permissively-infected S2 cells would reveal host genes that contribute to the replication of *Ehrlichia*. We used the Affymetrix *Drosophila* 2.0 array to identify a subset of genes that were exclusively expressed during *Ehrlichia*-permissive conditions in the infected S2 cells. A search of the *Drosophila* Flybase (www.flybase.org) for viable, fertile adult flies that contained mutations in genes from the narrowed data set revealed over 100 possible targets for investigation. In this paper we describe our initial screening outcomes. In particular, we screened a total of 15 genetic mutants and identified five in which survival was not impacted by *Ehrlichia* challenge and bacterial replication in the fly was hindered. We describe those genes and some follow up experiments that attempt to elucidate how these genes contribute to replication. To our knowledge this is the first description of host genes essential for replication of *E. chaffeensis*.

MATERIALS & METHODS

Maintenance of cell lines and *Ehrlichia chaffeensis* infections.

The canine macrophage cell line, DH82, was maintained at 37°C in Dulbeccos Modified Eagle's Medium with 3.5% fetal bovine serum and 3.5% Nu Serum (DMEM-7). The *E. chaffeensis* Arkansas isolate was continuously cultivated in the DH82 cell line at 37°C, 8% CO₂ in DMEM-7 medium. Bacteria were passaged when infectivity reached 80-90% as visualized using cytopsin prepared slides (stained with Hema3 fixative and Dif-Quik stain) to monitor formation of morulae in the cells. Infected cells were removed by scraping each plate with a cell scraper, transferring the culture to a conical tube, and vortexing the suspension with glass beads. The freed bacteria were purified by centrifuging the suspension at 600 x g for twenty minutes to

remove cell debris. The bacteria-containing supernatant was removed, transferred to a sterile conical tube, and was centrifuged at 15,000 x g for twenty minutes to pellet the free bacteria. The final supernatant was removed and the pellet was re-suspended in an appropriate amount of sterile PBS. Purified bacteria were used to re-infect DH82 and S2 cells. *Drosophila* S2 cells were cultivated at 28°C in Schneider's *Drosophila* medium (Gibco, Grand Island, NY, #11720) supplemented with 10% fetal bovine serum (Atlanta Biologicals, #S11150).

Incubation of S2 cells with Lipopolysaccharide (LPS).

To determine if LPS incubation would inhibit the growth of *E. chaffeensis*, S2 cells were plated at a concentration of 1×10^6 cells per plate in 6-well plates and were allowed to adhere for at least 30 minutes. LPS (from *Salmonella minnesota*; Sigma #L-6261) was sonicated for one hour and then added to each well at a concentration of 10 µg per ml. The cells plus LPS were incubated for five hours. S2 cells were then infected with *E. chaffeensis* purified from DH82 cells.

Infections in Permissive and Non-Permissive *Drosophila* S2 cells.

Drosophila S2 cells were plated in 6-well tissue culture plates (60-mm, Techno Plastic Products AG, #92406) at a concentration of 1×10^6 cells per ml. The cells were allowed to adhere for at least 30 minutes but no longer than 24 hours. To create non-permissive samples, cells were treated with LPS as described above. Bacteria purified from infected DH82 (85-100% of the cells infected) cells were added to S2 cell cultures and then were monitored for infection at 24 and 96 hours post-infection (hpi). Uninfected S2 cells and S2 cells activated with LPS (no *Ehrlichia* added) were used as a control for each infection experiment. At each time point, RNA was isolated using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, #TR118). S2 cells were removed by pipetting the cells off the dish and pelleted by centrifugation at 300 x g

for 5 minutes. The supernatants were discarded and 1 ml of TriReagent was used to lyse the pellet. The TriReagent/cell mix was transferred to 2.0 ml, Heavy Phase Lock Gel tubes (5 Prime/Eppendorf; Westbury, New York; #2302830). 300 μ l of chloroform was added and the mixture was shaken (not vortexed) for 15 seconds. The samples were then centrifuged at 12,000 x g for 10 minutes at 4°C and the aqueous phase was transferred to clean 1.5 ml tubes. 500 μ l of isopropanol was added and RNA was precipitated at -20°C for 24 hours. Samples were subsequently centrifuged at 12,000 x g for 10 minutes. The RNA pellet was washed with 1 ml of 70% ethanol and samples were centrifuged at 7.4 x g for 5 minutes. The 70% ethanol was decanted from the pellet, the pellet was allowed to slightly air dry and was resuspended in 50 μ l of nuclease-free water. RNA concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

Determination of infection by RT-PCR and quantitative real time reverse transcription – PCR (qRT-PCR).

Infections were assessed by using the reverse transcriptase polymerase chain reaction (RT-PCR) using the Promega Access One-Step RT-PCR kit (Madison, WI). 500-1000 ng of RNA were used for each reaction. Each reaction contained the following components: 1X buffer, 0.2 mM dNTPs, 2 μ M forward primer, 2 μ M reverse primer, 1.5mM MgSO₄, 1U per μ l DNA polymerase, 1U per μ l reverse transcriptase, and nuclease free water for a final reaction volume of 25 μ l. RT-PCR reactions were performed in a (Eppendorf Mastercycler Gradient) thermocycler, based on primers specific for the 16S ribosomal RNA gene of *E. chaffeensis*. Primers (Integrated DNA Technologies, Coralville, IA) used for detecting *E. chaffeensis* were RRG27 (5' GTATTACCGCGGCTGCTGGCAC 3') and RRG3

(5' CAATTGCTTATAACCTTTTGGTTATAAAT 3') (accession #M73222) (23). Cycling conditions for these primers were as follows: 48° C for 5 minutes, 42° C for 5 minutes, 45°C for 5 minutes, 48°C for 30 minutes, 94°C for 4 minutes, then 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute. Total RNA input was assessed using housekeeping genes for *Drosophila* ribosomal protein 49 (rp49)(accession #U92431). Sequences for housekeeping primers rp49 were (5' ATCGGTTACGGATCGAACAA 3'; 5' GACAATCTCCTTGCGCTTCT 3') for *Drosophila* S2 cells. Cycling conditions for rp49 primers were as follows: 48°C for 45 minutes, 94°C for 2 minutes, and then 35 cycles of 94°C for 45 seconds, 50°C for 1 minute, and 72°C for 1.5 minutes. RT-PCR reactions were also performed without reverse transcriptase in order to ensure that DNA was absent from the sample(s). RT-PCR products were identified on a ChemiImager after electrophoresis in 2% agarose gels and staining with ethidium bromide. The sized amplicons for each primer set were: RRG 27 & 3 (430 bp) and rp49 (165 bp).

To quantitate bacterial and housekeeping transcript levels, flies were anesthetized using CO₂, placed in 1.5 ml tubes (Kimble Kontes #749510-1500), and homogenized with disposable pestles in 1 ml of TriReagent (Molecular Research Center) (Kimble Kontes #749521-1590). Homogenates were transferred to 2.0 ml, Heavy Phase Lock Gel tubes and processed as described above.

The number of bacteria used for infection experiments of cells and flies was estimated using TaqMan-based real-time reverse transcriptase PCR (RT-PCR) as previously described (49). This TaqMan-based assay targets the *E. chaffeensis* 16S rRNA gene. Real-time PCR was performed on 10-fold serial dilutions of RNA extracted from 80- to 100%-infected DH82 cells (three different samples) using a Smart Cycler system (Cepheid, Sunnyvale, CA). Standard curves were generated by plotting the log number of bacteria versus the corresponding threshold

cycle value (mean of the results of three experiments). The lowest detection limit, or the presence of 1 bacterium, was considered to be the dilution at which the threshold cycle value approaches 40 (zero). In addition, we compared the ratio of *Ehrlichial* 16S rRNA and *Drosophila* housekeeping gene ribosomal protein 15a, using qPCR and qRT-PCR and DNA and RNA made from a single infection experiment. We found no differences in the ratio of 16S to 15a.

qRT-PCR was performed using the Invitrogen's One-Step Platinum qRT-PCR kit (#11732) or Invitrogen's Superscript III Platinum SYBR Green One-Step qRT-PCR kit (#11732) in a Cepheid Smart Cycler. *E. chaffeensis* was detected as described above. Sequences for *Drosophila ribosomal protein 15a* (Accession #NM_136772) were 5' TGGACCACGAGGAGGCTAGG 3', 5'GTTGGTGCATGGTCGGTGA 3', and Taqman probe 5'TGGGAGGCAAAATTCTCGGCTTC3' (9).

Analysis of gene expression based on qRT-PCR results was performed using the method described by Pfaffl (2001) (42). In short, primer efficiencies were calculated by performing serial dilutions of RNA, using the corresponding Ct values to generate standard curves for each unique set of primers, and using the equation:

$$\text{Efficiency} = 10^{(-1/\text{slope of standard curve})}$$

The change in Ct values for both genes of interest and housekeeping controls were calculated by subtracting the Ct of the "treated" sample from that of the "control/untreated" sample:

$$\Delta \text{ Ct interest} = \text{ Ct}_{\text{treated}} - \text{ Ct}_{\text{control/untreated}}$$

The differences were then applied back to the calculated efficiencies to determine the percent change in gene expression from control samples using the following equation:

(Efficiency of gene interest $\frac{\text{Gene interest:}\Delta\text{Ct control} - \text{treated}}{\text{Housekeeping gene:}\Delta\text{Ct control} - \text{treated}}$)

(Efficiency of housekeeping gene $\frac{\text{Housekeeping gene:}\Delta\text{Ct control} - \text{treated}}{\text{Housekeeping gene:}\Delta\text{Ct control} - \text{treated}}$)

Control samples were set at 100% expression of the gene of interested and all calculated values from treated samples were compared to 100%.

Microarray Analysis.

Microarray analysis was performed at the University of Kansas Medical Center Microarray Facility (Kansas City, KS) using Affymetrix (Santa Clara) *Drosophila* 2.0 Gene chips according to manufacturer's specifications. Analysis was performed on four treatment groups (each submitted in triplicate at 24 and 96 hpi): (1) S2 cells infected with *E. chaffeensis*; (2) S2 cells incubated with LPS and then infected with *E. chaffeensis*; (3) S2 cells incubated with LPS; and (4) untreated/uninfected S2 cells. CHP files were received from the University of Kansas Medical Center Microarray Facility and were analyzed using GeneSpring 7.3 and normalized by "Per Gene: Normalize to median". The "Filter on volcano plot" was applied fold change at 1.5 and one-way ANOVA at significant level $\alpha = 0.05$. Gene expression levels of permissive and non-permissive cells at 24 hpi were compared to uninfected controls at 24 hpi. Genes up-regulated 1.5 fold or higher above basal expression levels were identified in both the permissive and non-permissive conditions. These gene sets were then compared and those exclusively up-regulated in either permissive or non-permissive conditions at 24hpi were identified. Microarray data and the MIAMI compliant data are publicly accessible by creating an account and logging into bioinformatics.kumc.edu/mdms/login.php, and accessing the experiment titled "Differential Gene Expression in *Ehrlichia chaffeensis*-infected S2 cells".

Drosophila melanogaster.

Flies were maintained on standard dextrose/molasses/yeast medium at 18-29°C. For all experiments, flies of the appropriate background were used as wild-type (WT) controls.

w;Hemese-Gal4, UASGFP flies (GFPHeme) (from Dr. Michael J. Williams; Umea Centre for Molecular Pathogenesis; Umea University; Umea, Sweden), *yellow-white* (*yw*) (maintained in our stock collection at Kansas State University), and/or *white ocelli* (*wo* [1]) (Stock #634, from Bloomington *Drosophila* Stock Center at Indiana University; Bloomington, IN) were used as WT in these experiments. *Withered* (stock #441, *whd*[1]), *dumpy* (stock #276, *dp*[*ov1*]), and *tilt* (stock #623, *tt*[1]*wo*[1]) mutants are all the result of spontaneous mutations (33, 37, 55) and were obtained from Bloomington *Drosophila* Stock Center at Indiana University, Bloomington, IN.

The stock numbers, genotypes, and associated genes of adult *Drosophila* (obtained from Bloomington Stock Center; Bloomington, IN) screened by microinjection can be found in Table 4.1.

Adult *Drosophila* Infections.

Flies were transferred to fresh food at least 24 hours prior to injection/infection. For injection/infection, adult male and female flies were anesthetized with CO₂ (for no longer than 15 minutes at a time). Flies were injected with approximately 50 nl of sterile PBS with or without an average of 50-80 bacteria, using pulled, glass capillary needles. Injections were made in the abdomen of the fly, close to the junction of the thorax, and ventral to the junction between the dorsal and ventral cuticles. Following injection, flies were maintained in clean bottles with molasses caps that were changed every other day throughout the course of the experiments. Survival was monitored daily.

Cyclopentenylcytosine (CPEC) treatment of S2 cells.

CPEC was obtained from the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD) through a Materials Transfer Agreement. The CPEC was prepared to a final concentration of 15.06 mM using sterile water and DMSO. Two different infection protocols were used for testing the effect of CPEC on the growth of *E. chaffeensis* in the S2 cells. For the first protocol: S2 cells were plated at a concentration of 1×10^6 cells/ml in 6-well plates and CPEC was added at a final concentration of 100, 10, 1, or 0.1 μM per well; cell-free *Ehrlichia* was added to the treated cells 2 days later, and RNA was subsequently extracted from the cells 2 days post infection. For the second protocol: S2 cells were plated at a concentration of 1×10^6 cells/ml in 6-well plates and were infected with cell-free *E. chaffeensis* for 2 days; CPEC was then added to the infected cells and RNA extractions were performed 2 days following the CPEC treatment. S2 cells treated with the diluent only and infected with *Ehrlichia*, and uninfected S2 cells were used as controls. qRT-PCR was used to analyze transcript levels of *Ehrlichia* 16S rRNA and *Drosophila* ribosomal protein *15a*, as described earlier.

Addition of exogenous cytosine to *Drosophila* S2 cells.

Cytosine was obtained from Sigma-Aldrich Co. (St. Louis, MO) (#C3506-5G) and prepared using sterile water and 0.5M hydrochloric acid to a final concentration of 100 mM. The cytosine was first dissolved in 20 ml of hydrochloric acid and then brought to a final volume of 100 ml with sterile water, as recommended by the manufacturer. For infection experiments, S2 cells were plated in 6-well plates at a concentration of 1×10^6 cells/ml and incubated for 24 hours. Cytosine was then added to a final concentration of 25 mM and the cells were incubated for 24

hours. Cell-free *E. chaffeensis* was added to the cytosine-treated cells, to untreated S2 cells, and to S2 cells treated with the cytosine diluent. Untreated, uninfected S2 cells were used as a negative control. Following addition of cell-free *E. chaffeensis*, RNA was extracted from cells at 24, 48, 72, and 96 hpi. qRT-PCR was used to measure transcript levels of *Ehrlichia* 16S rRNA and *Drosophila* ribosomal protein *15a*, as described earlier.

Methyl- β -Cyclodextrin (M β CD) treatment of *Drosophila* S2 cells.

M β CD was obtained from Sigma-Aldrich Co. (St. Louis, MO) (#C4555-5G) and prepared using sterile water. S2 cells were plated at a concentration of 1×10^6 cells per ml in 6-well plates. M β CD was added to the cells at a final concentration of 2.5 mM for approximately 1.5 hours prior to infection with *E. chaffeensis*. Following infection, RNA was extracted from the cells at 24, 48, and 72 hpi and qRT-PCR was used to measure transcript levels of *Ehrlichia* 16S rRNA and *Drosophila* ribosomal protein *15a*, as described earlier. Infections were compared between S2 cells that received the M β CD treatment and were then infected with *Ehrlichia* to those that were treated with sterile water and then infected with *Ehrlichia*. Uninfected S2 cells were used as negative controls.

Statistics.

Data are presented as the means \pm standard errors of the means (SEM) or standard deviation (SD) of independent experiments. SEM was used when the means of 3 or more independent experiments were being compared. SD was used when less than 3 independent replicate experimental values were being compared. Differences in means and replicates were determined by using Students's *t* test (two tailed, general) (StatMost statistical package; Data XIOM, Los Angeles, CA). Survival data were analyzed for significance using the log rank test of Kaplan-

Meier plots using Prism Graphpad software (La Jolla, CA). *P* values of <0.05 were considered highly significant.

RESULTS

Microarray data analysis reveals “permissive-exclusive” genes.

We chose to use microarrays to identify *Drosophila* host genes that have a role during *E. chaffeensis* infections. We tested four different conditions of S2 cells using the microarray at both 24 and 96 hpi: (1) uninfected S2 cells; (2) S2 cells activated with LPS; (3) S2 cells activated with LPS and then infected with *E. chaffeensis*; and (4) S2 cells infected with *E. chaffeensis*. Uninfected cells were used to discern what the basal transcript levels were in the S2 cells and comparisons across the different conditions were based on this basal level of expression. In order to understand which gene transcripts were specific to our “non-permissive” S2 cells, we used the S2 cells that were treated only with LPS as a comparison. Cells that received only LPS treatment revealed activation specific gene transcripts. Comparing those genes to the genes that had increased transcript levels in our cells infected under conditions that were “non-permissive” for bacterial growth allowed us to deduce which genes up-regulated transcripts exclusive to non-permissive conditions. Finally, by utilizing the “permissive” and “non-permissive” infection conditions generated in S2 cells, we were able to determine which gene transcripts were exclusively up-regulated during conditions that were “permissive” for the growth of *Ehrlichia*. The genes in which transcript levels were up-regulated 1.5 fold or higher compared to uninfected cells in both “permissive” and “non-permissive” conditions were compared. We then focused on the genes that had transcript levels up-regulated only in the “permissive” subset of genes. We hypothesized that these genes would have some type of role in

the ability of *E. chaffeensis* to replicate in *Drosophila* S2 cells. Although we assessed transcript levels 24 and 96 hours post infection, we limited this analysis to just the 24 hour time point. As described in the material and methods, we focused on genes that had transcript levels 1.5 fold higher or above compared to uninfected cells. 2227 gene transcripts were up-regulated in S2 cells infected under “permissive” conditions and 1811 gene transcripts were up-regulated in S2 cells infected under “non-permissive” conditions. No genes were identified that had lower transcript levels when S2 cells were infected under either permissive or non-permissive conditions. Among the 2,227 genes with up-regulated transcript levels in permissive conditions, 2,128 of the genes were 1.5 fold higher than uninfected controls. Of the 1,811 genes that had increased transcript levels, 1,742 of the genes were 1.5 fold higher than uninfected S2 cells. When we compared genes that were up-regulated during permissive growth conditions compared to genes that were up-regulated under non-permissive growth conditions, we identified 517 genes that had higher transcript levels exclusively during permissive conditions. Of these 517 genes, 210 of them had previously been ascribed some function and had some characterization. However, 317 of the genes had yet to be defined and had “CG” gene designations. Among the 517 genes, we determined that viable, fertile, adult mutant stocks were available for 118 of the genes (37 stocks for defined genes and 81 stocks for undefined genes). These flies had appropriate mutations in coding exons in the genes of interest, and allowed us to test whether the absence of a functional gene would affect fly survival and/or bacterial replication *in vivo*.

***Ehrlichia chaffeensis* infection of selected mutant *Drosophila*.**

We screened 15 *Drosophila* lines with mutations in the gene of interest (Table 4.1) for fly survival and bacterial replication. Flies (WT or mutant) were injected with cell-free *E. chaffeensis* or sterile PBS and monitored for survival for 96-120 hpi (20 flies per treatment group

per experiment; results averaged from 3 independent experiments). We were interested in the mutants that displayed increased survival after bacterial challenge compared to the wild-type flies. These mutants included stocks 20231 (gene *CG6364*, FBgn0039179), 18540 (gene *CG3044* (*CHT11*), FBgn0029913), 13981 (gene *separation anxiety*, FBgn0024188), 15228 (gene *CG6479*, FBgn0036710), and 20026 (gene *CG6543*, FBgn0033879) (Figure 1). Stocks 20231, 18540, 13981 and 20026 were all created by transposable element insertion. The sequence location of the insertions for each stock was: (1) 20231 (3R:20,115,939); (2) 18540 (X:6,742,691); (3) 13981 (2R:7,248,253); and (4) 20026 (2R:9,741,819). Stock 15228 has been discontinued since the start of our experiments and our own stock of these flies became ill and died. Our hypothesis is that the gene affected in the mutants allowed increased survival because its expression was needed for successful replication of *Ehrlichia*. We found that *CG3044*, *san*, *CG6364*, *CG6479*, and *CG6543* all displayed significantly increased survival compared to WT flies after infection with *E. chaffeensis* (Figure 4.1; panels A-E respectively). Moreover, knock-out/down-regulation of the gene in these mutants did not allow for bacterial replication as measured by qRT-PCR (Figure 4.2).

Stock Number	Stock Genotype	Associated Gene
1124	al[1] Gpdh[n1-4]/SM1	<i>Glycerol 3 phosphate dehydrogenase</i>
1125	al[1] Gpdh[n0]/SM1	<i>Glycerol 3 phosphate dehydrogenase</i>
2402	al[1] Gpdh[n5-4]/SM1	<i>Glycerol 3 phosphate dehydrogenase</i>
14560	y[1] w[67c23]; P {y[+mDint2] w[BR.E.BR]=SUPor- P}CG10672[KG07864] ry[506]	CG10672
18615	w[1118]; PBac {w[+mC]=WH} CG4743[f03065]	CG4743
20231	y[1] w[67c23]; P {w[+mC] y[+mDint2]=EPgy2} CG6364[EY10942]	CG6364
18540	w[1118] PBac {w[+mC]=WH} Cht11[f02328]	Cht11
14880	y[1]; P {y[+mDint2] w[BR.E.BR]=SUPor- P} Map60[KG00506]/SM6a; ry[506]	Map60
13981	y[1]; P {y[+mDint2] w[BR.E.BR]=SUPor- P} san[KG04816]/CyO; ry[506]	san
24723	w[1118]; Mi {ET1} CG9300[MB04640]	CG9300
15228	NO LONGER AVAILABLE	NO LONGER AVAILABLE
18141	PBac {w[+mC]=RB} Tsp3A[e03287] w[1118]	Tsp3A
15434	y[1] w[67c23] P {w[+mC] y[+mDint2]=EPgy2} CG10992[EY03339]	CG10992
15676	y[1] w[67c23]; P {w[+mC] y[+mDint2]=EPgy2} Gap69C[EY03771]	Gap69C
20026	y[1] w[67c23]; P {w[+mC] y[+mDint2]=EPgy2} CG6543[EY08499]	CG6543

Table 4.1 Stock numbers (Bloomington Stock Center; Bloomington, IN), genotypes, and associated genes of viable, fertile, adult *Drosophila* screened by microinjection.

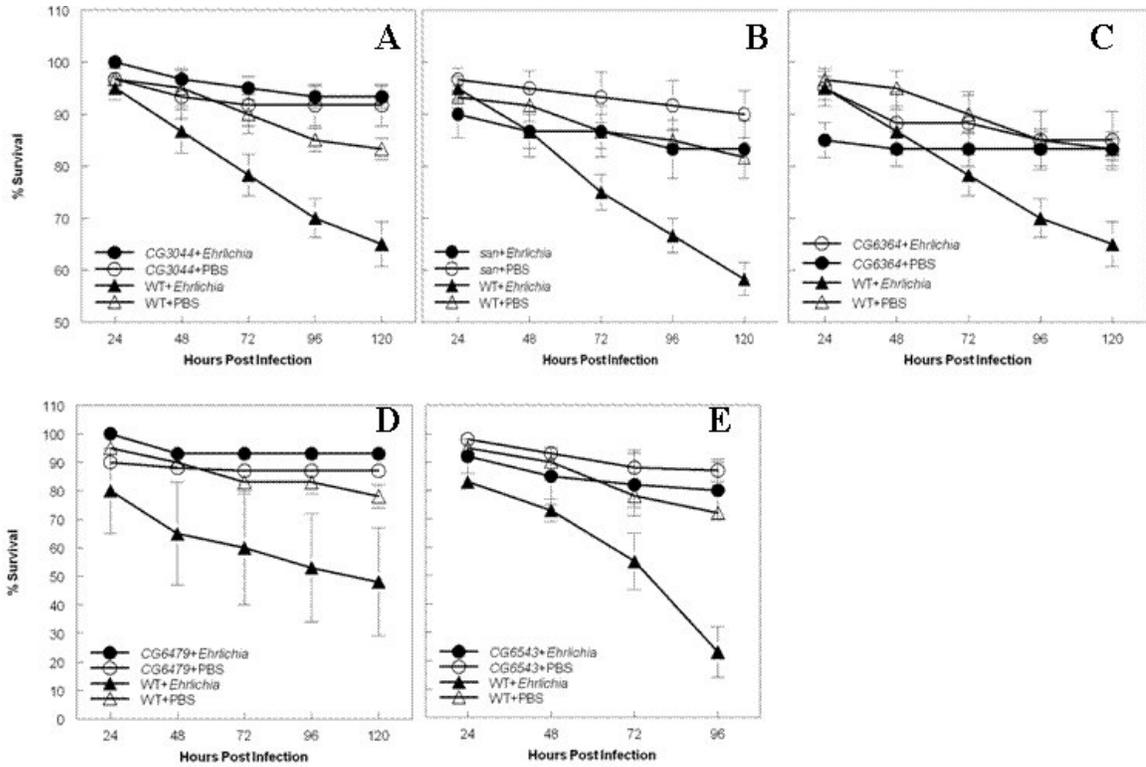


Figure 4.1 Comparison of survival of adult wild-type to mutant flies challenged with *E. chaffeensis*. Flies were injected with PBS or cell free *E. chaffeensis*. Data presented represent the mean \pm SEM of 3 independent experiments. 20 flies were injected per treatment group per experiment.

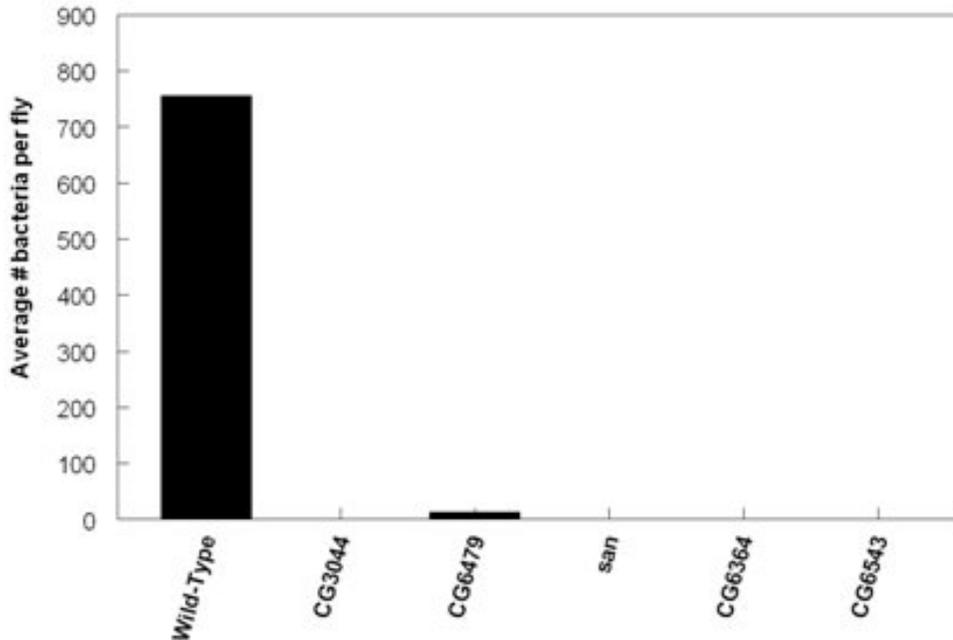


Figure 4.2 Average number of *Ehrlichiae* present in mutant flies (*CG3044*, *CG6479*, *san*, *CG6364*, and *CG6543*) compared to wild-type flies at 120 (*CG3044*, *CG6479*, *san*, *CG6364*) or 96 hours post infection (*CG6543*). Gene name on the X-axis designates flies with a mutation in that gene.

Uridine/Cytidine Kinase mutations affect fly survival and bacterial replication.

To better understand the role of the genes identified in our screen, we examined the gene *CG6364*. The molecular function of this gene is predicted to be uridine/cytidine kinase activity (putatively uridine 5'-phosphotransferase, E.c. 2.7.1.48) (15). Disruption of *CG6364* from an insertion at the sequence location 3R:20,115,939 resulted in poor bacterial replication *in vivo* (Figure 4.1; panel C). To confirm the impact of the uridine/cytidine kinase on *E. chaffeensis* growth, we obtained the mutant *Drosophila* stocks *dumpy* (FBgn0053196), *tilt* (FBgn0003868), and *withered* (FBgn0004012). All of these flies have been previously reported to carry mutation(s) which affect uridine/cytidine kinase function (50). Each stock was infected with *E. chaffeensis* and assessed for bacterial replication as described in the materials and methods. All

three fly lines were significantly more resistant to *E. chaffeensis* challenge than WT flies (Figure 4.3). For instance, at 96 hpi 61% of the *dumpy* flies were alive compared to 42% of WT flies; 66% of *withered* flies compared to 38% of WT flies; and 60% of *tilt* flies compared to 46% of WT flies. The increased survival of the mutants was accompanied by a decrease in the replication of the *Ehrlichial* organisms in these flies, as measured by qRT-PCR. At 96 hpi, WT flies contained an average of 575 *E. chaffeensis* organisms. Comparatively, *withered*, *tilt*, and *dumpy* mutants respectively contained an average of 27, 248, and 8 bacteria, respectively (Figure 4.4).

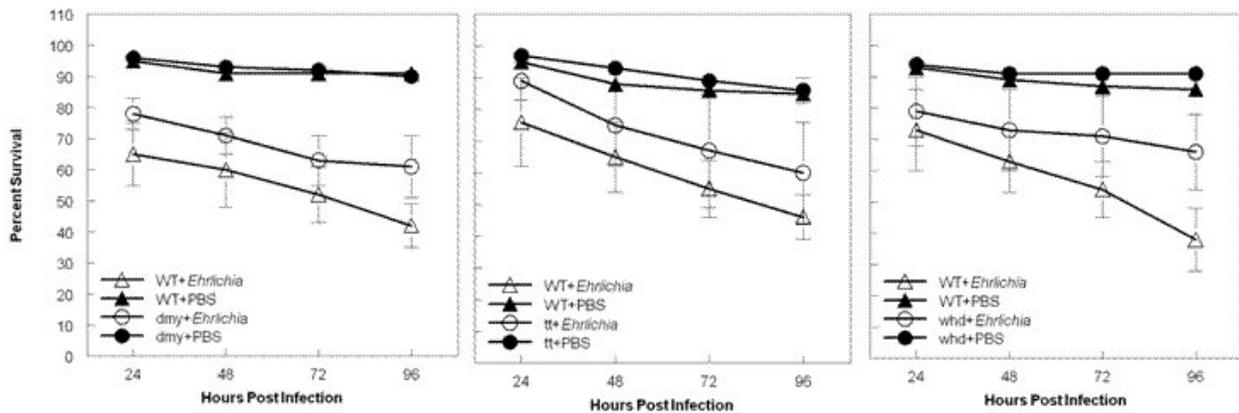


Figure 4.3 Comparison of survival of adult wild-type (WT) to mutant flies (*dmy=dumpy*; *tt=tilt*; *whd=withered*) challenged with *E. chaffeensis*. Flies were injected with PBS or cell free *E. chaffeensis*. Data presented represent the mean \pm SEM of 4-5 independent experiments. 20 flies were injected per treatment group per experiment.

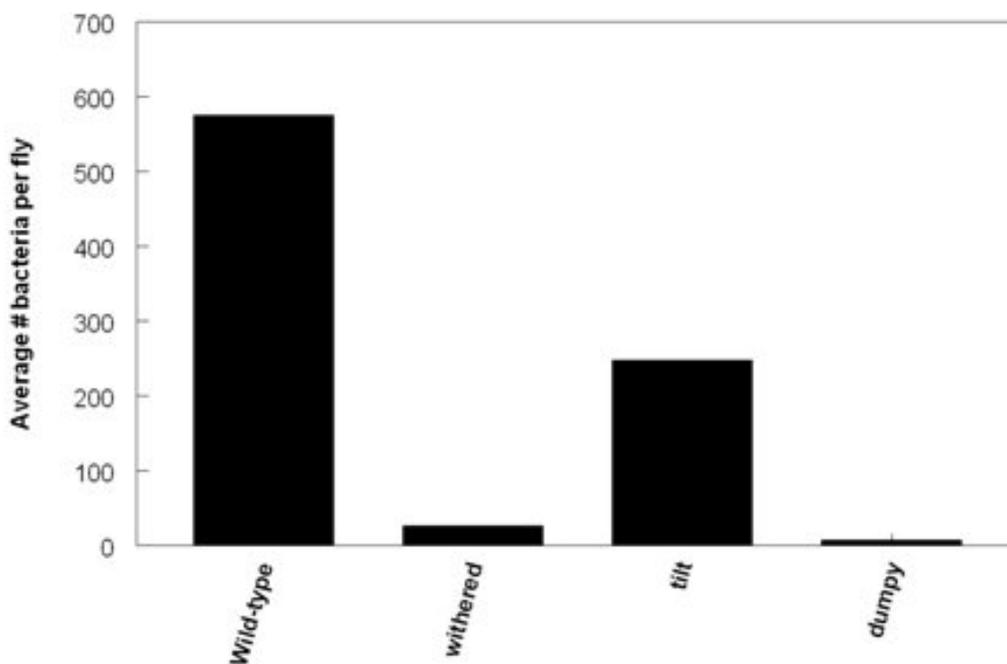


Figure 4.4 Average number of *Ehrlichiae* present in mutant flies compared to wild-type flies at 96 hours post infection. Data presented represent the mean of 3 independent experiments.

CPEC treatment increases *E. chaffeensis* infection.

The uridine/cytidine kinase enzyme functions in pyrimidine synthesis pathways, being specifically involved in the conversion of uridine to uridine monophosphate and/or cytidine to cytidine monophosphate and *vice versa* (46, 52, 53). Deoxycytidine triphosphate can be synthesized from glutamine through the de novo pathway (Figure 4.5). Alternatively, deoxycytidine triphosphate can be synthesized through the salvage pathway, which is dependent upon the uridine/cytidine kinase to convert cytidine or uridine to cytidine monophosphate or uridine monophosphate, respectively (Figure 4.5) (46, 52, 53). We obtained the drug CPEC, which inhibits the conversion of [³H]-UTP to [³H]-CTP (26). Therefore it is an inhibitor of cytidine triphosphate synthetase (CTP) (38, 46, 52) (Figure 4.5). Consequently this drug effectively inhibits de novo synthesis of pyrimidines, leaving only the salvage pathway with

cytidine as the substrate for pyrimidine synthesis. To explore the impact of CPEC on *E. chaffeensis* growth, S2 cells were treated with CPEC for 48 hours at final concentrations of 100, 10, 1, or 0.1 μM . We then infected those cells with *Ehrlichia* for an additional 48 hours. We found a significant increase in bacterial growth in the treated cells compared to cells treated with diluent only (Figure 4.6). Similarly, when S2 cells were first infected with *E. chaffeensis* for 48 hours and then treated with different concentrations of CPEC for 48 hours, we also observed increases in bacterial growth compared to cells treated with diluent only (Figure 4.6). Therefore, the de novo synthesis pathway was not needed for bacterial growth in the S2 cells.

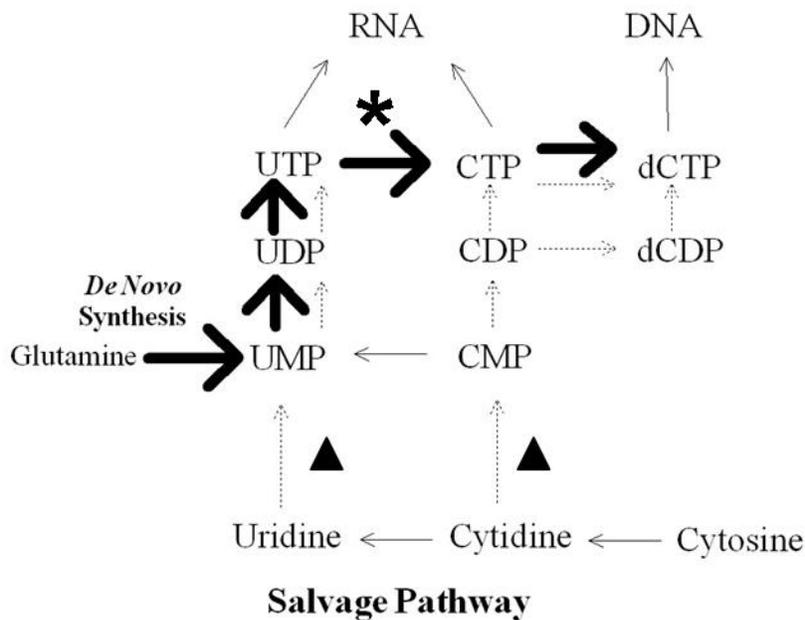


Figure 4.5 *De novo* and salvage pathways for production of dCTP (deoxycytidine triphosphate). *De novo* synthesis through glutamine and UMP (uridine monophosphate) is represented by bold arrows. The salvage pathways through uridine or cytidine are represented by thin/hatched arrows. The star (*) represents the enzyme CTP synthetase; and the black triangles represent uridine/cytidine kinase.

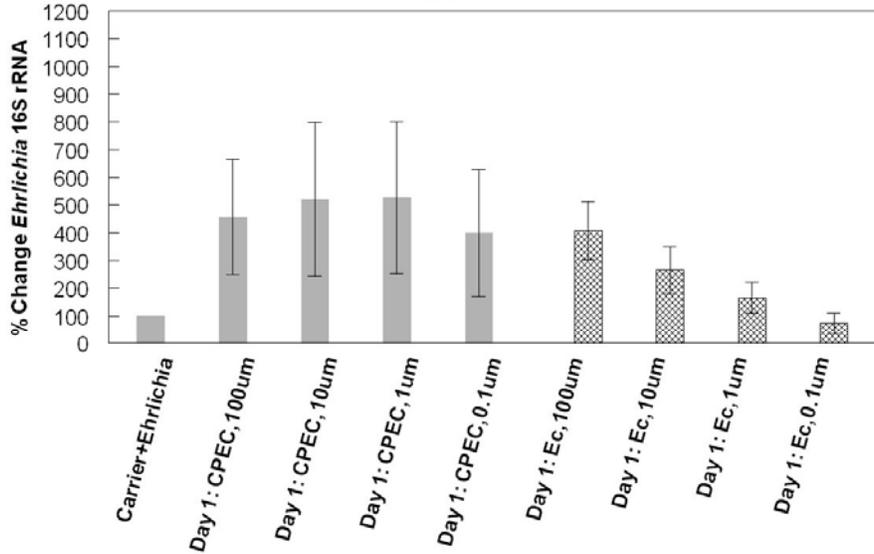


Figure 4.6 Percent change in *Ehrlichia* 16S rRNA copies in S2 cells treated with CPEC compared to S2 cells treated with carrier only, then infected with *Ehrlichia*. Two treatment schemes are represented in the figure. For the samples labeled “Day 1: CPEC”, S2 cells were first treated with CPEC (at indicated concentration) for 48 hours, and then infected with *E. chaffeensis* for an additional 48 hours. For the samples labeled “Day 1: Ec”, S2 cells were first infected with *E. chaffeensis* for 48 hours, and then treated with CPEC (at indicated concentration) for 48 hours. RNA extraction was performed following both treatment schemes. Data presented represent the mean \pm SEM of 3 independent experiments.

Cytosine treatment increases *E. chaffeensis* infection.

Since cytosine combines with ribose to form cytidine in the salvage pathway (Figure 4.5), we supplemented S2 cell culture medium for 24 hours with 25 mM of cytosine in order to determine if it was an important substrate for bacterial growth. The S2 cells were then infected with cell-free *E. chaffeensis* and bacterial replication was assessed at 24, 48, 72, and 96 hpi (Figure 4.7). The cytosine-treated, infected cells contained an average of 456, 1423, 3352, and 2465 percent

more copies of *Ehrlichia* 16S rRNA at 24, 48, 72, and 96 hpi compared to infected cells treated with diluent only. Taken together with the CPEC experiments, these data support the hypothesis that utilization of cytidine through the salvage pathway occurs during the replication of *E. chaffeensis*.

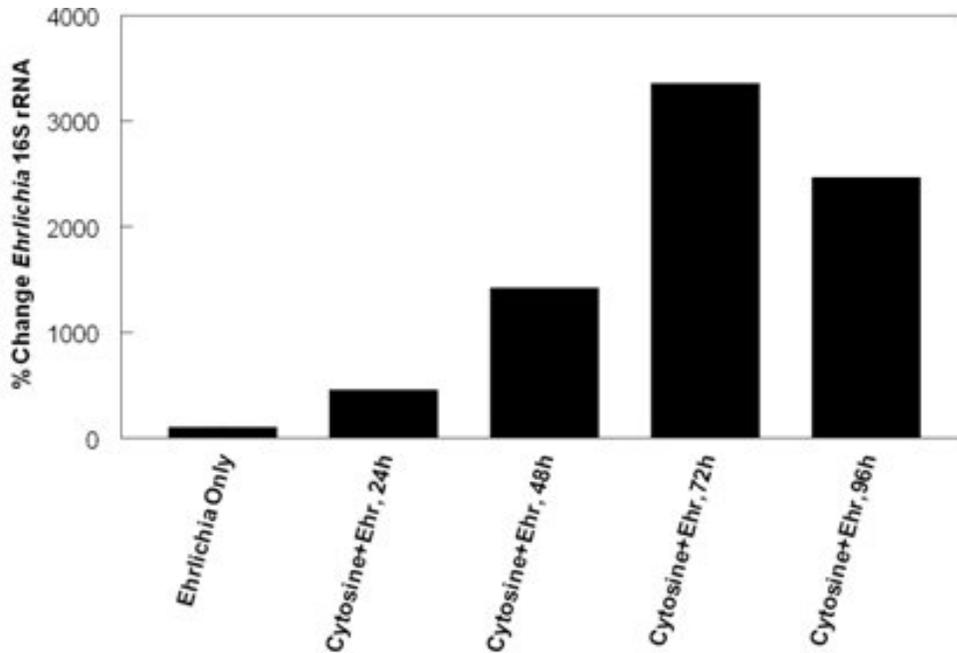


Figure 4.7 Percent change in *Ehrlichia* 16S rRNA copies in S2 cells treated with cytosine and infected with *Ehrlichia* compared to S2 cells treated with carrier only and infected with *Ehrlichia*. Data presented represent the mean of 3 independent experiments.

M β CD treatment does not affect *Ehrlichia* replication.

It is known that chitin derivatives can lower cholesterol levels (29) and that increased levels of cholesterol enhance *Anaplasma phagocytophilum* infection in mice (57). *CG3044* (*Cht11*) was described as having potential chitinase activity (18). We hypothesized that the up-regulation of chitinase could be a subversive virulence mechanism that the bacteria use to control cholesterol

levels through the control of chitin. To test this hypothesis, we treated S2 cells with the cholesterol-sequestering agent, M β CD (27, 28, 40), and then infected them with *E. chaffeensis* (Figure 4.8). No difference in bacterial load was observed between the infected cells that received M β CD and those that did not.

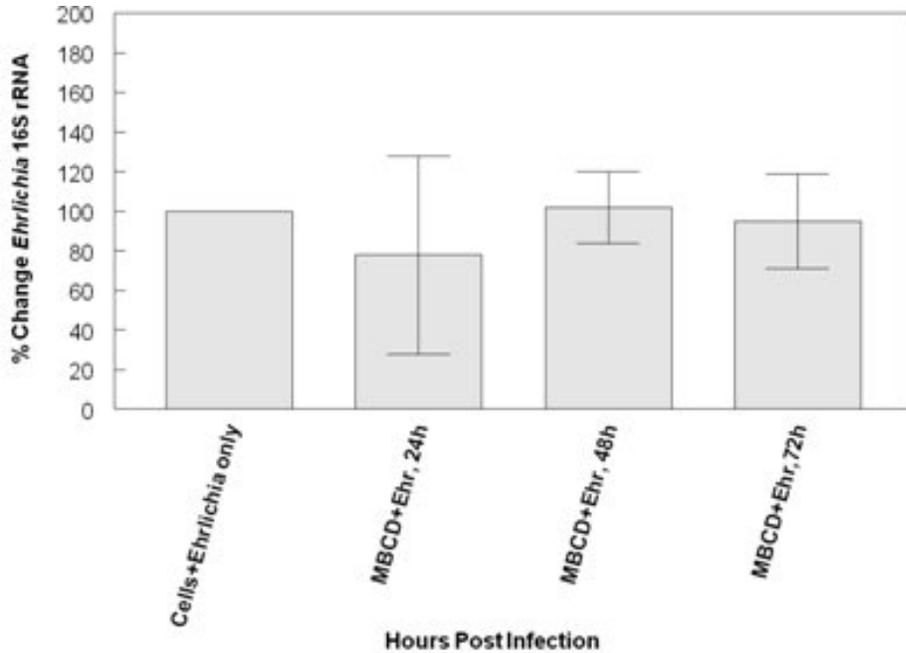


Figure 4.8 Percent change in *Ehrlichia* 16S rRNA copies in S2 cells treated with M β CD and infected with *Ehrlichia* compared to S2 cells treated with carrier only and infected with *Ehrlichia*. Data presented represent the mean \pm SD of 2 independent experiments.

DISCUSSION

We have successfully demonstrated the power of the *Drosophila* system for elucidating host factors that are important for the replication of *E. chaffeensis*. We combined microarray analysis and mutant screening to successfully identify five genes that contribute to the replication

of *E. chaffeensis* *in vivo*. The functions of the identified genes are diverse and include: (1) phagocytosis/engulfment (*CG6479*); (2) fatty acid binding/mitotic sister chromatid binding (*san*); (3) chitinase activity (*CG3044 (Cht11)*); (4) uridine/cytidine kinase activity (*CG6364*); and (5) enoyl –CoA hydratase activity (*CG6543*). To our knowledge, this is the first demonstration of host genes that control *Ehrlichia* replication.

Our data suggest that bacteria require cytosine or cytidine as a substrate to make deoxycytidine triphosphate and DNA. This hypothesis is supported by data showing that flies with mutations in *CG6364* were significantly less susceptible to *Ehrlichia* challenge compared to WT flies. In addition, blocking of the synthesis of deoxycytidine triphosphate with the CTP synthetase inhibitor, CPEC, resulted in increased replication of *E. chaffeensis* in S2 cells. This suggested that the bacteria do not require the host to use glutamine as a substrate during replication. Supplementation of cytosine to cells actively replicating bacteria also enhanced *Ehrlichia* replication and further implicates the salvage pathway through cytidine.

Stroman found that *tilt* and *withered* larvae accumulate uridine when supplemented with uracil (50), which is toxic to the *Drosophila*. However, uridine monophosphate was not toxic. Those data suggested that these mutants had a defect in the pathway that converted uracil to uridine monophosphate (50). Indeed, *tilt* and *withered* mutants were more resistant to infection by *E. chaffeensis* than WT flies. *dumpy* mutants also had apparent mutations in pyrimidine synthesis (50) and these flies were also more resistant to infection than our WT flies. While the *dumpy* mutation has been annotated, the *tilt* and *withered* mutations have not been characterized. We have attempted to approximate the cytogenetic locations of *tilt* and *withered*. Interestingly, all of these genes map to different loci from *CG6364*. *CG6364* maps cytogenetically to chromosome 3 at 95F10 (15) and *dumpy* maps to chromosome 2 between 24F4-25A1 (19).

Based on recombination maps (6) and the annotation by Lindsley and Zimm (33), we estimate that *withered* maps to chromosome 2 between 46-47 (22), and *tilt* maps to chromosome 3 between 40-41 (21). Therefore, additional work will be needed to characterize where in the pathway of host pyrimidine synthesis each of these genes function to regulate *E. chaffeensis* replication.

It is known that depletion of the cytidine pools can disrupt the balance of ribonucleotides in cells, leading to alterations in cellular homeostasis and apoptosis (46). Therefore, it is possible that the sequestering of cytidine by *Ehrlichia* affects the cell, making it more vulnerable to infection by the bacteria. Because cytidine is the least abundant nucleoside in cells (31), its use by the *Ehrlichia* may be particularly stressful on the host cell processes. In that case, the bacteria would be hijacking the normal cellular pyrimidine/purine metabolism, which may alter host cell defenses and make the environment more amenable to their own replication. *Ehrlichia* disseminate by lysing host cells in order to spread to uninfected cells (41), and the up-regulation of apoptotic genes during *Ehrlichia* infections has been observed (59). Therefore, a correlation between cell lysis, apoptosis and cytidine utilization by the bacteria is a possibility.

The predicted molecular function of *CG3044* is chitinase activity (18). *CG3044* (*Cht11*) may be related to the *Chitinase 11* gene of *Tribolium castaneum*, which has been assigned to a separate classification group (group VII) from other identified chitinases (3), (personal communication, Dr. Subbaratnam Muthukrishnan, Kansas State University). This indicated that *CG3044* may have a function other than those classically described for chitinases. Chitins and their derivatives regulate cholesterol when used as diet supplements (29, 58). Mice with high blood cholesterol have more severe *Anaplasma phagocytophilum* infections in their blood, livers, and spleens (57). *E. chaffeensis* and *A. phagocytophilum* (a Rickettsial pathogen closely related

to *E. chaffeensis*) cannot synthesize cholesterol and scavenge it from host cells during infections (32). Therefore, chitinase *CG3044* (*Cht11*) may function to regulate cholesterol levels to facilitate infections. Alternatively, chitin has been found to activate macrophages as measured by the phosphorylation of p38 and Erk (Figure 4.9) (39). It is possible that chitinase is involved in regulating cellular activation. Incubation of S2 cells with LPS activates MAPKinase (Von Ohlen et al., Kansas State University, unpublished observations) and makes the cells non-permissive to infection by *E. chaffeensis* (34). Since M β CD did not affect *E. chaffeensis* infection in S2 cells, and because M β CD inhibits the ability of cholesterol to activate MAPK (Figure 4.9) (39), it might be that the chitinase role could be to regulate activation of the parasitized cells (Figure 4.9). Reese et al. (2007) have shown that chitin can regulate inflammation and the activation of Th1 versus Th2 responses (44). Therefore, these data support this hypothesis. However, additional experiments will be needed to confirm that regulation of chitinase affects cellular activation and *E. chaffeensis* replication.

In conclusion, we have successfully used *Drosophila* to identify host genes that are involved in *E. chaffeensis* infections. Among those genes, we further tested the roles of *CG6364* and *CG3044* in the replication of *E. chaffeensis*. The human homolog of *CG6364* is uridine cytidine kinase 2 (*UCK2*) and is located on Chromosome 1 (12, 15). It functions in the same manner as *CG6364*, acting to phosphorylate uridine and/or cytidine (51). *UCK2* has been found to be more active in certain types of cancers (48), and also acts to phosphorylate nucleoside analog drugs used for treatment of cancers and hepatitis C virus (24, 51). A human homolog of *CG3044* has not been identified, but homologs have been identified in deer ticks and body lice (18). Both of these species serve as vectors of different *Rickettsial* bacteria (4), so it would be interesting to better understand the function of this host chitinase in those organisms. The human

homologs of the genes that we did not investigate include human N(alpha)-acetyltransferase 50, NatE catalytic subunit for *Drosophila* san (13, 20); human enoyl Coenzyme A hydratase for CG6543 (11, 17); and human transmembrane protein 209 for CG6479 (14, 16). More work will be necessary to better define the distinct roles and biochemistry of the processes and pathways regulated by these genes during *E. chaffeensis* infections. Nonetheless, this work provides a starting point for those experiments. Most importantly, the translation of these genes to mammalian systems will hopefully contribute to development of therapies, vaccines, and/or immunodiagnostics that can be used in the treatment, prevention, and diagnosis of *E. chaffeensis*/HME.

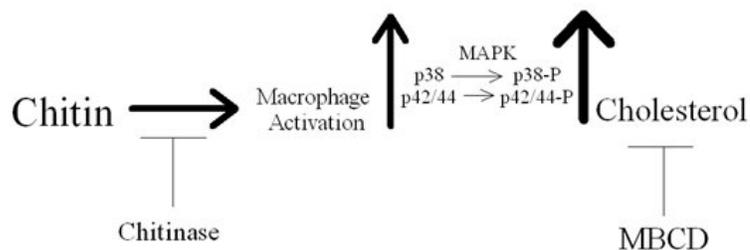


Figure 4.9 Role of host chitinase in *E. chaffeensis* infections. MAPK levels increase as a result of activation of macrophages by chitin, which is enhanced by cholesterol exposure. Chitinase putatively stops the activation of macrophages by degrading the chitin agonist, making them more permissible to infection. MβCD sequesters cholesterol, putatively lowering MAPK activation and making the environment less hostile to bacteria.

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CHAPTER 5 - Conclusion

The research accomplished for this dissertation effectively demonstrates that *Drosophila melanogaster* can be used for studying *Ehrlichia chaffeensis* infections. *E. chaffeensis* is an emerging, tick-borne pathogen that causes human monocytic ehrlichiosis (HME). Reports of the disease are on the rise and a 44% increase in reported HME cases was observed in just one year (2006-2007) (13). Moreover, the reports of HME have increased 159% overall since the year 2003 (13). The increased cases of HME may be the result of growing vector populations, increases in human contact with tick populations, and/or heightened surveillance and detection techniques. In any case, a model system for studying *E. chaffeensis* infections will be most helpful in better understanding the pathogenesis and resulting disease of these bacteria.

We have successfully used *D. melanogaster* as a model to determine host genes that are necessary for *E. chaffeensis* pathogenesis. The importance of better understanding tick-borne illness should not be underestimated. *E. chaffeensis* belongs to the Rickettsial group of diseases. It was recently shown that 2% of imported fevers are the result of infection with Rickettsial organisms and 20% of these fevers require hospitalization (14). The ticks and lice that vector these diseases have wide ranging habitats and are adapted to living in ever-changing conditions. The tick vector of *E. chaffeensis* is *Amblyomma americanum*, it has been expanding its range for the past 30 years, and has been reported as far northeast as Maine (15). In addition, Rickettsial diseases have a history of being considered for use as bioterrorism agents. In fact *Rickettsia prowazekii* and *Coxiella burnetii* have been tested as weapons by the former Soviet Union, the United States, and Japan (2). The consequential increasing incidence of disease, spread of disease vectors, and potential for the creation of weapons of mass destruction warrants careful study of these organisms.

To our knowledge, our description of host genes that contribute to the replication of *E. chaffeensis* is the first to be provided. Most importantly, these genes can be compared to their mammalian homologs. For instance, the human homologs of the genes we have pinpointed in playing a role in *E. chaffeensis* replication include human N(alpha)-acetyltransferase 50, NatE catalytic subunit for *Drosophila* san (5, 11); human enoyl Coenzyme A hydratase for CG6543 (3, 9); human transmembrane protein 209 for CG6479 (6, 8); and human uridine cytidine kinase 2 (UCK2) for CG6364 (4, 7). No human homolog exists for CG3044. As a next step, it would be interesting to determine the role of these genes during infections in a mammalian host or even in the tick vector. Experiments that utilize RNAi based silencing would ultimately confirm the role of the corresponding homologs during *Ehrlichia* infections in mammalian or tick systems. Our contributions to the body of knowledge surrounding *Ehrlichial* and *Rickettsial* diseases should ultimately lead to better characterization of the genes involved in infections in the mammalian host(s). These are the types of studies that will be necessary to contribute to the development of highly effective treatments, diagnostic procedures, and initial vaccines.

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