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Ehrlichia chaffeensis replication sites in adult *Drosophila melanogaster*

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Abstract

Ehrlichia chaffeensis is a Gram-negative, obligate intracellular bacterium which causes the tick-borne disease human monocytic ehrlichiosis. In vertebrates, *E. chaffeensis* replicates in monocytes and macrophages. However, no clear cell or tissue tropism has been defined in arthropods. Our group identified two host genes that control *E. chaffeensis* replication and infection in vivo in *Drosophila*, *Uridine cytidine kinase* and *separation anxiety*. Using the *UAS-GAL4* RNAi system, we generated F1 flies (*UAS-gene of interest RNAi* x *tissue-GAL4* flies) that have *Uck2* or *san* silenced in ubiquitous or tissue-specific fashion. When *Uck2* or *san* were suppressed in the hemocytes or in the fat body, *E. chaffeensis* replicated poorly and caused significantly less severe infections. Silencing of these genes in the eyes, wings, or the salivary glands did not impact fly susceptibility or bacterial replication. Our data suggest that in *Drosophila*, *E. chaffeensis* replicates within the hemocytes, the insect homolog of mammalian macrophages, and in the fat body, the liver homolog of mammals.

Keywords

Hemocytes; RNAi; Host-bacteria interactions; Intracellular bacteria

Introduction

Human monocytic ehrlichiosis (HME) is a tick-borne, zoonotic disease caused by the Gram-negative, obligate intracellular bacterium, *Ehrlichia chaffeensis*. *E. chaffeensis* is primarily vectored by *Amblyomma americanum* (lone star tick) (Anderson et al., 1993) and is transmitted transstadially in ticks (Parola et al., 2005). *A. americanum* has a 3-host life cycle. For progression from one stage to the next (larva to nymph to adult), the tick requires a vertebrate blood meal (Parola et al., 2005). The major natural reservoir of *E. chaffeensis* in the United States is white-tailed deer (*Odocoileus virginianus*) (Dawson et al., 1994; Lockhart et al., 1997). However, several other vertebrates, too, act as reservoirs including domestic dog (Yu et al., 2008), domestic goat (Dugan et al., 2000), white-footed mouse (Magnarelli et al., 1997), red fox (Davidson et al., 1999), raccoon (Dugan et al., 2005), and coyote (Kocan et al., 2000). Humans can also become accidental hosts when bitten by ticks.

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In humans, the bacteria are monocytotropic, meaning they are primarily found in monocytes and macrophages, and there is a good understanding of where the bacteria replicate (Sotomayor et al., 2001). Although it is clear that ticks can transmit *Ehrlichia* organisms to vertebrate hosts (Ewing et al., 1995; Varela-Stokes, 2007), and *Ehrlichia* bacteria have been detected in the salivary glands microscopically (Smith et al., 1976) and by PCR (Karim et al., 2012), it is less clear about bacteria replication in ticks. Although genetic tools for working with ticks are currently being developed (Pagel Van Zee et al., 2007), the available tools do not begin to approach those that are available in other arthropods such as *Drosophila melanogaster*.

In adult *D. melanogaster*, hemocytes contribute to host immune defenses against *E. chaffeensis* (Luce-Fedrow et al., 2009). In vertebrates, monocytes and macrophages contribute to host resistance (Chapes and Ganta, 2008; Ganta et al., 2002) even though the bacteria are monocytotropic. Our group has discovered that *D. melanogaster* genes, *separation anxiety* (*san*) and *Uridine cytidine kinase 2* (*Uck2*), are required for *E. chaffeensis* infections in flies (Von Ohlen et al., 2012). Flies carrying mutations in the coding regions of these genes do not support infection after needle injection (Von Ohlen et al., 2012). Therefore, to see if bacterial replication in arthropods parallels bacterial replication in vertebrates after needle injection, we took advantage of *Drosophila UAS-GAL4* RNAi system (Dietzl et al., 2007) that allowed for tissue-specific silencing of *san* and *Uck2* to determine host sites of bacterial replication. Here, we show that similar to vertebrate hosts, in *Drosophila* the bacteria require optimal host conditions (i.e. expression of *Uck2* or *san*) in the immune tissues, hemocytes, and fat body, for optimal replication.

Materials and methods

Cell lines and *E. chaffeensis* infections

E. chaffeensis (Arkansas isolate) was propagated in DH82 cells (American Type Culture collection, #CRL-10389, Rockville, Md.). The DH82 cells were grown in Eagle's minimal essential medium supplemented with 3.5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 3.5% NuSerum (BD, Franklin Lakes, NJ), and Glutamine plus (2 mM, Atlanta Biologicals) ($EMEM_7$). Cells were grown at 37°C in an 8% CO_2/92% air atmosphere. The level of infection was determined by examining cyto-centrifuged cells stained with Dif-Quik stain (Fisher Scientific Company, Kalamazoo, MI). When more than 80% of cells were infected, the cells were removed with a cell scraper and frozen at −80°C in cryogenic vials. Bacteria numbers were quantified using a TaqMan-based quantitative reverse transcriptase PCR (qRT-PCR) assay as described below. Purification of host cell-free bacteria was carried out as follows. Cells were scraped from tissue culture dishes. The recovered infected cells were placed in a 50-ml, sterile centrifuge tube and shaken with glass beads. The preparation was centrifuged at 600 × g for 20 min. The supernatant with host cell-free bacteria was transferred into a sterile tube and centrifuged at 15,000 × g for 20 min. The bacterial pellet was resuspended in sterile phosphate-buffered saline (PBS) mixed with blue food dye at a ratio of 0.6 ml dye for every 1 ml of PBS to help us visualize the inoculum during fly injections. Most flies were injected with 6000 bacteria per fly, however, some early experiments were done with 1500 bacteria per fly. Although the severity of infections was different with the different inocula, the experimental trends were similar.

D. melanogaster

D. melanogaster flies were raised on standard dextrose/molasses/yeast medium at 18–29°C. The following fly lines were used: *yellow white* (*yw*) and *arm-GAL4*, *Hml-GAL4*, *YP1-GAL4*, *MS1096-GAL4*, *Gmr-GAL4*, *Fhk-GAL4*, *UAS-Uck2RNAi* (w[1118]; P{GD2761}v16719)and *UAS-sanRNAi* (w[1118]; P{GD7580}v31742). The *yw* fly line

was maintained at Kansas State University and used as a wild type in these experiments (Von Ohlen et al., 2009). The *armadillo* (*arm*)-*Gal* fly line (Sanson et al., 1996) was obtained from Joan Hooper (University of Colorado, Anchutz Medical campus, Denver, CO). The *Fhk-GAL4* and *YP1-GAL4* were obtained from Tony Ip (The University of Massachusetts Medical School, Worcester, MA). The *Hml-GAL4* fly line was obtained from Michael Galko (MD Anderson Cancer Center, Houston, TX). The *MS1096* and *Gmr-GAL4* fly lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, Bloomington, IN. *UAS*-dsRNA transgenic fly lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). Five to seven virgin females carrying the *GAL4* promoter were crossed with 3–4 males carrying different *UAS*-dsRNA transgenes to generate F1 flies that had tissue-specific or ubiquitous knockdown of *Uck2* or *san* genes.

RNA extraction

The TriReagent (Molecular Research Center, Cincinnati, OH) RNA extraction method, as was previously described by our group (Luce-Fedrow et al., 2009), was used to extract RNA from flies or host cell-free bacteria. Pelleted bacteria or fly homogenates were resuspended in 1 ml of TriReagent. Preparations were transferred to 2.0 ml, Heavy Phase Lock Gel tubes (5 Prime/Eppendorf, Westbury, New York; #2302830). Three hundred microliters (300 μl) of chloroform were added, and the mixture was vortexed for 15 s. The samples were centrifuged at 12,000 × g for 10 min at 4°C, and the aqueous phase was transferred to clean 1.5 ml tubes. Five hundred microliters (500 μl) of isopropanol were added, and RNA was precipitated at −20°C for 24 h. Samples were subsequently centrifuged at 12,000 × g for 10 min at 4°C. The RNA pellet was washed with 1 ml of 70% ethanol, and samples were centrifuged at 7400 × g for 5 min at 4°C. The 70% ethanol was decanted from the pellet, and residual ethanol was allowed to evaporate for 5 min. RNA was resuspended in 50 μl of nuclease-free water. RNA concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

Quantification of bacterial numbers for infection/injection and in infected flies

RNA from host cell-free *Ehrlichia* was extracted as described above. A TaqMan-based, real-time reverse transcriptase PCR (RT-PCR) was used to quantify bacterial numbers (Sirigireddy and Ganta, 2005). A total of 500–1000 ng of RNA was used for each reaction. Real-time quantitative RT-PCR (qRT-PCR) was performed using the Invitrogen's One-Step Platinum qRT-PCR kit (#11732) in a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). *E. chaffeensis* RNA was detected using primers specific for the 16S ribosomal RNA gene (Sirigireddy and Ganta, 2005) (NCBI Accession # M73222). Custom-synthesized primers and probes were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and were used. The sequence of the primers used were: forward primer, RRG3 (5′ CAATTGCTTATAACCTTTTGGTTATAAAT 3′) and reverse primer, RRG27 (5′ GTATTACCGCGGCTGCTGGCAC3′). Serial 10-fold dilutions of the RNA from infected DH82 cells were used to generate standard curves plotting log number of bacteria versus the corresponding Ct value. The cycling conditions used for the assay were: 48°C for 30 min, 94°C for 4 min, then 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. To quantify bacterial numbers in injected/infected flies, anesthetized flies were transferred to 1.5-ml tubes (Kimble Chase, Vineland, NJ) and crushed with disposable pestles in 1 ml of TriReagent as previously described (Luce-Fedrow et al., 2009). Bacteria numbers were estimated from RNA samples using quantitative RT-PCR (qRT-PCR) as described above. *Drosophila* ribosomal *protein 15a* (NCBI Accession #NM_136772) gene was used as used as housekeeping. The sequence of the primers used were: forward primer (5′ TGGACCACGAGGAGGCTAGG 3′) and reverse primer (5′ GTTGGTGCATGGTCGGTGA 3′) and Taqman probe (5′-56-FAM/

TGGGAGGCAAAATTCTCGGCTTC/36-TAMsp-3′). The cycling conditions used for the assay were: 48°C for 45 min, 94°C for 2 min, and then 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1.5 min.

Estimation of silencing efficiency

To quantify the level of transcriptional silencing in RNAi flies, whole-flies or specific fly tissues were used to probe for specific transcript levels. Fly tissues were transferred to 1.5-ml tubes and crushed with disposable pestles in 1 ml of TriReagent for RNA extraction. Transcript levels of the gene of interest in F1 flies were assessed in the following whole-flies: ubiquitous (UAS-*sanRNAi* X *arm-GAL4* or *UAS-Uck2RNAi* X *arm-GAL4*); hemocytes (*UAS-sanRNAi* X *Hml-GAL4* or *UAS-Uck2RNAi* X *Hml-GAL4*); fat body (*UAS-sanRNAi* X *YP1-GAL4* or *UAS-Uck2RNAi* X *YP1-GAL4*). For wing-specific and eye-specific knockdown, fly heads or wings were used. For fat body-specific and salivary gland-specific knockdown, fat body or salivary glands dissected from wandering third-instar larvae were used. RNA from homogenates was extracted as described above. Transcript levels were determined in RNA samples using qRT-PCR using the Invitrogen's Superscript III Platinum SYBR Green One-Step qRT-PCR kit in. Primers were obtained from IDT. *Drosophila ribosomal protein 15a* was used as used as the housekeeping gene. *Drosophila Uck2* (NCBI Accession #NM_142984) was detected using forward primer (5′ TGTCCATCAGTCAGGACAGC 3′) and reverse primer (5′ CTCCACTTTGTGGCCCTTTA 3′). The cycling conditions used for the assay were 48°C for 30 min, 95°C for 3 min, and then 45 cycles of 95°C for 15 s, 56°C for 30 s, and 60°C for 1 min. The *Drosophila san* gene (NCBI Accession # NM_080040) was detected using forward primer (5′ ACCCGAACAATCAGGAACAG 3′) and reverse primer (5′ ACCCGAACAATCAGGAACAG 3′). The cycling conditions used for the assay were 48°C for 30 min, 95°C for 3 min, and then 45 cycles of 95°C for 15 s, 50°C for 30 s, and 60°C for 1 min.

To calculate knockdown efficiency serial 10-fold dilutions of RNA and corresponding Ct values were used to plot standard curves (mean of 3 experiments). Primer efficiency was calculated using the following equation (Pfaffl, 2001):

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

Primer efficiency values were used to calculate the relative change in gene expression by the following equation (Pfaffl, 2001):

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

Parental lines (*UAS* and *GAL4* constructs) and *yw* flies served as normal controls and were set at 100% expression for the gene of interest. Relative level of gene expression in RNAi flies as compared to controls was calculated to estimate the knockdown efficiency.

Infections

Adult flies were used to assess the effect of gene knockdown on *E. chaffeensis* infections. Male and female flies were anesthetized on a CO_2 anesthesia pad (Genesee Scientific, San Diego, CA; model# 59-119). Flies were injected in the thorax with 51 nl of sterile PBS-blue food dye solution or *Ehrlichia* resuspended in PBS-blue food dye solution. Injections were made in the abdomen of the fly with pulled glass capillary needles using a Nanoject II

(Drummond Scientific Company, Broomall, PA). Following injection, flies were maintained in clean bottles with molasses/yeast caps. Survival was monitored daily for 4 days. Twenty flies were injected per treatment group per experiment with 3–4 independent experiments for survival data. Four to five flies were used to determine bacterial infection.

Statistics

Survival data were analyzed for significance using the log-rank test of Kaplan Meier plots using Prism Graphpad software (La Jolla, CA). Data are presented as the mean ± standard error (SE) of independent experiments unless otherwise stated. *P* values of <0.05 were considered highly significant. Bacterial numbers were analyzed by Student's *t* test (two-tailed, general) by using the StatMost Statistical Package (Data XIOM, Los Angeles, CA, USA). Unless indicated, data are presented as the mean ± standard error (SE) of independent experiments.

Results

Uck2 and *san* are required for in vivo *E. chaffeensis* replication in adult *D. melanogaster*

E. chaffeensis is capable of infecting and completing its life cycle in *Drosophila* S2 cells and adult flies (Luce-Fedrow et al., 2008, 2009). Recent work by our group found that several host genes control the replication of *Ehrlichia* in vivo(Von Ohlen et al., 2012). We found that flies carrying mutations in coding regions of these genes were poorly infected by *E. chaffeensis*. In particular, 2 genes that are relevant to humans and ticks were chosen for this study, *Uck2* and *san. Uck2* functions as a uridine-cytidine kinase and is orthologous to the mammalian *UCK2* gene (McQuilton et al., 2012) and the *Ixodes scapularis* SCW003812 gene (Lawson et al., 2009). *san* is the ortholog to the human *NAA50* gene and encodes the N(α)-acetyltransferase 50 protein. It is orthologous to the *I. scapularis* SCW002560 gene (Lawson et al., 2009).

In vertebrates, *E. chaffeensis* exhibits a tropism for monocytes and macrophages (Paddock and Childs, 2003; Sotomayor et al., 2001). However, no clear requirements for cell or tissue tropism have been defined in ticks or other arthropods. To investigate where *E. chaffeensis* replicates in arthropods, we employed whole-organism in vivo RNAi in *D. melanogaster* to do tissue-specific inactivation of *Uck2* and *san* using a fly collection that consists of expressed RNAi transgenes where the inverted repeats produce a dsRNA hairpin that produces the RNAi effect. These tools allowed us to silence *Uck2* and *san* genes in a tissue-specific manner through the binary *GAL4-UAS* system (Brand and Perrimon, 1993; Dietzl et al., 2007) to determine what tissues must be permissive (via their expression of *Uck2* or *san*) for *E. chaffeensis* replication.

To determine whether *Uck2* and *san* could be efficiently silenced using the *UAS-GAL4* system, we individually crossed transgenic flies carrying inverted repeats of the *Uck2* and *san* genes under the control of *UAS* to flies carrying *armadillo (arm)-GAL4* (ubiquitous *GAL4* insertions) to silence *Uck2* or *san* in the F1 progeny. We consistently averaged 78% or better transcript reduction of *Uck2* or *san* in the RNAi flies in comparison to wild-type flies and parental lines using qRT-PCR (Table 1). When wild-type flies, parental line flies, and *Uck2*- and *san*-RNAi flies were experimentally challenged with *E. chaffeensis* in the abdomen, we observed that there was approximately 48% death in wild-type and parental lines after 96 h. This rate of death was significantly increased compared to control flies injected with PBS or flies which had ubiquitous tissue expression of *Uck2*-or *san*-RNAi (Fig. 1A and 1B) ($P<0.05$, log rank test). When we assessed the effect of infection on RNAi flies where *Uck2* or *san* were knocked down ubiquitously, we detected significantly fewer bacteria at 96 hpi ($P<0.05$, *t*-test) compared to wild-type or parental fly controls (Fig. 2A

and 2B). These experiments confirmed that the *UAS-GAL4* technique could be used to efficiently silence *Uck2* and *san* in adult flies and that *E. chaffeensis* infection in flies was dependent on functional *Uck2* and *san* genes.

E. chaffeensis replication in adult *D. melanogaster*

Ubiquitous tissue silencing of *Uck2* and *san* impaired *E. chaffeensis* infection in adult *D. melanogaster*. Therefore, we screened an array of tissues to determine if *Uck2* or *san* expression were necessary in those tissues for bacterial replication. In vertebrates, *E. chaffeensis* exhibits a tropism for monocytes and macrophages (Paddock and Childs, 2003). Therefore, we hypothesized that *E. chaffeensis* replicates in hemocytes, the insect equivalent to mammalian macrophages. To test this hypothesis, we silenced *Uck2* and *san* in the eyes, wings, hemocytes, fat body, and salivary glands in a tissue-specific manner in adult flies. We used eye-specific (*Gmr-GAL4*), wing-specific (*MS1096-GAL4*), hemocyte-specific (*Hml-GAL4*), fat body-specific (*YP1-Gal*), or salivary gland-specific (*Fhk-GAL4*) *GAL4* fly lines to generate F1 flies. To confirm the tissue-specific knockdown of targeted genes, we dissected whole heads and wings from adult flies to assess eye-specific and wing-specific adult knockdown. We observed an average ≥74% reduction in transcript levels of *Uck2* and *san* in whole heads or wings of F1 transgenic flies using eye- or wing-specific *GAL4* constructs (Table 2). In comparison, the average knockdown of *Uck2* or *san* in the whole body minus the respective eyes or wings never averaged >15% knockdown (Table 2). These data confirmed the tissue-specific targeting of both *Uck2* and *san* genes.

We used whole adult flies to assess hemocyte-specific knockdown using the hemocyte-specific *GAL4* line to express *Uck2* and *san*-RNAi transgenes specifically in hemocytes. We found that RNA levels were reduced by an average of 79±6% and 62±4%, respectively, when compared to experimental controls (Table 1). Because the *Hml-Gal4* line is highly specific for expression in the hemocytes, we can reasonably assume that the reduction in RNA levels in these whole flies is due to specific knockdown of the transcript in hemocytes and not other tissues (Dietzl et al., 2007). The fat body and salivary glands are difficult to dissect from adult flies. Since the RNAi effect is applicable at all stages of the *Drosophila* lifespan (Dietzl et al., 2007), we isolated the fat bodies and salivary glands from 3rd instar larvae to assess the silencing efficiency specifically in the fat body and salivary glands of our RNAi flies. In the dissected fat body from fat body-specific *Uck2* and *san*-RNAi third instar larvae, there was an average of 77±8% and 66±12% reduction in transcript levels, respectively, compared to dissected fat body from 3rd instar control larvae from wild-type or parental flies (Table 1). Similarly, transcript levels in dissected salivary glands from salivary gland-specific *Uck2* and *san*-RNAi 3rd instar larvae showed that RNA levels were reduced >90% in comparison to dissected salivary glands from control larvae from wild-type or parental flies (Table 2). In contrast, there was <29% knockdown of *san* or *Uck2* in RNA samples extracted from non-salivary gland tissue isolated from 3rd instar larvae in comparison to non-salivary gland tissue from control larvae from wild-type or parental flies (Table 2).

After confirming tissue-specific RNAi silencing effect of *Uck2* and *san*, control and RNAi flies were experimentally challenged with *E. chaffeensis*. We observed that eye- (Fig. 3A and 3B), salivary gland- (Fig. 3C and 3D), and wing- (Fig. 3E and 3F) specific knockdown of *Uck2* and *san* did not impact fly survival. These RNAi flies were as susceptible to *E. chaffeensis* infection as wild-type and parental controls (Fig. 3; $P>0.05$, log-rank tests). In contrast, hemocyte- and fat body-specific *Uck2*- (Fig. 4A and 4C)and *san*- (Fig. 4B and 4D) RNAi flies survived significantly better ($P<0.05$, log-rank test) than the control flies after infection with *Ehrlichia* (Fig. 4A–D). Ninety-six hours after infection, >80% of hemocyte-specific *Uck2*-RNAi and *San*-RNAi flies survived in comparison to an average of 50% of wild-type and parental control flies. When we assessed F1 RNAi flies for bacteria load,

there were no differences in bacteria number in eye-specific (Fig. 5A and 5B), salivary gland-specific (Fig. 5C and 5D), and wing-specific (Fig. 5E and 5F) *Uck2* and *san* F1 RNAi flies compared to control flies ($P>0.05$, *t*-test). However, F1 RNAi flies with hemocyte-specific (Fig. 6A and 6B) or fat body-specific (Fig. 6C and 6D) knockdown of *Uck2* or *san* had significantly fewer bacteria ($P<0.05$, *t*-test). At the 96-h time point, there were greater than 95% fewer bacteria in hemocyte-specific (Fig. 6A and 6B) or fat body-specific (Fig. 6C and 6D) *Uck2*- or *san*-RNAi flies, respectively, compared to controls.

Discussion

E. chaffeensis is transmitted from ticks to vertebrate hosts when the tick takes a blood meal (Paddock and Childs, 2003). One remaining question is where do the bacteria replicate in the tick? Because the genetic tools in ticks have not been developed to the same extent as they have in *Drosophila*, we addressed a more general question about where the bacteria replicate in dipteran arthropods. Indeed, this study provides insights about *E. chaffeensis* replication in arthropods. Our data confirm that *Uck2* and *san* are needed for *E. chaffeensis* replication (Von Ohlen et al., 2012) and support the hypothesis that the bacteria replicate in the hemocytes or the fat body in adult *D. melanogaster*. Infection was poorer in flies that had hemocyte- or fat body-specific knockdown of *Uck2* or *san.* We concede that fruit flies and ticks have different life cycles, feeding habits, and environments, and this may not allow extrapolation of the results to the tick system. Nevertheless, this is a valuable study because it identifies sites where the bacteria replicate in a model arthropod system using experimental methods which would not have been possible in the tick system.

We demonstrated that *E. chaffeensis* replicated when *Uck2* and *san* were expressed in hemocytes and the fat body, but not when these genes were silenced in those tissues. Silencing of *Uck2* and *san* in the eyes, wings, or the salivary glands of adult *D. melanogaster* did not affect infection. Because of the tissue-specific knockdown of the genes in these tissues (Dietzl et al., 2007), the data suggest that bacterial replication occurs predominantly in the hemocytes and the fat body. These data are consistent with the vertebrate tropism for macrophages and monocytes (Paddock and Childs, 2003; Sotomayor et al., 2001). Hemocytes are the arthropod host equivalent of macrophages (Lemaitre and Hoffmann, 2007). The fat body is functionally equivalent to mammalian liver (Lemaitre and Hoffmann, 2007). These data are also consistent with the previous observation that *Rickettsia rickettsii* invade hemocytes in ixodid ticks (Socolovschi et al., 2009). Previously, our group found that *E. chaffeensis* is capable of replicating in hemocyte-like phagocytic S2 cells (Luce-Fedrow et al., 2008). S2 cells have hemocyte-like properties and express a variety of hemocyte markers such as Hemolectin *(Hml)* (Charroux and Royet, 2009), Hemese *(He)* (Lebestky et al., 2000), *Drosophila* scavenger receptor-CI (*dSR-CI*) (Pearson et al., 1995), and croquemort, a member of the CD36 superfamily (Franc et al., 1999). However, because these cells have combined properties of plasmatocytes and crystal cells, we could not conclude that they are hemocytes (Cherbas et al., 2011). We also found that *E. chaffeensis* labeled with the pH-sensitive dye pHrodo fluoresced red after injection into adult flies, presumably because they were in the low pH environment of the hemocytes (Luce-Fedrow et al., 2009). Therefore, these new in vivo data add to the growing evidence that *E. chaffeensis* is capable of replicating in the hemocytes of adult *D. melanogaster*. Furthermore, these data are consistent with the behavior of other intracellular pathogens which are capable of avoiding the phagocytic pathway and replicate within *Drosophila* hemocytes. These pathogens include *Salmonella typhimurium* (Brandt et al., 2004), *Listeria monocytogenes* (Mansfield et al., 2003), *Mycobacterium marinum* (Dionne et al., 2003), *Legionella pneumophila* (Dorer et al., 2006), and *Francisella tularensis* (Vonkavaara et al., 2008).

The expression of *Uck2* or *san* in the salivary glands of adult *D. melanogaster* was not necessary for *E. chaffeensis* infection. These findings were intriguing since *A. phagocytophilum*, a bacterium closely related to *E. chaffeensis*, and *E. chaffeensis* have been detected in the salivary glands of experimentally infected ticks (Karim et al., 2012; Rikihisa, 2011). In our experiments, *Drosophila* were infected by needle injection in the abdomen; not the natural route of infection in a tick. Ticks acquire the infection from a vertebrate host during their blood meal (Paddock and Childs, 2003). Thus, the dissemination of *E. chaffeensis* to assorted tissues may vary due to differences in route of infection. Interestingly, in ticks, needle injection does result in bacteria in the salivary glands (Karim et al., 2012). However, it is possible that salivary gland and host physiology may vary among arthropods depending on the life cycle. In nature, *Drosophila* live on yeast growing on decaying fruit and food. This could suggest that their salivary glands are better adapted to fight potential pathogenic microorganisms and extrapolation of the data from *Drosophila* to ticks needs to be done cautiously.

We observed 97% fewer bacteria 96 h post infection in fat body-specific *Uck2* or *san*- F1 RNAi flies compared to wild-type and parental controls. Because there is tissue-specific knockdown in the fat body, some residual bacterial replication should occur in the hemocytes of the flies. Indeed, this might have been the case. In general, when one looks at the bacteria growth kinetics in tissues that are not affected by *Uck2* or *san* knockdown (eyes, wings, salivary glands), bacterial numbers generally increase over time after infection (Fig. 5). In contrast, when *Uck2* or *san* genes were silenced in hemocytes, bacteria failed to grow (Fig. 6A and 6B). However, when *Uck2* or *san* genes were silenced specifically in the fat body, the bacteria growth kinetics showed an initial increase in bacterial numbers for 24 h that paralleled the growth of the bacteria in the parental (non-RNAi control) fly lines (Fig. 6A and 6B). Subsequently, the bacterial numbers were reduced (Fig. 6C and 6D). These data suggest that the bacteria were replicating during the early part of the infection in the hemocytes, but because the bacteria were not replicating in the fat body, the activation of the fly immune system was able to respond more effectively against *E. chaffeensis* than when the bacteria replicate in both hemocytes and the fat body. We have previously found that hemocytes in adult flies as well as toll and IMD defense pathways are actively involved in host defense against *E. chaffeensis* (Luce-Fedrow et al., 2009). Alternatively, the failure of bacteria to thrive even in the presence of functional *Uck2* and *san* genes in hemocytes might be because there is coordination between the hemocytes and the fat body. Several observations indicate that hemocytes can signal to the fat body to regulate the humoral immune response (Agaisse et al., 2003; Brennan et al., 2007; Dijkers and O'Farrell, 2007; Shia et al., 2009). Thus, signaling between the hemocytes and other immunocompetent tissue such as the fat body may play a critical role in coordinating the cellular and humoral immune response to ensure efficient defense of the organism.

Our results suggest that the tissue tropism of *E. chaffeensis* for phagocytic cells arose approximately 600 million years ago, since that is when mammals and dipterans last shared a common ancestor (Gordon and Waterhouse, 2007; Pesole et al., 1991). Ticks and dipterans last shared a common ancestor approximately 500 million years ago (Ullmann et al., 2005). Therefore, one would expect the tropism for hemocytes to be similar in ticks. However, genetic drift could have occurred in the Ecdysozoa, Arthropoda, or later in the Chelicerata (Gordon and Waterhouse, 2007). There is 59.5% identity between *D. melanogaster* and *I. scapularis Uck2* gene products and 83.6% identity between *D. melanogaster* and *I. scapularis san* gene products (Lawson et al., 2009). Therefore, it is not clear, whether this tropism will also be seen in *A. americanum*, the vector of *E. chaffeensis*. Additional experiments are needed to confirm this hypothesis.

In conclusion, we have used *UAS-GAL4* RNAi system in *D. melanogaster* to show that functional *Uck2* and *san* genes are required for in vivo *E. chaffeensis* replication. Moreover, we have successfully identified intracellular niches where *E. chaffeensis* replicates in dipteran arthopods; the hemocytes and fat body of adult *D. melanogaster*. We hope that understanding the tissue targets of *E. chaffeensis* in arthropods will help in drug design and RNAi therapeutics for HME and other tick- and arthropod-borne diseases.

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References

Agaisse H, Petersen UM, Boutros M, Mathey-Prevot B, Perrimon N. Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. Developmental Cell. 2003; 5:441–450. [PubMed: 12967563]

Anderson BE, Sims KG, Olson JG, Childs JE, Piesman JF, Happ CM, Maupin GO, Johnson BJ. *Amblyomma americanum*: a potential vector of human ehrlichiosis. American J Trop Med Hyg. 1993; 49:239–244.

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993; 118:401–415. [PubMed: 8223268]

Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, Schneider DS. Secreted bacterial effectors and host-produced Eiger/TNF drive death in a *Salmonella*-infected fruit fly. PLoS biology. 2004; 2:e418. [PubMed: 15562316]

Brennan CA, Delaney JR, Schneider DS, Anderson KV. Psidin is required in *Drosophila* blood cells for both phagocytic degradation and immune activation of the fat body. Current Biology. 2007; 17:67–72. [PubMed: 17208189]

Chapes SK, Ganta RR. Defining the immune response to *Ehrlichia* species using murine models. Veterinary Parasitology. 2008; 158:344–359. [PubMed: 19028013]

Charroux B, Royet J. Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response. Proc Natl Acad Sci USA. 2009; 106:9797–9802. [PubMed: 19482944]

Cherbas L, Willingham A, Zhang D, Yang L, Zou Y, Eads BD, Carlson JW, Landolin JM, Kapranov P, Dumais J, Samsonova A, Choi JH, Roberts J, Davis CA, Tang H, van Baren MJ, Ghosh S, Dobin A, Bell K, Lin W, Langton L, Duff MO, Tenney AE, Zaleski C, Brent MR, Hoskins RA, Kaufman TC, Andrews J, Graveley BR, Perrimon N, Celniker SE, Gingeras TR, Cherbas P. The transcriptional diversity of 25 *Drosophila* cell lines. Genome Res. 2011; 21:301–314. [PubMed: 21177962]

Davidson WR, Lockhart JM, Stallknecht DE, Howerth EW. Susceptibility of red and gray foxes to infection by *Ehrlichia chaffeensis*. J Wildl Dis. 1999; 35:696–702. [PubMed: 10574528]

Dawson JE, Childs JE, Biggie KL, Moore C, Stallknecht D, Shaddock J, Bouseman J, Hofmeister E, Olson JG. White-tailed deer as a potential reservoir of *Ehrlichia* spp. J Wildl Dis. 1994; 30:162–168. [PubMed: 8028099]

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature. 2007; 448:151–156. [PubMed: 17625558]

Dijkers PF, O'Farrell PH. *Drosophila* calcineurin promotes induction of innate immune responses. Current Biology. 2007; 17:2087–2093. [PubMed: 18060786]

Dionne MS, Ghori N, Schneider DS. *Drosophila melanogaster* is a genetically tractable model host for *Mycobacterium marinum*. Infect Immun. 2003; 71:3540–3550. [PubMed: 12761139]

Dorer MS, Kirton D, Bader JS, Isberg RR. RNA interference analysis of *Legionella* in *Drosophila* cells: exploitation of early secretory apparatus dynamics. PLoS Pathogens. 2006; 2:e34. [PubMed: 16652170]

Dugan VG, Gaydos JK, Stallknecht DE, Little SE, Beall AD, Mead DG, Hurd CC, Davidson WR. Detection of *Ehrlichia* spp. in raccoons (*Procyon lotor*) from Georgia. Vector Borne Zoonotic Dis. 2005; 5:162–171. [PubMed: 16011433]

Dugan VG, Little SE, Stallknecht DE, Beall AD. Natural infection of domestic goats with *Ehrlichia chaffeensis*. Journal of Clinical Microbiology. 2000; 38:448–449. [PubMed: 10618139]

Ewing SA, Dawson JE, Kocan AA, Barker RW, Warner CK, Panciera RJ, Fox JC, Kocan KM, Blouin EF. Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). J Med Entomol. 1995; 32:368–374. [PubMed: 7616529]

Franc, NC.; Heitzler, P.; Ezekowitz, RA.; White, K. Science. Vol. 284. New York, N.Y: 1999. Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*; p. 1991-1994.

Ganta RR, Wilkerson MJ, Cheng C, Rokey AM, Chapes SK. Persistent *Ehrlichia chaffeensis* infection occurs in the absence of functional MHCII genes. Infect Immun. 2002; 70:380–388. [PubMed: 11748204]

Gordon KHJ, Waterhouse PM. RNAi for insect-proof plants. Nat Biotech. 2007; 25:1231–1232.

Karim S, Browning R, Ali L, Truhett R. Laboratory-infected *Ehrlichia chaffeensis* female adult *Amblyomma americanum* salivary glands reveal differential gene expression. Journal of Medical Entomology. 2012; 49:547–554. [PubMed: 22679861]

Kocan AA, Levesque GC, Whitworth LC, Murphy GL, Ewing SA, Barker RW. Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. Emerg Infect Dis. 2000; 6:477–480. [PubMed: 10998377]

Lawson D, Arensburger P, Atkinson P, Besansky NJ, Bruggner RV, Butler R, Campbell KS, Christophides GK, Christley S, Dialynas E, Hammond M, Hill CA, Konopinski N, Lobo NF, MacCallum RM, Madey G, Megy K, Meyer J, Redmond S, Severson DW, Stinson EO, Topalis P, Birney E, Gelbart WM, Kafatos FC, Louis C, Collins FH. VectorBase: a data resource for invertebrate vector genomics. Nucleic Acids Res. 2009; 37:D583–D587. [PubMed: 19028744]

Lebestky T, Chang T, Hartenstein V, Banerjee U. Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. Science. 2000; 288:146–149. [PubMed: 10753120]

Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. Annu Rev Immunol. 2007; 25:697–743. [PubMed: 17201680]

Lockhart JM, Davidson WR, Stallknecht DE, Dawson JE, Howerth EW. Isolation of *Ehrlichia chaffeensis* from wild white-tailed deer (*Odocoileus virginianus)* confirms their role as natural reservoir hosts. J Clin Microbiol. 1997; 35:1681–1686. [PubMed: 9196173]

Luce-Fedrow A, Von Ohlen T, Boyle D, Ganta RR, Chapes SK. Use of *Drosophila* S2 cells as a model for studying *Ehrlichia chaffeensis* infections. Applied Environ Microbiol. 2008; 74:1886–1891.

Luce-Fedrow A, Von Ohlen T, Chapes SK. *Ehrlichia chaffeensis* infections in *Drosophila melanogaster*. Infect Immun. 2009; 77:4815–4826. [PubMed: 19687202]

Magnarelli LA, Anderson JF, Stafford KC 3rd, Dumler JS. Antibodies to multiple tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in white-footed mice. J Wildl Dis. 1997; 33:466–473. [PubMed: 9249691]

Mansfield BE, Dionne MS, Schneider DS, Freitag NE. Exploration of host-pathogen interactions using *Listeria monocytogenes* and *Drosophila melanogaster*. Cell Microbiol. 2003; 5:901–911. [PubMed: 14641175]

McQuilton P, St Pierre SE, Thurmond J, FlyBase C. FlyBase 101 – the basics of navigating FlyBase. Nucleic Acids Res. 2012; 40:D706–714. [PubMed: 22127867]

Paddock CD, Childs JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin Microbiol Rev. 2003; 16:37–64. [PubMed: 12525424]

Pagel Van Zee J, Geraci NS, Guerrero FD, Wikel SK, Stuart JJ, Nene VM, Hill CA. Tick genomics: The *Ixodes* genome project and beyond. International J Parasitol. 2007; 37:1297–1305.

Parola P, Davoust B, Raoult D. Tick- and flea-borne rickettsial emerging zoonoses. Vet Res. 2005; 36:469–492. [PubMed: 15845235]

Pearson A, Lux A, Krieger M. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. Proc Natl Acad Sci USA. 1995; 92:4056–4060. [PubMed: 7732030]

Pesole G, Bozzetti MP, Lanave C, Preparata G, Saccone C. Glutamine synthetase gene evolution: a good molecular clock. Proc Natl Acad Sci USA. 1991; 88:522–526. [PubMed: 1671172]

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Rikihisa Y. Mechanisms of obligatory intracellular infection with *Anaplasma phagocytophilum*. Clin Microbiol Rev. 2011; 24:469–489. [PubMed: 21734244]

Sanson B, White P, Vincent JP. Uncoupling cadherin-based adhesion from wingless signalling in *Drosophila*. Nature. 1996; 383:627–630. [PubMed: 8857539]

Shia AKH, Glittenberg M, Thompson G, Weber AN, Reichhart JM, Ligoxygakis P. Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spätzle secreted by haemocytes. Journal of Cell Science. 2009; 122:4505–4515. [PubMed: 19934223]

Sirigireddy KR, Ganta RR. Multiplex detection of *Ehrlichia* and *Anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. J Mol Diagn. 2005; 7:308–316. [PubMed: 15858156]

Smith RD, Sells DM, Stephenson EH, Ristic MR, Huxsoll DL. Development of *Ehrlichia canis*, causative agent of canine ehrlichiosis, in the tick *Rhipicephalus sanguineus* and its differentiation from a symbiotic *Rickettsia*. Am J Vet Res. 1976; 37:119–126. [PubMed: 944000]

Socolovschi C, Mediannikov O, Raoult D, Parola P. The relationship between spotted fever group Rickettsiae and ixodid ticks. Vet Res. 2009; 40:34. [PubMed: 19358804]

Sotomayor EA, Popov VL, Feng HM, Walker DH, Olano JP. Animal model of fatal human monocytotropic ehrlichiosis. Am J Pathol. 2001; 158:757–769. [PubMed: 11159213]

Ullmann AJ, Lima CM, Guerrero FD, Piesman J, Black WC 4th. Genome size and organization in the blacklegged tick, *Ixodes scapularis* and the Southern cattle tick, *Boophilus microplus*. Insect Mol Biol. 2005; 14:217–222. [PubMed: 15796755]

Varela-Stokes AS. Transmission of *Ehrlichia chaffeensis* from lone star ticks (*Amblyomma americanum*) to white-tailed deer (*Odocoileus virginianus*). J Wildl Dis. 2007; 43:376–381. [PubMed: 17699076]

Von Ohlen T, Luce-Fedrow A, Ortega MT, Ganta RR, Chapes SK. Identification of critical host mitochondrial-associated genes during *Ehrlichia chaffeensis* infections. Infect Immun. 2012; 80:3576–3586. [PubMed: 22851751]

Von Ohlen T, Moses C, Poulson W. *Ind* represses *msh* expression in the intermediate column of the *Drosophila* neuroectoderm, through direct interaction with upstream regulatory DNA. Developmental Dynamics. 2009; 238:2735–2744. [PubMed: 19795518]

Vonkavaara M, Telepnev MV, Ryden P, Sjöstedt A, Stöven S. *Drosophila melanogaster* as a model for elucidating the pathogenicity of *Francisella tularensis*. Cell Microbiol. 2008; 10:1327–1338. [PubMed: 18248629]

Yu DH, Li YH, Yoon JS, Lee JH, Lee MJ, Yu IJ, Chae JS, Park JH. *Ehrlichia chaffeensis* infection in dogs in South Korea. Vector Borne Zoonotic Dis. 2008; 8:355–358. [PubMed: 18399775]

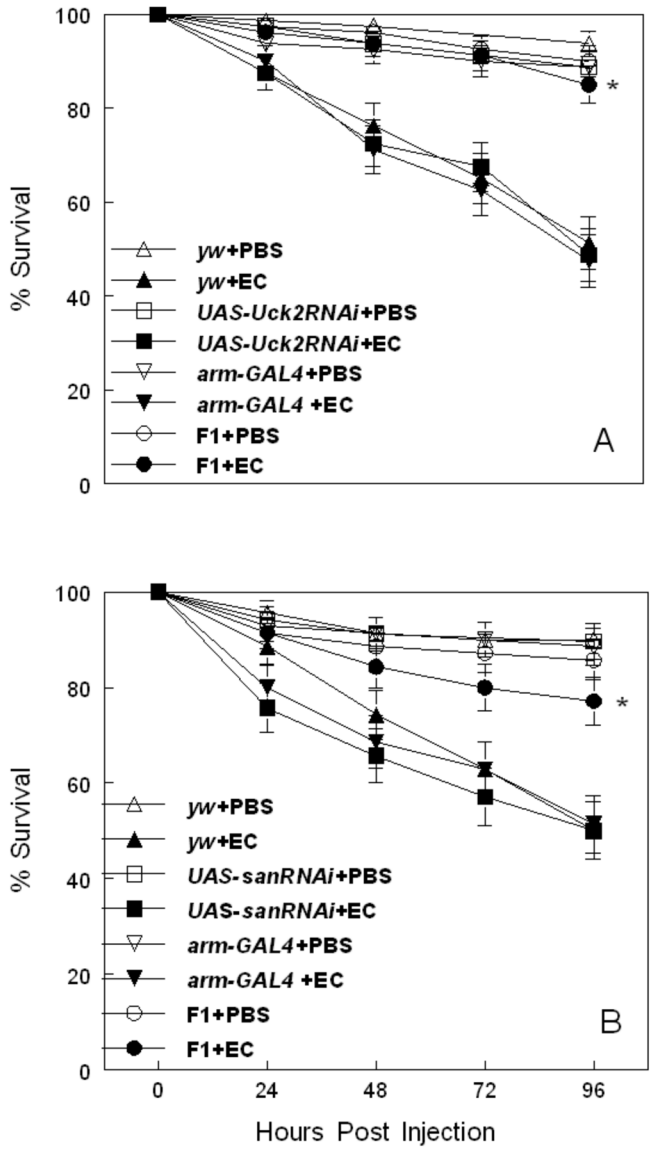

Fig. 1.

Impact of ubiquitous expression of *Uck2* (A) or *san* (B) hairpin RNA on *E. chaffeensis* infection. Ubiquitous knockdown of *Uck2* or *san* was accomplished using *UAS* and *GAL4* constructs as described in 'Materials and methods'. Flies were injected with PBS or cell-free *E. chaffeensis* (EC). Data presented represent the mean ± SE of 3–4 independent experiments. Twenty flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1 + EC (●) compared to other EC-treated flies is represented by * ($P<0.05$).

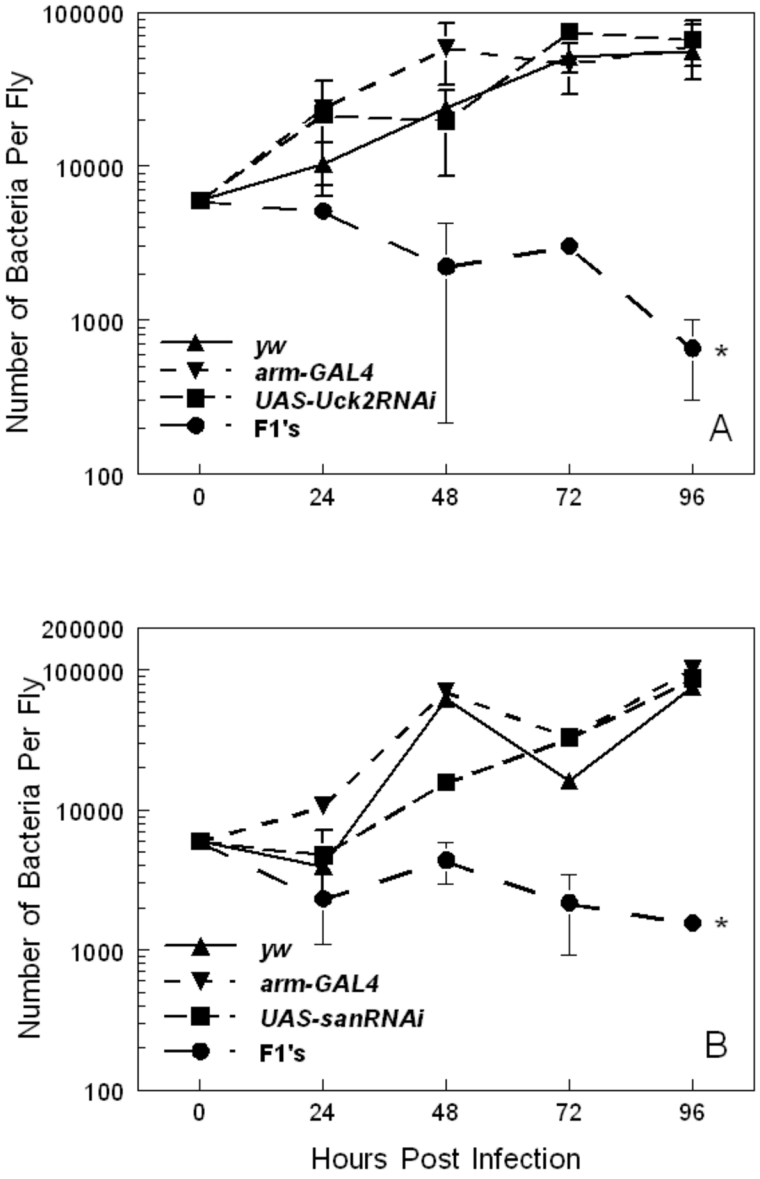

Fig. 2.

Impact of ubiquitous expression of *Uck2* (A) or *san* (B) hairpin RNA on bacterial clearance. Ubiquitous knockdown of *Uck2* and *san* was accomplished using *UAS* and *GAL4* constructs as described in the 'Materials and methods'. Bacterial load was estimated by qRT-PCR for ehrlichial 16S rRNA as described in the 'Materials and methods'. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4–5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1's(●) compared to other EC -infected flies is represented by * (P<0.05).

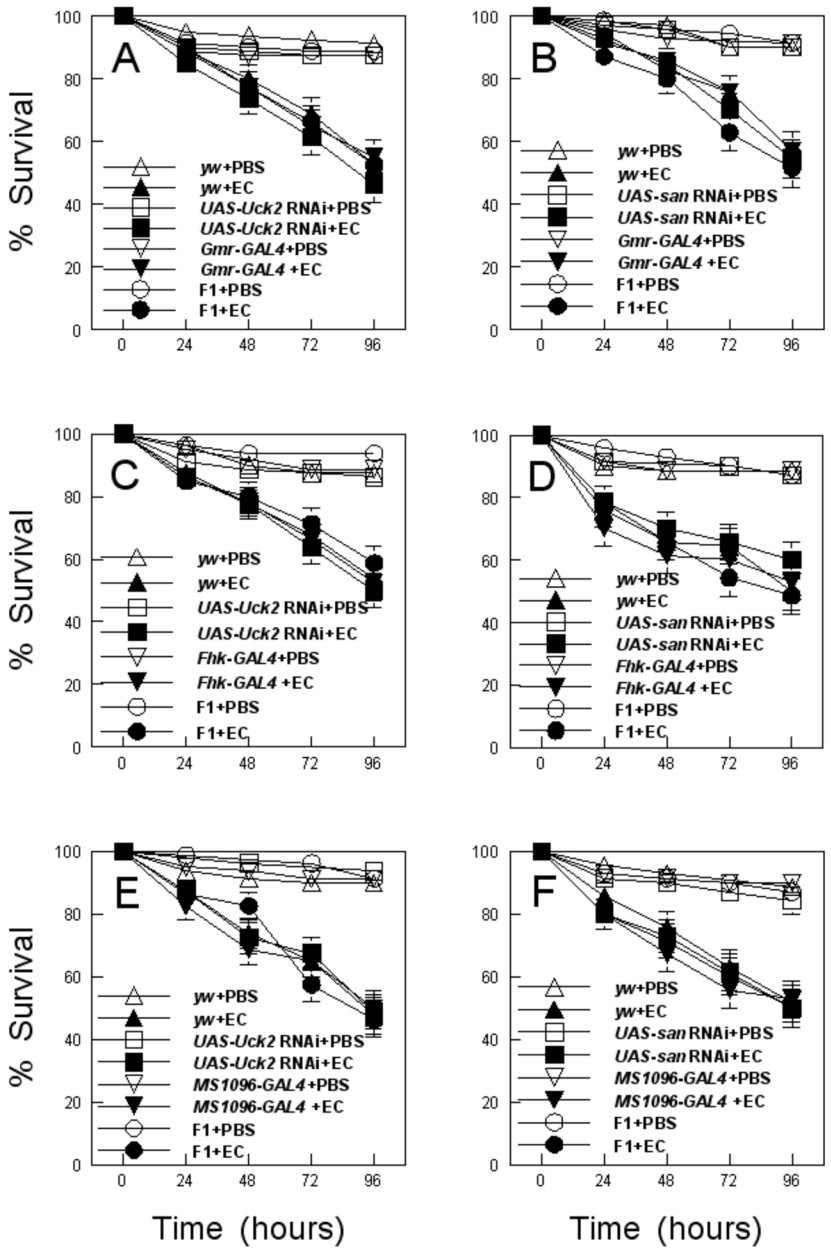

Fig. 3.

Impact of eye-, salivary gland- or wing-specific expression of *Uck2* or *san* hairpin RNA on *E. chaffeensis* infection. Eye (A and B), salivary gland (C and D), or wing (E and F) - specific knockdown of *Uck2* (A, C, and E) or *san* (B, D, and F) was accomplished using *UAS* and *GAL4* constructs as described in the 'Materials and methods'. Flies were injected with PBS or cell-free *E. chaffeensis* (EC). Data presented represent the mean ± SE of 3–4 independent experiments. Twenty flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1 + EC (●) compared to other EC-treated flies is represented by * (P<0.05).

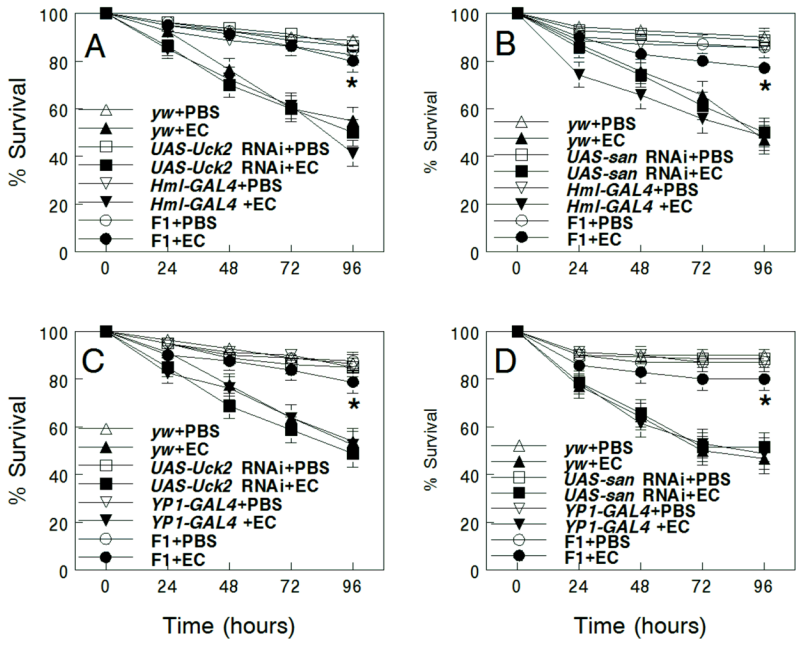

Fig. 4.

Impact of hemocyte or fat body expression of *Uck2* or *san* hairpin RNA on *E. chaffeensis* infection. Hemocyte-specific (A and B) or fat body (C and D) -specific knockdown of *Uck2*(A and C) or *san*(B and D) was accomplished using *UAS* and *GAL4* constructs as described in the 'Materials and methods'. Flies were injected with PBS or cell-free *E. chaffeensis* (EC). Data presented represent the mean ± SE of 3–4 independent experiments. Twenty flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1 + EC (●) compared to other EC-treated flies is represented by * (P<0.05).

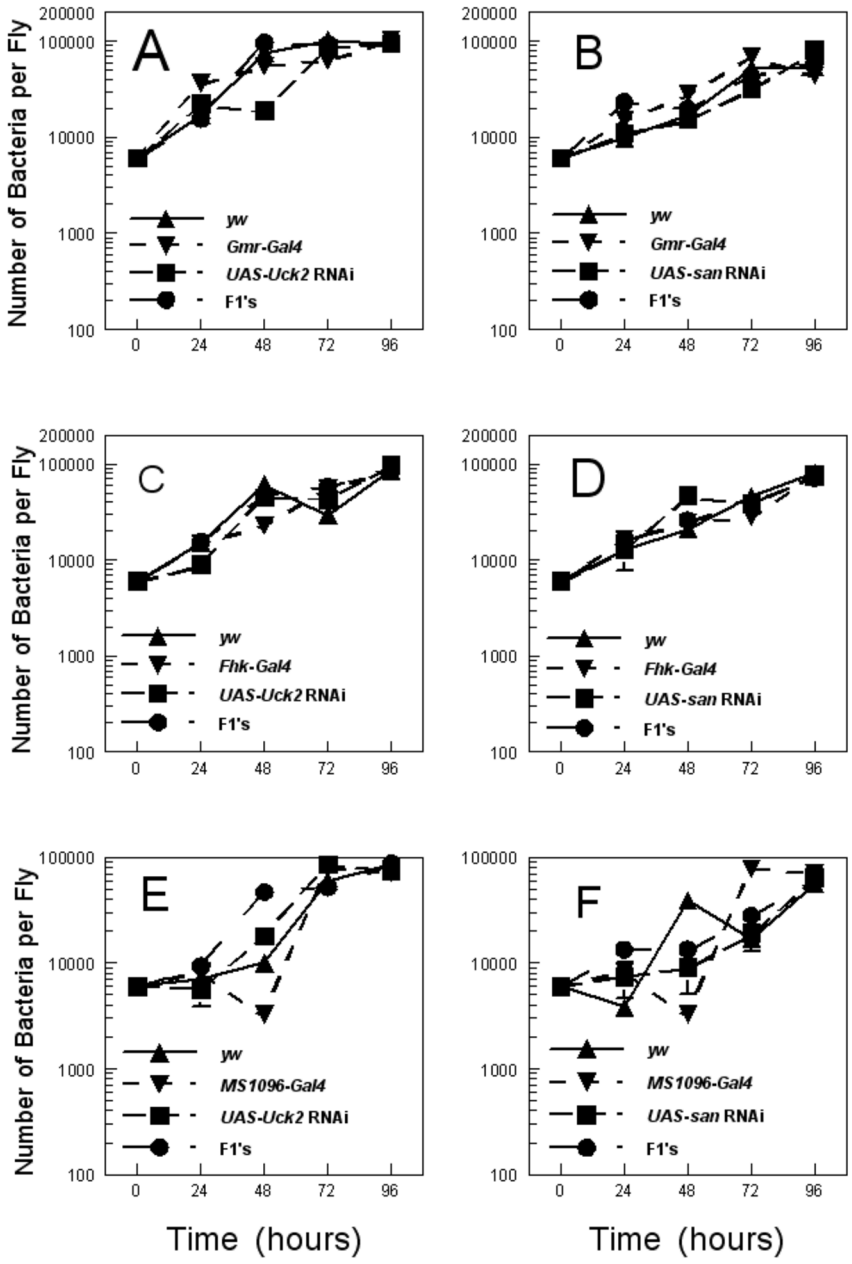

Fig. 5.

Impact of eye-, salivary gland-, or wing-specific expression of *Uck2* or *san* hairpin RNA on bacterial clearance. Eye (A and B), salivary gland (C and D), or wing (E and F) -specific knockdown of *Uck2* (A, C, and E) or *san* (B, D, and F) was accomplished using *UAS* and *GAL4* constructs as described in the 'Materials and methods'. Bacterial load was estimated by qRT-PCR for ehrlichial 16S rRNA as described in the 'Materials and methods'. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4–5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1's(●) compared to other EC -infected flies is represented by * (P<0.05).

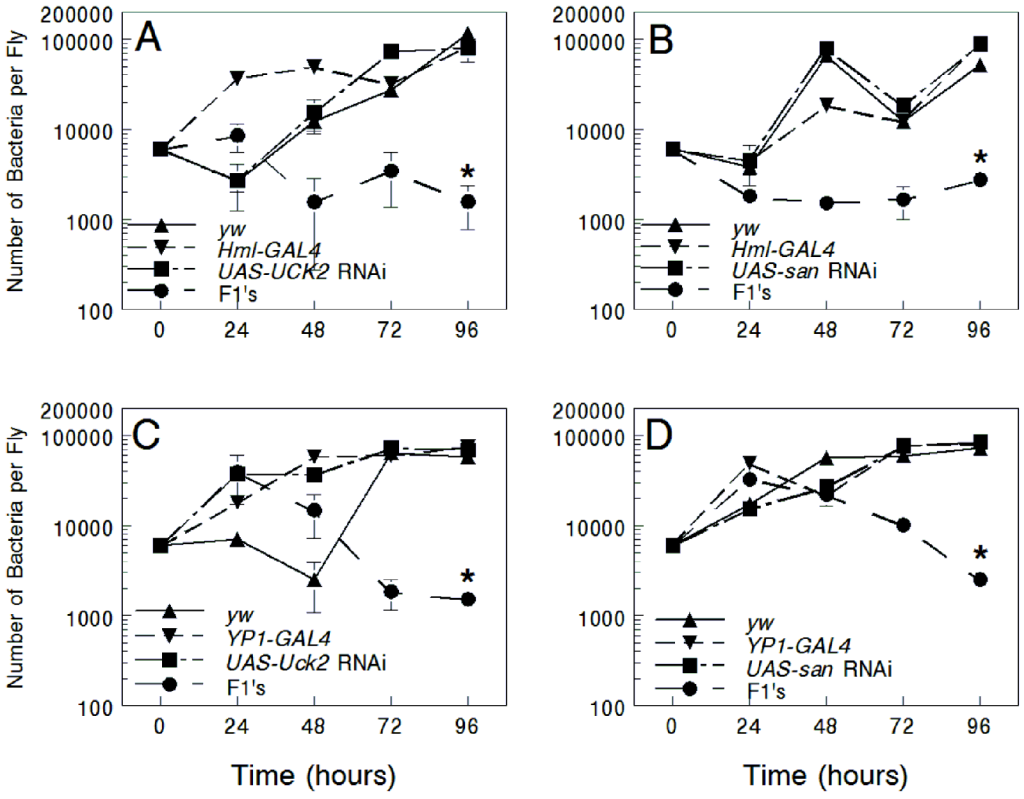

Fig. 6.

Impact of hemocyte or fat body expression of *Uck2* or *san* hairpin RNA on bacterial clearance. Hemocyte-specific (A and B) or fat body (C and D)-specific knockdown of *Uck2*(A and C) or *san*(B and D) was accomplished using *UAS* and *GAL4* constructs as described in the 'Materials and methods'. Bacterial load was estimated by qRT-PCR for ehrlichial 16S rRNA as described in the 'Materials and methods'. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4–5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance of F1's (●) compared to other EC-infected flies is represented by * (P<0.05).

Table 1

Silencing. Silencing efficiency of target genes that had ubiquitously expressed hairpin RNA or hairpin RNA expressed specifically in hemocytes or the fat body[a].

Tissues in which the target gene was knocked down	Tissue assayed[b]	Target Gene	% knockdown efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls[c]	% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (parental line 1)[c]	% knockdown efficiency of target gene in F1 progeny compared to *GAL4* construct (parental line 2)[c]	Average % knockdown efficiency of target gene in F1 progeny compared to control flies[d]
Ubiquitous (*arm-Gal4*)	Whole adult body	*Uck2*	86 ± 4	90 ± 5	89 ± 9	88 ± 2
	Whole adult body	*san*	86 ± 2	86 ±5	78 ± 4	83 ± 5
Hemocytes (*Hml-Gal4*)	Whole adult body	*Uck2*	76 ± 8	85 ± 5	73 ± 3	79 ± 6
	Whole adult body	*san*	61 ± 0	59 ± 2	66 ± 1	62 ± 4
Fat Body (*YP1-Gal4*)	Whole adult body	*Uck2*	55 ± 1	26 ± 9	23 ± 1	35 ±18
	Whole adult body	*san*	46 ± 2	28 ± 4	52 ± 3	42 ± 12
Fat body (*YP1-Gal4*)	Fat body, 3rd instar larvae	*Uck2*	75	85	70	77 ± 8
Fat body (*YP1-Gal4*)	Fat body, 3rd instar larvae	*san*	79	57	62	66 ± 12

[a] Percent (%) knockdown efficiency of target gene in RNAi flies (F1's) compared to wild type and parental lines (*UAS* and *GAL4* constructs).

[b] RNA was isolated from a pool of 10–12 whole flies or isolated fat bodies. Analysis of transcript level was done using qRT-PCR results as described in the 'Materials and methods'.

[c] Data presented represent the mean ± SD of one or 3 independent experiments.

[d] Numbers presented represent the mean ± SD of *yw* and parental fly lines 1 and 2 (columns 4–6).

Table 2

Determination of off-target silencing in wings, eyes, and body[a,b].

Tissues in which the target gene was knocked down	Experiment	Target gene	% knockdown efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls[c]	% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (parental line 1)[c]	% knockdown efficiency of target gene in F1 progeny compared to *GAL4* construct (parental line 2)[c]	Average % knockdown efficiency of target gene in F1 progeny compared to control flies[d]
Wings (*MS1096-GAL4*)	1	*Uck2*	81 ±2	66 ± 6	81 ± 7	76 ± 9
	1	*san*	77 ± 7	82 ± 22	86 ± 14	82 ± 5
Body (*MS1096-GAL4*)	1	*Uck2*	−26 ± 11	−8 ± 3	11 ± 2	−8 ± 19
	1	*san*	−41	17	20	−1 ± 34
Whole heads (*Gmr-GAL4*)	2	*Uck2*	86 ± 4	88 ± 5	89 ± 9	88 ± 2
	2	*san*	57 ± 2	85 ±7	81 ± 7	74 ±15
Body (*Gmr-GAL4*)	2	*Uck2*	23 ± 4	12 ± 3	8 ± 3	14 ± 8
	2	*san*	10 ± 0	25 ± 6	9 ± 3	15 ± 10
Salivary glands (*Fhk-GAL4*)	3	*Uck2*	92	97	96	95 ± 2
	3	*san*	93	96	82	90 ± 6
Body (*Fhk-GAL4*)	3	*Uck2*	16	32	36	28 ± 9
	3	*san*	18	22	30	23 ± 5

[a]Comparison of knockdown efficiency of target genes in F1 RNAi flies in wings compared to whole body (without wings) generated using *MS1096-GAL4* flies (Experiment 1), whole heads compared to whole-body tissue (abdomen and thorax) generated using *Gmr-GAL4* flies (Experiment 2), or salivary glands from 3rd instar larvae compared to whole-body tissue (without salivary glands) generated using *Fhk- GAL4* flies (Experiment 2) in comparison to wild type and parental lines (*UAS* and *GAL4* constructs).

[b]Analysis of transcript level was done using qRT-PCR as described in the 'Materials and methods'. Negative numbers indicate higher transcript levels.

[c]Numbers presented represent the mean ± SD of one or 3 independent experiments.

[d]Numbers presented represent the mean ± SD of yw and parental lines 1 and 2 (columns 4–6).