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The effect of bacterial challenge on ferritin regulation in the yellow fever mosquito, *Aedes aegypti*

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Abstract

Secreted ferritin is the major iron storage and transport protein in insects. Here we characterize the message and protein expression profiles of yellow fever mosquito (*Aedes aegypti*) ferritin heavy chain homologue (HCH) and light chain homologue (LCH) subunits in response to iron and bacterial challenge. *In vivo* experiments demonstrated tissue specific regulation of HCH and LCH expression over time post-blood meal (PBM). Transcriptional regulation of *HCH* and *LCH* was treatment specific, with differences in regulation for naïve versus mosquitoes challenged with heat-killed bacteria (HKB). Translational regulation by iron regulatory protein (IRP) binding activity for the iron responsive element (IRE) was tissue specific and time-dependent PBM. However, mosquitoes challenged with HKB showed little change in IRP/IRE binding activity compared to naïve animals. The changes in ferritin regulation and expression *in vivo* were confirmed with *in vitro* studies. We challenged mosquitoes with HKB followed by a blood meal to determine the effects on ferritin expression, and demonstrate a synergistic, time-dependent, regulation of expression for HCH and LCH.

Keywords

Aedes aegypti; bacteria; ferritin; iron; IRP; mosquito

Introduction

Female mosquitoes must blood feed for oogenesis, and as a consequence, they are exposed to a high iron load, and potentially, to blood-borne pathogens. Mosquitoes can be infected by bacteria, fungi, viruses and parasites. The yellow fever vector, *Aedes aegypti*, also transmits dengue. WHO currently estimates 40% of the world's population are now at risk from dengue, and there are approximately 50 million dengue infections each year (WHO, 2009a). While approximately 900 million people are at risk for yellow fever in Africa and Latin America; 200 000 people contract the disease worldwide annually (WHO, 2009b).

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Disclosure

All authors declare there are no relationships, financial or otherwise, that may pose a conflict of interest.

Mosquito immune response to pathogens induces ovarian follicular epithelial cell resorption during oocyte development and decreases egg production in the African malaria mosquito, *Anopheles gambiae* (Jiang *et al.*, 2009). Reduced access to iron also decreases egg numbers (Kogan, 1990). Proteins involved in the processing of dietary iron in *A. aegypti* may play a significant role in the mosquito immune response and ovarian follicular cell absorption supporting the importance of further research into iron metabolism in this vector (Fallon & Sun, 2001; Hua *et al.*, 2007; Jiang *et al.*, 2009; Magalhaes *et al.*, 2008; Yu *et al.*, 2007).

Since iron is an essential nutrient for many organisms, including pathogens, a novel method of sequestering iron during infection is needed to ensure the survival of the organism and to compromise the viability of the invading pathogen (Ganz, 2009; Ong *et al.*, 2006; Wang & Cherayil, 2009; Weinberg & Miklossy, 2008). One mechanism is the iron-withholding strategy of the innate immune response (Ganz, 2009; Ong *et al.*, 2006; Wang & Cherayil, 2009; Weinberg & Miklossy, 2008). In insects, hemolymph ferritin is found in mg/L concentration (Ong *et al.*, 2006; Yoshiga *et al.*, 1997). Current evidence supports that secreted ferritin serves as the primary iron transport protein in insects (Geiser *et al.*, 2009; Nichol *et al.*, 2002; Zhou *et al.*, 2007). Zhou *et al.* (2007) found radiolabeled iron fed to *A. aegypti* in a blood meal is absorbed, and the iron transported to tissues, particularly ovaries and eggs, by secreted ferritin. Insect ferritin consists of 24 heavy and light chain subunits, homologues of the vertebrate heavy and light chains (HCH and LCH, respectively), configured as a sphere that can hold up to 4500 atoms of iron per molecule (Hamburger *et al.*, 2005; Kalgaonkar & Lonnerdal, 2009). This property makes it a very efficient transport protein for acquiring iron from the diet when available and needed for rapid egg production. Since ferritin serves this role in insects, we hypothesized that it would be responsive to infections as an iron-withholding protein.

Insects are able to defend themselves against a variety of pathogens through the rapid induction of their innate immune response by humoral and cell-mediated mechanisms (Bartholomay *et al.*, 2004; Dong *et al.*, 2006; Fallon & Sun, 2001; Hoffmann, 1997; Hughes, 1998; Lowenberger, 2001; Masova *et al.*, 2010). This response involves induced expression and secretion of antibacterial and antifungal molecules (i.e. defensin, cecropin, transferrin (Tsf), and lysozyme (Bartholomay *et al.*, 2004; Bartholomay *et al.*, 2007; Castillo *et al.*, 2006; Dimopoulos *et al.*, 2000; Hillyer *et al.*, 2003a; Hillyer *et al.*, 2003b; Magalhaes *et al.*, 2008). These factors are a critical component of the humoral response of insects to fight infection. It takes as little as three hours to up-regulate the abundant expression of defensin in insects following bacterial challenge; the expression of defensin can plateau 12 to 36 hours post infection and remain elevated for 21 days (Cho *et al.*, 1996; Fallon & Sun, 2001; Gao *et al.*, 1999; Hoffmann, 1997; Lowenberger, 2001; Masova *et al.*, 2010). Defensins are expressed in insect fat body and secreted into the hemolymph (Blandin *et al.*, 2002; Cho *et al.*, 1997; Eggleston *et al.*, 2000; Masova *et al.*, 2010). They are also expressed in the gut of mosquitoes as well as the salivary glands (Blandin *et al.*, 2002; Dixit *et al.*, 2008; Dong *et al.*, 2006; Hoffmann, 1997; Luplertlop *et al.*, 2011). Expression of defensin can occur at any developmental stage in mosquitoes (Eggleston *et al.*, 2000; Lowenberger, 2001; Lowenberger *et al.*, 1999).

Many insect antimicrobial peptide gene promoter regions have several κ B-like motifs including that for *A. aegypti* and *A. gambiae defensin* (Antonova *et al.*, 2009; Eggleston *et al.*, 2000; Meredith *et al.*, 2006; Xi *et al.*, 2008). *Cis*-regulatory elements that bind NF- κ B factors also are found in the promoter regions of the mosquito ferritin heavy and light chains (Dunkov & Georgieva, 1999; Ong *et al.*, 2006; Pham *et al.*, 2003; Pham & Chavez, 2005; Recalcati *et al.*, 2008). We hypothesized that an increase in transcription of the insect ferritin HCH and LCH subunits could occur as part of the immune response.

In mammals, translation of both the ferritin light and heavy chain subunits is activated by cytokines and synthesis is increased via disassociation of *trans*-acting RNA poly(C)-binding proteins from the acute box, a *cis*-acting translational enhancer, downstream of the iron responsive element (IRE) in the 5'UTR of the message for either of the subunits (Rogers, 1996; Thomson *et al.*, 2005; Thomson *et al.*, 1999). The IRE allows translational control of subunit synthesis by iron through the action of the iron regulatory proteins (IRPs). Under intracellular low iron conditions, IRP binding to the IRE effectively blocks ferritin subunit translation. IRP/IRE binding activity is decreased during an immune response allowing increased ferritin subunit synthesis. Translational control by cytokines is not independent from iron-regulated translation of the subunit mRNA transcripts through the IRP/IRE interaction (Rogers, 1996; Thomson *et al.*, 2005; Thomson *et al.*, 1999).

In insects, one or both ferritin subunits have a 5'UTR IRE in the mRNA sequence which can respond to intracellular iron availability by either binding insect IRP under low intracellular iron to inhibit ferritin subunit translation or disassociating from insect IRP with high intracellular iron to increase ferritin subunit translation (Birney *et al.*, 2004; Du *et al.*, 2000; Geiser *et al.*, 2006; Hajdusek *et al.*, 2009; Nichol & Locke, 1999; Zhang *et al.*, 2001; Zhang *et al.*, 2002). The effect of the immune response on IRP/IRE binding activity in insects has only been demonstrated in *A. gambiae* where exposure to LPS increased binding activity in Sua1B cells. Of the two ferritin subunits characterized from *A. aegypti*, HCH has a 5'UTR IRE (Fiuza *et al.*, 1996; Geiser *et al.*, 2003) and since insect IRP/IRE binding activity in response to immune challenge has been examined in a singular insect (Zhang *et al.*, 2002), translational regulation of ferritin by IRP during an immune response is difficult to predict, warranting further analysis.

Much is known about the immune response in mosquitoes (Bartholomay *et al.*, 2004; Bartholomay *et al.*, 2010; Dimopoulos *et al.*, 2001; Fallon & Sun, 2001; Hillyer *et al.*, 2003a; Lowenberger, 2001; Michel & Kafatos, 2005; Osta *et al.*, 2004; Schnitger *et al.*, 2007; Vernick *et al.*, 2005; Xi *et al.*, 2008) and the effect of the response on fecundity (Hua *et al.*, 2007; Jiang *et al.*, 2009; Yu *et al.*, 2007). However, the regulation of ferritin and IRP in infection and following a blood meal is less clear. We report challenging mosquitoes with heat-killed bacteria (HKB) followed by a blood meal to determine the effects of these treatments in tandem on ferritin expression, and we demonstrate a synergistic, time-dependent, up-regulation of expression for HCH and LCH, as well as decreased IRP/IRE binding activity with iron and HKB challenge.

Materials and methods

Mosquito Rearing

Aedes aegypti (UGAL/Rockefeller strain) adult mosquitoes were routinely maintained at 27°C, 70%–80% relative humidity with a photoperiod of 16:8 h (L:D) on 10% sucrose solution *ad libitum*. As previously described (Zhou *et al.*, 2009), several hundred females four days old were injected with 69 nL of autoclaved gram-positive *Bacillus subtilis* suspension (6.9×10^5 bacteria/animal; heat-killed bacteria (HKB)) in mosquito saline (150 mmol/L NaCl, 4 mmol/L KCl, 3 mmol/L CaCl₂, 1.8 mmol/L NaHCO₃, 0.6 mmol/L MgCl₂, 25 mmol/L HEPES, pH 7.0) or 69 nL of mosquito saline as a naïve control. The injected mosquitoes were allowed to recover for 3 h and then fed on porcine blood administered by membrane feeding. Three independent samples of 15 female mosquitoes for each group were drawn at random from each treatment population at each time interval of 0, 24 and 72 h PBM; for each sample, fat body (FB), midgut (MG) and ovary (OV) tissues were dissected into and pooled in RLT Buffer (Qiagen) containing β -ME for total RNA extraction or disruption buffer (10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L KCl, 10 mmol/L NaCl, freshly added 2 \times Protease Inhibitor Cocktail Set I (Calbiochem) and 0.5 mM dithiothreitol) for protein analysis and frozen in liquid nitrogen, stored at –80°C.

Cell culture

Aedes aegypti (L.) epithelial-like, larval cells (CCL-125) were obtained from the American Type Culture Collection (ATCC). The cells were maintained as previously described (Geiser *et al.*, 2007). Briefly, at the start of each experiment the complete medium (75% DMEM high glucose (Invitrogen), 25% Sf-900 II SFM (Invitrogen), 15% heat-inactivated fetal bovine serum (Gemini Bio-Products) and 1% of 100 \times the antibiotic-antimycotic formula provided in the Invitrogen kit (Invitrogen)) was removed and the cells were washed twice with Hank's Balanced Salt Solution (HBSS; Invitrogen). Serum-free, antibiotic-antimycotic-free medium was placed on the cells and the cells were incubated for 1 h (28°C with 5% CO₂). Following this incubation the medium was replaced with fresh serum-free, antibiotics/antimycotics-free medium. An autoclaved bacterial mixture of *B. subtilis* and gram-negative *E. coli* (100 bacteria/cell) in HBSS was prepared (HKB). Experiments were conducted on thirty-six flasks of passage 85 subcultured CCL-125 cells. Flasks of cells taken at random were used for each treatment in an experiment and three experiments were conducted. Immune challenged cells were treated with HKB and HBSS (H), 100 μ mol/L ferric ammonium citrate (F or FAC, Sigma, 18.3% iron, 1 μ g Fe/ μ g FAC) dissolved in HBSS or 100 μ mol/L FAC with 100 μ M deferoxamine mesylate salt (F/D or FAC/DES, Sigma) prepared in HBSS. Naïve cells were treated with HBSS, FAC or FAC/DES and additional HBSS, the vehicle for the HKB, was added to make the treatment volume the same for all flasks. Cells were incubated for 4 or 22 h at 28°C in vented 75 cm² tissue culture flasks (Corning Incorporated). Since all cells at the time of harvest did not adhere, the medium was removed from the flask of cells and transferred to a 15 mL conical tube and centrifuged at 900 g for 10 min, 4°C. The supernatant was removed, flash frozen in liquid nitrogen and stored at –80°C for subsequent use. The remaining cells in the flasks were scraped into 5 mL HBSS, added to the cell pellet from the medium and suspended. The cell suspension was centrifuged at 900 g for 10 min, 4°C. The supernatant was removed and the cells were

suspended in 5 mL fresh HBSS. One ml ($\sim 1.5 \times 10^6$ cells) of the cell suspension was taken from each sample and microcentrifuged at 9000 g for 2 min, 4°C. The supernatant was removed and the cell pellet was frozen in liquid nitrogen and stored at -80°C for RNA isolation. Aliquots of cells were immediately taken to perform cell viability; total cell number and calcein quench assays. The remaining cells were centrifuged at 900 g for 10 min, 4°C. The supernatant was removed and the cell pellet was suspended in 250 μL hypotonic buffer (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl_2 , 10 mmol/L KCl, freshly added 2 \times Protease Inhibitor Cocktail Set I (Calbiochem) and 0.5 mmol/L dithiothreitol), transferred to a 1.5 mL microcentrifuge tube and frozen in liquid nitrogen for cell lysis.

Cell viability and cell number

Cell viability was determined in triplicate for all flasks by CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay per manufacturer's instructions (Promega Corporation). This assay measures the mitochondrial activity produced by metabolically active cells. Total cell number was measured in triplicate for all flasks by the LIVE/DEAD[®] Viability/Cytotoxicity Assay per manufacturer's instructions (Molecular Probes).

Iron determination

Calcein fluorescent quench by iron was used to measure iron uptake into cells as previously described (Geiser *et al.*, 2007). Fluorescent quench was measured in triplicate for all flasks by the LIVE/DEAD[®] Viability/Cytotoxicity Assay per manufacturer's instructions (Molecular Probes) by adding calcein to a final concentration of 1 $\mu\text{mol/L}$ and incubating for 45 min, 28°C.

Real-Time RT-PCR

Total RNA was isolated from $\sim 1.5 \times 10^6$ CCL-125 cells and mosquito tissues using the RNeasy[®] Mini Kit (Qiagen Inc.). Equal volume of each purified total RNA sample was treated with DNase (Invitrogen) according to the manufacturer's instructions for 30 min at 37°C and for 10 min at 65°C. Equal volume of each DNase-treated total RNA sample was used for real-time RT-PCR. Reverse transcription was done according to the manufacturer's instructions using M-MuLV Reverse Transcriptase from the First Strand cDNA Synthesis Kit (Fermentas, Inc.). The primers for the PCR reactions were designed to obtain specific PCR products of similar size for the ORF of each message: HCH (198 bp): 5'-ccagcccggaacaacag-3' and 3'-tcaaaagaaggtcggcg-5'; LCH (173 bp): 5'-ttcaccgcccagtttctca-3' and 3'-ctagaggctgtccggac-5'; Defensin A1 (DefA1; 211 bp): 5'-tgtcattgttctgctctg-3' and 3'-gcaaccactatcacgaacacga-5'; IRP (115 bp): 5'-aatcgaaggacagcgtgaag-3' and 3'-ccacagccaccataactcg-5'; S7 (184 bp): 5'-ggagatgaactcggacctga-3' and 3'-caagaggccgttcgtgca-5'. Real-time RT-PCR reactions were conducted using iQ[™] SYBR[®] Green Supermix (BIORAD) with the buffers provided, at: 94°C, 3 min, 1 cycle; 94°C, 10 sec; 60°C, 30 sec and 72°C, 30 sec, 40 cycles; with a melt curve over a temperature range starting at 55°C and ending at 95°C in a MyiQ[™] Cyclor (BIORAD). PCR product quality was monitored using post-PCR melt curve analysis. A standard curve for each product, performed simultaneously with experimental samples, demonstrated the experimental samples fell within the linear range. Data analysis of each

sample run twice (duplicates) for fold change was quantified using the Pfaffl (2001) method to calculate for relative quantification. The data are reported as fold change relative to a baseline of 1 established from the expression of these messages in HBSS-treated naïve cells incubated for either 4 or 22 h during the *in vitro* HKB treatment experiment or naïve females 0 h PBM during the *in vivo* HKB challenge experiment. All PCR products were cloned and sequenced to determine that the product sequence represents that of the desired message.

Protein extracts

Animal tissue samples suspended in ~150 μ L disruption buffer were frozen at -80°C , thawed on ice and homogenized, three times. The tissue samples were then centrifuged at $100000\times g$ for 30 min, 4°C ; after spinning the supernatant was transferred to a new tube labeled cytoplasmic extract and the pellet in the original tube was suspended in 100 μ L disruption buffer and labeled membrane extract. Cell media samples were concentrated five-fold by centrifuging samples in Centricon[®] Ultracel YM-30 centrifugal filter devices (Millipore) at $5000 g$ for ~20 min, 4°C . Cell extracts were prepared as previously described with some modification (Geiser *et al.*, 2009). Briefly, cell pellets suspended in hypotonic buffer were frozen at -80°C , thawed on ice and homogenized, three times. Animal tissue, and cell extracts and medium protein concentrations were determined by the method of Bradford (1976). Membrane and cytoplasmic extracts from OV were recombined after determining the protein content of the membranes was low. The membrane extracts from FB and MG were further processed by Triton extraction to release proteins from the membranes (Patton *et al.*, 2005). Briefly, Triton X-100 (Sigma) was added to the membrane samples to a concentration of 0.5% (v/v) in disruption buffer with $2\times$ Protease Inhibitor Cocktail Set I (Calbiochem), incubated for 1 h at 4°C and centrifuged at $20000\times g$ for 5 min, 4°C . Cytoplasmic and membrane extracts from MG were treated with methyl ethyl ketone (MEK) to remove the remaining heme from the blood meal that could potentially interfere with protein detection in the samples. Briefly, MEK was added to samples 3:1 (v/v), vortexed, allowed to incubate for 10 min at RT, centrifuged at $20000\times g$ for 5 min at RT and the aqueous, lower phase was collected. The concentrated media samples, cell and animal tissue extracts were frozen in small aliquots and held at -80°C until use.

Western blots

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 17% homogeneous slab gels, run at 60 volts overnight ($>1000 \text{ Vh}$), 4°C . Cell extracts were loaded at 15 μ g total protein and concentrated media samples at 6 μ g total protein. Animal tissue equivalents were loaded as follows: OV extracts (2 animals), FB cytoplasmic extracts (4 animals for 0 and 72 h; 1 animal for 24 h), FB membrane extracts (4 animals), MG cytoplasmic extracts (2 animals) and MG membrane extracts (1 animal for 24 h, 2 animals for 0 and 72 h). Ferritin and IRP bands were normalized to actin as an internal control for cell extracts and FB cytoplasmic and membrane extracts. However, since no internal control protein is available for use for cell culture medium, and OV and MG actin was not detectable, purified FLAG with bacterial alkaline phosphatase (25 ng media samples; 75 ng OV and MG samples; Sigma) was added as an exogenous loading control. Proteins were transferred to nitrocellulose membranes in the Electrophoretic Blotting System (C.B.S. Scientific Company). Efficient transfer of proteins was confirmed by

SYPRO® Ruby protein blot stain (BIORAD) and Kaleidoscope molecular weight markers (BIORAD). The nitrocellulose membranes were cut for analysis of the individual proteins. The ferritin nitrocellulose membranes were blocked overnight in 4.0% non-fat dry milk, 25 mmol/L phosphate, 25 mmol/L acetate buffer, 0.02% sodium azide pH 7.0 at 4°C. After blocking, the nitrocellulose membranes were incubated with anti-*A. aegypti* ferritin-specific rabbit serum (1:2000 v/v, a kind gift from Dr. John Law) diluted in blocking buffer for 2 h, RT. The nitrocellulose membranes were washed three times in 25 mmol/L phosphate, 25 mmol/L acetate, 0.02% sodium azide, pH 7.0 and developed with anti-rabbit alkaline phosphatase conjugate antibody (1:1000 v/v; Jackson Immuno) according to the manufacturer's methods (BIORAD). The IRP, FLAG and actin membranes were blocked overnight in 10 mmol/L phosphate-buffered saline and 0.05% Triton X-100 (PTX) with 3% BSA and 7% non-fat dry milk at 10°C. Membranes were incubated with either anti-KLH-conjugated-IRP-epitope-specific rabbit serum (1:2000 v/v; CKNQDLEFERNKERF; Sigma Genosys), anti-FLAG-specific rabbit serum (1:4000 v/v; Sigma) or anti- β -actin-specific mouse serum (1:2000 v/v; Abcam) diluted in PTX with 3% BSA for 2 h, RT. The membranes were washed three times and developed with either anti-rabbit alkaline phosphatase conjugate antibody (1:1000 v/v; Jackson Immuno) or anti-mouse alkaline phosphatase conjugate antibody (1:1000 v/v; Jackson Immuno). Digital images were accessed using the VersaDoc™ 3000 Imaging System (BIORAD) and quantified with Quantity One 4.5.1 software (BIORAD). Data were analyzed by densitometry of a defined volume of all the ferritin subunits, IRP, FLAG and actin proteins and corrected for background. Ferritin or IRP concentrations were evaluated by taking their volumes in ratio to an exogenous (FLAG) or endogenous (actin) loading control. FB, OV and MG samples volumes were corrected for the number of animal tissue equivalents. Volume units are arbitrarily assigned.

Electrophoretic mobility shift assays (EMSA)

EMSA were performed as described by Schalsinske and Eisenstein (1996) with modifications for mosquito samples (Geiser *et al.*, 2007; Geiser *et al.*, 2006; Zhang *et al.*, 2002). IRP1/IRE interactions were measured by incubating a molar excess of radio-labeled probe with cell or tissue extract. Briefly, 2.5 μ g of cell extract or animal tissue equivalents (1 animal for OV extracts, 1 animal for FB cytoplasmic extract, 1 animal for FB membrane extracts, 1 animal for MG cytoplasmic and membrane extracts combined) and 50 fmol of *Aedes* ferritin heavy chain IRE [³²P] labeled-transcript were incubated in 37.5 mmol/L KCl-Hepes, 12.5 mmol/L DTT, 1.9 mmol/L MgCl₂, 6.3% glycerol and 0.6 U RNase Inhibitor (Invitrogen) for 20 min at RT. RNase T₁ (6 Units) was added and the mixture incubated for 10 min at RT. Finally, heparin (100 μ g) was added and the sample incubated for 10 min at RT. All samples were run on 6.5% polyacrylamide gels, and IRP/IRE binding activity was assessed by autoradiography exposure to Blue XB-1 film (Eastman Kodak Company). Digital images were accessed using a VersaDoc™ 3000 Imaging System (BIORAD) and quantified with Quantity One 4.5.1 software (BIORAD). Data were analyzed by peak density of defined bands and corrected for background. Density units are arbitrarily assigned.

Data analysis

Protein extracts of samples were analyzed by Western blot or EMSA and combined data for a given time and treatment was reported as the mean and standard deviation (StD). For real time PCR, RNA extracts were analyzed twice, and for a given time and treatment both results were included in calculations of the mean and StD. Significance of time differences (0, 24, 72 h PBM) in animals or iron treatments (H, F, F/D) in cells was determined by one-way analysis of variance (ANOVA) using the Tukey's multiple comparisons test. Treatment differences (Naïve, HKB Challenge) in means for a given time point or iron treatment were determined by a one-tailed unpaired *t*-test for comparison of selected data sets (Graph Pad Software, Inc.). All cell culture experiments were conducted in triplicate and the data for a given variable were analyzed at the same time.

Results

Heat-killed bacteria induce an immune response in CCL-125 cells and mosquito tissues

Challenge with heat-killed bacteria (HKB) provoked an immune response in CCL-125 cells and female yellow fever mosquitoes as demonstrated by an increase in message levels of the antimicrobial peptide defensin A1 (DefA1) in cells and tissues. HKB challenge up-regulated DefA1 message of CCL-125 cells at 4 h ($P < 0.008$) and 22 h ($P < 0.03$) and confirmed the presence of an immune response (Fig. 1A). Iron-treatment with 100 $\mu\text{mol/L}$ ferric ammonium citrate (FAC) alone also increased *DefA1* at 22 h ($P < 0.05$). *DefA1* in mosquitoes increased in fat body (FB) with time post-blood meal (PBM) by 24 h in both naïve and HKB challenged animals. The response was significantly greater in HKB challenge relative to that of naïve animals at 24 h PBM ($P < 0.005$; Fig. 1B). Mosquitoes challenged with HKB also showed a significant increase in DefA1 message at 24 h relative to that of naïve mosquitoes for both midgut (MG; $P < 0.04$) and ovary (OV; $P = 0.067$; Fig. 1B). *DefA1* increased by 24 h in all three tissues, but remained elevated at 72 h only in FB. Thus, HKB challenge in cells and mosquitoes induces an immune response as measured by an increase in DefA1 mRNA where FB is the primary site of transcript expression.

HKB challenge differentially regulates ferritin transcript expression in CCL-125 cells and mosquito tissues

HKB challenge in the presence of iron significantly up-regulated LCH message at 22 h relative to levels for cells treated with either HKB or FAC alone ($P < 0.02$), and the addition of a ferric iron chelator, 100 $\mu\text{mol/L}$ deferoxamine mesylate salt (DES), reduced the up-regulation ($P < 0.05$) indicating iron was partially responsible for this effect (Fig. 2A). HCH message also showed significant up-regulation for cultures treated with HKB and iron relative to those treated with iron alone ($P = 0.05$; Fig. 2B). At 22 h, HCH message was only partially reduced by the addition of DES indicating that HKB and iron synergistically up-regulated this message (Fig. 2B). The observed regulation of LCH and HCH transcript expression was not a result of detrimental effects of the treatments on these cells since differences in cell numbers were less than 10% as a result of any treatment (Fig. 1SA). Although the addition of iron reduced viability by not more than 20% in both naïve and HKB challenged cells, this condition is resolved by 22 h (Fig. 1SB). Calcein quench analyses confirmed uptake of iron into the cells by 4 h and retention at 22 h in the presence

or absence of HKB and showed that HKB challenge did not alter the levels of the iron pool accessible by calcein (Fig. 1SC).

Comparison of the means for naïve and HKB challenged mosquitoes showed a synergistic increase for MG HCH and LCH message following blood feeding with HKB challenge. In MG, ferritin LCH message increased with time PBM and peaked at 24 h (Fig. 2C). Using 0 h for naïve mosquitoes as controls, HKB challenged MG LCH message doubled at 0 h, increased significantly to 56-fold by 24 h, and then declined by 72 h. The increase in LCH message appeared to be greater for HKB challenged mosquitoes versus naïve at 24h PBM; however values did not reach significance. Message levels for the ferritin HCH also increased with time PBM for naïve and infected animals; within either group the increase reached significance at 24 h (9-fold increase in naïve mosquitoes and a 12-fold increase in HKB challenged mosquitoes), then declined significantly by 72 h. A comparison of infected to naïve mosquitoes at each time interval suggested a greater positive message response occurred in infected animals, but values did not reach significance (Fig. 2D). Thus, both HCH and LCH messages show a positive response to iron exposure and to bacterial challenge. MG is the primary site of ferritin subunit transcription and message levels for either the LCH or HCH PBM were an order of magnitude greater than those for FB or OV (Fig. 2C–D). HCH and LCH messages also generally increased over time in FB and OV, and reached significance for the *HCH* at 24 h for FB for HKB challenged mosquitoes relative to 0 h for the same treatment group.

Mosquito ferritin is up-regulated in tissues PBM; HKB challenge and iron cause synergistic up-regulation of ferritin expression in CCL-125 cells

Ferritin protein levels were examined in three tissues. FB cytoplasmic ferritin increased by 72 h PBM, and was greatest for animals treated with HKB (Fig. 3A). FB membrane ferritin increased significantly 24 h PBM in naïve ($P = 0.053$) and HKB challenged ($P < 0.05$) animals and levels were sustained at 72 h in naïve animals and further increased in animals treated with HKB (Fig. 3A). Thus, despite that LCH and HCH message levels declined by 72 h in FB for animals treated with HKB, protein levels were maintained or increased in this tissue. In OV, ferritin increased with time PBM, was maximal at 72 h ($P < 0.0001$) and HKB challenge appeared to reduce this response at 72 h PBM ($P < 0.02$; Fig. 3B). Ferritin increased in MG in both cytoplasmic ($P < 0.001$) and membrane ($P < 0.01$) fractions 24 h PBM and decreased by 72 h (Fig. 3C). Levels of membrane ferritin were greater for mosquitoes treated with HKB 72 h PBM ($P = 0.053$) than for naïve animals indicating a synergistic up-regulation of ferritin by blood feeding and HKB treatment (Fig. 3C) for this tissue. We conclude from these data that ferritin protein expression is tissue specific and sensitive to blood feeding and HKB challenge.

In CCL-125 cells, ferritin increased significantly with time for all treatments (Fig. 4A). By 22 h, ferritin was greatest for cells treated with HKB and iron together ($P < 0.02$; Fig. 4A). Secreted ferritin also was greatest for cells treated with HKB and iron at 22 h ($P < 0.02$; Fig. 4B). From these data we conclude, that challenge of CCL-125 cells with HKB in the presence of iron provokes a synergistic up-regulation of ferritin that is reflected by an increase in cell-associated and secreted protein.

Mosquito IRP/IRE binding activity increases early in midgut and fat body and later in ovary PBM

We have previously demonstrated in CCL-125 cells that IRP/IRE binding activity is generally not responsive to iron dose or chelation (Geiser *et al.*, 2007; Geiser *et al.*, 2006). We also confirmed that IRP expression is not sensitive to these treatments by a separate set of experiments (Fig. 2SA–B; Geiser *et al.*, 2009). However, IRP/IRE binding activity is a sensitive measure of active IRP protein in tissues. We have previously shown that reducing agents, such as β -mercaptoethanol, do not alter binding activity of mosquito IRP (Geiser *et al.*, 2006), which is consistent with earlier studies characterizing *Drosophila* IRP (Gray *et al.*, 1996). Thus, we quantify only total available binding activity in these samples.

MG IRP/IRE binding activity increased at 24 h PBM, and was sustained at 72 h (Fig. 5A). Evaluation of MG IRP transcript indicated message increased 24 h PBM, but then declined (Fig. 3SA). This implies that a change occurs in the protein that allows sustained IRP/IRE binding activity despite decreased transcript levels at 72 h PBM. In MG, neither IRP/IRE binding activity nor IRP message levels were responsive to HKB treatment. In OV, IRP/IRE binding activity increased with time PBM and was unresponsive to HKB treatment (Fig. 5B). OV IRP message levels increase 72 h PBM and HKB treatment reduced this response (Fig. 3SA).

For FB, we evaluated protein and IRP/IRE binding activity in each fraction. Cytoplasmic IRP increased with time PBM, while membrane IRP increased and stabilized 24 h PBM (Fig. 5C). FB IRP levels were unresponsive to HKB challenge. IRP/IRE binding activity also increased in cytoplasmic and membrane fractions 24 h PBM, but decline by 72 h and was unresponsive to HKB treatment (Fig. 5D). In FB, IRP mRNA levels increased and were maximal by 72 h PBM ($P < 0.05$) and as with the other tissues HKB treatment reduced this response ($P < 0.002$; Fig. 3SA). In summary, IRP/IRE binding activity and IRP expression increase PBM in FB, MG and OV, but that each tissue follows a different time course. IRP/IRE binding activity and FB IRP is unresponsive to HKB treatment PBM in all tissues we examined. However, IRP message up-regulation PBM is reduced in each tissue by HKB.

HKB challenge and iron decreases IRP/IRE binding activity with no effect on IRP expression in CCL-125 cells

In CCL-125 cells, IRP increased with culture time, but was not responsive to stimulation with iron or HKB (Fig. 6A). Cell IRP/IRE binding activity was reduced by HKB challenge in the presence of iron at 4 h relative to cells treated with either HKB or iron. The administration of DES with FAC partially mitigated this effect (Fig. 6B). By 22 h, HKB challenge reduced IRP/IRE binding activity in the presence ($P < 0.03$) or absence ($P < 0.04$) of iron treatment compared to that of naïve cells (Fig. 6B). Neither HKB challenge nor iron-treatment altered cell IRP mRNA levels by 4 h (Fig. 3SB). By 22 h, iron administration down-regulated IRP message, while levels were unresponsive to HKB challenge (Fig. 3SB). While IRP expression increased with culture time, it was unresponsive to stimulation with iron or HKB. HKB challenge reduces IRP/IRE binding activity and has an early effect in the presence of iron. With time, the iron effect is stabilized and HKB down-regulation of IRP/IRE binding activity is sustained in the presence or absence of iron. This could be a

factor in the up-regulation of HCH synthesis in response to HKB challenge in the presence of iron.

Discussion

Mosquitoes are exposed to a variety of pathogens (i.e. viruses, bacteria, parasites, fungi). For these insects to survive and to thrive in this hostile environment an adequate immune response is required at all developmental stages. Insects are equipped with a highly efficient immune system that is activated quickly after a pathogenic invasion and encompasses many defensive strategies including phagocytic cells in the hemolymph; opsonization, clotting and melanization propagated by proteolytic cascades, and induced synthesis of potent antimicrobial peptides (Chenet *et al.*, 2010). Several insightful reviews have been published discussing the various aspects of insect immunity including those focused on vectors of human disease, such as mosquitoes (Abraham & Jacobs-Lorena, 2004; Chen *et al.*, 2010; Christophides *et al.*, 2004; Fallon & Sun, 2001; Lowenberger, 2001; Michel & Kafatos, 2005; Osta *et al.*, 2004). However, relatively few focus on the role of iron metabolism during the course of an immune response. Hematophagous insects consume a blood meal for a variety of reasons including egg development. Blood feeding provides the nutrients required for oogenesis, yet this practice is inherently dangerous to insects in that they are exposed to pathogens, oxidative stress and a high concentration of heme in the blood meal (Sanders *et al.*, 2003). Here we examined the effects of an immune response to HKB on ferritin, the major insect iron storage and transport protein, in *A. aegypti* larval cells and adult female tissues.

Our results indicate that the synthesis of DefA1 is a product of HKB challenge and not iron treatment, in CCL-125 cells as well as adult tissues where the magnitude of DefA1 mRNA up-regulation is substantially greater for tissues from infected animals relative to those from naïve animals. It has previously been observed that DefA1 expression in *Aedes* FB is similar in blood-fed and non-blood-fed mosquitoes (Xiao *et al.*, 2010). In HKB challenged mosquitoes, FB DefA1 transcript increased over time PBM, this has been demonstrated previously and is expected since DefA1 is one of several antimicrobial peptides produced by the FB in response to pathogens (Gao *et al.*, 1999; Magalhaes *et al.*, 2008; Meredith *et al.*, 2006). In HKB challenged mosquitoes, a differential expression pattern for DefA1 mRNA response is observed in MG and OV that likely reflects that these tissues are not typically responsible for the majority of immune defense, and are generally dedicated to processing the nutrients from the blood-meal for the purpose of oogenesis. This also may indicate that limited resources are dedicated to mounting an immune response in the MG and OV tissues in order to promote fecundity. This phenomenon has been reported in *A. gambiae* inoculated with lipopolysaccharide (LPS; (Jiang *et al.*, 2009)), mosquito vector for Western Equine encephalitis (*Culex tarsalis*) infected with West Nile virus (Yu *et al.*, 2007), and *A. aegypti* orally infected with dengue virus serotype 2 (Hua *et al.*, 2007). Thus, data support that the synthesis of DefA1 in tissues is sensitive to HKB challenge, while the blood meal has little effect on transcript expression.

As reported, ferritin is the major protein for sequestering and trafficking iron in insects and expression is induced by the availability of iron in cells and tissues. Previous work in

mosquitoes showed that *iron* treatment provokes an increase in ferritin expression (Li *et al.*, 2002). In order to confirm this and to determine whether *iron* was involved in the mechanism for the regulation of ferritin expression in response to blood feeding and HKB challenge; we evaluated ferritin expression of mosquito cells following treatment with iron, HKB or both. In CCL-125 cells, both the LCH and HCH subunit transcripts are increased by iron exposure. HKB challenge with iron supplementation significantly up-regulates transcripts for both subunits. In MG, LCH and HCH transcripts are increased at 24 h PBM and subsequently decreased by 72 h PBM. Whereas, FB and OV tissues demonstrate an increase in both subunit transcripts in naïve animals over time PBM; this pattern is suppressed in the HKB challenged mosquitoes. The findings in bumblebee (*Bombus ignitus*) demonstrated HCH message is up-regulated during conditions of wounding; bacterial challenge and iron overload (Wang *et al.*, 2009). Transcription of *B. ignitus* HCH is regulated in a time-dependent manner as well (Wang *et al.*, 2009).

The complex picture of ferritin message expression in mosquitoes could be a product of several regulatory schemes. Ferritin transcript expression is clearly a function of tissue specific regulation and MG LCH and HCH transcripts are an order of magnitude higher 24 h PBM than FB and OV. The MG is the major tissue for ferritin expression and secretion that is responsible for detoxifying and processing iron from the blood meal and transporting it to other tissues (Zhou *et al.*, 2007). The transcriptional regulation of both subunits is complicated by the head-to-head orientation of the *LCH* and *HCH* on the same chromosome and both share a common promoter sequence with no intervening insulator (Geiser *et al.*, 2003). This suggests the expression of the *Aedes ferritin* genes are most likely coordinately controlled (Pham & Chavez, 2005).

The influence of the immune response on the transcriptional regulation of the *Aedes LCH* and *HCH* may account for some of the differential regulation observed between naïve and HKB challenged cells and tissues. Further, it has been demonstrated in *Aedes* as well as *Anopheles* mosquitoes that the primary *cis*-regulatory elements (CRE) responsible for the induced expression of *defensin* in immune regulation bind NF κ B (nuclear factor κ B) and C/EBP (CCAAT/enhancer binding protein) (Meredith *et al.*, 2006) while NF-IL6 (nuclear factor interleukin 6), ICRE (interferon consensus response elements), NF-ELAM1 (nuclear factor endothelial leukocyte adhesion molecule 1), HNF-5 (hepatic nuclear factor 5) and GATA may also bind putative CREs identified in the *defensin* promoter (Cho *et al.*, 1997; Eggleston *et al.*, 2000). Several canonical immune-related CREs are identified in the shared promoter regions of the *Aedes* and *Drosophila LCH* and *HCH* genes that could interact with GATA, C/EBP, NF κ B/c-rel (Relish), and IRFs (interferon regulatory factors) (Fig. 4S; Dunkov & Georgieva, 1999; Pham *et al.*, 2003; Pham & Chavez, 2005). Other CREs in the shared promoter also could influence transcript regulation. Canonical moieties that bind transcription factors involved in the response to metals and ecdysone also have been identified in both the *Aedes* and *Drosophila* ferritin shared promoters (Fig. 4S; Dunkov & Georgieva, 1999; Pham *et al.*, 2003; Pham & Chavez, 2005).

A clear picture of the impact of the innate immune response on the regulation of *Aedes LCH* and *HCH* genes requires an examination of protein expression. The divergent expression of ferritin for different tissues undoubtedly reflects the timing of iron delivery from the blood

meal, the levels of iron delivery from the blood meal and the role of the tissue in iron homeostasis. Within 72 h PBM, absorption of iron from the diet is complete and unabsorbed iron is excreted accounting for the decline in ferritin in MG at this time point (Zhou *et al.*, 2007). Our data confirm that the high levels of ferritin in MG 24 h PBM reflect the requirement for ferritin as an iron delivery protein as well as early exposure of this tissue to the high iron levels of the blood meal. More than half of absorbed iron is transported to the ovaries and eggs, while the remainder is distributed among the other tissues (Zhou *et al.*, 2007). Thus, the peripheral tissues show an increase in ferritin at a later time when sufficient iron is delivered to the tissue to induce ferritin for iron storage. Once iron storage in ferritin is achieved we might expect that the primary stimulus for sustaining message levels would be reduced and message levels would decline while protein levels would be sustained or increased. Ferritin message levels are likely more sensitive to iron levels than protein levels, as the latter reflects not only storage requirements, but also synthesis, degradation and secretion. Ferritin is a relatively stable protein (Jensen *et al.*, 2002; Jensen *et al.*, 2003).

We evaluated ferritin in either the cytoplasmic or membrane compartments for MG and FB because our previous work established expression in both compartments in mosquito cells (Geiser *et al.*, 2007; Geiser *et al.*, 2009). Ferritin found in cell cytoplasm in insects probably functions to sequester and to store iron reducing potential oxidative stress and making iron available when needed as a cofactor in newly synthesized proteins. Whereas ferritin in cell membrane compartments could serve a role in long term iron storage, but more likely provides secreted ferritin for iron transport among tissues as needed. In keeping with these hypotheses, ferritin in MG compartments followed a time course reflecting iron levels PBM for this tissue, while ferritin expression in FB suggests this tissue could be the primary site of longer term iron storage for mosquitoes. We did not evaluate carcass as part of this work, and thus, the primary site of iron storage in mosquitoes remains inconclusive.

The variable response of ferritin expression and regulation observed *in vivo* was further analyzed *in vitro* with cell culture experiments to observe the mechanistic regulatory effects of HKB challenge in the presence of iron. Both the *in vivo* and *in vitro* work support a synergistic effect of HKB challenge and iron in up-regulation of ferritin. This was observed in cells and in animals, and supports the hypothesis that ferritin is cytoprotective, and in hemolymph could serve a role in sequestering iron away from pathogens in the hemocoel. This is consistent with our previous work that showed that ferritin is up-regulated as a cytoprotective compound (Geiser *et al.*, 2003; Geiser *et al.*, 2006), as well as that of others who demonstrated increased ferritin secreted into the humoral fluids of the invertebrate, *Amphioxus* (*Branchiostoma belcheri*), when exposed to iron and lipopolysaccharide (LPS) challenge (Li *et al.*, 2008). Although up-regulation of ferritin for iron delivery PBM is an early response, ferritin up-regulation by HKB challenge in cells or animals occurs later. This could support a role for ferritin in sequestering iron from pathogens. This hypothesis is supported by findings of others in flesh fly *Sarcophaga bullata* (Masova *et al.*, 2010), sea bass *Dicentrarchus labrax* (Ishiguro *et al.*, 2007), red flour beetle *Tribolium castaneum* (Vila *et al.*, 2005) and sand flies *Lutzomyia longipalpis* (Yuan *et al.*, 2006).

We found that IRP/IRE binding activity in FB, OV and MG was unresponsive to HKB challenge and, in fact, was up-regulated following blood feeding. These data are similar to

our earlier *in vitro* work where iron treatment of CCL-125 cells did not provoke a reduction in IRP/IRE binding activity (Geiser *et al.*, 2007; Geiser *et al.*, 2006). In contrast to the IRP1 of mammals, the mosquito IRP does not form an iron sulfur cluster and is neither down-regulated nor degraded in response to increasing iron levels (Fig. 2SA–B; Geiser *et al.*, 2007; Geiser *et al.*, 2006). Differential regulation of IRP by cell compartment has been demonstrated in mammals (Patton *et al.*, 2005). Although IRP levels in FB were greater in the cytoplasm than membranes, expression in response to blood feeding and HKB challenge followed a similar time course. However, the changes in IRP protein were not reflected in IRP/IRE binding activity for the cytoplasmic and membrane compartments in this tissue implying a change in the mosquito IRP allowing for a reduction in IRP/IRE binding activity 72 h PBM for both naïve and HKB challenged mosquitoes in the presence of increasing levels of protein. The results in mosquitoes were similar to observations *in vitro* (mosquito cells), where IRP protein expression increased overtime for all treatments; however, while IRP/IRE binding activity is refractory to HKB challenge in mosquito tissues, CCL-125 cell binding activity decreases in the presence of HKB. This evidence supports that IRP/IRE binding activity does not necessarily reflect IRP message or protein levels and implies a modification in the protein that regulates binding activity (Vardhanet *et al.*, 2009). The unique behavior of mosquito IRP could be related to post-translational modifications, specifically phosphorylation of the protein. Mammalian IRP1/ 2 are phosphoproteins. Potential PKC phosphorylation sites in the mosquito IRP are found at residues 157-159 and Ser 711 (Zhang *et al.*, 2002), sites known to be phosphorylated in mammals (Brown *et al.*, 1998; Eisenstein, 2000; Eisenstein *et al.*, 1993; Schalinske & Eisenstein, 1996).

Complicating the picture for ferritin regulation in insects is the finding that the *Drosophila* HCH transcript has two different sequences, one with an IRE and one without, that occur by alternative splicing of the 5'-UTR (Dunkov & Georgieva, 1999). On examination, the HCH sequence predicts a similar configuration for the *A. aegypti* transcript that also shows two putative sequences, one with an IRE in the 5'-UTR and one without. Although splicing would influence the IRE presence, the ORF would remain unaffected. We have not yet completed RACE analysis of these transcripts to confirm that splicing occurs and under what conditions. Nonetheless, the expression of the HCH transcript without an IRE could allow for increased translation of this subunit even in the face of increased IRP/IRE binding activity and could explain the up-regulation of ferritin in cells and tissues exposed to HKB or iron in the presence of sustained or increased IRP/IRE binding activity. The *A. aegypti* LCH transcript has no IRE and regulation of expression is at the transcriptional level (Geiser *et al.*, 2003). The unpredictable responsiveness of IRP to HKB challenge lends support to the notion that although this protein can repress HCH translation, in insects control of ferritin synthesis is primarily transcriptional and the synergistic effects we observed in ferritin up-regulation with iron and HKB challenge are most likely mediated by transcriptional control factors (Dunkov & Georgieva, 1999; Pham *et al.*, 2003; Pham & Chavez, 2005). In any case, taken together, available evidence supports that the roles of IRP in mosquitoes have yet to be elucidated, and that its functions and response in these animals clearly differs from those for mammals (Geiser *et al.*, 2007; Geiser *et al.*, 2006; Zhang *et al.*, 2002).

We have characterized the message and protein expression profiles of ferritin subunits and IRP from the invertebrate disease vector, *A. aegypti*, in response to iron and bacterial challenge through *in vivo* and *in vitro* experimentation. Mammalian ferritin acts as a positive acute phase protein in reaction to infection, mosquito ferritin responds similarly. However, the regulatory pathway is not as defined or well understood in mosquitoes and requires further investigation into the activity of CREs in the shared *ferritin* promoter, the implications of alternatively spliced variants of the HCH subunit and the unique behavior of mosquito IRP to elucidate the role of iron and immunity in *A. aegypti*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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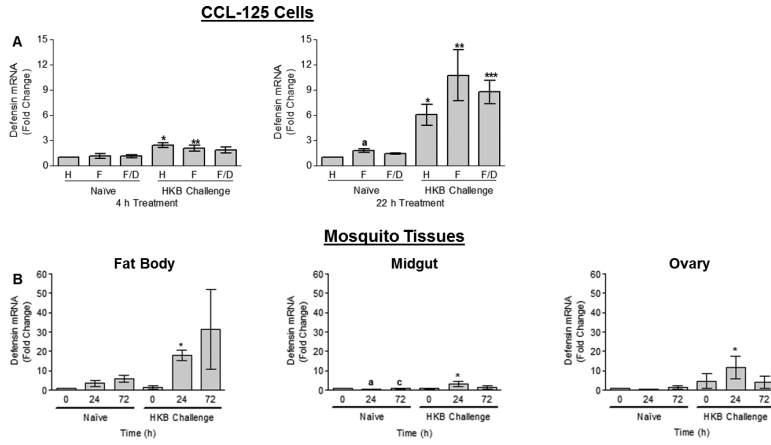
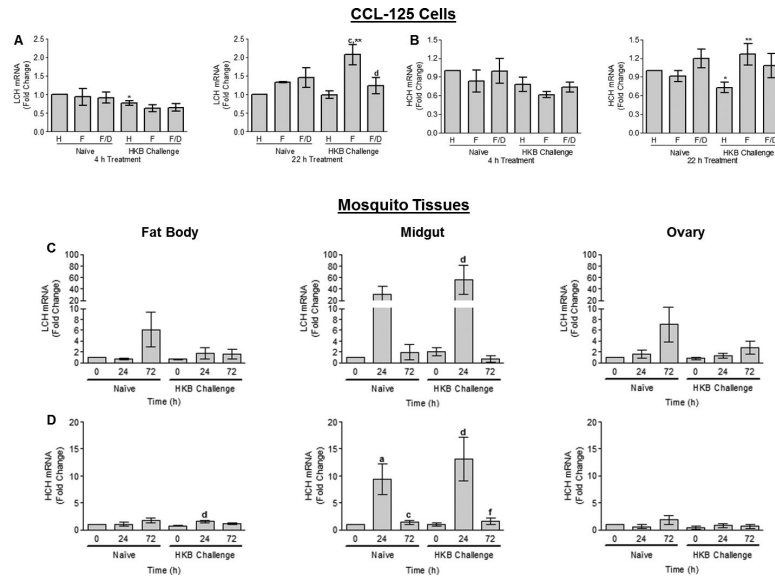
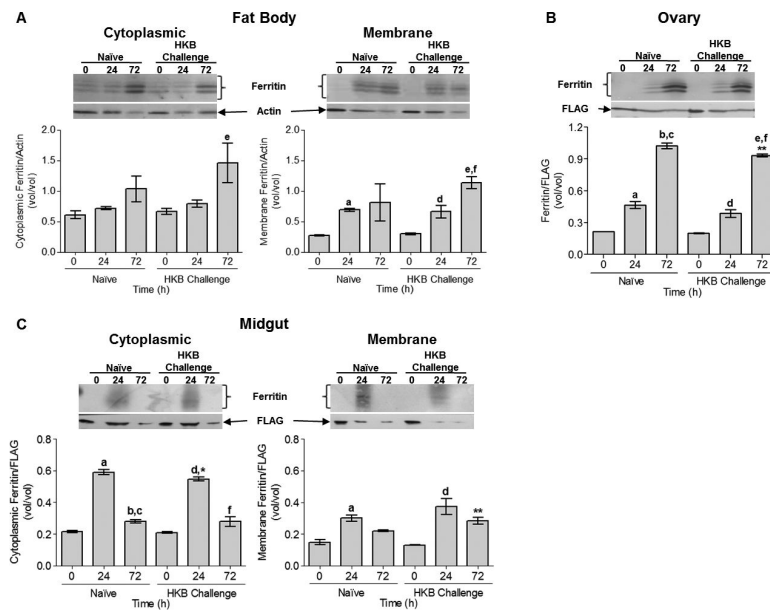


Fig. 1. Quantification of defensin A1 (DefA1) transcript expression by real-time RT-PCR in CCL-125 cells (A) and female yellow fever mosquito, *Aedes aegypti*, tissues (B) exposed to iron or a blood meal and heat-killed bacteria (HKB). (A) CCL-125 cells were treated with HBSS (H), 100 $\mu\text{mol/L}$ FAC (F) or 100 $\mu\text{mol/L}$ FAC/ 100 $\mu\text{mol/L}$ DES (F/D) as either naïve or HKB challenged cells. Cells were collected at 4 and 22 h post-treatment. *SD relative to naïve HBSS-treated cells 4 h ($P < 0.008$) and 22 h post-treatment ($P < 0.02$). **SD relative to naïve FAC-treated cells 4 h ($P = 0.056$) and 22 h post-treatment ($P < 0.03$). ^aSD relative to naïve 22 h HBSS-treated cells ($P < 0.05$). ^{**}SD relative to naïve 22 h FAC/DES-treated cells ($P < 0.007$). (B) Female mosquitoes were injected with either mosquito saline (naïve) or heat-killed bacteria (HKB challenge) 3 h prior to blood feeding. Fat body (FB), midgut (MG) and ovary (OV) tissues were collected at 0, 24 and 72 h post-blood meal (PBM) and processed. *Significantly different (SD) relative to 24 h PBM naïve animals in FB ($P < 0.005$), MG ($P < 0.04$) and OV ($P = 0.067$). ^aSD relative to naïve animals 0 h PBM ($P < 0.01$). ^cSD relative to naïve animals 24 h PBM ($P < 0.05$). Graphed data represent means \pm standard deviation (StD) of three independent cell culture samples and animal samples.

**Fig. 2.**

Quantification of ferritin light chain (LCH) and heavy chain homologue (HCH) transcript expression by real-time RT-PCR in CCL-125 cells (A–B) and female *A. aegypti* tissues (C–D) exposed to iron or a blood meal and HKB. (A–B) CCL-125 cells were treated with H, F or F/D as either naïve or HKB challenged cells. Cells were collected at 4 and 22 h post-treatment and processed. (A) CCL-125 cell LCH mRNA levels. ^{*}SD relative to naïve HBSS-treated cells ($P < 0.002$). ^cSD relative to HKB HBSS-treated cells ($P < 0.01$). ^{**}SD relative to naïve FAC-treated cells ($P < 0.02$). ^dSD relative to HKB FAC-treated cells ($P < 0.05$). (B) CCL-125 cell HCH mRNA levels. ^{*}SD relative to naïve HBSS-treated cells ($P < 0.009$). ^{**}SD relative to naïve FAC-treated cells ($P = 0.05$). (C–D) Female mosquitoes were either naïve or HKB challenged 3 h prior to blood feeding. FB, MG and OV tissues were collected at 0, 24 and 72 h PBM and processed. (C) Female mosquito tissues LCH mRNA levels. ^dSD relative to HKB treated animals 0 h PBM ($P = 0.057$). (D) Female mosquito tissues HCH mRNA levels. ^dSD relative to HKB animals 0 h PBM in FB ($P < 0.05$) and MG ($P < 0.05$). ^aSD relative to naïve animals 0 h PBM ($P < 0.05$). ^cSD relative to naïve animals 24 h PBM ($P < 0.05$). ^fSD relative to HKB animals 24 h PBM ($P < 0.05$). Graphed data represent means \pm StD of three independent cell culture samples and animal samples.

**Fig. 3.**

Tissue expression of ferritin protein from female *A. aegypti* exposed to a blood meal and HKB was analyzed by Western blot. Female mosquitoes were naïve or challenged with HKB 3 h prior to blood feeding. FB, MG and OV tissues were collected at 0, 24 and 72 h PBM and processed. (A) FB cytoplasmic and membrane ferritin protein levels. ^eSD relative to HKB animals 0 h PBM for cytoplasmic ($P = 0.052$) and membrane extracts ($P < 0.001$). ^aSD relative to naïve animals 0 h PBM ($P = 0.053$). ^dSD relative to HKB animals 0 h PBM ($P < 0.05$). ^fSD relative to HKB animals 24 h PBM ($P < 0.05$). (B) OV tissue ferritin protein levels. ^aSD relative to naïve animals 0 h PBM ($P < 0.01$). ^bSD relative to naïve animals 0 h PBM ($P < 0.001$). ^cSD relative to naïve animals 24 h PBM ($P < 0.001$). ^dSD relative to HKB animals 0 h PBM ($P < 0.01$). ^eSD relative to HKB animals 0 h PBM ($P < 0.001$). ^fSD relative to HKB animals 24 h PBM ($P < 0.001$). (C) MG cytoplasmic and membrane ferritin protein levels. ^aSD relative to naïve animals 0 h PBM for cytoplasmic ($P < 0.001$) and membrane extracts ($P < 0.01$). ^bSD relative to naïve animals 0 h PBM ($P < 0.05$). ^cSD relative to naïve animals 24 h PBM ($P < 0.001$). ^dSD relative to HKB animals 0 h PBM for cytoplasmic ($P < 0.001$) and membrane extracts ($P < 0.01$). ^fSD relative to HKB animals 24 h PBM ($P < 0.001$). Cytoplasmic ferritin levels are lower in HKB animals 24 h PBM ($*P = 0.052$) relative to naïve animals and membrane ferritin levels are greater in HKB animals 72 h PBM ($**P = 0.052$) relative to naïve animals. A representative Western blot is shown above the graphed data. Graphed data represent means \pm StD of three independent animal samples.

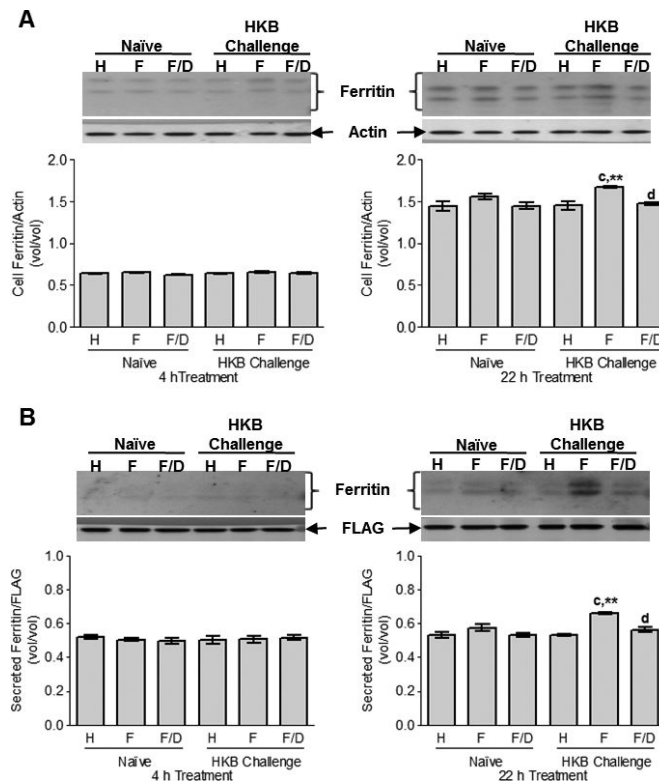


Fig. 4. CCL-125 cell-associated expression (A) and secretion (B) of ferritin protein with exposure to iron and HKB was analyzed by Western blot. CCL-125 cells were treated with H, F or F/D as either naïve or HKB challenged cells. Cells and medium were collected at 4 and 22 h post-treatment and processed. (A) Cell-associated ferritin levels. ^cSD relative to HKB HBSS-treated cells ($P < 0.01$). ^{**}SD relative to naïve FAC-treated cells ($P < 0.02$). ^dSD relative to HKB FAC-treated cells ($P < 0.05$). (B) Cell secreted ferritin levels in the media. ^cSD relative to HKB HBSS-treated cells ($P < 0.001$). ^{**}SD relative to naïve FAC-treated cells ($P < 0.02$). ^dSD relative to HKB FAC-treated cells ($P < 0.01$). A representative Western blot is shown above the graphed data. Data represent means \pm StD of three independent cell culture samples.

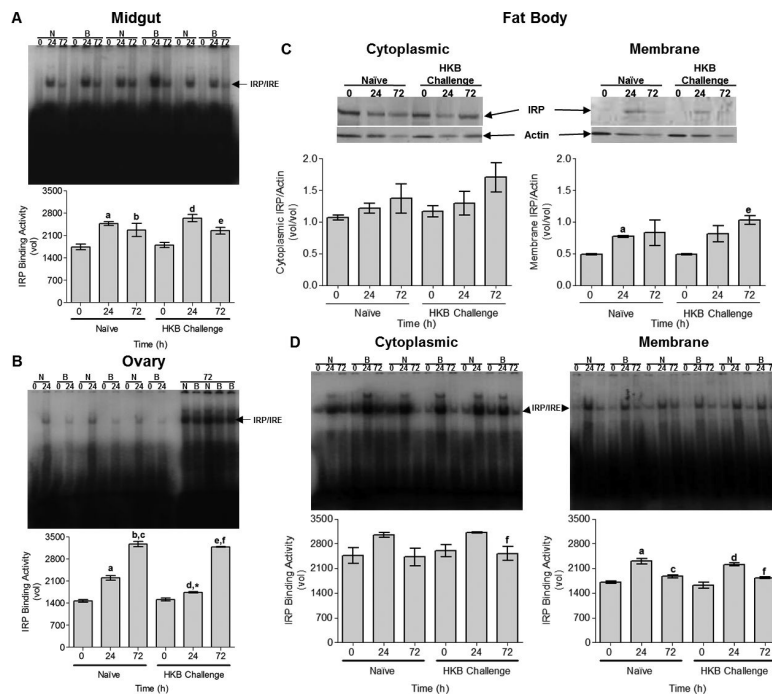


Fig. 5. Tissue iron regulatory protein (IRP)/ iron responsive element (IRE) binding activity (A–B, D) and FB IRP expression (C) from female *A. aegypti* exposed to a blood meal and HKB was analyzed by electrophoretic mobility shift assay (EMSA) and Western blot, respectively. Female mosquitoes were naïve or challenged with HKB 3 h prior to blood feeding. FB, MG and OV tissues were collected at 0, 24 and 72 h PBM and processed. (A) MG tissue IRP/IRE binding activity. ^aSD relative to naïve animals 0 h PBM ($P < 0.01$). ^bSD relative to naïve animals 0 h PBM ($P < 0.05$). ^dSD relative to HKB animals 0 h PBM ($P < 0.01$). ^eSD relative to HKB animals 0 h PBM ($P < 0.05$). (B) OV tissue IRP/IRE binding activity. ^aSD relative to naïve animals 0 h PBM ($P < 0.001$). ^bSD relative to naïve animals 0 h PBM ($P < 0.001$). ^cSD relative to naïve animals 24 h PBM ($P < 0.001$). ^dSD relative to HKB animals 0 h PBM ($P < 0.01$). ^{*}SD relative to naïve animals 24 h PBM ($P < 0.002$). ^eSD relative to HKB animals 0 h PBM ($P < 0.001$). ^fSD relative to HKB animals 24 h PBM ($P < 0.001$). (C) FB cytoplasmic and membrane IRP expression levels. ^aSD relative to naïve animals 0 h PBM ($P = 0.054$). ^eSD relative to HKB animals 0 h PBM ($P < 0.02$). A representative Western blot is shown above the graphed data. (D) FB cytoplasmic and membrane IRP/IRE binding activity. ^fSD relative to HKB animals 24 h PBM in cytoplasmic ($P = 0.057$) and membrane extracts ($P < 0.01$). ^aSD relative to naïve animals 0 h PBM ($P < 0.01$). ^cSD relative to naïve animals 24 h PBM ($P < 0.05$). ^dSD relative to HKB animals 0 h PBM ($P < 0.001$). Each tissue EMSA gel autoradiographs are shown above the graphed data. Graphed data represent means \pm StD of three independent animal samples.

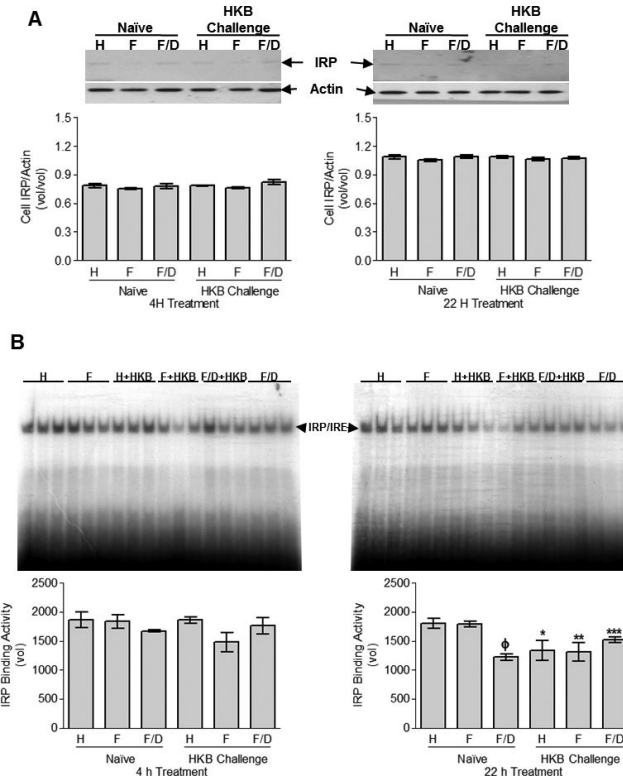


Fig. 6. CCL-125 cell-associated IRP expression (A) and IRP/IRE binding activity (B) with exposure to iron and HKB was analyzed by Western blot and EMSA, respectively. CCL-125 cells were treated with H, F or F/D as either naïve or HKB challenged cells. Cells were collected at 4 and 22 h post-treatment and processed. (A) Cell-associated IRP protein levels. A representative Western blot is shown above the graphed data. (B) Cell-associated IRP/IRE binding activity. Φ SD relative to naïve HBSS ($P < 0.01$) and FAC-treated cells ($P < 0.01$). *SD relative to naïve HBSS-treated cells ($P < 0.04$). **SD relative to naïve FAC-treated cells ($P < 0.03$). ***SD relative to naïve FAC/DES-treated cells ($P < 0.008$). The EMSA gel autoradiographs are shown above the graphed data. Graphed data represent means \pm StD of three independent cell culture samples.