Description of a Second Ferritin Light Chain Homologue From the Yellow Fever Mosquito (Diptera: Culicidae)

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Abstract

Ferritin is required for iron storage in vertebrates and for iron transport and storage in invertebrates, specifically insects. Classical ferritins consist of 24 subunits configured as a polyhedron wherein iron is held. The 24 subunits include light and heavy chains, each with specific functions. Several homologues of the light and heavy chains have been sequenced and studied in insects. In addition to iron transport and storage, ferritin has a role in dietary iron absorption, and functions as a protective agent preventing iron overload, decreasing oxidative stress, and reducing infection in these animals. The expression profile and regulation of a second ferritin light chain homologue (LCH2) in Aedes aegypti [Linnaeus (Diptera: Culicidae), yellow fever mosquito] was characterized in cells, animal developmental stages, and tissues post bloodmeal (PBM) by real-time PCR and immunoblot. Two previously studied ferritin subunits from Ae. aegypti, HCH and LCH1, along with LCH2, have different expression profiles and regulation with iron exposure, developmental stage, and tissue response PBM. Ae. aegypti expresses multiple and unique ferritin light chain subunits. Ae. aegypti, the vector for Zika, Dengue, and yellow fever, requires iron for oogenesis that is transported and stored in ferritin; this vector expresses a second light chain ferritin subunit homologue unlike any other species in which ferritin has been studied to date.

Key words: CCL-125 cell, development, ferritin, iron, mosquito

Introduction

Ferritins are a superfamily of ancient proteins found in species from all kingdoms that self-assemble as nanocages generally of 24 or 12 catalytic heavy chain and inactive light chain polypeptide subunits (Theil 2013). Iron is stored inside the nanocage, and thereby, iron insolubility and toxicity are minimized. Ferritin has been studied in mosquitoes and other insects (Pham 2000, Hamburger et al. 2005). Only a single LCH has been sequenced from several insect species. Analyses show that ferritin contains light and heavy chain homologues (Hamburger et al. 2005), the only insect ferritin structure currently available, shows the assembled protein consists of 12 HCH subunits and 12 LCH subunits configured in tetrahedral 3,2-fold symmetry. Cysteine residues found in the Tr. ni LCH subunits allow formation of intra- and interdisulfide bridges that help stabilize the tetradimensional structure (Pham 2000, Hamburger et al. 2005). Only a single LCH has been sequenced for each insect species to date, and we previously cloned and sequenced the LCH (LCH1; GenBank: AAO41698.1) and HCH (GenBank: AAA99996.1) subunits from Aedes aegypti [Linnaeus (Diptera: Culicidae), yellow fever mosquito; (Dunkov et al. 1995, Geiser et al. 2003)]. We recently identified a second unique LCH subunit, LCH2 (GenBank: KX986283), using shotgun proteomics of expressed proteins present in the ovaries of this animal following blood feeding (unpublished). To our knowledge, this is the first identification of the expression of a second light chain subunit in any species studied to date.

Iron requirement for development of mammals is well known (Gambling et al. 2011, Lipinski et al. 2013). Our previous work demonstrated that following blood feeding ferritin is expressed and iron-loaded in the midgut, secreted into hemolymph, and transports meal iron from the midgut to the ovaries and developing eggs.
(Zhou et al. 2007). Iron-loaded ferritin in ovaries increases post blood meal production in Aedes (Clements and Boocock 1984, Kogan 1990, Gonzales et al. 2015). Given the unique presence of an LCH2, we are interested in the expression profile of this protein in mosquitoes, and whether, like the HCH and LCH1 subunits, expression is responsive to cellular iron levels. We report the expression of LCH2 in response to iron, in comparison to HCH and LCH1 during development of the mosquito, as well as the temporal expression in various mosquito tissues PBM.

Materials and Methods

Cell Culture and Collection

_Ae. aegypti_ larval cells (CCL-125) were obtained from the American Type Culture Collection (Manassas, VA). Stock cell cultures were maintained as described previously (Geiser et al. 2009). Briefly, CCL-125 cells were maintained in 75% DMEM high glucose (Catalogue # 11960092, Invitrogen Corporation, Carlsbad, CA) and 25% SF-900 II SFM (Catalogue # 10902096, Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (Catalogue # 100-500, Gemini Bio-Products, Calabasas, CA) and 0.15% antibiotics/antimycotics (Catalogue # 15240626, Invitrogen), as stock cultures in vented 75-cm² tissue culture flasks (Corning Inc., Corning, NY) in a water-jacketed incubator (10% humidity, 95% air–5% CO₂ atmosphere, 28°C). Confluent cells were split 1:2 and cells were 80% confluent in 3 d. All experiments were performed on cells at >80% confluence under sterile conditions and BSL2 containment protocols. At the start of each experiment, the complete medium was removed and the cells were washed twice with Hank’s Balanced Salt Solution (HBSS; Catalogue # 12098050, Invitrogen). Serum-free, antibiotics/antimycotics-free medium was placed on the cells and incubated for 1 h (28°C). Following this incubation the medium was replaced with fresh serum-free, antibiotics/antimycotics-free medium and supplemented with HBSS (0, control), 50- to 500-µM ferric ammonium citrate (F, FAC; Catalogue # F5879, Sigma, St Louis, MO, 18.3% iron, ~1 µg Fe/µg FAC) in HBSS, 200-µM FAC and 200-µM deferoxamine mesylate salt (DFO; Catalogue # D9533, Sigma) or 500-µM FAC plus 500-µM DFO (F/D, FAC/DFO) in HBSS, or 200-µM DFO or 500-µM DFO (D) in HBSS, and incubated for 18 h (28°C). Since not all cells at the time of harvest adhere, the medium was removed from the cell flask, transferred to a 15-ml conical tube, and centrifuged at 900 g for 10 min, 4°C; the supernatant was removed. 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 900g for 10 min, 4°C. The supernatant was removed and the cells were suspended in 5-ml fresh HBSS. Aliquots were taken for RNA isolation and protein for immunoblot analyses as previously described (Geiser et al. 2009). Briefly, 1 ml (~1.5 × 10⁶ cells) of the cell suspension was taken from each sample and centrifuged at 9,000g for 2 min, 4°C. The supernatants were removed and the cell pellets were frozen in liquid nitrogen and stored at −80°C for total RNA extraction. The remaining cell suspensions were centrifuged at 900 g for 10 min, 4°C. The supernatants were removed and the cell pellets were suspended in hypotonic buffer (10-mM HEPES, pH 7.9, 1.5-mM MgCl₂, 10-mM KCl, freshly added 1× Protease Inhibitor Cocktail Set I [Catalogue # 539131, EMD Millipore, Billerica, MA] and 0.5 mM DTT), transferred to fresh 1.5-ml microcentrifuge tubes, frozen in liquid nitrogen, and stored at −80°C until protein extraction. All experiments were conducted in triplicate.

Mosquito Rearing

_Ae. aegypti_ (Rockefeller strain (L.)); a kind gift from Dr. Michael Richle (Department of Entomology, University of Arizona), eggs were hatched and animals raised as previously described (Zhou et al. 2007, Corby-Harris et al. 2010) with modifications. Briefly, larvae were fed on a diet of Purina Cat Chow Complete Cat Food Formula pellets (UPC 1780046572, Nestle’ Purina PetCare Company, St. Louis, MO) and maintained in trays of approximately 100 mosquito larvae. Larvae were kept in water-filled trays for approximately 7 d until pupation where they were transferred into water-filled cups inside small cages. From the onset of pupation, adult mosquitoes emerged approximately 1–2 d later and were provided 10% sucrose solution ad libitum; animals were kept at 27°C, 70–80% relative humidity with a photoperiod of 16:8 (L:D) h. Mated female mosquitoes, 4–5 d postemergence, were fasted for 12 h with sterile water ad libitum and then membrane-fed a diet of warmed (37°C), defratted porcine blood supplemented with 3-mM ATP (Sigma) for 2 h in glass feeders (Zhou et al. 2007, Geiser et al. 2015).

Animal and Tissue Collection

Collection of animals for developmental stage RNA analysis was performed as previously described (Zhou et al. 2009). Briefly, cold anesthetized 900 eggs (E), 45 fourth instar larvae (L), 45 pupae (P), and 15 males (M) at day 5 postemergence were collected. For time point blood feeding analysis 15 females at day 5 postemergence were collected representing time zero, 0 h. A cohort of 5-d postemergence females were blood fed (B) and samples of 15 engorged, cold anesthetized females were collected at days 6 (representing a 24 h post blood fed cohort, 24 hB) and 8 (representing a 72 h post blood fed cohort, 72 hB) postemergence. As a control cohort, cold anesthetized sugar-fed (S) females (15) were collected on each of days 6 (24 hS) and 8 (72 hS) postemergence. Collection of animals for developmental stage protein analysis was performed as previously described (Zhou et al. 2007, Geiser et al. 2015) with modifications. Briefly, 900 eggs (E), 900 first instar larvae (1L), 45 fourth instar larvae (4L), 45 pupae (P), cold anesthetized 45 males (M), and 45 females (non-blood fed, 0 h) at day 4 postemergence were collected. Cold anesthetized sugar-fed females (12) were collected on each of days 5 (24 hS) and 7 (72 hS) postemergence. After blood feeding, 12 engorged, cold anesthetized females were collected at days 5 (24 hB) and 7 (72 hB) postemergence.

Collection of PBM tissues for RNA analysis was performed as previously described (Zhou et al. 2009). Briefly, cold anesthetized females (45) at 24- and 72-h PBM were dissected for ovaries (OV), midguts (MG), and fat bodies (FB). Cold anesthetized females (15) at 24- and 72-h PBM were collected for whole animal (WA) RNA. Collection of PBM tissues for protein analysis was performed as previously described (Zhou et al. 2007, Geiser et al. 2015) with modifications. Briefly, cold anesthetized females (45) at 24- and 72-h PBM were collected for hemolymph (HM), and dissected for ovaries (OV), midguts (MG), and fat bodies (FB).

Animal and tissue RNA samples were placed into 1.5-ml microcentrifuge tubes, suspended in Buffer RT (Catalogue # 79216, Qiagen Inc., Valencia, CA) containing β-mercaptoethanol (β-ME), and stored at −80°C until total RNA extraction. Animal and tissue protein samples were placed into 1.5-ml microcentrifuge tubes, suspended in disruption buffer (10-mM Tris–HCl, pH 8.0, 10-mM KCl, 10-mM NaCl, freshly added 1x Protease Inhibitor Cocktail Set I (EMD Millipore) and 0.3-mM DTT), frozen in liquid nitrogen, and stored at −80°C until protein extraction. All experiments were conducted in triplicate.
Cell, Animal, and Tissue

Total RNA Extraction

Total RNA was isolated from CCL-125 cells, animals, and tissues using the RNeasy Mini Kit (Catalogue # 74104, Qiagen) as previously described (Geiser et al. 2009, Zhou et al. 2009). Purified total RNA was treated with DNase I (Catalogue # AM2222, Invitrogen) according to the manufacturer’s instructions for 30 min at 37°C and for 10 min at 65°C. The DNase-treated total RNA was used for real-time RT–PCR.

Cell, Animal, and Tissues Protein Extracts

CCL-125 cytoplasmic extracts were prepared as previously described with some modification (Ausubel et al. 1998, Geiser et al. 2009). Briefly, cell pellets suspended in hypotonic buffer and stored at –80°C underwent three cycles of freezing in liquid nitrogen, thawing on ice with homogenization for 30 s, and then centrifugation at 100,000 g for 30 min, 4°C, to rupture and separate out the cell membranes from the cytoplasmic extracts. The cytoplasmic extract supernatants were transferred to fresh 1.5-ml microcentrifuge tubes, protein concentrations were determined by Bradford assay and stored at −80°C until use. These pellets contain plasma membranes, Golgi, and other cell membrane components and are referred to as membrane extracts. We attempted to extract the membrane proteins using a detergent as previously described by others (Patton et al. 2005). Briefly, membrane extracts were thawed on ice and Triton X-100 (Catalogue # X-100, Sigma) was diluted in each buffer and stored at −80°C until use.

Real-Time RT–PCR

DNase-treated total RNA from CCL-125 cells, animals, and tissues was analyzed by 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 (Catalogue # K1612, Fermentas, GlenBurnie, MD). The primers for the PCRs were designed to obtain specific PCR products of similar size for the ORF of each message: ferritin heavy chain homologue (GenBank: AA999996.1; HCH, 198 bp): \( 5'-\text{ccagccagcgacgcaagc-3'} \) and \( 3'-\text{tcaaaaaagagggcgcgg-5'} \); ferritin light chain homologue 1 (GenBank: AA041698.1; LCH1, 173 bp): \( 5'-\text{tcacagccgagttcctct-3'} \) and \( 3'-\text{ctagagctgctcggacc-5'} \); ferritin light chain homologue 2 (GenBank: KX986283; LCH2, 126 bp): \( 5'-\text{gctctcgttctgattccag-3'} \) and \( 3'-\text{ccggccgtctgctgc-5'} \); and ribosomal protein S7 (GenBank: AAQ88428.1; RPS7, 184 bp): \( 5'-\text{ggagataacagctgacgtaagc-3'} \) and \( 3'-\text{caagagggctcttgca-5'} \). Real-time RT–PCRs were conducted using iQ SYBR Green Supermix (Catalogue # 12002500, BIORAD, Hercules, CA) with the buffers provided: at 94°C, 3 min, 1 cycle; 94°C, 10 s; 60°C, 30 s, and 72°C, 30 s, 40 cycles; with a melt curve over a temperature range starting at 55°C and ending at 95°C in a MyiQ Cycler (BIORAD). PCR product quality was monitored using post-PCR melt curve analysis, while a standard curve for each product showed the experimental samples fell within the linear range. Primer pair efficiencies for each message are indicated as follows: HCH, 96.9%; LCH1, 105%; LCH2, 100%; RPS7, 103%. Data were analyzed by MyiQ Optical System Software (Version 1, BIORAD) and fold change was quantified using the Pfaffl method to calculate for relative quantification (Pfaffl 2001) utilizing RPS7 transcript as the housekeeping gene. All PCR products were cloned and sequenced to determine that the product sequence represents that of the desired message.

Tissue Collection and Immunoprecipitation of Ferritin Subunits

On six different occasions, 15 animals were collected at 72-h PBM. The animals were cold anesthetized, and ovaries were dissected into a modified disruption buffer (10-mM Tris–HCl, pH 7.9; 1.5-mM MgCl₂; 0.5-mM DTT added fresh; 2× Protease Inhibitor cocktail [EMD Millipore] added fresh). Ovaries were pooled; thus, samples represent a total of 90 ovary pairs collected from six different, laboratory-raised Ae. aegypti mosquito populations.

Ovary protein extraction was performed as follows: samples underwent three cycles of freezing in liquid nitrogen, thawing on ice with homogenization for 30 s, and then centrifugation at 100,000 g for 30 min, 4°C, to separate soluble and insoluble fractions. Insoluble fractions were separated and washed three times in 1 M NaCl, centrifuged at 15,000 g for 5 min, 4°C. After washing, the insoluble fractions were separated in extraction buffer (50-mM Tris–HCl, pH 7.8; 2% w/v SDS; 100-mM NaEDTA; 20-mM DTT), boiled for 5 min and centrifuged at 15,000 g for 5 min, 4°C. The supernatants from the insoluble fractions were pooled with the soluble fractions. Protein concentration for the pooled sample was determined by the SDS–Lowry method.

After protein concentrations were determined, the pooled sample was divided into several 1.5-ml microtube tubes of 30- or 15-µg protein per tube. All samples were frozen and stored at –80°C until analysis. This method enabled us to freeze the samples subjected to analysis only once and to analyze the samples immediately on thawing.

Immunoprecipitation (IP) of each ferritin subunit was accomplished using the Pierce Crosslink Immunoprecipitation Kit (Catalogue# 26147; Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions. All steps were performed at 4°C, unless otherwise indicated. Briefly, three aliquots of Protein A/G Plus Agarose resin and 95 µl of each ferritin subunit rabbit antisera (HCH [epitope: ATLKKMKKSAPKL], LCH1 [epitope: YNLDSIEKEDKTL], and LCH2 [epitope: QPDMMKYTKQMLDK]) were crosslinked separately to permanently bind the HCH, LCH1, and LCH2 antibodies to the resin. Following crosslinking, the resins were washed extensively according to the manufacturer’s protocol to remove any free or nonspecifically bound antisera from the resins. IP resins for HCH, LCH1, and LCH2 were each incubated with 100 µg of precleared 72-h PBM ovary extracts overnight with agitation, 4°C. Approximately 1 ml of flow through and 180 µl of eluent for each resin were collected and stored at –20°C until immunoblot analysis.
Immunoblots

CCL-125 cell, animal, and tissue protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Any kD Mini-PROTEAN TGX Precast Protein Gels (Catalogue # 4569304, BIORAD) under denaturing conditions for approximately 133.3 Vh at RT. Protein samples were boiled for 20 min in 4× Laemml Sample Buffer (Catalogue # 161-0747, BIORAD; 277.8 mM Tris-HCl, 44.4% glycerol, 4.4% LDS, 0.02% bromophenol blue; pH 6.8) containing βME, and centrifuged prior to applying samples onto gels. CCL-125 cell extracts showed insufficient total protein concentration such that the gels were loaded by the maximum volume, 32.8 μl. Animal and tissue protein extracts were loaded by the equivalent of 1 animal per lane except for eggs and first instar larvae which were loaded by the equivalent of 10 animals per lane. As noted in previous publications (Geiser et al. 2013, Geiser et al. 2015), purified FLAG-containing bacterial alkaline phosphatase protein (FLAG; Catalogue # P7457, Sigma, 25 ng, 50 kDa) was added to each sample as an exogenous loading control because there currently is no available cytoplasmic protein that does not change in Aedes that can serve as a good endogenous loading control, to track gel resolution and transfer of proteins to the nitrocellulose membrane. IP flow through (FT) and eluent samples (E) were loaded at 20 μl per lane, and the original experimental sample (O), 72-h PBM ovary extracts, was loaded at 30 μg per lane.

Proteins were transferred to nitrocellulose membranes in the Electrophoretic Blotting System (C.B.S. Scientific Company, Inc., Del Mar, CA). Efficient transfer of proteins was confirmed by SYPRO Ruby protein blot stain (Catalogue # 179-3127, BIORAD) and Spectra Multicolor Broad Range Protein Ladder (K; Catalogue # 26634, Thermo Fisher Scientific). The nitrocellulose membranes were cut for analysis of the individual proteins of interest. The nitrocellulose membranes were blocked overnight, 4°C, in the appropriate buffering conditions for each protein of interest: HCH and FLAG (140-mM NaCl, 2.7-mM KCl, 5.4-mM Na2HPO4∙7H2O, 1.8-mM KH2PO4, 0.05% Triton x-100; pH 7.4 with 7% nonfat dry milk (NFDM) and 3% bovine serum albumin [BSA]); LCH1 (140-mM NaCl, 2.7-mM KCl, 5.4-mM Na2HPO4∙7H2O, 1.8-mM KH2PO4; pH 7.4 with 7% NFDM and 3% BSA), LCH2 (140-mM NaCl, 2.7-mM KCl, 24.8-mM Tris base, 0.1% Tween-20, pH 7.4 with 7% NFDM and 3% BSA). After blocking, the nitrocellulose membranes were incubated with anti-Ae. aegypti HCH-specific rabbit serum (diluted in 50% glycerol; 1:250 v/v), anti-Ae. aegypti LCH1-specific rabbit serum (diluted in 50% glycerol; 1:250 v/v), anti-Ae. aegypti LCH2-specific rabbit serum (1:1,000 v/v), or anti-FLAG-specific rabbit serum (1:4,000 v/v; Catalogue # F7425, Sigma) diluted in the appropriate incubation buffer system for 2 h, RT, HCH, LCH1, LCH2, and FLAG were detected on the nitrocellulose membranes with antirabbit alkaline phosphatase-conjugated antibody (1:1,000 v/v; Catalogue 711-055-152, Jackson Immuno, West Grove, PA) according to the manufacturer’s protocol. Digital images were assessed using the UVP Bioimaging Systems Epichemi II Darkroom (Upland, CA) and LabWorks analysis software (Version 4.6; UVP).

Bioinformatics Analysis of a Secondary Ferritin Light Chain Homologue

Ae. aegypti ferritin light chain sequences were aligned with Homo sapiens [Linnaeus (Primates; Hominidae), human] and Tr. ni light chain sequences using Clustal Omega (version 1.2.1; Goujon et al. 2010, Sievers et al. 2011, McWilliam et al. 2013), where an asterisk (*) indicates positions which have a single, fully conserved residue, a colon (:) indicates conservation among groups of strongly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix), and a period (.) indicates conservation among groups of weakly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix). The SignalP 4.1 Server (Petersen et al. 2011) predicted the presence and location of signal peptide cleavage sites in the Ae. aegypti ferritin light chain amino acid sequences. The theoretical MW and isoelectric point (pI) for both Ae. aegypti ferritin light chain amino acid sequences was provided by Compute pI/Mw (Gasteiger et al. 2005). Ferritin subunit amino acid alignment similarities and identities were determined by BLASTP Suite-2sequences analysis (NCBI-Resource-Coordinators 2014) for Ae. aegypti, Anopheles gambiae [Giles (Diptera: Culicidae), common malaria mosquito], and Xenopus laevis [Daudin (Anura: Pipidae), African clawed frog].

Statistical Analyses

Treatment differences were determined by one-way analysis of variance using the Tukey’s HSD test or one-tailed unpaired t-test for comparison of selected data sets (Graph Pad Software, Inc., San Diego, CA). Experiments were conducted simultaneously in triplicate and the data for a given variable were analyzed at the same time.

Results

Alignment of Ae. aegypti LCH1 and LCH2 shows that LCH2 has a signal peptide, similar to LCH1 (Fig. 1A; Geiser et al. 2003). Although there are exceptions (Collin et al. 1988, Nichol and Locke 1990, Adams et al. 2000), most of the insect ferritin subunits identified to date have hydrophilic leader sequences that signal secretion. Insect ferritin is generally vacuolar (Geiser et al. 2009) and secreted ferritin serves a role in iron transport in mosquitoes (Zhou et al. 2007). Cys4 and Cys24 of the Tr. ni LCH formed intrasubunit disulfide bridges, while LCH-Cys12 formed intersubunit disulfide bridges (Fig. 1A). Only one of these residues is conserved in the mosquito LCH2. The mosquito LCH2 also contains two conserved Cys residues found in Lepidoptera, Coleoptera, Hemiptera, and Diptera LCH sequences, but not found in Ae. aegypti LCH1 (Fig. 1A; Hamburger et al. 2005). Other residues have been proposed to participate in the structural integrity of ferritins (Geiser et al. 2003); recent work in vertebrate homo-polymer ferritins that have an octahedral structure indicates that a cluster of E, D, and K residues (human light chain; β in Fig. 1A) contributes to protein structural stability (Tosha et al. 2012). These residues also are partially conserved in the mosquito LCH1 and LCH2. Taken together, partial conservation of residues in the mosquito sequences could suggest that the structure of the mosquito nanocage differs from that of vertebrates as well as other insects. Vertebrate L subunits have a porphyrin-binding pocket associated with ferrihydride nucleation (Andrews et al. 1992, Gallois et al. 1994); this pocket is not conserved in the mosquito LCH subunits; however, there are two predicted N-linked glycosylation sites in the mosquito LCH1 and LCH2. Partial conservation of the mosquito nanocage differs from that of vertebrates as well as other insects. Vertebrate L subunits have a porphyrin-binding pocket associated with ferrihydrite nucleation (Andrews et al. 1992, Gallois et al. 1994).
is consistent with *An. gambiae*, African malaria mosquito) HCH versus LCH (22%). Since the LCH2 was identified from the *Aedes* ovary proteome, we compared the amino acid sequence against the *Xe. laevis* [Daudin (Anura: Pipidae), African clawed frog] oocyte ferretin subunits (Huang et al. 2003); the *Aedes* LCH2 subunit has low identity and similarity to *Xe. laevis* light chain (LC, 27%/48%) and heavy chain (HC, 25%/46%).

According to VectorBase (https://www.vectorbase.org/, last accessed November 29, 2017), the genes for LCH1 and LCH2 are located on two different supercontigs (1.252 and 1.50, respectively), with LCH1 laying in a head-to-head configuration with HCH (Geiser et al. 2003), while no other ferritin genes are found near the LCH2 gene (Supp Fig. 1 [online only]). RNA Analyzer (http://rnaanalyzer.bioapps.biozentrum.uni-wuerzburg.de/, last accessed November 29, 2017) predicted that neither LCH transcript contains an iron responsive element that is found in the 5′UTR of the HCH transcript (Dunkov et al. 1995). Both LCH transcripts are predicted to be alternatively spliced, with LCH1 having four different transcripts (Geiser et al. 2003) and LCH2 having two different transcripts. The LCH1 transcripts have one to two introns (Geiser et al. 2003), while the LCH2 transcripts have one intron.

We obtained antiserum for the individual ferritin subunits; the epitopes for which are shown in Supp Fig. 2 (online only). A search of the current translated protein database from NCBI showed no other *Aedes* proteins with sequences that matched these epitopes. Specific antiserum for HCH and LCH2 identified bands of mass >260 kDa
in samples taken from 72-h PBM ovaries for native ferritin (Supp Fig. 3 [online only]). In keeping with our previous work using an antiserum against purified Aedes larval ferritin (whole molecule), mosquito native ferritin exists at ≥260 kDa (Supp Fig. 3, A Ferritin [online only]), and as ferritin subunits detected at approximately 28, 26, and 24 kDa (Dunkov et al. 1995, Pham and Winzerling 2010). Our previous work showed by N-terminal sequencing that the 24 and 26 kDa bands (Dunkov et al. 1995; Supp Fig. 3, A Ferritin [online only]) identified the HCH subunit, while that of the 28-kDa band was LCH1 (Dunkov et al. 1995). Although the LCH1 antiserum did not detect ferritin >260 kDa, it did detect subunits of the appropriate mass. This could suggest that although the LCH1 subunits are present, they are not incorporated into the whole ferritin molecule; or that the epitope is not available for antibody binding in the whole ferritin molecule; further work would be required to substantiate these speculations. The Coomassie blue stained gel (Supp Fig. 3, A Coomassie [online only]) shows that there are numerous proteins present in the ovarian sample which are not detected by any of our antisera. To further document the specificity of the antisera, proteins of interest were resolved under denaturing conditions and detected by immunoblot. Ferritin is a molecule highly resistant to denaturation that can withstand heating to 100°C as well as proteinase K digestion (Dunkov et al. 1995); subsequent to denaturation the ferritin subunits begin to reassemble as a sample cools and form multimers (Pham and Winzerling 2010). In addition, subunits can be post-translationally processed (Geiser et al. 2003). Under our denaturing conditions, specific antiserum detected bands at masses expected (20–28 kDa) based on our previous work for each ferritin subunit (Supp Fig. 4A [online only]); those identified for LCH1 and HCH were seen in our previous work (Geiser et al. 2015) as noted earlier, and the subunits at 24 and 26 kDa were previously documented as HCH by N-terminal sequencing (Dunkov et al. 1995). The approximate masses for the subunits identified by specific antiserum are shown in the table insert (Supp Fig. 4B [online only]). The calculated mass for each subunit from the annotated sequences without the secretion signal sequence is 20.9, 23, and 22.3 kDa for HCH, LCH1, and LCH2, respectively (Supp Fig. 4C [online only]).

Although each antiserum was produced using a unique epitope, the immunoblots obtained yielded expected results identifying specific bands; to further document the specificity of the ferritin subunit antiserum, we immunoprecipitated ferritin from 72-h PBM ovary extracts with each subunit antiserum. For each sample, we detected ferritin in the eluent (immunoprecipitate), but not in the flow through using the ferritin subunit antiserum used to perform the respective IP (Fig. 2). Each immunoprecipitate was resolved by 12% SDS–PAGE and in each, bands were detected by Coomassie blue stain in the range of 20–25 kDa. This region for each immunoprecipitate eluent was cut out and subjected to mass spectrometry to determine the protein sequences present (Supp Fig. 5 [online only]). In the sample precipitated with HCH antiserum, we identified only the HCH subunit (estimated mass of 24 kDa). Likewise, only the LCH1 subunit (estimated mass of 24 kDa) was found in the sample precipitated by the LCH2 antiserum, and only the LCH1 subunit (estimated mass of 18 kDa) was present in the LCH1 immunoprecipitate. Thus, the specific antiserum immunoprecipitated only the respective subunit from the sample as identified by mass spectrometry, and we conclude that each antiserum is specific for the subunit of interest (Fig. 2). The differences in the mass of the subunits obtained by immunoblotting, mass spectrometry, and primary sequence indicate that the subunits are post-translationally processed (Supp Fig. 4C [online only]). The LCH1 subunit is glycosylated (Geiser et al. 2003), although the glycan(s) structure(s) is (are) not known; however, the HCH subunit was not found to be glycosylated in previous work (Dunkov et al. 1995). Thus, the post-translational processing of the HCH and LCH2 subunits remains unknown and likely accounts for the additional bands detected by SDS–PAGE.

In our previous work, we showed by ICP-MS that iron is taken up by CCL-125 cells in direct proportion to iron dose, and we found that LCH1 message expression in CCL-125 cells significantly increased in response to iron dose and this response was rescued by the administration of iron chelating agent, DFO (Geiser et al. 2006, 2009). In contrast, here we show that expression of LCH2 in CCL-125 cells does not significantly change in response to iron dose (Fig. 3).

Surprisingly, although in our previous work, we found that HCH and LCH1 protein expression increases in CCL-125 cells with iron exposure (Geiser et al. 2006, 2009), we found that the expression of LCH2 is not only unresponsive to increasing iron dose in these cells (Fig. 4) but also expressed primarily in the absence of iron whether by withholding iron from the culture medium (FAC/DFO treatment) or by the addition of DFO in these cells.

Given the differences in subunit expression in response to iron in cells, we explored transcript and protein expression of the various subunits during development and in whole animals following blood feeding. As we anticipated, transcripts for all subunits are present...
at all life stages (Fig. 5). No significant differences were found in a comparison of the ferritin subunit transcripts within each developmental stage. A comparison of RNA expression for each ferritin subunit across all developmental stages showed that LCH1 (Fig. 5, white bars) expression tended to be greatest in pupae and males, as well as PBM relative to sugar feeding; however, differences were not significant. HCH expression (Fig. 5, gray bars) follows a similar pattern and appears to increase following blood feeding relative to sugar feeding; however, values did not reach significance due to high variance. LCH2 expression (Fig. 5, hatched bars) is significantly greater in males than in eggs, larvae, and pupae. LCH2 expression also appears to increase PBM, but this did not reach statistical significance.

In keeping with transcript expression, LCH2 is present in fourth instar larvae, pupae, males, and females prior to blood feeding; the 26-kDa subunit predominates (Fig. 6A). LCH2 protein is not increased in whole animals with blood feeding despite an increase in message expression. LCH1 shows low expression in eggs and increases with larval stage (Fig. 6B). It is expressed in pupae, males, and females prior to blood feeding. LCH1 is also expressed in sugar fed females, and in contrast to LCH2, expression increases following blood feeding by 72 h. The 28-kDa subunit predominates, and a band near 23 kDa also is observed in sugar-fed females suggesting that LCH1 expressed during this time exists in a nonglycosylated form, while both the 23- and 28-kDa subunits are expressed post blood feeding by 72 h. The expression of multiple bands suggests variation in glycosylation or additional post-translational processing. In contrast to the LCH subunits, HCH is strongly expressed in fourth instar larvae and pupae and is expressed in males (Fig. 6C). Expression here was not increased with blood feeding or sugar feeding in whole animals.

In order to further explore the expression of the ferritin subunits in response to blood feeding, we evaluated the expression in various tissues (Fig. 7). Messages for the various subunits are expressed in all tissues’ PBM. Subunit transcripts are low in ovaries at 24-h PBM. Expression of LCH1 (Fig. 7, white bars) and HCH (Fig. 7, gray bars) is increased twofold in midgut at this time, while expression in fat body remains stable. By 72-h PBM, expression of LCH2 (Fig. 7, hatched bars) in ovary increases dramatically by almost 15-fold, while that of HCH and LCH1 remains low and stable. LCH2 also shows about a fold increase in fat body at 72-h PBM.

In keeping with transcript expression, LCH2 expression increases in ovaries and fat body by 72-h PBM (Fig. 8A). It is also detected in midgut at 24-h, but not at 72-h PBM. LCH1 (Fig. 8B) and HCH (Fig. 8C) are expressed at very low levels 24-h PBM and expression of both subunits increases in ovaries and fat body by 72 h PBM.

**Discussion**

While analyzing the proteome of *Ae. aegypti* ovaries, we identified a second ferritin light chain subunit (unpublished). Long ago, Dunkov and Georgieva (2006) analyzed expressed sequence tags (ESTs) and full-length cDNA collections and reported four putative LCH subunits in *Ae. aegypti*; this report demonstrates that at least two of these subunits are expressed proteins. Whether the other two LCH subunits are expressed and the reason for this redundancy remains unknown. Lower vertebrates have been shown to express three unique ferritin subunits, heavy (H), middle (M), and light (L), where the M subunit has both the ferroxidase center of the H subunit and the nucleation site of the L subunit (Dickey et al. 1987, You et al. 2015), while fish seems to only express the H and M subunits (Giorgi et al. 2008, Lee et al. 2014). Work in mollusks has discovered transcript expression of four ferritin subunits, where two subunits are predicted to be secreted while the other two subunits are not (Zhang et al. 2013, Huan et al. 2014). In *Drosophila*, three ferritin subunits are expressed, Fer1HCH, Fer2LCH, and Fer3HCH, where Fer1HCH and Fer2LCH are secreted proteins and Fer3HCH is a mitochondrial ferritin (Dunkov and Georgieva 2006, Missirlis et al. 2006). *Ae. aegypti* expresses a second light chain ferritin subunit homologue unlike any other species in which ferritin has been studied to date.

The mosquito LCH2 showed partial conservation of the Cys residues found in the *Tr. ni* structure (Hamburger et al. 2005), as well
Fig. 5. *Ae. aegypti* developmental stage affects ferritin subunit mRNA expression. The effect of *Ae. aegypti* developmental stage on ferritin subunit transcript expression was measured as described in the Materials and Methods. RPS7 transcript was used as the housekeeping gene for relative quantification by the Pfaffl method (Pfaffl 2001). Data are triplicates from one experiment. Error bars indicate SD. LCH2 ANOVA ($F = 3.127; df = 8, 43; P < 0.01$), significance was found for M verses E ($F = 4.701; df = 5, 4; P < 0.05$), L ($F = 7.248; df = 5, 5; P < 0.05$), P ($F = 16.08; df = 5, 5; P < 0.01$), 24 hS ($F = 10.96; df = 5, 5; P < 0.05$), and 72 hS ($F = 7.838; df = 5, 4; P < 0.05$); HCH ANOVA ($F = 2.890; df = 8, 16; P < 0.05$), significance was found for E verses 24 hB ($F = 17.14; df = 2, 2; P < 0.05$); Key: E = eggs; L = fourth instar larvae; P = pupae; M = 5-d-old males; 0h = 5-d-old females; 24h = 6-d-old females; 72h = 8-d-old females; S = sugar fed; B = blood fed.

Fig. 6. *Ae. aegypti* developmental stage affects ferritin subunit protein expression. The effect of *Ae. aegypti* developmental stage on ferritin subunit protein expression was measured by separation of animal protein using SDS–PAGE and detection of ferritin subunits by immunoblot assay as described in the Materials and Methods. Representative panels of an experiment performed in triplicate. (A) LCH2 was detected by immunoblot assay. (B) LCH1 was detected by immunoblot assay. (C) HCH was detected by immunoblot assay. Key: K = Spectra Protein Ladder (Thermo Fisher Scientific); E = eggs (10 animal equivalents); 1L = first instar larvae (10 animal equivalents); 4L = fourth instar larvae (one animal equivalent); P = pupae (one animal equivalent); M = 4-d-old males (one animal equivalent); 0 = 4-d-old females (one animal equivalent); 24h = 5-d-old females (one animal equivalent); 72h = 7-d-old females (one animal equivalent); S = sugar fed; B = blood fed.
as partial conservation of a cluster of residues that contributes to vertebrate ferritin structural stability (Tosha et al. 2012). Both Aedes LCH2 and LCH1 subunits have a conserved porphyrin-binding pocket associated with ferrihydrite nucleation (Andrews et al. 1992, Trikha et al. 1994, Gallois et al. 1997, Hempstead et al. 1997) and although both lack an N-linked glycosylation site found in the loop region in the Tr. ni ferritin structure, we previously showed glycosylation of the LCH1 (Geiser et al. 2003). In keeping with other insect ferritins, LCH2 has a secretion signal (Collin et al. 1988, Nichol and Locke 1990, Adams et al. 2000); insect cellular ferritin is generally vacuolar (Geiser et al. 2009) and secreted (Zhou et al. 2007). LCH1 and LCH2 are 55% identical and show very low identity with the HCH subunit (LCH1, 31% and LCH2, 27%).

In our previous work, we demonstrated that following blood feeding, ferritin is expressed in midgut and transports iron to ovaries and developing eggs (Zhou et al. 2007). Our data indicate expression of all three subunits in midgut following feeding and that ferritin from midgut could be composed of LCH2, LCH1, and HCH subunits. The increased requirement for ferritin to transport iron to the ovaries PBM suggests that expression in this tissue could account for the increase in HCH and LCH observed PBM in whole animals (Zhou et al. 2007).

Ferritin is detected in developing tissues; protein found in ovaries could originate from subunits expressed in the ovarian tissues or from hemolymph ferritin entering the tissue. The latter source could account for the low transcript expression for HCH and LCH1 observed in ovaries following blood feeding. Each ferritin molecule is capable of transporting a high iron load of 2,000–4,000 atoms of iron (Theil et al. 2016) (an advantage of ferritin as an iron transport protein), so expression of ferritin need not be high to accommodate the iron requirement for development. Once iron is delivered to the ovaries, it may be utilized in the development processes or stored in eggs for use by the developing larvae. LCH1 appears to be the predominating subunit of eggs and Dunkov et al. (2002) reported HCH in 0- to 3-h post-oviposition eggs and 4-d eggs per first instar larvae using the whole molecule ferritin antiserum.

What is the role and importance of the LCH2? In contrast to the other subunits, LCH2 expression is not increased by the presence of iron in CCL-125 cells, and, in fact, it is greater in the absence of iron. Further, in contrast to the other subunits, expression of the LCH2 message is dramatically increased in ovaries following blood feeding. We speculate that, taken together, these data suggest control of the
expression of LCH2 message differs from that of the other subunits and that LCH2 message expression is transcriptionally controlled by changes during vitellogenesis and egg development rather than by the iron load of the bloodmeal. Although the LCH1 and HCH lie head-to-head on the chromosome and share expression control elements, LCH2 is found on a different chromosome and subject to different control elements. In our previous work as well as work by Pham et al. (Pham and Chavez 2005, Pham et al. 2005), HCH and LCH1 were predicted to have responsive elements in the promoter region for ecdysone, metals, hypoxia, antioxidants, and inflammation, while the LCH2 promoter has predicted responsive elements for ecdysone, hypoxia, antioxidants, and inflammation; we found no metal response element (data not shown). Further, LCH2 levels increase in both ovaries and fat body following blood feeding again suggesting this subunit is involved in the vitellogenic process. The tissue expression specificity for the ferritin subunits is a subject for further research.

As noted in the introduction, the structural work of Hamburger et al. (2005) showed that ferritin molecules from the insect, Tr. ni, consist of two types of ferritin subunits, the HCH and LCH. We modeled the LCH2 amino acid sequence against other proteins using the SWISS-MODEL database and found that the structure of LCH2 was consistent with the other ferritin subunits in having five alpha helices and a long internal loop region (data not shown). In mammals, ferritin in various tissues displays different isoforms depending on how many of each type of subunit, heavy or light chain, are incorporated into the 24-subunit molecule. The Tr. ni structure consisted of 12 subunits of each type, HCH or LCH. We speculate that mosquitoes could form various isoforms of ferritin by selecting different LCH subunits rather than varying number of HCH and LCH subunits in the molecule. If this is the case, then it implies that modest alterations in ferritin structure can influence ferritin use by these animals.

At this point, we do not know the importance or role of the LCH2. It may be involved in holding iron for use by the developing tissues. Perhaps, it enhances the storage of iron within the ferritin molecule, an advantage in eggs that can remain dormant for long periods prior to hatch. The expression of this subunit in the absence of iron implies that it could serve different and unexpected roles in cells and probably during development.

Supplementary Data
Supplementary data are available at Journal of Insect Science online.

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