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Aedes aegypti ferritin heavy chain homologue: feeding of iron or blood influences message levels, lengths and subunit abundance

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Abstract

Secreted ferritin in the mosquito, *Aedes aegypti*, has several subunits that are the products of at least two genes, one encoding a homologue of the vertebrate heavy chain (HCH) and the other the light chain homologue (LCH). Here we report the developmental and organ specific pattern of expression of the ferritin HCH messages and of both subunit types in control sugar-fed mosquitoes, in those exposed to high levels of dietary iron, and after blood feeding.

When Northern blots were probed with a HCH cDNA, two bands were observed, representing at least two messages of different sizes that result from the choice of two different polyadenylation sites. Either raising mosquito larvae in an iron-enriched medium, or blood feeding adult female mosquitoes resulted in a marked increase in the HCH message level, particularly of the shorter message. Changes in the amount and length of messages and amount of ferritin subunits were studied over the life span of the mosquito and in different organs of female mosquitoes after blood feeding. The midgut of blood-fed insects is the main site of increased ferritin message synthesis. Ferritin protein levels also increase in midgut, fat body and hemolymph after blood feeding. Ferritin messages and subunits are synthesized in the ovaries and ferritin is found in the eggs. These observations are discussed in terms of translational and transcriptional control of ferritin synthesis and are compared to similar events in the regulation of *Drosophila melanogaster* ferritin.

Keywords: polyadenylation, expression, induction

Abbreviation:

ABM	artificial blood meal
FAC	ferric ammonium citrate
HCH	heavy chain homologue
LCH	light chain homologue
NTA	nitrilotriacetic acid, an iron chelator
UTR	untranslated region
rp49	ribosomal protein #49 mRNA of *Drosophila melanogaster*

Introduction

Much has been learned about iron metabolism in insects in the past few years (reviews: Winzerling and Law, 1997; Nichol et al., 2002). Iron is both essential and toxic, and organisms employ specialized proteins to sequester iron and prevent its toxic effects but also keep it in a bioavailable form. Of special interest is the iron storage protein, ferritin, which in most animals is a cytosolic component, but in insects is an exported protein found in the vacuolar system of cells and in the hemolymph. It consists of several subunits that combine to form a large hollow sphere in which iron is stored in the form of a ferrihydrite crystal. The subunits of secreted ferritins in insects are products of two genes encoding light chain and heavy chain homologues of the vertebrate cytosolic ferritins. Earlier studies have shown that *Aedes aegypti* ferritin is composed of three major subunits, encoded by two different genes, one encoding a 28 kDa chain (LCH) and the other encoding 26 and 24 kDa chains (HCH) (Dunkov et al., 1995). The multiplicity of the HCH ferritin subunits seems to be the result of as yet unknown posttranslational modifications. In many animals iron regulates ferritin synthesis at the translational level through an interesting mechanism involving the interaction of a stem loop structure in the 5'UTR, which is an

iron responsive element, with an iron sensor, which contains an iron regulatory protein. Messages encoding *Ae. aegypti* HCH contain an iron responsive element (Dunkov et al., 1995) suggesting that this regulatory mechanism might be conserved.

In *Drosophila melanogaster* messages for both HCH and LCH increase dramatically when larvae are fed a diet rich in iron (Georgieva et al., 1999, 2002). Multiple messages encoding both subunits result from alternative splicing in the 5' UTR and choice of alternative polyadenylation signals in the 3' UTR (Lind et al., 1998; Georgieva et al., 1999, 2002). The proportion of the messages is strongly influenced by iron feeding (Georgieva et al., 1999, 2002). The increase in message abundance is paralleled by increases in the encoded polypeptides (Georgieva et al., 2002).

Since adult female mosquitoes feed on a diet rich in iron, they have an increased need to sequester iron. We investigated whether iron or blood feeding would cause changes in ferritin HCH messages similar to those seen in *D. melanogaster*. Indeed, we found two HCH messages of different lengths, the result of choice of one of two polyadenylation signals, upregulation of HCH messages by iron and blood feeding, and a concomitant increase in ferritin subunit proteins.

Materials and Methods

Mosquito culture, treatment, and blood meals

Aedes aegypti (Rockefeller strain) were maintained at 29°C, 80% humidity with a 12h:12h light:dark photoperiod. Larvae were raised according to Shapiro and Hagedorn (1982). For iron loading the culture water was supplemented with $FeCl_3$ (0.05 and 0.5 mM), $FeCl_3$ (0.1 mM) and the iron chelator nitrilotriacetic acid (NTA) (1 mM), NTA (1 mM), ferric ammonium citrate (FAC) (0.5 mM), or hemin (0.5 mM). Mosquitoes were blood-fed on a rabbit or on the following meals: bovine blood (supplemented with 2 mg/ml isoleucine); 125 mg/ml bovine hemoglobin in 150 mM NaCl and 100 mM $NaHCO_3$; 150 mM NaCl; 150 mM NaCl and 3.7 mg/ml FAC (9.3 mM Fe); artificial blood meal (ABM) (Kogan, 1990); ABM supplemented with 9.3 mM Fe (3.7 mg/ml FAC or 2.5 mg/ml $FeCl_3.6H_2O$) or with 9.3 mM hemin. All meals also contained 1 mM ATP.

RNA isolation and Northern blotting

Total RNA was isolated from insects at different developmental stages, from control or iron-loaded larvae, from female mosquitoes fed different meals, and from organs and body parts dissected from blood-fed female mosquitoes using a powdered glass affinity matrix (RNAid matrix, Bio 101, La Jolla, CA, USA) as described (Noriega and Wells, 1993). RNA samples (10 - 30 µg) were run on a 1.2% agarose/formaldehyde gels and transferred to nylon membranes (GeneScreen Plus, NEN Life Science Products, Boston, MA, USA). Blots were hybridized at high stringency (16-18 h at 42°C) in 50% formamide, 5xSSPE (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5x Denhardt's solution, 1% SDS, after pre-incubation (2-4 h) in the above solution supplemented with 100 µg/ml salmon sperm DNA. Two *Ae. aegypti* ferritin cDNA probes were used. Probe FerHCH (~900 bp) was the complete insert of cDNA clone F (Dunkov et al., 1995), comprising two thirds of the coding region and the 3' UTR of 426 bp. Probe FerHCH 3'UTR was a 233 bp PCR fragment of the 3'UTR amplified using the primers 5' AATACAATAGACTTAACGCACTCC 3' (forward) and 5'GTGTCACTCGACAGCTCTAC 3' (reverse), (Fig.1). Blots were also hybridized with a fragment of the *D. melanogaster* ribosomal protein #49 mRNA (rp49) gene as a loading control (O'Connel and Rosbash, 1984) or in some experiments with an *Ae. aegypti* rRNA probe. Probes were gel purified with the Sephaglas BandPrep kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), labeled with [alpha-^{32}P]dCTP using the RadPrime DNA labeling system (Gibco BRL, Grand Island, NY, USA), and purified with the ProbeQuant G-50 micro columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Relative abundance of the ferritin messages was estimated by normalizing the ferritin hybridization signals to the rp49 or rRNA signals using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA), a Beta Scope (Betagene, Waltham, MA, USA), or scanning the autoradiograms and using the Scion Image computer program (Scion Corporation, Frederick, MD, USA).

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1    taaAACGCGAGGCAGTATGGGCGACAGGAGTGCGACGGTGCTTCACTGTGTCATCATTCT   60

61   AGTTTAGGAGAAGCGATCTTGTTATCTTTGGAGATTTCGAACCGATGAAGACCACCGGAA   120

121  AATGCTTGAATAATAACAATACAATAGACTTAACGCACTCCTATTGGATCTCGTTCTATC   180

181  TTTTAGTTGTGTATTTTTTTTATTTCCTCTCTCAAGTGACGTCCTCAATTTCCTTGTGGG   240

241  TCCGTGGTGCTCTGAATTGGTGGTCACACGTGTGACGTAAGATTCGTCATTCAATTGGCG   300

301  TAATATTCAACTATAATTTATGTAGATACATGATGTGAATCCGTTAGATTGTAGAGCTGT   360

361  CGAGTGACACAAACTTTCCAATAAATTTAAATGGTTCTTCAAGAGACGGGATTCTTTACA   420

421  TGGAAAG   427
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Figure 1. Nucleotide sequence of the 3'UTR of an *Aedes aegypti* ferritin HCH cDNA (Dunkov et al., 1995). The stop codon is shown in lower case. The distal polyadenylation signal (nt 380 – 385) and four overlapping proximal polyadenylation signals (nt 129 – 148) are underlined. Arrows show the location and the direction of PCR primers used for generating the 3'UTR-specific probe.

Western blot

Whole insects were homogenized in 0.25 ml PBS (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.0) containing 4 mM phenylmethanesulfonylfluoride (PMSF). Organs from sugar-fed and from blood-fed mosquitoes, 24h and 48h after feeding were dissected in PBS. Organs from several insects were pooled, homogenized in PBS containing PMSF and centrifuged for 15 min at 12,000 g. Aliquots of the supernatants were mixed with 2x SDS-PAGE sample buffer, boiled for 5 min and loaded on a 12.5% homogenous slab gel (Laemmli, 1970). Hemolymph was collected in the drop of PBS used for dissection. Fat bodies were collected together with the abdominal carcass. For early embryos, eggs were collected within 3h after oviposition; for pharate 1st instar larvae, eggs 4 days after oviposition were used. Purified pupal ferritin (Dunkov et al., 1995) was also loaded on the gels along with the samples, or in some experiments it was added during the extraction of the midgut samples to serve as a protein degradation control. After the electrophoresis polypeptides were transferred to a nitrocellulose membrane. Blots were blocked with 1% non-fat dry milk in 0.02 M Tris, 0.1 M NaCl, pH 7.5, incubated with a rabbit anti-*Ae. aegypti* ferritin serum (1:2,000), washed, incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories, Hercules, CA, USA), and ferritin was visualized using alkaline phosphatase stain. The anti-*Ae. aegypti* ferritin antiserum used in these experiments was generated against a mixture of pupal HCH and LCH subunits. It recognizes all ferritin subunits in larvae, pupae and adults (Dunkov et al., 1995).

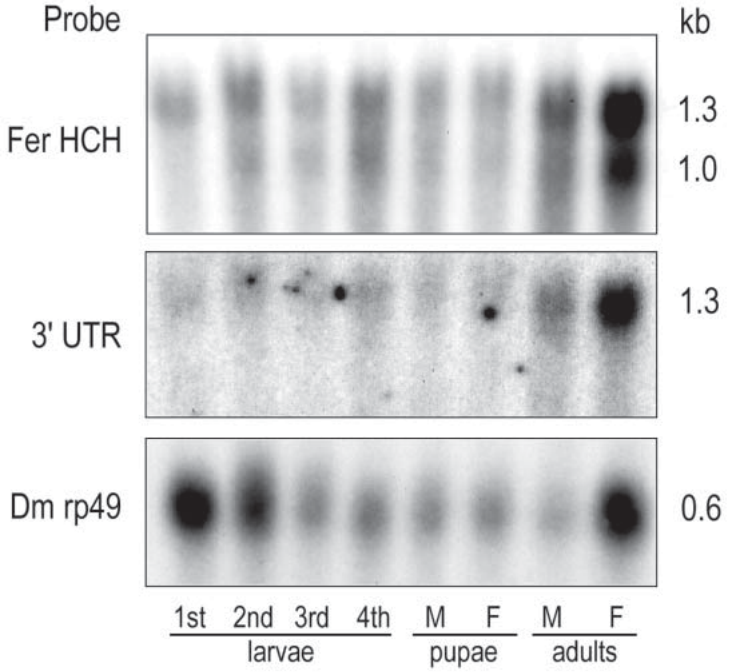

Figure 2. Developmental expression of *Aedes aegypti* ferritin HCH messages. Northern blot hybridized with probes from the ferritin HCH coding region and 3'UTR (Fer HCH), from the 3' UTR between the two polyA signals (3' UTR), and with *D. melanogaster* rp49 (Dm rp49). The RNA samples (30 ug) were isolated from the four larval instars, from male (M) and female (F) pupae, and adult mosquitoes.

Results

Ae. aegypti ferritin HCH messages are of two lengths

Northern blots hybridized with a probe spanning most of the coding region and the 3' UTR of the ferritin HCH cDNA revealed the presence of messages of two distinct lengths, ~1.3 and ~1.0 kb (Fig. 2). Based on previous observations that both ferritin HCH and LCH messages of *D. melanogaster* can be polyadenylated using two alternative signals (Lind et al., 1999; Georgieva et al., 1999, 2002), we examined the sequence of the *Ae. aegypti* HCH cDNA (Dunkov et al., 1995). This revealed the presence of four overlapping non-canonical putative polyadenylation signals proximally to the coding region (Fig. 1). To test the possibility that the shorter messages result from the choice of these signals, we designed a probe that represents the sequence between the proximal and distal polyadenylation signals (Fig. 1). This probe hybridized only with the longer (1.3 kb) messages, thus confirming the alternative use of the two signals (Fig. 2).

Developmental expression of ferritin HCH messages and induction by iron

As seen in Fig. 2, the abundance of both ferritin HCH messages varied during mosquito development. In 1st instar larvae (and in pharate 1st instar larvae, data not shown) only the 1.3 kb message is seen. The 1.0 kb mRNA is first detected in 2nd instar larvae.

Figure 3 compares the abundance of both ferritin HCH messages in *Ae. aegypti* larvae cultured in control and iron supplemented water. No increase in message abundance was detected in larvae grown in water supplemented with $FeCl_3$. A significant accumulation of messages, especially of the shorter message, is evident in larvae grown in media supplemented with an iron chelator (NTA). This increase was more dramatic when the culture water was supplemented with chelated iron (NTA+$FeCl_3$).

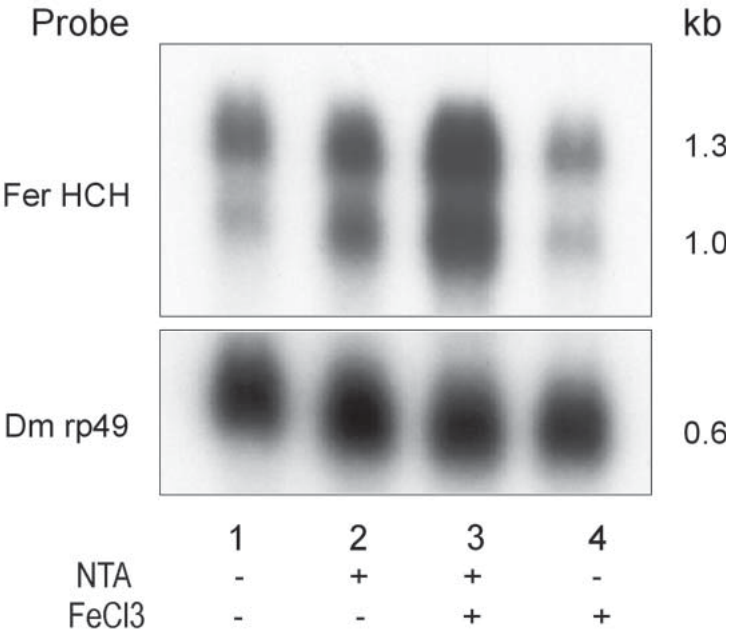

Figure 3. Expression of ferritin HCH messages in *Aedes aegypti* larvae. Northern blot hybridized with a probe containing Fer HCH coding region and 3'UTR and with *D. melanogaster* rp49. RNA samples (10 ug) were prepared from 4th instar larvae raised on control diet (1), and on diets supplemented (+) with 1mM NTA (2), 1mM NTA + 1 mM $FeCl_3$ (3), and 0.05 mM $FeCl_3$ (4).

Induction of transient and organ specific accumulation of ferritin HCH message by a blood meal

Feeding adult female mosquitoes with a natural blood meal or with several artificial blood meals containing different iron sources greatly increased the abundance of HCH messages (Fig. 4A,B). All artificial meals, including saline, induced accumulation of ferritin messages. When the meals (saline meal included) contained iron at the concentration of heme iron in human blood (9.3 mM, Kogan, 1990) this accumulation was enhanced. The most dramatic increase in ferritin HCH messages was seen after feeding on an artificial blood meal containing ferric chloride or ferric ammonium citrate.

The time course of accumulation of the ferritin messages was studied after a natural blood meal on a rabbit (Fig. 5A). While there was an increase in the abundance of both ferritin messages following blood feeding, the transient accumulation of the 1.0 kb mRNA is particularly interesting. The 1.0 kb message increased in relative abundance by 12h after feeding, was more abundant than the 1.3 kb message 48h after feeding (Fig. 5B), persisted at a high level until 72h after feeding, and then returned to the level seen before blood feeding.

Figure 6 shows the ferritin HCH messages in dissected organs and body parts from female mosquitoes 24h after blood feeding. This Northern blot demonstrates that the increase in HCH messages seen in whole insects is particularly dramatic in the midgut but is also apparent in the thorax/head and ovaries. In ovaries the ratio of the 1.0 and 1.3 kb messages was 1.4 suggesting that the 1.0 kb message may be more abundant in the ovary. Fat body and Malpighian tubules appear to be relatively minor sites of HCH message synthesis.

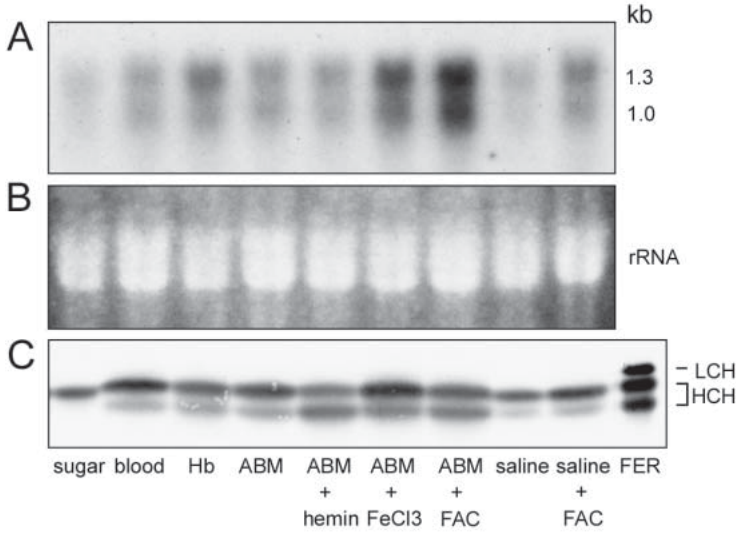

Figure 4. Expression of ferritin HCH messages and subunits in female *Aedes aegypti* fed various meals. A. Northern blot of total RNA prepared from mosquitoes 24 h after feeding, hybridized with a probe from the Fer HCH coding region and 3'UTR. B. Ethidium bromide stained rRNA on the Northern blot to show loading. C. Western blot of extracts (one insect per lane) prepared from mosquitoes 24 h after feeding and probed with antibodies against ferritin. Mosquitoes were sugar-fed or fed on the meals as indicated. Hb, bovine hemoglobin; ABM, artificial blood meal; FAC, ferric ammonium citrate; FER, ferritin purified from iron loaded pupae (Dunkov et al., 1995). The protein bands corresponding to LCH (28 kDa) and HCH (26 and 24 kDa) are indicated on the right.

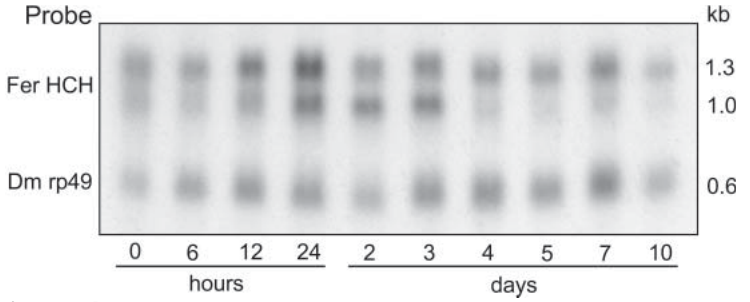

Figure 5A. Time course of ferritin HCH message expression after a natural blood meal in *Aedes aegypti*. Northern blot of total RNA prepared from female mosquitoes fed on a rabbit, hybridized with a probe from the ferritin HCH coding region and 3'UTR (Fer HCH) and with *D. melanogaster* rp49 (Dm rp49). The time points in hours or days after the blood meal are indicated.

Western blot analysis of ferritin expression.

Initial studies and effect of iron supplementation of the diet on ferritin synthesis

A Western blot of extracts prepared from *Ae. aegypti* larvae, female pupae and female adults raised in control or iron supplemented water is shown in Figure 7. The LCH ferritin subunit (28 kDa band) is present only in iron-loaded larvae and pupae; it is absent or present in very low amounts in adults, even after iron loading. In contrast, HCH ferritin subunits are present in varying amounts at all stages. The 26 kDa HCH subunit is most abundant and its amount usually increased in iron-loaded insects. The abundance of the 24 kDa HCH subunit also increased in larvae and pupae raised in iron-rich water; in adults it was seen only in insects raised in hemin or $FeCl_3$ supplemented water.

Figure 4C shows a Western blot of extracts prepared from whole mosquitoes 24 h after feeding on several types of meals. All meals, including saline, resulted in an increase in ferritin HCH synthesis. The 26 kDa subunit is usually dominant. The 24 kDa HCH subunit is not present in sugar fed female mosquitoes, but it appeared after feeding natural or substitute blood meals. Both 26

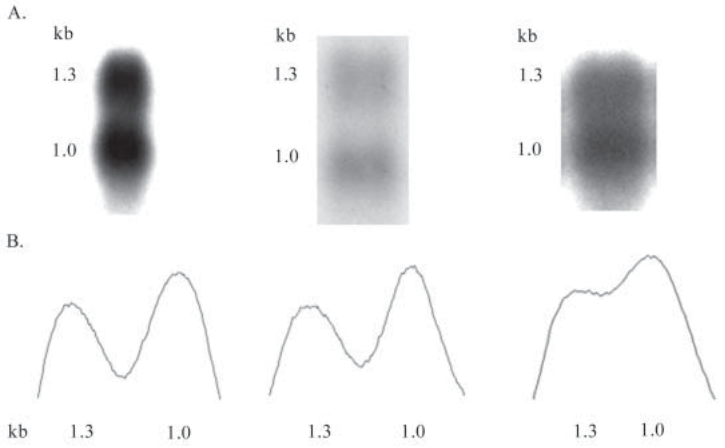

Figure 5B. Relative abundance of the 1.3 and 1.0 kb HCH messages in female *Aedes aegypti* 48h after blood feeding. A. Northern blots prepared with total RNA isolated from female mosquitoes 48h after blood feeding on a rabbit and hybridized with a ferritin HCH probe. The blots are from three different experiments; the blot in the middle is from the blot shown in Fig. 5. B. Plots of the areas of the 1.3 and 1.0 kb bands measured from the Northern blots using the Scion Image computer program. The ratios of the 1.0 to the 1.3 kb band calculated from these plots were 1.41, 1.29, and 1.41 respectively.

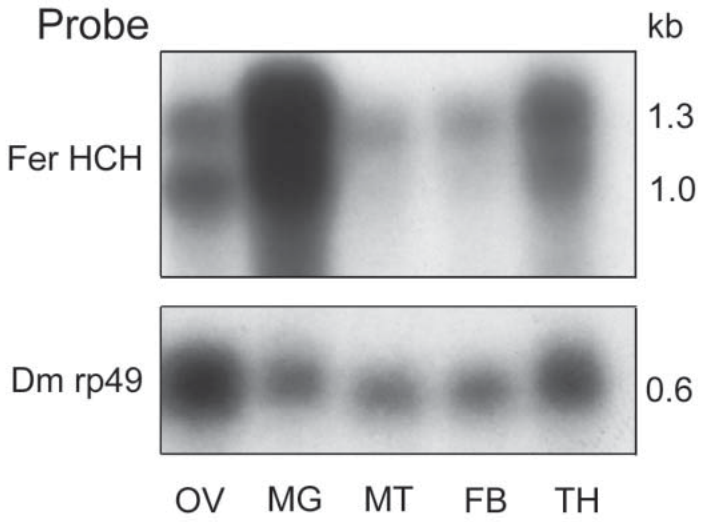

Figure 6. Organ-specific expression of Fer HCH messages in blood-fed female *Aedes aegypti*. Northern blot of total RNA isolated from organs dissected from mosquitoes 24 hours after blood feeding. The blot was hybridized with probes from the ferritin HCH coding region and 3'UTR (Fer HCH) and with *D. melanogaster* rp49 (Dm rp49). OV, ovaries; MG, midgut; MT, Malpighian tubules; FB, fat body + abdominal carcass; TH, thorax + head +legs.

kDa and 24 kDa HCH subunits increased after a meal of blood, hemoglobin, ABM, or saline supplemented with ferric ammonium citrate. This increase was even greater when the ABM was supplemented with iron salts. However, when hemin and FAC were added, the levels of the 24 and 26 kDa subunits were more equivalent, suggesting that the relative level of the 24 kDa subunit increased. In contrast, the LCH ferritin subunit (28 kDa) is not detectable in either fed or non-fed females.

Organ distribution of ferritin subunits in blood-fed mosquitoes.

We investigated the distribution of ferritin in different organs and body parts of mosquitoes non-fed (sugar-fed) or fed a natural blood meal (Fig. 8). Ferritin HCH is present as a faint 26 kDa band in midgut wall, fat body and hemolymph of sugar-fed insects and the abundance of this band increased after blood feeding. This HCH subunit is very abundant in the thorax of blood-fed mosquitoes. A faint 24 kDa HCH band appeared in the fat body 48h after blood-feeding. The LCH subunit is seen in extracts of the thorax, hemolymph, and fat body dissected from blood-fed females. A higher molecular weight band of ~30 kDa is also seen in the midgut wall of blood-fed females. Since a dramatic increase in ferritin HCH messages was seen in the midgut, and because the midgut is rich in protease activities, purified ferritin was added during the extraction and we found no evidence of protein degradation (Fig. 9). This blot also demonstrates that all of the midgut ferritin is found in the midgut wall and not in the lumen.

Ferritin HCH and LCH subunits accumulate in the ovaries 24h after a blood meal (Fig. 10). The 30 kDa band is also seen. This ferritin subunit pattern persists in ovaries 48h and 77h after blood feeding. In early embryos the 30 kDa ferritin band almost disappears and it is seen again in eggs containing pharate 1st instar larvae.

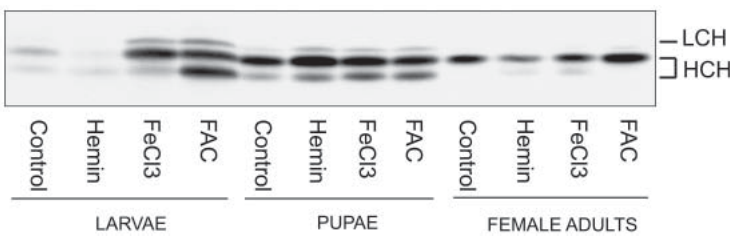

Figure 7. Expression of ferritin subunits in control and iron-loaded *Aedes aegypti*. Western blot of protein extracts (one insect per lane) probed with antibodies against ferritin. Fourth instar larvae, female pupae, and adults raised in control water or in water supplemented with hemin, FeCl3, or FAC were collected from mass cultures. The protein bands corresponding to LCH (28 kDa) and HCH (26 and 24 kDa) are indicated on the right.

Discussion

In this paper we report the temporal, organ specific, and induction expression pattern of the HCH messages as well as of the HCH and LCH subunits of *Ae. aegypti* secreted ferritin. HCH messages were of two lengths as a result of alternative polyadenylation. The abundance of both messages increased after raising larvae in iron-supplemented water or blood feeding of female mosquitoes. A more prominent increase in messages with a short 3'UTR was evident. A concomitant increase in ferritin polypeptide abundance was also observed. HCH was the predominant ferritin subunit type at all developmental stages and in all adult organs that were examined. The blood meal-induced increase in ferritin message and polypeptide abundance was transient and organ specific. While midgut and ovaries of blood-fed females were major sites of ferritin HCH message synthesis, HCH polypeptides became very abundant in the hemolymph, accumulated in the ovaries and were found in the eggs.

Alternative polyadenylation: a common feature of ferritin messages with possible function in ferritin regulation.

The Northern blots clearly demonstrated the presence of *Ae. aegypti* ferritin HCH messages of two lengths. Detailed examination of the sequence of the HCH cDNA (Dunkov et al., 1995) indicated that the shorter messages seen on the blots result from the use of four overlapping proximal non-canonical polyadenylation signals. This is also supported by the recent cloning of two short *Ae. aegypti* HCH cDNAs (GenBank accession numbers AF326341 and AF326342; Morlais and Severson, personal communication) that are derived from messages with poly A tails starting eight nucleotides after the last of the putative proximal signals. Interestingly, in agreement with the results presented here,

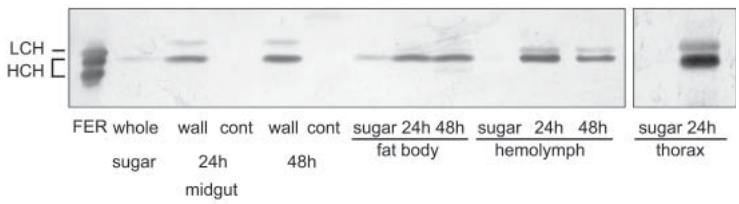

Figure 8. Organ distribution of ferritin subunits in sugar- and blood-fed female *Aedes aegypti*. Western blots prepared with extracts of organs dissected from 4 insects, and of thoraces from 2 insects and probed with antibodies against ferritin. Whole midgut from sugar fed females was used. Midgut from blood fed females was separated into the gut wall and gut contents. FER, ferritin purified from iron-loaded pupae; wall, midgut wall; cont, midgut content. Organs and body parts were dissected from sugar-fed or from blood-fed female mosquitoes 24h and 48h after the blood meal, as indicated.

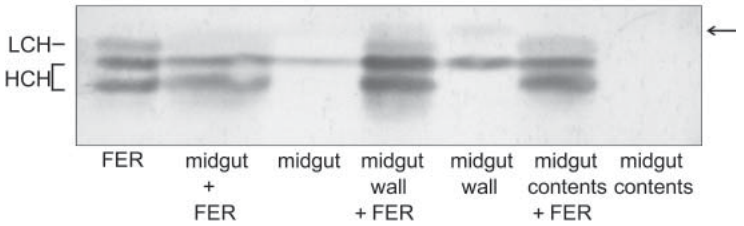

Figure 9. Ferritin expression in the midgut of blood-fed female *Aedes aegypti*. Western blot of extracts prepared from midguts dissected from mosquitoes, 24 hours after feeding on bovine blood and probed with antibodies against ferritin. Ferritin purified from iron-loaded *A. aegypti* pupae (FER) was added during the preparation of the extracts where indicated to serve as a protein degradation control. Protein extracts were prepared from whole midguts from 4 insects, midgut walls from 4 insects, and midgut contents from 3 insects. The protein bands of ferritin HCH and LCH subunits are indicated on the left; the arrow on the right points to a higher molecular weight band.

these cDNAs have been cloned from midguts of blood-fed mosquitoes and are induced by blood meal (Morlais and Severson, personal communication). We previously demonstrated that *D. melanogaster* also produced HCH and LCH messages of different lengths by use of two polyadenylation sites. The shorter messages in *D. melanogaster* were also predominant in the midgut and their abundance increased after iron supplementation of the diet (Georgieva et al., 1999, 2002). We examined cDNA sequences coding for ferritin subunits from several insects for the presence of putative proximal polyadenylation signals. Either canonical (AATAAA) or modified putative polyadenylation signals were found in cDNAs encoding HCH and/or LCH subunits from *Manduca sexta* (Pham et al., 1996; Zhang et al., 2001), *Calpodes ethlius* (Nichol and Locke, 1999), *Nilaparvata lugens* (Du et al., 2000) and *Galleria mellonella* (Kim and Yun et al., 2001). Ferritin heavy chain messages of different lengths resulting from alternative polyadenylation have been described in humans (Dhar and Joshi, 1993; Percy et al., 1998), and several ferritin heavy chain messages presumably resulting from differential processing have been also observed in rats (Gould et al., 2000). The human liver, an organ with high iron and ferritin content, is enriched in the shorter messages while the longer messages are predominant in fetal brain (Percy et al., 1998). Thus, alternative polyadenylation appears to be a common feature of ferritin mRNAs in various animals. It is noteworthy that in both mammalian and insect systems ferritin messages with short 3'UTRs are much more abundant in tissues with high constitutive (mammalian liver) or inducible (insect midgut) synthesis of ferritin subunits. Various sequence determinants mostly in the 3'UTRs of mRNAs have been implicated in mRNA stability (Ross, 1995). It has been noted that a pyrimidine-rich sequence in the 3'UTR of the ferritin heavy chain messages (with short 3'UTR) in a human monocytic cell line plays a destabilizing role via interaction with a cytosolic protein (Ai and Chau, 1999). It is tempting to speculate that ferritin messages with long 3'UTRs might contain additional copies of similar instability cis-elements. Thus, a shift toward shorter messages would increase message stability and result in ferritin mRNA and protein up-regulation. Indeed, polypyrimidine sequences are present in all insect ferritin messages with extended 3'UTRs, but their role in message stability remains to be tested.

Regulation of ferritin synthesis by iron and blood.

While this study focussed on the induction pattern of ferritin mRNAs and polypeptides, the results obtained give an insight into possible regulatory mechanisms and allow comparison with *D. melanogaster* and vertebrate systems. Raising larval mosquitoes on a $FeCl_3$ supplemented diet led to an increase of ferritin subunit abundance (Dunkov et al., 1995). Here we demonstrate that iron enrichment of the diet results in increased abundance of HCH messages in *Ae. aegypti* larvae, particularly of messages with short 3' UTR. Interestingly, even when an iron chelator (NTA) alone is added to the diet, a significant increase in ferritin messages is seen (Fig. 3). A possible explanation is that NTA makes iron that is already present in the diet available for absorption, which then leads to ferritin upregulation. The abundance of ferritin HCH and LCH polypeptides also increased in larvae and pupae raised in iron rich water; elevated ferritin levels persist in adults derived from such larvae. This upregulation of ferritin messages and protein by iron closely resembles the situation in another dipteran, *D. melanogaster* (Georgieva et al., 1999, 2002). However in contrast to *D. melanogaster* ferritin, in which LCH predominates, 26 kDa HCH is the major subunit in *Ae. aegypti*.

In an attempt to dissect the mechanisms of ferritin induction by iron, experiments with *Ae. aegypti* cells in culture have demonstrated that ferritin HCH messages and subunits are induced threefold after iron treatment and that this induction is under transcriptional control (Pham et al., 1999). The presence of an iron reponsive element in the 5' UTR of the HCH mRNA (Dunkov et al., 1995) and the failure of transcription inhibitors to completely block ferritin induction by iron in *Ae. aegypti* cells (Pham et al.1999) suggest that translational control is also involved in the regulation of this ferritin subunit. Since the structure of the *Ae. aegypti* LCH mRNA is not known at present, the expression data for the LCH subunit cannot be adequately interpreted.

Of particular interest are the results obtained after feeding adult female mosquitoes with natural and modified blood meals. A natural blood meal, a hemoglobin meal, ABM, and even a saline meal all led to ferritin upregulation, but ABM supplemented with heme or non-heme iron resulted in even greater accumulation of ferritin. These results are consistent with observations made with various mammalian cells where both heme and iron salts led to ferritin induction at transcriptional and posttranscriptional level (Eisenstein et al., 1991; Coccia et al., 1994, 1995; McGahan et al., 1994). The apparent lack of correlation between the HCH message and protein levels (Fig. 4) most probably reflects the fact that the

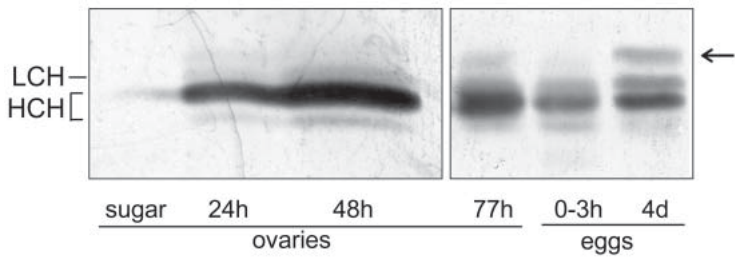

Figure 10. Presence of ferritin subunits in ovaries and eggs of *Aedes aegypti*. Western blots probed with antibodies against ferritin. Protein extracts were prepared from ovaries of 4 mosquitoes sugar fed, 24h and 48h after blood feeding, from ovaries of 2 mosquitoes 77h after blood feeding, from 40 embryos (eggs 0-3 h after oviposition), and from 40 pharate 1st instar larvae (eggs 4 days after oviposition). The protein bands of ferritin HCH and LCH subunits are indicated on the left; the arrow on the right points to a ~30 kDa band.

synthesis of *Ae. aegypti* HCH subunits is under both transcriptional and translational control by iron. This can be further explained with uncoupling of the transcription and translation under high iron conditions, as we have suggested for *D. melanogaster* LCH (Georgieva et al., 2002).

Physiological role of ferritin upregulation after a blood meal.

Natural blood meals are rarely enriched in non-heme iron, but they are very rich in heme (Kogan 1990), a potent prooxidant. The results presented here indicate that ferritin, which accumulates in female mosquitoes after a blood meal, probably serves a dual function: 1) to sequester iron released from heme and thus prevent oxidative damage; and 2) to provide an iron store in the eggs to be used by the developing embryos. The role of ferritin as an antioxidant in various mammalian tissues is well established (Balla et al., 1992, 1995; Vile and Tyrrell, 1993; Borman, et al., 1999). Our results, demonstrating that the midgut of blood-fed mosquitoes is a major site of ferritin message and subunit synthesis, are consistent with such antioxidant role of ferritin.

The dramatic increase of ferritin HCH messages in the midgut after a blood meal could be the source of ferritin in the hemolymph. This is supported by studies with *D. melanogaster* where high hemolymph ferritin concentrations were also attributed to increased ferritin message synthesis in the midgut (Georgieva et al, 2002). Secretion of ferritin from the fat body could also contribute to the hemolymph ferritin pool, as suggested by the increased ferritin abundance in this tissue after a blood meal. A portion of the midgut ferritin in insects might be secreted into the lumen, as suggested by Nichol and Locke (1990). However, as the peritrophic matrix is not permeable for ferritin (Perone and Spielman, 1988) any ferritin that enters the lumen would remain between the gut wall and peritrophic matrix. Our results (Fig. 9) indicate that no detectable amounts of excreted ferritin were present in the midgut 24h after a blood meal. Consistent with these results, we did not detect ferritin in extracts from whole hindguts 24h after a blood meal (data not shown).

It is interesting that a protein band of ~30 kDa, most likely corresponding to unprocessed HCH or LCH ferritin polypeptides, is seen on Western blots prepared from both midgut and ovaries (Figs. 8, 9, 10). This is consistent with the increased ferritin HCH message abundance in these organs observed after a blood meal, which probably results in elevated ferritin synthesis and thus allows detection of unprocessed polypeptides. Interestingly, a 30 kDa band was observed in eggs with pharate 1st instar larvae as well, but not in early embryos. Most likely, ferritin synthesis is low during early embryogenesis but resumes in pharate 1st instar larvae, resulting in the detection of the 30 kDa band. The observed accumulation of ferritin in the ovaries and eggs of *Ae. aegypti* is in accord with similar findings in *D. melanogaster* (Georgieva et al., 2002). These results are also in agreement with studies on *Sarcophaga peregrina* (Kurama et al. 1995) suggesting that ferritin is the main iron storage protein in the eggs of this fly. In addition, recently Kim and Lee et al. (2001) demonstrated the presence of ferritin in the yolk and in the vitelline membrane of the oocyte in the moth *Galleria mellonella*. Ferritin is also the major yolk protein in snails (Bottke, 1982) and it is found in the eggs of amphibians (Brown and Caston, 1962; Kandror et al., 1992) and sea urchins (Infante et al., 1993).

It is noteworthy that in contrast to *D. melanogaster*, HCH is the predominant ferritin subunit type in *Ae. aegypti*. While the roles of HCH and LCH chains in the function of insect secreted ferritins are not clear, the observed differences might reflect specific iron storage requirements of these insects. Indeed, *D. melanogaster* is rarely exposed to high iron diets while *Ae. aegypti* females take blood meals rich in heme iron. HCH ferritin chains may be somehow more effective in meeting the increased need for iron sequestration after a blood meal.

In conclusion, the results reported here describing the constitutive and inducible temporal and spatial pattern of ferritin expression provide a background for further investigation of the molecular mechanisms that control ferritin function in the mosquito *Ae. aegypti*. Two observations deserve special attention: the transient upregulation of ferritin HCH gene and the accumulation of secreted ferritin in the hemolymph after a natural blood meal. Together with the already known gene structure (Pham et al., 2000), and with the available technology for producing transgenic mosquitoes (Coates et al., 1998; Jasinskiene et al., 1998), these results should stimulate studies designed to use the ferritin HCH gene promoter for directing expression of antiparasite genes in a time and tissue specific manner.

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