Doctoral Thesis

Studies on the functions of *Haemaphysalis longicornis* ferritins and their potential as target molecules for tick control

フタトゲチマダニ由来フェリチンの機能とマダニ
コントロールへの標的分子としての可能性に関する研究

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ABBREVIATIONS

cDNA	-	complementary DNA
dsRNA	-	double-stranded RNA
ELISA	-	enzyme-linked immunosorbent assay
EST	-	expressed sequence tags
FAC	-	ferric ammonium citrate
Fe^{2+}	-	ferrous iron
Fe^{3+}	-	ferric iron
FER	-	ferritin
GFP	-	green fluorescence protein
HE	-	hematoxylin & eosin
Hlfer1	-	*Haemaphysalis longicornis* ferritin 1 gene
HlFER1	-	*H. longicornis* ferritin 1 protein
Hlfer2	-	*H. longicornis* ferritin 2 gene
HlFER2	-	*H. longicornis* ferritin 2 protein
HlMRC	-	*H. longicornis* short C-type mannose receptor homolog protein
HlPrx	-	*H. longicornis* peroxiredoxin2 protein
HlVg	-	*H. longicornis* vitellogenin genes
HRP	-	horseradish peroxidase
IFAT	-	indirect immunofluorescent antibody test
IPTG	-	isopropyl β-D-1-thiogalactopyranoside
IRE	-	iron-responsive element
IRP	-	iron-regulatory protein
LB	-	Luria-bertani
Luc	-	firefly *luciferase* gene
MDA	-	malondialdehyde
mRNA	-	messenger RNA
ORF	-	Open reading frame
PBS	-	phosphate-buffered saline
PBS-T	-	PBS with 0.05% Tween 20
PC	-	protein carbonyl
PVDF	-	polyvinylidene difluoride
R_H	-	Reduction of hatching

R_O	-	Reduction of oviposition
R_W	-	Reduction of tick engorged weight
rHlFERs	-	recombinant *H. longicornis* ferritins
RNAi	-	RNA interference
ROS	-	reactive oxygen species
RT-PCR	-	reverse transcriptase-polymerase chain reaction
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFTS	-	Severe fever with thrombocytopenia syndrome
TAE	-	Tris-acetate EDTA
TBARS	-	thiobarbituric acid reactive species
TBDs	-	tick-borne diseases
TBS	-	Tris-buffered saline
TEM	-	transmission electron microscopy

GENERAL INTRODUCTION

Ticks are obligate blood-sucking arthropods known to transmit a wide variety of infectious diseases worldwide. While the blood-feeding behaviour of ticks, which causes anemia, irritation, allergic reactions, and (in the case of some tick species) toxicoses [1] can be already harmful in its own rite, particularly in heavy infestation, further harm is done because transmission of pathogens also occurs during this process [2]. They are considered second to mosquitoes in terms of their impact on public health, but they are the most important vectors of different pathogens in both domestic and wild animals [3]. Tick-borne diseases continue to have great economic impact on livestock production, particularly on cattle and small ruminants, in several continents [1]. Tick-borne infections are commonly shared by animals and humans, and mostly are categorized as emerging and re-emerging infectious diseases [4]. The hard tick *Haemaphysalis longicornis,* mainly distributed in East Asia and Australia, is a known vector of babesiosis caused by *Babesia ovata, B. major*, *B. gibsoni*, and possibly *B. bigemina* and of theileriosis caused by *Theileria sergenti*, *T. orientalis*, and *T. buffeli* [5]. Recently, *H. longicornis* has been strongly implicated as a vector of severe fever with thrombocytopenia syndrome (SFTS) virus affecting humans, which has been reported in China [6], Japan [7] and South Korea [8].

In the Philippines, tick-borne diseases (TBDs) still threatens livestock and companion animal health. Although tick-resistant breeds are commonly raised, the risk for TBDs is still high. The Philippine National Dairy Authority has identified tick fever as the major cause of disease outbreaks that usually occur in imported dairy cattle, often resulting to substantial loss of valuable breeding stock. In companion

animals particularly dogs, tick-borne infections, such as ehrlichiosis and babesiosis, are also commonly encountered. The common practice for tick control is the application of chemical acaricides.

Tick control is essential in controlling TBDs. Different methods of tick control, including the use of chemical acaricides and vaccines, are presented with several challenges [9]. Problems with the use of chemical acaricides arise from emergence of resistant tick strains and environment and animal product contamination. Studies on the identification of tick protective antigens that can be used on the formulation of potent vaccines continue to gain much interest.

Hematophagous arthropods, including ticks and mosquitoes, utilize blood for nutrients and reproduction. The blood meal of female mosquitoes provides iron, which is required for optimal egg development and offspring viability [10]. In female ticks, initiation of the reproductive cycle necessitates a blood meal. Host-derived heme is bound to vitellogenin and further incorporated into developing oocytes [11]. Most hematophagous arthropods ingest enormous amounts of blood in a single meal [12]. Similarly, ticks have developed several mechanisms that allow them to efficiently exsanguinate their hosts, evading hemostatic, inflammatory, and immune responses [13]. Aside from their sophisticated feeding mechanism, the adaptation of ticks to potentially toxic molecules in their hosts' blood has enabled them to thrive in their exclusively hematophagous lifestyle. Remarkably, adult female hard ticks can ingest blood more than a hundred times their unfed body weight in a single blood meal [14]. Host blood contains pro-oxidants that may induce oxidative stress. A number of antioxidant enzymes have been identified in the tick midgut that likely are responsible for protecting ticks from oxidative stress [15,16]. Iron is a component of the host blood that has both beneficial and harmful effects on ticks.

Iron is an essential element required for various physiological processes in most living organisms. Iron metabolism involves a continuous redox cycling between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. Fe^{2+} is potentially toxic due to its ability to catalyze the formation of reactive oxygen species (ROS) through Fenton reaction [17]. High levels of ROS can lead to cellular damage and death, resulting from damage to biomolecules including lipid peroxidation, DNA and protein oxidation, which is collectively known as oxidative stress [18]. Oxidative stress occurs when the level of ROS overwhelms the antioxidant defense mechanisms, accompanied by the accumulation of oxidative stress products. These products of oxidative damage to biomolecules can be used as indicators in evaluating oxidative stress, termed biomarkers [19].

Iron in the blood occurs as heme, which is bound to hemoglobin, and non-heme or inorganic iron. Non-heme iron accounts for the smaller proportion of circulating iron in the host blood compared to hemoglobin and is usually bound to metalloproteins, such as iron–sulfur clusters, or to an iron-binding protein transferrin as ferric-transferrin [20]. Compared to heme iron, ferric-transferrin is more bioavailable and a higher percentage of ingested ferric-transferrin is utilized by female mosquitoes, primarily stored in the eggs [10]. Several proteins have already been identified in mosquitoes [21,22] and *Drosophila melanogaster* [23] that function similarly to the proteins involved in mammalian iron metabolism, including the regulation of absorption, transport, and storage of non-heme iron obtained from their diet. In comparison, little is known about the regulation of non-heme iron in hard ticks.

Iron-binding proteins, such as transferrin and ferritin, are present in most living organisms that function to regulate iron levels and prevent iron toxicity. Mammalian ferritins serve mainly as intracellular iron storage proteins, while insect

ferritins also function in iron transport [24]. Aside from iron transport and storage functions, ferritin was also implicated in immune response [25] and oxidative stress [26].

Iron metabolism and iron-binding proteins obviously have a protective role in ticks, preventing toxicity when faced with high levels of iron during their blood meal, and is therefore an attractive target for controlling ticks. This dissertation describes studies on ferritin molecules of *H. longicornis* with the following specific objectives:

1. To identify and characterize ferritin molecules in the hard tick *Haemaphysalis longicornis*.
2. To determine the functions of *H. longicornis* ferritins (HlFERs) on tick physiology, survival, and reproduction.
3. To determine if HlFERs has a function on establishment of pathogen within the tick.
4. To evaluate the potential of HlFERs as target molecules for tick control using recombinant protein vaccines.

CHAPTER 1

Multiple ferritins are vital to successful blood feeding and reproduction of the hard tick *Haemaphysalis longicornis*

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1.1. INTRODUCTION

Ferritin is an iron storage protein that plays an important role in the iron metabolism of most organisms. In general, ferritins consist of 24 subunits, which fold in a 4-helical bundle, with a large cavity that can accommodate up to 4000 Fe atoms [27]. Vertebrate ferritins are composed of two types of subunits, the heavy (H) chain, associated with Fe^{2+} oxidation, and the light (L) chain, which assists in iron nucleation and core formation [28]. Similarly, insect ferritins have two types of subunits, referred to as a heavy-chain homolog (HCH) and a light-chain homolog (LCH) [24]. Ferritin functions both in iron homeostasis and protection against oxidative damage. Ferritin is considered to be a critical cytoprotective protein that comprises an essential part of the antioxidant response [29]. In insects, it also plays a role in iron transport [24].

In ticks, iron metabolism remains poorly understood. The hard tick *Rhipicephalus (Boophilus) microplus* lacks a heme synthetic pathway [30]. A heme-binding lipoprotein, HeLp, has been described in this tick as a special adaptation. HeLp transports heme in the hemolymph and peripheral tissues, primarily the ovaries [31]. The ferritin gene from different species of ticks [32] has already been identified. Knockdown of two ferritins from *Ixodes ricinus* caused adverse effects on tick feeding and reproduction [33].

Here, I describe the characterization of a newly identified secretory ferritin from *H. longicornis*, ferritin 2 (HlFER2), together with the previously reported intracellular ferritin, herein referred to as ferritin 1 (HlFER1). I examined the distribution and expression pattern of both HlFERs in different tissues and developmental stages of the tick and further evaluated their physiological importance

in blood feeding and reproduction of *H. longicornis* through a reverse genetic approach.

1.2. MATERIALS AND METHODS

1.2.1 *Ticks and experimental animals*

The parthenogenetic, Okayama strain of *H. longicornis* was used in all experiments throughout this study. Ticks were maintained by feeding on ears of Japanese white rabbits (Kyudo, Kumamoto, Japan) for several generations at the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan [34]. Rabbits were also used in all tick infestation experiments. Mice (strain ddY, 6 weeks old, female) were used for HlFER anti-serum preparation. The experimental animals were kept in a temperature- and humidity-controlled room, with a constant supply of water and commercial feeds. The care and use of experimental animals in this study has been approved by the Animal Care and Use Committee of Kagoshima University (Approval number VM13007).

1.2.2 *Identification and characterization of Hlfer cDNA clones*

The full-length cDNA library of *H. longicornis* was constructed using the vector-capping method [35], and expressed sequence tags (EST) were prepared in our laboratory [36]. cDNA clones encoding ferritin were identified and selected from the EST database for further analysis. Plasmids containing *Hlfer* gene-encoding inserts were extracted using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and were subsequently analyzed using plasmid-specific primers through an automated sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) to determine the full-length sequences. The deduced amino acid translation of *Hlfer* genes was determined using GENETYX software (Genetyx, Tokyo, Japan). Homologous search of the full-length *Hlfer* sequences was performed using the

BLAST program, through which putative conserved domains were also identified. The presence of signal peptide and its cleavage site was determined using the prediction server SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), and the predicted molecular weight and isoelectric points (pIs) were determined using the ExPASy server (http://web.expasy.org/peptide_mass/). Analyses for *N*-glycosylation, domain structure and ligand binding were performed using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), PROSITE software (http://prosite.expasy.org/), and PDBe motif and site software (http://www.ebi.ac.uk/pdbe-site/pdbemotif/), respectively. A phylogenetic tree was also constructed based on the amino acid sequences of ferritins from selected species by the neighbor-joining method using MEGA software version 5.0 (Tempe, AZ, USA).

1.2.3 *Expression and purification of recombinant proteins*

The open reading frame (ORF) of *Hlfer1* and *Hlfer2* was extracted from pGCAP1 vector using gene-specific primers (Table 1-1). For *Hlfer2*, a forward primer without signal peptide sequence was used. PCR products were purified using the GENECLEAN II kit (MP Biomedicals, Solon, OH, USA) and then subcloned into the pRSET A vector (Invitrogen, Carlsbad, CA, USA). The resulting plasmids were checked for accurate insertion through restriction enzyme analysis and purified using the Qiagen Plasmid Mini Kit (Qiagen). Purified plasmids were expressed in *Escherichia coli* BL21 cells, grown in Luria–Bertani (LB) broth medium with ampicillin. Synthesis of recombinant HlFERs tagged with histidine was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mmol l^{-1}. Cells were collected by centrifugation, and protein was extracted through ultra-sonication. Purification was carried out through chromatography using a HisTrap

column (GE Healthcare, Uppsala, Sweden) and then dialysis against phosphate-buffered saline (PBS). Purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Concentration of proteins was also determined through SDS-PAGE using bovine serum albumin as standard. The Micro BCA Assay kit (Thermo Scientific, Rockford, IL, USA) was also used for soluble proteins.

1.2.4 *Preparation of mouse anti-ferritin sera*

To prepare mouse anti-HlFER sera, each mouse was injected intraperitoneally with 100 μg of recombinant HlFER and completely mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St Louis, MO, USA). Immunization was repeated 14 and 28 days after the first immunization, using an incomplete adjuvant (Sigma-Aldrich). All sera were collected 14 days after the last immunization.

1.2.5 *Total RNA extraction and reverse-transcriptase PCR analysis*

Total RNA was extracted from different developmental stages (egg, larva, nymph and adult) and tissues of adult female ticks, including the midgut, salivary glands, ovary, fat body and hemocytes, during blood feeding. For different developmental stages, whole tick samples were homogenized using a mortar and pestle in TRIzol reagent (Invitrogen). Tissues were dissected and washed in PBS and then placed directly in tubes with the TRIzol reagent. Hemocytes were collected as described previously [37]. RNA extraction was performed based on the manufacturer's protocol. Single-strand cDNA was prepared by reverse transcription using the Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's protocol. RT-PCR was carried out using *Hlfer* gene-specific primers (Table 1-1). Primers for actin, used for control amplification, and vitellogenin genes (*HlVg*s) are described elsewhere [38]. PCR products were

subjected to electrophoresis in 1.5% agarose gel in a Tris-acetate EDTA (TAE) buffer, and bands were visualized after staining the gel with ethidium bromide using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy).

1.2.6 *Protein extraction and western blot analysis*

Protein extraction was performed as described previously [39,40]. Tick protein extracts were separated with 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA, USA). The membrane was blocked overnight with 5% skim milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T) and then incubated with a primary antibody using mouse anti-ferritin sera (1:500 dilution). For detection of protein in the whole and different tissues of adult ticks, an antiserum previously prepared for β-actin [41] was used as the control. Alternatively, β-tubulin antiserum [42] was used for experiments that include the egg, wherein actin band was difficult to detect, particularly in the early stage of embryogenesis. After incubation with peroxide-conjugated sheep anti-mouse IgG (1:50,000 dilution; GE Healthcare, Little Chalfont, Buckinghamshire, UK), a signal was detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and analyzed using FluorChem FC2 software (Protein Simple, Santa Clara, CA, USA).

1.2.7 *Indirect immunofluorescent antibody test*

An indirect immunofluorescent antibody test (IFAT) was performed to demonstrate the endogenous localization of HlFERs, following the method previously described [39,43]. Briefly, the midgut, salivary glands and ovaries from 4-day partially fed adult ticks were fixed overnight in 4% paraformaldehyde in PBS with 0.1% glutaraldehyde added, and then washed with different concentrations of sucrose

in PBS. The organs were embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan, Tokyo, Japan) and then frozen using liquid nitrogen. Tissue sections of 10 μm thickness were cut using a cryostat (Leica CM 3050; Leica Microsystems, Wetzlar, Germany) and placed on MAS-coated glass slides (Matsunami Glass, Osaka, Japan). After overnight blocking with 5% skim milk in PBS at 4°C, sections were incubated with a 1:50 dilution of anti-ferritin sera for 2 h at room temperature. Normal mouse serum was used as a negative control at the same dilution. Sections were washed with PBS and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000; Invitrogen) for 1 h at room temperature. Following washes with PBS, the tissue sections were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken using a fluorescence microscope mounted with a DP71 camera and processed using DP Controller software (Olympus, Tokyo, Japan).

1.2.8 *RNA interference (RNAi)*

The PCR primers used for the synthesis of double-stranded RNA (dsRNA) are listed in Table 1-1. The *Hlfer* fragments were amplified by PCR from cDNA clones using oligonucleotides, including T7-forward and T7-reverse primers, to attach the T7 promoter recognition sites on both the forward and reverse ends. The firefly *luciferase* (*Luc*) was amplified from a vector DNA of pGEM-luc (Promega, Madison, WI, USA) through PCR using oligonucleotides containing T7-forward and T7-reverse primers. PCR products were purified using the GENECLEAN II kit (MP Biochemicals). The T7 RiboMAX Express RNA System (Promega) was used to synthesize double-stranded RNA (dsRNA) by *in vitro* transcription following the manufacturer's protocol. Successful construction of dsRNA was confirmed by running 1 μl of the dsRNA products in a 1.5% agarose gel in a TAE buffer. Microinjection of

dsRNA was performed as previously described [39,40]. Briefly, 1 μg of *Hlfer1* or *Hlfer2* dsRNA in 0.5 μl of PBS was injected into the hemocoel of unfed adult female ticks through the fourth coxae. *Luc* dsRNA was injected in the control group. A total of 40 ticks per group were injected. After injection, the ticks were held for 18 h in a 25°C incubator to check for mortality resulting from possible injury during injection. Injected ticks from both the experimental and control groups were simultaneously infested on rabbit ears, with one group in each ear. To confirm gene-specific silencing, 10 ticks from each group were collected 4 days after attachment, and then total RNA and protein lysates were prepared for RT-PCR and western blot analysis, respectively. The remaining ticks were allowed to feed to repletion until they naturally dropped off from the host. The success of tick feeding was determined by measuring the weight and mortality after detachment from the host. The success of reproduction was determined by oviposition and hatching of eggs. This experiment was performed four times.

1.2.9 *Histological and ultrastructural characterization of the midgut after RNAi*

Four-day partially fed ticks from *Luc* and *Hlfer* dsRNA-injected groups were collected and fixed overnight in 10% formalin and then embedded in paraffin. After deparaffinization and rehydration, sections were stained with hematoxylin & eosin (HE), mounted and observed under the microscope. Images were taken using a microscope mounted with a DP71 camera (Olympus).

For transmission electron microscopy (TEM), sections were prepared as previously described [44]. Briefly, midgut samples were fixed in cold 3% glutaraldehyde in a sodium cacodylate buffer followed by post-fixation with 1% OsO_4 in the same buffer for approximately 2 h. After dehydration with an ethanol series, fixed samples were embedded in epon resin, and sections were cut using a

Leica UCT ultramicrotome (Leica Microsystems) with a diamond knife. Sections were observed under a Hitachi H7000KU electron microscope (Hitachi High-Technologies, Tokyo, Japan).

1.2.10 *Iron-binding activity of recombinant HlFER2*

The iron-binding activity of recombinant HlFER2 was determined and compared with that of commercially prepared horse apoferritin (MP Biomedicals LLC) using the ferrozine-based assay [45,46]. The recombinant HlFER2 and horse apoferritin were dissolved in 1 ml water and mixed with 20 µl of 2 mmol l^{-1} $FeCl_2$ to reach final concentrations ranging from 75 to 600 nmol l^{-1}. Another *His*-tagged protein purified in our laboratory, the recombinant short C-type mannose receptor homolog (HlMRC) (Maeda et al., unpublished results), was used as negative control. After incubation at 30°C for 30 min, 40 µl of 6 mmol l^{-1}ferrozine (Sigma-Aldrich) was added and incubated further for 30 min with constant shaking. Each mixture was transferred to three wells in a microtiter plate, placing 300 µl in each well, and absorbance was measured at 550 nm using a microplate reader (Bio-Rad). The average absorbance reading was obtained, and the experiment was performed four times. The recombinant HlFER2 and horse apoferritin were also subjected to 12% SDS-PAGE and 3–10% native gradient polyacrylamide gels (Pagel, ATTO, Tokyo, Japan).

1.2.11 *Statistical analysis*

All statistical analyses were performed using Student's *t*-tests, with significant difference defined by $P<0.05$.

1.3. RESULTS

1.3.1 *Identification and characterization of Hlfer cDNAs*

Two full-length cDNAs encoding ferritin were identified and cloned from the EST database of *H. longicornis*. The full-length *Hlfer1* we identified contains 814 bp, with the predicted start codon at 142–144 bases, the stop codon at 664–666 bases, and an ORF extending from position 142 to 666, encoding for 174 amino acid polypeptides (Fig. 1-1A). In agreement with a previous report, the 5′ untranslated region (UTR) of*Hlfer1* contains a putative iron-responsive element (IRE), with the highly conserved CAGUGA loop [47]. The calculated molecular mass based on this sequence is ~20 kDa and has a pI of 4.97. *N*-glycosylation site prediction analysis of HlFER1 revealed a potential *N*-glycosylation site at position 109 NQS. Meanwhile, *Hlfer2* has 1635 bp, with the predicted start codon at 43–45 bases, the stop codon at 640–642 bases, and an ORF extending from position 43 to 642, coding for 199 amino acid polypeptides (Fig. 1-1B). The nucleotide sequence data of *Hlfer2* have been deposited in the DDJB/EMBL/GenBank database under accession number AB734098. Unlike *Hlfer1, Hlfer2* does not have IRE in its 5′ UTR. A signal peptide was identified from the HlFER2 sequence using SignalP 3.0 Server prediction software, and a cleavage site was predicted between positions 17 and 18. The calculated molecular mass of HlFER2 without the signal peptide is ~20.8 kDa and has a pI of 5.29. No *N*-glycosylation site was identified in HlFER2. A ferritin-like diiron domain was identified between 30 and 180 amino acids. Ligand-binding statistics showed that both HlFER1 and HlFER2 have a high tendency for iron-binding. Both ferritin nucleotide sequences end with a 20 bp polyadenylation tail at the 3′ UTR.

BLAST analysis showed that HlFER1 has 100% homology with the previously identified *H. longicornis* ferritin (GenBank Accession number AY277905). It also showed high homology with ferritin of other hard ticks and the soft tick *Ornithodoros moubata*, while HlFER2 showed high homology with secreted ferritin of *I. ricinus*. Analysis of putative conserved domains showed that both ferritins contain the seven conserved amino acid residues in the ferroxidase center loop, which are important for metal binding, and a conserved ferrihydrite nucleation center, where ferric atoms are stored [28]. Multiple alignment of amino acid sequences of ferritins from different ticks and selected species (Fig. 1-2) demonstrated that HlFER1 has high identity with intracellular ferritin of the ticks *Rhipicephalus sanguineus* (AY277907.1), *O. moubata* (AF068225.2) and *I. ricinus* (AF068224.1), followed by the human ferritin H-chain (NM_002032.2),*Drosophila melanogaster* ferritin1 HCH (AF145125.1) and the *Aedes aegypti* putative ferritin subunit (XM_001654469.1). In contrast, HlFER2 shares high identity with *I. ricinus*-secreted ferritin (EU885951.1), followed by intracellular ferritin of the ticks *R. sanguineus, O. moubata* and *I. ricinus*, the human ferritin H-chain, *D. melanogaster* ferritin1 HCH and the *A. aegypti* ferritin subunit. A phylogenetic tree was also constructed using amino acid sequences of ferritins from different species by the neighbor-joining method (Fig. 1-3). HlFER1 was found to be closely related to intracellular ferritin of *I. ricinus*, whereas HlFER2 was found to be closely related to *S. mansoni* ferritin (XM_002576034.1).

1.3.2 *Expression profile of ferritin transcripts from different tick organs and developmental stages*

RT-PCR was performed to determine the expression profile of *Hlfer1* and*Hlfer2* from different organs of adult female ticks (Fig. 1-4) and

different developmental stages of the tick during blood feeding (Fig. 1-5A). The organs included midgut, salivary glands, hemocytes, fat body and ovary. The amount of cDNA was indexed based on control amplification using actin-specific primers. Results showed that *Hlfer1* was constitutively expressed in all five organs during blood feeding as well as in different developmental stages. Whereas *Hlfer2* was also constitutively expressed in the midgut and hemocytes, decreasing expression was observed in the fat body and the salivary glands towards engorgement, and limited expression was observed in the ovary only in unfed ticks and on the first day of feeding. *Hlfer2* expression was strong in unfed and engorged larvae, nymphs and adults, weak in the partially fed stages, and barely detectable in the egg.

1.3.3 *Detection of native tick protein using anti-ferritin sera*

The expression of endogenous HlFER in different organs of adult female tick and different developmental stages was determined through western blot analysis using specific anti-ferritin sera. In different developmental stages, HlFER1 and HlFER2 were detected in partially fed and engorged but not in unfed larvae, nymphs and adults (Fig. 1-5B). Interestingly, HlFER2 was detected in the egg, but HlFER1 was not. A similar pattern of expression of HlFER1 and HlFER2 was observed in the midgut and salivary gland (Fig. 1-6A). In the midgut, HlFER expressions remained unchanged regardless of the degree of feeding. In salivary glands, the expressions increased during blood feeding. Meanwhile, in the hemolymph including hemocytes from partially fed adults, HlFER2 but not HlFER1 was detected (Fig. 1-6A). To evaluate the importance of HlFER during oviposition and embryonic development, protein expression was checked in the ovary at different times during oviposition (Fig. 1-6B) and in the eggs at different stages of embryonic development and immediately after hatching (Fig. 1-6C). HlFER1 showed strong expression only in unfed ovary but

none in the egg at any stage of development (data not shown). HlFER2, meanwhile, was expressed in unfed ovary, and expression decreased during feeding but increased again during the oviposition period. In the egg, HlFER2 was constitutively expressed throughout embryonic development and decreased in newly hatched larvae.

1.3.4 *Demonstration of HlFER in different organs using IFAT*

The endogenous localization of HlFER1 and HlFER2 in adult midgut, salivary glands and ovary was demonstrated using IFAT (Fig. 1-7). Both HlFERs were found in the cytoplasm of digestive cells in the midgut. However, HlFER1 showed stronger fluorescence than HlFER2, which showed punctate fluorescence in the cytoplasm of digestive cells, suggesting that HlFER2 is located inside granules within the cells. In salivary glands, both HlFER1 and HlFER2 showed a dot-like pattern of fluorescence along the basement membrane of salivary acini and salivary duct. In the ovary, HlFER1 fluorescence was distributed throughout the oviduct and, to some extent, in the oocytes, whereas HlFER2 showed strong fluorescence on the surface of oocytes.

1.3.5 *Impact of Hlfer silencing on feeding, survival and reproduction*

To evaluate the importance of HlFERs on blood feeding and reproduction of ticks, gene silencing through RNAi was performed. Ticks were individually injected with either *Hlfer1* or *Hlfer2* dsRNA or with *Luc* dsRNA for the control group. Silencing of both *Hlfer1* and *Hlfer2* had significant effects on feeding and fecundity, as shown in Fig. 1-8. The average body weight of both *Hlfer1*- and *Hlfer2*-silenced ticks was 72.1 and 63.6% lower than that of the control *Luc* group, respectively (Table 1-2). Clearly, silencing of both *Hlfer1* and *Hlfer2* caused failure to feed to repletion. *Hlfer*-silenced ticks showed high mortality after a blood meal, the *Hlfer1*-silenced group having more than 90% mortality. Furthermore, most ticks died without laying eggs. The control group did not have any mortality until the oviposition period

had finished. *Hlfer* silencing also significantly reduced the fecundity of ticks. *Hlfer2*-silenced ticks that laid eggs had lower egg weight to body weight ratios, and the eggs had abnormal morphology and lower hatchability. Gene silencing from whole ticks and different organs was confirmed to be successful using RT-PCR, western blotting and IFAT (Figs. 1-9 & 1-10). Interestingly, we found that silencing of *Hlfer1* led to a reduction of HlFER2 expression in whole ticks and the midgut and its absence in the salivary glands and ovary, whereas silencing of *Hlfer2* reduced the expression of HlFER1 in the salivary glands and ovary.

To clarify the decreased reproductive capacity after silencing of *Hlfer1* and*Hlfer2*, I determined whether there was an effect on the expression of different *HlVg*s using RT-PCR (Fig. 1-11). Three *HlVg*s (*HlVg-1, HlVg-2* and *HlVg-3*) have been identified from *H. longicornis* and have been shown to be essential for the tick's reproduction [36]. Interestingly, I found that silencing of *Hlfer1* led to silencing of*HlVg-1* and reduced the expression of *HlVg-3*, whereas silencing of *Hlfer2* reduced the expression of both *HlVg-1* and *HlVg-3*. No effect on *HlVg-2* was observed from silencing of either *Hlfer1* or *Hlfer2*. Due to the marked effect of*Hlfer1* silencing on *HlVg-1*, we further examined its expression in the midgut, where it was reported to be mainly expressed [36]. Nevertheless, I only found reduction in expression after *Hlfer1* knockdown, in contrast with the result using whole tick cDNA (Fig. 1-11).

1.3.6 *Microscopic analyses of the midgut after RNAi*

To understand the effect of *Hlfer* knockdown on blood feeding, I performed histological and ultrastructural examination of the midgut. Morphological characteristics of midgut cells were observed based on previous reports [48,49]. In ticks, blood meal components are phagocytosed, and digestion occurs inside digestive

cells [50]. Based on HE sections and TEM of partially fed midgut, knockdown of either *Hlfer1* or *Hlfer2* resulted in abnormal morphology of digestive cells and apparently reduced their digestive activity (Fig. 1-12). HE sections revealed digestive cells with altered shape, vacuolated cytoplasm, and disrupted microvilli and cell membranes. These abnormalities were more pronounced in midguts of *Hlfer1*-silenced ticks. Eosinophilic granules were also observed in the cytoplasm of digestive cells, which may indicate cell degeneration. Diminished production of hematin was observed in HE sections, particularly from *Hlfer1*-silenced ticks. From TEM, fewer digestive cells containing hematin granules were observed in*Hlfer1*-silenced ticks, in contrast to control ticks. Furthermore, the microvilli of digest cells were short and sparsely distributed in *Hlfer1*-silenced ticks. No significant difference was observed between control and *Hlfer2*midgut sections (data not shown).

1.3.7 *Iron-binding activity of recombinant HlFER2*

A colorimetric assay using ferrozine as the iron-detecting reagent was used to determine whether the recombinant HlFER2 purified from *E. coli* has iron-binding activity. The absorbance reading is directly proportional to the available ferrous ion in the solution that reacted with ferrozine. As shown in Fig. 1-13A, the absorbance reading decreased with increasing concentration of both recombinant HlFER2 and horse apoferritin compared with the recombinant short HlMRC (P<0.05) used as a negative control, which did not show iron-binding activity. The recombinant HlFER2 displayed a sharp decrease in absorbance at 75 nmol l^{-1}, but this decrease became smaller at 150 nmol l^{-1}. These results suggested that recombinant HlFER2 has iron-binding activity similar to that of horse apoferritin and that the *His*-tag did not induce this activity. Based on SDS-PAGE (Fig. 1-13B), the purified *His*-tagged recombinant HlFER2 has only one subunit, with a molecular weight of ~26 kDa, as compared with

horse apoferritin with H- and L-subunits, with a molecular weight of ~20 and 19 kDa, respectively. With regards to native PAGE analysis (Fig. 1-13C), the recombinant HlFER2 does not form the typical 24-mer folding of most ferritins, in contrast to horse apoferritin.

1.4. DISCUSSION

Ticks are completely dependent on host blood for survival and reproduction. They consume great amounts of blood, which exposes them to potentially toxic levels of iron. Hence, ticks must have a protective mechanism against the detrimental effects of iron overload. Iron from the blood meal may be in the form of heme or host-transferrin-bound iron. Excessive heme resulting from hemoglobin degradation aggregates in specialized organelles called hemosomes within the digestive cells of the midgut as a detoxification mechanism [51]. In the hard tick *H. longicornis*, intracellular ferritin has been previously identified, but its function has not been described [47]. Here, I reported a new secretory ferritin from *H. longicornis*, HlFER2, and characterized it with the intracellular ferritin, HlFER1. Through RNAi, I demonstrated the importance of both HlFERs in successful tick feeding and reproduction.

Sequence analysis showed that *Hlfer2* mRNA lacks an IRE, similar to the secretory ferritin of *I. ricinus* [33], the yolk ferritin of *L. stagnalis* [52], *S. mansoni* ferritins [53] and *Haliotis discus discus Abf1* [54]. In contrast to HlFER1, HlFER2 contains a signal peptide and was detected in the hemolymph using a specific antiserum. Insect ferritins are synthesized with a signal peptide [24] that accumulates in the vacuolar system and the secretory pathway, except in some insect species in which cytosolic ferritin also occurs [55]. IFAT demonstrated punctate distribution of

HlFER2 in the cytoplasm of digestive cells, suggesting that HlFER2 is located inside granules and possibly within the secretory pathway. Further examination is necessary to confirm this observation.

The difference in mRNA and protein expression patterns of the two HlFERs reflects the difference in regulatory mechanism. *Hlfer1* is regulated by the interaction of IRE with iron-regulatory protein (IRP), demonstrated by the absence of protein bands despite the presence of mRNA transcripts. IRE-IRP binding is known to regulate iron-binding proteins in mammals, controlling intracellular iron homeostasis [56]. IRE-IRP binding has also been reported in the translational regulation of mosquito HCH [57]. In contrast, *Hlfer2* seems to be transcriptionally regulated. Interestingly, HlFER2 was detected in the salivary glands of engorged ticks and the egg despite the very low mRNA transcript level. This suggests that HlFER2 is synthesized elsewhere in the adult. Based on gene and protein expression, the midgut is the source of HlFER2, from which it is secreted to the hemolymph for transport to other organs, including maturing oocytes, and later incorporated in the eggs upon laying. This was further supported by the presence of HlFER2 in the ovary during the oviposition period and the strong fluorescence in the surface of oocytes revealed by IFAT. Moreover, the difference in the pattern of gene and protein expression of the two HlFERs among organs may indicate variation in the role of these organs in iron metabolism. The midgut, being the organ involved in blood digestion and the first to be exposed to large amounts of iron, would also be the major organ for iron metabolism. The expression of both HlFERs was unchanged from unfed to engorged midgut. IFAT also revealed the abundance of both HlFERs in the cytoplasm of digestive cells.

Genetic manipulation through RNAi has been shown to be a very useful method in assessing gene function in ticks and a valuable tool for the characterization of tick protective antigens [58,59]. In the present study, knockdown of both ferritin genes reduced the capacity of the ticks to fully engorge, and greatly impaired reproductive capacity. A study has been previously conducted in the ixodid tick *I. ricinus*, wherein knockdown of two ferritins affected feeding and reproduction of this hard tick [33]. Here I also showed that knockdown of both HlFERs reduced the feeding capacity of *H. longicornis*. *Hlfer* RNAi could have altered iron homeostasis and resulted in iron overload. In the midgut, fewer digestive cells containing hematin were observed in HE sections and TEM after *Hlfer* knockdown. Hematin is the most prominent end-product of intracellular blood digestion, particularly of hemoglobin [48]. This reduction was marked in the*Hlfer1*-silenced group, suggesting decreased digestive activity. Disrupted microvilli were also noted, which could have impaired the phagocytic activity of digestive cells. Microvilli increase the cell surface area and absorptive capacity of digestive cells [60] and are possibly involved in receptor-mediated endocytosis [61]. Silencing of *Hlfer1* prevented the intracellular storage of iron in digestive cells, whereas silencing of *Hlfer2* prevented iron sequestration for delivery to other organs, including developing oocytes. In both cases, accumulation of iron within digestive cells decreased both the uptake and digestion of blood. Thus, ticks were unable to take up large amounts of blood and feed to repletion.

Ferritin responds to oxidative stress in mammalian cells [26] and functions in preventing oxidative challenge and iron toxicity in hematophagous insects [62]. High mortality after silencing of *Hlfer*s can be attributed to iron-induced cytotoxicity and oxidative stress. Iron is known to induce lipid peroxidation [63,64]. Iron toxicity is largely due to its ability to catalyze the Fenton reaction, which leads to the generation

of radicals that attack and damage cellular macromolecules and promote cell death and tissue injury [65]. Abnormal morphologic features observed in HE sections of the midgut, particularly from *Hlfer1*-silenced ticks, suggested possible cell damage. These abnormal morphologic features found in HE sections could not be confirmed through TEM, but decreased phagocytic and digestive activity was evident.

I hypothesized that HlFER1 is the primary HlFER in the midgut cells responsible for iron storage and that it interacts with HlFER2. This was confirmed by the results of western blot analysis after *Hlfer1* silencing, wherein HlFER2 expression was reduced in the midgut and abolished in salivary glands and ovary. These data suggest that HlFER2 secretion from the midgut might be affected by HlFER1 expression. Moreover, *Hlfer1* silencing had more distinct effects on midgut cell morphology and digestive activity, implying a greater role in protection against iron toxicity. The previous study on *I. ricinus* also showed that silencing of *fer2* prevented the expression of FER1 in salivary glands and ovary [33]. Interestingly, although *Hlfer2* was silenced, HlFER2 was still detected in the midgut by western blot analysis, possibly due to its secretory nature.

Female ixodid ticks are known for their high fecundity, as they are able to lay thousands of eggs in a single batch. Failure of ferritin-silenced ticks to lay eggs may be due to insufficient nutrients, including iron, required for optimum egg production [10]. Western blot analysis of ovary and eggs suggest that HlFER2 is important during oviposition and embryonic development. This may supply iron or protect the developing oocytes and embryo from excessive iron. This was further supported by the abnormal morphology of eggs laid from *Hlfer2*-silenced females. In another study in our laboratory, host-derived transferrin was detected in the ovary during oviposition; however, it was not detected in the eggs after laying [66], suggesting an

interplay between host transferrin and the tick's HlFER2. In addition, the expression of two vitellogenin genes, *HlVg-1* and*HlVg-3*, was affected by silencing of ferritin genes. Boldbaatar et al. [36] showed that multiple vitellogenins are essential for oocyte development and oviposition in *H. longicornis*. Vitellogenin production was previously shown to be dependent on the degree of feeding [67], and surpassing critical mass is important in vitellogenesis in ixodid ticks [68]. In mosquitoes, vitellogenesis is a complex event involving several hormones, triggered by the blood meal. Midgut distension from the blood meal sends a signal to the brain and starts the cascade for vitellogenesis. Amino acids from the blood meal also activate vitellogenesis [69]. The same could be true for ticks. Weiss and Kaufman [70] showed that attainment of critical weight in the female hard tick *Amblyomma hebraeum* is necessary for production of 20-hydroxyecdysone, the hormone responsible for the initiation of vitellogenin synthesis. Moreover, they also showed that higher mass is required for ovary maturation. In both *Hlfer*-silenced groups, the body mass after a blood meal was less than half of that of the control group, and the midgut contained less blood meal. *Hlfer*-silenced ticks also have smaller ovaries after detachment from the host. Although some of the *Hlfer2*-silenced ticks laid eggs, the number was very small compared with that laid by control ticks, possibly as a result of failure of the ovary to mature. Nevertheless, the results here cannot fully demonstrate the direct relationship between vitellogenin and ferritin or vitellogenin and iron.

Recombinant HlFER2 demonstrated apparent iron-binding activity, comparable to that of commercial horse apoferritin. During purification of recombinant HlFERs from *E. coli*, insoluble recombinant HlFER1 and soluble recombinant HlFER2 were obtained. All ferritins have high iron-binding capacity and

can readily interact with Fe^{2+} ions in a solution under aerobic conditions [28]. SDS-PAGE analysis showed that recombinant HlFER2 consists only of H-subunits, in contrast to horse apoferritin, which has H- and L-subunits. Interestingly, the recombinant HlFER2 did not exhibit the typical 24-mer folding of most ferritins, but still it demonstrated iron-binding activity like other recombinant ferritins [54,71]. This may indicate a highly functional H-chain, which possesses the catalytic activity necessary for Fe^{2+} oxidation and iron storage.

Both HlFER1 and HlFER2 have high homology and identity with heavy-chain ferritins, and possess a conserved ferroxidase diiron center and a ferrihydrite nucleation center. Thus, both HlFERs must have the same capacity for iron uptake and storage. Conversely, HlFER2, being secretory, may circulate throughout the entire tick body. Aside from iron storage and transport, HlFER2 may have additional roles. Mammals have a relatively low concentration of extracellular ferritin in the serum and synovial and cerebrospinal fluids, which has been implicated as an inflammatory indicator and possibly a component of immune response [72,73]. In insects and other invertebrates, ferritin has been implicated in response to bacterial infections [71,74,75]. The blood meal of ticks may also expose them to various pathogens. As vectors, several pathogens undergo multiplication in ticks before they are transmitted. It would be interesting to evaluate whether ferritin also plays a role in pathogen multiplication and transmission. Moreover, ticks are capable of long periods of starvation and may survive without feeding. The results here showed that HlFER1 and HlFER2 are both expressed in unfed midgut and ovary. It would be interesting to determine the utilization of iron during starvation periods in the ticks and may be considered in future research.

In summary, this chapter described the identification of a secretory ferritin, HlFER2, from *H. longicornis* and its characterization with the intracellular HlFER1. The results here suggest that HlFER1 is the main iron storage ferritin and the midgut is the primary organ involved in iron metabolism. Secretory HlFER2 may play a role in iron transport and is very important in oviposition and embryonic development. RNAi experiments further demonstrated the critical importance of the iron storage function of ferritin for successful blood feeding and reproduction of the hard tick. Furthermore, knockdown of HlFERs induced abnormalities in midgut cell morphology and apparently decreased their digestive activity. I also found that silencing *Hlfer*s decreased the expression of *HlVg*s, which is integral in reproduction. To my knowledge, this is the first report demonstrating the protective function of ferritin against iron in arthropod digestive cells and suggesting the correlation of the iron metabolism and vitellogenesis in hematophagous arthropods. The critical importance of HlFERs makes them attractive target molecules for anti-tick vaccination. The succeeding chapters will focus on further studies on the functions of HlFERs and the evaluation of their potential as anti-tick vaccine targets.

Table 1-1. Gene-specific primers used for RT-PCR and dsRNA synthesis.

Name	Sequence (5'→3')
Hlfer1 forward	ATGGCCGCTACTCAACCCCG
Hlfer1 reverse	TCAGTCGTCTCCTCCGAGGG
Hlfer2 forward	ATGCTCCCGATCCTGATCTT
Hlfer2 reverse	TTATTTGTCGCTGTCTTCGC
Hlfer1 RNAi forward	CTGTACGCGAGCTACGTCTA
Hlfer1 RNAi reverse	ACACGCTTCAGGTTGGTCA
Hlfer1 T7 forward	GGATCCTAATACGACTCACTATAGGCTGTACGCGAGCTACGT
Hlfer1 T7 reverse	GGATCCTAATACGACTCACTATAGGACACGCTTCAGGTTGGT
Hlfer2 RNAi forward	CAAGTATTTCCTGCACGACC
Hlfer2 RNAi reverse	GAGCCGCTGCAGCTTGTCGA
Hlfer2 T7 forward	GGATCCTAATACGACTCACTATAGGCAAGTATTTCCTGCACG
Hlfer2 T7 reverse	GGATCCTAATACGACTCACTATAGGGAGCCGCTGCAGCTTGT
Luc T7 forward	GTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG
Luc T7 reverse	GTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC

Table 1-2. Effects of *Hlfer1* and *Hlfer2* silencing on blood feeding and reproduction of *H. longicornis.*

Groups	Body weight of ticks post-detachment (mg)	Mortality of ticks post-detachment (%)[a]	Egg weight / body weight (%)[b]	Percentage (%) of ticks that laid eggs	Hatched eggs
Luc dsRNA (control)	259.3 ± 52.3	0	51.6 ± 6.9	100	25 of 25
Hlfer1 dsRNA	72.5 ± 34.7*	91.67	55	4.16*	1 of 1
Hlfer2 dsRNA	94.5 ± 22.8*	54.54	40.3 ± 0.59	36.36*	1 of 9

[a]Ratio of ticks that died to the total number of ticks until 20 days after ticks dropped off from the host.
[b]Mean calculated from ticks that laid eggs ± s.d. For the *Hlfer1* dsRNA group, only 1 tick laid eggs.
*Significantly different vs. control (P <0.05) as calculated by the Student's t-test.

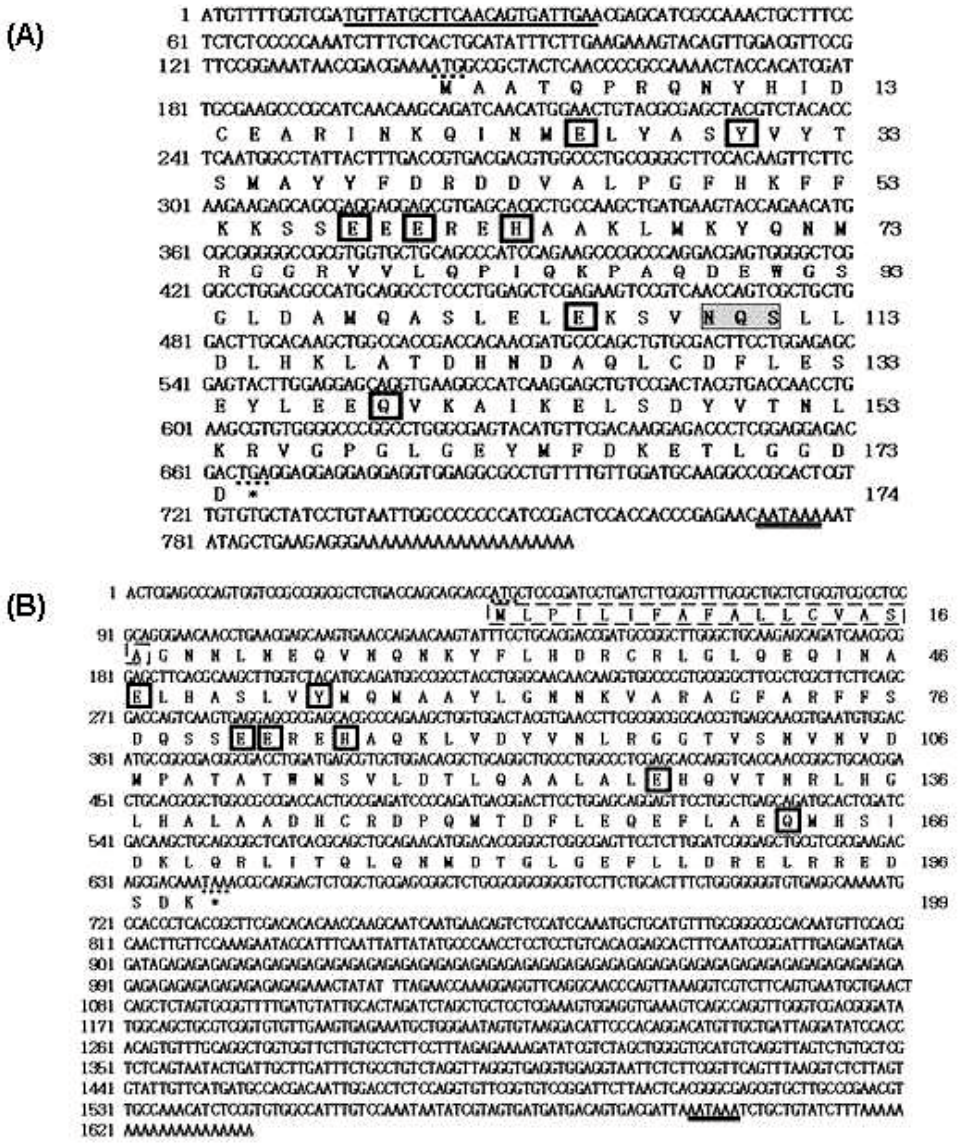
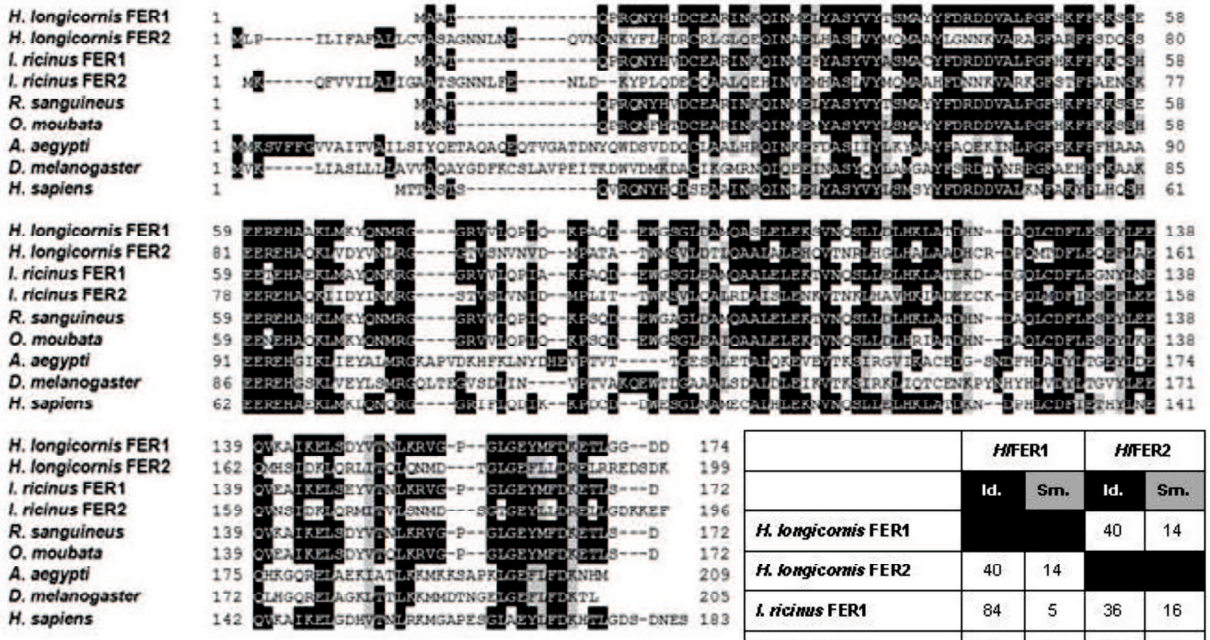


Fig. 1-1. Complete nucleotide and amino acid sequences of *H. longicornis* ferritin1 (A) and ferritin2 (B). The start and stop codons are underlined by dotted lines. The amino acid residues within the ferroxidase center loop are enclosed in boxes. The 3' UTR ends with a 20-bp polyadenylation tail after the putative polyadenylation signal, AATAAA (double-underlined). For (A), the putative iron-responsive element (IRE) in the 5' UTR is underlined, and the predicted glycosylation site is enclosed in a gray-shaded box. For (B), the signal peptide is enclosed in a dashed box.



	*Hl*FER1		*Hl*FER2	
	Id.	Sm.	Id.	Sm.
H. longicornis FER1			40	14
H. longicornis FER2	40	14		
I. ricinus FER1	84	5	36	16
I. ricinus FER2	36	15	56	20
R. sanguineus	94	2	38	16
O. moubata	87	5	37	31
A. aegypti	28	19	29	22
D. melanogaster	30	16	31	38
H. sapiens	66	12	34	21

Fig. 1-2. Multiple alignment of the amino acid sequences of *H. longicornis* ferritins with other tick ferritins, human ferritin, and selected invertebrate ferritins. Identical residues are shaded with black, while similar residues are shaded with gray. The percent identity (Id.) and similarity (Sm.) were calculated and are summarized in a table. The GenBank accession numbers for the ferritin sequences are as follows: *H. longicornis* FER1, AY277905; *H. longicornis* FER2, AB734098; *I. ricinus* FER1, AF068224.1; *I. ricinus* FER2, EU885951.1; *R. sanguineus*, AY277907.1; *O. moubata*, AF068225.2; *A. aegypti*, XM_001654469.1; *D. melanogaster*, AF145125.1; and *H. sapiens*, NM_002032.2.

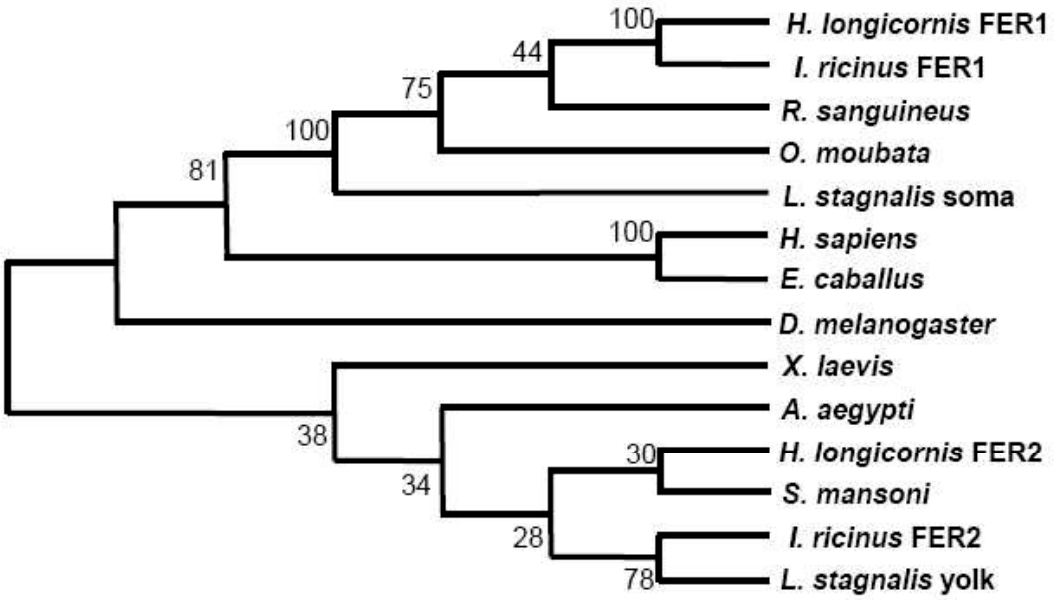


Fig. 1-3. Phylogenetic tree of ferritins from different species of ticks, selected vertebrates, and invertebrates. A dendogram was constructed by the neighbor-joining method based on the amino acid sequences of ferritins. Bootstrap values are shown on the lineage of the tree. GenBank accession numbers are as follows: *H. longicornis* FER1, AY277905; *H. longicornis* FER2, AB734098; *I. ricinus* FER1, AF068224.1; *I. ricinus* FER2, EU885951.1; *R. sanguineus*, AY277907.1; *O. moubata*, AF068225.2; *A. aegypti*, XM_001654469.1; *D. melanogaster*, AF145125.1; *H. sapiens*, NM_002032.2.; *E. caballus*, AY112742.1; *L. stagnalis* yolk ferritin, AY112742.1; *L. stagnalis* soma ferritin, X56778.1; *S. mansoni*, XM_002576034.1; and *X. laevis*, NM_001086111.1.

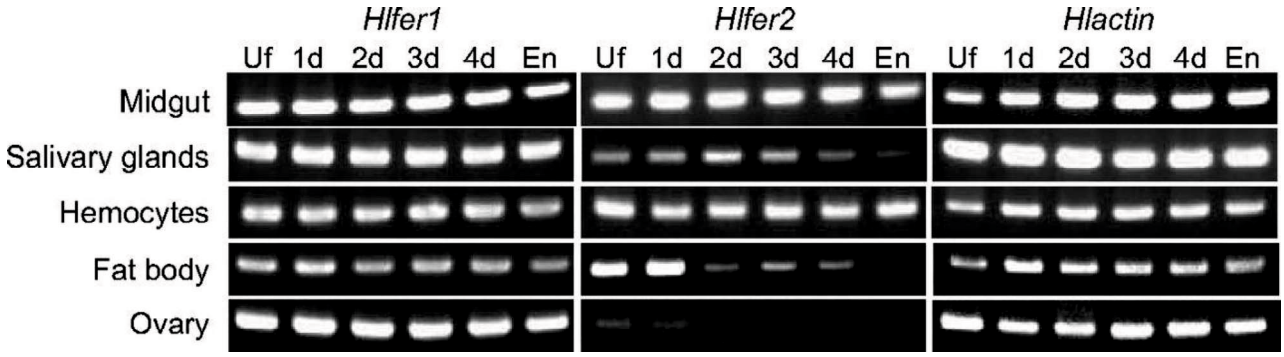


Fig. 1-4. Transcription profiles of *Hlfer1* and *Hlfer2* in different tissues of adult female *H. longicornis* during blood feeding. Gene-specific primers are indicated on the topmost row. Actin was used as a loading control. Uf, unfed; 1d, 1-day fed; 2d, 2-day fed; 3d, 3-day fed; 4d, 4-day fed; En, engorged tick.

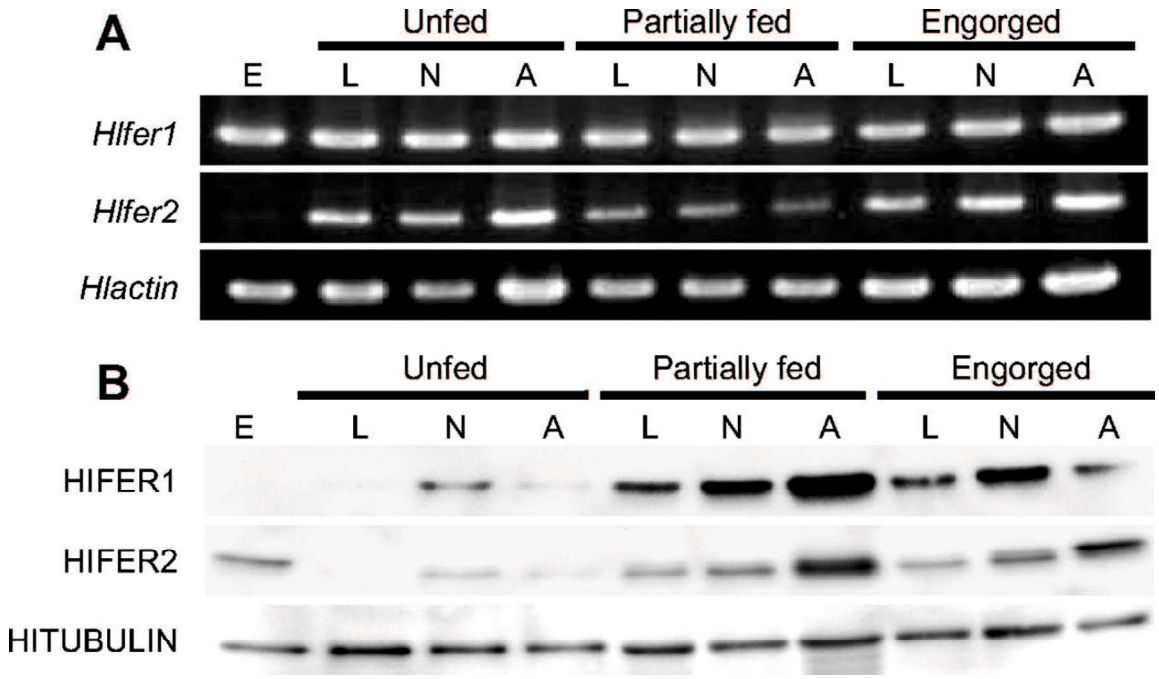


Fig. 1-5. RT-PCR (A) and Western blot (B) analyses in different developmental stages of *H. longicornis* using ferritin-specific primers and mouse sera. Total RNA or protein lysates were prepared from the egg and whole nymph, larva, and adult in different states of feeding (unfed, partially fed, and engorged). Actin primer and mouse anti-tubulin serum were used as a loading control for RT-PCR and Western blot analysis, respectively. E, egg; L, larva; N, nymph; A, adult.

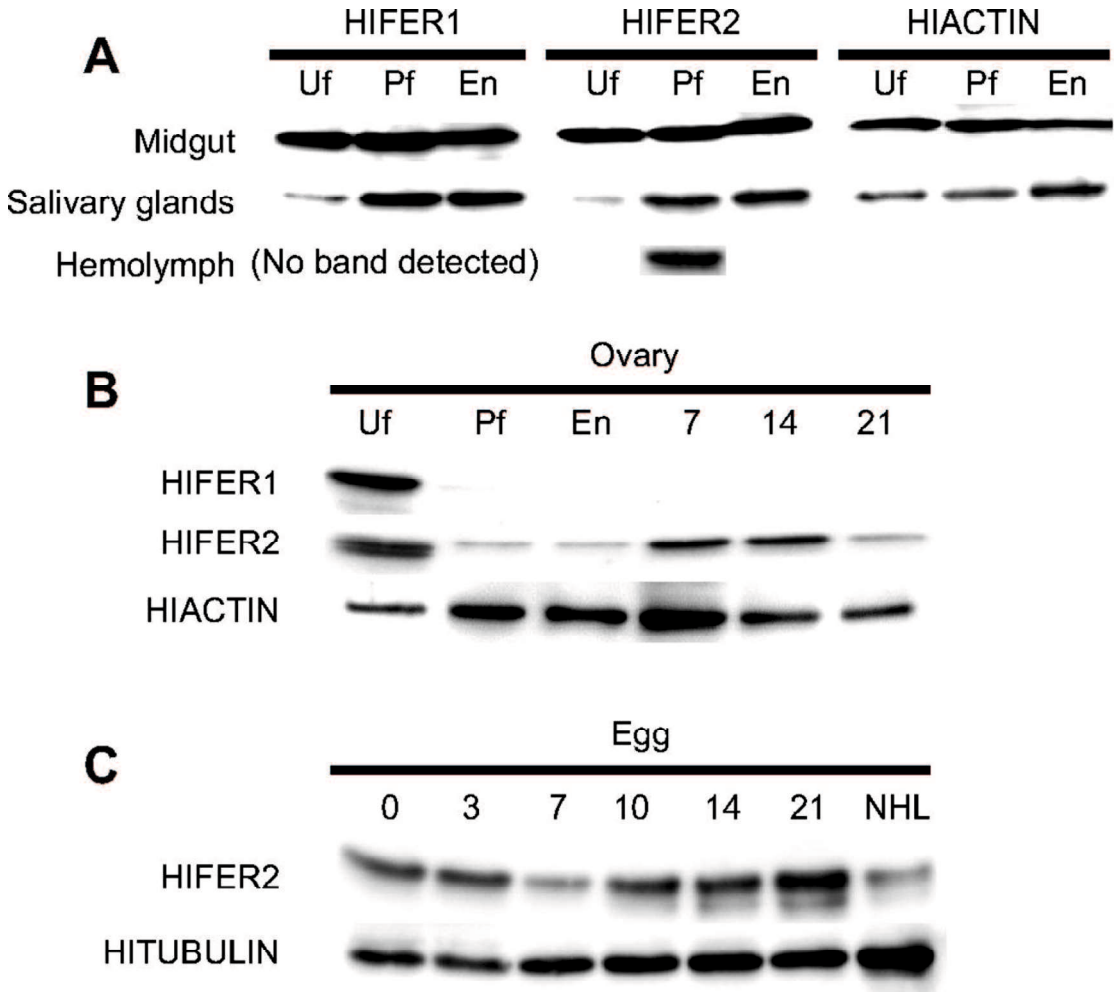


Fig. 1-6. Western blot analyses for HlFER1 and HlFER2 using specific mouse anti-HlFER sera. Actin or tubulin antiserum was used as a control. (A) Protein expression in the midgut and salivary glands from unfed (Uf), 4-day partially fed (Pf), and engorged (En) adult ticks and hemolymph including hemocytes from partially fed adult ticks. (B) Protein expression in the ovary during feeding and oviposition. The numbers represent the days after engorgement, also corresponding to the oviposition period of the tick. (C) Protein expression in the egg during embryonic development and in newly hatched larva (NHL). The numbers represent the days after which the eggs were laid.

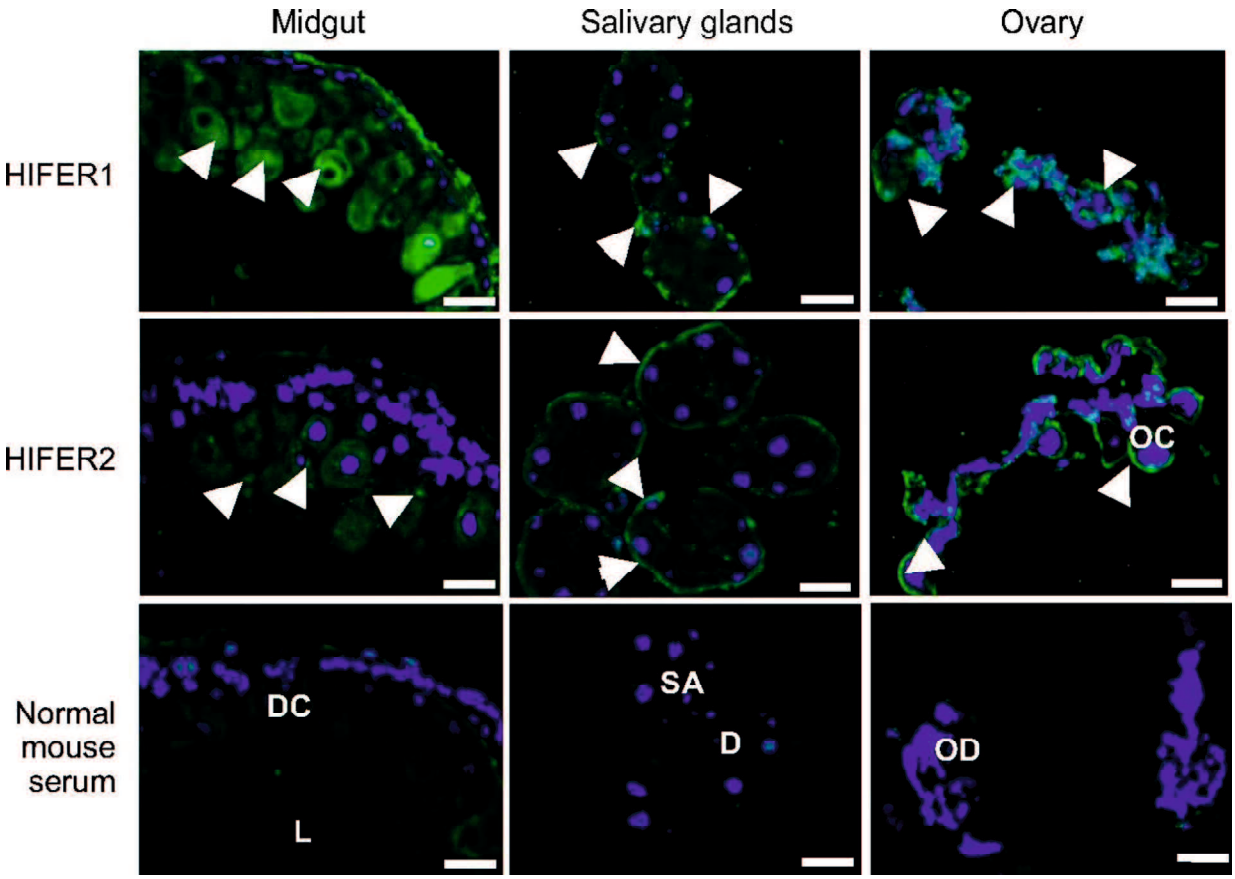


Fig. 1-7. Indirect fluorescent antibody test (IFAT) for the localization of endogenous HlFER1 and HlFER2 in partially fed midgut, salivary glands, and ovary. Frozen sections were incubated with specific mouse anti-HlFER sera as primary antibodies. Normal mouse serum was used as a control. Mouse anti-IgG conjugated with Alexa 488 was used as a second antibody. Arrowheads point to positive fluorescence. DC, digestive cells; L, lumen; SA, salivary gland acinus; D, duct; OC, oocyte; OD, oviduct. Bars = 50 µm.

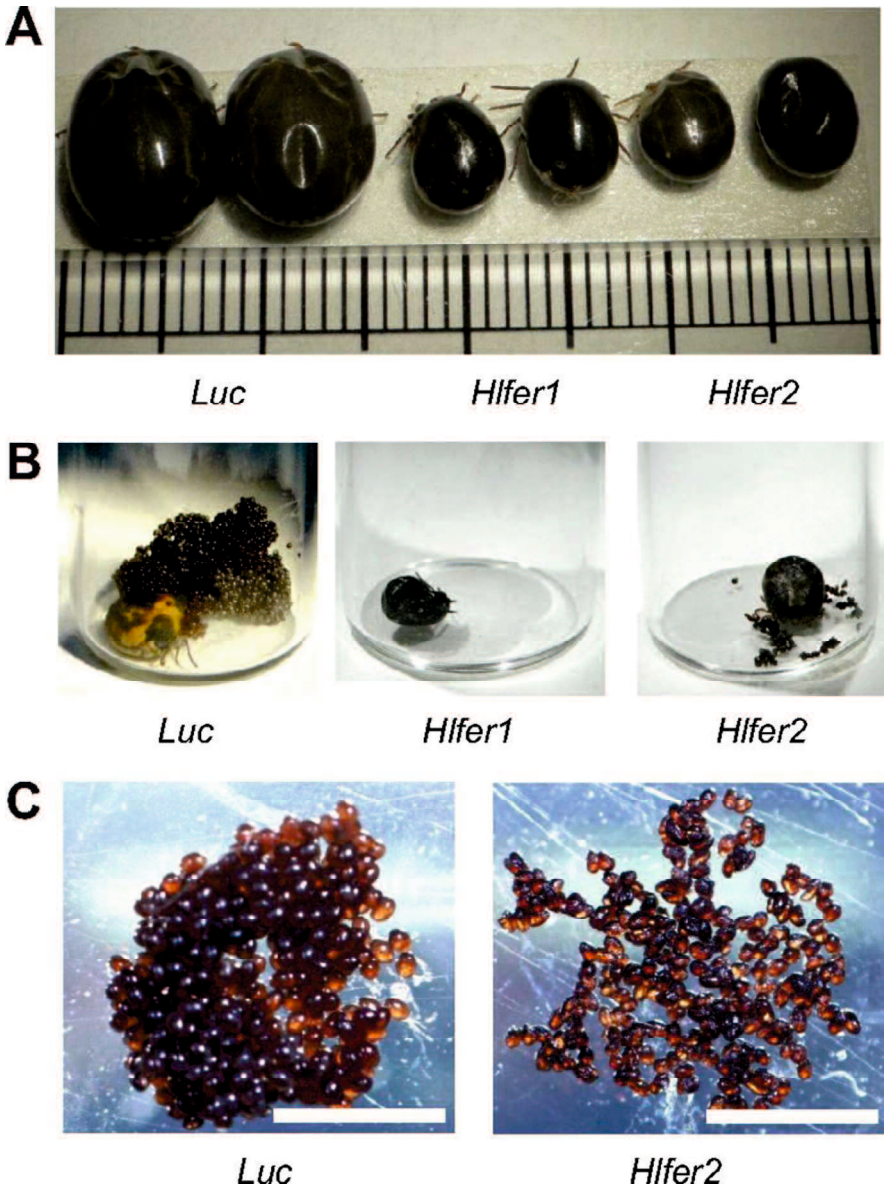


Fig. 1-8. Effects of RNAi-mediated silencing of *Hlfer* on the feeding and reproduction of *H. longicornis*. *Hlfer1* or *Hlfer2* dsRNA was injected into female ticks. *Luciferase* dsRNA was injected into the control group. Ticks were allowed to feed and naturally drop off from the host. (A) Both *Hlfer1* and *Hlfer2* dsRNA-injected ticks were smaller than the control upon detachment due to failure to fully engorge. (B) Mortality was high during oviposition for both *Hlfer* dsRNA-injected groups. Most *Hlfer1* dsRNA-injected ticks died without laying eggs, while some *Hlfer2* dsRNA-injected ticks laid very few eggs. (C) Eggs were examined under a steromicroscope. Eggs from *Hlfer2* dsRNA-injected ticks have an abnormal morphology. Bars = 1 mm.

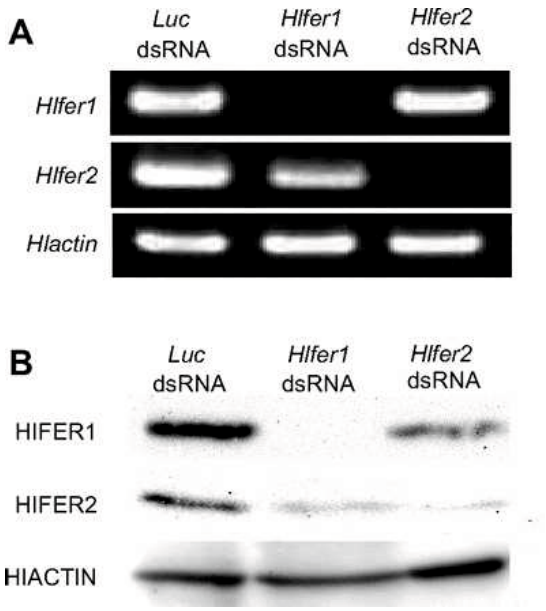


Fig. 1-9. Confirmation of *Hlfer* silencing. (A) Total RNA was extracted from whole 4-day fed ticks, and cDNA was subjected to RT-PCR using *Hlfer*-specific primers. (B) Western blot analysis of protein lysates prepared from whole 4-day partially fed ticks. Bands were detected using specific mouse anti-HlFER sera. Actin was used as a loading control.

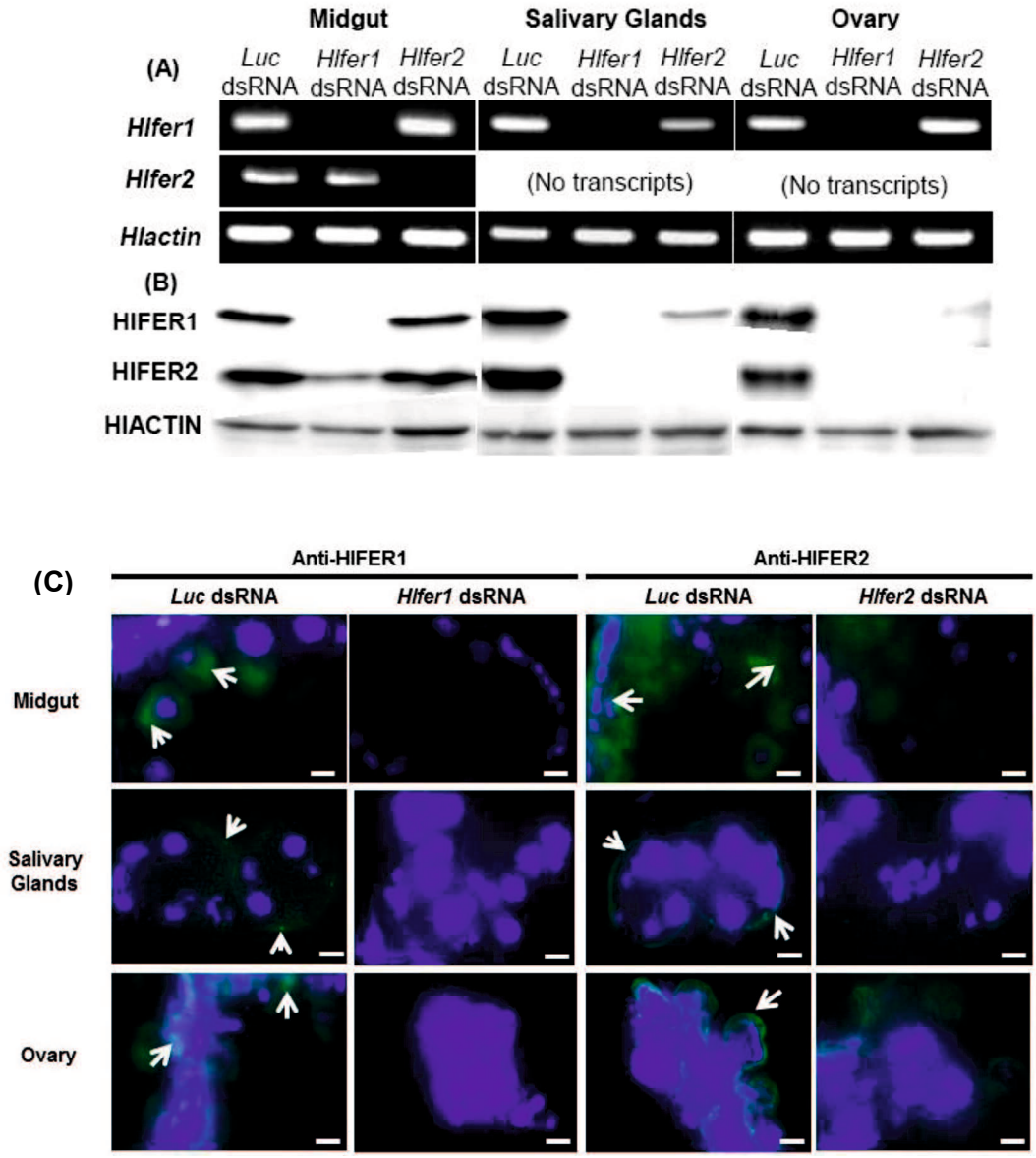


Fig. 1-10. Confirmation of *Hlfer* silencing by RT-PCR (A), Western blot analysis (B) and IFAT (C) in different organs of adult *H. longicornis*. After injection of *Hlfer1*, *Hlfer2*, or *Luc* dsRNA, ticks were allowed to feed and collected at 4 days post-attachment. The midgut, salivary glands, and ovary were collected for RNA and protein extraction. Frozen sections were also prepared for IFAT. For RT-PCR and western blot, specific primers and anti-sera were used, respectively, indicated on the left. No transcript was detected in the salivary glands and ovary using the *Hlfer2* primer (please refer to Fig. 1-4). Arrows in IFAT images point to positive fluorescence. Bars = 20 μm.

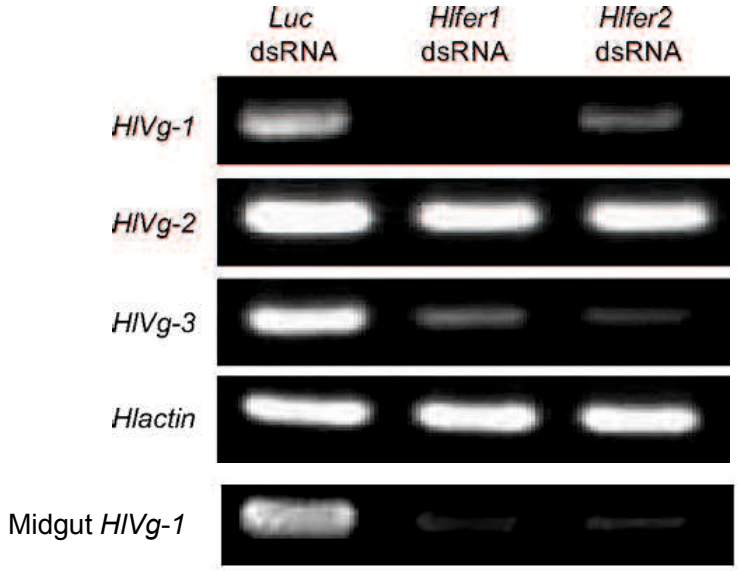


Fig. 1-11. Effect of *Hlfer* silencing on the expression of vitellogenin genes (*HlVg*). cDNA from whole 4-day fed ticks injected with either *Hlfer1* dsRNA or *Hlfer2* dsRNA or with *Luciferase* dsRNA for the control group was amplified by three sets of primers of *HlVg*-1, *HlVg*-2, and *HlVg*-3. The actin primer was used as a loading control. *HlVg*-1 expression in 4-day fed midgut was also checked.

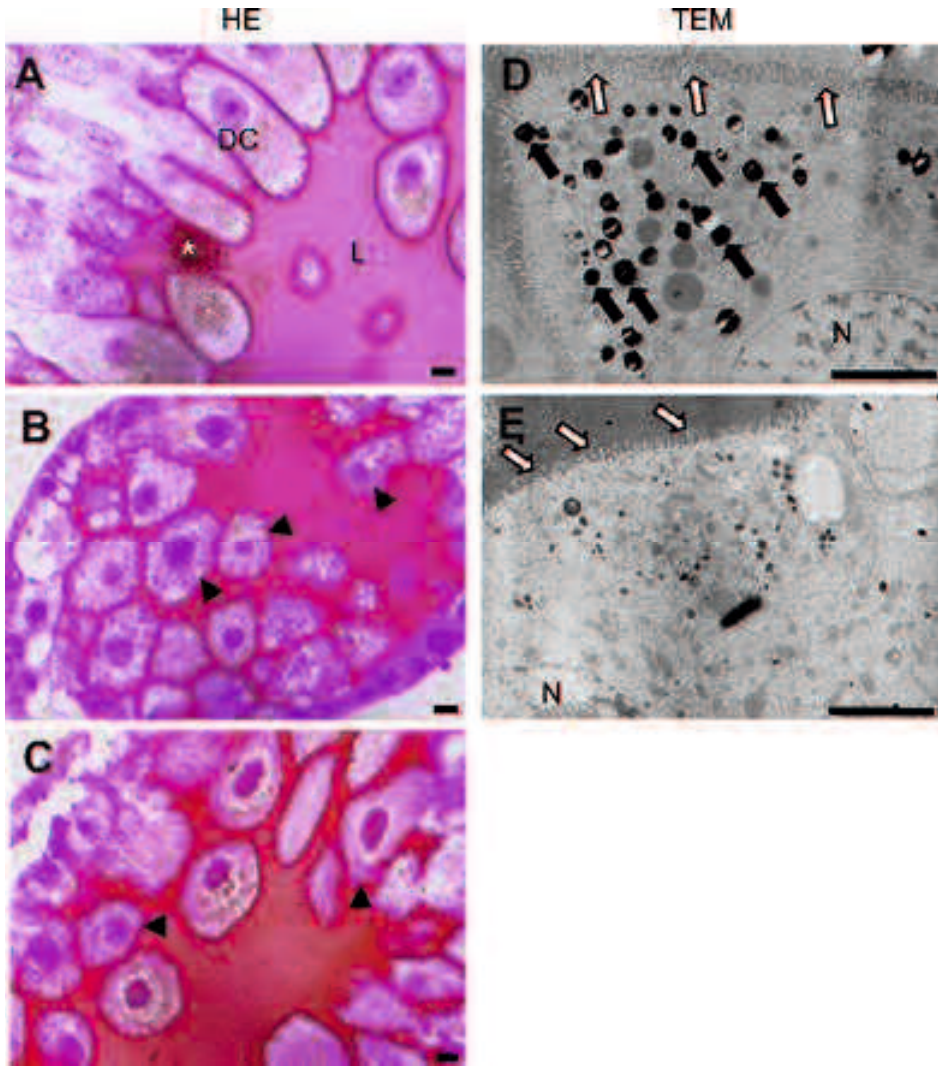


Fig. 1-12. Histological and ultrastructural examination of the midgut after knockdown of *Hlfer1* and *Hlfer2*. Hematoxylin-eosin (HE) staining of paraffin sections from *Luciferase* (A), *Hlfer1* (B), and *Hlfer2* (C) dsRNA-injected ticks was performed. Arrowheads point to disrupted microvilli and cell membrane. An asterisk indicates hematin granules released in the lumen. TEM micrographs of midgut from *Luciferase* (D) and *Hlfer1* (E) dsRNA-injected ticks are also shown. Black arrows point to hematin granules within the cell. White arrows point to microvilli. DC, digestive cells; L, lumen; N, nucleus. Bars= 10 µm (A - C), 0.5 µm (D & E).

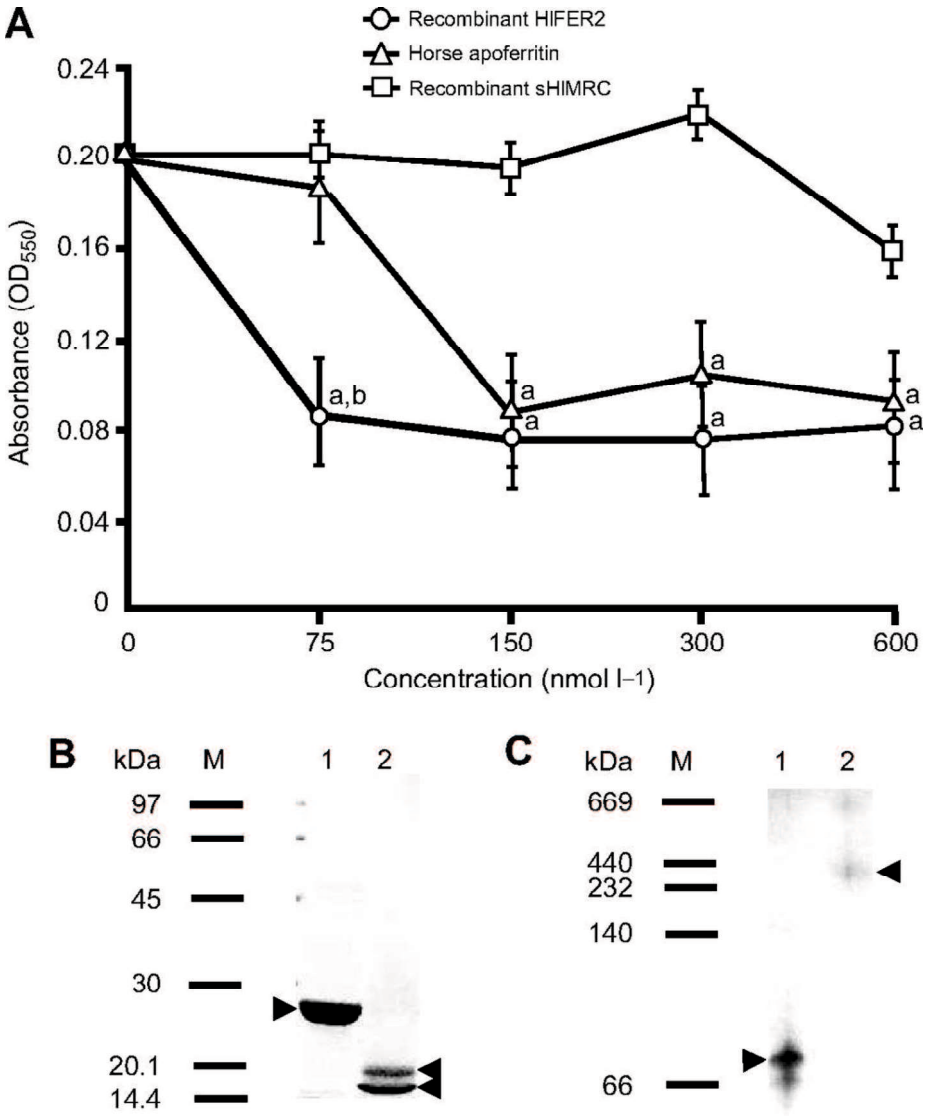


Fig. 1-13. Iron-binding activity and electrophoretic analyses of recombinant HlFER2. (A) Different concentrations of recombinant HlFER2 and horse apoferritin dissolved in water were incubated with $FeCl_2$. Recombinant short HlMRC was used as negative control. Ferrozine was used as an indicator agent, and absorbance at 550 nm was measured. Different superscripts indicate significant difference ($P < 0.05$) vs. negative control (a) or horse apoferritin (b). (B) SDS-polyacrylamide gel electrophoresis (12%) and (C) Native PAGE (3-10%) of recombinant HlFER2 (lane 1, 5 μg) and horse apoferritin (lane 2, 10 μg). Gels were stained with Coomasie brilliant blue. kDa, molecular weight in kilodaltons; M, molecular weight marker. The recombinant HlFER2 and horse apoferritin bands are indicated by arrowheads.

CHAPTER 2

Two kinds of ferritin protect ixodid ticks from iron overload and consequent oxidative stress

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2.1. INTRODUCTION

Ticks have successfully developed counteractive means against their hosts' immune and hemostatic mechanisms [76], but their ability to cope with potentially toxic molecules in the blood remains unclear. Iron is important in various physiological processes but can be toxic to living cells when in excess [65]. In the previous chapter, I reported that the hard tick *H. longicornis* has an intracellular (HlFER1) and a secretory (HlFER2) ferritin, and both are crucial in successful blood feeding and reproduction. Silencing *Hlfer*s by RNA interference caused reduced feeding capacity, low body weight and high mortality after blood meal, decreased fecundity and morphological abnormalities in the midgut cells. Similar findings were also previously reported after silencing of *fer* genes in another hard tick*, I. ricinus* [33]. In this chapter, I further demonstrate the role of ferritin in protecting hard ticks from oxidative stress by measuring the levels of malondialdehyde (MDA) produced during lipid peroxidation and protein carbonyl (PC) produced during protein oxidation, after exposing the ticks to high levels of iron through blood feeding or injection of ferric ammonium citrate (FAC). IFAT and iron staining evaluation after FAC injection also suggest that iron injected in the hemocoel may stimulate midgut HlFER expression.

2.2. MATERIALS AND METHODS

2.2.1 *RNA interference and tick infestation*

The silencing of *Hlfer* in unfed adult female ticks was induced by injection of gene-specific dsRNA prepared as described in Chapter 1. Briefly, ticks were attached to glass slides and then injected with 1 µg per 0.5 µl of *Hlfer1* or *Hlfer2* dsRNA through the fourth coxae using an IM 300 Microinjector (Narishige, Tokyo, Japan).

Control ticks were injected with the same amount of firefly *Luciferase* (*Luc*) dsRNA. To confirm silencing, total RNA was extracted from whole ticks 4 days post-injection of dsRNA for RT-PCR analysis. Ticks injected with dsRNA were held in a humidity chamber kept in a 25°C incubator for 18 h before infestation to rabbits or for 4 days before injection with ferric ammonium citrate (FAC).

For rabbit infestation, a total of 50 ticks per dsRNA injected group were attached in separate ears of rabbits, individually covered with an ear bag. Attached ticks were allowed to feed until they naturally dropped off. From the total number of engorged ticks, 30 ticks from each group were used for the thiobarbituric acid reactive species (TBARS) assay in the whole ticks. Five pooled midgut samples, comprising of three ticks each for *Hlfer1*and *Hlfer2*-silenced ticks and two ticks each for *Luc*-injected group, were also prepared for the TBARS assay. The remaining ticks were used for immunoblot detection of oxidative stress biomarkers, described in the succeeding sections. All ticks were stored in -80°C until use.

2.2.2 *Injection of ferric ammonium citrate* (*FAC*)

To further investigate the effect of high levels of iron on ticks, different concentrations of FAC were injected into unfed adult ticks, with or without dsRNA injection. To check the survival rate of *Hlfer*-silenced ticks after exposure to iron, 100 μM FAC was injected in the same manner as dsRNA injection. Likewise, sterilized high-purity water was injected to dsRNA-injected ticks for additional control. Thirty ticks for each group were used for this experiment. After injection of FAC, ticks were held as mentioned above and monitored for mortality every 12 h for 11 days. The survival experiment was repeated three times to confirm the reproducibility of results. Otherwise, unfed adult ticks not injected with dsRNA were injected with 50 or 100

μM FAC or sterilized high-purity water for control to evaluate mRNA and protein expression and iron staining in response to iron treatment.

2.2.3 *Protein extraction*

Blood-fed or FAC-injected whole ticks were homogenized in PBS. Midguts and salivary glands were also collected and homogenized in Tris-buffered saline (TBS) with a protease inhibitor (Complete Mini EDTA-free, Roche, Manheim, Germany). Hemolymph was collected from the amputated legs of immobilized ticks. Hemocytes were separated by centrifugation. Protein from whole ticks and organs was extracted as described previously [39,40]. Protein samples were kept at -80°C until use.

2.2.4 *Electrophoresis, Western blot analysis and gel iron staining*

To investigate the protein expression, protein samples were separated in 12% SDS-PAGE and subjected to Western blot analysis as described in Chapter 1. Specific mouse anti-HlFER sera described in Chapter 1 or anti-β-tubulin serum for control [42] were used as primary antibodies. Protein signals were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and images were taken using the FluorChem FC2 Imaging System (Protein Simple). Western blotting was performed at least three times. To accurately determine differences in the protein expression, band densitometry analysis was performed using Alpha View Software (Alpha Innotech, Protein Simple). The band densitometry analysis results shown in this study represent the mean of three trials of Western blot analysis.

To stain native HlFER for ferric iron, protein extracts were separated in 6% native PAGE. Protein concentration was adjusted after determination of protein concentration using a Micro BCA Assay kit (Thermo Scientific) or based on control immunoblotting with β-tubulin as described above. The gel was stained in a freshly

prepared Prussian blue staining solution (equal volume of 10% $K_4[Fe(CN)_6]$ and 10% HCl) at room temperature for 48 h as previously reported [77]. The high molecular weight marker (GE Healthcare), which contains ferritin from equine spleen for the 440 kDa band, as well as the commercially-prepared horse holoferritin (Sigma-Aldrich) were used as positive controls.

2.2.5 *Immunofluorescent examination of organs after FAC injection*

An IFAT was performed as described in Chapter 1. Briefly, midguts and salivary glands were dissected from unfed adult ticks then fixed overnight in 4% paraformaldehyde in PBS with 0.1% glutaraldehyde and washed with a sucrose series before being embedded in Tissue-Tek® O.C.T Compound (Sakura Finetek Japan, Tokyo, Japan). After cutting, tissue sections were air-dried and then blocked overnight with 5% skim milk in PBS at 4°C. Sections were incubated with a 1:50 dilution of anti-HlFER sera or normal mouse serum as a control for the primary antibody and a 1:1,000 dilution of Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen) for the secondary antibody for an hour each at room temperature. Following washes with PBS, sections were mounted in Vectashield with DAPI (Vector Laboratories) and then viewed on a fluorescence microscope mounted with a DP71 camera (Olympus).

2.2.6 *Assessment of oxidative stress*

Oxidative stress was evaluated by detecting oxidative stress biomarkers including MDA and PC. MDA was demonstrated through immunoblotting using the Oxiselect Malondialdehyde Immunoblot Kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's recommendation. Engorged whole ticks were homogenized individually, whereas midguts and unfed adult ticks injected FAC were pooled. Protein was adjusted based on tubulin profile. Bands were viewed using

Clarity™ Western ECL Substrate (Bio-rad Laboratories, Hercules, CA, USA) and the MDA level relative to tubulin was calculated after band densitometry analysis. TBARS assay was also performed to quantify MDA [78]. For the TBARS assay, tick homogenates were mixed with TBARS reagent (0.37% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid in 0.25 M HCl) and then placed in boiling water bath for 15 min and allowed to cool. Absorbance was measured at 532 nm and MDA content was calculated using the molecular extinction coefficient for MDA. PC was also demonstrated following the immunoblot assay using the Oxiselect Protein Carbonyl Immunoblot Kit (Cell Biolabs) according to the manufacturer's instruction and analyzed similar to MDA.

2.2.7 *Measurement of total ferrous iron*

The ferrozine assay for measuring non-heme iron was adapted to determine the amount of ferrous iron in whole ticks injected with FAC after *Hlfer* knockdown [79,80]. Ten whole ticks from each group were collected 72 h after the injection of FAC and homogenized in lysis buffer (20 mM Tris, 137 mM NaCl, 1% Triton X-100, 1% glycerol). Protein concentration was measured using a Micro BCA Assay Kit (Thermo Scientific). Concentrated HCl was added and then heated to 95 °C. After cooling to room temperature, the mixture was centrifuged and the supernatant was obtained, to which 10 mM ferrozine (Sigma-Aldrich) was added. Color development was accomplished by the addition of saturated ammonium acetate. Absorbance was measured at 550 nm and iron concentration was calculated based on a molar extinction coefficient of the iron-ferrozine complex of 27 900 M^{-1} cm^{-1} and based on protein concentration.

2.2.8 *Statistical analyses*

For band densitometry analysis, Student's *t*-test or the Mann-Whitney U test was performed, depending on data distribution. For the survival experiment, the Mantel-Cox log-rank test was performed using GraphPad Prism software. In all statistical analyses, significant difference between groups is defined by $P < 0.05$.

2.3. RESULTS

2.3.1 Hlfer-*silenced ticks had low survival rate after FAC injection*

In Chapter 1, I reported high mortality in *Hlfer*-silenced ticks after blood feeding. To further demonstrate that the low survival rate was related to iron overload in the absence of HlFERs, here I exposed unfed *Hlfer*-silenced adult female ticks to iron by injecting 100 μM FAC into the hemocoel. Silencing was confirmed by RT-PCR analysis as seen in Chapter 1. After FAC injection, ticks were kept at 25°C and survival was monitored every 12 h. No mortality was observed in the control group injected with *Luc* dsRNA (Fig. 2-1A). In contrast, both *Hlfer1*- and *Hlfer2*-silenced groups had a continuously decreasing survival rate ($P < 0.0001$). Eleven days after FAC injection, the *Hlfer2* dsRNA-injected group showed the lowest survival rate. As an additional negative control, high-purity sterilized water was similarly injected after RNAi but this did not result in high mortality as in the case of FAC injection (Fig. 2-1B).

2.3.2 *FAC injection has no effect on transcription but stimulates protein expression of HlFERs*

Next, I evaluated whether injection of FAC as exogenous iron source can affect *Hlfer* transcript level and HlFER protein expression. Different concentrations of FAC were injected to the hemocoel of normal unfed female ticks or sterilized high-

purity water for the control group. The transcript level in whole ticks was then checked at 24 h and 72 h after FAC injection, whereas protein expression was examined from 24 h to 96 h after FAC injection. RT-PCR analysis showed no difference among the groups at any time point (Fig. 2-2A). However, increased protein expression particularly of HlFER1 was observed in both concentrations of FAC from 24 h to 96 h post-injection (Fig. 2-2B). Band densitometry analysis was performed to accurately determine the differences in protein expressions (Fig. 2-2C). I also examined the HlFER expression in some organs 24 h and 72 h post-injection and found that both HlFER1 and HlFER2 levels were higher in the midguts (Fig. 2-3A) of FAC injected ticks but not in the salivary glands (Fig. 2-3C). In the hemolymph where only HlFER2 is present, its expression is also higher after FAC injection compared to the control (Fig. 2-3B).

2.3.3 *FAC injection led to ferric iron accumulation in HlFER in the whole tick and the midgut*

After observing that FAC can stimulate HlFER expression in the whole tick and in the midgut, I determined whether there was a corresponding accumulation of Fe^{3+} on native HlFER. After separating the tick protein in native PAGE, HlFER was stained using Prussian blue staining to indicate Fe^{3+}. Both the high molecular weight marker containing ferritin from equine spleen as the 440 kDa band, and the commercial horse holoferritin strongly stained for Fe^{3+} (Fig. 2-4A). In whole ticks, increased staining was observed at 72 h and 96 h after injection of any concentration of FAC (Fig. 2-4A). Fe^{3+} staining also increased in the midgut and hemolymph at 72 h post-injection of FAC but not in the salivary glands (Fig. 2-4B) or ovary (data not shown). In all experiments, only one band was stained with Prussian blue, with an estimated molecular weight of around 440 kDa. The bands that stained for ferric iron

were confirmed to be HlFERs through Western blot analysis after native PAGE (Fig. 2-5). HlFER1 and HlFER2 had almost the same molecular weight on native PAGE.

2.3.4 *FAC injected into the hemocoel stimulated HlFER expression of digestive cells in the midgut*

It is interesting that injection of FAC to the hemocoel stimulated HlFER expression in the midgut, as shown by Western blot analysis, and that the midgut can also store the iron from the hemolymph, as demonstrated by Fe^{3+} staining on native PAGE. I wanted to know the extent of the effect of FAC on HlFER expression of digestive cells, therefore I performed IFAT. The salivary gland was also examined for comparison. Midguts and salivary glands were collected from normal unfed adult female ticks 72 h after injection of FAC or sterile high-purity water. Increased fluorescence was observed in digestive cells 72 h after injection of 50 μM and 100 μM FAC (Fig. 2-6A). For HlFER1, extensive fluorescence was observed throughout the midgut, from the basal lamina up to the inner digestive cells lining the lumen. For HlFER2, much of the fluorescence was observed along the basal lamina. In contrast, very weak fluorescence for both HlFERs was observed in the salivary glands (Fig. 2-6B).

2.3.5 Hlfer-*silenced ticks had higher levels of oxidative stress biomarkers after blood feeding or FAC injection*

Iron is known to catalyze the formation of ROS in living cells, thus promoting oxidative stress. In the previous chapter, I described abnormal midgut morphology and high mortality after blood feeding in *Hlfer*-silenced ticks and I hypothesized that this was caused by oxidative stress. Here I evaluated the oxidative status of *Hlfer*-silenced ticks after blood feeding or exposure to exogenous iron through

demonstration of known oxidative stress biomarkers: MDA, a known product of lipid peroxidation [19]; and PC resulting from the oxidation of proteins [81].

Immunodetection using specific antibodies against MDA (Fig. 2-7) and PC (Fig. 2-8) showed that *Hlfer*-silenced ticks have significantly higher (P <0.05) levels of these oxidative stress biomarkers than the control group after blood feeding or FAC injection. Band densitometry analysis was performed to calculate the relative MDA or PC content of the samples based on tubulin. *Hlfer1*-silenced ticks showed the highest levels of MDA and PC, including engorged whole ticks and midguts and unfed ticks injected with FAC.

The level of MDA in *Hlfer*-silenced ticks after blood feeding was further evaluated using the TBARS assay, the most common technique employed in studying lipid peroxidation and oxidative damage [19]. The results showed that lipid peroxidation was higher in both *Hlfer*-silenced groups as compared to the *Luc*-injected control group, either in whole ticks or in midguts (Fig. 2-9). The highest level of MDA was observed in *Hlfer1*- and *Hlfer2*-silenced groups in whole ticks and midguts, respectively.

2.3.6 Hlfer1-*silenced ticks did not accumulate ferric iron after blood feeding or injection of FAC*

Iron is stored in ferritin as Fe^{3+}. I hypothesized that Fe^{3+} accumulation should be reduced in the *Hlfer*-silenced ticks after blood feeding or FAC injection. To evaluate this hypothesis, staining of HlFERs for Fe^{3+} after native PAGE was performed. Protein concentration was adjusted based on the tubulin level. In whole ticks and midguts after blood feeding, as well as in whole ticks injected with FAC, *Hlfer1*-silenced ticks weakly stained for ferric iron (Fig. 2-10A). Interestingly, the *Hlfer2*-silenced group still showed strong staining.

2.3.7 *Higher level of ferrous iron was detected in* Hlfer2*-silenced ticks injected with FAC*

I also hypothesized that in the absence of HlFER, Fe^{2+}cannot be stored as Fe^{3+} and should accumulate. Thus, the ferrozine assay for measuring non-heme iron was performed to determine the amount of ferrous iron in whole ticks injected with FAC after *Hlfer* knockdown. In performing the ferrozine assay, the addition of ascorbic acid was omitted to avoid the reduction of ferric to ferrous iron. The ferrozine assay showed that *Hlfer1*-silenced ticks had only a slightly higher ferrous iron level, while *Hlfer2*-silenced ticks had a significantly higher (P <0.05) Fe^{2+} level 72 h after FAC injection than the control group (Fig. 2-10B).

2.4. DISCUSSION

Ticks are known for their ability to ingest large amounts of blood from their host, reaching more than a hundred times their unfed body weight. The numerous bioactive molecules in their saliva allow them to evade the host's immune and hemostatic mechanisms, which is important for successful attachment and feeding [82]. However, they also must cope with potentially toxic molecules in the host blood, including iron. Ferritin is an iron-storage protein involved in iron homeostasis in most living organisms. The physiological importance of ferritin in blood feeding and reproduction of the hard ticks *I. ricinus* [33] and *H. longicornis* (Chapter 1) has been demonstrated through RNAi; however, the specific role of tick ferritins has not been demonstrated. In this study, we showed that HlFERs act as antioxidant molecules, minimizing oxidative stress during blood feeding.

Aside from the effects of *Hlfer* silencing on blood feeding and reproduction, I reported in the previous chapter that *Hlfer*-silenced ticks had high mortality after

blood feeding. In this chapter, I show that this mortality is related to the iron-storage function of ferritin. For the first time, ticks were exposed to exogenous iron by injecting FAC into the hemocoel. The silencing of *Hlfer* alone (data not shown) or with injection of water in unfed adult female ticks did not result in any mortality; however, mortality increased with each day after FAC injection. The group injected with *Hlfer2* dsRNA showed a more rapid increase in mortality and a lower survival rate at the end of the observation period compared to both *Hlfer1*-silenced and *Luc*-injected control groups. The injection of FAC introduced high levels of free Fe^{2+} in the hemocoel. Only the secretory HlFER2 is present in the tick's hemolymph. After *Hlfer2* knockdown, excessive Fe^{2+}, as we have demonstrated through the ferrozine assay, could have caused oxidative damage in the ticks that eventually lead to mortality. Conversely, the absence of HlFER1 after its knockdown could have led to high levels of intracellular Fe^{2+}.

To confirm that the mortality after FAC injection in *Hlfer*-silenced ticks is related to HlFER function, additional experiments were performed after injecting FAC into normal unfed adult ticks, including RT-PCR, Western blotting and IFAT. In contrast to reports of ferritin up-regulation on mosquitoes following artificial feeding and *in vitro* exposure of cells to iron [57,83,84], and after injection of iron in *Macrobrachium rosenbergii* [85] and *Bombus ignitus* [75], the transcript level of either *Hlfer* did not change in response to iron injection. Meanwhile, Western blot analyses showed an increasing protein level in whole ticks, particularly of HlFER1, in a time-dependent manner after FAC injection. In agreement with the conclusion in Chapter 1, these results demonstrated the translational regulation of HlFER1 through the binding of the iron-responsive element (IRE) to the iron-regulatory protein (IRP) [56].

Interestingly, Western blot analysis of different organs showed that FAC injection stimulated the expression of both HlFER1 and HlFER2 in the midgut but not in the salivary glands or ovary. IFAT also showed the extensive fluorescence of digestive cells for HlFER1, extending from the basal lamina to the cells close to the lumen, whereas HlFER2 was strong particularly along the basal lamina in the midgut. In contrast, no fluorescence was found in the salivary glands. These results suggest that the iron in the hemolymph may cross the basal lamina of the midgut for storage in HlFER1 of digestive cells. In mammals, circulating iron bound to transferrin can enter the basolateral membrane of enterocytes through transferrin receptor 1 [17]. However in the ticks, the function of transferring and transferrin receptor in iron metabolism remains to be elucidated. Meanwhile, the increased fluorescence of HlFER2 along the basal lamina of the midgut after FAC injection may imply that iron in the hemolymph stimulated its expression with subsequent secretion, since the HlFER2 level in the hemolymph also increased after FAC injection. In mosquitoes, iron treatment resulted in an increase in the secretion of ferritin [57]. Here, since a high level of iron was present in the hemolymph, HlFER2 could have been secreted to sequester iron. Moreover, I previously concluded that HlFER2 is secreted from the midgut to remove iron and distribute it to other organs of the tick, in agreement with the model of iron metabolism in ticks proposed by Hajdusek et al. [33]. Presently, the other components of iron metabolism in ticks, as well as the regulatory signals in iron distribution, remain to be elucidated. Iron traffic during blood feeding in ticks must be systemically regulated, involving complex signal pathways. Whereas a series of signal pathways are known to be involved in iron traffic aside from the iron-binding proteins in mammals [20] and several proteins have already been identified in arthropods such

as *Drosophila melanogaster* [23] and *Anopheles gambiae* [22], to function in iron absorption and homeostasis, these aspects require further investigations in the ticks.

The Prussian blue staining for Fe^{3+} in native HlFER after native PAGE was useful in the assessment of Fe^{3+} accumulation. I observed increased staining after FAC injection, which may reflect the increased HlFER level and iron uptake of HlFER molecules at these time points. The increased Fe^{3+} accumulation, together with the increased levels of both HlFERs in the midgut I mentioned earlier, supports the conclusion in Chapter 1 that the midgut is the primary organ for iron metabolism, most likely being the first organ exposed to large amounts of iron during blood feeding. Interestingly, staining for Fe^{3+} was weakened after *Hlfer1*-silencing but not after the silencing of *Hlfer2*. In the previous chapter, I reported that HlFER1 was still expressed after *Hlfer2* silencing, particularly in the midgut. Thus, the present result on Fe^{3+} staining in *Hlfer2*-silenced ticks implicates HlFER1.

Iron is known to promote the formation of ROS that can result in damage to macromolecules, including DNA, proteins and lipids—the condition collectively termed oxidative stress [18]. Iron was particularly reported to induce lipid peroxidation [63,64] and oxidation of several amino acid residues in proteins [81]. Thus, the function of ferritin as a repository for excess iron is crucial to preventing oxidative damage. Here I showed that the knockdown of either *Hlfer* resulted in oxidative stress in ticks exposed to high levels of iron, either from blood meal or FAC injection. Similar to the observed results of tick infestation in the previous chapter, *Hlfer*-silenced ticks infested on rabbits failed to engorge, weighing less than half of the *Luc*-injected ticks' engorged body weight, meaning they ingested a lower amount of blood. Oxidative stress was confirmed by the detection of MDA and PC, which are products of lipid peroxidation and protein oxidation, respectively [18], and

observation of higher levels in *Hlfer1*- and *Hlfer2*-silenced ticks than in *Luc*-injected ticks after blood feeding or injection of FAC. The TBARS assay was also employed to assess lipid peroxidation after blood feeding and similarly, it showed that *Hlfer*-silenced ticks had a higher degree of lipid peroxidation compared to the control. I also attempted to perform TBARS assay on unfed *Hlfer*-silenced ticks injected with FAC but due to the low sensitivity of this test [86], I was unable to detect the presence of MDA on the samples. Taken together, these results imply that without HlFER1 or HlFER2, free Fe^{2+} predisposed the ticks to oxidative stress that led to death.

In Chapter 1, I described abnormalities in the digestive cell morphology in *Hlfer*-silenced ticks during blood feeding, including altered shape, disrupted microvilli and cell membrane and vacuolated cytoplasm. I hypothesized that these abnormalities resulted from oxidative damage. Here I show that the midgut of *Hlfer*-silenced ticks had high levels of MDA and PC. Lipid peroxidation leads to alterations of biological membranes and gives rise to several products that are known to induce diverse biological effects [87]. MDA, which is one of the most known and most studied products of lipid peroxidation, is highly toxic and can interact with DNA and proteins and thus can impair physiological functions [88]. Aside from the direct injury caused by reactive oxygen species, products of lipid peroxidation including MDA can promote further injury. Carbonylation of proteins is another hallmark of oxidative stress resulting from irreversible oxidative modification of proteins that can be induced by transition metals including iron, ROS, products of lipid peroxidation including MDA, and glycoxidation. PCs cannot be repaired and accumulation may lead to cell death [81]. The results in this chapter also show that the midguts from *Hlfer1*-silenced ticks had the highest level of either oxidative stress biomarker, which corresponds my previous observation of more severe abnormalities in the digestive

cells of *Hlfer1*-silenced ticks. Moreover, I also reported in Chapter 1 a decrease in hematin production after *Hlfer1* silencing, indicative of impaired digestive activity. Oxidative stress can also alter physiological processes including heme detoxification in the midgut [89].

Several antioxidant enzymes that counteract reactive oxygen species, such as superoxide dismutase, glutathione S-transferase and thioredoxin, have been identified in hard ticks [15,16]. These enzymes prevent oxidative stress by keeping the level of free radicals to a minimum. In the hard tick *D. variabilis*, these antioxidant enzymes were found in the midgut at day 6 of blood feeding, corresponding to the rapid feeding stage [15]. In this chapter, I demonstrated that, by sequestering Fe^{2+} and keeping it in the oxidized Fe^{3+} form, ferritin is also an important antioxidant molecule in the hard tick during blood feeding because it prevents oxidative stress.

In summary, this chapter reported that the silencing of two *Hlfer*s resulted to increased levels of oxidative stress biomarkers after a blood meal or injection of iron. The results here provide concrete evidence for the first time that two kinds of ferritin act as antioxidant molecules in a hard tick that prevent oxidative stress during blood feeding, thus ensuring tick survival. This chapter provides further explanation on the crucial importance of ferritin in the ticks that was reported in Chapter 1, and also the other work in another hard tick, *I. ricinus* [33]. Moreover, the iron-injection experiment, which to my knowledge was employed for the first time in ticks, demonstrated that iron in the hemocoel can stimulate HlFER expression of the midgut and that iron molecules can be apparently transported from the hemolymph to digestive cells. However, further experiments are needed to elucidate this aspect of iron transport mechanism in ticks. Moreover, the iron-sequestration function of ferritin is implicated in immune response in many organisms [25]; thus, I am

interested in the possible role of HlFERs in the tick immunity that will be dealt with in the next chapter. Together with the results in Chapter 1, the results in this chapter show that ferritin is an important protective antigen of ticks that can be utilized to design a control strategy.

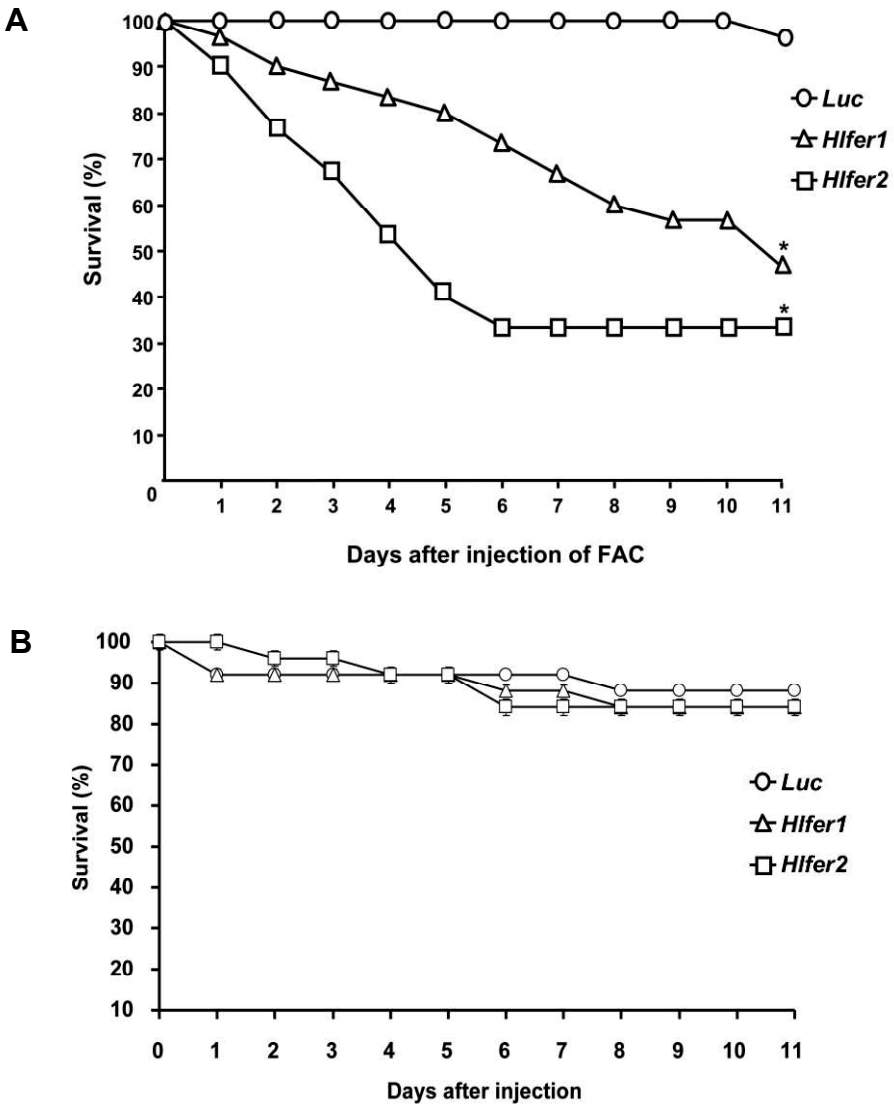


Fig. 2-1. Survival rate of *Hlfer*-silenced ticks after injection of FAC (A) or sterilized high-purity water (B). Unfed adult female ticks were injected with *H. longicornis fer1* (*Hlfer1*), *H. longicornis fer2* (*Hlfer2*) or *Luciferase* (*Luc*) dsRNA for the control to induce RNAi. Silencing was confirmed through RT-PCR. After 4 days, 100 μM FAC or sterilized high-purity water was injected, and mortality was monitored. Both *Hlfer1*- and *Hlfer2*-dsRNA-injected groups had a lower survival rate compared to *Luc* after injection of FAC, whereas low mortality without significant difference in the survival rate of the three groups was observed after injection of sterilized high-purity water. n=30 ticks per group. The graph here represents the result of a single independent trial. Bars represent standard error. Significant difference was determined using the log-rank Mantel-Cox test (P <0.0001, *Luc* vs. *Hlfer1* or *Hlfer2*).

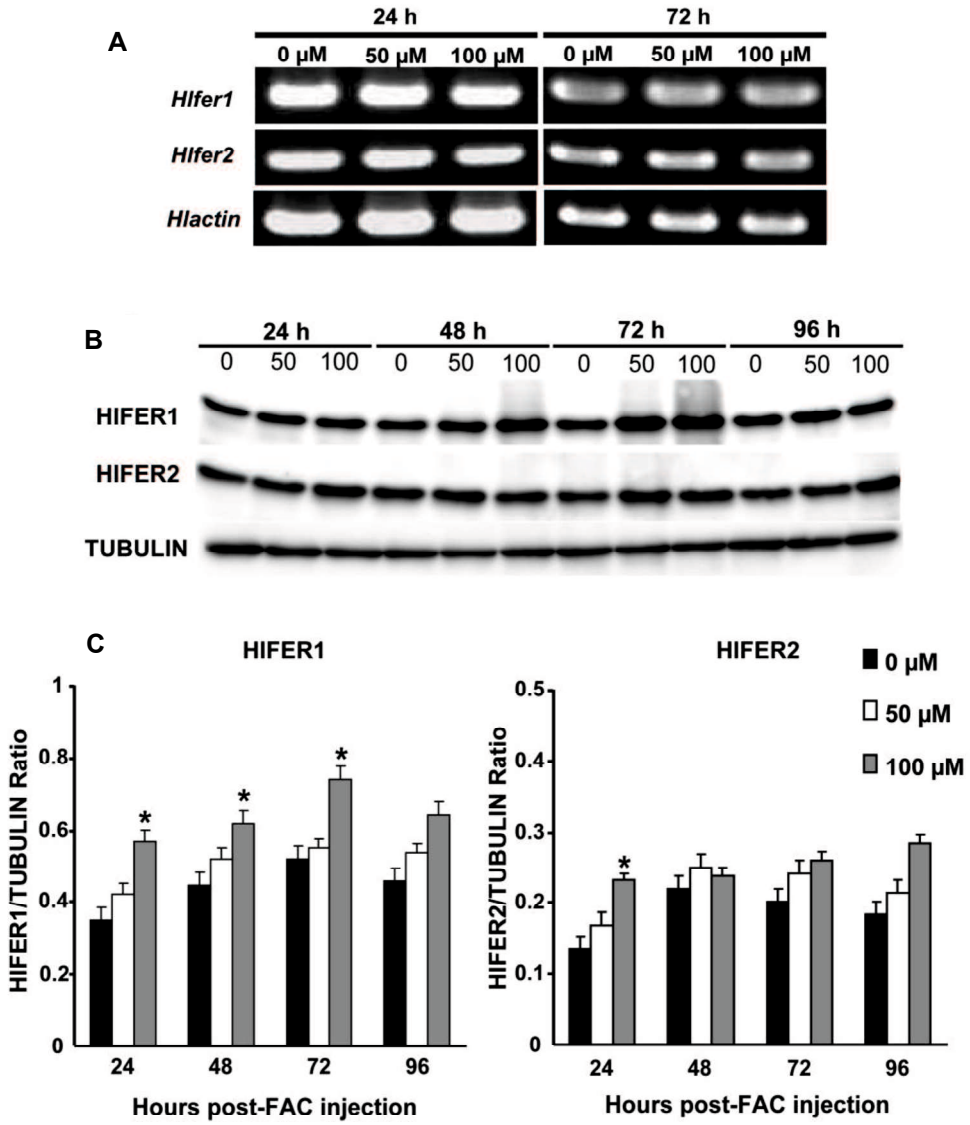


Fig. 2-2. Transcription and protein expression profile of *H. longicornis* ferritins in whole bodies of adult ticks injected with FAC. Unfed adult ticks were injected with 50 µM or 100 µM FAC. Sterilized high-purity water was injected into the control group (0 µM). (A) Total RNA was extracted from whole ticks at 24 h and 72 h after injection and RT-PCR analysis was performed using specific primers for *Hlfer1* and *Hlfer2*. cDNA was adjusted based on control amplification for *Hlactin*. No significant difference was observed among groups. (B) Protein was extracted and Western blot analysis was performed using specific antisera against HlFER1 or HlFER2. Tubulin antiserum was used as an internal control. (C) Band densitometry analysis was performed to determine the relative expression of HlFERs to tubulin. Significant increase in expression was particularly found in HlFER1. Data represent the means of three independent trials ± SE. Statistical significance (*P <0.05) was determined using the Mann-Whitney test.

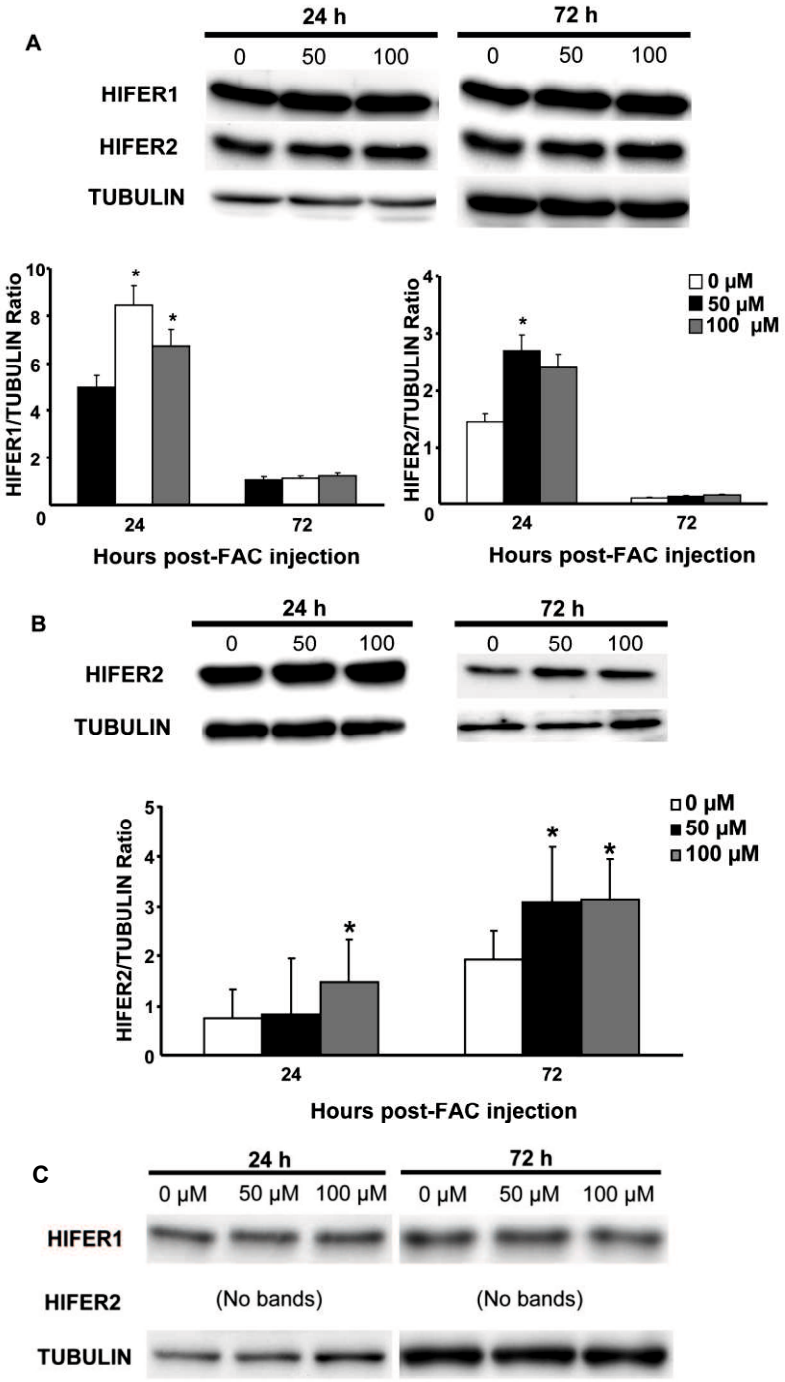


Fig. 2-3. Protein expression of *H. longicornis* ferritins (HlFERs) in the midguts (A), hemolymph (B) and salivary glands (C) of unfed ticks injected with different concentrations of FAC. Organs were collected at 24 h and 72 h after injection of 50 µM or 100 µM FAC or sterilized high purity water for the control group (0 µM). Hemocyte was separated from the hemolymph by centrifugation. Western blot analysis was performed using specific anti-sera against HlFER1 or HlFER2. Tubulin antiserum was used as an internal control. The relative expression of HlFER1 and HlFER2 was calculated based on tubulin after band densitometry analysis. Significant increase in expression was particularly found in midgut HlFER1 and hemolymph HlFER2. No change in HlFER1 level was observed in salivary glands. Data represent the means of three independent trials ± SE. Statistical significance (**P* <0.05) was determined using the Mann-Whitney test.

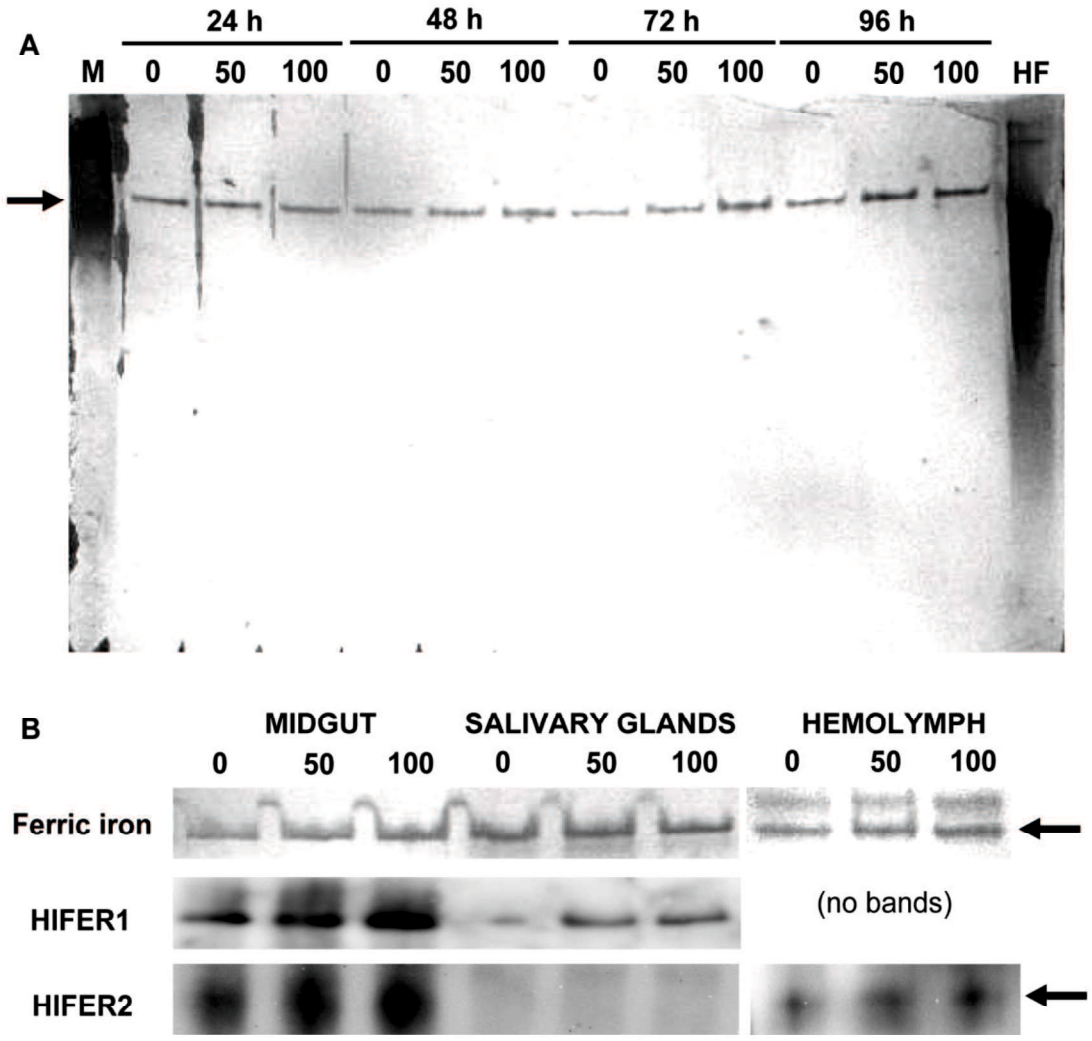


Fig. 2-4. Staining of HlFER for ferric iron on native PAGE gel after FAC injection. Ticks were injected with 50 or 100 μM FAC or sterilized high-purity water (0 μM) for the control group. Whole ticks were collected from 24 h to 96 h and total protein was extracted (A). Each lane of native PAGE gel was loaded with 20 μg of total protein and then the gel was stained with an equal volume of 10% $K_4[Fe(CN)_6]$ and 10% HCl for up to 48 h. A single band stained for ferric iron. The high molecular weight marker (M), which contains ferritin from equine spleen, also stained for ferric iron, as well as the horse holoferritin (HF), which was used as a positive control. Midguts, salivary glands, and the hemolymph (B) were also collected from ticks 72 h after injection of FAC or sterilized high-purity water. Each lane was loaded with 10 μg of protein. Western blot analysis using specific anti-HlFER sera was also performed to confirm that the band stained for ferric iron is HlFER. Arrows indicate approximately 440 kDa, which is the theoretical molecular weight of native ferritin.

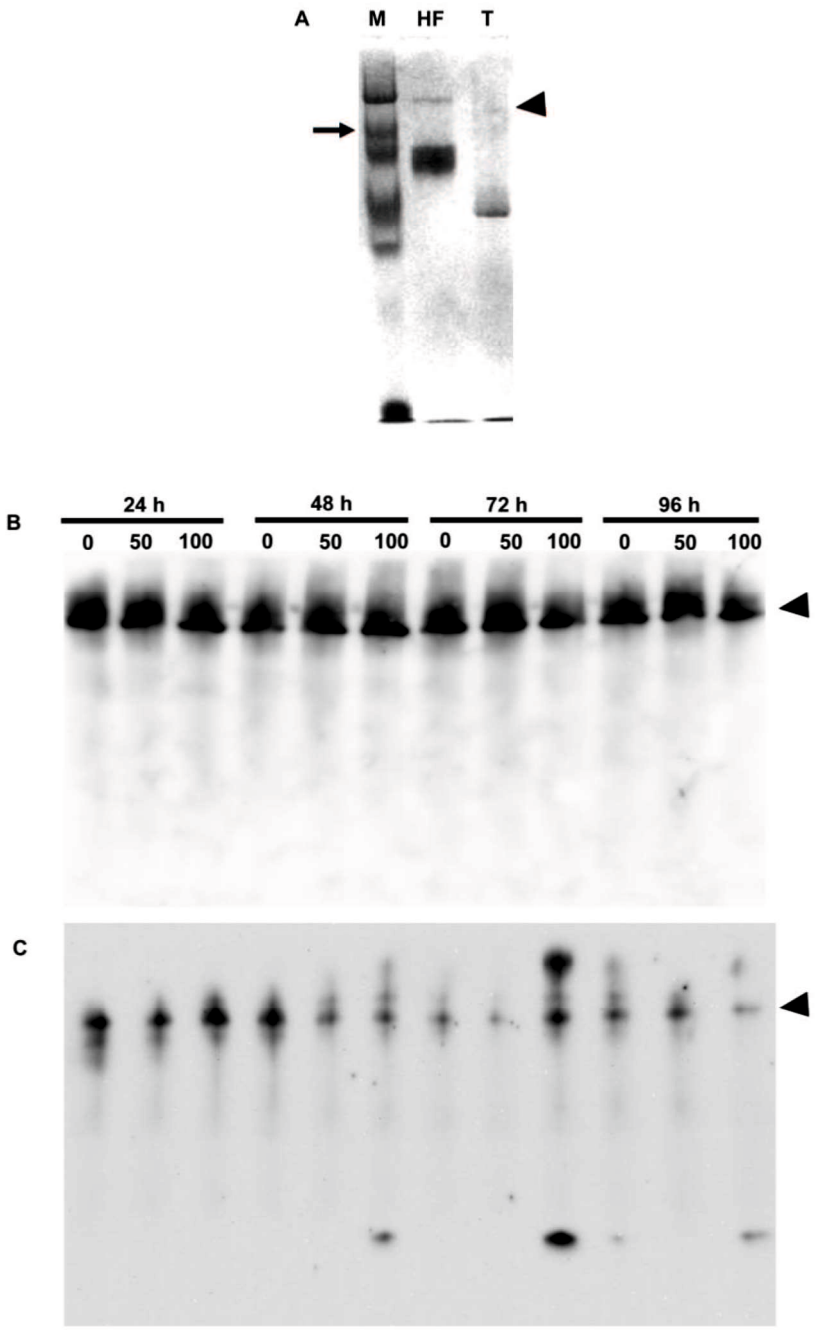


Fig. 2-5. Coomassie blue staining and Western blot analysis after native PAGE for HlFERs. To further confirm that the bands stained for ferric iron from tick protein samples were HlFER, Coomassie blue staining and Western blot analyses using specific anti-HlFER sera were performed. (A) Coomassie blue staining showed all the bands of high molecular weight marker (M), the commercially prepared horse holoferritin (HF) and the tick protein (T). The weak band of approximately 440 kDa in the tick protein sample was presumed to be HlFER. Western blot analyses for HlFER1 (B) and HlFER2 (C) showed a single band of approximately 440 kDa. Arrow indicates the 440 kDa band in the high molecular marker while arrowheads point to HlFER.

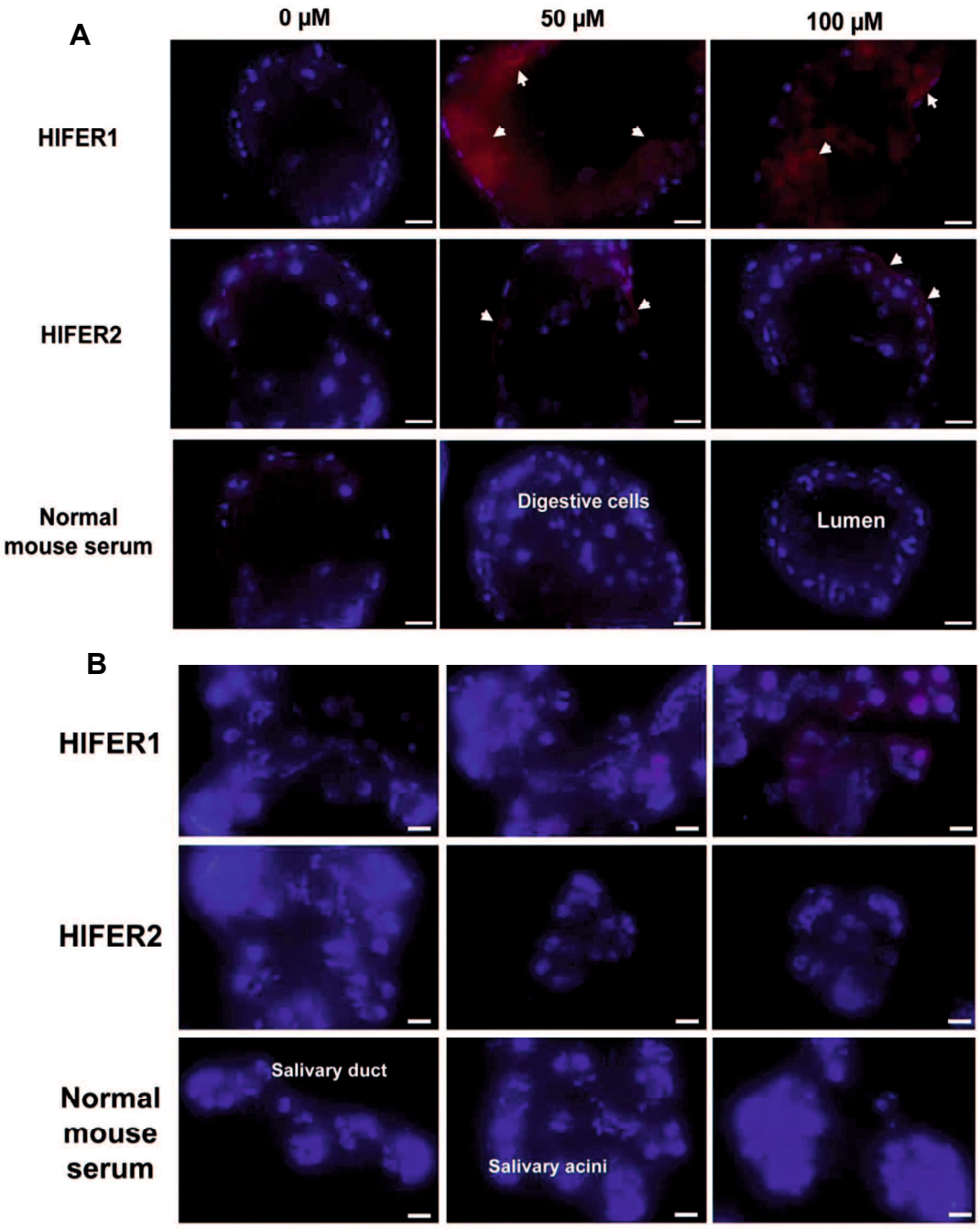


Fig. 2-6. An indirect immunofluorescent antibody test (IFAT) in the midgut (A) and salivary glands (B) after injection of FAC. Different concentrations of FAC or sterilized high-purity water (0 μM) were injected to unfed adult ticks and organs were collected after 72 h. To visualize the extent of the stimulation of HlFER expression caused by FAC injection, frozen sections of the midgut and salivary glands were incubated with specific mouse anti-HlFER1 or anti-HlFER2 sera. Mouse normal serum was used as a negative control. Anti-mouse IgG conjugated with Alexa 594 was used as secondary antibody and nuclei were visualized using DAPI. Arrowheads point to areas with increased fluorescence in the midguts. No positive fluorescence was observed in the salivary glands (Bars = 20μm).

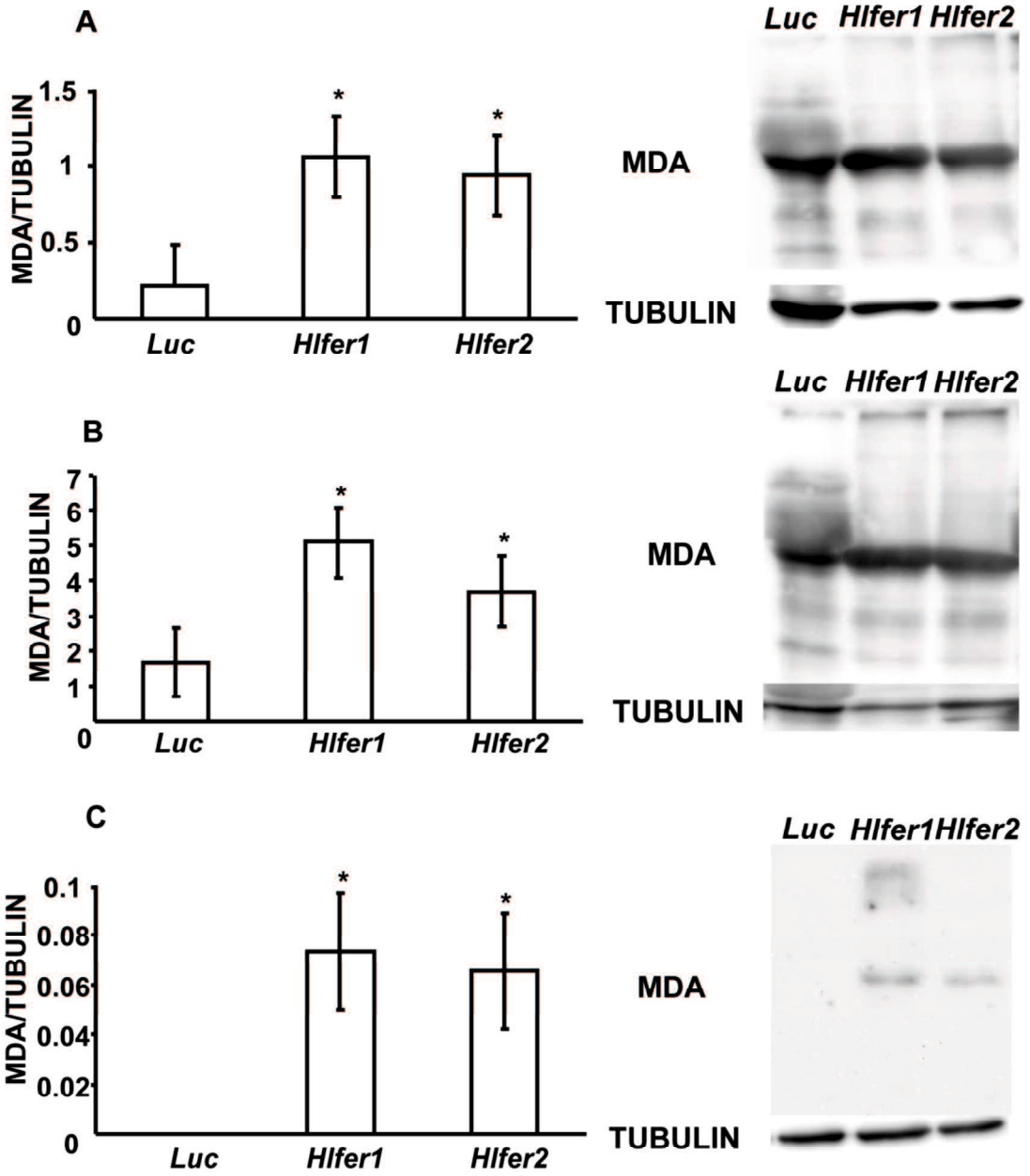


Fig. 2-7. Detection of malondialdehyde (MDA) from *Hlfer*-silenced ticks after blood feeding or injection of FAC. Total protein was extracted from whole ticks (A) and midguts (B) after blood feeding and whole ticks 72 h after injection of 100 µM FAC (C). Western blot analysis was performed and the membrane was incubated with a specific anti-MDA antibody. Tubulin was used as internal control. The relative content of MDA (clearest band) to tubulin was calculated after band densitometry analysis. Both *Hlfer1*- and *Hlfer2*-silenced ticks had significantly higher MDA compared to the control (*Luc*) group. Data represent the means of three independent trials ± SE. **P* <0.05, significantly different vs. *Luc*, Student's *t*-test.

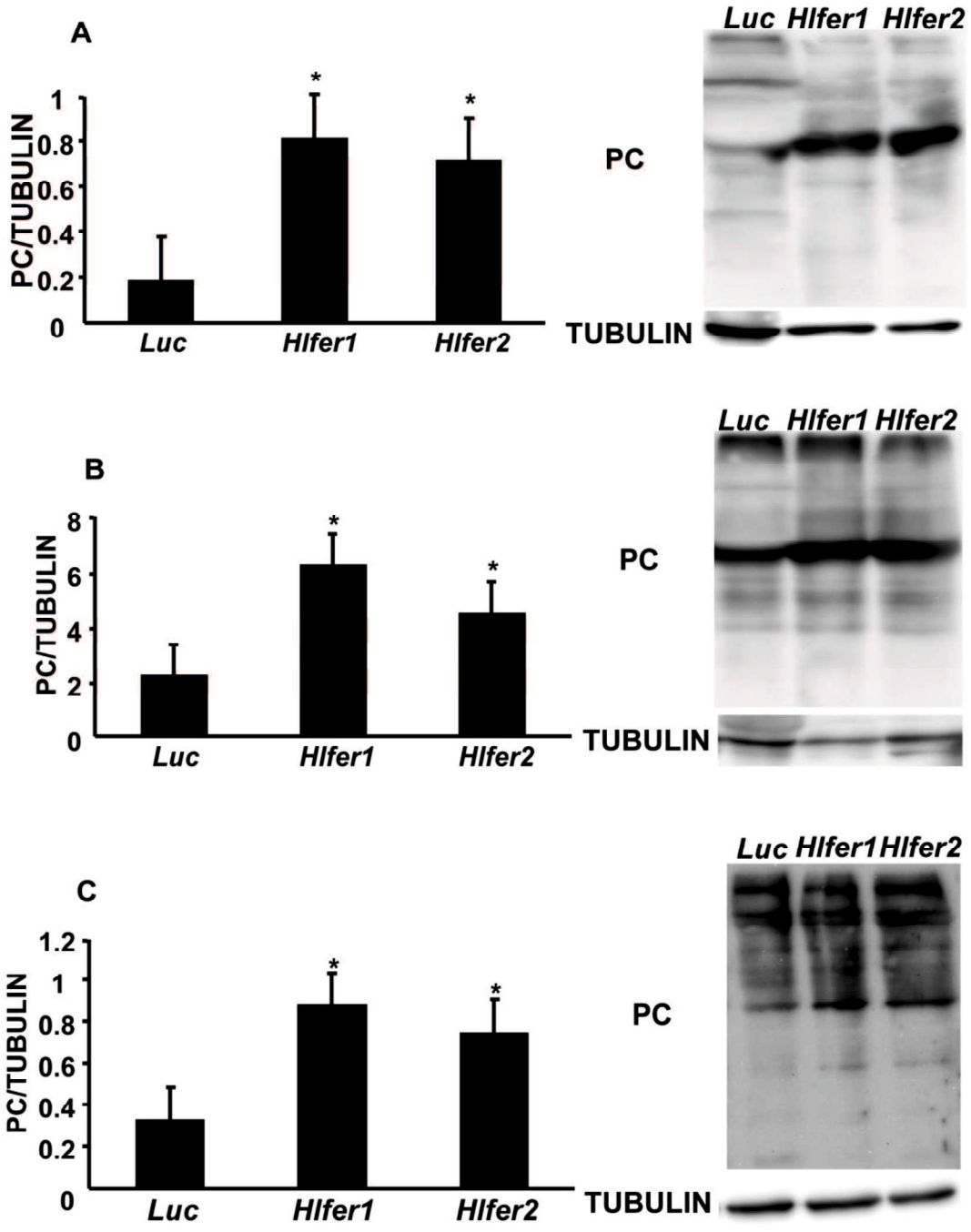


Fig. 2-8. Detection of protein carbonyl groups (PC) from *Hlfer*-silenced ticks after blood feeding or injection of FAC. Total protein was extracted from whole ticks (A) and midguts (B) after blood feeding and whole ticks 72 h after injection of 100 μM FAC (C). After the transfer of proteins to a membrane through Western blot, the membrane was incubated first with dinitrophenylhydrazine (DNPH) for pre-derivitazation of the carbonyl group, before incubation with a specific anti-DNP antibody. Tubulin was used as internal control. The relative content of PC (strongest band) to tubulin was calculated after band densitometry analysis. Both *Hlfer1*- and *Hlfer2*-silenced ticks had significantly higher PC levels compared to the control (*Luc*) group. Data represent the means of three independent trials ± SE. **P* <0.05, significantly different vs. *Luc*, Student's *t*-test.

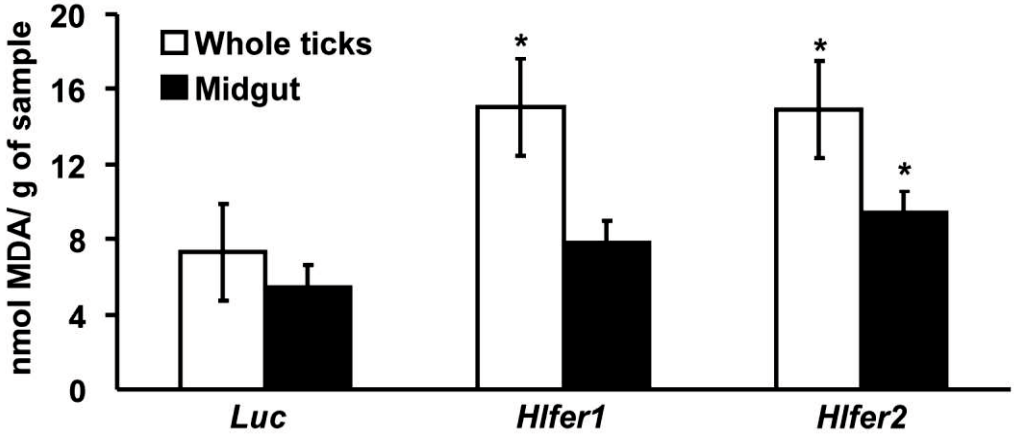


Fig. 2-9. Thiobarbituric acid reactive species (TBARS) assay for *Hlfer*-silenced ticks after blood feeding. Whole bodies or midguts of ticks injected with *Hlfer1*, *Hlfer2*, or *Luc* dsRNA were collected after dropping from the host. Individual whole ticks or pooled midguts were weighed before being homogenized. After ultrasonication, the supernatants were obtained and boiled with TBARS reagent. Upon cooling and centrifugation, the absorbance of the supernatants was measured at OD_{532}. The relative amount of MDA was calculated based on the sample weight and expressed as nmol/g. Both *Hlfer1*- and *Hlfer2*-silenced ticks had higher MDA levels in both whole ticks and midguts than the control (*Luc*) ticks. Values are means of 30 samples for each group ± SE. **P* <0.05, significantly different vs. control, Student's *t*-test.

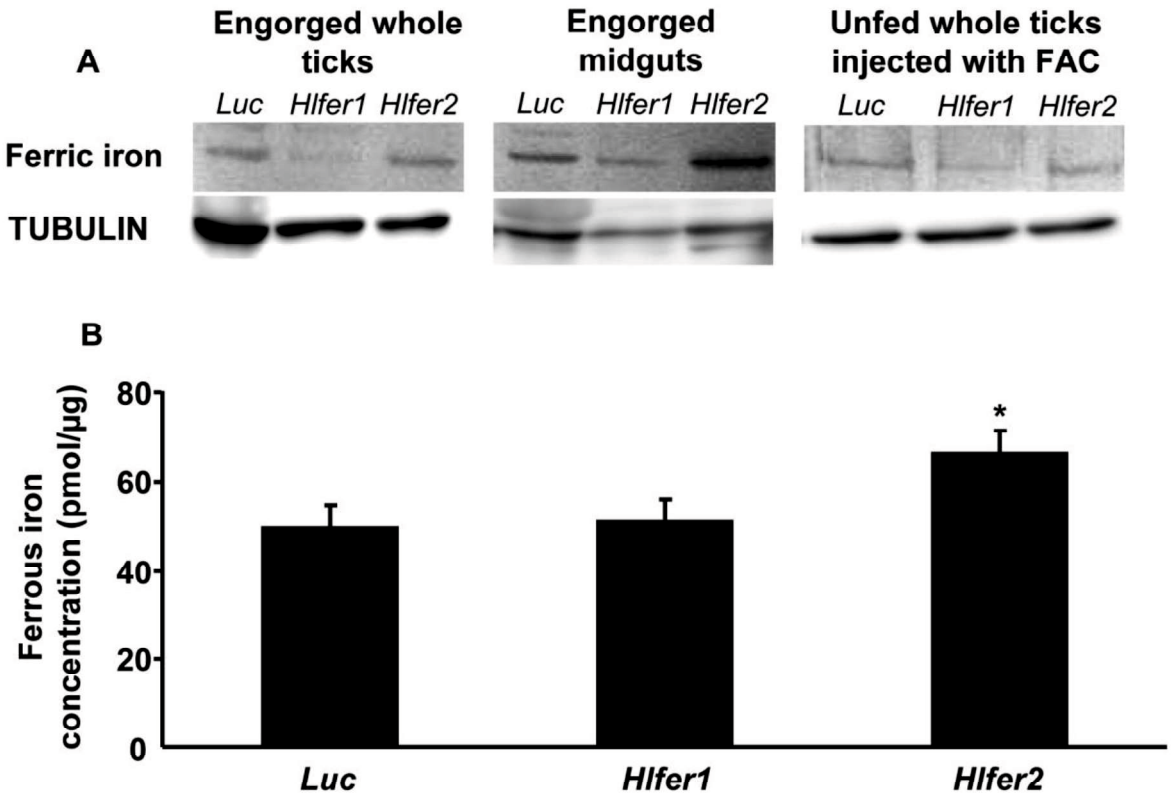


Fig. 2-10. Evaluation of iron accumulation in *Hlfer*-silenced ticks. Ferric iron accumulation was evaluated by staining HlFER on native PAGE (A). Total protein was extracted from whole bodies and midguts after blood feeding and whole bodies 72 h after FAC injection. The amount of protein was adjusted based on the tubulin profile after Western blotting. Weak staining was observed in *Hlfer1*-silenced ticks. Ferrozine assay for ferrous iron 72 h after injection of 100 µM FAC to unfed *Hlfer*-silenced ticks (B). Ten ticks from each group were homogenized and total protein concentration was measured. Ferrous iron was extracted with concentrated HCl and then detected using ferrozine. Absorbance was measured at OD_{550}. Relative ferrous iron content was calculated based on protein concentration. *Hlfer2*-silenced ticks had significantly higher ferrous iron content than the control (*Luc*) group. **P* <0.05, significantly different vs. *Luc*, Student's *t*-test.

CHAPTER 3

Knockdown of ferritin genes increases the susceptibility of the hard tick *Haemaphysalis longicornis* to bacterial infection

3.1 INTRODUCTION

Iron is an essential element required for several cellular processes in many organisms [17]. Except for some species, most pathogens require iron to establish infection within their hosts [90], and thus the host and pathogens compete for iron. Withholding of nutrients, including iron-sequestration is a well-recognized part of vertebrate innate immunity to suppress pathogen multiplication, and has been particularly studied in bacterial infections [25,91]. Iron also regulates several components of host immune effector pathways, and these components such as cytokines, IFN-γ and TNF-α can affect iron homeostasis [92]. As a countermeasure, many bacterial pathogens evolved several mechanisms that allow them to access iron from their host, such as siderophore production and heme acquisition [93].

Iron-binding proteins are utilized by the vertebrate hosts to either deprive the pathogen of iron or to concentrate the iron that will facilitate iron-mediated pathogen-killing [94]. These proteins include transferrin, lactoferrin, lipocalin-2 and Nramp [25,92]. Ferritin is another iron-binding protein that primarily functions for intracellular iron storage in most organisms but its role in immunity is not yet well-established. Ferritin gene expression has been reported to respond to bacterial challenge in several organisms, including mosquitoes [74,95], the turbot fish, *Scophthalmus maximus* [71] and the bumblebee *Bombus ignitus* [75].

Ticks serve as vectors of a wide range of pathogens, including several species of rickettsiae. Moreover, ticks may encounter bacterial pathogens when they feed on a septicemic host. Therefore, tick innate immunity is crucial in limiting pathogen multiplication. A single report on the hard tick, *D. variabilis* showed up-regulation of a ferritin gene following *E. coli* injection [96]. However, the role of ferritin in pathogen multiplication in ticks has not been elucidated. This chapter demonstrates

the importance of the two types of ferritins in protecting the hard tick *H. longicornis* from bacterial infection.

3.2 MATERIALS AND METHODS

3.2.1 *Preparation and injection of EGFP-expressing* E. coli

The ampicillin-resistant enhanced green fluorescence protein (EGFP)-expressing *E. coli* previously prepared in our laboratory [97] was grown overnight in 2 ml LB broth medium with ampicillin (50 μg/ml) at 37°C, and then added to 25 mL LB medium with ampicillin the next day and further incubated until OD_{600}= 0.5. IPTG was then added to the bacterial culture at 1 mM final concentration and further incubated for 4 h to induce EGFP expression. Bacterial cells were first examined under a fluorescence microscope to confirm EGFP expression before harvested by centrifugation at 760 *g* for 15 min at 4°C. After removing the culture medium, *E. coli* cells were re-suspended in 1 mL PBS. Gram-staining was performed to visualize the bacteria and to ensure the absence of contamination. A bacterial suspension with an OD_{600}=20 was injected to unfed female ticks at 0.5 μl per tick in the same manner as dsRNA injection described in the previous chapters. Conversely, 0.5 μl heat-killed *E. coli*, prepared by heating the bacterial suspension at 90°C for 7 min, was injected to each tick. As an additional control, PBS was also injected to ticks.

To compare the survival rate of *Hlfer*-silenced ticks after bacterial challenge, 30 unfed female ticks per group were injected with *Hlfer1*, *Hlfer2* or *Luc* dsRNA, as described in the previous chapters, and then kept in a humidity chamber at 25°C for 4 d before injection with live or heat-killed EGFP-*E. coli*. After injection, ticks were kept as described above and monitored for mortality or until use for other experiments. The survival experiment was repeated three times to confirm its reproducibility.

3.2.2 *Gene and protein expression analyses*

The gene and protein levels of HlFERs at different hours after injection of live or heat-killed GFP-*E. coli* or PBS were evaluated. Total RNA and protein were extracted from three whole ticks collected 0, 6, 12, 24, 48, 72 and 96 h after injection with live or heat-killed EGFP-*E. coli* or PBS as described in the previous chapters. Specific primers and anti-sera described in the previous chapters were used for RT-PCR and Western blot analysis, respectively.

3.2.2 *Preparation and examination of hemocytes*

Hemolymph, collected from *E.coli*-injected ticks by amputating the legs as previously described [37], was smeared directly on glass slides and air-dried. After drying, hemocyte smears were fixed with either 4% paraformaldehyde in PBS or methanol for IFAT and Giemsa staining, respectively, and then washed with tap water. For IFAT, hemocyte smears were blocked with 5% skimmed milk in PBS, and then incubated with mouse anti-HlFER sera described in Chapter 1 or normal mouse serum for control in a blocking solution (1:50 dilution) as primary antibody for 1 h at room temperature. After washing, the smears were incubated with Alexa-594 conjugated goat anti-mouse Ig (Invitrogen) in a blocking solution (1:1000 dilution) for 1 h at room temperature. After washing for several times, the smears were mounted in a medium containing DAPI (Vectashield, Vector Laboratories). The smears were then examined using the LSM 700 confocal microscope (Carl Zeiss Microscopy, Jena, Germany). For Giemsa staining, hemocyte smears from three ticks each group were covered with a 3% Giemsa solution for 30 min, washed with tap water and then air-dried before being observed under a light microscope.

3.2.3 *Culture of hemolymph for bacterial count*

To determine whether EGFP-*E. coli* can multiply within the ticks after injection, 0.5 µl hemolymph was collected from each tick at 48 h after *E. coli* injection and individually suspended in 100 µl LB medium broth with ampicillin. This suspension was further diluted to 1:10,000 before applying in an LB agar medium with 1 µg/mL ampicillin and 1 mM IPTG. After incubating at 37°C overnight, images of LB agar plates were taken using FluorChem FC2 Imaging System (Protein Simple) and bacterial colonies were counted using the colony count tool of Alpha View Software (Alpha Innotech, Protein Simple). Five ticks per dsRNA group were used for this experiment, which was repeated twice.

3.2.4 *Ferrozine assay for measurement of total ferrous iron*

Total ferrous iron from whole ticks was measured through ferrozine assay as described in the previous chapter. Briefly, 15 ticks per group were pooled and homogenized 4 d after dsRNA injection and suspended in 200 µl lysis buffer. Protein concentration was determined using a Micro BCA Assay kit (Thermo Scientific). After addition of concentrated HCl, the mixture was heated to 95°C and then cooled to room temperature. After centrifugation, the supernatant was recovered to which 10 mM ferrozine (Sigma-aldrich) was added. Saturated ammonium acetate was finally added to facilitate color development and absorbance was measured in a microplate reader (Bio-rad) at 550 nm. Iron concentration was calculated using the formula described by Missirlis et al [79], and was expressed based on the protein concentration.

3.2.5 *Statistical analyses*

The Mantel-Cox log-rank test was performed using GraphPad Prism software for analyzing the results of the survival experiment. Student's *t*-test was used for

analyzing the data from hemocyte count and colony count. In the hemocyte count, values reflect the average count from three ticks per group. In the colony count, the values reflect the average from two trials, using five ticks per group for each trial. In all statistical analyses, significant difference is defined by $P < 0.05$.

3.3 RESULTS

3.3.1 *EGFP*-E. coli *injection affected the transcription level of* Hlfer2 *but not of* Hlfer1

Total RNA was extracted from whole ticks at 0, 6, 12, 24, 48, 72 and 96 h after injection with live or heat-killed EGFP-*E. coli* or PBS. RT-PCR was performed using specific primers for *Hlfer1* and *Hlfer2* (Fig. 3-1A). Actin primer was used as an internal control. RT-PCR showed that injection of live or heat-killed EGFP-*E. coli* did not affect the level of *Hlfer1* transcripts in comparison to that of PBS-injected ticks. In contrast, *Hlfer2* transcription of ticks injected with live EGFP-*E. coli* was higher relative to that of PBS-injected ticks right after injection until 12 h, decreased from 24 h until 72 h, and then increased again at 96 h. Meanwhile, the *Hlfer2* level of ticks injected with heat-killed EGFP-*E. coli* was relatively higher than that of PBS-injected ticks right after injection until 24 h.

3.3.2 *Live EGFP*-E. coli *stimulated the expression of both HlFER1 and HlFER2*

Protein expression in the whole ticks after injection of live or heat-killed EGFP-*E. coli* was also evaluated, parallel to the sampling time for checking the mRNA level. Tubulin level was also determined for internal control. As shown in Fig. 3-1B, the expression of HlFER1 and HlFER2 at 48 and 72 h seems to be higher in ticks injected with live EGFP-*E. coli* as compared to ticks injected with PBS, whereas

no change in HlFER1 and HlFER2 level was observed after injection of heat-killed EGFP-*E. coli*.

3.3.3 Hlfer-*silenced ticks had lower survival rate after live GFP*-E. coli injection

To better demonstrate the function of HlFERs on tick immunity, *Hlfer* genes were silenced, and then the ticks were challenged with bacteria also through injection in the hemocoel. The success of gene silencing was confirmed by RT-PCR, as shown in Chapter 1. The survival rate (Fig. 3-2) of *Hlfer2*-silenced ticks began to greatly decrease from 60 hours after live GFP-*E. coli* injection and continued to drop by 96 h without any tick surviving (P <0.0001, Mantel-Cox log-rank test vs. *Luc*), whereas the survival rate of *Hlfer1*-silenced ticks gradually decreased from 60 h after live GFP-*E. coli* injection and all the ticks are dead by 192 h (P <0.0001, Mantel-Cox log-rank test vs. *Luc*). Conversely, both *Hlfer*-silenced groups had the same trend of survival rate after injection of heat-killed GFP-*E. coli*, decreasing greatly from 72 h until no ticks are surviving by 108 h. Interestingly, even the *Luc* group had gradually decreasing survival rate from 60 h after injection of heat-killed GFP-*E. coli* but with a final survival rate of 30 % by the end of monitoring.

3.3.4 *Hemocytes from* Hlfer-*silenced ticks did not show positive fluorescence for HlFERs on IFAT examination*

To examine the presence of HlFERs in the haemocytes and whether bacterial injection can induce their expression, smears of hemocytes were prepared from *Hlfer*-silenced ticks at 24 h and 48 h after injection injection of live (Fig. 3-3) or heat-killed (Fig. 3-4) GFP-*E. coli* for IFAT examination. Live GFP-*E. coli* were seen within the cytoplasm and outside of hemocytes as green fluorescence. Positive fluorescence for HlFER1 and HlFER2 with a granular distribution was observed in the cytoplasm of hemocytes from *Luc* group, which was greater in number in the hemocytes from ticks

injected with live GFP-*E. coli* than from those injected with heat-killed *E. coli*. Meanwhile, no fluorescence for HlFER1 and HlFER2 were seen in the hemocytes from *Hlfe1*- and *Hlfer2*-silenced ticks, respectively, after injection of either live or heat-killed *E. coli*.

3.3.5 *Hemocytes from* Hlfer-*silenced ticks had increasing EGFP-E. coli cells*

To examine the fate of live EGFP-*E. coli* after injection in the hemocoel, hemocyte smears stained with Giemsa were prepared at 1, 24, 48 and 72 h after injection of live EGFP-*E. coli* in dsRNA-injected ticks. The two main types of hemocytes, the plasmatocyte and granulocyte, were examined [98]. No significant difference in the number of plasmatocyte and granulocyte was observed among the three groups (Fig. 3-5). *E. coli* cells can be seen inside plasmatocytes as early as 1 h post-injection in all groups indicating phagocytosis, without significant difference in the percentage of plasmatocytes with and without *E. coli* from 1 h through 48 h after injection. However, 72 h post-injection, *dsLuc*-injected ticks had significantly fewer plasmatocytes with EGFP-*E. coli*. In addition, the number of EGFP-*E. coli* within the cytoplasm of plasmatocytes differed between the *dsLuc* and *dsHlfer* groups. In *dsLuc*-injected ticks, the number of bacteria within a majority of the observed plasmatocytes increased from 1 h to 24 h and then decreased afterwards. In contrast, both *dsHlfer1*- and *dsHlfer*-injected groups had fewer bacteria within plasmatocytes at 24 h and 48 h, but greatly increased at 72 h.

3.3.6. *Cultures of hemolymph from Hlfer-silenced ticks yielded more EGFP-*E. coli *colonies*

Examination of Giemsa-stained hemocytes revealed that EGFP-*E. coli* injected to *Hlfer*-silenced ticks increased in number. To further demonstrate that the bacteria in the ticks retained its viability, hemolymph was collected at 48 h after

injection of EGFP-*E. coli* and inoculated in LB agar plates. Colonies were counted after overnight incubation. Both *Hlfer1* and *Hlfer2*-silenced groups yielded significantly greater number of colonies (P= 0.0393 and 0.0135, respectively) than the control group (Fig. 3-6A). The colonies were examined under fluorescent microscope to confirm that these were EGFP-*E. coli*.

3.3.7 *Hlfer-silenced ticks had higher ferrous iron levels than control ticks*

In Chapter 2, *Hlfer2*-silenced ticks had higher level of Fe^{2+} after injection of ferric ammonium citrate. Here I hypothesized that after *Hlfer*-silencing, the highly soluble Fe^{2+} became more available to *E. coli* and sustained its multiplication. To test this hypothesis, ferrozine assay was performed after silencing *Hlfer*s as described in Chapter 2. Significantly higher Fe^{2+} was detected from *Hlfer1*-silenced ticks compared control ticks (Fig. 3-6B; P= 0.09). Meanwhile, *Hlfer2*-silenced ticks also had greater Fe^{2+} level than control ticks but it was not statistically significant.

3.4 DISCUSSION

The previous two chapters demonstrated that HlFERs have critical functions in blood feeding and reproduction of *H. longicornis* by preventing the occurrence of oxidative stress that may arise from iron overload. Here I investigated whether HlFERs have function in tick innate immunity, particularly in sequestering iron that might be utilized by pathogens to establish infection. *E. coli* was used as a representative bacterium for this study since it is widely known that this bacterium utilizes iron for its multiplication, facilitated by several iron uptake mechanisms [99]. I first examined the response of HlFERs to bacterial injection by determining their gene and protein levels. *Hlfer1* level was unchanged after injection of live *E. coli*, contrary to the previous reports in the bumblebee *B. ignitus* [75] and the hard tick *D.*

variabilis [96] where the transcript level of intracellular ferritin increased after bacterial injection. Meanwhile, *Hlfer2* transcript seemed to respond to the introduction of live *E. coli* in the hemocoel. Interestingly, both HlFERs seems to be up-regulated at 48 h and 72 h after bacterial challenge, as compared to the levels of HlFERs after PBS injection. Conversely, injection of heat-killed *E. coli* seemed to have no effect on the transcription of *Hlfer1* but caused transient upregulation of *Hlfer2*. In contrast to a study of mosquito ferritins, wherein exposure to heat-killed bacteria caused upregulation of transcript levels of cytoplasmic, membrane and secreted ferritins in the presence of iron or blood meal [95], the results here show that heat-killed *E. coli* only upregulates the transcription of the secretory *Hlfer2* but not of the intracellular *Hlfer1*.

Transcription and protein expression analyses, however, did not show a clear function of HlFERs in response to bacterial challenge, hence I employed gene silencing to determine whether HlFERs are important to ticks during bacterial infection. Results in this chapter showed that the absence of HlFERs significantly decreased the survivability of ticks when exposed to live *E. coli*, implying that HlFERs are involved in the immune defence of ticks. Interestingly, control ticks and *Hlfer*-silenced ticks had a similar survival rate after injection of heat-killed *E. coli*, with only a few ticks surviving in the control group. Dead bacteria may have released endotoxins that adversely affected the ticks.

Hemocytes comprise the cellular immune response in arthropods, including ticks, playing an important role in defense against microbial infections. The phagocytic hemocytes in ticks, plasmatocytes and granulocyte type I [100] were examined in this chapter. For the first time, it was demonstrated through IFAT that both HlFERs are present in the cytoplasm of these hemocytes, the expressions of

which are apparently induced by injection of live bacteria, and to a lesser extent by heat-killed bacteria in the case of HlFER1, but not by injection of PBS (data not shown). Similar to RNAi experiments described in the previous chapters, the injection of gene-specific dsRNA induced successful silencing in the hemocytes, shown by the absence of positive fluorescence. Examination of Giemsa stained smears showed that only plasmatocytes phagocytosed *E. coli* cells and that *Hlfer*-silencing did not affect their phagocytic ability. Interestingly, the number of *E. coli* inside and outside the hemocytes of *dsHlfer1*- and *dsHlfer2*-injected ticks was very high at 72 h after injection, in contrast to that of *Luc* ticks that have only few *E. coli*. This suggests that *E. coli* injected to *Hlfer*-silenced ticks survived the antibacterial action of plasmatocytes and even multiplied, overwhelming these cells and eventually destroying them. Moreover, cultures of the hemolymph from *Hlfer*-silenced ticks yielded significantly higher colony counts, further indicating that bacterial cells observed in hemocyte smears retained their viability. The most likely explanation for these findings is that in the absence of HlFER1 or HlFER2, iron, particularly Fe^{2+}, became more accessible to *E. coli*, which might have supported its multiplication within the cell. Ferrozine assay showed that *Hlfer1*- and *Hlfer2*- silenced ticks had higher ferrous iron concentrations than control ticks. In contrast to Fe^{3+}, Fe^{2+} is more soluble and can be easily taken up by bacteria [99]. Several Fe^{2+} uptake mechanisms have been reported in *E. coli* [101,102].

Ticks naturally harbour a wide range of bacterial species as part of their microflora [103]. Furthermore, ticks are known hosts of pathogenic intracellular bacteria belonging to order Rickettsiales [104], and a recent study showed that many bacterial species aside from the already known pathogens can be transmitted to the vertebrate host during blood feeding [105]. Except for the Lyme disease agent,

Borrelia burgdorferi [106], other bacteria requires iron for their multiplication. The hard tick *H. longicornis* is known to host *Rickettsia japonica* [5], and while no report demonstrates that this bacterial pathogens utilizes iron in the ticks in the course of infection, the results presented in this study suggest that HlFERs serve an iron-depriving role to limit the bacterial population. In nature, ticks acquire pathogens during blood feeding. One of the limitations of this study is the method of introducing bacteria to the ticks. However, it can be speculated that iron sequestration by HlFERs may also occur in the natural course of infection since HlFERs are abundant in the midgut, as described in Chapter 1.

In conclusion, this chapter presented the role of HlFERs in tick immunity, further demonstrating that these molecules are crucial to tick survival. It is still unknown whether HlFERs are involved in the acquisition and transmission of tick-borne pathogens, including viruses and protozoa, requiring further investigations.

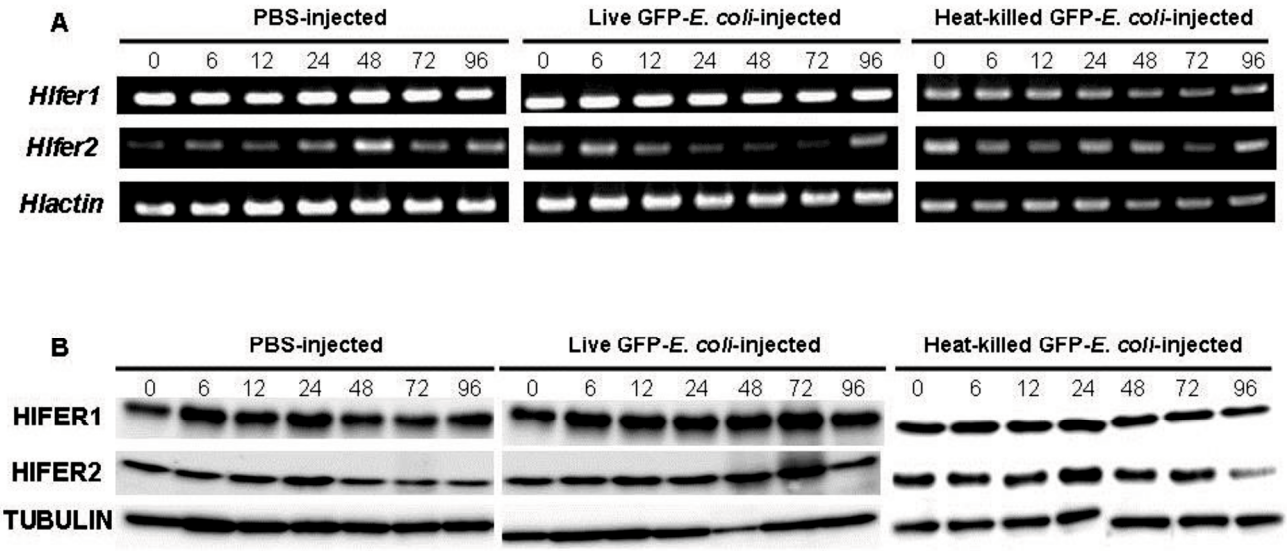


Fig. 3-1. Gene (A) and protein (B) expression of *H. longicornis* ferritins after injection of live or heat-killed EGFP-*E. coli*. Total RNA and protein was extracted from whole bodies of unfed ticks at different hours after injection of live or heat-killed EGFP-*E. coli*. PBS was also injected for control. (A) RT-PCR was performed using gene-specific primers, with actin as an internal control, to check gene expression. (B) Western blot analysis using specific mouse antisera, with tubulin as an internal control, was performed to check protein expression. Numbers indicate the hours after injection.

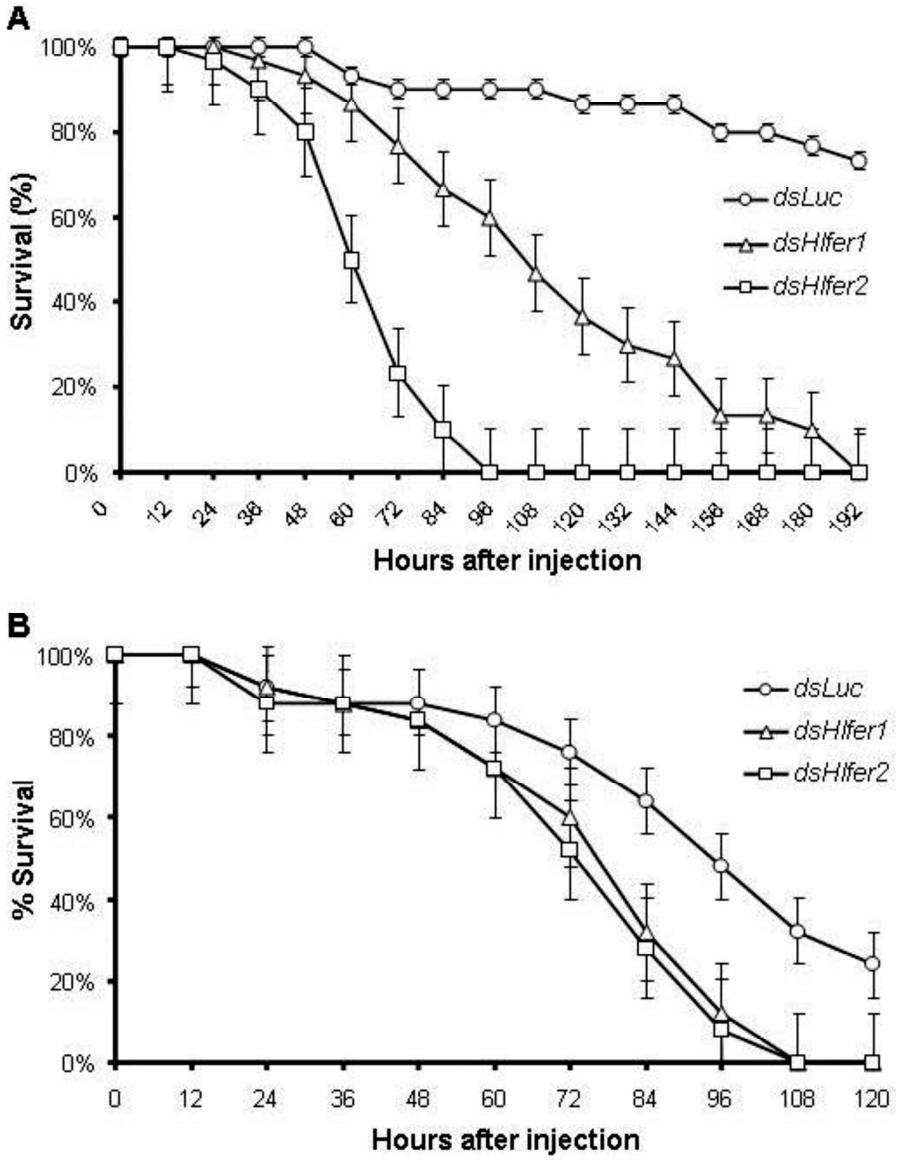


Fig. 3-2. Survival rate of *Hlfer*-silenced ticks after injection of live (A) or heat-killed EGFP-*E. coli* (B). Unfed adult female ticks were injected with *H. longicornis fer1* (*Hlfer1*), *H. longicornis fer2* (*Hlfer2*) or *Luciferase* (*Luc*) dsRNA for the control to induce RNAi. Silencing was confirmed through RT-PCR. After 4 days, a suspension of live or heat-killed EGFP-*E. coli* ($O.D._{450}$=20) was injected at 0.5 µl per tick, and mortality was monitored every 12 h. Both *Hlfer1*- and *Hlfer2*-silenced groups had a lower survival rate compared to *Luc* by the end of monitoring period, in which all the ticks are dead in these groups. n=30 ticks per group. The graph here represents the result of a single independent trial. Bars represent standard error. Significant difference was determined using the log-rank Mantel-Cox test (P <0.0001, *Luc* vs. *Hlfer1* or *Hlfer2*).

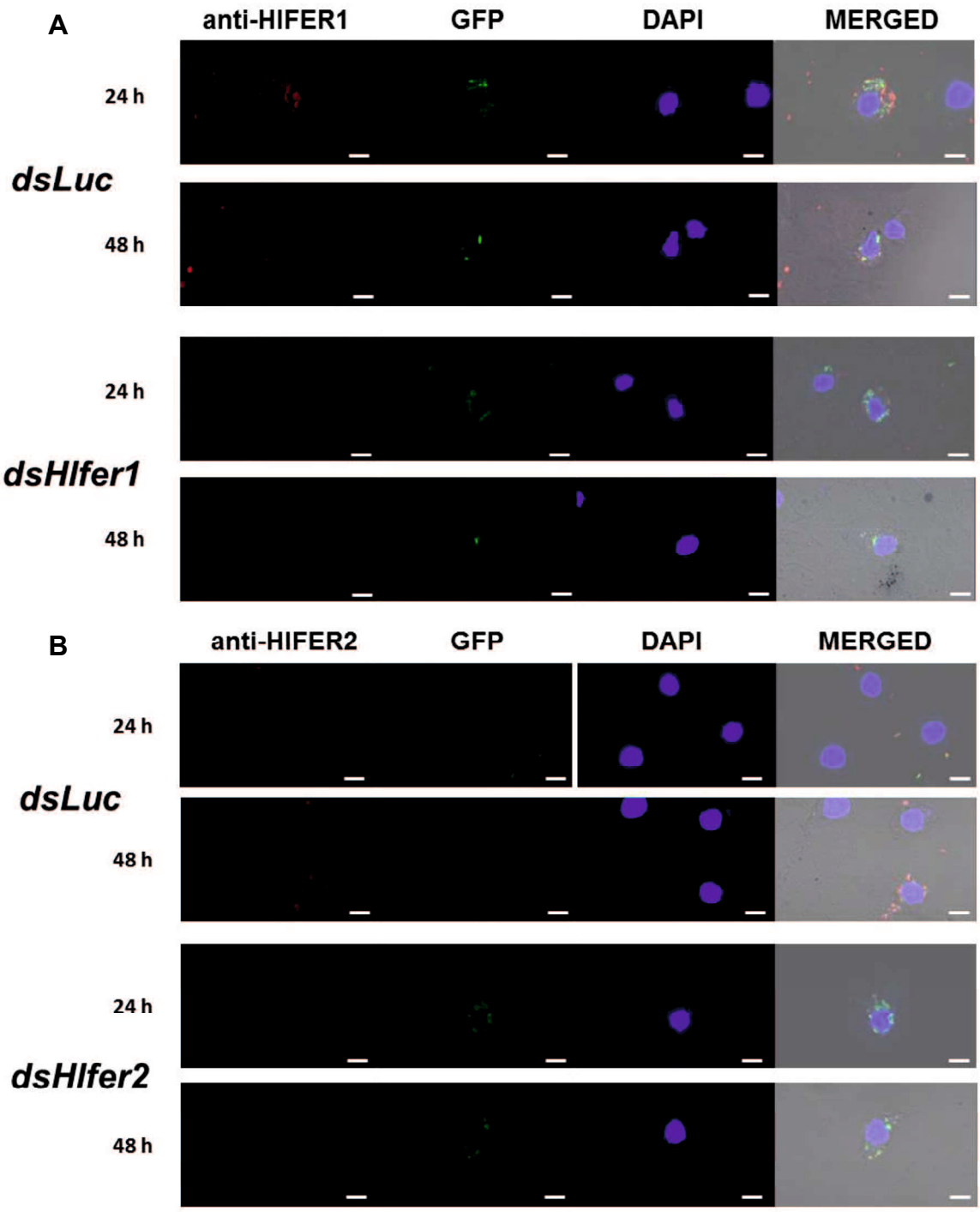


Fig. 3-3. Immunofluorescent examination of hemocytes after injection of live EGFP-*E. coli*. IFAT was performed to detect the expression of HlFER1 (A) and HlFER2 (B) in hemocytes of ticks injected with *Luc, Hlfer1*, or *Hlfer2* dsRNA at 24 h and 48 h after live EGFP-*E. coli* injection. Alexa-594 (red fluorescence) was used as a second antibody to locate HlFER1 and HlFER2. GFP panel shows EGFP-*E. coli*. Bars= 10 μm

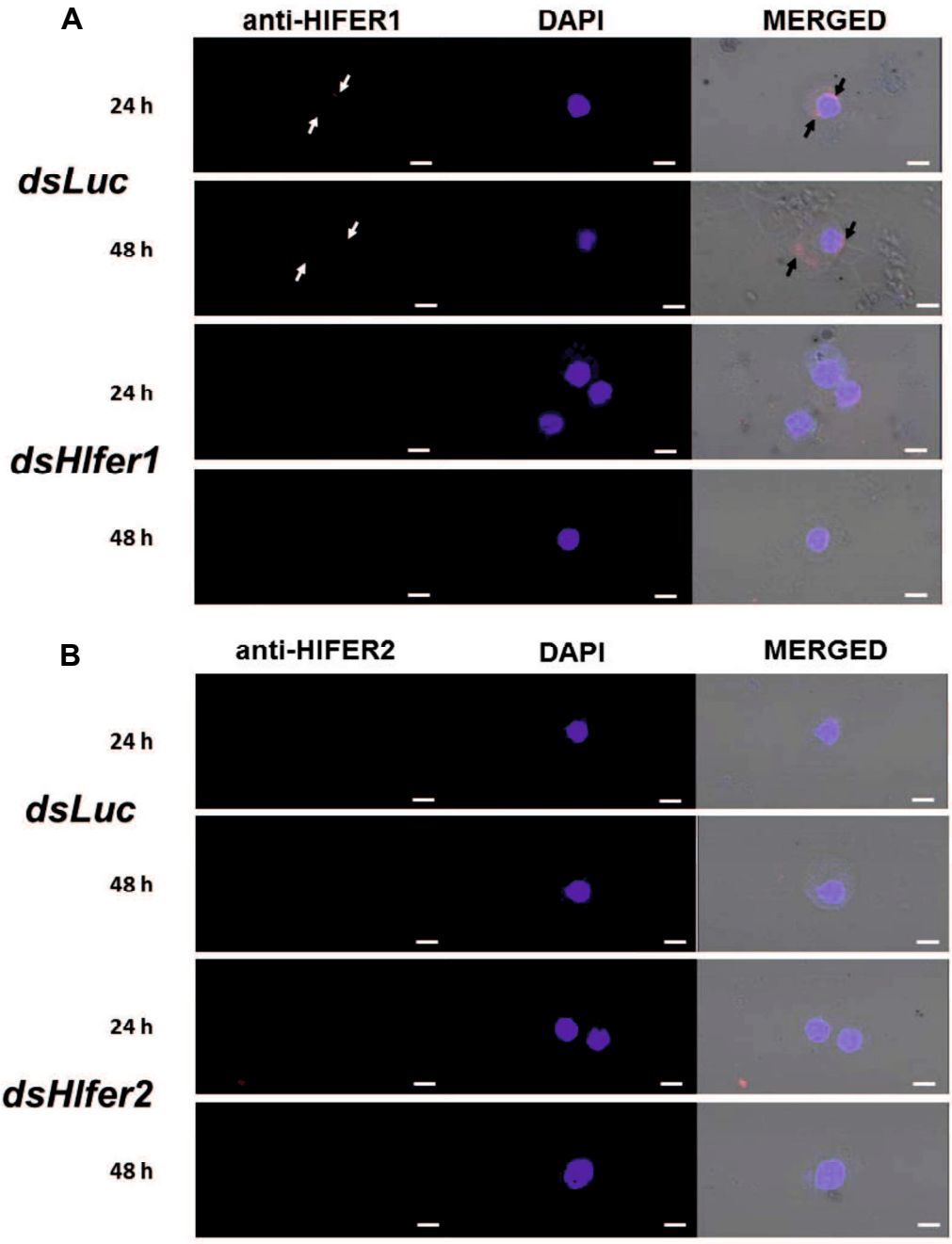
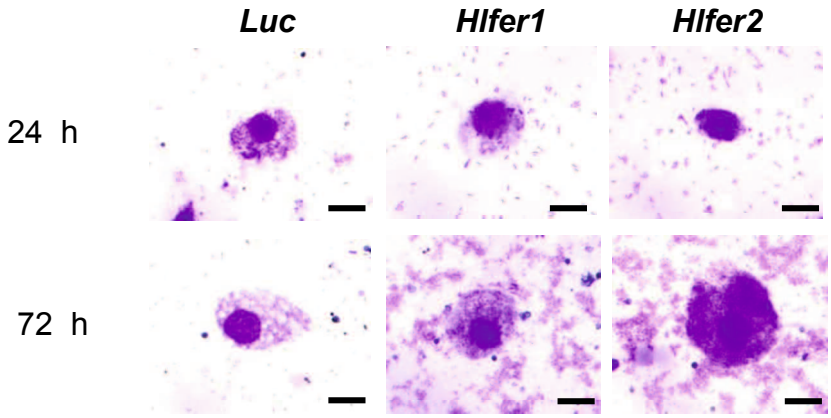
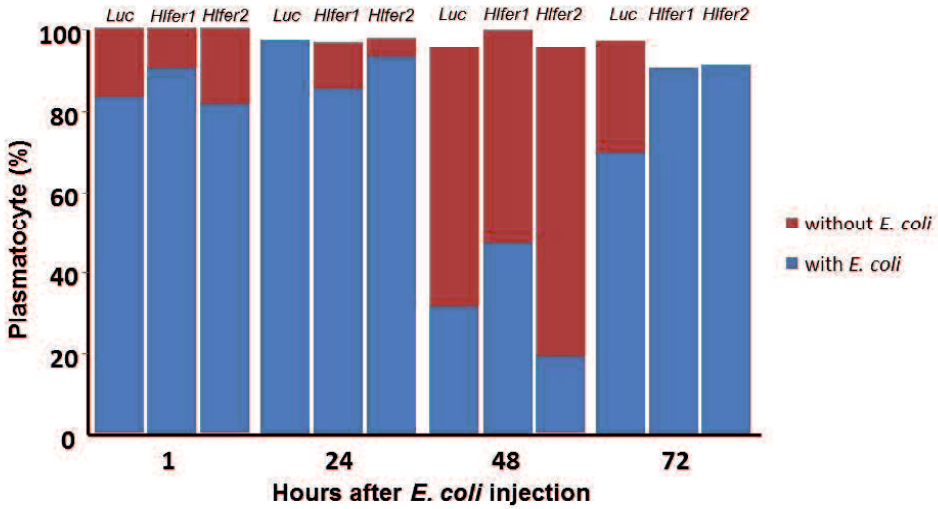


Fig. 3-4. Immunofluorescent examination of hemocytes after injection of killed EGFP-*E. coli*. IFAT was performed to detect the expression of HlFER1 (A) and HlFER2 (B) in hemocytes of ticks injected with *Luc, Hlfer1*, or *Hlfer2* dsRNA at 24 h and 48 h after heat-killed EGFP-*E. coli* injection. Alexa-594 (red fluorescence) was used as a second antibody to locate HlFER1 and HlFER2. Arrows point to positive fluorescence. Bars= 10 µm





	Plasmatocyte (%)			Granulocyte (%)		
dsRNA	24 h	48 h	72 h	24 h	48 h	72 h
Luc	97	95	96.5	3	5	3.5
Hlfer1	96.5	99.5	90	3.5	0.5	10
Hlfer2	98.5	95	94.5	1.5	5	5.5

Fig. 3-5. Examination of phagocytic activity of hemocytes from *Hlfer*-silenced ticks injected with live EGFP-*E. coli*. Hemocytes from unfed adult ticks injected with *Hlfer1*, *Hlfer2* or *Luc* dsRNA were collected at different hours after injection of live EGFP-*E. coli* and stained with 3% Giemsa staining solution. Representative images of hemocytes at 24 h and 72 h are shown in the top. Plasmatocytes but not granulocytes phagocytosed *E. coli*. The graph shows the percentage of plasmatocytes with *E. coli* in the cytoplasm. Table at the bottom shows the percentage of plasmatocytes and granulocyte at different time points. Bars= 10 μm

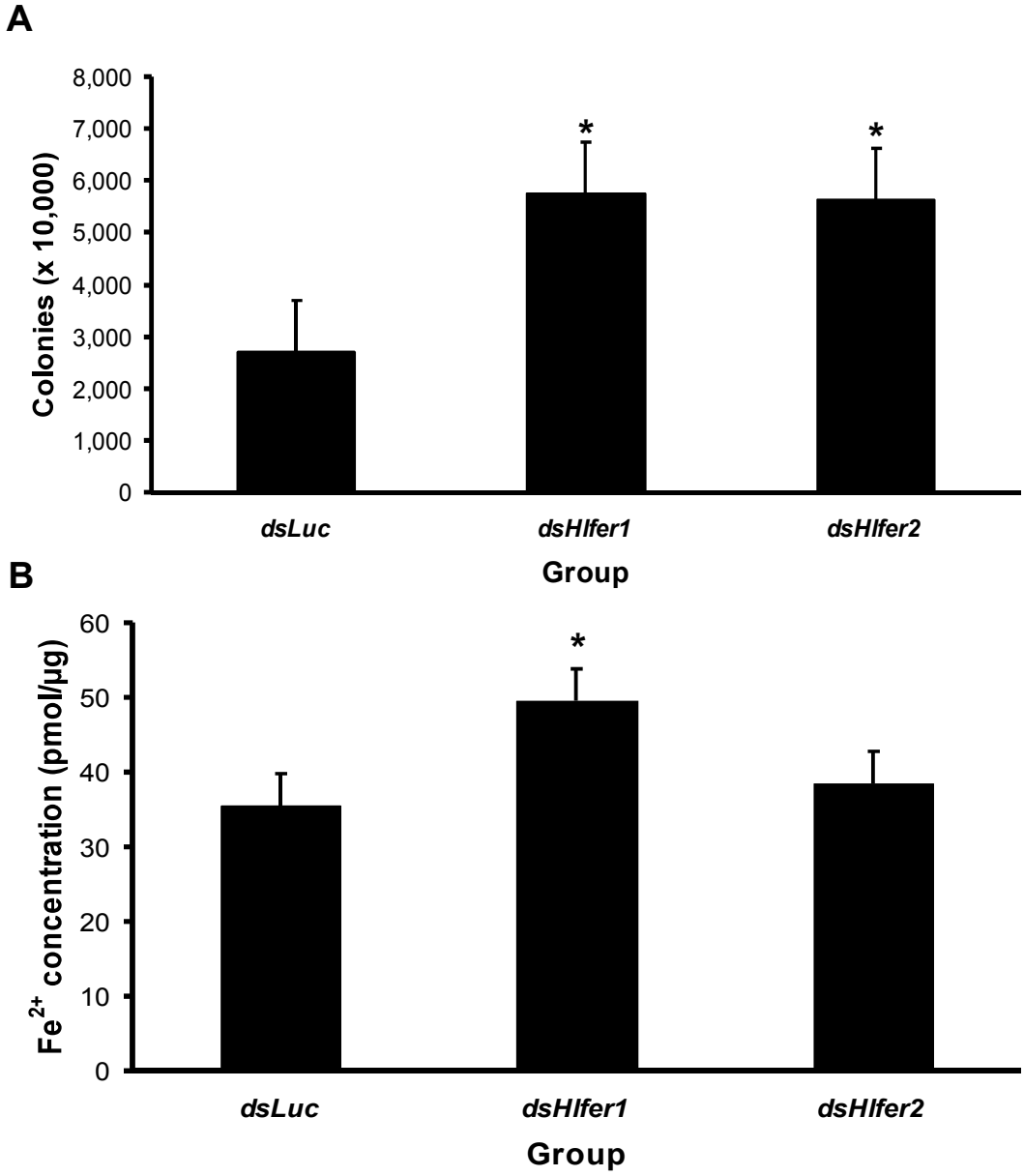


Fig. 3-6. Colony count of EGFP-*E. coli* from the hemolymph of *Hlfer*-silenced ticks (A) and ferrous iron (Fe2+) concentration in the whole bodies of unfed *Hlfer*-silenced ticks (B). (A) Hemolymph was collected from ticks at 48 h after injection of EGFP-*E. coli* and cultured in LB agar plate containing ampicillin and IPTG. Colonies that emerged after overnight incubation were counted. Colony count was expressed according to 10,000x dilution of the hemolymph before application in the LB agar plate. (B) Whole ticks were homogenized 4 d after injection of *Luc, Hlfer1* or *Hlfer2* dsRNA and ferrozine assay was performed. Iron concentration was calculated based on the protein concentration of tick homogenates. *Significantly different, $P< 0.05$

CHAPTER 4

Evaluation and comparison of the potential of two ferritins as anti-tick vaccines against *Haemaphysalis longicornis*

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4.1 INTRODUCTION

Effective tick control is essential in preventing tick infestation and, subsequently, the spread of tick-borne pathogens. Until now, the use of chemical acaricides was the primary measure of controlling ticks worldwide. However, concerns about limited efficacy, the emergence of resistant ticks, and contamination of the environment and animal products are among the disadvantages of acaricide application. Vaccination is a promising control alternative that will avoid the drawbacks of acaricide application [107]. Ideally, these vaccines should reduce tick infestation and pathogen transmission [108]. For about 20 years, the only commercially available anti-tick vaccine has utilized the midgut protein Bm86 from *R. microplus* as the antigen [109]; however, it is only effective against a limited number of tick species [107]. Although numerous antigens have been studied as candidates for a tick vaccine, no other tick vaccine has progressed to commercial development [9]. The main challenge in anti-tick vaccine development is the identification of a suitable tick protective antigen that can be effective against all developmental stages and a wide range of tick species. Many studies on ticks are now focused on the identification of antigens using combined approaches [58] aimed at targeting multiple tick species and multiple tick-borne pathogens at the same time [110,111,112].

In the previous chapters, the various functions of two ferritin molecules in the hard tick *H. longicornis* were studied. RNA interference experiments shown in the previous chapters demonstrated that HlFERs are important in tick survival and reproduction (Chapter 1), protecting the ticks against oxidative stress that might occur from the high iron content of their blood meal (Chapter 2), and also plays role in innate immunity against bacteria (Chapter 3). This chapter shows the potential of targeting HlFERs for tick control by immunizing the host with recombinant HlFERs

(rHlFER1 and rHlFER2). I also attempted to demonstrate how vaccination using these rHlFERs can affect ticks by examining whether induced antibodies can block the function of native HlFERs.

4.2 MATERIALS AND METHODS

4.2.1 *Expression and purification of recombinant HlFERs*

The ORF of *Hlfer1* (GenBank: AY277905) or *Hlfer2* (GenBank: AB734098) was extracted from their respective pGCAP1 vector using the following primer sets with *Bam*HI recognition sites: Hlfer1-F 5'-ACGGATCCAAAATGGCCGCTACT-3' and Hlfer1-R 5'-ACGGATCCTCCTCAGTCGTCTCC-3' for rHlFER1 or Hlfer2-F 5'- ACGGATCCACCATGCTCCCGATC-3' and Hlfer2-R 5'-ACGGATCCGGTTTATTTGTCGCT-3' for rHlFER2. After cutting with *Bam*HI, the amplified DNA fragments were purified using GENECLEAN II kit (MP Biomedicals LLC) and then subcloned into the *Bam*HI cutting site of pRSET A vector (Invitrogen). The constructs, pRSETA/HlFER1 and pRSETA/HlFER2, were expressed in *E. coli* BL21 (DE3) cells grown in Luria-Bertani broth medium with ampicillin. The expression of His-tagged rHlFERs was induced with 1 mM final concentration of isopropyl IPTG. After overnight induction, cells were collected by centrifugation at 3,350x*g* for 30 min, and the proteins were extracted through ultrasonication. Purification was done through affinity chromatography using a His-trap^{TM} FF column (GE Healthcare) at denatured condition with 6 M urea, followed by dialysis, first against PBS containing 0.5 M arginine for refolding overnight, and then against PBS alone overnight. The purity of the rHlFERs was confirmed by SDS-PAGE, and the concentration was determined through SDS-PAGE using bovine serum albumin as the

standard and Micro BCA Assay kit (Thermo Scientific). The rHlFERs were kept at -30°C until use.

4.2.2 *Rabbit immunization*

A total of three rabbits from each group were used for two separate vaccination trials. For each immunization, rHlFER1 or rHlFER2 was thoroughly mixed with an equal volume of incomplete Freund's adjuvant (Sigma-Aldrich) to a final concentration of 100 µg per 1.5 mL. The mixture was administered subcutaneously using a sterilized glass syringe and a 21G needle and repeated three times at two-week intervals. Control rabbits were immunized with adjuvant alone. Sera were collected before the first immunization and a week after each immunization for confirmation of antibody titer (days 0, 7, 21, and 35).

4.2.3 *Measurement of serum antibody levels through ELISA*

The antigen-specific serum antibody titer was determined by enzyme-linked immunosorbent assay (ELISA). ELISA plates (F96 Maxisorp, Nunc, Roskide, Denmark) were coated with either rHlFER1 or rHlFER2 dissolved in a carbonate buffer (pH 9.6) at 100 ng/100 µl per well concentration at 4°C overnight. Another recombinant protein prepared in our laboratory with His-tag, recombinant peroxiredoxin2 of *H. longicornis* (HlPrx2), was used as a control antigen (Kusakisako *et al.,* unpublished results). After washing with PBS-T, each well was blocked with 150 µl of 5% skimmed milk in PBS-T at 37°C for 1 h. The plates were incubated with 100 µl/well of rabbit sera in the blocking solution, diluted serially starting at 1:50, at 37°C for 1 h. ELISA plates were washed several times with PBS-T before applying 100 µl of HRP-conjugated polyclonal goat anti-rabbit immunoglobulins (Dako Cytomation, Glostrup, Denmark) in the blocking solution (1:2,000 dilution) in each well and then incubated at 37°C for 1 h. After another series of washing, 100 µl of

TMB One Component HRP Microwell substrate (SurModics, Inc., Eden Prairie, MN, USA) was placed in each well and then incubated at 37°C for 30 min. The reaction was stopped by adding 100 µl of a mixture of 0.6 N H_2SO_4 and 1 N HCl (1:1) in each well. Absorbance was measured using a microplate reader (Bio-Rad) at OD_{450}.

4.2.4 *Tick infestation and evaluation of vaccination efficacy*

Two weeks after the final immunization, 30 adult female ticks were infested on the ears of each rabbit as described in Chapter 2 until they fed to repletion. After dropping, ticks were weighed and then monitored for survival rate, egg laying and subsequent hatching to larvae as described in Chapter 1. The effect of immunization was evaluated based on the reduction of the tick's engorged body weight, oviposition, and hatching. Calculations were made using formulas adapted from previous tick vaccination reports [113].

- Reduction of tick engorged weight (R_W)= 100[1 – (BWV/BWC)], where BWV is the average engorged weight of ticks infested on rHlFER vaccinated rabbits and BWC is the average engorged weight of ticks infested on the control rabbits.
- Reduction of oviposition (R_O)= 100[1 – (EWV/EWC)], where EWV is the average weight of the eggs from ticks infested on rHlFER vaccinated rabbits and EWC is the average weight of the eggs from ticks infested on the control rabbits.
- Reduction on hatching (R_H)= 100[1 – (AHV/AHC)], where AHV is the percent of ticks with completely hatched eggs from the total number of ticks that laid eggs from rHlFER vaccinated rabbits and AHC is the percent of ticks with completely hatched eggs to the total number of ticks that laid eggs from the control rabbits.

Finally, the overall vaccine efficacy (E) for each group was calculated as $100[1 - (E_W \times E_O \times E_H)]$, where E_W = BWV/BWC, E_O = EWV/EWC, and E_H = AHV/AHC.

4.2.5 *Tick protein preparation and Western blot analyses*

Tick protein samples were prepared from unfed whole adults, partially fed midguts, eggs (20 days after laying), and newly hatched larvae by homogenizing them and suspending in PBS. After sonication, the tick homogenates were centrifuged and the supernatants were collected. The protein concentration of eggs and larvae was determined using Micro BCA kit (Thermo Scientific). To confirm the reactivity of rabbit sera to native tick HlFERs, whole adult and midgut protein samples were separated through SDS-PAGE and then transferred to a PVDF membrane (Millipore). After blocking with 5% skimmed milk in PBS-T overnight, the membrane was incubated with rabbit sera collected after the third immunization (1:100 dilution) as primary antibodies. Western blot analysis was also performed to further demonstrate the reactivity of rabbit antibodies to rHlFER1, rHlFER2, and rHlPrx2 as a control, with the primary antibodies diluted to 1:3,000. To detect the presence of antibodies in the eggs, rHlFERs were used as protein samples, and then egg homogenates were used as primary antibodies (20 μg/500 μL). To demonstrate oxidative stress in eggs and larvae, MDA and PC were detected using specific kits for these oxidative stress markers (OxiSelect, Cell Biolabs, Inc.) as described in Chapter 2. Tubulin was detected as an internal control using a mouse-derived serum [42]. After incubation with HRP-conjugated goat anti-rabbit or anti-mouse immunoglobulin as a secondary antibody (1:30,000 dilution; Dako Cytomation), protein signals were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and images were taken using the FluorChem FC2 Imaging System (Protein Simple).

4.2.6 *Statistical analysis*

The average values of two vaccination trials were calculated and statistical significance was determined using the Student's *t*-test, with significant difference defined by $P < 0.05$.

4.3. RESULTS

4.3.1 *Purification of recombinant HlFERs and rabbit immunization*

rHlFERs were expressed and purified from *E. coli* cells. SDS-PAGE showed that the purified rHlFER1 and rHlFER2 have an almost similar molecular mass of around 26 kDa (Fig. 4-1) as described in Chapter 1. An antigen-specific ELISA was conducted to monitor the antibody titer of individual rabbits, and then the average antibody titer for each group from the two trials was calculated (Fig. 4-2). Whereas no changes were observed in the antibody titers of the control group, the antibody titers of the groups immunized against rHlFER1 (Fig. 4-2A) and rHlFER2 (Fig. 4-2B) significantly increased after the second immunization. Furthermore, the antibodies also exhibited cross-reactivity to each antigen. rHlFER1-immunized rabbits showed an abrupt increase in antibody titer against rHlFER1 after the second immunization, which further increased after the third immunization, while the titer for rHlFER2 only significantly increased after the third immunization. For rHlFER2-immunized rabbits, the antibody titer against rHlFER2 abruptly increased after the second immunization but did not significantly increase further after the third immunization, while the level and the trend of the antibody titer against rHlFER1 were similar to those of the rHlFER1-immunized rabbits. Cross-reactivity of the antibodies was also observed on ELISA using rHlPrx2 as antigen but it was lower than that observed in HlFERs (Fig. 4-2C).

4.3.2 *Reactivity of rabbit antibodies to recombinant and native tick HlFERs*

To further evaluate the reactivity of rabbit antibodies, Western blot analysis was performed using rHlFERs, whole tick homogenates, and a midgut homogenate as protein samples. The results showed that sera from immunized rabbits reacted with rHlFERs and the respective native HlFERs from whole ticks and the midgut (Fig. 4-3). Non-specific bands were seen, particularly on partially-fed midguts, which may be due to the reactivity of secondary antibody to rabbit blood proteins, since the ticks were fed to rabbits. In addition, cross-reactivity was further demonstrated here, as the sera from the rHlFER1-immunized rabbit showed a positive band against rHlFER2, while the sera from the rHlFER2-immunized rabbit showed a positive band against rHlFER1. Both immune sera also reacted with rHlPrx2 but to a lesser extent. No positive bands were detected in any of the tested protein samples using sera from control rabbits.

4.3.3. *Tick infestation challenge*

The rabbits were infested with adult *H. longicornis* after two weeks from the third immunization. The total number of attached and engorged ticks from rHlFER1-immunized, rHlFER2-immunized, and control rabbits was 78, 80 and 78, respectively, which was not significantly different. After dropping, the ticks were collected and evaluated for blood feeding and reproduction parameters (Table 4-1). Ticks infested on rHlFER1- and rHlFER2-immunized rabbits had a lower engorged weight compared to that of the control. However, a significant difference ($P < 0.0001$) was only observed in ticks infested on rHlFER2-immunized rabbits, with a 16% mean reduction in engorged weight (R_W). Moreover, the engorged weight of ticks from rHlFER2-immunized rabbits was also significantly lower ($P= 0.0377$) compared to that of ticks from rHlFER1-immunized rabbits. No mortalities were observed in any

of the groups until the completion of egg laying. Eggs with abnormal features, such as irregular shape, wrinkled surface and darker colour, were observed from ticks infested on rHlFER2-immunized rabbits (Fig. 4-4A) but not from the ticks infested on control and rHlFER1-immunized rabbits. Upon completing oviposition, the eggs were weighed and the average egg weight for each group was calculated. Ticks from both rHlFER1- and rHlFER2-immunized rabbits had a significantly lower egg weight ($P < 0.05$). The ticks from rHlFER2-immunized rabbits had the least egg weight among the three groups, significantly lower (22.4 % reduction, $P < 0.0001$) compared to the control group, but not compared to the rHlFER1 group. The effect of vaccination on hatching was evaluated by the number of ticks with completely hatched eggs. Both rHlFER groups had a reduced number of ticks with completely hatched eggs (~20% reduction) compared to the control group. Furthermore, larval mortality was observed from some ticks in these groups, but not from control. Based on these parameters, the calculated vaccine efficacy (E) for rHlFER1 is 34% and for rHlFER2 is 49%.

4.3.4. *Effects of antibodies on eggs and larvae*

Among the functions of ferritins in ticks is the prevention of iron-mediated oxidative stress, as demonstrated in Chapter 2. Compared with the previous results of *Hlfer* gene silencing, less pronounced effects on the blood feeding, survival, and egg production of adults were observed. Therefore, I analyzed the eggs and larvae to elucidate the mechanism by which antibodies against HlFERs can affect the ticks. I first detected whether the host antibodies against rHlFERs are present in the eggs (Fig. 4-5). Western blot analysis showed positive bands for rHlFER1 when egg homogenates from ticks infested on rHlFER1- and rHlFER2-immunized rabbits were used as primary antibodies. Meanwhile, a positive band for rHlFER2 was detected when the egg homogenate from ticks infested on rHlFER2-immunized rabbits was

used as the primary antibody. No bands were detected when egg homogenates from ticks infested on control rabbit, and secondary antibody alone was used. I then evaluated whether the antibodies can block the function of native HlFER2. Western blot analysis showed that only HlFER2 is present in the eggs and larvae from all groups as previously described in Chapter 1. Next, I detected two oxidative stress biomarkers, MDA from lipid peroxidation and PC from protein oxidation using specific antibodies. MDA was detected in the eggs from ticks infested on rHlFER1- and rHlFER2-immunized rabbits and in larvae from ticks infested on rHlFER2-immunized rabbits (Fig. 4-4B). Meanwhile, the amount of protein with the carbonyl group was higher in eggs and larvae from ticks infested on rHlFER1- and rHlFER2-immunized rabbits than from the control group (Fig. 4-4B). These results suggest the occurrence of oxidative stress in these samples.

4.4 DISCUSSION

The use of vaccines for tick and tick-borne pathogen control has many advantages over the application of chemical acaricides in terms of resistance development and environmental and animal product contamination concerns. Thus, numerous tick studies are now focused on developing anti-tick vaccines, but most have limited effectiveness in a wide range of tick-species. An anti-tick vaccine affecting multiple tick species and targeting both the ticks and pathogens is still far from reality [110]. Multiple approaches are currently being employed to screen tick antigens and evaluate their potential [58]. Chapters 1 and 2 showed that both HlFERs are physiologically important in blood feeding and reproduction by preventing iron overload and the occurrence of oxidative stress. Furthermore, a previous study showed that FER2 vaccination had considerable effects on infestation of *I. ricinus* and

R. microplus [113]. These prompted us to evaluate and compare the potential of two HlFERs as targets for the control of *H. longicornis*.

As described in Chapter 1, HlFERs are abundant in different tissues of the tick, with HlFER1 being intracellular in nature, while HlFER2 is secretory. Being concealed antigens, these proteins are not normally encountered by the host immune system during blood feeding, and thus a high level of host antibodies against HlFERs is probably necessary for the blockade of the HlFER function [114]. HlFERs also have complex structures that might require more antibodies binding on them before their function is blocked. The increasing antibody titer after immunization with rHlFERs showed that these proteins are immunogenic. Furthermore, the antibody titer in the immunized rabbits after infestation was as high as the titer after the third immunization, implying that the ticks must have ingested a high amount of anti-rHlFER antibodies during blood feeding. The effects of the antibodies on adult ticks were seen in the reduced engorged bodyweight and oviposition, although these were lower compared to the results of *Hlfer* gene silencing in Chapter 1, demonstrating differences in the mechanisms by which RNAi and host antibodies exert their blockade effects against a certain molecule. In contrast to the vaccination result against *I. ricinus* and *R. microplus* using FER2 [113], no effect on attachment and number of engorging ticks was observed in either group. Nevertheless, the ability of host antibodies to react with native tick HlFERs in the whole tick and midgut shown in Western blot analysis suggests that the antibodies can bind with native HlFERs within the ticks and possibly interfere with their crucial function on blood feeding and egg production. The cross-reactivity of the antibodies to recombinant proteins seen in ELISA and Western blot analyses may be partly attributed to the His-tag, since the antibodies also reacted to HlPrx2. However, since the amino acid sequences of

HlFER1 and HlFER2 have 40% identity, there is still a possibility that the antibodies against tick HlFER1 may cross-react with tick HlFER2, and vice versa.

I wanted to elucidate the mechanism by which antibodies against HlFERs can affect the ticks. It has been demonstrated previously that host antibodies can pass through the midgut barrier of ticks and circulate in the hemolymph [115,116,117]. Detection of positive bands for recombinant HlFERs on Western blot analysis using egg homogenates as primary antibodies may indicate the presence of antibodies against rHlFERs in the eggs of ticks infested on rHlFER-vaccinated rabbits. However, further examination is needed to confirm this result. I also observed eggs with abnormal morphology from ticks infested on recombinant HlFER2 rabbits, similar to the reported observation after *Hlfer2* gene silencing in Chapter 1. Only HlFER2 that comes from the adult ticks is present in the eggs, which may serve to supply iron and/or protection against iron overload during embryonic development. The tick embryo and larvae are normally challenged with oxidative stress as embryogenesis and aging progresses [118]. Therefore, the function of antioxidants, including HlFER2, which keeps iron from promoting the formation of reactive oxygen species, is crucial. While it is unclear whether these anti-HlFER antibodies in the eggs are in the free form or are already bound to HlFER2, its presence and the higher level of oxidative biomarkers in the eggs of the ticks from rHlFER-vaccinated rabbits, indicating the occurrence of oxidative stress, are highly suggestive of interference in native HlFER function, eventually leading to embryonic death and, hence, reduced hatching. Furthermore, the persistence of these antibodies in the larvae after hatching most likely caused oxidative stress and larval mortality. Thus, there is also a possibility that these antibodies may interfere with HlFERs when the larvae feed on a host.

The results of the tick challenge after vaccination showed that rHlFER2 has higher vaccination efficacy than rHlFER1. Although the findings in Chapter 1 and 2 suggested that HlFER1 is the major iron-storage HlFER, abundant in most tick tissues, and that silencing the *Hlfer1* gene seemed to have a greater impact on blood feeding, survival and reproduction, the importance of HlFER2 as an iron transporter in hard ticks should not be overlooked. Moreover, the mainly intracellular localization of HlFER1 might have made it inaccessible to anti-HlFER1 antibodies, with the exception of the midgut, where the antibody may interact with HlFER1 within digestive cells. On the other hand, HlFER2, being a secretory protein, may be more accessible to the antibodies after passing through the midgut barrier. HlFER2 is abundant in the hemolymph, circulating within the tick's body, and as mentioned earlier, can be passed to the eggs. This systemic function of HlFER2, as well as its exclusive presence in the eggs, may have contributed to its higher vaccine efficacy. Furthermore, the antibodies against HlFER2 showed a higher cross-reactivity compared to antibodies against HlFER1.

Ferritin is a highly conserved molecule among different tick species that is ubiquitous in most tick tissues and in all developmental stages. HlFERs have high homology with other tick ferritins. This makes tick ferritins a highly preferable candidate target antigen for the formulation of a multi-species anti-tick vaccine [110]. In contrast to HlFER1, HlFER2 has a lower identity/similarity, less than 40%, to vertebrate ferritins. The secretory FER2 is also considered unique to ticks [33]. In the light of the results in this study, HlFER2 is a better antigen than HlFER1, supporting the previous vaccination study in other tick species [113].

Table 4-1. Effect of vaccination using recombinant HlFERs on tick feeding and reproduction parameters

Experimental group	Engorged weight (mg)	R_W[a] (%)	Egg weight (mg)	R_O[a] (%)	Ticks with hatched eggs (%)	R_H[a] (%)	Ticks with dead larvae (%)	E[b] (%)
Adjuvant (control)	261.2 ± 51.9	0	170.0 ± 40.4	0	100.0	0	0	-
rHlFER1	243.4 ± 77.3	6.8	142.8 ± 62.5*	12.4	82.0*	18.0	5.5	34.0
rHlFER2	218.0 ± 66.0*	16.5	126.5 ± 55.8*	22.4	80.0*	20.0	5.7	49.0

Data represent average values from ticks infested on three rabbits for each group, from two separate vaccination trials.
[a]Formulas for the calculation of reductions in engorged weight (R_W), oviposition (R_O), and hatch (R_H) are described in the Methods section.
[b]Vaccine efficacy (E) was calculated by comparing tick engorged weight, tick egg weight, and the number of ticks with completely hatched eggs from the recombinant HlFER1 (rHlFER1) or recombinant HlFER2 (rHlFER2) group with those from the control group. The formula is described in the Methods section.
*Significantly different vs. control ($P < 0.05$, Student's t-test).

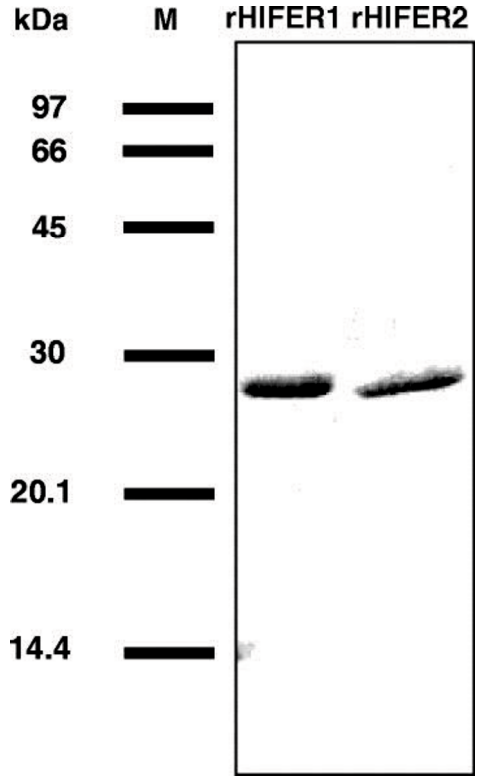

Fig. 4-1. Purification of recombinant HlFERs (rHlFERs). His-tagged rHlFERs (rHlFER1 and rHlFER2) were expressed in *E. coli* and then purified through Ni-affinity chromatography and dialysis against PBS. After refolding, 2 μg per protein was subjected to SDS-PAGE, and then the gel was stained with Coomassie brilliant blue. M, low molecular weight marker

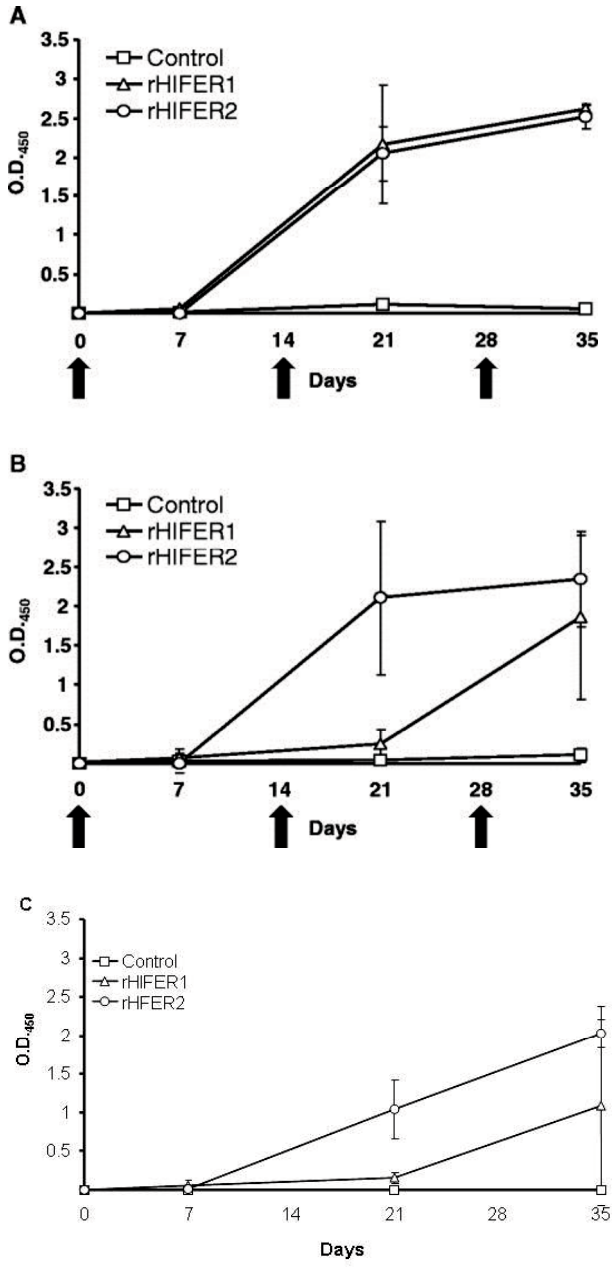

Fig. 4-2. Antigen-specific antibody titer against recombinant HlFER1 (rHlFER1) (A) and recombinant HlFER2 (rHlFER2) (B). Rabbit sera were collected for serum ELISA at days 0, 7, 21, and 35. Antibody titers reflect the mean absorbance at OD_{450} of sera (10^4 dilution) for each vaccinated group from two separate trials (n=3). The times of immunizations are indicated by arrows. Recombinant *H. longicornis* peroxiredoxin (HlPrx2), another his-tagged protein, was used as a control antigen (C).Control, rabbits injected with adjuvant only; rHlFER1, rabbits injected with rHlFER1; rHlFER2, rabbits injected with rHlFER2. Bars represent standard deviation.

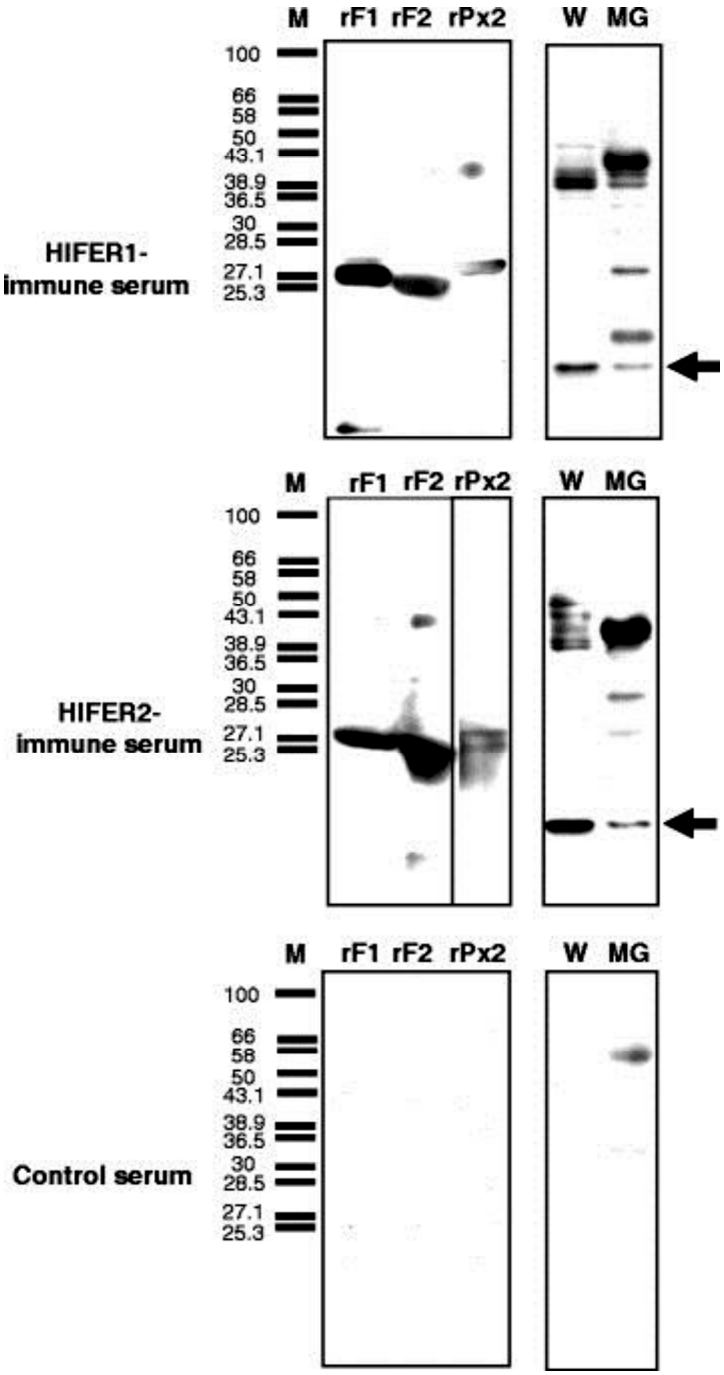

Fig. 4-3. HlFER detection using immunized rabbit sera. Western blot analysis was performed to examine the reactivity of antibodies from vaccinated rabbits against recombinant HlFER1 (rF1), recombinant HlFER2 (rF2), recombinant HlPrx2 (rPx2) and native HlFERs of unfed whole ticks (W) and partially-fed midguts (MG). M, low molecular weight marker. Arrows point to positive bands for native tick HlFER1 or HlFER2 with around 20 kDa molecular weight.

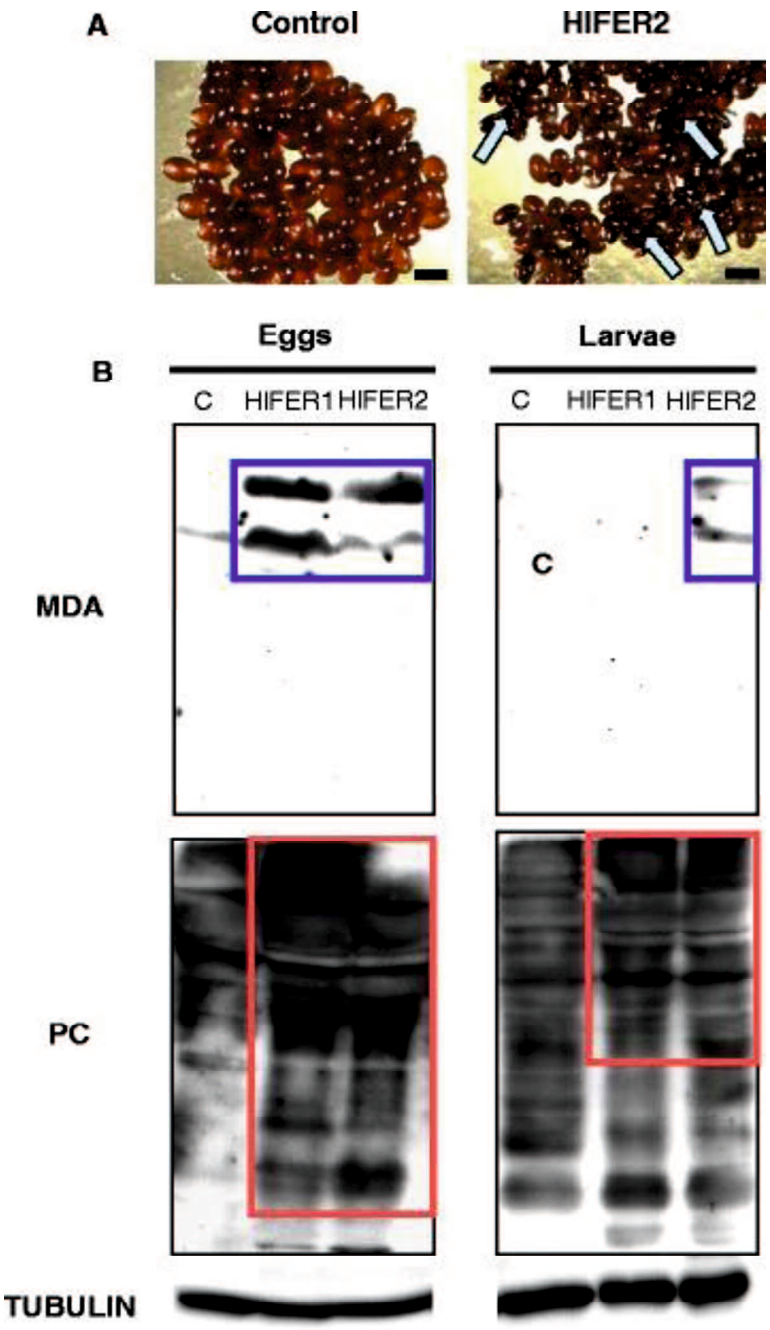

Fig. 4-4. Effect of recombinant HlFER vaccination on the eggs and larvae. (A) The morphology of eggs laid by ticks infested on immunized rabbits was compared. Eggs with abnormal morphology, indicated by arrows, were observed from ticks infested on the rHlFER2-vaccinated group but not from the ticks from the control group. Bars= 200 μm. (B) Detection of oxidative stress in the eggs and larvae. Malondialdehyde (MDA)and protein carbonyl (PC) were detected in the eggs and larvae of ticks from vaccinated rabbits using specific immunoblot detection kits. Tubulin was detected as internal control. Proteins with increased MDA and PC are enclosed in blue and red boxes, respectively. C, eggs/larvae from a tick infested on a control rabbit; HlFER1, eggs/larvae from a tick infested on a rHlFER1-vaccinated rabbit; HlFER2, eggs/larvae from a tick infested on a rHlFER2-vaccinated rabbit.

SUMMARY AND CONCLUSION

The importance of ticks as ectoparasites of humans and animals and vectors of several diseases is widely known. Vaccination is highly anticipated to overcome the drawbacks of chemical acaricide control. Thus, numerous tick studies are aiming to identify a single or multiple target antigens that can affect multiple tick species and, ideally, also target tick-borne pathogens [58,110,111]. Most studies on tick vaccination focus on controlling *Rhipicephalus* and *Ixodes* species. Iron metabolism is an interesting aspect of tick physiology and could be a good target for tick control, since this allows them to survive on obligatory blood-feeding habit [119]. This dissertation presents my studies on the functions of two kinds of the iron-binding protein ferritin in the hard tick *H. longicornis*, an intracellular HlFER1 and a secretory HlFER2 and their potential as targets for the control of this hard tick.

RNAi is a good technique for screening candidate antigens for vaccine development [58]. In Chapter 1, silencing *Hlfer*s by injection of gene-specific dsRNA, which resulted to reductions in feeding capacity and fecundity, showed that HlFERs are crucial to the blood feeding and reproduction of *H. longicornis*. Morphological analyses of the midgut during blood feeding revealed abnormalities in digestive cells and diminished digestive activity. Vitellogenesis was also indirectly affected due to suppressed expression of *HlVg* genes.

In Chapter 2, I demonstrated that HlFERs act as antioxidant molecules, providing protection against iron-mediated oxidative stress. Silencing *Hlfer*s predisposed the ticks to oxidative stress during blood feeding or injection of iron. High levels of oxidative stress biomarkers MDA and PC, produced during lipid

peroxidation and protein oxidation, respectively, were detected in whole bodies of *Hlfer*-silenced ticks after a blood meal or iron injection. High levels of MDA and PC was also detected in the midguts of blood-fed *Hlfer*-silenced ticks, confirming that the morphological abnormalities described in Chapter 1 resulted from oxidative stress.

In Chapter 3, I demonstrated that HlFERs are important in tick innate immunity through its iron-sequestration function. *Hlfer*-silenced ticks had lower survival rate after injection of live *E. coli*. The phagocytic and antimicrobial action of plasmatocytes in *Hlfer*-silenced ticks was apparently overwhelmed by the increasing number of *E. coli*. The higher level of Fe^{2+} that was available in *Hlfer*-silenced ticks most likely favored the multiplication of *E. coli*.

RNAi results showed the critical importance of HlFERs on ticks, making them good candidate vaccine antigens. The vaccination experiments in Chapter 4 showed that both rHlFER1 and rHlFER2 are highly immunogenic, inducing host antibody production. The tick infestation challenge showed that immunizing the host with rHlFER2 significantly reduced the engorged weight of the infested ticks. Immunization with either of the rHlFERs reduced the number of eggs and the number of ticks with completely hatched eggs, with rHlFER2 producing a greater reduction. Based on these tick parameters, rHlFER2 showed a higher vaccine efficacy of almost 50%. Moreover, eggs with abnormal morphology were observed from ticks infested on rHlFER2-immunized rabbits. I also attempted to elucidate the mechanism of anti-HlFER antibody protection against ticks. Induced antibodies against rHlFERs were detected in the eggs. The presence of a higher level of molecules produced during lipid and protein oxidation in the eggs and larvae from ticks infested on rHlFER-vaccinated rabbits indicates the occurrence of oxidative stress, suggesting that the antibodies interfered with the HlFER2 function. Collectively, results show that the

secretory HlFER2 is a good target for the control of *H. longicornis*, supporting the findings of a previous study targeting FER2 in other hard tick species[113]. While its vaccine efficacy may still be lower compared with those of other studied vaccine antigens, inclusion of HlFER2 in the vaccine with other antigens may yield better results than immunizing with a single kind of antigen and may provide multi-species protection since FER is a highly conserved molecule.

Taken altogether, the results in this dissertation show that both types of HlFERs prevent oxidative stress in *H. longicornis* during blood feeding, which is essential for reproduction. Through their iron storage function, the intracellular HlFER1 also serves as an "in-house" antioxidant of cells, whereas the secretory HlFER2, which can be transported within the tick, may also serve as a systemic antioxidant molecule. The results of vaccination trial suggest that HlFER2 can be a good candidate molecule as an anti-tick vaccine antigen that may possibly affect multiple tick species.

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