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Cloning and molecular characterization of two ferritins from red abalone *Haliotis rufescens* and their expressions in response to bacterial challenge at juvenile and adult life stages

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Abstract

Ferritins are ubiquitous proteins with a pivotal role in iron storage and homeostasis, and in host defense responses during infection by pathogens in several organisms, including mollusks. In this study, we characterized two ferritin homologues in the red abalone *Haliotis rufescens*, a species of economic importance for Chile, USA and Mexico. Two ferritin subunits (*Hrfer1* and *Hrfer2*) were cloned. *Hrfer1* cDNA is an 807 bp clone containing a 516 bp open reading frame (ORF) that corresponds to a novel ferritin subunit in *H. rufescens*. *Hrfer2* cDNA is an 868 bp clone containing a 516 bp ORF that corresponds to a previous reported ferritin subunit, but in this study 5'- and 3'-UTR sequences were additionally found. We detected a putative Iron Responsive Element (IRE) in the 5'-UTR sequence, suggesting a posttranscriptional regulation of Hrfer2 translation by iron. The deduced protein sequences of both cDNAs possessed the motifs and domains required in functional ferritin subunits. Expression patterns of both ferritins in different tissues, during different developmental stages, and in response to bacterial (*Vibrio splendidus*) exposure were examined. Both *Hrfer1* and *Hrfer2* are most expressed in digestive gland and gonad. *Hrfer1* mRNA levels increased about 34-fold along with larval developmental process, attaining the highest level in the creeping post-larvae. Exogenous feeding is initiated at the creeping larva stage; thus, the increase of *Hrfer1* may suggest and immunity-related role upon exposure to bacteria. Highest *Hrfer2* expression levels were detected at trochophore stage; which may be related with early shell formation. Upon challenge with, the bacteria an early mild induction of *Hrfer2* (2 h post-challenge), followed by a stronger induction of *Hrfer1* at 15 h post-challenge, was observed in haemocytes from adult abalones. While maximal upregulation of both genes in the whole individual occurred at 24 h post-challenge, in juveniles. A significant increase in ferritin protein levels from 6 h to 24 h post-challenge was also detected. Our results suggest an involvement of *Hrfer1* and *Hrfer2*, and of ferritin proteins in the immune response of *H. rufescens* to bacterial infection.

Key words: Ferritin; *Haliotis rufescens*; abalone immunity; *Hrfer1*; *Hrfer2*

1. Introduction

Ferritins are ubiquitous proteins that play a crucial role in iron storage and homeostasis, regulating intracellular iron concentration and avoiding iron-induced oxidative damage [1,2]. Typical vertebrate ferritins are multimeric proteins composed of 24 subunits forming a nanocage structure with a capacity for storing up to 4500 atoms of iron in its central cavity [2]. Each subunit is a protein chain folded in 4-helical bundles, a fifth short helix and a long-extended loop [3]. In mammals, there are primarily two cytosolic ferritin subtypes, the heavy (H) and light (L) chain types. H- and L-subunits can assemble in different ratios to form the nanocage. The H chain has iron binding sites with ferroxidase activity, oxidizing and actively sequestering iron. The L chain lacks ferroxidase activity, but contains the ferrihydrite nucleation center, that is, several negative charged residues in the inner surface that facilitate the transfer of oxidized iron from the ferroxidase center to the iron core of the nanocage, iron hydrolysis, mineralization and storage [4]. An additional ferritin middle (M) chain was characterized in amphibians and bony fishes; M chain exhibits both, ferroxidase activity and the ferrihydrite nucleation site [5,6]. Regarding their subcellular location in animals, ferritins can be cytosolic, mitochondrial or secreted [2]. A post-transcriptional regulation mechanism of ferritin expression in animals is mediated by the Iron Regulatory Element (IRE), a regulatory sequence located at the 5´ untranslated region (5´-UTR) of ferritin mRNA. IRE forms a hairpin structure for binding to regulatory proteins, which regulate translation of ferritin mRNAs depending on intracellular iron concentrations [7,8].

Up to date, ferritin genes and functions were poorly studied in marine invertebrates compared to vertebrates. In mollusks, H-type cytosolic and secretory ferritins have been identified [9-14]. It was postulated that mollusk ferritins were involved in different functional roles besides iron storage and release, as growth [15,16], shell development [9,13,17], heavy metal detoxification [11], tolerance to thermal stress [18] and innate immunity [10-12,14,16].

Abalone species of the genus *Haliotis* have a wide geographical distribution, and several species are very important in fisheries and aquaculture industries because of their high commercial value as food and jewelry, being cultured worldwide [19,20]. Global abalone production from legal fisheries has decreased over the last decades, due

to over-exploitation, disease and habitat degradation, promoting an important increase in farm production in several countries [21]. The culture of red abalone (*Haliotis rufescens*) was introduced in Chile in 1977, being currently this Country one of the greatest producers of cultured abalone in the world [22,23]. However, natural and cultured *Haliotis* spp. and other mollusks can suffer massive mortalities at larval, post-larval and juvenile stages due to vibriosis, a deadly hemorrhagic septicemic disease caused by pathogenic bacteria *Vibrio* spp. [24-27]. Thus, a better characterization of the mechanisms of defense of red abalone to *Vibrio* spp. infections is necessary for the development of strategies to control diseases and the development of its sustainable farming.

The aim of the present study was to characterize *H. rufescens* ferritin genes and their expression patterns related with early development and immune response. Here we report the identification and characterization of two ferritin homologues cloned from *H. rufescens*, designated as *Hrfer1* and *Hrfer2*. Ferritin cDNA sequences were analyzed and gene transcriptional patterns were studied in different tissues, along larval development and in juvenile and adult abalones challenged with *Vibrio splendidus*. In order to analyze ferritin expression patterns during the bacterial challenge we further produced and validated an anti-ferritin polyclonal antibody for *H. rufescens*.

2. Materials and methods

2.1. Animals, bacterial challenge and sample collection

Red abalone (*Haliotis rufescens*) individuals were grown at the abalone production center (AWABI), Universidad Católica del Norte (Coquimbo, Chile). They were maintained in a 1000 L raceway with flow-through seawater (mean temperature of 16ºC) and fed *ad libitum* with *Macrocystis pyrifera* twice a week. Healthy adult individuals (2 years old, shell length-ranged between 5 and 6 cm) were collected and samples of several tissues (mantle, adductor muscle, gonads, gills and digestive tract) were extracted and fixed in RNA*later* Stabilization Reagent (Ambion Inc., Austin, Texas, USA) and stored at -80ºC.

Larvae at different stages of development were also collected. Adult red abalone individuals were induced to spawn [28] and gametes were mixed together at

concentrations sufficient to allow for fertilization. Samples of gastrulae, trochophores, veligers and creeping larvae were respectively collected at 6 h, 24 h, 3 days and 6 days after fertilization; fixed in RNA*later* and stored at -80ºC for subsequent RNA purification.

Challenge experiments were performed with the marine bacterial strain *Vibrio splendidus*. *V. splendidus* was heat-attenuated in order to expose the abalones to a pathogen associated molecular pattern (PAMP) immune stimulus and eliminate the virulent component of the bacteria that could inhibit the immune response activation. Bacteria were inactivated by heating and pellet was obtained as described in Coba de la Peña et al. [16]. Pellet was then resuspended in sterile sea water at a final concentration of $1x10^7$ cells/mL. Forty-four adult or juvenile (12 mo. old, shell length of 1.0-1.5 cm) abalones were randomly divided into three groups. One group consisted on 20 individuals that were challenged by injection of 100 µL of the *V. splendidus* suspension in sterile seawater ($1x10^7$ cells/mL) in the adductor muscle using a 25 G syringe. The second group consisted on 20 individuals that were injected with 100 µL of sterile seawater and were used as controls. One mL of hemolymph from 4 individuals in each group was collected after 2, 6, 15 and 24 h of injection at the adult stage. An additional control group (basal status) of four individuals not subjected to injection was assessed, and from which haemolymph was collected at time 0 h. Collected haemolymph samples were immediately centrifuged at 600 *g* at 4ºC for 10 min to harvest the haemocytes. Haemocytes were then immediately fixed in RNA*later* and stored at -80ºC. In juvenile abalones was not possible to collect enough haemolymph, thus whole individuals from each group were sampled after 6, 15, 24 and 48 h post injection. Their tissues were deep frozen and pulverized with liquid nitrogen and stored at -80ºC for the analyses of ferritin mRNA and protein levels.

Animal maintenance and handling were carried out in strict accordance with the recommendations in the CCAC guidelines (http://www.ccac.ca/Documents/Standards/Guidelines).

2.2. RNA extraction, first strand cDNA synthesis and isolation of Hrfer1 and Hrfer2 full-length cDNAs

Total RNA extraction, RNA quantification and intactness, and reverse transcription of RNAs from samples were performed as described in Coba de la Peña et al. [16].

In order to isolate at least two different *H. rufescens* ferritin homologues, two different pairs of oligonucleotides were used. The first primer pair, 5´-CCCACAAAGCACGTAACGAA-3´ (forward) and 5´-CACGCCATACTGTTATAGGG-3´ (reverse), were designed from conserved ferritin sequences that are available at the NCBI GenBank Sequence Database (http://www.ncbi.nlm.nih.gov/genbank) using the Primer3web program v4.0.0 (http://bioinfo.ut.ee/primer3/). A 485-bp PCR product from mantle cDNA of adult individuals was amplified, purified and sequenced by Servicio de Secuenciación de la Pontificia Universidad Católica de Chile. In order to isolate the full coding DNA sequence of the *H. rufescens* ferritin homologue, RACE (Rapid Amplification of cDNA ends) was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Approximately 1 µg of RNA from *H. rufescens* mantle was used for first-strand DNA synthesis, obtaining 5´-RACE-Ready cDNA and 3´-RACE-Ready cDNA. Specific primers for 5´- and 3´-RACE PCR were designed to recognize a region within the cDNA previously isolated and sequenced. Primer sequences were: 5´-GTCAGTGTTGCAGTCCGCCACCTTGTG-3´ (5´-RACE) and 5´-GGCGGGCGTCAACAAACAGATTAACGT-3´ (3´-RACE). Thermal cycling parameters were set following manufacturer's instructions. The amplification products were visualized, cloned, sequenced and aligned as described in Coba de la Peña et al. [16].

In order to obtain a second *H. rufescens* ferritin homologue, a second primer pair was designed based in a published *H. rufescens* ferritin coding sequence (GenBank accession number GU191936; [18]). Primers were: 5´-GATGGCCCAAACTCAACC-3´ (forward) and 5´-GGTTATGTCAGCCCAAACAAA-3´ (reverse). PCR performed with these primers generated a 700-bp PCR product that was cloned and sequenced. In order to obtain the 5´ untranslated region (5-UTR), 5´-RACE PCR was performed using 5´-RACE-ready cDNA and a specific primer designed to recognize a sequence within the 700-bp cDNA previously isolated and sequenced. Primer sequence was 5´-TCAGCTTCTCGGCATGCTCTCGCTCCT-3´. The obtained PCR product was cloned, sequenced and aligned.

2.4. Sequence analyses

Sequences were analyzed by BLAST algorithm at the National Center for Biotechnology Information. Nucleotide sequences were translated to predicted protein sequences and aligned with known ferritin sequences using the Expasy web server tools (http://www.expasy.org/).The iron responsive element (IRE) and stem-loop structure of *Hrfer2* was predicted by the SIREs Web Server v2.0 (http://ccbg.imppc.org/sires/) [29]. The tertiary structures of deduced proteins codified by *Hrfer1* and *Hrfer2* were predicted using the Phyre^2 server [30]. Phylogenetic analysis was performed with MEGA (Molecular Evolutionary Genetic Analysis) software v6.06 [31] using the neibourg-joining method [32], with bootstrap values calculated from 2,000 pseudo-replicates.

2.5. Quantitative real-time PCR

Primers for RT-qPCR were designed using Primer Express v3.0 software (Applied Biosystems, CA, USA). The amplified region included a partial 3´-UTR sequence, in order to amplify specifically the gene homologs under study. β-actin was used as endogenous control in order to normalize experimental results [18,33]. Primer sequences were as follows: *Hrfer1* (forward, 5´-CAGGTCTCGGAGAGTACCAGTTC-3´; reverse, 5´-GGCCGCGGACCAATTAAC-3´); *Hrfer2* (forward, 5´-ACGACAAGGAGTCCATGGAGTAG-3´; reverse, 5´-GGCCACGTGCGACTATGC-3´); β-actin (forward, 5´-GAGAGGTTCCGTTGTCCAGAGT-3´; reverse, 5´-CCAGCAGATTCCATACCCAAGA-3´).

RT-qPCR assays were run as described in Coba de la Peña et al. [16]. The comparative C_T method [34] was applied for relative quantification. Experiments included five biological replicates except in larvae expression (three biological replicates), and three technical replicates were performed.

2.6. Production and validation of anti-ferritin polyclonal antibody, ELISA and Western Blotting

Polyclonal antibodies were generated in CF-1 mice (6 weeks old) against a mix of three synthetic antigenic epitopes of ferritin from *H. rufescens* (UniProtKB - Q0PKG1) (Fig. S1). These epitopes were designed using a method described by Bethke et al. [35] and they were chemically synthesized by the solid phase multiple peptide system using Fmoc amino acids (Iris Biotech); and purified through HPLC. The peptides were

lyophilized and analyzed by matrix assisted laser desorption/ionization mass spectrometry to confirm molecular mass [36]. For antibody production, CF1 mice were subcutaneously injected at 1, 14 and 28 days with 300 μg of ferritin peptide mix (100 μg each one) diluted 1:1 in FIS peptide (peptide sequence: FISEAIIHVLHSR), a T helper cell activator [37], and 1:1 in Freund's adjuvant (Thermo). The antiserum was collected on day 44, centrifuged at 800 x g for 10 min and the supernatant was stored at -20 °C. Antibody affinity was determined by indirect ELISA [38] and antibody specificity was verified by Western blot using a total protein extract of *H. rufescens* following Schmitt et al. [39] (Fig. S1).Indirect ELISA was used to quantify the ferritin expression in the juveniles from the challenge experiment following Morales-Lange et al. [38], but using 1:2500 ferritin antisera.

2.6. Statistical analyses

Data were analysed with the STATISTICA v7.0 software package (StatSoft Inc., Tulsa, OK, USA). The following statistical tests were performed: one-way analysis of variance (one-way ANOVA) and Tukey post-hoc test for data of gene expression in tissues and larvae. Data of gene expression upon bacterial challenge were analysed by factorial ANOVA (in order to assess the effect of two factors: presence or absence of bacteria and time post-challenge) and Tukey post-hoc test. We applied a sequential Bonferroni procedure to correct for type I error in multiple simultaneous tests. Differences were considered significant at $P < 0.05$. For ANOVAs, normality of the dependent variable was tested with the Shapiro-Wilks test [40] and homogeneity of variances with the Levene test [41] to verify that the data met model assumptions.

3. Results and discussion

3.1. cDNA cloning and sequence characterization of Hrfer1 and Hrfer2

Two full-length cDNA clones were isolated from mantle of *H. rufescens*, and they were designated as *Hrfer1* (for *H. rufescens* ferritin 1, GenBank accession no. MH006611) and *Hrfer2* (GenBank accession no. MH006612). They were 807 and 868 bp length, respectively. The open reading frames (ORFs) of *Hrfer1* and *Hrfer2* are 516 bp length, encoding 171 amino acids. Molecular weights of *Hrfer1* and *Hrfer2* predicted proteins are 19.9 and 19.8 kDa, respectively. Predicted isoelectric points are 5.04 and 5.09,

242 respectively. By homology search, it was observed that *Hrfer2* coding sequence and
243 part of the 3'-UTR sequence corresponded to a ferritin gene (designed as *Abf2*)
244 previously identified in *H. rufescens* (Genbank accession number GU191936; [18]).
245 Seven conserved residues, corresponding to the ferroxidase diiron center, which
246 catalizes Fe(II) oxidation in mammalian H ferritins, were identified in both *Hrfer1*
247 (L25, Y32, K59, K60, F63, E105, E139) (Fig. 1A) and *Hrfer2* ([18]; Fig. 1B) predicted
248 proteins. Four conserved amino acids corresponding to the ferrihydrite nucleation center
249 of mammalian L-type ferritin subunits were observed in both *Hrfer1* (D55, C58, K59,
250 E62) and *Hrfer2* (K55, E58, E59, E62) predicted proteins (Fig. 1A and 1B). Both
251 sequences also displayed conserved iron ion channel amino acids (H116, D129, E132)
252 (Fig. 1A and 1B). *Hrfer1* predicted protein has a putative N-glycosylation site
253 (27NCSY30). Thus, *Hrfer1* presented all sequence motifs determining ferritin function
254 [1,18,42]. As M-type ferritins, both genes have both the ferroxidase activity observed in
255 H-type ferritins and the ferrihydrite nucleation center observed in L-type ferritins [2].
256 No putative signal peptide was detected, which suggested that both predicted ferritins
257 are cytosolic proteins.
258 Multiple sequence alignment showed that Hrfer1 and Hrfer2 displayed high amino acid
259 identities with other mollusk, invertebrate and vertebrate ferritin homologues (Fig. S2).
260 Hrfer1 corresponded to a new ferritin homologous sequence and showed 98% amino
261 acid identity with a ferritin of *Haliotis discus hannai* (GenBank accession no.
262 ADK60915). Hrfer2 corresponded to a sequence previously described by Salinas-Clarot
263 et al. [18] with high amino acid homology with other *H. discus hannai* ferritin
264 homologue (GenBank accession no. ABH10672). Hrfer1 showed 64% amino acid
265 identity with Hrfer2.

266 Hrfer1 and Hrfer2 3D models consisted of four α helixes from the N-terminal that were
267 parallel with each other, binding together through random coils and turns (Fig. S3A and
268 S3B). A fith short C-terminal α helix was also present in both proteins. Thus, predicted
269 3D models of Hrfer1 and Hrfer2 resembled the typical spatial features of known H- and
270 M-type ferritin protein structures [3,42].

271 Phylogenetic analysis of most known ferritin nucleotide coding sequences of mollusks
272 revealed that both *Hrfer1* and *Hrfer2* sequences are included into a clade of cytosolic,
273 non-secretory ferritins (Fig. S4). *Hrfer1*, with other *Haliotis* sequences, is into a cluster
274 that is separated from a big cluster containing all other non-secretory ferritins. *Hrfer2* is

into is included into a clade with other *Haliotis* sequences, and this clade further clustered together with other *Argopecten*, *Mizuhopecten* and *Azumapecten* sequences in the main (Fig. S4).

A putative iron responsive element (IRE) was identified by analysis of the 5'-UTR of the *Hrfer2* cDNA clone. Alignment of the *Hrfer2* IRE sequence showed 100% identity to known IREs of *H. discus discus*, *H. discus supertexta*, *H. discus hannai*, and other mollusks (Fig. S5A). This IRE of *Hrfer2* is predicted to be folded in a typical stem-loop secondary structure, which matches all IRE characteristics, (Fig. S5B). No IRE was detected in the case of *Hrfer1*, probably because *Hrfer1* cDNA clone lacks whole 5´-UTR sequence. The presence of this IRE suggests a posttranscriptional regulation of *Hrfer2* translation [7,8].

3.2. Tissue-specific expressions of Hrfer1 and Hrfer2

Transcriptional levels of both genes were detected in all tissues examined (Fig. 2). Levels of mRNA of both genes were higher in digestive gland (36.9- and 35.5-fold higher compared to muscle for *Hrfer1* and *Hrfer2*, respectively) and gonad (21.3- and 30.9-fold higher, respectively), and lowest level was observed in mantle, adductor muscle and gill (Fig. 2A and 2B). Our results differed from those reported by Salinas-Clarot et al. [18] for *Hrfer2* mRNA levels in the tissues. This could be associated to differences in the age or general physiological status of the analyzed abalone in each study.

Relative high basal levels of *ferritin* transcripts in digestive gland were also observed in other mollusks, as *Crassotrea gigas* [43], *Argopecten purpuratus* [16], *H. discus discus* [44], *H. diversicolor supertexta* [45] and *H. diversicolor* [46]. In abalone and other mollusks, the digestive gland is involved in iron storage, metal accumulation and detoxification [47,48]. Digestive gland is also an important immune organ involved in defense functions and in the integration of metabolism and immunity [48,49]. Thus, *Hrfer1* and *Hrfer2* may play an important role in iron storage and immune functions in digestive glands.

Relative high basal levels of ferritin expression in gonad were also observed in *H. discus discus* [44]. Abalone and mussels can uptake iron from the environmental seawater, and this iron can be accumulated in several organs, including gonads [47,50].

It was also observed that expressions of two ferritin genes are induced in gonad of the freshwater pearl mussel *Hyriopsis schlegelii* upon bacteria or heavy metal challenge [51]. Thus, *Hrfer1* and *Hrfer2* expression in gonad may be related with iron homeostasis and/or immune defense. Also, high levels of ferritin in gonads could be associated to maternal transfer of immune proteins through the eggs (i.e., passive immunity) to protect newly hatched larvae in abalone. This strategy has been observed in other mollusks [52], but further studies would be necessary to elucidate if it is present in abalone.

3.3. Hrfer1 and Hrfer2 expression in different developmental stages

Ferritin may be involved in mollusk shell formation during larval development [9,13,17]. The first steps of the larval shell formation take place during the trochophore stage [53]. In gastropods, ferritin genes are expressed in the edge of the shell field during the trochophore stage [54,55]. A role of ferritin in construction of the operculum was also suggested [54]. Transcriptional analysis of *Hrfer1* during larval development of *H. rufescens* showed an increase of its level along the different developmental stages, from gastrula (1-fold) to trochophore (about 4.3-fold higher than in gastrula), veliger (18.4-fold) and creeping larva (33.8-fold) (Fig. 3A). A similar ferritin expression pattern from eggs or gastrulae to juveniles was observed in the gastropod *H. asinine* [54] and *Aplysia californica* [56]. *Hrfer1* expression pattern suggests that this ferritin homologue may be involved in both protoconch (larval shell) and teleconch (juvenile/adult shell) formation from trochophore to creeping larval stage, being the continuous increase in expression from gastrulation to post-metamorphosis correlated with an increasing demand in biomineralization. Other putative functional roles of *Hrfer1* cannot be excluded. Development of the buccal apparatus in abalone post-larvae (creeping larva stage) initiates exogenous feeding, which include bacteria [57]. Thus, the observed significant increase of *Hrfer1* transcription level in abalone creeping larvae may suggest and immunity-related role upon exposure to bacteria, as suggested for some ferritin homologues characterized in different developmental stages of *C. gigas* [13] and *A. purpuratus* [58].

In contrast, *Hrfer2* expression was higher in gastrula (1-fold) and in trochophore stage (1.56-fold higher than in gastrula), and decreased in subsequent veliger (0.58-fold) and creeping larva (0.26-fold) stages (Fig. 3B). *Hrfer2* may play a role as mitogen for cell

proliferation, as it was observed in animal models [59,60]. *Hrfer2* expression may also be related with the first steps in larval shell formation at the trochophore stage [61].

3.4. Expression of Hrfer1 and Hrfer2 in response to bacterial challenge

In haemocytes from adult abalone *Hrfer1* expression was significantly up-regulated after 15 h post-injection with *Vibrio splendidus* (4.5-fold compared to 1-8-fold of its control injected with SSW or not injected), and declined afterwards (Fig. 4A). *Hrfer2* was found to be slightly but significantly upregulated after 2 h, and a down-regulation of *Hrfer2* was observed at 6 h post-injection with the bacteria (1.6-fold), compared with its control (2.6-fold) (Fig. 4B). *Ferritin* transcriptional induction in response to bacterial challenge was also observed in haemocytes of bivalve mollusks as *A. irradians* [10,62], *A. purpuratus* [16] and *Hyriopsis schlegelii* [51], among others. Qiu et al. [63] observed an upregulation of a *ferritin* homologue in haemocytes of *H. discus hannai* upon infection with the bacterial pathogen *Vibrio anguillarum* in a time-dependent manner, with a maximum expression level at 6 h post-inoculation. Our results suggest an involvement of *Hrfer1* and *Hrfer2* in the immune response of *H. rufescens* to pathogen infection. Ferritin upregulation upon bacterial infection may be related with the iron withholding activity of ferritins, preventing host iron acquisition by bacteria [64]. A putative antioxidant activity of ferritins, by preventing the generation of free radicals and reactive oxygen species by iron, was also proposed [2].

In juvenile abalones, significant up-regulations of *Hrfer1* and *Hrfer2* levels were observed after 24 h (respectively, 5.1 and 6.6-fold compared to 2.6 and 3.3-fold of their control injected with sterile sea water) and 48 h (respectively, 3.4 and 2.7-fold compared to 1.7 and 1.5-fold) post-injection with the bacterial suspension (Figs. 4C and 4D). *Ferritin* upregulation upon pathogen challenge was also observed in different tissues of bivalves as *Mizuhopecten yessoensis* [11,14], and *Hyriopsis schlegelii* [51]; and of gastropods as *H. tuberculata* [65], *H. diversicolor* [46] and *H. discus hannai* [63]. Thus, ferritin homologues may be induced upon pathogen inoculation in several organs in different time-manners. Our results suggest that both *Hrfer1* and *Hrfer2* genes are probably induced in one or several tissues (besides hemocytes) upon infection, and maximum upregulation of both genes in the whole individual occurs at 24 h post-injection.

3.5. Ferritin protein levels in response to bacterial challenge

Further investigation to detect and to estimate ferritin protein levels during bacterial challenge was performed. The produced polyclonal antibody cannot discriminate between ferritin isoforms of *H. rufescens*; thus considering the similar transcriptional pattern observed for *Hrfer1* and *Hrfer2* in juveniles, ferritin protein levels were assessed in extracts from whole individual at this stage. Significant higher amounts of ferritin protein were detected in individuals challenged with bacteria at 6, 15 and 24 h post-injection, compared with their respective controls injected with sterile seawater (or not injected juveniles). No significant higher amount of protein was observed in challenged individuals 48 h post-injection (Fig. 5).

We have identified two ferritin homologues in *H. rufescens*, but more ferritin homologues may be expressed in this organism. For instance, two cytosolic and two secretory ferritin homologues were identified in *C. gigas* [13], and four cytosolic and two secretory ferritin homologues were identified in *Mizuhopecten yessoensis* [13,14]. Thus, our results suggest that there is a significant overexpression of ferritin protein homologues (possibly including Hrfer1, Hrfer2 and other ferritin homologues) in whole individuals of *H. rufescens* from 6 to 24 h post-injection. This result suggests that, besides Hrfer1 and Hrfer2, other ferritin homologues are induced and involved in the immune response to bacterial infection in *H. rufescens*.

In summary, our results showed that both ferritin homologues *Hrfer1* and *Hrfer2* participate in the immune response against bacterial challenges in haemocytes and probably in other tissue organs; and that the novel *Hrfer1* could be implicated in post-larvae immunity. These results indicate that these ferritins are potential candidate genes to be used as molecular markers for enhancement of immune capacity in *H. rufescens* through genetic programmes. However, functional characterization experiments involving the interference of expression of both genes and/or the expression of the recombinant ferritin proteins will be necessary to verify the involvement of these genes in *H. rufescens* immunity.

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404 **References**

405 [1] E.C. Theil, Ferritin: structure, gene regulation and cellular functions in animals,
406 plants and microorganisms. Annu. Rev. Biochem. 56 (1987) 289-315.

407 [2] P. Arosio, R. Ingrassia, P. Cavadini, Ferritins: a family of molecules for iron storage,
408 antioxidation and more. Biochim. Biophys. Acta 1790 (2009) 589-599.

409 [3] R.R. Crichton, J.P. Declerq, X-ray structures of ferritins and related proteins.
410 Biochim. Biophys. Acta 1800 (2010) 706-718.

411 [4] P. Arosio, S. Levi, Cytosolic and mitochondrial ferritins in the regulation of cellular
412 iron homeostasis and oxidative damage. Biochim. Biophys. Acta 1800 (2010) 783-
413 792.

414 [5] L.F. Dickey, S. Sreedharan, E.C. Theil, J.R. Didsbury, Y.H. Wang, R.E. Kaufman,
415 Differences in the regulation of messenger RNA for housekeeping and specialized-
416 cell ferritin. A comparison of three different ferritin complementary DNAs, the
417 corresponding subunits, and identification of the first processed in amphibia. J.
418 Biol. Chem. 262 (1987) 7901-7907.

419 [6] A. Giorgi, G. Mignogna, G. Bellapadrona, M. Gattoni, R. Chiaraluce, V. Consalvi,
420 E. Chiancone, S. Stefanini, The unusual co-assembly of H- and M-chains in the
421 ferritin molecule from the Antartic teleosts *Trematomus bernachii* and *Trematomus*
422 *newnesi*. Arch. Biochem. Biophys. 478 (2008) 69-74.

423 [7] E.C. Theil, Iron regulatory elements (IREs): a family of mRNA non-coding
424 sequences. Biochem. J. 304 (1994) 1-11.

425 [8] E.C. Theil, IRE mRNA riboregulators use metabolic iron (Fe^{2+}) to control mRNA
426 activity and iron chemistry in animals. Metallomics 7 (2015)15-24.

[9] X. Wang, B. Liu, J. Xiang, Cloning, characterization and expression of ferritin subunit from clam *Meretrix meretrix* in different larval stages. Comp. Biochem. Physiol. B 154 (2009)12-16.

[10] X. He, Y. Zhang, X. Wu, S. Xiao, Z. Yu, Cloning and characterization of two ferritin subunit genes from bay scallop, *Argopecten irradians* (Lamark 1819). Mol. Biol. Rep. 38 (2011) 2125-2132.

[11] L. Zhang, W. Sun, W. Cai, Z. Zhang, Y. Gu, H. Chen, S. Ma, X. Jia, Differential response of two ferritin subunit genes (*VpFer1* and *VpFer2*) from *Venerupis philippinarum* following pathogen and heavy metals challenge. Fish Shellfish Immunol. 35 (2013a) 1658-1662.

[12] Y. Zhang, R. Zhang, J. Zou, X. Hu, S. Wang, L. Zhang, Z. Bao, Identification and characterization of four ferritin subunits involved in immune defense of the Yesso scallop (*Patinopecten yessoensis*). Fish Shellfish Immunol. 34 (2013b) 1178-1187.

[13] P. Huan, G. Liu, H. Wang, B. Liu, Multiple ferritin subunit genes of the Pacific oyster *Crassotrea gigas* and their distinct expression patterns during early development. Gene 546 (2014) 80-88.

[14] Y. Sun, Y. Zhang, X. Fu, R. Zhang, J. Zou, S. Wang, X. Hu, L. Zhang, Z. Bao, Identification of two secreted ferritin subunits involved in immune defense of Yesso scallop *Patinopecten yessoensis*. Fish Shellfish Immunol. 37 (2014) 53-59.

[15] Z. Bai, Y. Yuan, G. Yue, J. Li, Molecular cloning and copy number variation of a ferritin subunit (Fth1) and its association with growth in freshwater pearl mussel *Hyriopsis cumingii*. PLoS ONE 6 (2011) e22886.

[16] T. Coba de la Peña, C.B. Cárcamo, M.I. Díaz, K.B. Brokordt, F.M. Winkler, Molecular characterization of two ferritins of the scallop *Argopecten purpuratus* and gene expressions in association with early development, immune response and growth rate. Comp. Biochem. Physiol. B 198 (2016) 46-56.

[17] I. Zhang, Q. Meng, T. Jiang, H. Wang, L. Xie, R. Zhang, A novel ferritin subunit involved in shell formation from the pearl oyster (*Pinctada fucata*). Comp. Biochem. Physiol. B 135 (2003) 43-54.

[18] K. Salinas-Clarot, A.P. Gutiérrez, G. Núñez-Acuña, G. Gallardo-Escárate, Molecular characterization and gene expression of ferritin in red abalone (*Haliotis rufescens*). Fish Shellfish Immunol. 30 (2011) 430-433.

[19] R. Gordon, P.Cook, World abalone fisheries and aqua-culture update: supply and market dynamics. J. Shellfish Res. 23 (2004) 935–940.

[20] F. Lafarga de la Cruz, C. Gallardo-Escárate, Intraspecies and interspecies hybrids in *Haliotis*: natural and experimental evidence and its impact on abalone aquaculture. Rev. Aquacult. 3 (2011) 74-79.

[21] P.A. Cook, The worldwide abalone industry. Modern Economy 5 (2014) 1181-1186.

[22] R.A. Flores-Aguilar, A. Gutiérrez, A. Ellwanger, S. Searcy-Bernal, Development and current status of abalone aquaculture in Chile. J. Shellfish Res. 26 (2007) 705-711.

[23] FAO. 2016. The State of World Fisheries and Aquaculture: Contributing for Food Security and Nutrition for All. Food and Agriculture Organization of the United Nations. FAO, Rome.

[24] R. Elston, G.S. Locwood, Pathogenesis of vibriosis in cultured juvenile red abalone, *Haliotis rufescens* Swainson. J. Fish. Dis. 6 (1983) 111-128.

[25] C. Anguiano-Beltrán, R. Searcy-Bernal, M.L. Lizárraga-Partida, Pathogenic effects of *Vibrio alginolyticus* on larvae and postlarvae of the red abalone *Haliotis rufescens*. Dis. Aquat. Org. 33 (1998) 119-122.

[26] C. Rojas-Villalobos, Causas de mortalidad del abalón rojo (*Haliotis rufescens*) en etapa de engorda en el período otoño-invierno (2005) PhD. Valdivia, Chile.

[27] J. Cai, Y. Han, Z. Wang, Isolation of *Vibrio parahaemolyticus* from abalone (*Haliotis diversicolor supertexta* L.) postlarvae associated with mass mortalities. Aquaculture 257 (2006) 161-166.

[28] D.E. Morse, H.Duncan, N. Hooker, A. Morse, Hydrogen peroxide induces spawning in mollusks, with activation of prostaglandin endoperoxide synthetase. Science 196 (1977) 298-300.

[29] M. Campillos, I. Cases, M.W. Hentze, M. Sanchez, SIREs: searching for iron-responsive elements. Nucleic Acids Res. 38 (2010) W360-W367.

[30] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, J.E. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 10 (2015) 845-858.

[31] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30 (2013) 2725-2729.

[32] N. Saitou, M. Nei, The neiborg-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4 (1987) 406-425.

[33] F. Silva-Aciares, M. Zapata, J. Tournois, D. Moraga, C. Riquelme, Identification of genes expressed in juvenile *Haliotis rufescens* in response to different copper concentrations in the north of Chile under controlled conditions. Mar. Pollut. Bull. 62 (2011) 2671–2680.

[34] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (2001) 2002-2007.

[35] J. Bethke, V. Rojas, J. Berendsen, C. Cárdenas, F. Guzmán, J.A. Gallardo, L. Mercado, Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like protein in infected salmonids. J. Fish Dis. 35 (2012) 379-388.

[36] V. Rojas, B. Morales-Lange, F. Guzmán, J.A. Gallardo, L. Mercado, Immunological strategy for detecting the pro-inflammatory cytokine TNF-alpha in salmonids. Elect J Biotech. 15(5) (2012) 1-7.

[37] I. Prieto, S. Hervás-Stubbs, M. García-Granero, C. Berasain, J.I. Riezu-Boj, J.J. Lasarte, P. Sarobe, J. Prieto, F. Borrás-Cuesta, Simple strategy to induce antibodies of distinct specificity: application to the mapping of gp120 and inhibition of HIV-1 infectivity. Eur J Immunol. 25 (1995) 877-883.

[38] B. Morales-Lange, J. Bethke, P. Schmitt, L. Mercado, Phenotypical parameters as a tool to evaluate the immunostimulatory effects of beta-laminarin in *Oncorhynchus mykiss*. Aquac Res. 46 (2015) 2707-2715.

513 [39] P. Schmitt, J. Wacyk, B. Morales-Lange, V. Rojas, F. Guzmán, B. Dixon, L.
514 Mercado, Immunomodulatory effect of cathelicidins in response to a beta-glucan in
515 intestinal epitelial cells from rainbow trout. Dev. Comp. Immunol. 51 (2015) 160-
516 169.

517 [40] G. Snedecor, W. Cochran, Statistical methods. 8th ed. (1989) Iowa: Iowa State
518 University Press.

519 [41] P.M. Harrison, P. Arosio, The ferritins: molecular properties, iron storage function
520 and cellular regulation. Biochim. Biophys. Acta 1275 (1996) 161-203.

521 [42] J.P. Durand, F. Goudard, J. Pieri, J.M. Escoubas, N. Schreider, J.P. Cadoret,
522 *Crassostrea gigas* ferritin: cDNA sequence analysis for two heavy chain type
523 subunits and protein purification. Gene 338 (2004) 187-195.

524 [43] M. De Zoysa, J. Lee, Two ferritin subunits from disk abalone (*Haliotis discus*
525 *discus*): cloning, characterization and expression analysis. Fish Shellfish Immunol.
526 23 (2007) 624-635.

527 [44] J. Xie, X. Cao, L. Wu, M. Luo, Z. Zhu, Y. Huang, X. Wu, Molecular and
528 functional characterization of ferritin in abalone *Haliotis diversicolor supertexta*.
529 Acta Oceanol. Sin. 31 (2012) 87-97.

530 [45] J. He, J. Jiang, L. Gu, M. Zhao, R. Wang, L. Ye, T. Yao, J. Wang, Identification
531 and involvement of ferritin in the response to pathogen challenge in the abalone,
532 *Haliotis diversicolor*. Dev. Comp. Immunol. 60 (2016) 23-32.

533 [46] R.V. Hyne, J.D. Smith, G. Ellender, Tissue and sub-cellular distribution of Fe, Cu,
534 Zn and ^{210}Po in the abalone *Haliotis rubra*. Mar. Biol. 112 (1992) 75-8.

535 [47] I. Marigómez, M. Soto, M.P. Cajaraville, E. Angulo, L. Giamberini, Cellular and
536 subcellular distribution of metals in mollusks. Microsc. Res. Tech. 56 (2002) 358-
537 392.

538 [48] T. Roszer, The invertebrate midintestinal gland ("hepatopáncreas") is an
539 evolutionary forerunner in the integration of immunity and metabolism. Cell Tissue
540 Res. 358 (2014) 585-695.

[49] R. Eisler, Compendium of Trace Metals and Marine Biota: Volume 1: Plants and Invertebrates. 1st Edition. Eisler, R. (2010) Elsevier. The Netherlands.

[50] J.Q. Sheng, Q.C. Shu, J.W. Shi, J.H. Wang, K. Peng, S. Yuan, Y.J. Hong, Immunological function and antibacterial activity of two ferritin proteins from the freshwater pearl mussel *Hyriopsis schlegelii*. Genet. Mol. Res. 15(3) (2016) gmr.15038533.

[51] L. Wang, F. Yue, X. Song, L. Song, Maternal immune transfer in molluscs. Dev. Comp. Immunol. 48 (2015) 354-359.

[52] F. Marin, G. Luquet, B. Marie, D. Medakovic, Molluscan shell proteins: primary structure, origin and evolution. Curr. Top. Dev. Biol. 80 (2008) 209-276.

[53] D.J. Jackson, G. Wörheide, B. Degnan, Dynamic expression of ancient and novel molluscan shell genes during ecological transitions. BMC Evol. Biol. 7 (160) (2007) 1-17.

[54] N. Hashimoto, Y. Kurita, H. Wada, Developmental role of *dpp* in the gastropod shell plate and co-option of the *dpp* signaling pathway in the evolution of the operculum. Dev. Biol. 366 (2012) 367-373.

[55] A. Heyland, Z. Vue, C.R. Voolstra, M. Medina, L.L. Moroz, Developmental transcriptome of *Aplysia californica*. J. Exp. Zool. B (Mol. Dev. Evol.) 316 (2011) 113-134.

[56] K.O. Hahn, Nutrition and growth of abalone. In: Hahn, K.O. (Ed.), Handbook of Culture of Abalone and Other Marine Gastropods. CRC Press, Boca Raton, FL, (1989) pp. 135–156.

[57] K**.** Brokordt, I. Rojas, C. Cárcamo, R. Rojas, L. Mercado, P. Schmitt, Transcriptional patterns of antimicrobial effectors during early development of the scallop *Argopecten purpuratus* after exposure to the pathogen *Vibrio splendidus*. Proceedings of the Physiomar17 Conference. (2017) Cambridge, UK.

[58] A. Cozzi, B. Corsi, S. Levi, P. Santambrogio, G. Biasiotto, P. Arosio, Analysis of the biologic functions of H- and L-ferritins in HeLa cells by transfection with

siRNAs and cDNAs: evidence for a proliferative role of L-ferritin. Blood 103 (2004) 2377-2383.

[59] S. Li, Identification of iron-loaded ferritin as an essential mitogen for cell proliferation and postembryonic development in *Drosophila*. Cell Res. 20 (2010) 1148-1157.

[60] F. Marin, G. Luquet, B. Marie, D. Medakovic, Molluscan Shell Proteins: Primary Structure, Origin, and Evolution. Curr Top Dev Biol, 80 (2008) 209-276.

[61] J. Li, L. Li, S. Zhang, J. Li, G. Zhang, Three ferritin subunits involved in immune defense from bay scallop *Argopecten irradians*. Fish Shellfish Immunol. 32 (2012) 368-372.

[62] R. Qiu, Y. Kan, D. Li, Ferritin from the Pacific abalone *Haliotis discus hannai*: analysis of cDNA sequence, expression, and activity. Fish Shellfish Immunol. 49 (2016) 315-323.

[63] S.T. Ong, J.Z. Ho, B. Ho, J.L. Ding, Iron-withholding strategy in innate immunity. Immunobiol. 211 (2006) 295-314.

[64] M.A. Travers, A.L. Meistertzheim, M. Cardinaud, C.S. Friedman, S. Huchette, D. Moraga, C. Paillard, Gene expression patterns of abalone, *Haliotis tuberculate*, during successive infections by the pathogen *Vibrio harveyi*. J. Invert. Pathol. 105 (2010) 289-297.

Figure captions

Figure 1. Nucleotide and deduced amino acid sequences of *Hrfer1* (A) and *Hrfer2* (B) from *Haliotis rufescens*. IRE sequence is shaded in yellow. Non-codifying regions are shaded in dark grey. Start codon (ATG) and stop codons are underlined. Asterisk denotes the stop codon in the amino acid sequence. Conserved residues for the ferroxidase site are shaded in green. Conserved residues of the ferrihydrite nucleation centre are shaded in pink. Conserved iron channel residues are shaded in blue. Putative iron-binding regions are shaded in red. Putative N-glycosilaton site is shaded in light grey.

598 **Figure 2. Relative basal levels of *Hrfer1* (A) and *Hrfer2* (B) transcripts in different**
599 **tissues of adult *Haliotis rufescens***. Transcript levels of *Hrfer1* and *Hrfer2* in mantle
600 (Mn), adductor muscle (Mu), gill (Gi), gonad (Go) and digestive gland (Dg) were
601 detected by real-time PCR. β-actin was used as housekeeping gene. All values represent
602 the means ± S.E. (n = 5 biological replicates). Different letters denote significant
603 differences at $P<0.05$.

604 **Figure 3. Relative basal levels of *Hrfer1* (A) and *Hrfer2* (B) transcripts in different**
605 **developmental stages of *Haliotis rufescens***. Assessed developmental stages were
606 gastrula, trochophore, veliger and creeping larva. Metamorphosis stage is indicated by
607 an arrow. β-actin was used as housekeeping gene. All values represent the means ± S.E.
608 (n = 3 biological replicates). Different letters denote significant differences at $P<0.05$.

609 **Figure 4. Relative levels of *Hrfer1* and *Hrfer2* mRNA transcript levels in *Haliotis***
610 ***rufescens* after challenge with *Vibrio splendidus***. Time-course expression analyses of
611 *Hrfer1* and *Hrfer2* transcript levels were measured by RT-qPCR in haemocytes from
612 adult abalone (A and B); and in whole individuals at juvenile stage (C and D). β-actin
613 was used as housekeeping gene. Individuals injected with sterile seawater (SSW), as
614 control, and individuals injected with bacterial suspension are displayed. Values are
615 means ± S.E. (n = 5 biological replicates per treatment). Different letters denote
616 significant differences at $P<0.05$.

617 **Figure 5. Ferritin protein levels in juvenile *Haliotis rufescens* challenged with *V.**
618 ***splendidus***. Ferritin levels were assessed by ELISA in extracts from whole individuals
619 over a period of 48 h post-injection with sterile seawater (SSW), as control; and with
620 bacterial suspension. Values are means ± S.E. (n = 5 biological replicates per
621 treatment). Different letters denote significant differences at $P<0.05$.

622

623 **Captions of supplementary figures**

624 **Figure S1. Production and validation of an anti-ferritin polyclonal antibody for**
625 ***Haliotis rufescens*.** Sequences of the three synthetic peptides (A) used as antigens
626 during the immunization for obtaining antibodies. The best antibodies were obtained
627 against the peptide 1973. The 3D structure (B) of HrFer-1 shows in gold the localization
628 of p1973. (C) Left panel: Immunorecognition of synthetic peptide of ferritin (p1973) by

indirect ELISA (calibration curve of anti-HrFer serum against the synthetic peptide used for the immunization; R: Pearson correlation coefficient). Right panel: Determination of antibody specificity by Western blot against mantle protein profile (C: Control group sample; E: challenged group sample).

Figure S2. Multiple sequence alignment of vertebrate and invertebrate ferritin subunits. Conserved residues, important in iron binding and ferroxidation, are contained in boxes. Ferritin sequences shown are from: *Haliotis rufescens* (Hrfer1 and Hrfer2, GenBank: respectively MH006611 and MH006612); *H. discus hannai* (GenBank: ADK60915); *H. diversicolor* (GenBank: AMA34095); *Hyriopsis cumingii* (GenBank: ADZ04889); *Argopecten irradians* ferritin 1 (GenBank: AEN71558); *Rhipicephalus sanguineus* (GenBank: AAQ54715); *Daphnia pulex* heavy (H) chain (GenBank: EFX74776); *Callorhinchus milii* mitochondrial subunit (GenBank: AFM87687); *Danio rerio* middle (M) subunit-like (GenBank: XP_687175); *Homo sapiens* heavy (H; GenBank: NP_002023) and light (L; GenBank: NP_000137) ferritin subunits.

Figure S3. Predicted 3D structural models of Hrfer1 (A) and Hrfer2 (B) subunits from *Haliotis rufescens*. The four α helixes are coloured in brown, yellow, blue and green. A fifth short helix is coloured in red. Random coils and turns are also displayed. N, N-terminal; C, C-terminal.

Figure S4. Phylogenetic optimal tree of most known nucleotide sequences of mollusk ferritin. Subunits *Hrfer1* and *Hrfer2* references are contained in boxes. The evolutionary history was inferred from the neighbour-joining method. Bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accession numbers are indicated. Ferritins with GenBank accession numbers DQ821493, XM_011440581 and KC754752 (the three sequences at the bottom of the tree) correspond to secretory ferritins. All other sequences correspond to non-secretory ferritins.

Figure S5. Iron responsive element (IRE) analysis of *Hrfer2* and other ferritin homologues. (A) Alignment and sequence comparison with selected ferritin IREs from mollusk ferritin subunits (and the non-molluscan ferritins of *Xenopus laevis* and *Homo sapiens*). GenBank Acc No. of each ferritin homologue is indicated. Conserved 5'-

CAGTGN-3' loop structure residues and bulged cysteines located five bases upstream are shaded in light grey. The other homologous regions are shaded in dark grey. The percentage similarities of IREs are on the right side. (B) Predicted IRE stem-loop structure of *Hrfer2*, compared with IREs of *Argopecten purpuratus* 2 (GenBank: KT895279), *A. irradians* 2 (GenBank: HQ225741); *Lymnaea stagnalis* (GenBank: X56778) and *Hyriopsis cumingii* 1 (GenBank: HQ896721).

A

Hrfer1

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ATGCCAGAAAGTCAAGCACGTCAGAACTACCACGTCAACAGCGAGGCGGGCGTCAACAAACAG
 M  P  E  S  Q  A  R  Q  N  Y  H  V  N  S  E  A  G  V  N  K  Q
ATTAACGTCCTGCTGAACTGTAGCTATGTCTACCATTCCATGGCCTGGTATTTTGACCGGGAT
 I  N  V  L  L  N  C  S  Y  V  Y  H  S  M  A  W  Y  F  D  R  D
GACGTGGCCCTGAAAGGATTCTTTGAGTTTCTCAAGGATGCCTCTTGCAAGAAGCGCGAGTTC
 D  V  A  L  K  G  F  F  E  F  L  K  D  A  S  C  K  K  R  E  F
GCCGAGAAAATGATGAAGTACCAGAACCAGAGAGGTGGGCGCATCGTGCTGCAGGACATCAAG
 A  E  K  M  M  K  Y  Q  N  Q  R  G  G  R  I  V  L  Q  D  I  K
AAGCCGCCCCATGACGAATGGGGGACAGGCCTGGACGTGATGCAGTCTGCCCTGGCCCTGGAG
 K  P  P  H  D  E  W  G  T  G  L  D  V  M  Q  S  A  L  A  L  E
AAGAACGTCAACCAGGAGTTCCTGGACCTGCACAAGGTGGCGGACTGCAACACTGACCCACAG
 K  N  V  N  Q  E  F  L  D  L  H  K  V  A  D  C  N  T  D  P  Q
ATGATGGACTTCCTGGAGGATGAGTTCCTGGAAGAGGAGGTCGAGATGATCAAGAAGCTCTCC
 M  M  D  F  L  E  D  E  F  L  E  E  E  V  E  M  I  K  K  L  S
GACCACGTGACCAACCTGAAGCGCGTTGGACCAGGTCTCGGAGAGTACCAGTTCGACCACGAG
 D  H  V  T  N  L  K  R  V  G  P  G  L  G  E  Y  Q  F  D  H  E
ACTCTCAGTTAAATTGGTCCGCGGCCTTCCGGTCACCATGGTAACCGATGACAACGCCCGACA
 T  L  S  *
ACGCCTACTGCTTCTGTCCTATACTTTCTTGTAAGAACTAAAGCCCCGCTTCTTGTAACTGGC
AGCCTGCTTCACGTGACGCGTGACGTGAATTCCAAAATGAGATGAAACATTTAATTTTTATAC
GTGTCTTATCTTTTTATACATTTTGTCTGTTGAATTTTGTTAATTTTCCCTGTTATAAACTCC
TGTAAAACAACATCTGCAATAAATCCAAGCATGCAAAAAAAAAAAAAAAAAA
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B

Hrfer2

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GACCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACATGGGGAGATGTTTGTCTTGCT
GCGTCAGTGAACGTACGGGCAAAATCGACGCTATCAAAAGCACTTCTTCAACACCCATTTA
ATCTCATTTATTCCCAGTTGTCAGTCCGAGAATCTAGCAAGATGGCCCAAACTCAACCCCG
                                          M  A  Q  T  Q  P  R
CCAGAACTTCCACTGCGAGAGCGAAGCCGGCATCAACCGCCAGATCAATATGGAGCTGTAC
  Q  N  F  H  C  E  S  E  A  G  I  N  R  Q  I  N  M  E  L  Y
GCCAGCTATACCTACCAGTCAATTGGGTTCTACTTCGAGCGCGATGACGTTGCTCTGCCTG
 A  S  Y  T  Y  Q  S  I  G  F  Y  F  E  R  D  D  V  A  L  P
GCTTCAGCAAGTACTTCAAGAAGGCATCCGAGGAGGAGCGAGAGCATGCCGAGAAGCTGAT
G  F  S  K  Y  F  K  K  A  S  E  E  E  R  E  H  A  E  K  L  N
GAAGTACCAGAACACCCGAGGAGGTCGCATCGTCCTGCAGGACATCAAGAAGCCTGACCGG
  K  Y  Q  N  T  R  G  G  R  I  V  L  Q  D  I  K  K  P  D  R
GATGAGTGGGGTACAGCACTGGAATCCATGCAGGTGGCTCTGTCCCTGGAGAAGAACGTCA
 D  E  W  G  T  A  L  E  S  M  Q  V  A  L  S  L  E  K  N  V
ACCAGTCCTTGCTGGACCTCCACGCTGTGGCCAGCAAACACAGCGACGCACAGATGTGCGA
N  Q  S  L  L  D  L  H  A  V  A  S  K  H  S  D  A  Q  M  C  D
CTTCCTGGAGAGCGAGTACCTTGAGGAGCAGGTGAAGGCCATCAAGGAGATCTCGGACCAC
  F  L  E  S  E  Y  L  E  E  Q  V  K  A  I  K  E  I  S  D  H
ATCACCAACCTGAAGCGGGTTGGGGCTGGCCTGGGTGAATACATGTACGACAAGGAGTCCA
 I  T  N  L  K  R  V  G  A  G  L  G  E  Y  M  Y  D  K  E  S
TGGAGTAGTCCCCAACCGTCACGTGGGCATAGTCGCACGTGGCCGCACCCAGTCAGCCCAT
M  E  *
GTAATGCAATCGTTGATGAACGTTTTGTTTCTGTTGAACAGTTTTATATGTTTTGTTTGGG
CTGACATGTGAACATCTCTGCTTGTAATACTGGGGCCGCGAGGCCGTCCTTTGATTTCATT
CATATTAACCCCAA
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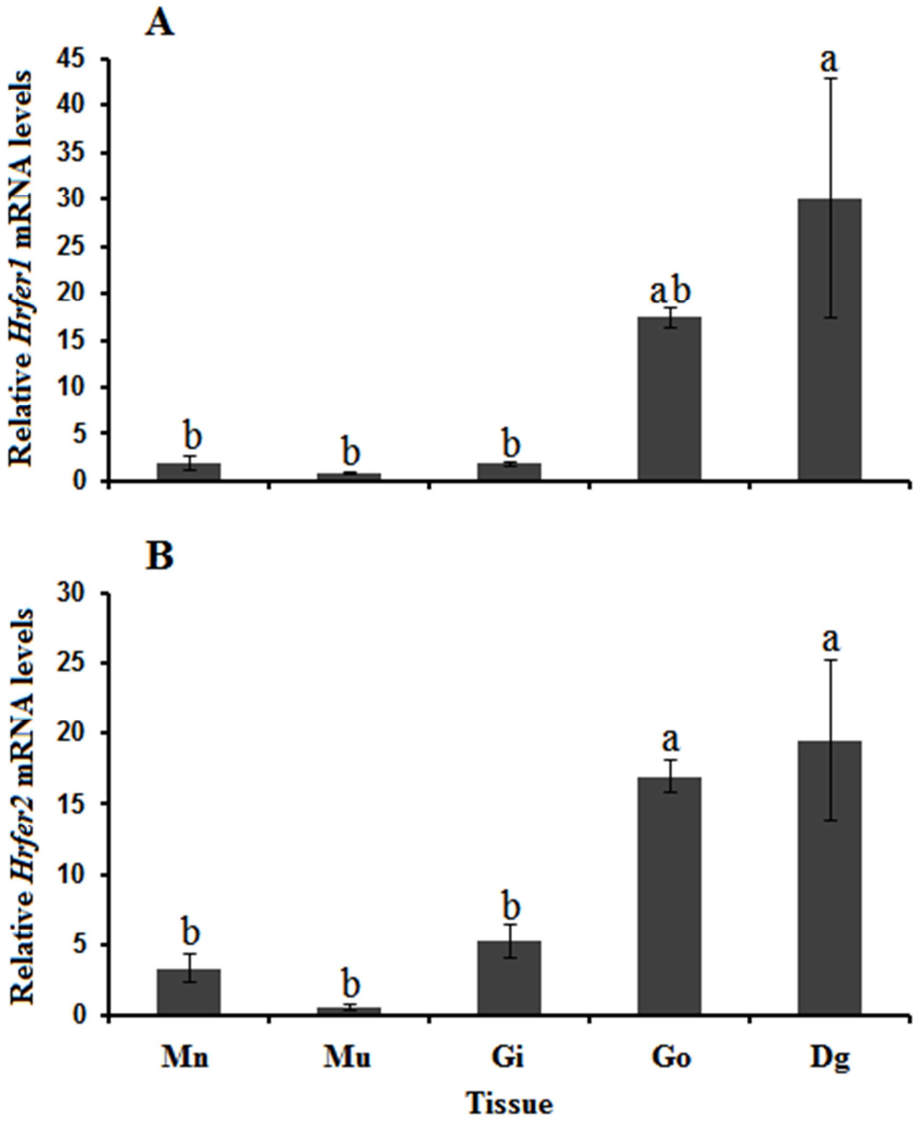

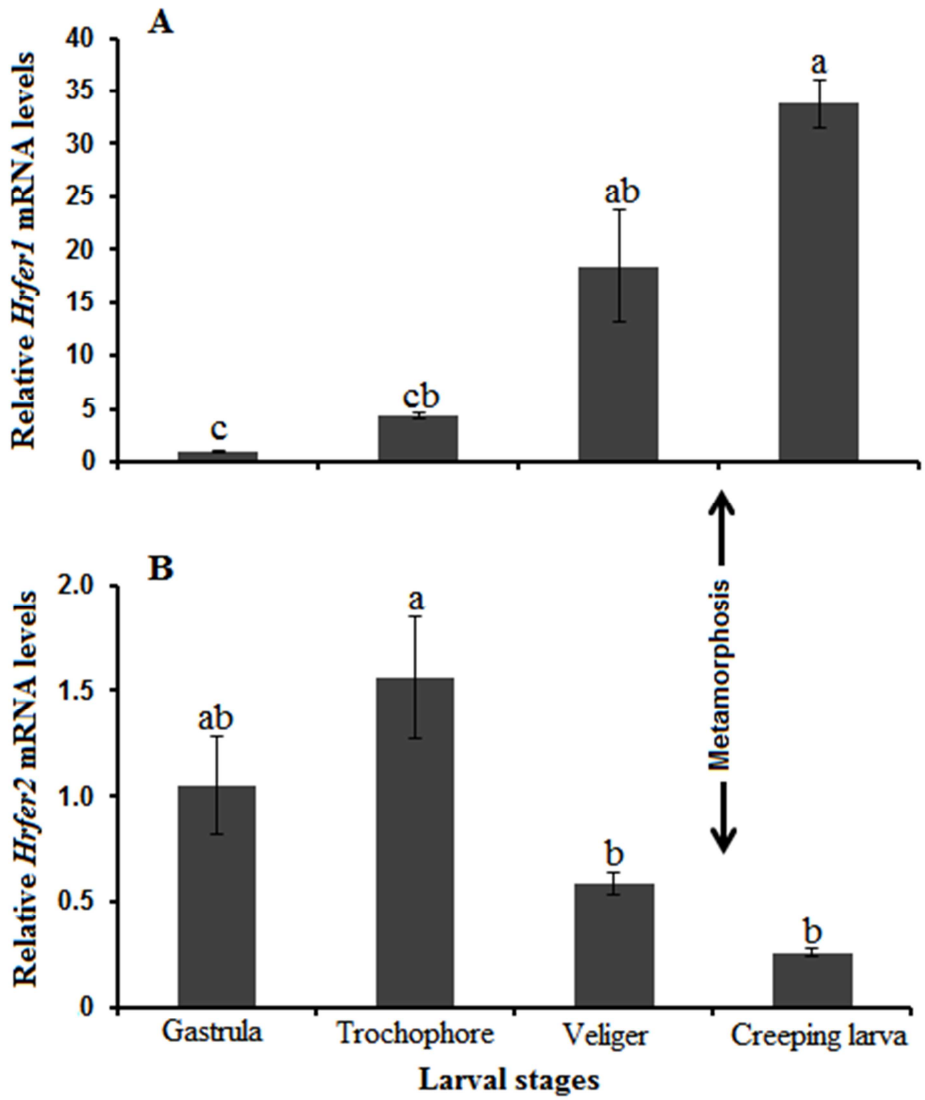

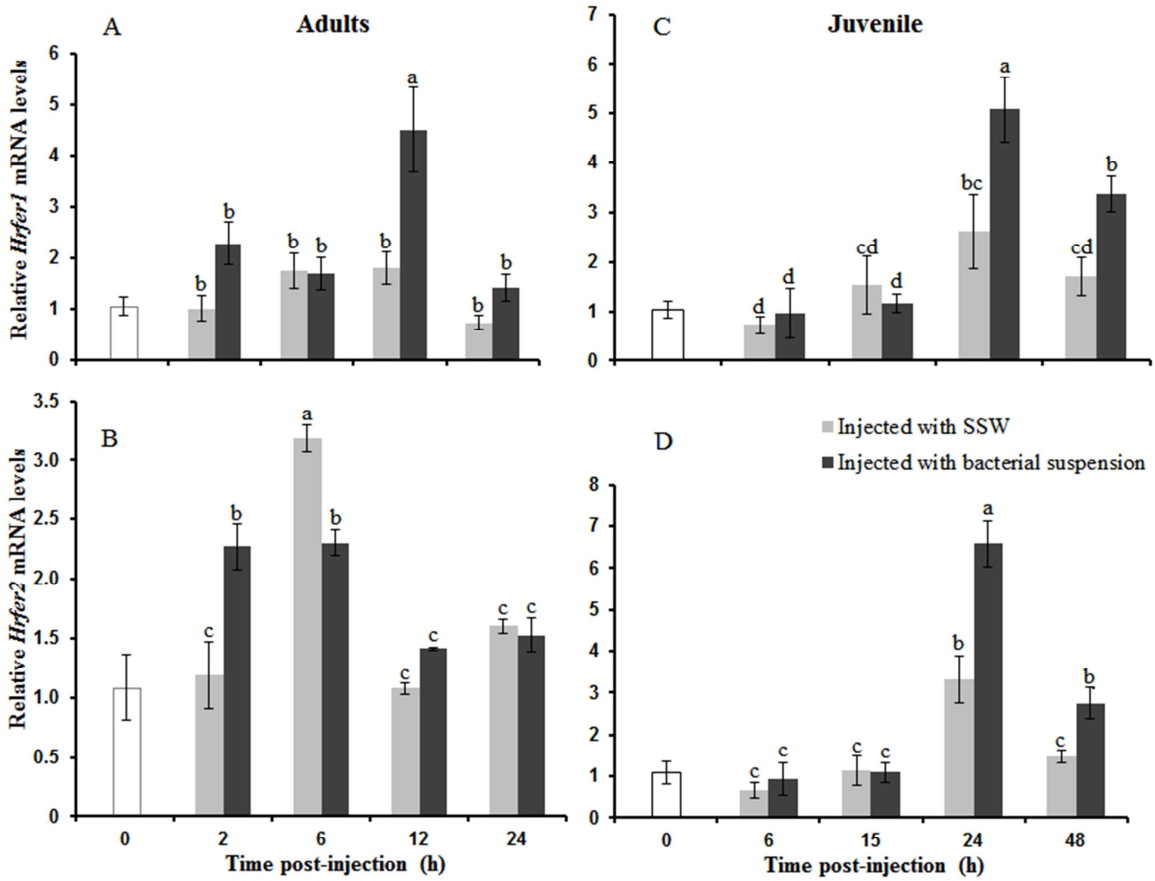

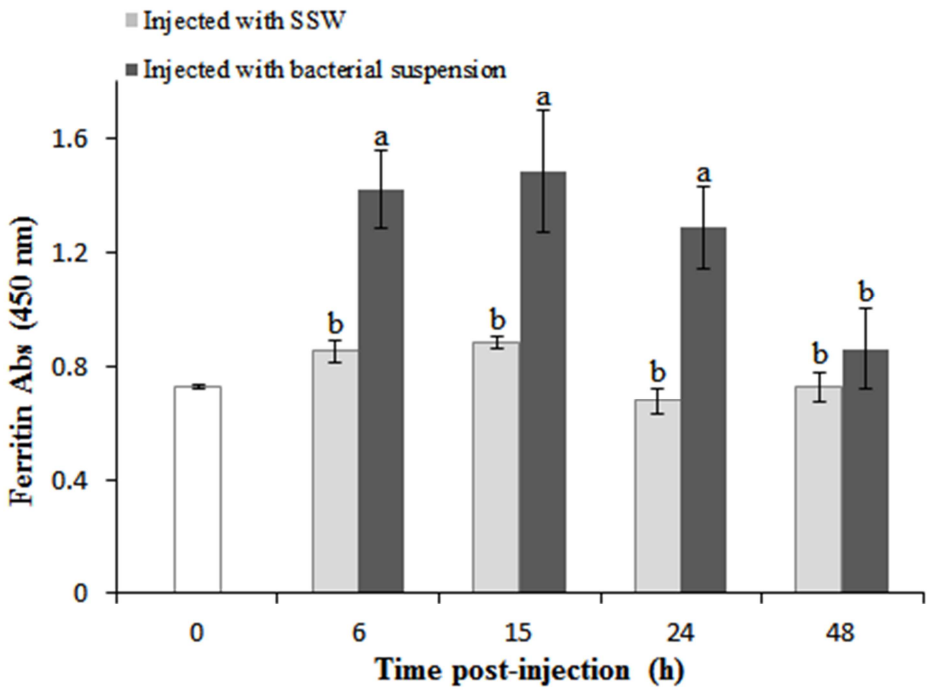

Highlights

- Two ferritin sequences were characterized in red abalone, Hrfer1 and Hrfer2
- Both *ferritins* are present in all tissues and strongly expressed in digestive gland and gonad
- *Hrfer1* transcripts increased along with larval development being highest in creeping post-larvae
- Both *ferritins* are upregulated in adults and juveniles challenged with *Vibrio splendidus*
- Ferritin is upregulated at protein level in the juveniles after bacterial challenge