Article

The Distribution of Ferritins in Marine Copepods

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Abstract: Iron is an essential element for the functioning of cellular processes. Ferritins, the major intracellular iron storage proteins, convert the free Fe^{2+} into the nontoxic Fe^{3+} which can be stored and transported where needed. To date, little is known about the iron metabolism in copepods; however, in these crustaceans, ferritins have been used as biomarkers of stress and diapause. A limiting factor of these studies has been the use of a single ferritin transcript as a biomarker. In this paper, we in silico mined the publicly available copepod transcriptomes to characterize the multiplicity of the ferritin transcripts in different orders and families. We also examined the expression of ferritin in three ecologically important copepods—*Calanus finmarchicus*, *C. helgolandicus* and *Temora stylifera*—during development and under stress conditions. A full-length transcript encoding ferritin heavy chain has been identified in all 27 mined transcriptomes, with 50% of the species possessing multiple transcripts. Ferritin expression increased in *C. finmarchicus* during the early–late development transition, and in *T. stylifera* females exposed to oxylipins at sea. Overall, our results suggest that copepod ferritins can be involved in iron storage, larval development and stress response, thus representing potential biomarker genes for ocean health status monitoring.

Keywords: zooplankton; transcriptome; gene discovery; iron metabolism; stress response; diapause

1. Introduction

In all animals, iron (Fe) is an essential element required for the functioning of many cellular processes and to meet nutritional requirements. It participates in various metabolic processes, including DNA synthesis, electron and oxygen transport and cellular oxidation mechanisms [1]. Iron must be absorbed from the diet into gut cells and then transported and stored for future use [2]. However, excess free Fe in living cells can be potentially toxic. In the presence of oxygen, Fe can be rapidly reduced to the ferrous ion (Fe^{2+}) form and this may catalyze the generation of reactive oxygen species (ROS). High levels of ROS cause oxidative stress, which is harmful to cellular compounds such as lipids, DNA and proteins [3,4]. Thus, the maintenance of a balanced Fe metabolism is essential for the organism's homeostasis [2].

In order to avoid cell harm, organisms possess iron storage proteins (transferrins and ferritins) that keep Fe in their cavity and transport it where needed [2,3]. Ferritins are globular proteins composed of multiple subunits, with 24 equivalent subunits assembled into a "cage-like" oligomer [2]. In the ferroxidase center, they convert the Fe^{2+} into a nontoxic, soluble and biologically non-reactive ferric ion (Fe^{3+}). In vertebrates, ferritins have been classified in heavy (H) chain and light (L) chain subunits; in arthropods, ferritins are homologs to the vertebrate proteins (heavy-chain homolog, HCH, and light-chain homolog, LCH) [2,5]. The major difference is that H-chain ferritins possess the ferroxidase center and are capable of ferroxidase activity, while L-chain ferritins, lacking the ferroxidase center, are characterized by amino acid residues that induce nucleation of iron and may be responsible for the electron transfer across the protein cage [6]. A third type of ferritin, mitochondrial ferritin—a homopolymer with ferroxidase activity targeted to mitochondria—has

been identified in mammals and *Drosophila melanogaster* (ferritin 3, heavy-chain homolog, Fer3HCH), (reviewed in [5]). Although intensively studied in mammals and yeast, much less is known about the structure and function of ferritins in arthropods. Three ferritins have been identified in the fruit fly *D. melanogaster*: the cytoplasmic ferritin 1 (HCH), the ferritin 2 (LCH) and the mitochondrial ferritin [2]. Their role in dietary iron efflux or delivery was confirmed by knockdown experiments of either HCH or LCH ferritin subunits by RNAi, which resulted in the downregulation of the protein level of both subunits and in Fe accumulation in the midgut (reviewed in [5]). In the cladoceran *Daphnia pulex*, genome annotation and phylogenetic analysis revealed that ferritins clearly expanded compared with insects with a total of seven distinct ferritin *loci* [7].

Scanty information is available on the iron metabolism in zooplankton organisms, particularly in copepods; however, in these crustaceans, ferritins have been intensively used as biomarkers of stress. In the neritic calanoid species *Acartia tonsa*, an increase in transcriptional expression of ferritin has been reported in individuals after exposure to nano-contaminants such as Ni and CdSe/ZnS quantum dots [8], after acclimation to high pCO_2 conditions (1200 ppm) [9], and in response to infestation by the epibiotic euglenid *Colacium vesiculosum* [10]. Higher expression of a ferritin transcript was also reported in *A. tonsa* quiescent eggs compared with the subitaneous stage [11]. In the calanoid *Calanus finmarchicus*, ferritin has been suggested as a biomarker of stress associated with the diapause phase; a significant upregulation of ferritin was found in copepodites (CV) and females collected from deep water compared with individuals from the surface [12,13]. However, in all these studies, only a single ferritin gene has been used as a biomarker. In a recent study, genome analysis revealed the presence of a total of four ferritin genes (LsFer1–4) in the salmon louse *Lepeophtheirus salmonis*, with three encoding heavy chains (LsFer1,3,4) and one (LsFer2) encoding a light chain [14]. Based on these results, we might suggest that in copepods, the use of a single biomarker for ferritin might be limiting.

In consideration of the critical role of ferritin in Fe homeostasis, the aim of this study is to expand the understanding of the ferritin diversity and function in copepods. Mining the publicly available high-quality transcriptomes for several calanoid families [15–17], we examined the presence of transcripts encoding ferritins and compared them to homologous ferritins in *D. melanogaster* and in salmon louse *L. salmonis*. To provide a better understanding of the functioning of these genes, the expression of ferritins was examined using existing RNASeq data *in C. finmarchicus*, *Calanus helgolandicus* and *Temora stylifera*. Expression was examined across development (*C. finmarchicus*) [18] and after exposure to toxic algae (*C. finmarchicus, C. helgolandicus, T. stylifera*) [19–22]. This study expands the knowledge of the diversity of the ferritin family in copepods and suggests species-specific and stage-specific functional roles in these organisms. Our results shed light on the need to characterize the gene family of interest in particular when the genes are widely used as biomarkers in eco-physiological studies.

2. Materials and Methods

2.1. In Silico Workflow

Searches for putative transcripts encoding ferritins (HCH and LCH) were performed using a well-established vetting workflow that includes a mining step, a reciprocal blast and an examination of the protein structural domain [23,24]. Query sequences from the fruit fly *Drosophila melanogaster* (NP_524873, AAF07876, NP_572854) were used to mine the transcriptome shotgun assembly (TSA) database on the National Center for Biotechnology Information (NCBI), limiting the searches (tblastn algorithm) to Copepoda (taxid:6830) (search February 2023). For *Rhincalanus gigas*, raw reads were downloaded from NCBI [25] and assembled as described in [26]. Additional mining was performed using queries from the copepod *Lepeophtheirus salmonis* (order Siphonostomatoida) (BT121711, BT121232, MK887318, BT077723, BT121164) [14]. The results of both searches were compared and integrated.

All resulting transcripts were fully translated using ExPASY [27] and blasted against the NCBI non-redundant (nr) protein database (blastp algorithm) limited to Arthropoda

(taxid:6656). The presence of the expected protein structural domain for ferritin (Pfam:PF00210) was examined using SMART software [28]. In the species with multiple transcripts, amino acid sequences were aligned and amino acid identity was calculated between pairs. Sequences with ≥95% amino acid identity were considered the same protein, and among those the longest sequence was kept. A final alignment was performed for the copepod transcripts identified in this study encoding full-length proteins and having the expected ferritin domain, with HCHs and LCHs from *D. melanogaster*, *L. salmonis* and *Homo sapiens*, in order to verify the presence of amino acids characterizing the ferroxidase center [14]. Amino acid sequence alignment was performed using MAFFT software [29].

2.2. Cladogram of Copepod Ferritin Genes

A phylogenetic analysis was performed to confirm the annotation of transcripts identified in this study and to establish their relationship to each other, to other copepod species and arthropods. An unrooted phylogenetic tree was generated with amino acid sequences from this study, and sequences previously identified from the copepods *Acartia pacifica* and *Calanus sinicus* (order: Calanoida) [8], *Caligus rogercresseyi*, *C. clemensi*, *L. salmonis* (order: Siphonostomatoida) [14] and *Tigriopus californicus* (order: Harpacticoida) [30]. We also considered sequences from the insects *D. melanogaster* and *Anopheles aegypti*, homologs from *Homo sapiens* [14] and the water flea *Daphnia pulex* [7]. Among the seven ferritin sequences identified in *D. pulex*, two partial sequences (DQ983427, DQ983426) were excluded from the analysis. All sequences were first aligned using ClustalW software (Galaxy version 2.1), and then a maximum-likelihood phylogenetic tree was built using the evolution model Kimura computing bootstrap for 1000 samples (RapidNJ, Galaxy version 2.3.2).

2.3. Expression of Ferritin in Calanus finmarchicus, Calanus helgolandicus and Temora stylifera

Relative expression of transcripts encoding for ferritins was examined in the copepods *Calanus finmarchicus*, *Calanus helgolandicus* and *Temora stylifera* (order: Calanoida), using existing RNASeq data. In *C. finmarchicus*, the expression of ferritins was examined across development [18] and in females exposed to a toxic dinoflagellate [19]. For the developmental dataset, adult *C. finmarchicus* and stage CV copepodites were collected from the Gulf of Maine in 2012. Wild-caught females were laboratory maintained to obtain the target developmental stages: embryos, early nauplii, early copepodites (CI) and late copepodites (CIV). From each stage, total RNA was extracted from three biological replicates (two for CI and CIV), and cDNA libraries were multiplexed and sequenced on an Illumina HiSeq 2000 platform (PE 100 bp) [18]. The second dataset includes *C. finmarchicus* females exposed to the saxitoxin-producing dinoflagellate *Alexandrium fundyense* [19]. Briefly, adult females collected from the Gulf of Maine (Mount Desert Rock, 2012) were laboratory incubated over a week with a low dose (LD: 50 cell mL^{-1}) and high dose (HD: 200 cell mL^{-1}) of the toxic dinoflagellate and with the non-toxic cryptophyte *Rhodomonas baltica* (8000 cells mL^{-1}) as the control diet. Copepods were kept under 10 °C, on a 14:10 light:dark cycle and at two and five days, three biological replicates (15 females each) were harvested and processed for RNASeq. The two time points were chosen to test the hypothesis that the toxic algae would induce a detoxification response after two days that would persist over time (five days). Total RNA was extracted from each sample, followed by the generation of multiplexed cDNA libraries that were sequenced on an Illumina HiSeq 2000 platform (PE 100 bp) [19].

In *C. helgolandicus*, ferritin expression was derived from RNASeq data of laboratory-incubated females feeding on the oxylipin-producing toxic diatom *Skeletonema marinoi* (SKE) and the dinoflagellate control diet *Prorocentrum minimum* (PRO) for five days [20]. Briefly, females were collected in the Gulf of Naples (Central Tyrrhenian Sea, Western Mediterranean Sea, 2012) and laboratory incubated for five days with either *S. marinoi* (45,000 cells mL^{-1}) or *P. minimum* (5000 cells mL^{-1}) (three replicates per diet, 10 females each). Copepods were kept under 18 °C, on a 12:12 light:dark cycle, fed daily either with SKE or PRO and harvested after five days for RNASeq. Total RNA was extracted

from each sample followed by the generation of multiplexed cDNA libraries that were sequenced on an Illumina platform HiSeq (PE 50 bp) [20]. Lastly, the *T. stylifera* dataset included females collected in the Gulf of Naples during two consecutive weeks in May 2017 (test: 23rd and control: 30th) when low–high reproductive fitness was associated to high–low oxylipin content in the natural phytoplankton assemblage, respectively [21]. Phytoplankton-derived oxylipins were measured from surface water samples collected on the two dates, as described in [22]. Wild-caught *T. stylifera* females were immediately flash-frozen in liquid nitrogen and stored for RNA extraction. Three replicates (10 females each) were harvested on both weeks, extracted for total RNA, processed for cDNA library preparation and sequenced on a HiSeq 2500 platform (PE 100 bp) [21]. For each copepod species, expression levels were quantified by mapping the RNASeq libraries against their species-specific reference transcriptome using Bowtie and reads were normalized by length using the RPKM methods reads per kilobase per million mapped reads (RPKM). RPKM in each species were compared among transcripts and among stages or treatments using two-way ANOVA ($p < 0.0001$) followed by Tukey's post hoc test and multiple unpaired *t*-tests ($p < 0.05$). Statistical analyses were performed using GraphPad Prism, version 9 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Identification of Ferritins in Copepods

Ferritin-encoding transcripts were identified in 27 copepod species, including 22 from the order Calanoida, three from the order Harpacticoida and two from the order Cyclopoida (Table 1). Within the Calanoida order, transcripts were identified among different families, the majority within the Calanidae (e.g., *Calanus finmarchicus*, *C. helgolandicus*, *C. propinquus*, *Calanoides acutus*, *Neocalanus flemingeri*, *N. cristatus* and *N. plumchrus*), followed by the Temoridae (*Temora stylifera*, *T. longicornis*, *Epischura baikalensis*), the Acartiidae (*Acartia clausi*, *A. tonsa*), the Pontellidae (*Labidocera madurae*), the Pseudodiaptomidae (*Pseudodiaptomus annandalei*), the Centropagidae (*Centropages hamatus*) and the Rhincalanidae (*Rhincalanus gigas*) (Table 1). For all transcripts, reciprocal blast confirmed their annotation as a heavy chain (HCH) or light chain (LHC), with the majority being highly similar (top hit reciprocal blast) to homologs from *L. salmonis* (LS Fe1; BT121711), *Calanus sinicus* (APC62655) and *Eurytemora affinis* (XM_023489461). For the majority of the transcriptomes mined in this study, the resulting transcripts encoded full-length proteins with the typical conserved ferritin domain (Pfam00210; *p*-values < 0.05) (Table S1). Few partial transcripts, with no significant domain, were identified in *N. flemingeri* (2), *C. helgolandicus* (1), *C. hamatus* (1) and *C. acutus* (1), and these were not included in the downstream analyses.

Table 1. **Summary of ferritins in copepods.** For each copepod, genus, species, order, family and number of transcripts encoding ferritin were listed. The table includes only ferritins that were identified as full length that passed the reciprocal blast and protein domain analysis (Table S1).

Species	Order	Family	Ferritin Transcripts
Acartia clausi	Calanoida	Acartiidae	1
Acartia tonsa	Calanoida	Acartiidae	2
Calanoides acutus	Calanoida	Calanidae	1
Calanus finmarchicus	Calanoida	Calanidae	4
Calanus glacialis	Calanoida	Calanidae	1
Calanus helgolandicus	Calanoida	Calanidae	4
Calanus hyperboreus	Calanoida	Calanidae	2
Calanus marshallae	Calanoida	Calanidae	3
Calanus pacificus	Calanoida	Calanidae	4
Calanus propinquus	Calanoida	Calanidae	2

Table 1. *Cont.*

Species	Order	Family	Ferritin Transcripts
Centropages hamatus	Calanoida	Centropagidae	3
Epischura baikalensis	Calanoida	Temoridae	1
Eucyclops serrulatus	Cyclopoida	Cyclopidae	3
Hemidiaptomus amblyodon	Calanoida	Diaptomidae	1
Labidocera madurae	Calanoida	Pontellidae	1
Neocalanus cristatus	Calanoida	Calanidae	1
Neocalanus flemingeri	Calanoida	Calanidae	6
Neocalanus plumchrus	Calanoida	Calanidae	1
Paracyclopina nana	Cyclopoida	Cyclopettidae	1
Platychelipus ittoralis	Harpacticoida	Laophontidae	4
Pleuromamma xiphias	Calanoida	Metridinidae	1
Pseudodiaptomus annandalei	Calanoida	Pseudodiaptomidae	2
Rhincalanus gigas	Calanoida	Rhincalanidae	3
Temora longicornis	Calanoida	Temoridae	6
Temora stylifera	Calanoida	Temoridae	4
Tisbe furcata	Harpacticoida	Tisbidae	1
Tisbe holothuriae	Harpacticoida	Tisbidae	1

The number of transcripts encoding ferritins changed across copepods, ranging from one to a maximum of six. A single ferritin transcript was identified in 12 copepod species, whereas the highest diversification was found in *N. flemigeri* and *T. longicornis*, both having six different transcripts (Table 1). *C. finmarchicus*, *Calanus pacificus*, *C. helgolandicus* and *T. stylifera* showed four different ferritins. Lastly, three transcripts were identified in *Calanus marshallae*, *C. hamatus* and *R. gigas*.

Alignment of the copepod sequences identified in this study with ferritins from *D. melanogaster*, *H. sapiens* and *L. salmonis* showed that 57 out of 64 transcripts identified in this study conserved all the amino acids of the ferroxidase site in HCH from the reference sequences (Figure S1). The exception to this is the single ferritin from *C. finmarchicus* (GAXK01169093), *C. helgolandicus* (GJFL01003552) and *N. flemingeri* (GFUD01021847), and two ferritins from *T. longicornis* (GINW01248260, GINW01100697) and *C. pacificus* (GJQY01004559, GJQY01232255). While in the *N. flemingeri* and *C. pacificus* ferritins only a few substitutions were observed, in the other sequences five (out of seven) of the amino acids making the ferroxidase center were not highly conserved; both *C. finmarchicus* and *C. helgolandicus* showed in all the sites the same substitutions (Figure S1). The amino acid variability found in the ferroxidase center for *C. finmarchicus*, *C. helgolandicus* and *T. longicornicus* sequences might suggest that these ferritins belong to the LCH category, although further analyses are needed.

The unrooted tree generated from a total of 100 transcripts showed that ferritins clustered into several phylogenetic groups (Figure 1). LCH clustered in a separate group (Figure 1 light blue, bootstrap 98) including LCHs from *D. melanogaster*, *Aedes aegypti*, the copepods *C. clemensi* and *L. salmonis* (previously known), two *T. longicornis* transcripts, one from *C. finmarchicus* and one from *C. helgolandicus* (this study). The sequences in this group were the ones showing changes in the ferroxidase center in Figure S1. HCH ferritins are separated into several groups. The largest group (Figure 1 yellow, bootstrap 85) included known HCHs from the insects *D. melanogaster* and *A. aegypti*, the cladoceran *D. pulex*, the copepods *L. salmonis*, *C. clemensi*, *C. rogergressery*, *E. affinis*, *T. californicus* and 17 sequences identified in this study; these included ferritins from several species such as *C. marshallae*, *N. flemingeri*, *L. madurae*, *A. clausi* and *A. tonsa*, *P. annanadalei*, *P. nana*,

T. longicornis and *T. stylifera* (two out of four). The second largest group (Figure 1 green, bootstrap 81) included only ferritins identified in this study (23) from *C. finmarchicus* (two out of four), *C. helgolandicus* (one), *C. marshallae*, (two out of three), *N. flemingeri* (three out of six), *T. furcata* and *Tisbe holoturiae*, *P. annandalei* and *Pleuromamma xiphias*. The remaining HCHs clustered in smaller groups. Among those, there was a small group including all *D. pulex* sequences (four out of five) and a single ferritin from *C. propinquus* (Figure 1, orange, bootstrap 58), and another group including six *T. longicornis* ferritins and two *H. sapiens* homologs (Figure 1, purple, bootstrap 51). None of the ferritin identified in this study clustered with the *D. melanogaster* mitochondrial ferritin which was separated from all other sequences.
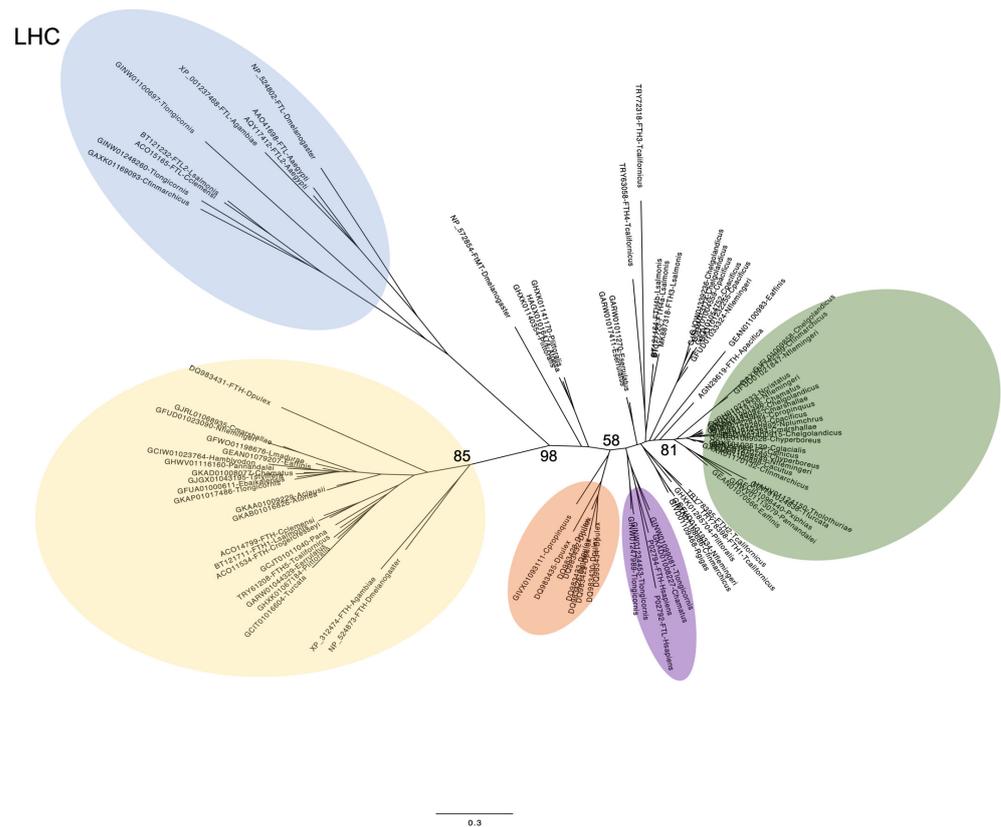
Figure 1. Cladogram of ferritin-related genes identified in this study. The analysis includes sequences from this study (Table 1) from the insect *D. melanogaster*, the crustacean *D. pulex* and other copepods previously identified (see text). For the analysis, amino acid sequences were aligned using ClustalW and then a maximum-likelihood phylogenetic tree was built using the evolution model Kimura computing bootstrap for 1000 samples (RapidNJ, Galaxy version 2.3.2). Colors refer to the groups described in the text. Bootstrap is only indicated for the groups described. Scale bars: 0.3 estimated substitutions per site. LCH: light chain subunit.

3.2. Expression of ferritin in C. finmarchicus, Calanus helgolandicus and Temora stylifera

The expression of ferritin-encoding transcripts significantly changed in *C. finmarchicus* across the different developmental stages; a significant difference was also found among the different transcripts in each stage (two-way ANOVA, $p < 0.001$) (ta 2). Two ferritins, the HCH (GAXK01142559) and the LCH (GAXK01169093), showed a similar and low expression in all developmental stages, whereas HCH ferritin (GAXK01168686) was always highly expressed in copepodites (C1-C5) compared to embryos, copepodites and adults (568 RPKM vs. 82 RPKM, on average; Tukey's multiple comparison test, $p < 0.0001$); similarly, the other HCH transcript (GAXK01170132) showed significantly higher expression in the later developmental stages (500 RPKM, on average, in C4 and AF), with a significant peak in expression in the C5 stage (Tukey's multiple comparison test, $p < 0.0001$) (Figure 2).
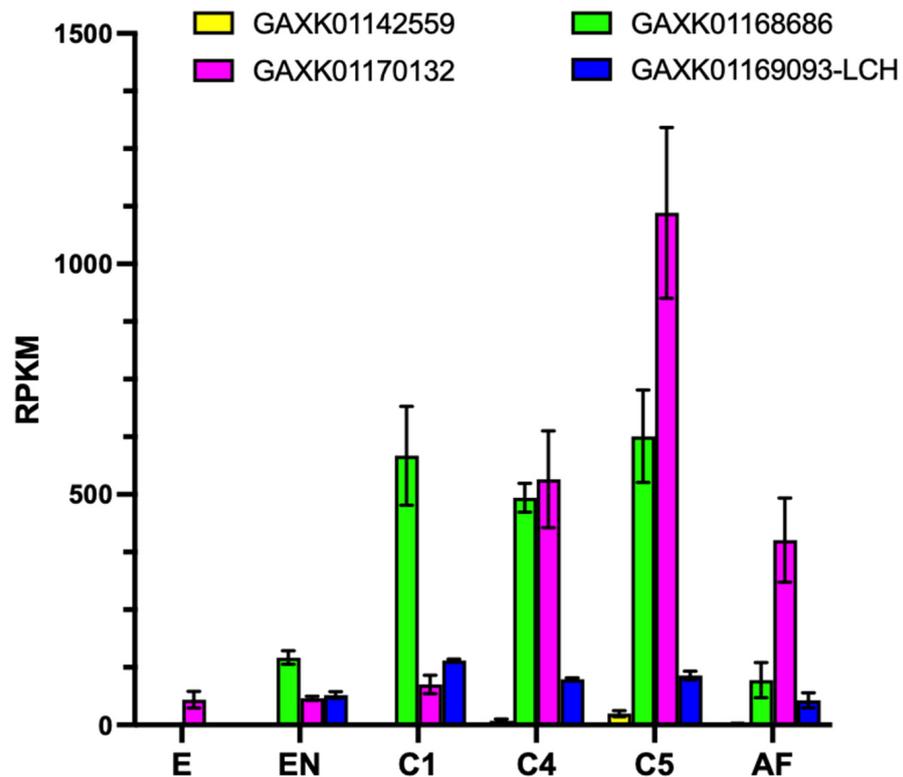
Figure 2. Relative expression of ferritin transcripts in *C. finmarchicus* across development. Relative expression normalized by length (RPKM) across six developmental stages: embryos, early nauplii (NII-NIII), copepodites I, IV and V (C1, C4 and C5), and adult females (AF). Bars are mean ± standard deviation (*n* = 3 replicates, *n* = 2 C1 and C4). Colors indicate different transcripts and their NCBI accession number.

No significant differences were found in the expression of all ferritins in *C. finmarchicus* females feeding for two days on the toxic *Alexandrium fundyense* at different concentrations (low and high dose) compared to control conditions (Figure 3a,b). However, in spite of treatments and time points, the expression of the different transcripts was significantly different (two-way ANOVA, *p* < 0.0001). At two days, similarly to the previous dataset, two transcripts showed a null or very low expression in all treatments, whereas the transcripts GAXK01168686 and GAXK01170132 were significantly more abundant (average 300 RPKM) compared to the other two (Tukey's multiple comparison test, *p* < 0.001) (Figure 3a). The same results were observed after five days of exposure, with the same two transcripts (GAXK01168686 and GAXK01170132) being significantly highly expressed in all conditions compared with the other two (Tukey's multiple comparison test, *p* < 0.001) (Figure 3b).

A similar result was also found in *C. helgolandicus*, where expression of the four ferritins did not change significantly between females feeding the toxic *Skeletonema marinoi* diet and the control treatment *Prorocentrum minimum*. However, similar to what was observed in *C. finmarchicus*, in spite of the treatments, one HCH ferritin (GJFL01006140) and the LCH ferritin (GJFL01003552) showed a significantly high expression (up to 20-fold and 60-fold difference) compared with the other two transcripts (Figure 4a). In *T. stylifera* females collected from the field during different weeks, the same transcript-dependent expression was observed: one transcript (GJGX01146428) showed ca. 100-fold higher expression compared to the other three; the same transcript was also significantly upregulated in females exposed to a high content of harmful oxylipins (test) (multiple unpaired *t*-test, *p* < 0.05) (Figure 4b).
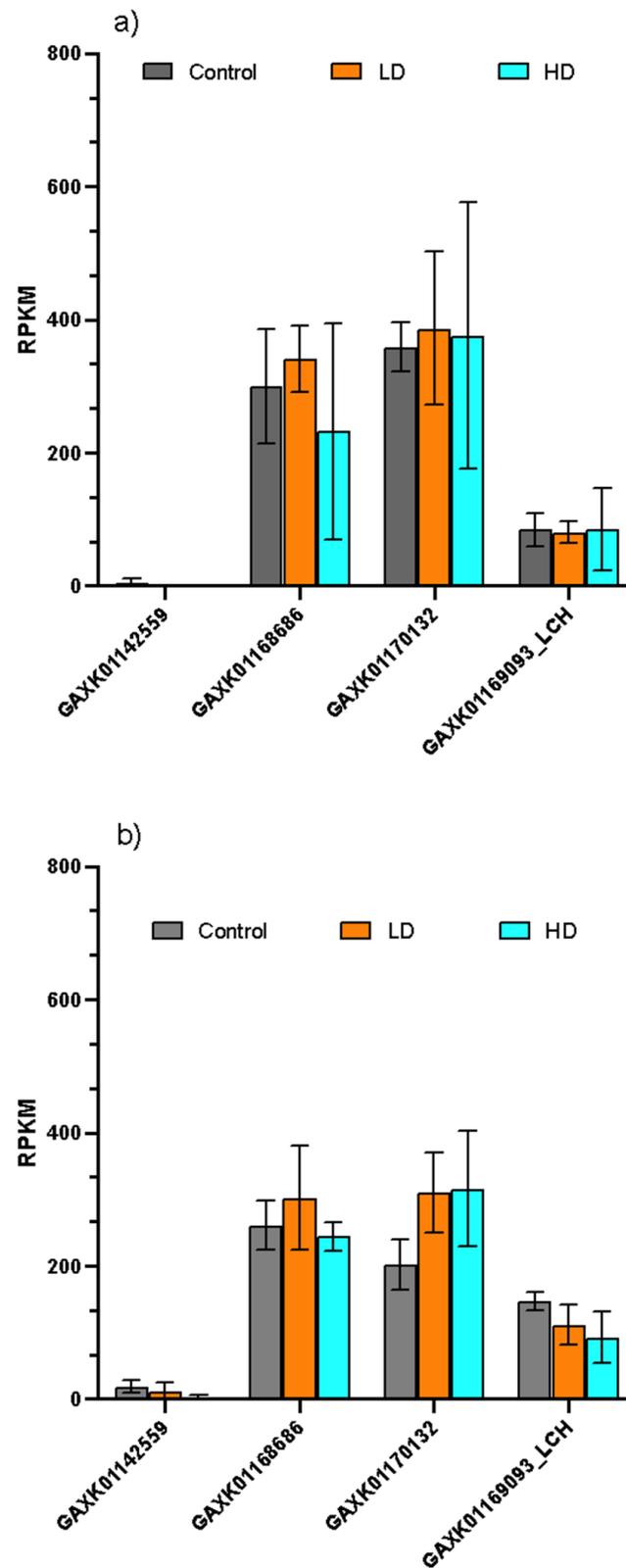
Figure 3. Relative expression of ferritin transcripts in *C. finmarchicus* feeding on a toxic diet. Relative expression normalized by length (RPKM) in *C. finmarchicus* females fed the non-toxic cryptophyte *Rhodomonas baltica* (control) and the toxic dinoflagellate *Alexandrium fundyense* at low (orange) and high (cyan) doses, for 2 (**a**) and 5 (**b**) days. Different names indicate different transcripts. Bars are mean ± standard deviation (*n* = 3 replicates).
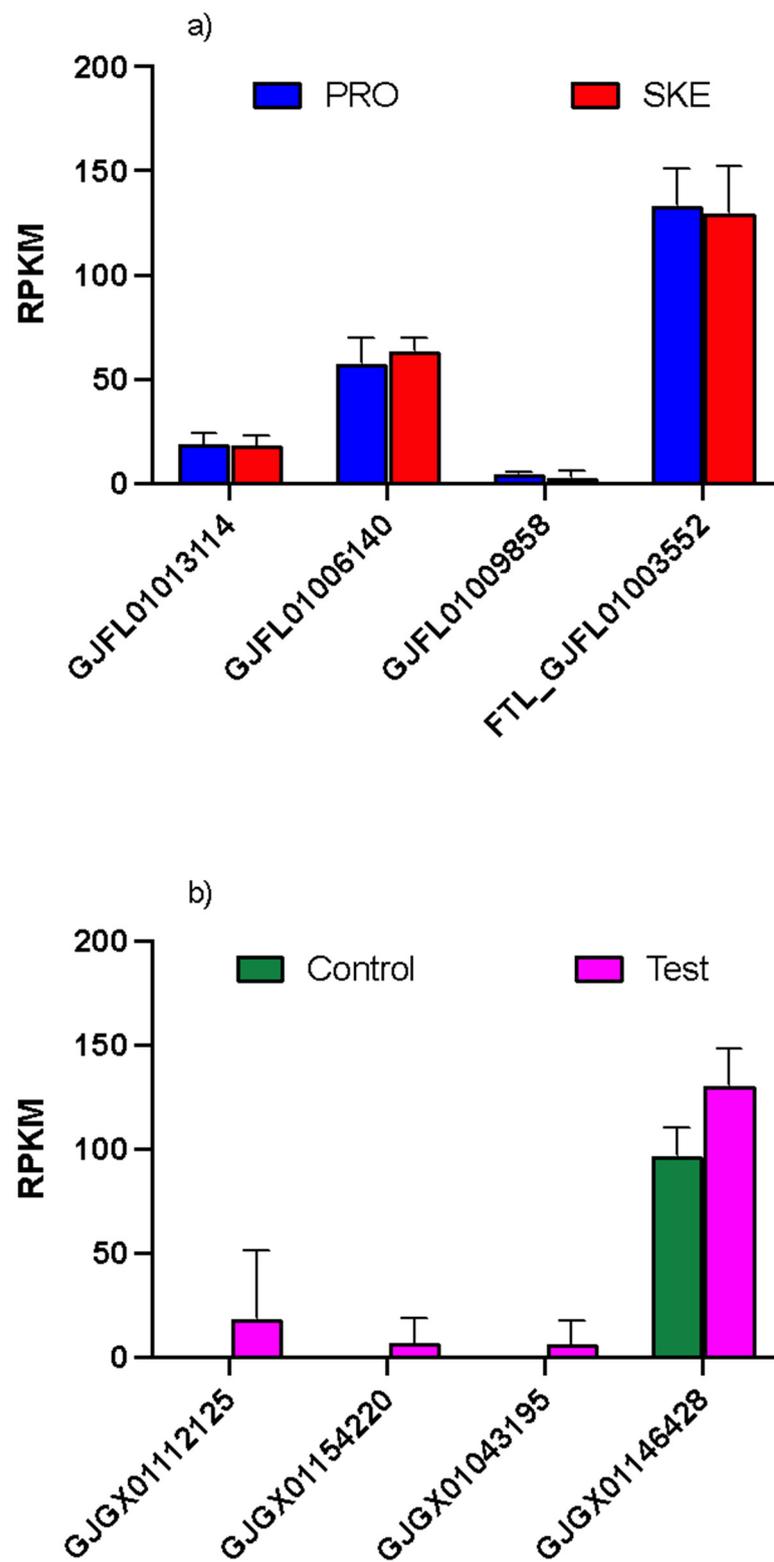
Figure 4. Relative expression of ferritin transcripts in *C. helgolandicus* and *T. stylifera*. (**a**) Relative expression normalized by length (RPKM) in *C. helgolandicus* females fed the dinoflagellate *Prorocentrum minimum* (PRO) and the toxic diatom *Skeletonema marinoi* (SKE) for 5 days. (**b**) Relative expression normalized by length (RPKM) in *T. stylifera* collected in the Gulf of Naples when low (control) and high (test) content of harmful oxylipins were measured in the phytoplankton. Different names indicate different transcripts. Bars are mean $\pm$ standard deviation (n = 3 replicates).

4. Discussion

The evolution of life on Earth has been dependent on the availability of iron, leading to the evolution of ferritins, multimeric iron storage proteins that are ubiquitous in the living kingdom [1]. Most cells express ferritin genes, but protein concentration can vary among cell types. Ferritin sequence and structures are highly conserved among plants and animals, suggesting evolutionary convergence in eukaryotes, while bacterial ferritins diverge in sequence but not in structure (as reviewed in [31]).

Ferritins are also present in copepods, the most abundant metazoans on the planet [32]. Gene expression changes in ferritin have been reported in studies in the literature (Table 2) when copepods are exposed to abiotic stress, such as metals [8,33], heat shocks [10], high pCO$_2$ levels [9] or water-accommodated fractions [34]. Changes in gene expression have also been recorded in response to biological drivers, namely, infestation by an epibiotic parasite [10], during different phases of dormancy, such as induction and recovery from quiescence [11], or during the diapause "stationary" phase [12,13]. In addition, it has been reported an impairment of feeding and reproduction upon the knockdown of ferritin, as well as a reduction in the protein level during starvation [14]. Interestingly, no changes in ferritin expression were associated with different crowding levels [35]. Overall, the reported differences in the regulation of ferritins opened questions related to stressor-specific responses associated with different transcripts or species-specific responses.

Table 2. Summary of ferritin gene expression changes in copepods reported in the literature and our study. For each study, the species tested, the stressor and the regulation (+ = upregulation, − = downregulation and ns = non-significant change) of the ferritin gene are shown.

Species	Stressor	Regulation	Reference Paper
P. annandalei	Ni nanoparticles	+	33
A. tonsa	Ni nanoparticles	+	8
	CdSe/ZnS quantum dots	+	8
	Heat shock (salinity dependent)	+	10
	Infestation *C. vesiculosum*	+	10
	Entry/emergence quiescence eggs	+	11
	Crowding	ns	11
	High pCO^2 (only costal)	+	9
	WAF	ns	34
Calanus spp.	Diapause (CV females)	+	12 13 42
L. salmonis	Starvation	−	14
C. finmarchicus	Diapause preparation (CV) vs. reproductive program	ns	45
Our study			
C. finmarchicus	Copepodites	+	
	A. fundyense	ns	
C. helgolandicus	Oxylipin (lab)	ns	
T. stylifera	Oxylipin (field)	+	
N. flemingeri	Diapause emergence	ns	

Indeed, the role of ferritin in the metabolism and physiology of copepods is presently understudied. Based on the relatively sparse available evidence (as summarized in Table 2), we speculate about different mechanisms potentially at play, which may differ not only among stressors but also among species. In this context, we interpret our results also by direct comparison with insect ferritins, considering their closeness in terms of absolute success [36].

Insect ferritins can be divided into three groups depending on their structure, location and function [37]. HCH and LCH differentiate based on the presence in HCH of the ferroxidase center, which confers the role of reducing the ferrous ion (Fe^{2+}) to its non-reactive ferric ion (Fe^{3+}). Insects also present a third type of protein, mitochondrial ferritin (HCH). Endoplasmic reticulum ferritins (HCH) act as iron transporters, mitochondrial ferritins act as antioxidants, while hemolymph ones (LCH) have a dual function [37]. In copepods, a full-length transcript encoding ferritin HCH has been identified in all 27 mined transcriptomes, with 50% of the species possessing multiple transcripts. Consistently with what was reported in the genome of *Lepeophtheirus salmonis* [14], mitochondrial ferritins were not found in any of the mined copepod transcriptomes. As opposed to insects [37], the location of ferritins has not been explored yet in copepods. From our results, mining transcripomes generated from whole or even pool of individuals, it is not possible to infer the specific location of the identified ferritins. Nonetheless, by analogy with insects, we can speculate that copepod ferritins are associated with the endoplasmic reticulum (HCH) and the hemolymph (LCH).

Arthropod immunity is based solely on an innate system [38]. In this framework, ferritin plays a key role, acting as a stress-induced protein and permitting the accumulation of iron which is no longer available to pathogens [38]. This process is compliant with the upregulation of ferritin in *Acartia tonsa* infested by the epibiotic euglenid *Colacium vesiculosum* [10]. Blood-sucking arthropods need further protection to reduce the risks associated with the massive ingestion of iron and heme [39]. In the yellow fever mosquito *Aedes aegypti*, ferritin expression is upregulated upon iron uptake, likely as cytotoxic protection [39,40]. Consistently, in the parasitic copepod *L. salmonis* ferritin levels in the midgut decrease during starvation [14], evidencing the direct link between gene expression and blood-feeding.

Involvement of ferritin has also been suggested during dormancy in the fly *D. melanogaster*, in the crustacean branchiopod *Artemia* sp., and in the copepods *C. finmarchicus* and *A. tonsa*. High expression of ferritins in *Artemia* sp. and *A. tonsa* embryos was hypothesized as preventing embryogenesis to continue and inhibiting development during the resting state [11,41]. Indeed, an increase in ferritin corresponds to higher chelation of iron stores and a reduction of iron available for processes such as embryogenesis [42]. Consistent with its role as an enhancer of stress resistance, high expression of ferritin has been found in both *D. melanogaster* and *C. finmarchicus* (C5, females) when at diapause [12,13,42,43]. Diapause, a type of dormancy, is characterized by a delay in development, a decrease in metabolism and an increase in stress resistance [44]. In *Calanus* spp., it has been reported that ferritin expression has its highest value during the early stages of diapause and it decreases at the beginning of the maintenance phase [12,13,42]. However, in another study, ferritin was not found among the genes differentiating C5s preparing for diapause from the ones on the reproductive program [45]. Furthermore, it seems that the ferritin role could be species-specific, as suggested by the fact that in *Neocalanus flemingeri* diapausing females ferritins were not among the genes characterizing the diapause phenotype [46].

In six out of the eleven papers published in the literature on copepod ferritins, the target species is the calanoid *A. tonsa* [8–11,34,35]. A renowned global-scale invasive species (e.g., [47,48]), *A. tonsa* is an established model organism in ecotoxicology studies (e.g., [49]) and it has proven valuable as feed for fish larvae in mariculture (e.g., [50]). The remaining papers surveyed are centered on two pelagic (*Calanus* spp.: [12,13,42]) and one parasitic (*L. salmonis*: [14]) species, relevant for their ecological (*Calanus* spp.) and economic (*L. salmonis*) impacts. Ferritin expression studies in these species allow the development of

sensitive biomarkers for marine pollution monitoring, but also for the implementation of ecological indicators. The present study surveys the ferritin distribution in 27 copepods from the Calanoida, Harpacticoida and Cyclopoida orders, and it explores the variability of ferritin expression in three key ecological species: *C. finmarchicus*, *C. helgolandicus* and *T. stylifera*. Our study highlights the fact that the use of a single transcript as a biomarker can be highly limiting. In more than 50% of the examined copepods, in fact, we found more than one transcript encoding ferritin and these included *C. finmarchicus*, *C. helgolandicus* and *T. stylifera*. Moreover, our expression studies highlighted the fact that in spite of the stressor or developmental stage, two transcripts (out of four) were always more abundant compared with the others in all three species. Most protein-coding genes have dominant transcripts that are expressed at a considerably higher level than any minor transcripts across different conditions [51,52]. It is likely that these dominant transcripts are the main contributors to the proteome of the individual; thus, they might be used as good candidates for physiological and ecological studies on copepods.

Based on our results, we suggest that the role of ferritin in stress is still unclear. Changes in the expression of ferritins were only found in *T. stylifera* in response to different levels of the cytotoxic oxylipins in the natural phytoplankton assemblage; in contrast to the two *Calanus* species exposed to toxic algae, expression of ferritin did not change compared with a control diet. However, in *C. helgolandicus,* exposure to the harmful *S. marinoi* was associated with the upregulation of detoxification (GSTs), protein repair (HSP60 and HSP70), and immune system (prophenoloxidase activating enzyme, PPAE) genes [20]. Thus, we can speculate that the lack of a significant regulation of the ferritins could be associated with the fact that the concerted over-expression of detoxification genes could be sufficient to protect the copepod from the direct toxic effect of the diatom. In contrast, in *T. stylifera,* a high concentration of oxylipins induced the downregulation of stress response and oxidation–reduction genes [21]. Therefore, the upregulation of ferritin transcripts in *T. stylifera* could act as a compensatory defensive mechanism protecting the copepods from the oxidative damage and apoptosis associated with the ingestion of oxylipins [48]. Concerning *C. finmarchicus*, the differences in the expression found across development and not in response to the toxic algae could suggest a role of ferritin as a developmental marker. Two dominant ferritins were always poorly expressed in embryos and nauplii, compared to copepodites, confirming that iron mobilization due to low ferritin expression might stimulate embryogenesis and early larval development in copepods [43]. These ferritin-encoding dominant transcripts, thus, could be used as potential biomarkers of early–late development transition in *C. finmarchicus*.

5. Conclusions

Ferritins are the major intracellular iron storage proteins, highly conserved and present in most prokaryotes and eukaryotes including fungi, algae, plants, and animals [1]. Their expression in copepods is regulated by numerous endogenous and exogenous factors and may also present species-specific modulations. Our study expands the knowledge on the ferritin diversity in copepods; in all species, we found a high chain ferritin (HCH) and we confirm the lack of mitochondrial ferritin in crustaceans. We suggest that copepod ferritins can be involved in multiple processes such as iron storage, larval development and stress, highlighting that ferritin regulation can be species-specific, stressor-specific and stage-specific.

and homologs from *H. Sapiens* (P02794, P02792). Copepod sequences are in alphabetical order as in Table 1. Light chain subunits (LCH) are indicated in green. Amino acids that make up the ferroxidase center of the heavy chain in *H. Sapiens* are bolded in red. Highlighted in yellow are the amino acids which were not conserved.

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