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RESEARCH PAPER

Molecular Characterization and Expression of Calpains and Cathepsins in Tilapia Muscle in Response to Starvation

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Abstract

Calpains are a class of calcium-dependent, non-lysosomal proteins. Two main calpain isoforms are expressed in skeletal muscle, m-calpain, and mu-calpain, which exert critical proteolytic activities related to muscle functions, muscle atrophy, and myogenesis. Cathepsins are lysosomal acid proteases. The main cathepsin isoforms implicated in muscle aging and atrophy are cathepsins B, L, and H. This study characterized calpain1, calpain2, cathepsin L, cathepsin B, and cathepsin H genes in the tilapia genome. In addition, the expressions of these genes were examined in the muscle tissues of starved versus fed; and refed versus control tilapia, *Oreochromis niloticus*. qPCR expression data showed that Calpain1 and 2 catalytic subunits and the regulatory subunit were significantly higher in starved compared to the fed tilapia. Similarly, cathepsin L, B, and H showed significant upregulation in the starved compared to the fed fish. Besides, calpain and cathepsin L enzymatic catalytic activity increased in the starved fish relative to the fed fish. The results indicate that the studied genes are involved in atrophying muscle and are considered to have a potential role as markers of tilapia muscle accretion.

Introduction

Fish is a good source of proteins and vitamins for the human body. Many countries are interested in increasing aquaculture production. Among them are many developing countries where increasing aquaculture production and profitability is an important goal for food security and social well-being. Tilapia is the second most important cultured fish globally after carp. The global aquaculture industry has been increased. Seventy percent of tilapia farming production comes from Nile tilapia (*Oreochromis niloticus*). The multiple introductions of several tilapia species, tilapias hybridization, and poor hatchery management complicate the genetic improvement of tilapia for aquaculture, particularly in developing countries (Mair et al., 1995).

Muscle is the essential edible part of fish; thus, muscle accretion is vital for the profitability of fish farming. Muscle growth/degradation is controlled by many factors involving highly orchestrated catalytic enzyme systems. The combination of the effects of lysosomal enzymes, ubiquitin-proteasome pathway, and calpain proteinases controls muscle degradation. Calpains, when activated, break down cytoskeletal proteins to release myofibrils for the ubiquitin-proteasome pathway (Zhu et al., 2017). It was suggested that calpain proteinases cooperate in the early stages of degradation while the other pathways proceed at late stages (Kumamoto et al., 1995). Calpains are calcium-activated cysteine proteases with three main subtypes (calpain1, calpain2, and calpain regulatory subunit) in muscle tissue. Calpain enzymes have specific catalytic domains (Bartoli et al., 2005). Calpain1 (μ-calpain) and

calpain2 (m-calpain) have four conserved domains: N-terminal domain, catalytic protease core domain (CysPc) domain, C2-like domain, and Penta-EF-hand (PEF) domains as previously reported in human (Ono et al., 2012). Human calpains need the small calpain subunit to be activated by Ca^{2+}, while it can be inhibited by calpastatin (Ono & Sorimachi, 2012).

They may be released into the cytoplasm and intracellular space because of lysosomal disorders or a pH fall after cell death.

The lysosomes are acidic cytoplasmic organelles that contain more than 50 acid hydrolases, including cathepsins. These hydrolases can degrade numerous biological macromolecules, suggesting a nonspecific role in intracellular degradation. cathepsins may be released into the cytoplasm and intracellular space because of lysosomal disorders or a pH fall after cell death. However, the localization of cathepsins outside the cells may indicate their involvement in specific functions. For example, cathepsins have a significant role in degrading essential neuron proteins (Stoke et al., 2001). Additionally, cathepsins are among the lysosomal cysteine proteases highly expressed during muscle atrophy (Deval et al., 2001) and have an essential role in protein turnover (Uchiyama et al., 1994). These enzymes usually are released in the cytoplasm and intracellular space and induce post-mortem degradation (Yates et al., 1983). Previous work on rainbow trout suggested that the cathepsins activities in rainbow trout are not affected by starvation (Salem et al., 2007). However, another study on rainbow trout recorded elevation in cathepsin D and other lysosomal enzymes under the effect of starvation (Martin et al., 2001).

The roles of the calpain and cathepsin enzymes in tilapia muscle degradation are still unclear. Therefore, this study was conducted, in order to determine and characterize the genes that code for the calpain proteinases and cathepsin lysosomal enzymes in tilapia and analyse their expression/activity under starvation conditions.

Materials and Methods

Starvation Experiment

The study was carried out at Middle Tennessee State University (MTSU) according to the institutional animal care and use committee's (IACUC) rules (protocol number 17-3008). As previously mentioned (Shaalan et al., 2019), fish (*Oreochromis niloticus*) fingerlings weighing 1.4g were obtained from Allen's company and acclimated for 15 days before beginning the experiments. Fish were randomly assigned to four experimental groups: 1) A starved group, the feed was withheld for two weeks; 2) a fed fish group, fed with a commercial diet (from Allen's company) for 2 weeks; 3) refed fish group, fish were starved for two weeks then fed for another 2 weeks; 4) control fish group, continuously fed for four weeks. Each group consisted of triplicate tanks of 18 fish/ tank. The parameters of the measured water quality were kept at temperature= 28–30°C, pH = 7–7.2, ammonia = 0– 0.25, nitrite = 0, and nitrate = 0. At the end of the experiments, overdose of MS222 (Syndel USA, Ferndale, WA) was used to euthanize fish (Shaalan et al., 2019). Tissue samples were immediately preserved in liquid nitrogen and maintained at -80°C.

RNA Isolation

TRIzol reagent (Invitrogen, Carlsbad, CA, cat: TR 118) was used to isolate total RNA from each fish muscle tissue sample for the starvation experiment. In addition, for the tissue distribution study, RNA was extracted from skin, gills, heart, liver, stomach, intestine, brain, kidney, eye, red muscle, and white muscle (7 fish per group). Nanodrop (Thermo Scientific, Hudson, NH) was used to measure the concentration of the samples at 260 nm. Gel electrophoresis was used to assess the integrity of the RNA. A verso cDNA synthesis kit (Thermo Scientific, Hudson, NH, USA, cat# AB-1453/B) was used to synthesize a complementary DNA (cDNA) according to the manufacturer's instructions and using a random primer. The cDNA synthesis kit's buffer was used to eliminate DNA contamination. Nanodrop was used to determine the concentration of cDNAs.

Real-time RT-PCR

Primer3 software (version 0.2.0) was used to design primers for the studied genes as seen in Table 1 (Koressaar & Remm, 2007). Bio-Rad iCycler PCR detection system was used for measuring gene expression. A master mix of SYBR green (Thermo Scientific, Hudson, NH, USA) was added to the cDNA templates at a concentration of 0.006 g/l. The forward

Table 1. Forward and reverse primers used in qPCR analyses of each gene.

Gene name/ NCBI accession	Forward primer	Reverse primer
Calpain1 (XM_003447550.3)	GATCCGAAAGTGGCTGGTCA	TTCCACAGCAAGTCGCATCT
Calpain2 (XP_003460107.1)	AACTCGATGACGAGACCGTG	ATGTCACTTCCGGCGAGTTT
Calpain regulatory subunit (XM_003449799)	CGAGCTGATGAACATCCTGA	TGACTGCTACCATGCTCCTG
Calpastatin (XM_013269965.2)	AGGCAAAGTGACGAAGGAGA	TGGTTTTGAAGGAGGCAGTT
Cathepsin L (XP_003444531.1)	CAGCTCATGAACGGCTACAA	CCTCCAGTCCAGAGAACGAG
Cathepsin B (XP_003454569.1)	AGGATCTGCTGACCTGCTGT	AGTCCTTCACTTGCCCAGAA
Cathepsin H (XP_003440460.1)	TCAACAACCATGGATGCAAT	ACCCTCAAAAGCCGTGTATG
β – actin (XM_003455949)	TCTCGGCTGTGGTGGTGAA	GACCCACACAGTGCCCATCT

and reverse primers concentration was µM/µl (Ali et al., 2018). During the first denaturation stage, the cycling procedure was performed at 95°C for 7.00 minutes. Following that, each gene was subjected to 40 cycles of amplification that included denaturing at 95°C for 0.1 min, annealing at 57–64°C, and a final extension step at 60°C for 5 min. The internal control for normalization was the tilapia β -actin gene (Yang et al., 2013). The 2^-ΔΔCT formula was used to calculate relative gene expression between each treatment and control samples (Panetu et al., 2018).

Protein Extraction and Enzyme Activity Measurements

The muscle samples were prepared by homogenization in phosphate buffer saline (PBS, pH 7.4) with 1% triton and centrifugation for 20 min at 10,000 rpm to preserve the supernatant (Fareed et al., 2006). A Pierce BCA protein assay kit was used under its guidelines (Thermo Scientific, Rockford, lL, USA) to determine protein concentration for each sample.

The calpain activity was measured using the substrate of BODIPY FL-casein (4,4-difluoro-5,7-dimethyl-4-bora3a,4a-diaza-s-indacene-3-propionic acid-labeled casein). The reaction buffer was prepared by mixing 20mM Tris/HCl (pH7.5), 1mM Ethylenediaminetetraacetic acid (EDTA), 100mM KCl, 1mM Dichlorodiphenyltrichloroethane (DTT), and 10mM $CaCl_2$. The substrate used was 4.6 µg/ml of BODIPY FL-casein in a total volume of 220µl. One time incubation was done in the presence of calcium and one time in the absence of calcium and the existence of 10 mM EDTA. Fluorescence produced was measured at an excitation of 485 nm and emission of 520 nm at 26°C. Calpain enzyme activity was calculated by the difference between the measured activities in the presence and absence of calcium and the presence of EDTA.

Measuring activity of cathepsin L was done using the fluorescent substrate Z-Phe-Arg-NMec. The hydrolysis of this substrate leads to the liberation of 7-amino-4methylcoumarine. The reagents used were prepared as mentioned in a previous study (Barret & Kirschke., 1981). The buffered activator was prepared using 340mM sodium acetate, 60mM acetic acid, and 4 mM disodium EDTA (pH 5.5). 8 mM dithiothreitol was added to the mixture shortly before usage. The substrate solution was prepared by dissoling a 1mM solution of Z-Phe-Arg-NMec HC1 in dimethyl sulfoxide (DMSO) and then stored at 4°C. The working substrate solution used in the reaction was prepared to have a concentration of 20 µM. The stopping reagent was prepared by mixing 100 mM sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid (pH 4.3). 125µl of diluent was added to the mixture of 31 µl buffered activator and the sample (0.3-3 ng). The mixture was incubated at a temperature of 30°C for 1 min to activate the enzyme. After that, 31µl of the substrate solution was added and incubated for 10 min before adding 250 µl of the stopping solution. A blank reaction was prepared by adding the stopping solution before adding the sample. A spectrophotometer was set to 370 nm for excitation and 460 nm for emission, and the fluorescence was determined. The enzyme activity was measured and normalized to the protein concentration.

Molecular Characterization of the Genes and Phylogenetic Analysis

The identities of the tested genes were identified using the Blastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) bioinformatics tool on the NCBI website. Homologous gene sequences from the annotated genome of rainbow trout were used to find the homologous tilapia calpain and cathepsin genes. ClustalW software was used for protein sequence alignment (https://www.ebi.ac.uk/tools/msa/clustalw2/). to calculate the best match of the tested genes against homologous sequences from other species. The results of the alignments were copied to BoxShade software (version 3.21) to prepare the figures showing protein similarities, identities, and differences between sequences. The Interpro tool (Mitchell et al., 2019) was used to predict the domains of each studied gene (http://www.ebi.ac.uk/interpro/). Phylogenetic analysis was performed using the MEGA7 program (version 7.0.26) for molecular and evolutionary analysis (Kumer et al., 2016).

Results

Starvation and Refeeding Effect on Body Weight Measurements

The changes in average body weight were reported previously (Shaalan et al., 2019). After two weeks of starvation, the average body weight was 0.839±0.154 g for the starved group, which was statistically different versus 5.621±0.942 g for the fed group. For the refed group, the mean body weight was 3.236±0.332 g, while the control group was (7.879±1.039 g).

Relative Gene Expression of Calpain and Cathepsin Genes

The abundance of genes encoding for calpain1, calpain2, calpain regulatory subunit, calpastatin, cathepsin-L, cathepsin-B, and cathepsin-H were measured by Real-time PCR. The expression of calpain1 was significantly higher in starved fish by 10.39 folds in comparison with the fed fish. Similarly, calpain2 showed a 2.94 -fold increase (P≤0.05). Calpain1 and calpain2 showed insignificant changes in the refed fish vs. control (P≥0.05, Figure 1a/b). Calpain regulatory subunit showed a significant increase by 13.34-fold in the starved fish over the fed fish, while it was not recorded a significant difference between the refed and the control fish groups (Figure 1c). The calpastatin gene showed a significant decrease in abundance in both
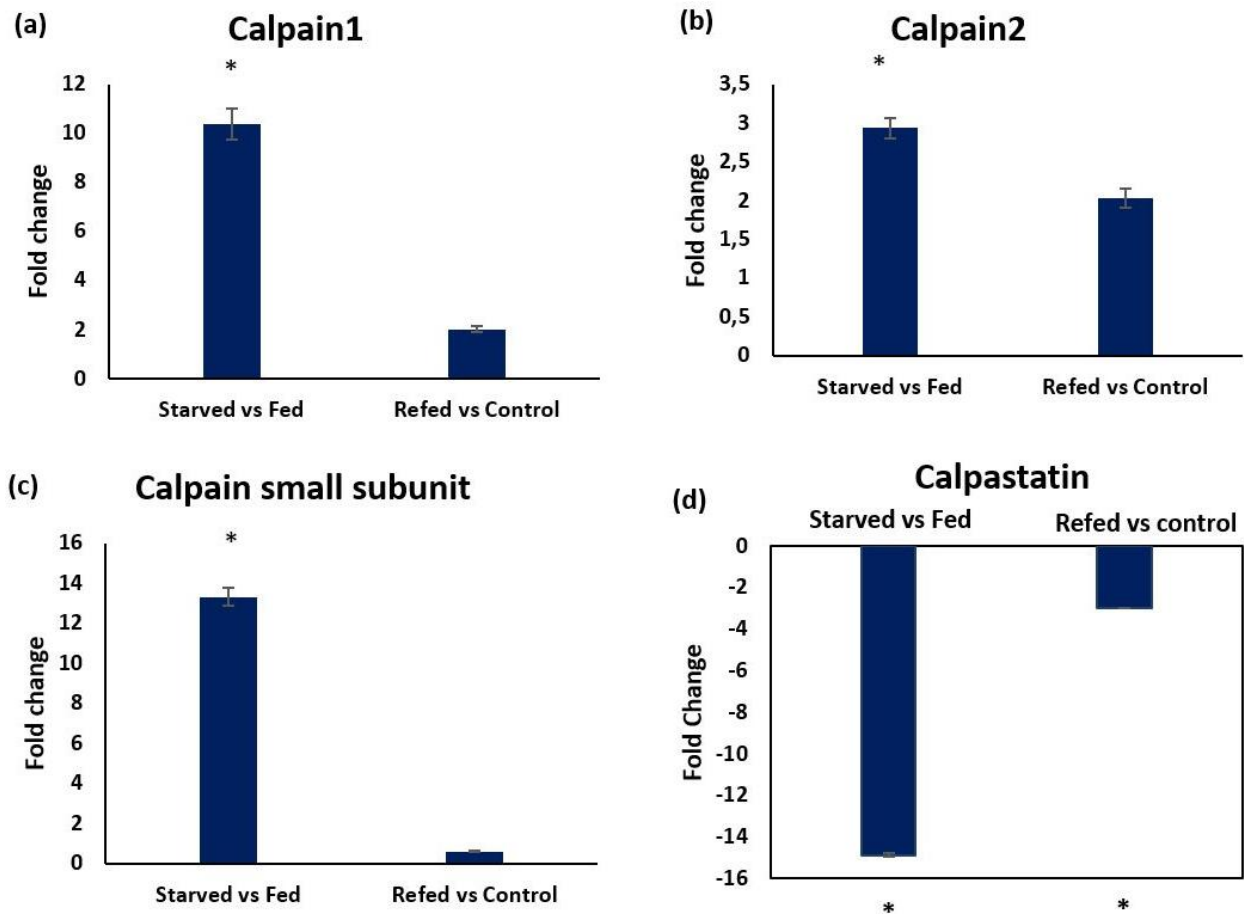
Figure 1. qPCR results showing differential gene expression of calpain1, calpain2, calpain regulatory subunit, and calpastatin genes in treated groups vs. their control groups. β-actin gene was used as the internal control for data normalization. The results were presented as fold change between groups ±standard deviation (* indicates statistical significance, n=7, and p≤0.05).

starved fish vs. fed fish; and refed fish vs. control by -14.28 and -2.94, respectively (Figure 1d). Altogether, calpains and their inhibitor calpastatin showed inversely coordinated expression in response to starvation.

Cathepsin L gene expression showed an enormous increase of 88.6-fold in the muscle of the starved fish versus the fed fish, while 2.6-fold in refed versus control fish. Similarly, the starved fish significantly increased cathepsin B gene expression 6-fold over the fed fish and a 2.7-fold increase in refed fish vs. control. In addition, cathepsin H showed a significant increase in starved fish vs. fed fish (26.8-fold), while it was not significantly changed in refed fish vs. control (Figures 2a/b/c).

Calpains and Cathepsin L Activity

The present study showed increased calpain activity in the starved fish relative to the fed group by 1.9-fold. It was noticed that the control group decreased significantly by 1.37 fold compared to the refed group, as shown in Figure 3. Cathepsin L activity was 2.3-folds higher in starved fish muscle than in the fed fish. Consistently, an increase of 1.3-fold in cathepsin L was observed in the muscle of the refed group versus the control one as shown in Figure 3.

Calpains and Cathepsins Tissue Distribution

Calpain1, calpain2, cathepsin L, B, and H genes showed ubiquitous expression among all studied tissues (gill, liver, white muscle, skin, intestine, heart, red muscle, stomach, brain, eye, and kidney) as shown in Figures 4 and 5.

Molecular Characterization of Calpian1/2 and Cathepsin L/B and H Genes and Phylogenetic Analysis

Calpain1 was found to have 3,135 nucleotides in length. The number of amino acids in the longest open reading frame (ORF) was 704. *Oreochromis niloticus (O. niloticus)* calpain1 gene shared 98% of its sequence identity with the *Mylandia zebra (M. zebra)*, 83% with *Danio rario (D. rario)*, 78% with *Gallus gallus (G. gallus)*, and 68% with *Homo sapiens (H. sapiens)* (Figures 6 and 7). The number of nucleotides recorded for calpain2 was 2,367 nt, and the longest ORF had 577 amino acids. The calpain2 sequence shared 97% of its sequence identity with *M. zebra*, 80% with *D. rario*, 65% with *G. gallus,* and 64% identical to *H. sapiens* (Figures 6 and 8). The predicted protein functional domains for calpain1 and calpain2 were peptidase C2 (catalytic domain), ranging from 30-355 and 27-349 amino acids. Peptidase C2

(large subunit domain III) spans amino acids 358-515 and 351-507 for calpain1 and calpain2, respectively. In addition, the 5 EF-hand domain was predicted in calpain1 and calpain2 in amino acids 529-702 and 520-696, respectively (Figures 7 and 8).

Cathepsin L, B, and H have predicted signal peptide regions in amino acids 1-116, 1-78, and 1-17, respectively, in addition to the mature peptide region (Figures 9, 10, and 11). Cathepsin L had 1,467 nucleotides in length, while the number of amino acids found in the longest ORF was 338 (Figure 9). Cathepsin L sequence was 93% identical to *M. zebra*, 83% identical to *D. rario*, 82% identical to *G. gallus*, and 68% identical to *H. sapiens* (figure 6). Cathepsin B, with a sequence

length of 1,717 nucleotides and the longest ORF had 330 amino acids (Figure 10), shared 97% of its sequence identity with *M. zebra*, 83% with *D. rario*, 77% with *G. gallus*, and 74% with *H. sapiens* (figure 6). The number of nucleotides for cathepsin H was 1,341, with the longest ORF having 324 amino acids (Figure 11). This gene sequence was 97% identical to *M. zebra*, 73% identical to *D. rario*, 68% identical to *G. gallus*, and 66% identical to *H. sapiens* (Figure 6).

Phylogenetic analysis was performed using MEGA7 software using sequences of the studied genes with homologous sequences from mammals, reptiles, amphibians, birds, and fish species. *O. niloticus* genes were nearly identical to those of the *M. zebra* as they
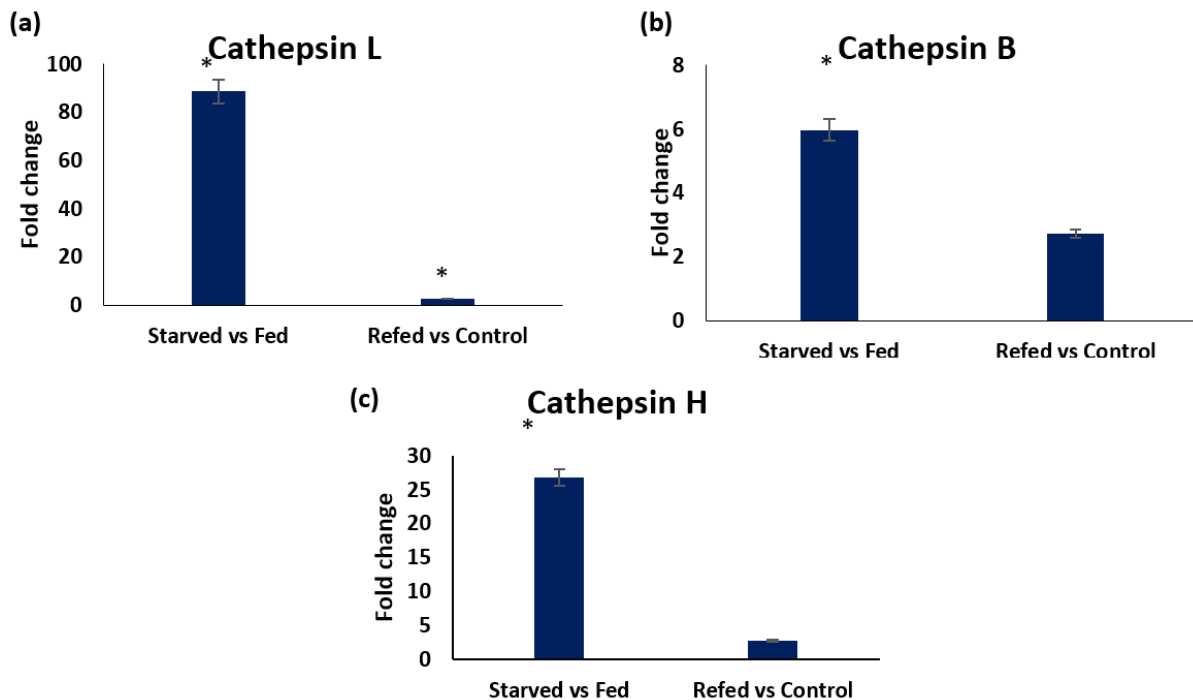

Figure 2. qPCR results showing differential gene expression of calpain1, calpain2, calpain regulatory subunit, and calpastatin genes in treated groups vs. their control groups. β-actin gene was used as the internal control for data normalization. The results were presented as fold change between groups ±standard deviation (* indicates statistical significance, n=7, and p≤0.05).
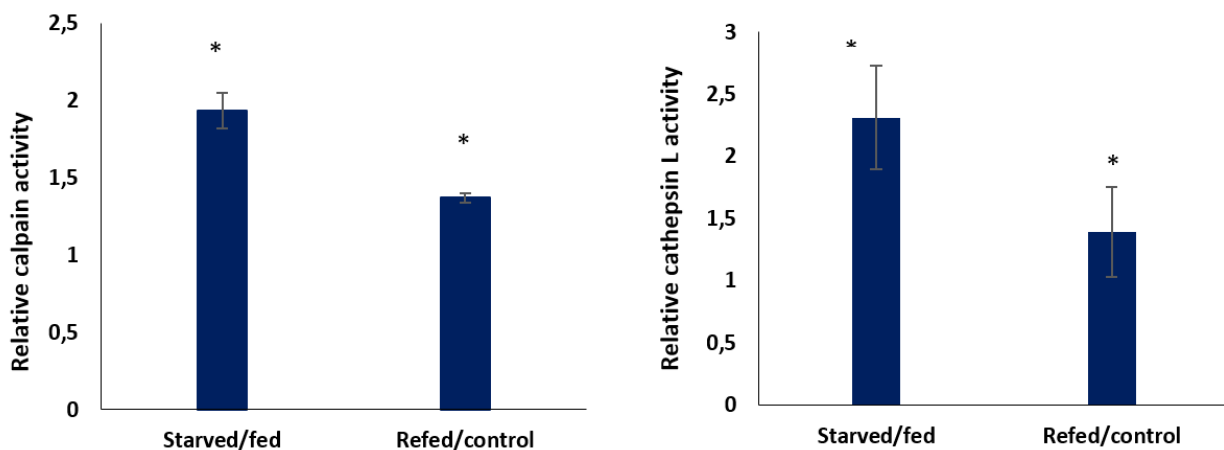

Figure 3. Calpain and cathepsin L enzyme activity in starved vs. fed and refed vs. control groups (P ≤0.05). The results were presented as relative fold change ± standard deviation, n=7.
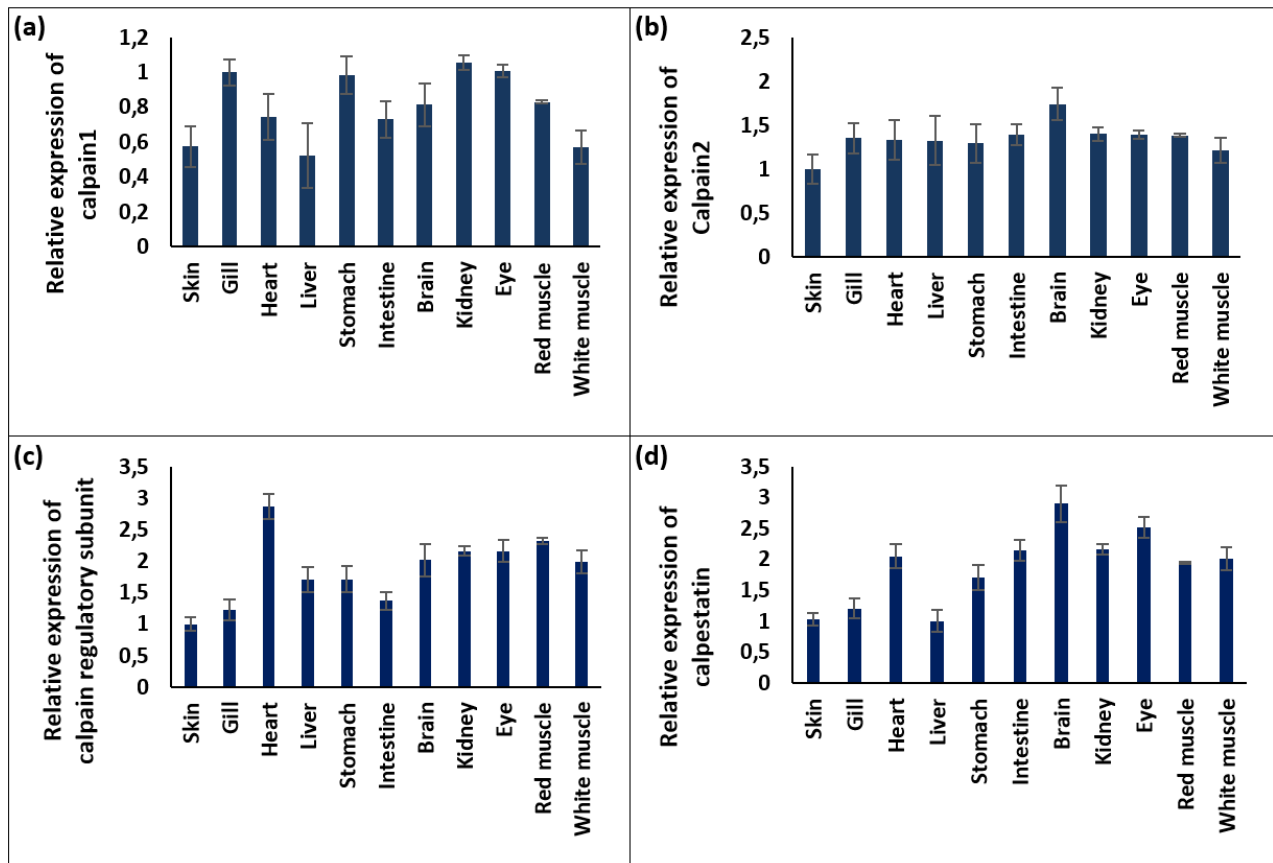
Figure 4. Ubiquitous expression of calpain genes in selected tissues of tilapia, (a) calpain1, (b) calpain2, (c) calpain regulatory subunit, and (d) calpastatin.
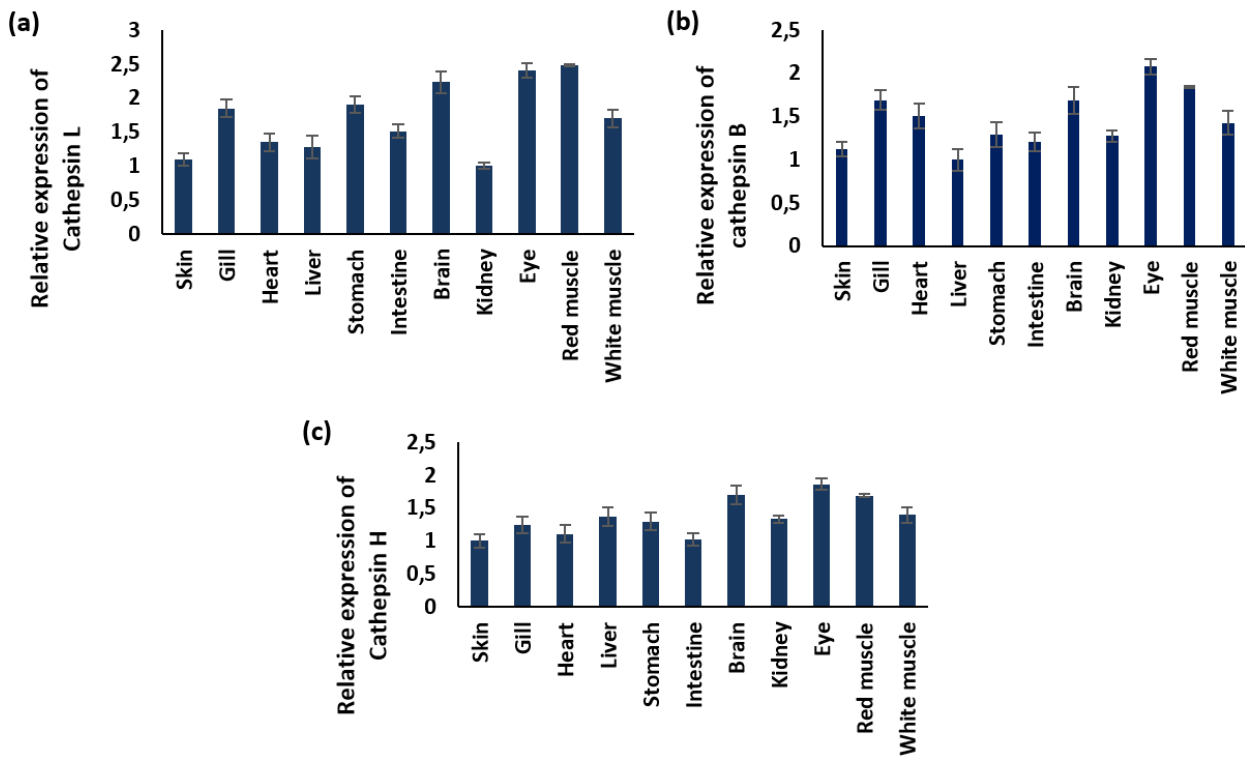

Figure 5. Ubiquitous expression of cathepsin genes in selected tissues of tilapia, (a) cathepsin L. (b) cathepsin B. (c) cathepsin H.
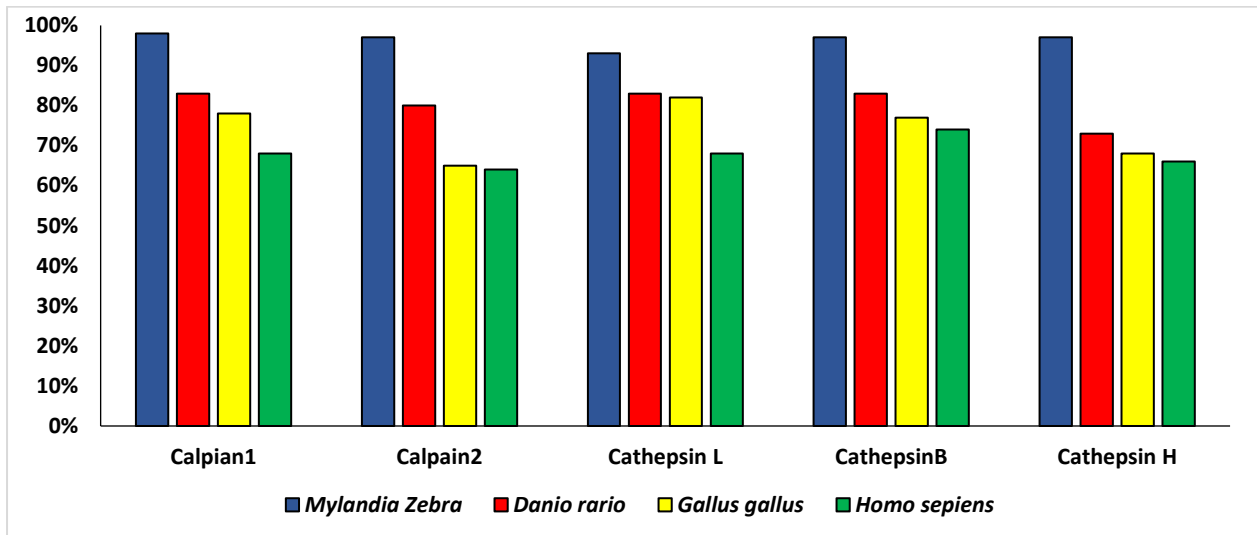
Figure 6. Amino acid sequence homology of calpain1/2, cathepsin L/B, and H genes in tilapia compared to homologous genes in different species.
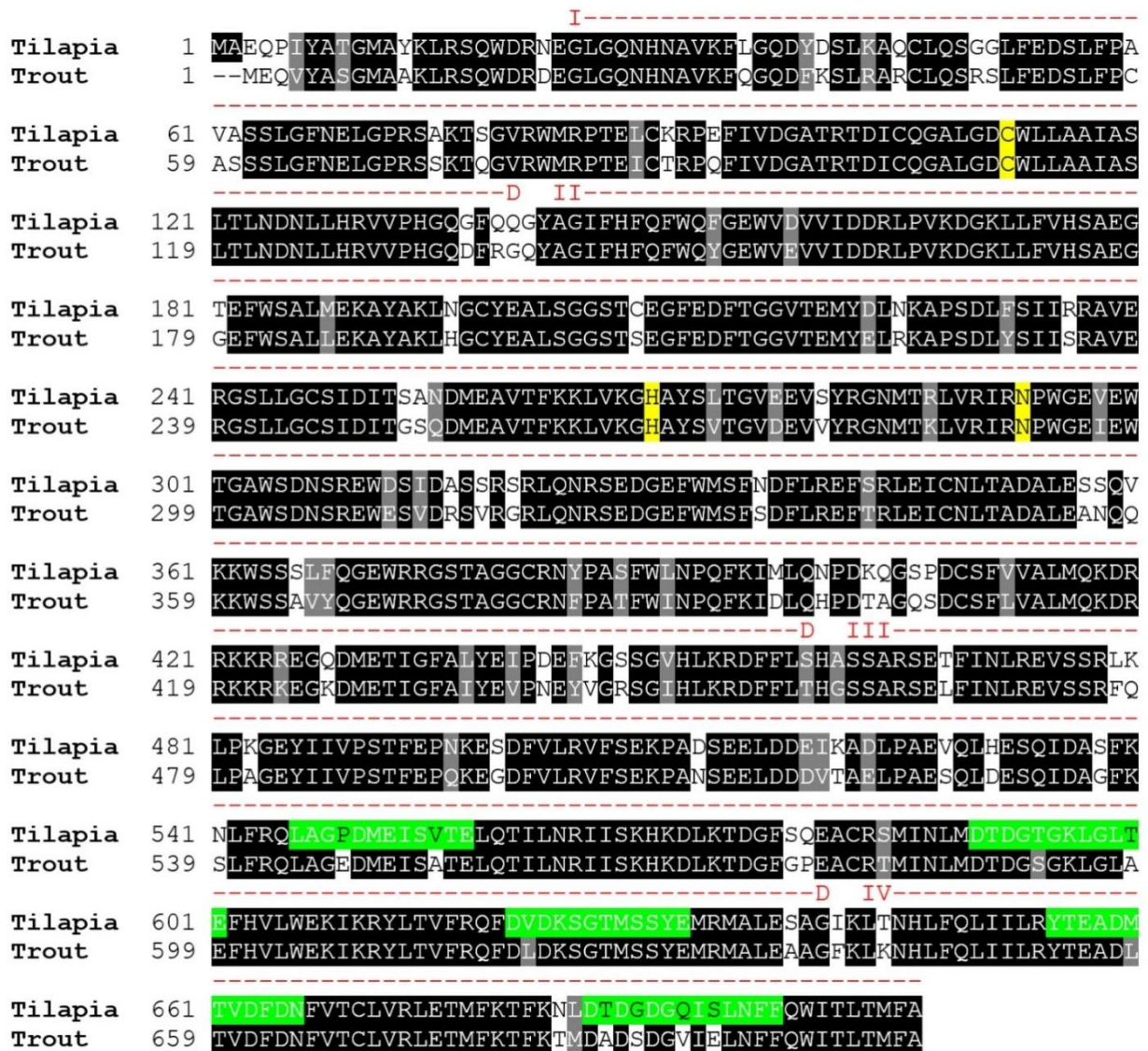

Figure 7. Multiple alignments of Calpain1 amino acid sequences of tilapia (*Oreochromis niloticus*) and trout with corresponding GenBank accession number: NP_001117962.1. Amino acids with a dark background are identical, while the amino acids with light backgrounds are different but have similar properties; no background indicates different amino acids with different properties. The distinct domains I through IV of calpain1 are indicated by dash lines above the sequence. The green highlights mark the 5 Ca2+ EF-hands.
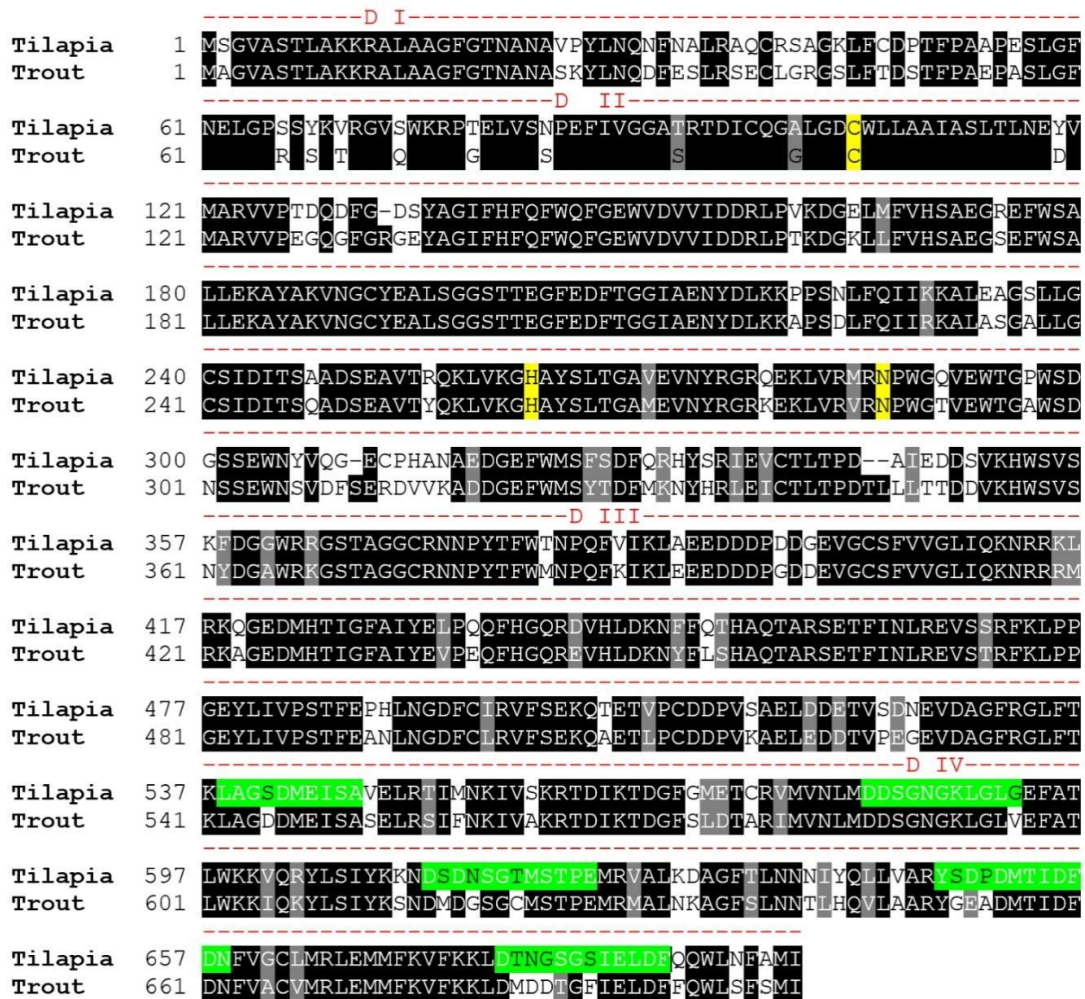
Figure 8. Multiple alignments of Calpain2 amino acid sequences of tilapia (*Oreochromis niloticus*) and trout with corresponding GenBank accession number: NP_001117963.1. Amino acids with a dark background are identical, while the amino acids with light background have the same properties; no background indicates different amino acids with different properties. The distinct domains I through IV of calpain1 are indicated by dash lines above the sequence. The green highlights mark the 5 Ca2+ EF-hands.

Figure 9. Multiple alignments of Cathepsin L amino acid sequences of tilapia (*Oreochromis niloticus*) and trout with corresponding GenBank accession number: AAK69706.1. Amino acids with a dark background are identical, while the amino acids with light background have the same properties; no background indicates different amino acids with different properties. Arrowheads mark the active sites. The yellow highlight marks a potential N-glycosylation signal.
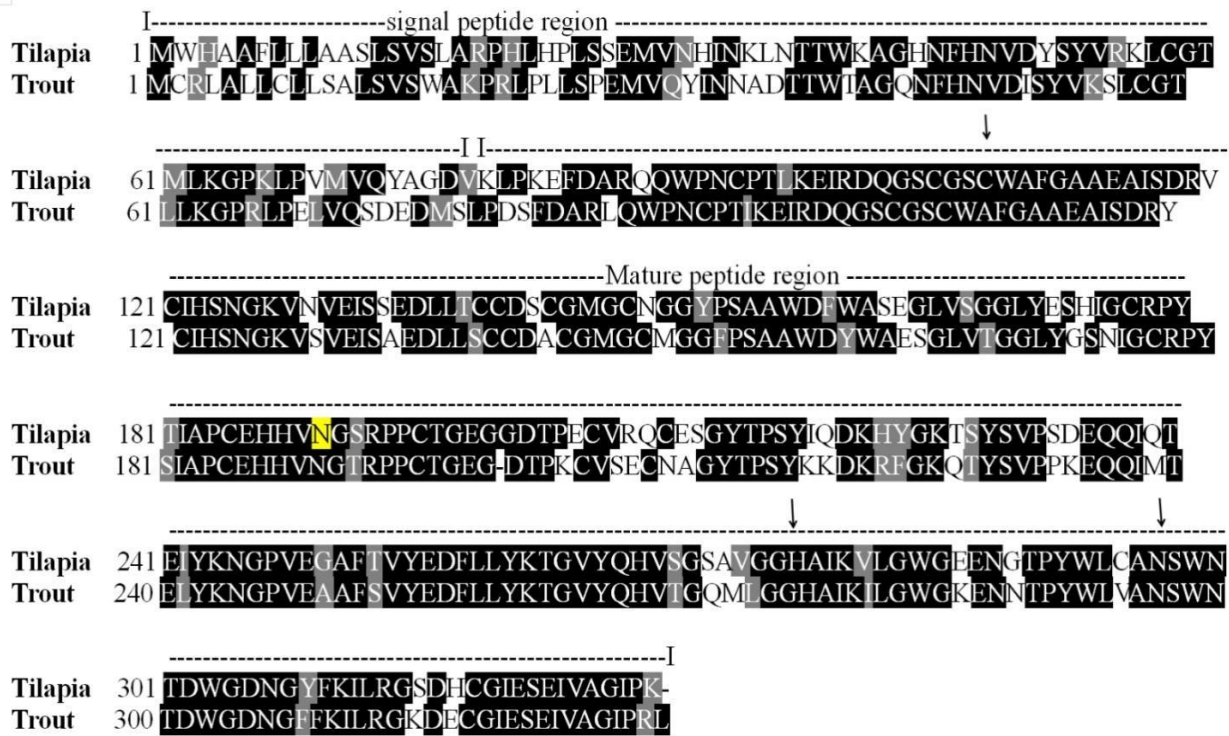
Figure 10. Multiple alignments of Cathepsin B amino acid sequences of tilapia (*Oreochromis niloticus*) and trout with corresponding GenBank accession number: AAK69705.1. Amino acids with a dark background are identical, while the amino acids with light backgrounds have the same properties; no background indicates different amino acids with different properties. Arrowheads indicate the active sites. The yellow highlight marks a potential N-glycosylation signal.
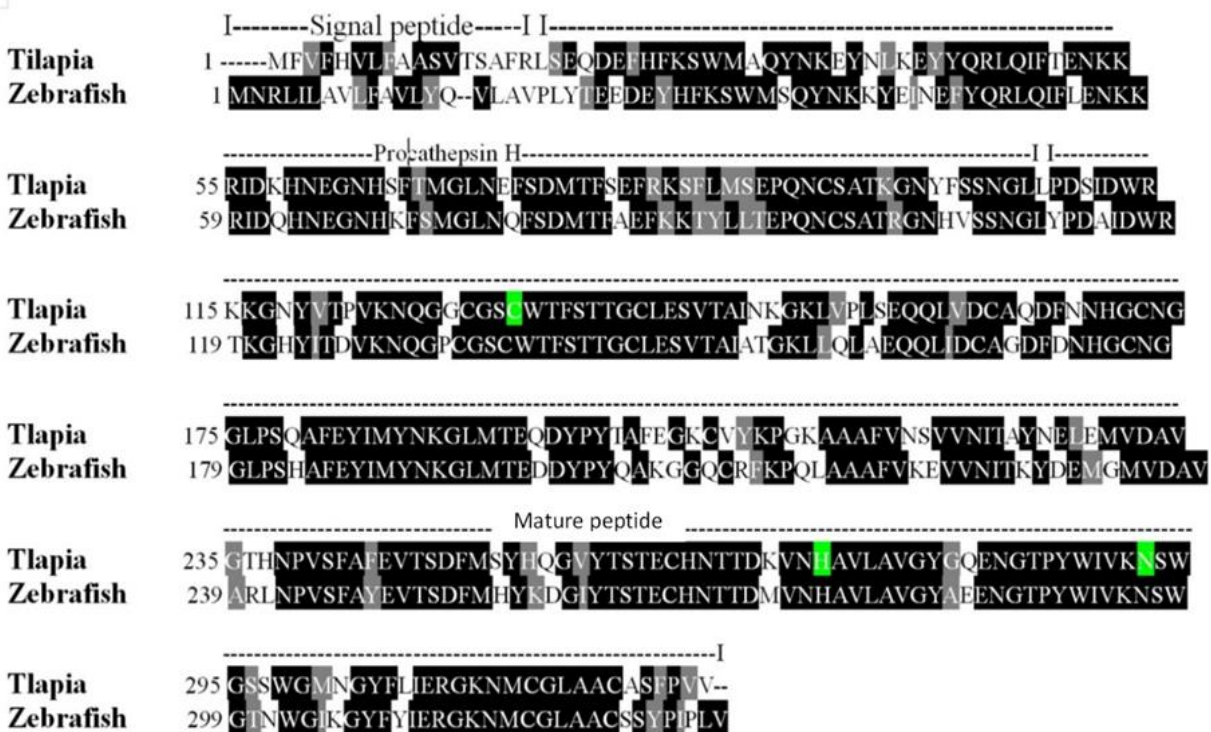

Figure 11. Multiple alignments of Cathepsin H amino acid sequences of tilapia (*Oreochromis niloticus*) and zebrafish with corresponding GenBank accession number: NP_997853.1. Amino acids with a dark background are identical, while the amino acids with light background have the same properties, but different amino acids and amino acids with no background are different in names and properties. The potential catalytic sites, substrate-binding sites, and disulfide linkage sites are highlighted in green.

relate to the same fish family. *D. rario*, as a bony fish, represents the second phylogenetically related species with protein sequences similar to those of tilapia. Calpain1 sequences were clustered together and separately from the calpain2 cluster. The same pattern was observed for cathepsin L, B, and H (Figures 12 and 13).

Discussion

The expression of calpain1 and calpain2 were significantly increased in the starved fish muscle by 10.39 and 2.94-folds, respectively. The calpain regulatory subunit also significantly increased by 13.34-fold in the starved fish over the fed fish. Consistent with our investigation, studying the effect of starvation on Atlantic halibut (*Hippoglossus hippoglossus* L.) revealed that calpain1 expression reaches its maximum level during starvation and begins to decrease with refeeding; meanwhile, there was little response for calpain2 during starvation (Macqueen et al., 2010). Similarly, in gilthead sea bream, calpains' expressions were modulated by fasting, diet composition, and carbohydrate-to-protein ratios. Fasting significantly increased calpain1; however, 7 days after refeeding, expressions of calpains were significantly reduced (Salmeron et al., 2013). In channel catfish, starvation increased the expression of muscle calpain2 and calpain3; however, calpain1 expression was downregulated. Simultaneously, the calpain catalytic increased by 1.2-1.4 fold (Preziosa et al., 2013). Calpains were suggested to be involved in muscle atrophy (Huang & Zhu 2016). It was found that calpain1 and calpain2 were increased significantly in starved rainbow trout fish compared to the fed ones and proved their prospective role in protein mobilization for energy supply (Salem et al., 2005).

On the other hand, the calpain inhibitor, calpastatin, gene showed a significant decrease in abundance in both starved fish vs. fed fish; and refed fish vs. control. Similar to our results, fasting reduced the mRNA abundance of the calpastatin with a simultaneous increase in the calpain catalytic activity in rainbow trout liver. Altogether, calpains and their inhibitor calpastatin showed inversely coordinated expression in response to starvation. Calpastatin's role was studied in mice and showed the ability to reduce muscle degradation (Tidball & Spencer., 2002). Also, calpastatin was positively correlated with rainbow trout growth (Salem et al., 2005).

Cathepsin L gene expression showed a massive increase of 88.6-fold in the muscle of the starved fish compared to the fed fish. Similarly, the starved fish significantly increased cathepsin B gene expression over the fed fish. In addition, cathepsin H showed a significant increase in starved fish vs. fed fish. Also, starved fish vs. fed fish showed a significant increase in cathepsin B gene expression. These results are consistent with data in mice that showed upregulated expression of cathepsin L in skeletal muscle due to starvation (Yamazaki et al., 2010). Other studies on mice indicated a significant increase of cathepsin B and L accompanying muscle atrophy (Tsujinaka et al., 1996). Also, consistent with our data, Atlantic salmon (*Salmo salar*) showed significant enhancement of cathepsins L, H, and B expression in muscle and liver due to starvation (Yabu et al., 2012). Conversely, starvation did not affect rainbow trout's mRNA levels of cathepsin D and L (Salem et al., 2007).

The present study showed increased calpain activity in the starved fish relative to the fed group by 1.9-fold, consistent with the increase in their mRNA abundance. Cathepsin L activity was also higher in starved fish muscle than in the fed fish. Similar results of increased calpain activity were shown in the liver of starved rainbow trout (Salem et al., 2007). Similarly, cathepsin B and L activities recorded a 3-times increase in salmon during the stress of migration (Yamashita & Konagaya, 1990). However, starvation did not increase rainbow trout's cathepsin D and L activities (Salem et al., 2007). The overall coordinated increase in mRNA expression and enzyme activity of members of the calpain and cathepsin systems suggest an essential role in muscle growth and degradation of tilapia.

Calpain1, calpain2, cathepsin L, B, and H genes showed ubiquitous expression among all studied tissues (gill, liver, white muscle, skin, intestine, heart, red muscle, stomach, brain, eye, and kidney). Previous studies recorded similar tissue expression distribution of calpain and cathepsin genes in different tissues of fish, which slightly vary from tissue to tissue (Salem et al., 2005; Salmeron et al., 2015).

Calpain1 and calpain2 genes of *O. niloticus* shared 98-97% of their sequence identity with the *M. zebra*, and 83-80% with *D. rario* as they originate from the same ancestral species, while the identity decreased to 78-65% with *G. gallus,* and 68-64% with *H. sapiens*. Previous studies reported that calpain2 is an intronless gene limited to specific tissues (Schád et al., 2002). The alignment was done for the studied sequence of tilapia fish, and trout or zebrafish that were characterized by homologous sequences with near evolutionary distances to facilitate the characterization process of the genes of interest (Pirovano & Heringa, 2008). Introns in the calpian1 gene were reported in fish, especially rainbow trout. The prediction of protein functional domains for calpain1 and calpain2 showed the presence of the 5 EF-hand calcium-binding motifs in calpain1 and calpain2 (Figures 7 and 8).

Cathepsin L sequence was 93% identical to *M. zebra*, 83% identical to *D. rario*, 82% identical to *G. gallus*, and 68% identical to *H. sapiens* (figure 6). Cathepsin B shared 97% of its sequence identity with *M. zebra*, 83% with *D. rario*, 77% with *G. gallus*, and 74% with *H. sapiens*. The cathepsin H gene sequence was 97% identical to *M. zebra*, 73% identical to *D. rario*, 68% identical to *G. gallus*, and 66% identical to *H. sapiens*. It was reported previously that all cathepsins share identical conserved residues that have a critical role in
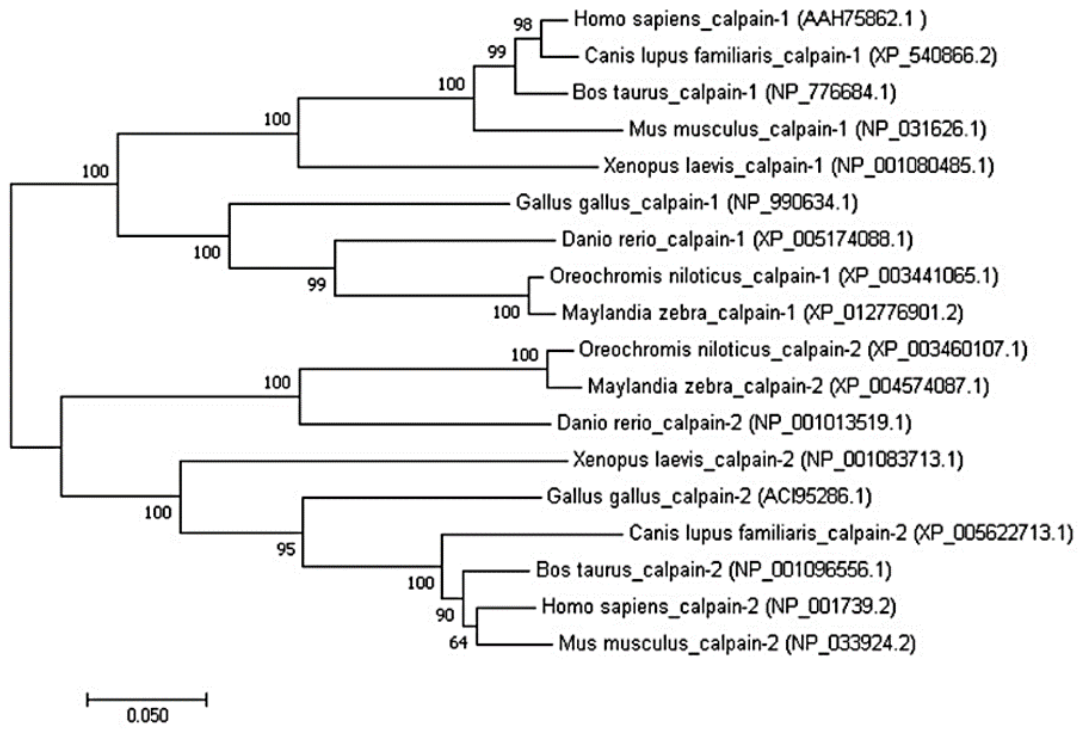
Figure 12. Phylogenetic tree by MEGA7 software. The tree showed the phylogenetic relationship between *Oreochromis niloticus* amino acids calpain1 and calpain2 with other species. The GenBank accession numbers are presented beside the species name.
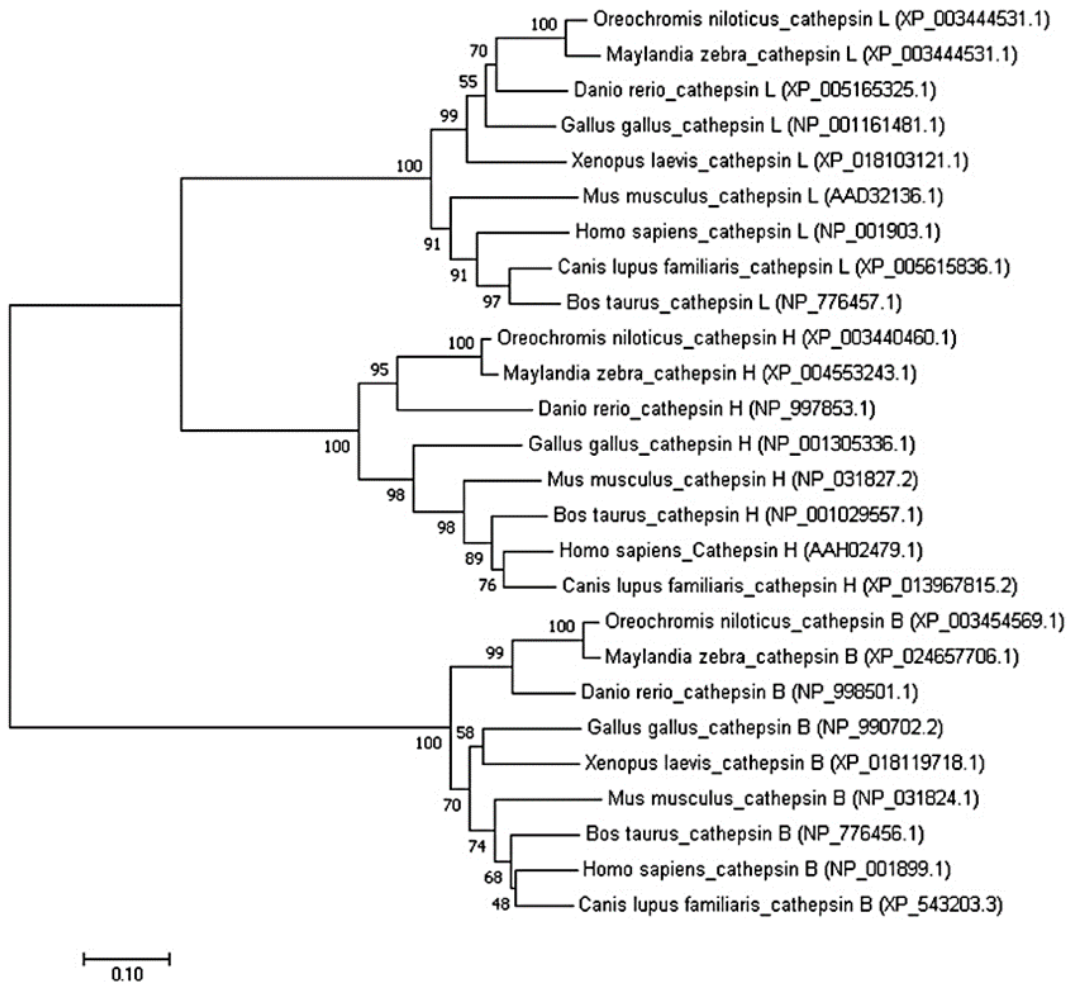

Figure 13. Phylogenetic tree by MEGA7 software. The tree showed the comparison between *Oreochromis niloticus* amino acids for cathepsin L, cathepsin B, and cathepsin H and other species. The GenBank accession numbers are presented beside the species name.

the stability of the catalytic site of the activated enzyme (Lecaille et al., 2002). This structure similarity proved its essential role in the physiological and pathological processes (Kim et al., 2013).

A phylogenetic comparison of the studied calpain and cathepsin genes was made with different mammals, reptiles, amphibians, birds, and fish species. *O. niloticus* genes were nearly identical to those of the *M. zebra* as they relate to the same fish family. *D. rario*, as a bony fish, represents the second phylogenetically related species with protein sequences similar to those of tilapia. Calpain1 sequences from all species were clustered together separately from the calpain2 cluster. Similar clustering profiles of the calpain genes were previously recorded (Salmeron et al., 2015; Lepage & Bruce, 2008). The same pattern was observed for cathepsin L, B, and H, consistent with other fish studies (Che et al., 2014).

Ethical Statement

Funding Information

Author Contribution

WS analyzed conducted the experiment and analyzed the data, and drafted the manuscript. NA and SE helped in interpreting the data. MS designed the experiment, helped in data analyses and contributed to writing the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

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