Genetics of Aquatic Organisms 6(3.Special Issue), GA509
https://doi.org/10.4194/GA509

RESEARCH PAPER

Heat-shock Protein 70: Sequence and Expression Analysis in African Catfish *Clarias gariepinus* after Bacterial Infection and Stress Exposures

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How to cite

Nasrullah, H., Layinatuzzain, L., Soelistiyowati, D.T., Widanarni, W., Alimuddin, A. (2022). Heat-shock Protein 70: Sequence and Expression Analysis in African Catfish *Clarias gariepinus* after Bacterial Infection and Stress Exposures. *Genetics of Aquatic Organisms, 6(3.Special Issue), GA509*.

Article History
Received 21 March 2022
Accepted 30 May 2022
First Online 13 June 2022

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Keywords
Hsp70
Catfish
Stress
Bacterial Infection
Transportation

Abstract

Heat-shock protein 70 (HSP70) is a member of the Heat-shock protein family that plays an important role in cellular homeostasis against various stressors in fish. This research aimed to identify the full length of cDNA sequence *HSP70* in African catfish *C. gariepinus* and assessed its mRNA expression under various tissues and stress conditions. The cDNA was cloned and amplified using the polymerase chain reaction (PCR) followed by bi-directional sequencing. The mRNA expression was quantified using the real-time PCR (qPCR) method. A full-length ORF of *C. gariepinus* HSP70 cDNA was 1932 bp long, encoded 643 amino acids. Three signature sequences of the HSP70 family were detected in the predicted amino acid sequence. The *C. gariepinus* HSP70 showed the highest similarity in nucleotide and amino acid sequence (>90%) to HSP70 from other Siluriformes catfish species. The HSP70 mRNA was expressed in all tested tissues, with the highest expression found in the liver and spleen, followed by the intestine, heart, head-kidney, and gill. The *C. gariepinus* HSP70 expression was significantly induced generally after 6 hours of bacterial infection, transportation, heat shock, and high nitrite exposure. The results indicated that the HSP70 gene could serve as an early stress biomarker in *C. gariepinus*

Introduction

Aquaculture and fisheries industries have several unavoidable stressors, such as handling, transportation, temperature, crowding, salinity, oxygen deprivation, and pollution that resulted in the stress responses of fish (Aleng et al., 2015; Yarahmadi et al., 2016). All of these negative environmental conditions have the potential to cause stress reactions in the fish as well as immunosuppression followed by pathogen infection. Stressed fish will experience a sequence of changes in the expression of associated genes, followed by protein synthesis that is involved in the adaptation process.

Heat shock proteins (HSPs) are a family of highly conserved proteins that play an important role in cellular homeostasis in humans, animals, and including fish (Genest et al., 2019; Yan et al., 2017). HSP has a crucial function as a cytoprotector in stressful situations, regulating protein homeostasis and assisting in the refolding of misfolded proteins (Genest et al., 2019; Roberts et al., 2010). HSPs are divided into several groups based on their molecular weight, amino acid sequence homologies, and activities. These families include the small HSP family, HSP40, HSP70, HSP90, HSP100, and chaperonins (Csermely, 1999). Among HSPs, the heat shock protein 70 (HSP70) family is one of the most conserved and is the most widely researched protein in the HSP family (Roberts et al., 2010). HSP70 cDNA has been cloned and studied in various fish species such as common carp (Sung et al., 2012), Asian seabass

(Han et al., 2017), mudskipper (Han et al., 2016), and loach (Yan et al., 2017) and reported to plays a critical role in fish stress responses such as heat stress, pathogen infection, crowding, and transportation and also to activate innate immunity (Eissa et al., 2017; Yarahmadi et al., 2016). HSP70 has recently been employed as a biomarker to monitor many sorts of environmental changes, as well as as a stress biomarker in fish (Eissa et al., 2017).

African catfish *C. gariepinus* is one of the most economically important aquaculture commodities in Southeast Asia countries including Indonesia. This fish is commonly cultured in the intensive system with highly exposed to various stressors and higher susceptibility to disease (Ekasari et al., 2016; Nasrullah et al., 2020). However, in this species, knowledge of stress-related genes like HSP70 is scarce. Therefore, there is an urgent need to identify the stress and immune-related genes in this species to monitor stress levels and investigate stress and immune-related pathways as the aquaculture stress biomarker.

In this study, HSP70 cDNA was cloned using RACE-PCR. The acquired sequences were examined and matched to homologs found in other fish species, and a phylogenetic tree was constructed. The mRNA expression of HSP70 was also investigated in different tissues, under bacterial infection and stress exposure to determine anti-stress roles of *C. gariepinus* HSP70. Fish subjected to the common yet strong stressors in the *C. gariepinus* culture: pathogenic Gram-negative bacteria *Aeromonas hydrophila* infection, temperature shock, transportation stress, and nitrite exposures. Our results provide some molecular insights into HSP70's response to various stressors in *C. gariepinus*.

Materials and Methods

Fish

C. gariepinus fingerlings were obtained from the National Centre for Freshwater Aquaculture (Sukabumi West Java, Indonesia) with an average body weight of 6 grams. Fish were acclimatized in the 1050 L fiber tanks for one week, fed with commercial feed two times a day. The water temperature during the acclimatization was ranged from 26-28 °C. The liver was isolated from the healthy fish for HSP70 cDNA identification. The liver, gills, head-kidney, spleen, intestine, and heart were dissected for HSP70 expression analysis in various tissues. Fish were euthanized using MS222 (250 mg L^{-1}) before each sample collection. The tissues were stored in *Genezol* reagent (Geneaid, Taiwan) at −80 °C before analysis. All animal experimental and rearing procedures were carried out in accordance with the national standard for fish experimentation guidelines (01-6489-2000) of the Republic of Indonesia.

Amplification of *C. gariepinus* HSP70 cDNA

Total RNA was extracted with *Genezol* reagent from the fish liver. RNA purification and reverse transcriptions were conducted following the *ReverTra Ace qPCR RT master mix* kit (Toyobo, Japan) procedures. A pair of primers (Table 1) that covered the full-length sequence of the HSP70 coding sequence was designed by aligning the HSP70 sequence of other fish species that are available at Genbank (www.ncbi.nlm.nih.gov/nuccore/?term=HSP70+fish). The PCR amplification was conducted in the 0.2 mL stripped PCR tubes (ExtraGene, Taiwan) using the *MyTaq HS™ Red Mix* (Bioline, UK). The reaction condition as follows: 5 min at 95 °C, 40 cycles (95 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s), and 6 min at 72 °C for the final extension. All molecular procedures were conducted using sterile filtered pipette tips (ExtraGene, Taiwan).

The amplification products were separated by 2% gel agarose electrophoresis and purified using *Wizard SV Gel and PCR Clean Up-System Kit* (Promega, USA). Purified PCR products were cloned into pTA2 vector (Toyobo, Japan) and then bi-directionally sequenced at 1st Base Laboratory, (Malaysia). Sequence similarity analyses were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequences of *C. gariepinus* HSP70 were analyzed at the ExPasy system site (http://www.expasy.org/). The multiple sequence alignments of *C. gariepinus* HSP70 and the phylogenetic tree construction were conducted in MEGA X software (http://www.megasoftware.net/) by the Neighbor-joining (NJ) method with 1000× bootstrap.

Table 1 Primers used in this study

Primer name	Primer sequence (5' – 3')	Purpose
HSP70-F	AAG**ATG**TCCTCTGCTAAAGG	HSP70 cloning
HSP70-R	TTAATCCACTTCTTCAATAG	HSP70 cloning
qHSP70-F	CAAACGCAACACCACTATTCC	HSP70 qPCR analysis
qHSP70-R	CATGGCTCTCTCACCTTCATAC	HSP70 qPCR analysis
qβactin-F	ACCGGAGTCCATCACAATACCAGT	Internal control, β-actin qPCR analysis
qβactin-R	GAGCTGCGTGTTGCCCCTGAG	Internal control, β-actin qPCR analysis

*Underlined sequence was the anchor sequence that randomly added to obtain the Start Codon Sequence (in bold) of the *C. gariepinus* HSP70 after sequencing

Bacterial Challenge

The bacterial challenge was conducted using pathogenic *Aeromonas hydrophila*, the main pathogen in African catfish culture. The pathogenic isolate was obtained from Fish Health Laboratory, Bogor Agricultural University, which was previously isolated and purified from infected fish (Nasrullah et al., 2020). The bacteria were diluted with sterile phosphate-buffered solution (PBS) to the lethal dose 50 (LD50) of 1.5×10^6 CFU mL^{-1} calculated from our preliminary study. Before injection, eighty fish were euthanized using MS222 (75 mg L^{-1}). Forty fish was injected with 100 µL of suspended bacteria intra-muscularly, and another forty fish were injected with the same amount of PBS as the control group. The liver was randomly collected from both groups at 0, 6, 12 and 24 h after infection (n=4) to evaluate the gene expression.

Fish Transportation

Fish were divided into two groups for the transportation procedure. The first group was the non-transported group, where the fish was kept in a normal culture condition after being subjected to handling and packing. Another group was transported fish. After netting and handling, fish was transferred into transparent plastic bags with a density of 100 fish in 1.5

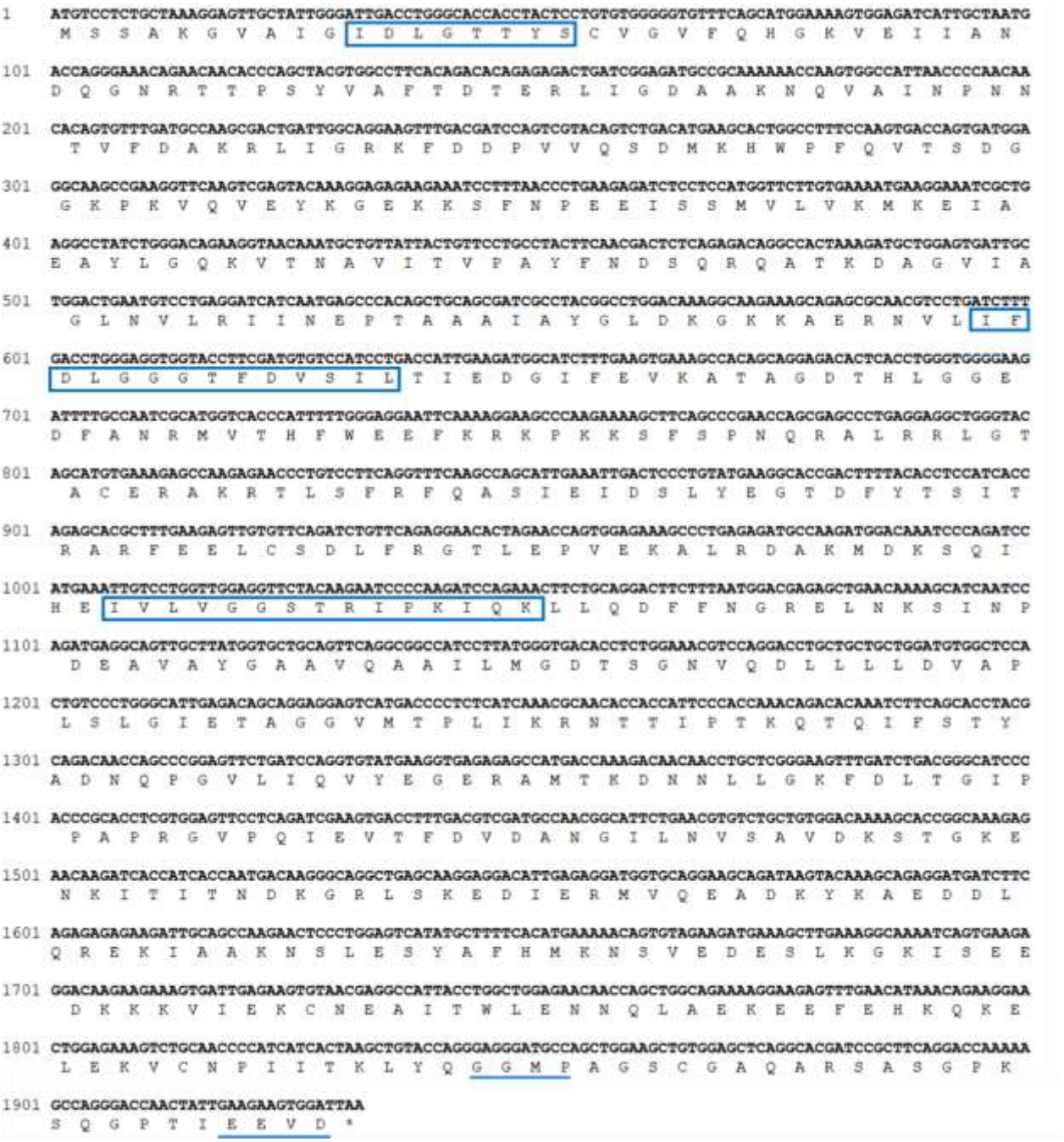

Figure 1 The *C. gariepinus* HSP70 cDNA and deduced amino acid sequence. The HSP70 cDNA sequence consists of 1932 bp ORF that encodes 643 amino acids. Three signature sequences of the HSP70 family indicated in boxes; Tetrapeptide motif of GGMP and the cytoplasmic HSP70 EEEVD at the c-terminal region are in underline.

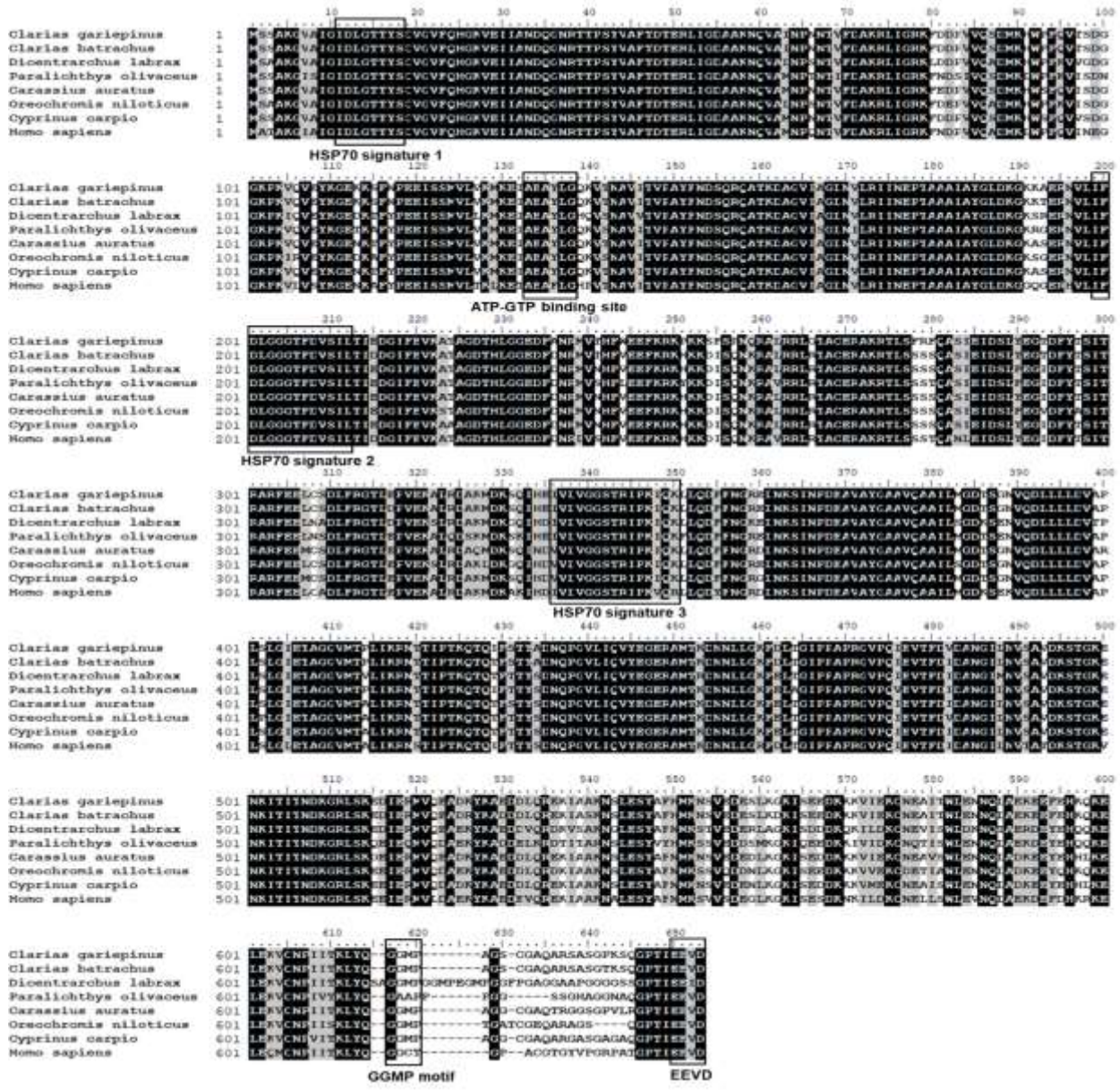

Figure 2 Multiple alignments of HSP70 amino acid sequence of *C. gariepinus* and other species. Identical and similar protein residues among species are indicated by black and grey highlights, respectively. Gaps are marked with periods. ATP/GTP-binding site, HSP70 family signatures, GGMP motif, and the EEVD are marked in box. The predicted protein model was presented.

Table 2 Percentage similarity of *C. gariepinus* HSP70 nucleotide and amino acid sequence compared to other fish species

Organism	% Nucleotide similarity	Accession No.	% Amino acid similarity	Accession No.
Clarias batrachus	95.19	LCO13677.1	96.73	BAQ00457.1
Pangasianodo hypophthalmus	93.53	XM_026916256.2	94.4	XP_034165815.1
Tachysurus fulvidraco	90.58	XM_027161171.1	94.25	XP_027016972.1
Carassius auratus	85.63	XM_026203619.1	92.38	XP_026063888.1
Cyprinus carpio	86.21	XM_019074376.1	92.38	AEO44578.1
Anabas testudienus	82.92	LR132046.1	89.42	XP_026205869.1
Oreochromis niloticus	82.16	NM_001311332.1	89.6	NP_001298261.1
Monopterus albus	82.45	XM_020613172.1	89.29	XP_020468877.1

L water and 4.5 L of oxygen. Plastic bags were subsequently placed in an open truck, and covered with a black-shading net. The fish were transported for five hours. After transportation, each bag was transferred into a 40 L glass tank. The fish liver was randomly collected from both control and transported groups after transportation (0 h), 6, 12, and 24 h after transportation (n=4) to evaluate the gene expression.

Thermal Stress and Nitrite Exposure

Eighty fish were transferred from the stock into 20 L aquariums for thermal stress. Forty fish were reared with a high temperature of 32 °C, and forty others were reared at 28 °C as control. The fish were subjected to thermal stress for 24 h, and no mortality was observed after thermal stress. For the nitrite stress, 200 fish were transferred into 40 L aquariums (100 fish per aquarium) from stock. The concentration for nitrite exposure was 24 mg L^{-1} $NaNO_2$ (MERCK, USA) which resulted in more than 50% fish mortality in 24 hours from our preliminary study. Fish without nitrite exposure was treated as a control (n=100 each group). The fish liver was randomly collected from both groups at 0, 6, 12, and 24 h after exposure (n=4).

Quantification of mRNA expression

Real-time quantitative PCR (qPCR) was used to quantify the mRNA levels of the *C. gariepinus* HSP70 gene after bacterial challenge and stress exposure. The qPCR primers were designed based on the obtained *C.*

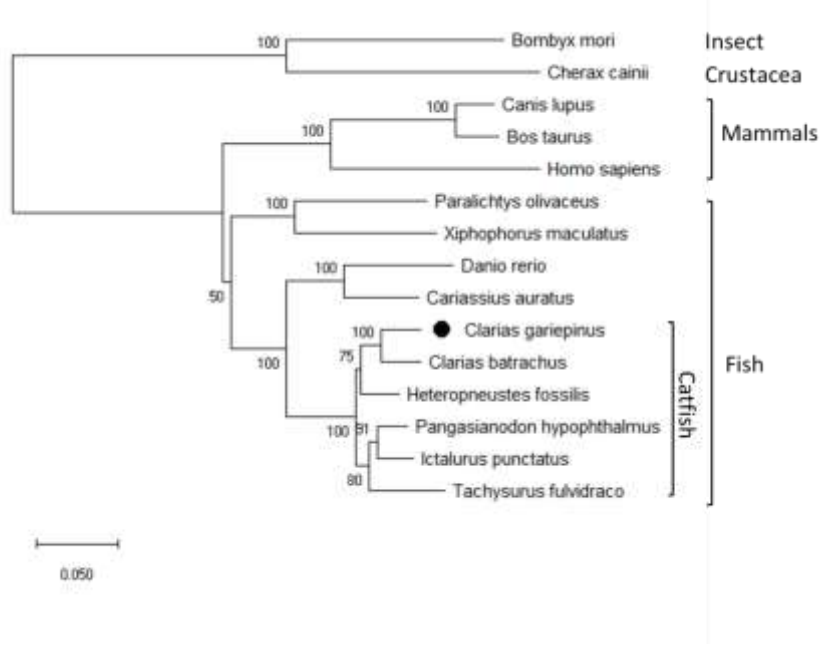

Figure 3 Phylogenetic tree of HSP70 amino acid sequence of *C. gariepinus* and other species. The three were constructed using the Neighbor-Joining method from the amino acid sequence with 1000× bootstrap.

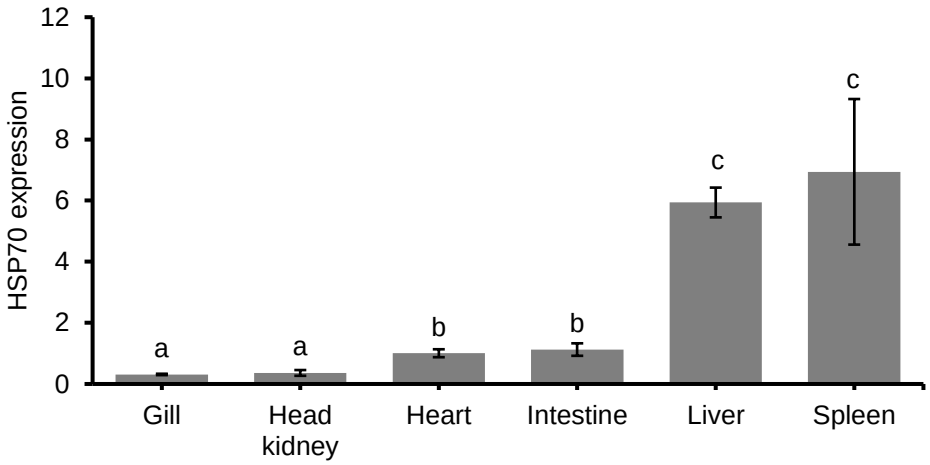

Figure 4 The HSP70 mRNA expression distribution in various tissues of healthy *C. gariepinus*. Data were presented as mean ± SD (n=4). Different letters indicate the significant expression difference between tissues.

gariepinus HSP70 cDNA sequence. Total RNA was purified and reverse-transcripted at the concentration of 50 ng μl^{-1}. The qPCR reaction was conducted in *Rotor-Gene 6000* machine (Qiagen, USA) using the *SensiFast SYBR kit* (Bioline, UK). qPCR program run as follows: 2 min at 95 °C, 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s for 40 cycles and ended with melting curves analysis from 72 to 95 °C to ensure the primer specificity. The qPCR reaction was performed from four biological replicates from each treated and control group. The gene expression ratios were determined by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) after normalized with the β-actin gene as an internal control. The expression level in the tissues was relative to the expression at the heart, and the expression of the challenged fish was relative to the expression at the corresponding control group at the same time points. All expression data were presented as the mean fold change ± SD. The one-way ANOVA analysis followed by the Duncan test was performed in SPSS v.20 software (SPSS Inc, USA) to determine the significant difference between the samples (α= 0.05).

Results

Cloning and sequence analysis of *C. gariepinus* HSP70 cDNA

The complete coding sequence of *C. gariepinus* HSP70 cDNA sequence was 1932 bp long, encoding 643 amino acids (Figure 1 and Figure 2). The obtained sequence of *C. gariepinus* HSP70 was submitted to the GenBank database and published under accession number MT263070. The theoretical isoelectric point of HSP70 was 5.71 and its predicted molecular mass was 70.78 kDa. The deduced amino acid sequences of HSP70

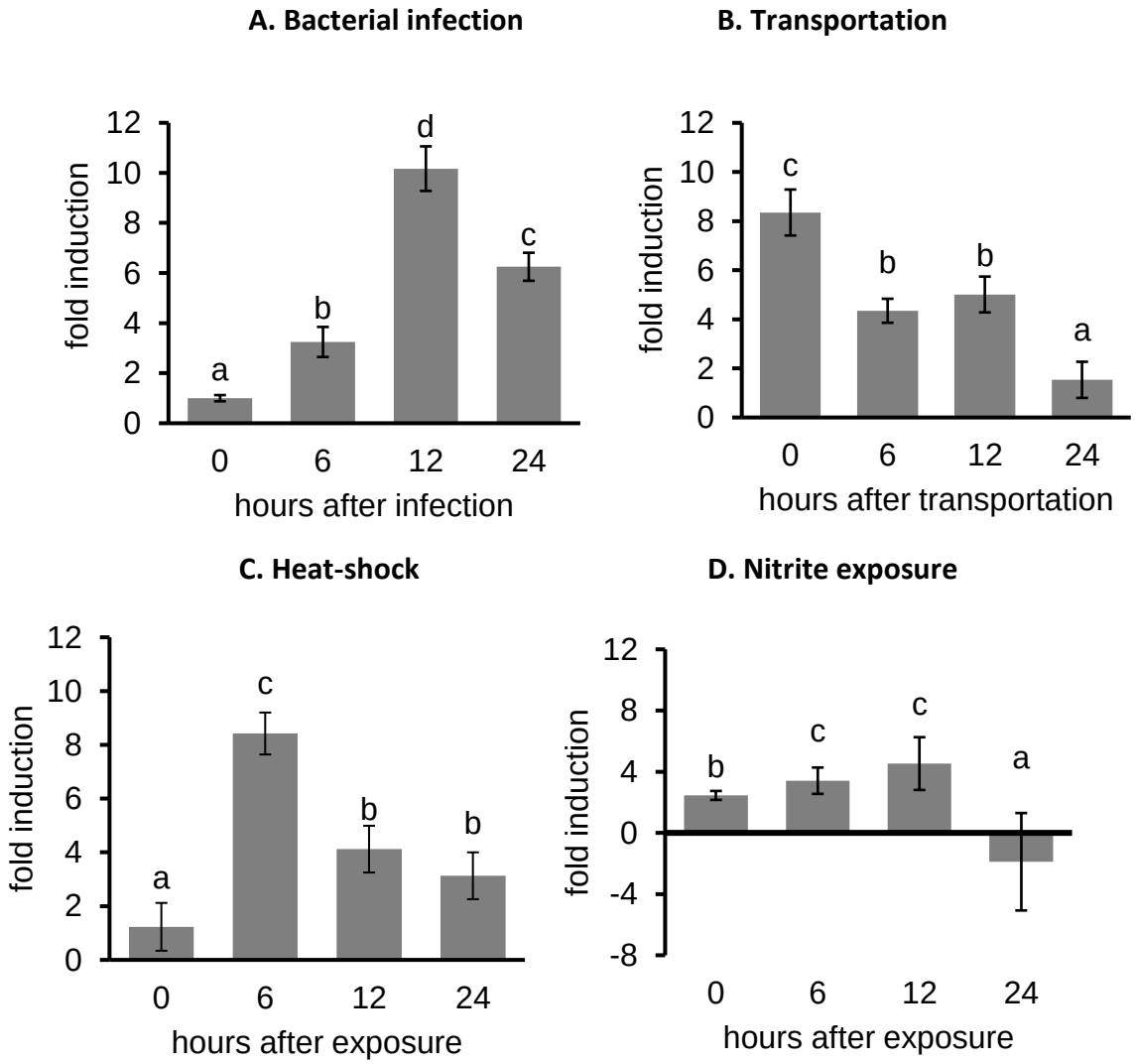

Figure 5 The *C. gariepinus* hepatic HSP70 mRNA expression after *A. hydrophila* infection (A), transportation (B), Heat-shock (C), and nitrite exposure (D). Data were presented as mean fold induction compared to the expression at the corresponding control group (control = 0). Different letters indicate the significant expression difference between time points (n=4).

of *C. gariepinus* contained three signature sequences of the HSP70 family: IDLGTTYS, IDLGGGTFD, and IVGGSTRIPKIQ. The consensus sequence of ATP-GTP binding site, GGMP motif, and EEVD at the C-terminus were also observed within the sequence (Figure 2).

Sequence similarity and phylogenetic analysis

The predicted amino acid sequence of *C. gariepinus* HSP70 shared high similarities with those of many other species (Figure 2). The nucleotide and amino acid residues shared high similarity with other fish species with the highest similarity was found at other catfish species, such as walking catfish *C. batrachus* (95. 19% and 96.73%), striped catfish *Pangasianodon hypophthalmus* (93.53% and 94.4%), and yellow catfish *Tachysurus fulvidraco* (90.38% and 94.25%) (Table 2). Phylogenetic analysis also revealed that *C. gariepinus* HSP70 was grouped in the same clade with other fish species, and separated from mammals, crustaceans, and insects (Figure 3). Within the fish clade, *C. gariepinus* HSP70 was grouped with HSP70 from other catfish species.

HSP70 mRNA expression

The qPCR analysis result showed that *C. gariepinus* HSP70 was expressed in all tested tissues. The HSP70 mRNA was highly expressed in the spleen and liver, moderately expressed in the intestine and heart, and low expressed in the gill and head kidney of healthy *C. gariepinus*. The HSP70 mRNA levels of *C. gariepinus* were also significantly modulated After bacterial infection and various stressors ($p<0.05$), shown in Figure 5. After bacterial infection (Figure 5A), hepatic HSP70 mRNA expression significantly increased at 6 h and reach a peak at 12 h (10-fold higher, $p<0.05$). The expression was decreased at 24 h but still at a higher level than control (6-fold, $p<0.05$). The HSP70 expression level in the liver was also significantly elevated after transportation by 8-fold ($p<0.05$, Figure 5B). Subsequently, the expression was downregulated at 6 and 12 h and then recovered to the same level after 24 h. Thermal stress by heat shock also modulated the *C. gariepinus* HSP70 mRNA expression (Figure 5C). The HSP70 mRNA expression did show significant elevation within the 6 h after heat shock (8-fold, $p<0.05$). The mRNA level remained high at 12 and 24 h by 3-4-fold compared to control. Nitrite exposures also modulated the HPS70 mRNA expression in *C. gariepinus* (Figure 5 D). The mRNA level in the liver was significantly increased at 6 and 12 h ($p<0.05$) during the exposure. After 24 h, the expression was significantly decreased ($p<0.05$).

Discussion

To the best of our knowledge, the information regarding the full-length cloning of HSP70 cDNA in *C. gariepinus* is not available yet. A recent study by Osman et al., (2019) cloned the short partial sequence of *C. gariepinus* HSP70 cDNA that only consisted of 410 bp nucleotides. This sequence is within the 1932 bp of full-length ORF sequence obtained by this study, at the nucleotide base number 120-530 bp. Interestingly, after comparing the amino acid residues between the sequence using BLAST, 19 from 136 amino acid residues was different (86% identities). However, this difference was not further discussed in this study. The molecular approach of stress responses study in *C. gariepinus* is limited, mainly by observing tissue histology, and blood plasma parameters after a various range of stressors (Akinsanya et al., 2016; Dauda et al., 2018; Manuel et al., 2013). This study helps to provide the molecular evaluation of stress responses in this species using the HSP70 mRNA expression. This study was the first to investigate the mRNA expression of *C. gariepinus* after being exposed to common aquaculture stressors such as bacterial infection, nitrite, transportation, and temperature shock.

In this study, a complete ORF sequence of HSP70 cDNA in *C. gariepinus* was obtained and showed high similarity with their homologs in other fish species (Table 2). The *C. gariepinus* HSP70 cDNA sequence contained three signature sequences of the HSP70 family: IDLGTTYS, IDLGGGTFD, and IVGGSTRIPKIQ which are highly conserved in all members of the HSP70 family (Han et al., 2016). *C. gariepinus* HSP70 also contains one tetra-peptide repeat (GGMP) that mediates the multi-chaperone complex formation between HSP70 and HSP90 molecules (Scheufler et al., 2000; Yan et al., 2017). The consensus sequence EEVD at the C-terminus was also observed within the sequence, suggesting that *C. gariepinus* HSP70 belongs to the family of the cytoplasmic HSP70 that enables the molecule to bind other co-chaperones (Scheufler et al., 2000).

The tissues distribution study showed that HSP70 expression was ubiquitous in *C. gariepinus*. Previous studies have also reported the ubiquitous expression of HSP70 in fish tissues, such as liver, testis, kidney, spleen, gill, ovary, adipose tissue, and brain (Han et al., 2016; Yan et al., 2017). High expression of *C. gariepinus* expression in the liver and spleen indicated their important role in the fish defense system.

HSPs have an important role as a cytoprotector in stressful situations, preventing irreversible protein loss and enabling their regeneration (Genest et al., 2019; Roberts et al., 2010). Bacterial infection, temperature shock, transportation, and nitrite exposures are considered as general and strong stressors in fish culture, including in *C. gariepinus* (Dauda et al., 2018; Manuel et al., 2013; Shoko et al., 2016). *A. hydrophila* is the main pathogen in *C. gariepinus* culture. The hepatic expression of HSP70 in *C. gariepinus* was strongly induced after infection that that of the control group. HSPs genes are reported to be involved in the bacterial defense system of aquatic animals (Aleng et al., 2015; Roberts et al., 2010). A recent study on sturgeon

fingerlings showed that hepatic mRNA expression of HPS70 was significantly enhanced after *A. hydrophila* infection. The reports also stated that the resistant group tends to have higher HSP70 expression that also correlates with higher immune parameters (Baharloei et al., 2020). The HSP70 expression significantly increased in the anterior kidney and spleen of channel catfish *Ictalurus punctatus* upon exposure to Gram-positive bacteria *Edwardsiella ictaluri* (Song et al., 2016). This result has confirmed the role of HSP in the fish bacterial defense system and suggests that HSP70 plays a role in the early stages of the response to bacterial infection in *C. gariepinus*.

Transportation might be a significant stressor for catfish, compounding other stresses and affecting the fish's well-being (Manuel et al., 2013). In the previous study, the mRNA expression of HSP70 was significantly elevated in sea bass *Dicentrarchus labrax* (Poltronieri et al., 2007) and channel catfish *I. punctatus* (Refaey & Li, 2018) after being subjected to transportation procedure. The same trend observed in this study, transport stress led to the increase of the HSP70 expression in *C. gariepinus* liver at 0,6, and 12 hours after transportation. The significant decrease of mRNA level of HSP70 after 24 h suggests that *C. gariepinus* started to recover after 24 h of transport stress. This response, however, is dependent on the fish species, the duration of the transport, the degree of stress, and the transport conditions. The results suggest that HSPs play a role in the cell-protection under transport stress.

Water temperature is thought to be one of the most important environmental elements influencing fish physiology and behavior (Yan et al., 2017). In this study, the water temperature was set at 32 °C for heat-shock, and 28 °C for control. No mortality was observed during the thermal stress. The expression of HSP70 in *C. gariepinus* liver was strongly induced after 6 h of exposure and maintained at a high level until 24 h of exposure. This result is also widely reported in other fish species. The heat shock (34 °C) significantly elevated the HSP70 mRNA since 2 h of exposure in loach *Misgurnus anguillicaudatus* (Yan et al., 2017). Heat (32°C) and cold shock (9 °C) up-regulated the HSP70 mRNA level significantly in yellow croaker *Larimichthys crocea* (Xu et al., 2018) after 12 h. This result indicates that HSP70 also had an important role in the thermal stress responses in *C. gariepinus*.

Nitrite could become toxic to aquatic animals at high concentrations (Kocour Kroupová et al., 2018). Because of the potential risk of nitrite build-up in both natural aquatic ecosystems and aquaculture systems, the toxicity and physiological abnormalities caused by nitrite have been extensively researched in fish (Jensen et al., 2015). Nitrite toxicity will disrupt the ion uptake mechanism in the gill (Jensen et al., 2015) that lead to the hypoxic condition/methemoglobinemia (Kocour Kroupová et al., 2018), oxidative stress, and liver damage (Padmini et al., 2009). Under high nitrite concentration, the *C. gariepinus* HSP70 mRNA was overexpressed at 6 and 12 h after exposure and decreased significantly after 24 h. Similar elevation of HSP70 in fish tissues was also reported in the brachial of brown trout *Salmo trutta* (Jensen et al., 2015) and liver of mugil *Mugil cephalus* (Padmini et al., 2009) as the cytoprotective mechanism against nitrative stress.

Conclusion

In summary, this study is the first to reveal the full-length cDNA sequence of HSP70 from the *C. gariepinus* liver and the expression profile in different tissues and stress exposures. The results indicated that the HSP70 gene of *C. gariepinus* could serve as an early stress biomarker in response to bacterial infection and various aquaculture stressors.

Ethical Statement

All animal experimental and rearing procedures were carried out in accordance with the national standard for fish experimentation guidelines (01-6489-2000) of the Republic of Indonesia.

Funding Information

This research was partially funded by the Ministry of Education, Culture, Research and Technology of the Republic of Indonesia through *Peningkatan Global Competitiveness Perguruan Tinggi Indonesia* program, managed by IPB University (No: 9/IT3/SP/WCU/2021).

Author Contribution

HN and LL: Conceptualization, Investigation, Formal analysis, Original draft preparation, Review, and Editing. DTS, WW, and AA: Conceptualization, Data Curation, Supervision, Writing - Review & Editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper

Acknowledgements

The authors thank Dr. Dian Novita Sari for the technical help in the manuscript preparation and submission.

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