Scientific African 3 (2019) e00056

Contents lists available at ScienceDirect

Scientific African

journal homepage: www.elsevier.com/locate/sciaf

Lead-induced heat shock protein (HSP70) and metallothionein (MT) gene expression in the embryos of African catfish *Clarias gariepinus* (Burchell, 1822)

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ARTICLE INFO

Article history:
Received 4 January 2019
Revised 26 February 2019
Accepted 21 March 2019

Keywords:
Expression of stress proteins
RT-QPCR
Fish embryos
Heavy metal pollution

ABSTRACT

The enhanced expression of heat shock proteins (HSPs) and metallothionein (MT) can be detected in response to many environmental stressors, including exposure to heavy metals. We identified partial mRNA sequences for beta2-microglobulin (ß2m), heat shock cognate 70 kDa protein (HSP70), and MT in embryos of the African catfish *Clarias gariepinus* (Burchell, 1822). Levels of these transcripts were assessed using reverse-transcription quantitative polymerase chain reaction (RT-QPCR) after embryos of various stages were exposed to different concentrations of lead nitrate. ß2m gene expression exhibited a continuous increase over time. Neither HSP70 nor MT were upregulated, supporting the well-known sensitivity of these organisms to environmental pollutants. Interestingly, the partial MT and HSP70 mRNA sequences revealed an intimate phylogenetic relationship with these proteins in siluriforms. These partial sequences were more closely related to salmoniform proteins than to cypriniform proteins. The expression levels of HSP70 and MT were lower in embryonic tissues exposed to high concentrations of lead (500 µg/L), which impaired the fish's coping strategy via toxication at the cellular level. In conclusion, early developmental stages appear to be particularly vulnerable to lead and fail to activate the typical coping mechanisms. Therefore, these early life stages may not be suitable to characterize concentration-dependent toxicity.

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Introduction

Heavy metals are hazardous pollutants to animal and human beings commonly found in aquatic environments [1]. These elements can have a significant ecological impact on ecosystems and can modify the water quality, affecting aquatic organisms. Lead (Pb) is one of the most common heavy metals found in the aquatic environment and one of the most dangerous pollutants because it accumulates in the body due to its low rate of elimination [17]. Lead appears to lack biological functions and is very toxic to aquatic organisms even in small doses [2,24,40,41]. To minimize this toxicity,

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https://doi.org/10.1016/j.sciaf.2019.e00056
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aquatic organisms synthesize a group of proteins known as heat shock proteins (HSPs) and metallothioneins (MTs) that serve to protect against environmentally induced cellular damage [43]. HSPs and MTs are commonly used as biochemical markers of exposure to metal toxicity [45].

HSPs are involved in several processes, including preventing the aggregation and improper folding of newly synthesized proteins and the remodeling of protein complexes [10,30]. However, when cells are exposed to proteotoxic stressors, organisms respond with the rapid activation of genes regulating these proteins. The subsequent synthesis of HSPs has been shown to protect the cells from harmful effects, ensuring the survival of the organism under stressful conditions [30,46]. These proteins have been classified into several families based on their molecular weight and include HSP90, HSP70, HSP60, and HSP47 [15,51]. Of the HSP families, the HSP70 family, which is the most widely studied, is also the most widely conserved and the largest [20,39,47]. Under adverse environmental conditions, HSP70 is responsible for the defense, repair and detoxification of the cell [5,38]. Although studies have investigated HSP70 at the protein level in fish, little is known about the genomic structure and protein sequences of fish HSPs [10]. HSP70 has been cloned in several fish species, including rainbow trout [23], zebrafish [25], tilapia (*Oreochromis mossambicus*) [28], and pufferfish (*Fugu rubripes*) [26].

In terms of protein metabolism induced by heavy metals, MTs are among the most widely studied proteins. MTs comprise a metal binding protein family of low molecular weight (6.0–7.0 KDa) and high cysteine content (>30%) [48]. These proteins function to detoxify non-essential metal ions such as lead [4] and to protect cells against oxidative stress [29]. The transcription of MTs in fish is induced by a variety of metals, providing an efficient tool for evaluating the biological effects of heavy metal in aquatic environments [45], and MTs are widely employed as metal-specific biomarkers to indicate the presence of heavy metals [13,42].

Therefore, HSP and MT expression are important mechanisms that help organisms cope with adverse environmental conditions such as metal toxicity [35]. The expression levels of HSP and MT genes in fish have been used as biomarkers for water pollution [15,22,39]. Expression of these genes is also considered an early warning for the degradation of water quality, such as increased levels of dissolved oxygen. Furthermore, molecular biology techniques (such as reverse-transcription quantitative polymerase chain reaction, RT-QPCR) have been applied in several species to study fish HSPs and MTs [20,45], presenting a tool to quantify organisms' responses to environmental stressors.

Fish are common sentinel organisms and are among the most popular test organisms for biomonitoring in aquatic environments because they accumulate toxic metal pollutants through their gills [3,21,42]. Developmental stage and fish size can have a significant effect on the impact of a toxicant. The time required to accumulate toxicants is likely shorter during early life stages than in later stages due to the shorter diffusion distances involved [36]. The sensitivity of embryonic stages to chemical-induced adverse effects is substantially influenced by specific developmental events, e.g., organogenesis [18]. *Clarias gariepinus* is an excellent candidate for aquaculture and is used for ecotoxicological studies [31–34]. However, the analysis of only one developmental stage is not adequate for risk assessments of toxic chemicals. Therefore, the use of different developmental stages in ecotoxicology research should be promoted [18].

The present work investigated the induction of HSP70 and MT gene expression in different embryonic stages of the African catfish and evaluated their use as a biomarker of lead exposure using RT-QPCR.

Materials and methods

Animals

Artificial reproduction of the African catfish *C. gariepinus* was performed according to De Graaf and Janssen [9]. Spawners (1–2 kg body weight) were fed a commercial pellet diet (3% of body weight per day) and were kept in a recirculation system, ensuring high water quality (conductivity 2000 µs/cm; pH ~7.5; oxygen 90–95% saturation; temperature 25 °C; photoperiod 12:12 light:dark). Male spawners were anesthetized with 400 mg/L MS-222 (tricaine methanesulfonate; Crescent Research Chemicals, Phoenix, AZ) and subsequently killed. Next, the testes were removed and sperm was transferred into a sterile, dry petri dish to be used for artificial reproduction. For the collection of eggs, ovulation was artificially induced by an intramuscular injection of carp pituitary suspension (Cps; Stoller Fisheries, Spirit Lake, IA) (4 mg/kg body weight). Approximately 11–12 h after the injection, eggs were stripped and collected in plastic containers for dry fertilization.

In vivo exposure and sampling

The fertilized eggs were incubated in dechlorinized tap water (conductivity 700 µs/cm; pH ~8; oxygen 90–95% saturation; temperature 24 °C). The eggs were divided into 12 aliquots and transferred to 12 aquaria (20 cm × 20 cm × 5 cm, 2 L), providing four experimental groups: one control group and the other groups exposed to 100, 300 and 500 µg/L of lead nitrate. Each group was assessed in triplicate. Lead nitrate standard solution (1000 mg/L, VWR; MERCK, Germany) was used as stock solution to establish the nominal concentrations. Static exposure started 6 h post-fertilization. For sampling, embryos were pooled (12 and 36 h; $n = 4$) or individually sampled (48 and 132 h), transferred to 1 mL RNAlater, incubated for 12 h at 4 °C and stored at −20 °C for further processing.

Table 1
List of primers used for the gene identification (cons), and quantification by real-time PCR (q). ß2m – ß-microglobulin, HSP70 – heat shock protein 70, MT – metallothionein, Ta – annealing temperature.

Target	Primer	Sequence	Ta
ß2m	ß2m consF	TTCTgCTTTCCTTCgTCgTTCTC	63 °C
	ß2m consR	ACgCTCTTggTCAgATgAAACTT	63 °C
	qClß2m F	CTTCgCTAAggAgTgTACACCAA	
	qClß2m R	gCTCTTggTCAgATgAAACTTCC	
MT	MT consF	gAACCTgCAACTgCggTACAT	60 °C
	MT consR	ggCAgCACTTggAATCgCAggTAT	60 °C
	qClMT F	MT consF	
	qClMT R	MT consR	
HSP 70	HSP70 consF	gACCAAggAAACCggACCAC	67 °C
	HSP70 consR	TAggCAATAgCAgCAgCAgTAggC	67 °C
	qClHSP70 F	TTTgATgCCAAgCgTCTgAT	
	qClHSP70 R	TTgACggTCTTTCCAAgATAgg	

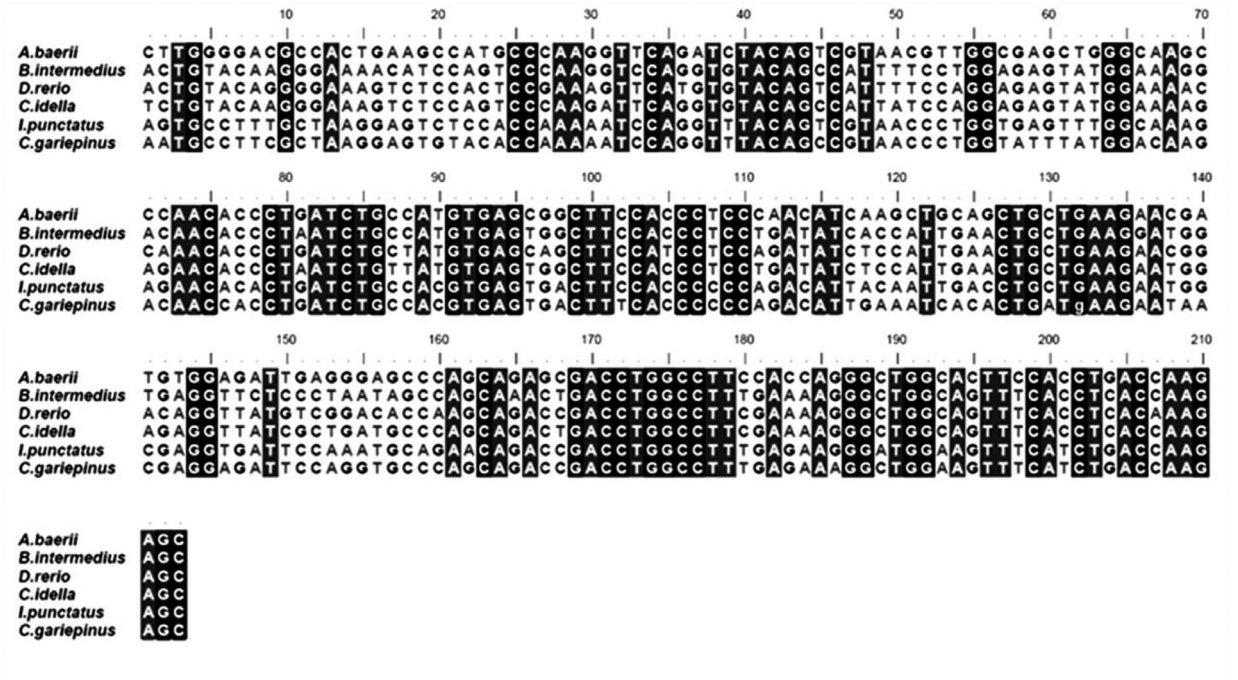

Fig. 1. Alignment of the partial ß-microglobulin (ß2m) coding sequence identified in African catfish *Clarias gariepinus* (DQ885943) and the sequences described in sturgeon *Acipenser baerii* (AJ132766), barb *Barbus intermedius* (AJ507011), grass carb *Ctenopharyngodon idella* (AB190816), zebrafish *Danio rerio* (BC062841), channel catfish *Ictalurus punctatus* (AF016042).

Reverse transcription

RNA extraction was performed with TRIzol (Invitrogen, Germany) with an additional DNAse digestion according to Wuertz et al. [50]. AMV-reverse transcriptase, AMV-RT (Finnzymes, Finland) was employed according to the protocol of Reiser et al. [37]. Selected sequence information from teleosts was used to calculate a multiple alignment using BioEdit 7.0.0 [14] and to design consensus primers using OLIGO 5.0 (National Biosciences Inc.). PCR primers for the identification of MT, HSP70 and β2m are listed in Table 1. After PCR amplification (95 °C for 3 min, 35 cycles of 95 °C for 30 s, Ta for 30 s, 72 °C for 1 min, 72 °C for 5 min; with 1.5 mM $MgCl_2$, 1× PCR buffer, 0.4 µM primer, 0.2 mM dNTPs, 0.7 U GO Taq, and 3 µL cDNA), sequencing was performed with the capillary sequencer CEQ 8800 (Beckman Coulter, Germany) according to the manufacturerś protocol. Sequences were analyzed with the database homology search tool BLAST and with multiple alignment sequence comparison using BioEdit.

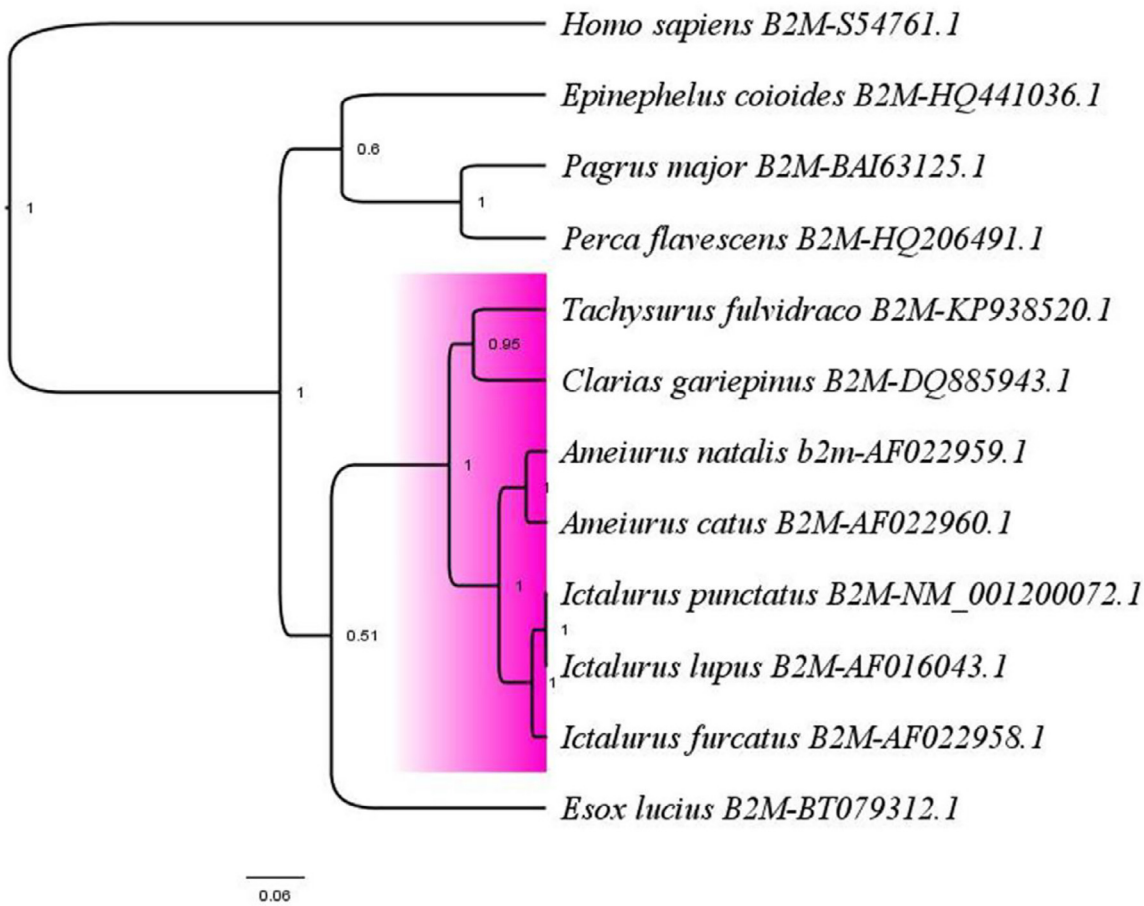

Fig. 2. Maximum Likelihood phylogenetic tree showing the relation between the partial sequence of *Clarias gariepinus* ß2m obtained in the current study and the same mRNA precursors in other fishes. H. sapiens ß2m sequence was applied as an outgroup. Accession numbers for each species ß2m are shown in the figure. The subclade where *C. garipinus* located is highlighted by dark pink color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Quantitative PCR (QPCR)

Using hot-start Platinum Taq polymerase (Invitrogen), QPCR was performed with SYBR Green in a Stratagene MX3000P cycler under the following conditions: 94 °C initial denaturation for 5 min followed by 40 cycles of 94 °C denaturation for 20 s, 58 °C primer annealing for 20 s, and 72 °C polymerization for 20 s [with 5 mM $MgCl_2$, 1× PCR buffer, 1× SYBR Green, 0.4 µM primer, 0.3 mM dNTP, 1 U Taq, and 4 µL cDNA]. For each run, the relative quantification was calculated from a co-amplified standard dilution series (with cDNA from a liver sample of *C. gariepinus*) to compensate for lot-to-lot and run-to-run variations [49]. All samples were performed in duplicate.

Phylogenetic analyses

A sequence alignment was generated from the ß2m, HSP70 and MT sequences we identified and from the GenBank database using the ClustalW algorithm. JModelTest software was used to detect the best nucleotide substitution model to construct a maximum likelihood phylogenetic tree, which was uploaded to Beauti software and calculated with 10,000,000, 50,000,000 and 100,000,000 Markov chains. Tree topologies were estimated using BEAST software uploaded to Tracer v. 1.6 to assess the quality of the results. Only trees with effective sample sizes (ESSs) above 200 were accepted. The resulting trees were combined using LogCombiner software and uploaded to TreeAnnotator to summarize the information retrieved from the tree samples produced through BEAST. The consensus tree was compiled using FigTree software V. 1.4.2.

Statistical analysis

Results are presented as the mean ± SD. Data were analyzed for normal distribution using the Kolmogorov-Smirnov test and for equal variance using the Kruskal-Wallis test. One-way analysis of variance was performed using SigmaStat 2.0

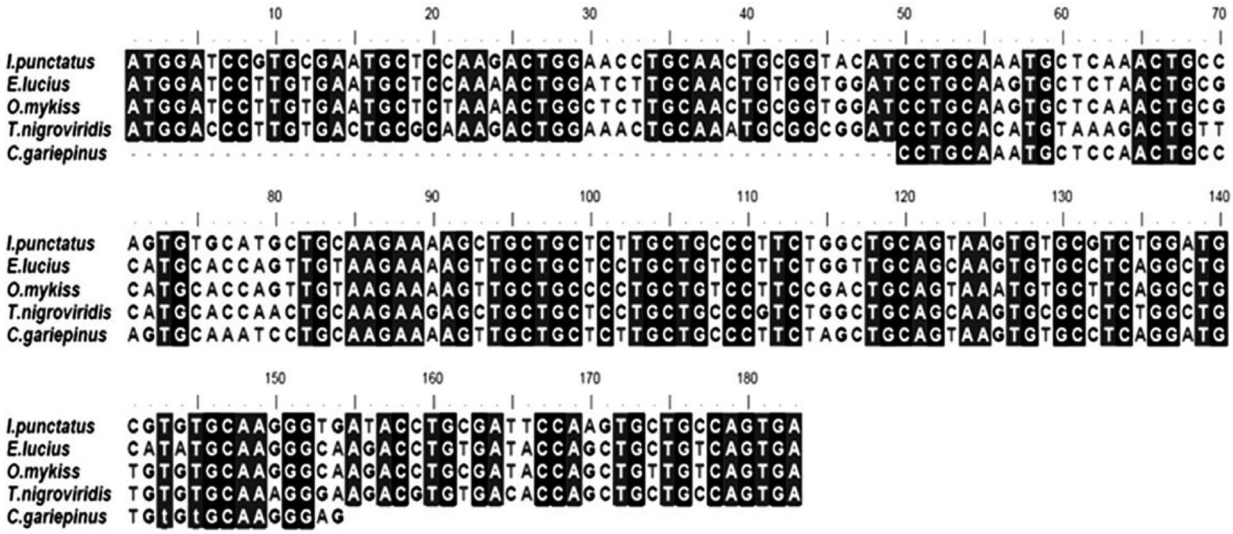

Fig. 3. Alignment of the partial metallothionein (MT) coding sequence identified in African catfish *Clarias gariepinus* (DQ885944) and the sequences described in channel catfish *Ictalurus punctatus* (AF087935), pike *Esox lucius* (gi|62780), Rainbow trout *Oncorhynchus mykiss* (gi|213816), Green pufferfish *Tetraodon nigroviridis* (CR651488). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

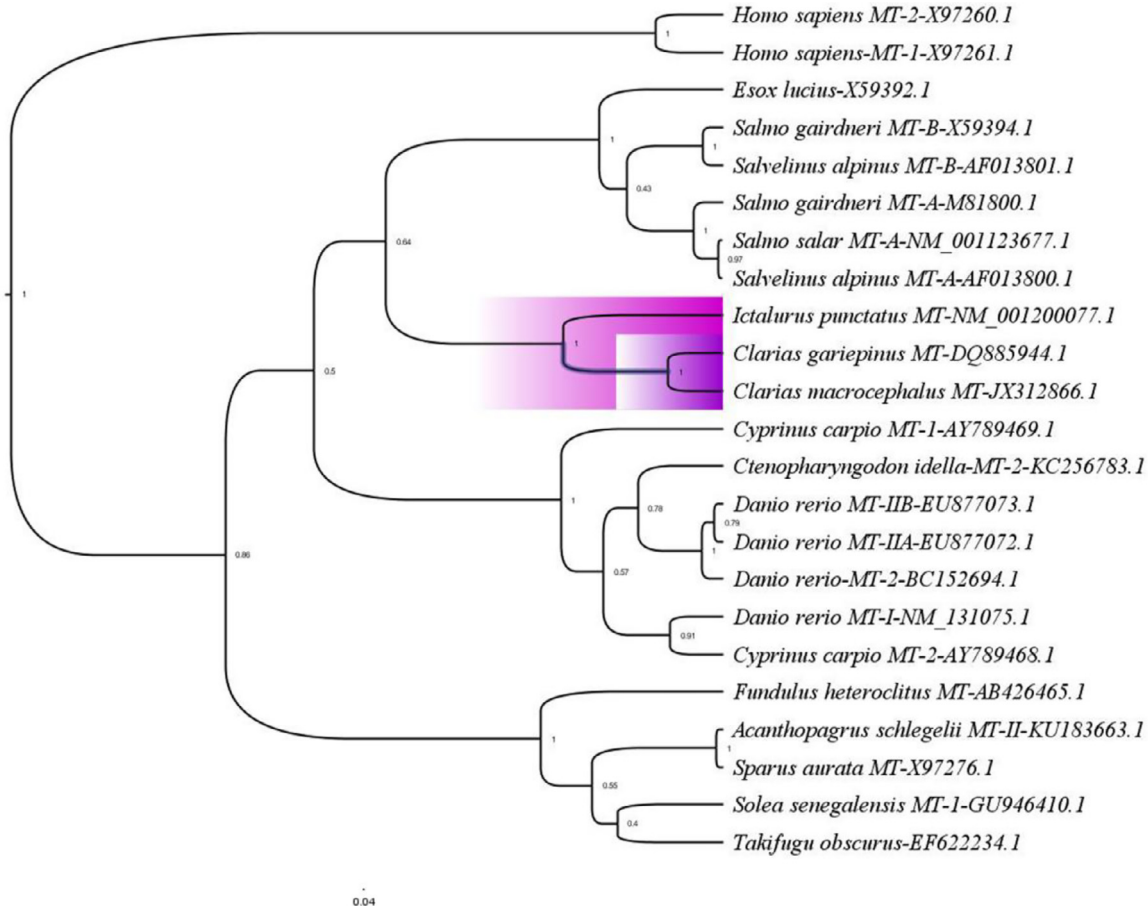

Fig. 4. Maximum Liklihood phylogenetic tree showing the relation between the partial sequence of *Clarias gariepinus* MT obtained in the current study and the same mRNA precursors in other fishes. H. sapiens MT isoformsśequences were applied as an outgroup. Accession numbers for each species MT are shown in the figure. The subclade where *C. garipinus* located is highlighted purple-bluish color, and the higher subclade where all siluriform exist is highlighted by purple one. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

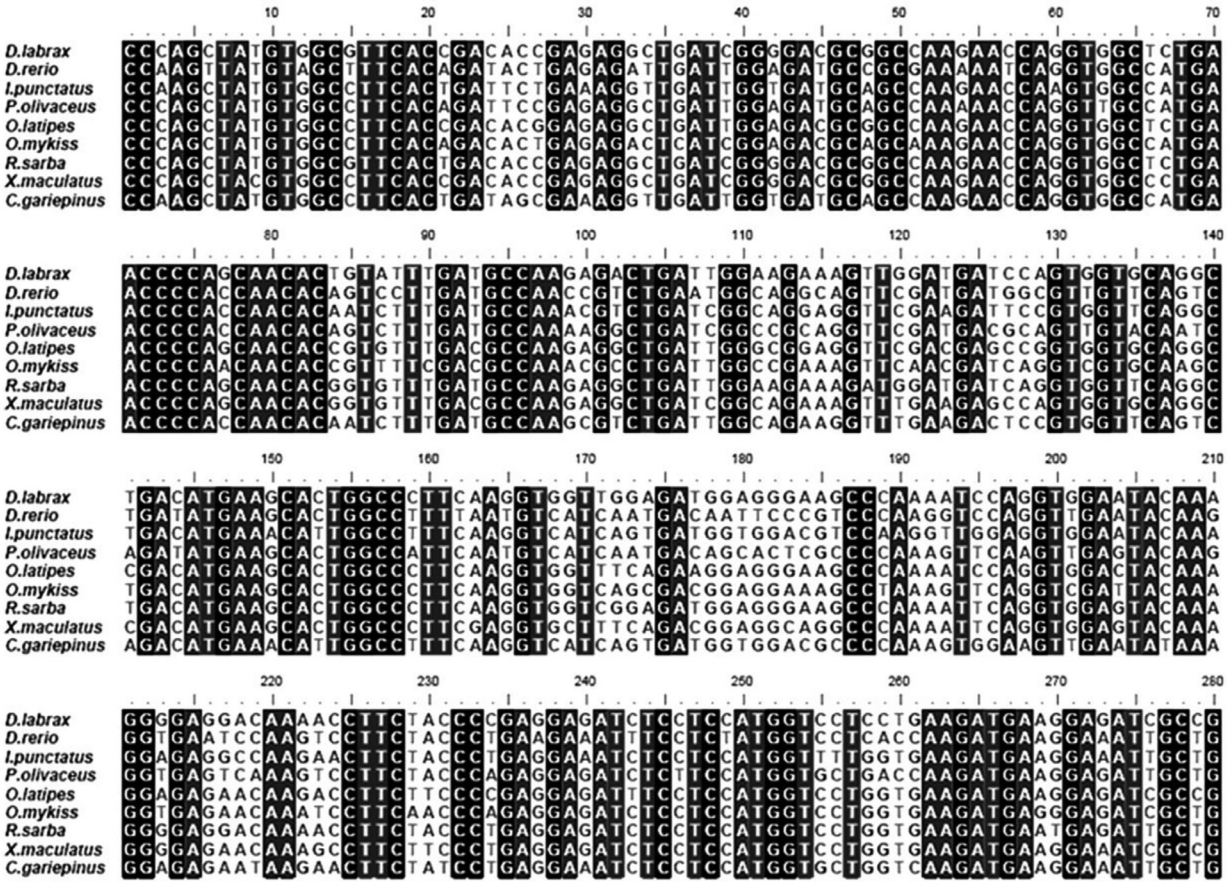

Fig. 5. Alignment of the partial heat shock protein 70 (HSP70) coding sequence identified in African catfish *Clarias gariepinus* (DQ885945) and the sequences described in sea bass *Dicentrarchus labrax* (AY423555), zebrafish *Danio rerio* (L77146), channel catfish *Ictalurus punctatus* (gi|758782), medaka *Oryzias latipes* (AF286875), trout *Oncorhynchus mykiss* (AB062281), *Paralichthys olivaceus* (AF053059), *Rhabdosargus sarba* (AY436787), platyfish *Xiphophorus maculatus* (AB062114).

software (Jandel Scientific, San Rafael, USA). A pairwise comparison was carried out using the non-parametric Mann-Whitney rank sum test and parametric *t*-test.

Results

Identification of genes

A 213 bp partial CDS of ß2m was identified, revealing 86% identity to *Ictalurus punctatus* (NM_001200072.1) and *Tachysurus fulvidraco* (KP938520.1) and 81% amino acid sequence identity to other siluriform species, including *Ameiurus catus* (AAC64996.1), *Ictalurus furcatus* (AAC64994.1) and *Pelteobagrus fulvidraco* (ALK16248.1) (Fig. 1). Conserved domain analysis [27] https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=KJ8HTT2T014&mode=all) confirmed that the non-covalently bound light chain of the class I major histocompatibility complex contained the putative signature of the Ig superfamily. Phylogenetic analysis revealed clustering with other siluriform fishes (Fig. 2).

The partial MT mRNA sequence (105 bp) exhibited 95% identity with *Clarias macrocephalus* (JX312865.1), 92% with *Pelteobagrus fulvidraco* (FJ418583.1) and 90% with *Ictalurus punctatus* (acc. no. NM_001200077.1) (Fig. 3). The constructed maximum likelihood tree revealed clustering of *C. gariepinus* with other siluriform species (Fig. 4).

The HSP70 (410 bp) sequence revealed 97%, 96% and 91% identity to *C. macrocephalus* (HM044876.1), *C. batrachus* (KC981647.1) and *I. punctatus* (NM_001200273.1), respectively (Fig. 5). Again, the maximum likelihood tree identified a siluriform cluster, which, surprisingly, appeared to be more closely related to salmoniform HSP70 than to Cypriniformes, Perciformes and other fish HSP70s (Fig. 6).

HSP70 and MT expression

In this study, ß2m expression increased steadily from 2.7 ± 1.9% (control), 3.4 ± 0.7% (100 µg/L lead nitrate), 3.4 ± 2.2% (300 µg/L), and 4.1 ± 2.7% (500 µg/L) to 73 ± 13%, 84 ± 12%, 88 ± 18%, and 70 ± 15%, respectively (Fig. 7). At each sampling

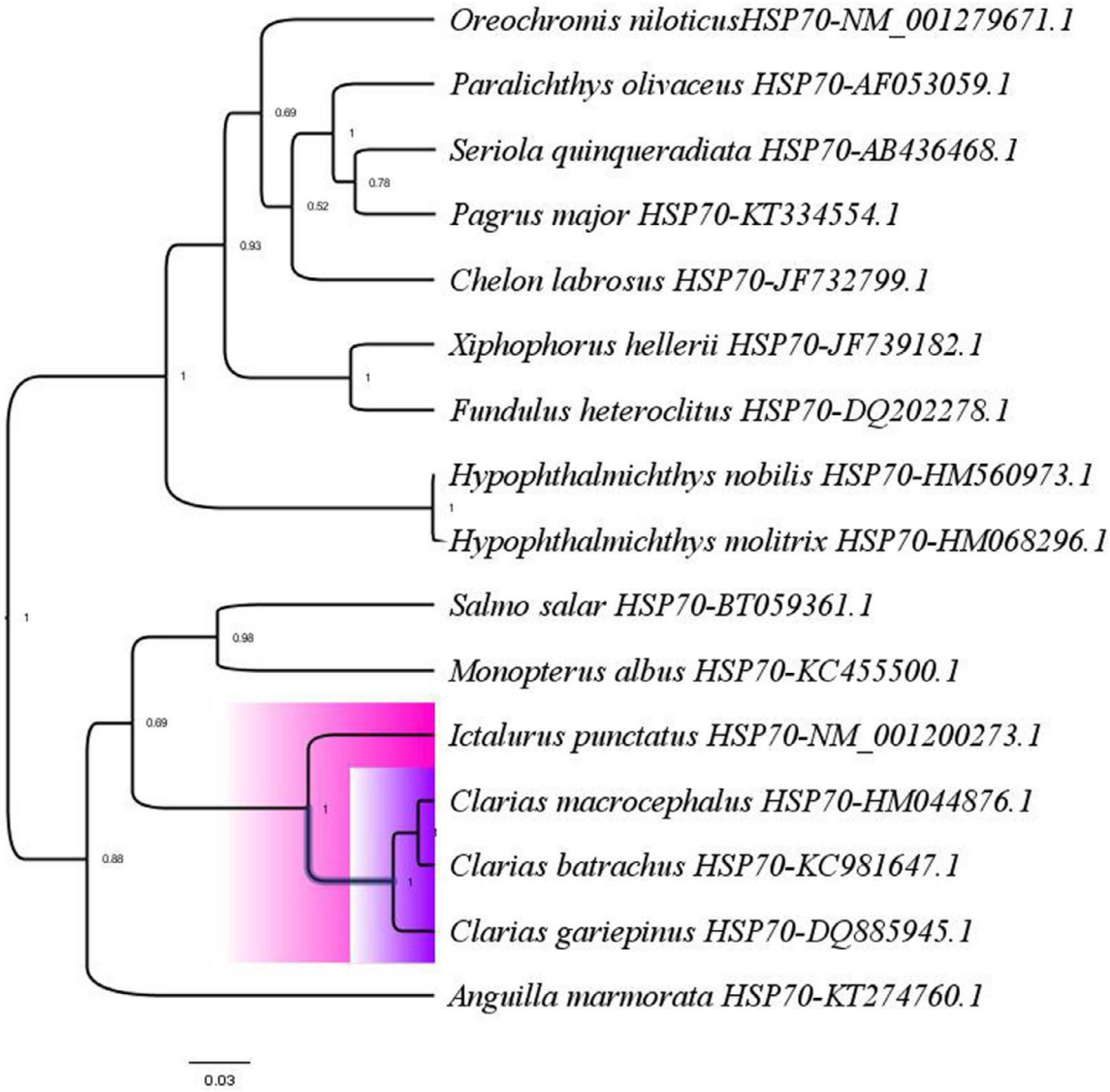

Fig. 6. Maximum Liklihood phylogenetic tree showing the relation between the partial sequence of *Clarias gariepinus* HSP70 obtained in the current study and the same mRNA precursors in other fishes. Accession numbers for each species HSP70 are shown in the figure. The subclade with *C. garipinus* is highlighted with purple and the higher subclade where all Siluriformes are included is highlighted in pink. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

point, consistent expression was observed. Thus, MT and HSP70 expression were normalized to ß2m expression, allowing a comparison of treatments at each sampling point although not over time. No significant differences (Dunn's, $p < 0.05$, $n = 6$) in HSP70 or MT expression were detected between the treatment and control groups (Figs. 8 and 9). A high variability between samples was observed in MT and HSP70 expression at 12 h and 36 h (Figs. 8 and 9).

Discussion

In general, HSPs and MTs are upregulated in cells exposed to environmental stressors, including heavy metals [20]. Recently, HSP70 and MT were used to assess environmental contamination using a contact assay [15]. The present study focused on two biomarkers commonly used to monitor the ecotoxicology of heavy metal pollution: HSP70 and MT. We identified the gene sequences of HSP70 and MT in different embryonic stages of the African catfish *C. gariepinus* after exposure to different concentrations of lead nitrate and performed a phylogenetic analysis based on the molecular data. Selection of the reference gene was critical because commonly used housekeeping genes are highly regulated during early development. However, expression of the ß2m gene was confirmed for each time point, and a continuous increase over time

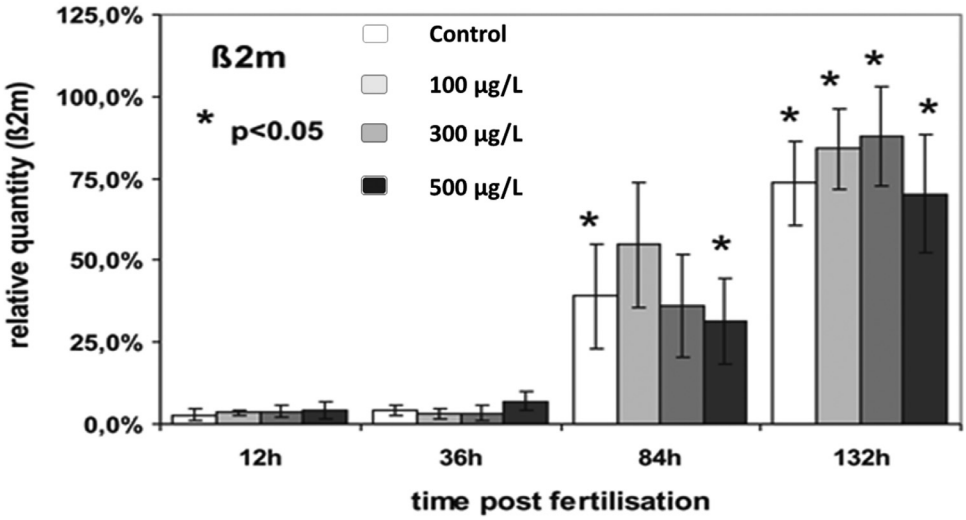

Fig. 7. Expression of ß microglobulin (ß2m) in African catfish larvae after 12 h, 36 h, 84 h and 132 h of exposition to lead nitrate (control, 100, 300 and 500 µg/L). Significant (Dunn's, $n = 6$) increases within a treatment are indicated*.

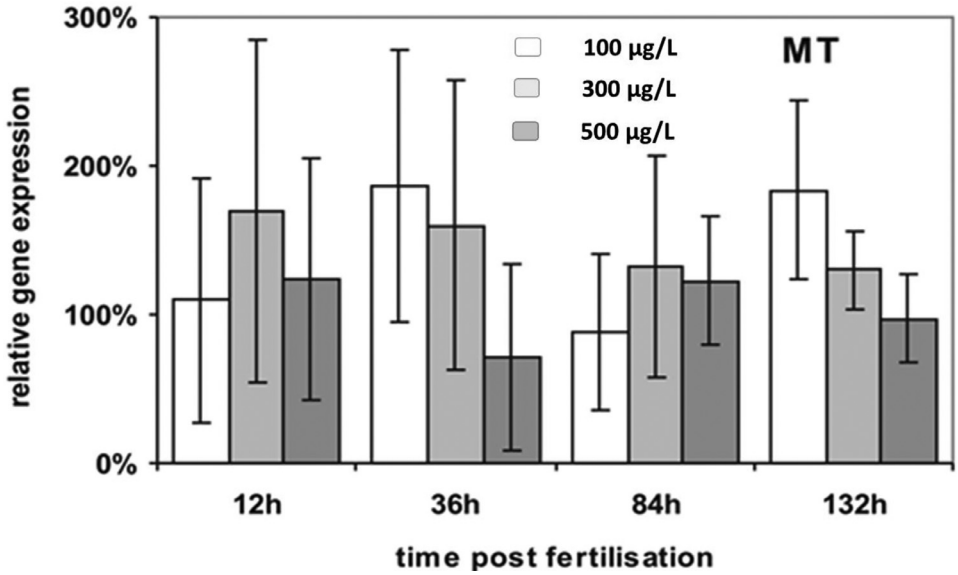

Fig. 8. Expression of metallothionein (MT) in African catfish larvae after 12 h, 36 h, 84 h and 132 h of exposition to lead nitrate (100, 300 and 500 µg/L) normalized to ß2m and the control treatment. No significant differences were detected (Dunn's, $n = 6$).

was observed. Comparative analysis revealed high identities to HSP70 and MT in other teleost species and to sequences identified in African catfish clustered within the Siluriformes.

Phylogenetic analysis of the isolated ß2m mRNA revealed its prevalence in the clade of catfishes. The precursor is closely related to that of *T. fulvidraco*, a siluriform species, and performs well as a reference gene in QPCR studies [48]. Neither HSP70 nor MT were upregulated in the present study. Therefore, coping strategies involving HSP70 and MT may not be effective as a consequence of overloading at the embryonic level. This result explains the well-known sensitivity to environmental pollutants and is even more significant for those protected against in later stages by more specific detoxification mechanisms, including heavy metals. Interestingly, the MT and HSP70 partial mRNA sequences we obtained showed an intimate phylogenetic relation to siluriform MT and HSP70 and were more closely related to the salmoniform proteins than to the cypriniform proteins. In both siluriformes and salmoniformes, the protein homologues showed increased expression levels in response to cadmium, copper, zinc and PCBs [12,21]. Additionally, specific lead-binding MTs have been reported in rats and may also be present in fish [19].

As shown for a variety of animal taxa [8,11,16], HSP70 and MT levels decline beyond a certain threshold with increasing lead concentrations. In contrast, expression of HSP70 and MT did not show consistent changes after exposure to lead nitrate

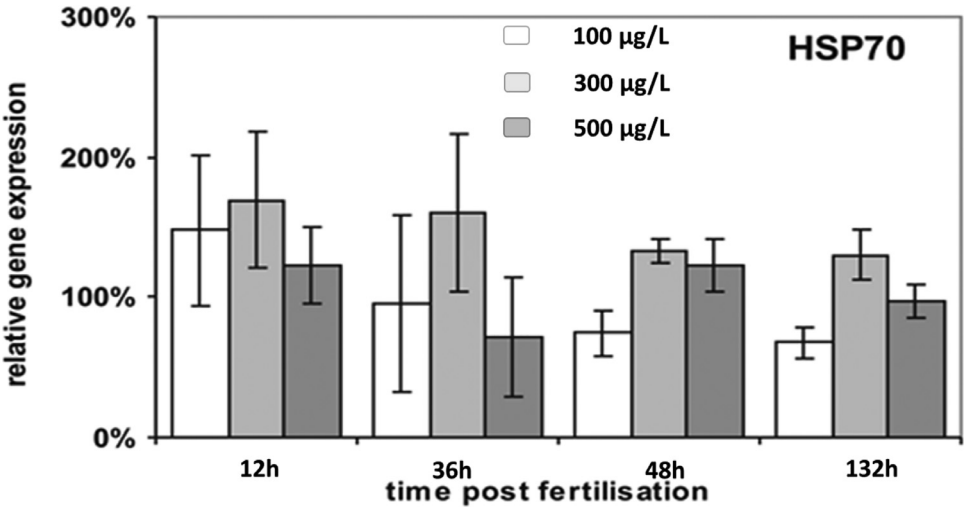

Fig. 9. Expression of heat shock protein 70 (HSP70) in African catfish larvae after 12 h, 36 h, 84 h and 132 h of exposition to lead nitrate (100, 300 and 500 µg/L) normalized to ß2m and the control treatment. No significant differences were detected (Dunn's, $n = 6$).

at any dose. Congruent with this study, Jing et al. [20] and Brulle et al. [6] reported that HSP and MT did not show variations in expression level after exposure to heavy metals. Additionally, Cheung et al. [7] demonstrated that Nile tilapia exposed to lead did not show increased MT expression. According to our results, the expression levels of HSP70 and MT were lower in the embryonic tissues exposed to high lead concentrations (500 µg/L) than in those exposed to low lead concentrations (100/300 µg/L) at each sampling point. Similarly, Tiwari et al. [44] reported a decrease in the MT level in tissues of the freshwater teleost *Channa punctate* with increasing metal concentrations. At higher lead concentrations, the coping strategy may be impaired by the intoxication process at the cellular level. Nevertheless, Tiwari et al. [44] suggested that the limited capacity of cells to bind metals via MT is not a direct effect of metal toxicity on the capacity to synthesize proteins.

In conclusion, early developmental stages appear particularly vulnerable to lead and fail to activate the typical coping mechanisms. Therefore, these early life stages may not be ideally suited to characterize concentration-dependent toxicity.

Conflict of interest

No potential conflict of interest was reported by the authors.

Acknowledgments

The first author is grateful for the continuous support from the Alexander von Humboldt Foundation (3.4 - RKS - AGY/1134716). This work was conducted in the framework of an Alexander von Humboldt foundation return fellowship for the first author, hosted by Faculty of Science, Al-Azhar University, Assiut, Egypt.

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