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Dietary supplementation of curcumin augments heat stress tolerance through upregulation of *nrf-2*-mediated antioxidative enzymes and *hsps* in *Puntius sophore*

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Abstract Heat stress is one of the major environmental concerns in global warming regime and rising temperature has resulted in mass mortalities of animals including fishes. Therefore, strategies for high temperature stress tolerance and ameliorating the effects of heat stress are being looked for. In an earlier study, we reported that Nrf-2 (nuclear factor E2-related factor 2) mediated upregulation of antioxidative enzymes and heat shock proteins (Hsps) provide survivability to fish under heat stress. In this study, we have evaluated the ameliorative potential of dietary curcumin, a potential Nrf-2 inducer in heat stressed cyprinid *Puntius sophore*. Fishes were fed with diet supplemented with 0.5, 1.0, and 1.5% curcumin at the rate 2% of body weight daily in three separate groups (n = 40 in each group) for 60 days. Fishes fed with basal diet (without curcumin) served as the control (n = 40). Critical thermal maxima (CTmax) was determined for all the groups (n = 10, in duplicates) after the feeding trial. Significant increase in the CTmax was observed in the group fed with 1.5% curcumin-supplemented fishes whereas it remained similar in groups fed with 0.5%, and 1% curcumin-supplemented diet, as compared to control. To understand the molecular mechanism of elevated thermotolerance in the 1.5% curcumin supplemented group, fishes were given a sub-lethal heat shock treatment (36 °C) for 6 h and expression analysis of *nrf-2*, *keap-1*, *sod*, *catalase*, *gpx*, and *hsp27*, *hsp60*, *hsp70*, *hsp90*, and *hsp110* was carried out using RT-PCR. In the gill, expression of *nrf-2*, *sod*, *catalase*, *gpx*, and *hsp60*, *hsp70*, *hsp90*, and *hsp110* was found to be elevated in the 1.5% curcumin-fed heat-shocked group compared to control and the basal diet-fed, heat-shocked fishes. Similarly, in the liver, upregulation in expression of *nrf-2*, *sod*, *catalase*, and *hsp70* and *hsp110* was observed in 1.5% curcumin supplemented and heat shocked group. Thus, this study showed that supplementation of curcumin augments tolerance to high temperature stress in *P. sophore* that could be attributed to *nrf-2*-induced upregulation of antioxidative enzymes *sod*, *catalase*, *gpx*, and the *hsps*.

Keywords Heat stress · *nrf-2* · Antioxidative enzymes · *hsp* genes · Curcumin · *Puntius sophore*

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Introduction

The ectothermic animals like fish can maintain their body temperature within a narrow temperature range by means of behavioral and physiological means. When the temperature increases beyond those limits, it can

cause physiological disturbances, ultimately leading to death (Neuheimer et al. 2011). The global increase in temperature has caused many incidences of mass mortalities of fishes in recent times (Kibria 2014). Such mass mortalities are the major threats to the aquaculture industry and could cause a huge economic loss in near future. Therefore, means of controlling death of fish upon a sudden increase in water temperature are being searched for.

It has long been known that the heat shock response represented by increased synthesis of heat shock proteins (Hsps) plays a central role in survival of organisms under heat stress (Feder and Hofmann 1999; Pirkkala et al. 2001; Tomanek and Somero 2002). The Hsps not only stabilize and refold the denaturing proteins but also facilitate the proteolysis of the denatured proteins (Tomanek and Somero 2002; Wang et al. 2015; Zunino et al. 2016). In combination with the Hsps, the antioxidative enzyme system is also activated as a survival strategy during heat stress to counteract the increased reactive oxygen species (ROS) production under heat stress condition (Madeira et al. 2013; Nakano et al. 2014; Mahanty et al. 2016a). In an earlier integrated proteomics and pathway analysis study, we have shown that increased synthesis of both Hsps and antioxidative enzymes superoxide dismutase (*Sod*), glutathione peroxidase (*Gpx*), and *Catalase* coordinated by a common transcription factor, i.e., Nrf-2 (nuclear factor (erythroid-derived 2)-like 2) helps fish *Channa striatus* to survive under heat stress condition (Mahanty et al. 2016a). Thus, it was hypothesized that external agents that can trigger the expression of *nrf-2* can induce the expression of heat shock proteins and thus can provide increased survivability to fish under heat stress.

Curcumin is a phenolic compound that naturally occurs in the rhizomes of the plant *Curcuma longa* and is used as a common food additive especially for human consumption in the Indian sub-continent. Besides many other biological effects such as anti-inflammatory, chemopreventive, and chemotherapeutic activities, curcumin has direct and indirect antioxidative effects by scavenging ROS and induces the expression of cytoprotective proteins in an Nrf-2-dependent pathway (Kou et al. 2013). Therefore, in the present study, we investigated whether curcumin supplementation can induce the expression of *nrf-2* and the antioxidative enzymes *sod*, *gpx*, and *catalase* in *Puntius sophore* and whether upregulation of these genes have any effect on overall heat stress tolerance and survivability of the fish. It has also been well studied that curcumin can activate the expression of *hsp70* and the antioxidative enzymes in human cancer cell lines and some other vertebrate models (Dunsmore et al. 2001; Teiten et al. 2009; Zhang et al. 2015). In contrast, curcumin has been reported to have differentially affecting the expression of *hsp27* depending upon the model/cell type chosen for the study; it has been found to upregulate the expression of *hsp27* in leukemia cells (Sarkar et al. 2014) whereas in nephrons of diabetic mouse, downregulation of *hsp27* has been observed following curcumin administration (Tikoo et al. 2008). However, its effect on expression of other *hsp* families have been scantily studied. Therefore, we carried out the gene expression analysis of a number of *hsp* genes, *hsp27*, *hsp60*, *hsp70*, *hsp90*, and *hsp110*, in the fishes fed with curcumin-supplemented diet, and heat-stressed fish (1.5%Cur+HS) and compared them with those of basal diet-fed heat-stressed (BD+HS) and non-heat-stressed groups (BD; control).

Materials and methods

Selection of test species

P. sophore, a minor carp of the family Cyprinidae, was chosen as the experimental model because of the following reasons: (a) phylogenetically, *Puntius* and zebrafish (*Danio rerio*) are in the same family and the proteogenomic information available for zebrafish could be used for studies on *P. sophore* (Mahanty et al. 2016b; Meyer et al. 1993). (b) It is a highly nutritious fish and owing to high nutritive value, attempts are being made to bring it under aquaculture practices and this study could be helpful in its culture and stress management (Mahanty et al. 2014; Wahab et al. 2003). (c) The study can have implications in stress management in other carps of the Cyprinidae family like *Catla catla*, *Labeo rohita*, and *Cirrihinus mrigala* which contributes to the majority of aquaculture production in the Indian sub-continent.

Collection of fish

Fishes were collected from the local aquaculture ponds (Barrackpore, Kolkata; 22.76° N 88.37° E) and were taken to laboratory in tanks with 30 l water holding capacity. Fishes were acclimatized in laboratory condition (temperature, 25–27 °C) and were fed twice daily at

the rate of 2% of their body weight with basal feed formulated to satisfy the protein requirement (Fig. 1).

Experimental design

Fishes of uniform size and length were transferred to aquarium tanks (30 l) and randomly distributed in four experimental dietary groups (A, B, C, D) containing 40 fishes in each group (Fig.1). Curcumin was purchased from Himedia laboratories (RM1449). Four isonitrogenous (crude protein 34%, crude fat 5.8%) feeds were prepared with graded levels of curcumin (0.5–1.5%) except for the control (Zheng et al. 2012). Feed was prepared using soyabean oil cake (290 g Kg^{-1}), musturd oil cake (524 g Kg^{-1}), fish meal (50 g Kg^{-1}), vitamin-mineral premix (20 g Kg^{-1}), and edible veg. oil (15 g Kg^{-1}). The quantity of de-oiled rice bran (100 g Kg^{-1} in control feed) was replaced with equal amount of curcumin in the supplemented feeds (5–15 g Kg^{-1}). Fishes in tank A were fed with basal diet (control); those in the dietary groups B, C, and D were fed with 0.5, 1, and 1.5% curcumin-supplemented feed, respectively, for a period of 60 days. The unconsumed feed and fecal matters were siphoned out from the tank bottom and 3/4th of the water was changed daily.

Determination of critical thermal maxima for *P. sophore*

After the 60-day regime of feeding, apparently, healthy fishes from each group (n = 10; in duplicates) were taken out and critical thermal maxima (CTmax) values were determined for each group (A, B, C, D) following earlier reported methods (Mahanty et al. 2016b). Briefly, fishes were exposed to gradual increase in temperature at the rate of 2 °C/h in metallic aquaria with temperature control system. The aquaria were monitored regularly to record the temperature at which the fishes lost equilibrium. CTmax was calculated as the arithmetic mean of these collective thermal points.

Sub-lethal heat shock treatment and collection of tissues for gene expression analysis

Ten fishes from the basal diet and 1.5% curcumin-supplemented feed were taken out and heat shocked at 36 °C for 6 h in two different aquariums (30 l capacity) fitted with thermostat. Basal diet-fed fishes kept at ambient temperature (25–27 °C; n = 10) served as the experimental controls. After heat shock treatment, fishes were dissected (after euthanization with 200 mg/l MS-222) and gill and liver tissues were collected in RNA Later (R0901, Sigma). Sub-lethal heat shock treatment was not performed for the fishes of the 0.5 and 1% curcumin-supplemented groups as there was no significant increase in the CTmax values of these fishes.

RNA extraction and cDNA preparation

Total RNA was extracted using the RiboZol kit (Himedia Laboratories, India) following the manufacturer's protocol. Following isolation of RNA, the concentration of each RNA sample was measured by Bioanalyzer (Agilent, USA). RNA samples were treated with the DNase 1 (NEB, UK) as per the manufacturer's

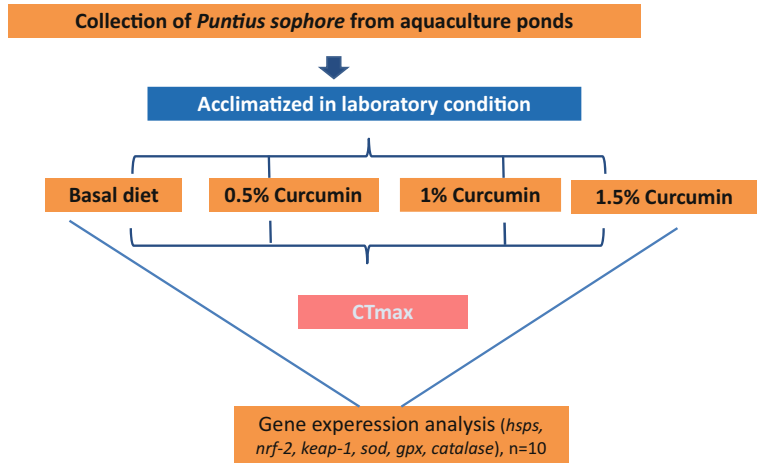

Fig. 1 A schematic representation of the study design

recommended standard protocol to remove potential genomic DNA carryover. RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Thermo Scientific, USA) according to the manufacturer's protocol.

Gene expression analysis

A total of five *hsp* genes (*hsp27*, *hsp60*, *hsp70*, *hsp90*, *hsp110*), two transcription factor genes (*nrf-2*, *keap-1*), and three antioxidative enzymes (*sod*, *gpx*, *catalase*) were analyzed in gill and liver tissues of three groups of *P. sophore* ($n = 10$ in each group): control (BD) (basal diet fed and kept at 25–27 °C), basal diet fed and heat shocked (BD+HS), and 1.5% Curcumin supplemented with basal diet and heat shocked (1.5%Cur+HS). Primers for amplification of different *hsps*, antioxidative enzyme genes, *nrf-2*, and *keap-1* genes were synthesized using the information available in the literature as mentioned in Table 1. PCR in a 50-μl mixture consisted of 20 ng of first strand complementary DNA (cDNA), 1× buffer (200 mM Tris-HCl pH 8.3, 500 mM $MgCl_2$ pH 8.5), 200 μM each of dNTPs, 10 μM of each gene-specific primer, and 5 U of hot start polymerase. PCR analysis was carried out by using a gradient thermal cycler (Veriti 96 well Thermal Cycler, Applied Biosystems, USA). The amplification conditions were as follows: 3 min of predenaturation at 95 °C followed by 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing for 45 s at temperatures optimized for specific genes, extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min. PCR products (10 μL) were electrophorosed in a 1.6% agarose gel and gel images were captured in ImageQuant LAS4000 (GE Healthcare). The linear range of amplification for each primer pair was calculated by plotting the band intensities of amplified products of 20–40 cycles of amplification (Meadus 2003). The linear range of amplification was observed between 30 and 35 cycles for the genes studied. The thirty-fifth cycle was chosen as the optimal number of cycle for all the genes as amplification of all the genes in samples of all conditions could be visually confirmed with this cycle number. Additionally, the band intensities of different genes studied were normalized with those of *tubulin* (internal control); the expression of which remained unaltered in all the experimental condition.

Densitometric quantification

Semi-quantitative analyses of mRNA expression were carried out by gel densitometric analysis software ImageJ (http://rsb.Info.nih.gov/ij/index.html) (Banerjee et al. 2015). Several studies have reported *tubulin* as a housekeeping gene in the cell (Williams et al. 2003; Mohindra et al. 2014; Mahanty et al. 2016a, b; Purohit et al. 2016). In the present study, also expression of *tubulin* remained constant at each of the experimental groups; no significant difference was observed. Hence, it was used as an internal control to correct for sample to sample variations. Target gene expression was normalized relative to *tubulin* after subtraction of the background pixel intensity. Expression of the different genes were analyzed in gill and liver tissues of 10 individual fishes from each experimental group. Comparison of target gene expression between individuals was adjusted with the internal standards which were previously normalized between samples (Meadus 2003). The procedure was repeated and the values were combined and expressed as mean ± standard deviation. Fold changes of gene expression are expressed in comparison with the control. One way analysis of variance (ANOVA) followed by Tukey's test was employed to compare the variation between the experimental groups ($p < 0.05$).

Network analysis

To visualize the interaction between genes studied in the present investigation, pathway analysis was carried out using freely available online tool String 10: functional protein association software (http://string-db.org/). All the genes were mapped to their human homolog before analysis was carried out; *nrf-2*, *keap-1*, *hsp110*, *hsp90*, *hsp70*, *hsp60*, *hsp27*, *sod*, *catalase*, and *gpx* were mapped to *nfe2l2*, *keap1*, *hsph1*, *hsp90aa1*, *hspa4*, *hspd1*, *hspb1*, *sod1*, *cat*, and *gstk1*, respectively. The physical (indirect) and functional (direct) interactions were considered when establishing the links, which are derived from genomic context, high-throughput experiments, co-expression analysis, and previous literature resources. Different colored edges were used to predict functional links between different molecules. Databases

Table 1 Primer sets used for semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR				
Gene	Primer sequence (5′-3′)	Annealing temperature (°C)	Accession no.	References
hsp90	F: 5′-GGAAATCTTCCTCCGAGAGC-3′ R: 5′-CCGAATTGACCGATCATAGA-3′	51	KC962223	Mahanty et al. 2016b
hsp70	F: 5′-GCATGGTGAACCACTTTGTG-3′ R: 5′-CTCTGCCGTTGAAGAAATCC-3′	53	JX401427	Mahanty et al. 2016b
hsp60	F: 5′-C(C/T)GTCACCATGGG(A/G/T)CCAAAGG-3′ R: 5′-C/G/T)GCCTCTCCATCCACATCC/T)TC(A/C)GC-3′	65	KC844065	Mahanty et al. 2016b
hsp27	F: 5′-CTTTGGGATGCCACACTTCT-3′ R: 5′-ATCTGGCGTCTGCTTGATCT-3′	50	JX844669	Mahanty et al. 2016b
hsp110	F: 5′-GCAGATCTCCGCTATGCTGT-3′ R: 5′-CGAATGACCCAAGTCCACGA-3′	56	KC915027	Purohit et al. 2014
nrf-2	F: 5′-TTCCCGCTGGTTTACCTTAC R: 5′-CGTTTCTTCTGCTTGTCTTT	60	JX462955	Jiang et al. 2015
keap-1	GCTCTTCGGAAACCCCT GCCCCAAGCCCACTACA	60	JX470752	Jiang et al. 2015
sod	5′GGACCAACCGATAGTGAAAGACAC CCTCTATGATTGGAGCAGGACACT	57	FJ458445	Zheng et al. 2012
catalase	F: CCACTTCTGGTCCAGGATGTGGT R:GCGAACAGCGATGGGTGTCGTCT	62	FJ560431	Zheng et al. 2012
gpx	F: GGCACAACAGTCAGGGATTACACT R: GGTGGGCGTTCTCACCATTCACT	59	EU828796	Zheng et al. 2012
tubulin	F:CCTGCTGGGAACTGTATTGT R: TCAATGAGTTCCT TGCCAAT	54	KC710731	Purohit et al. 2016

experimental, literature mining were the evidence of the interaction. The network is represented in molecular action view, but action and evidence view was also used in the case of biological relevance analysis.

Results

Critical thermal maxima

In the present study, significant difference between the CTmax values of the fishes fed with basal diet (A) and diet supplemented with 1.5% curcumin (D) was observed, while the difference in CTmax values of the groups fed with basal diet plus 0.5% (B) and 1% (C) curcumin as compared to control (A) was insignificant (Fig. 2). As there was no significant change in CTmax values of A, B, and C groups, the B and C groups of fishes were excluded from the sub-lethal heat shock treatment and subsequent gene expression analysis study.

Gene expression analysis

Expression of *nrf-2* and *keap-1*

Significant increase ($p < 0.05$) in expression of *nrf-2* gene was observed in both liver and gill tissues of the

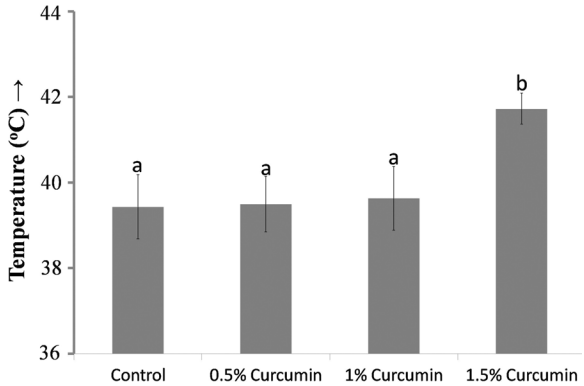

Fig. 2 CTmax of *Puntius sophore* fed with basal diet and 0.5, 1.0, and 1.5% curcumin-supplemented diet. Significant increase ($p = 0.05$) in CTmax was observed in the 1.5% curcumin-supplemented group as compared to the control group. *Different letters* above the bars indicate significant difference ($p < 0.05$)

1.5% curcumin-supplemented group compared to control (BD). In heat-stressed fish fed with basal diet, change in *nrf-2* expression was insignificant relative to the control. There was no significant changes in the expression of *keap-1* in all the three groups in the gill (Figs. 3 and 4) and its expression in the liver was very low and thus was not quantifiable.

Expression of *hsp* genes

In the gill, there was significant upregulation in the expressions of all the *hsps—hsp60*, *hsp70*, *hsp90*, and *hsp110* except *hsp27*—in 1.5% curcumin-supplemented group compared to control. The expression of all these *hsps*, in heat-shocked group fed with basal diet (BD+HS), was comparable to the control group and no significant changes were observed (Fig. 3).

In the liver, expressions of *hsp70* and *hsp110* were found to be significantly upregulated in 1.5% curcumin supplemented group relative to control. Although, there was upregulation in the expressions of *hsp27* and *hsp60*, these were statistically insignificant ($p < 0.05$). The expressions of *hsp90* were very low and thus were not quantifiable in the liver tissues (Fig. 4).

Expression of antioxidant enzyme genes

In the gill, significant upregulation in expressions of *sod*, *catalase*, and *gpx* were found in the 1.5% curcumin-supplemented group, while the changes in the basal diet-fed, heat-shocked group was insignificant compared to control (Fig. 3). In the liver, expressions of *sod* and *catalase* were found to be upregulated in the curcumin-supplemented group whereas no significant changes in the expression of *gpx* were observed in all the three groups (Fig. 4).

Network analysis

Network analysis showed that there could be direct binding and interaction between all the *hsps*. *hsp70* (*hspa4*; human homolog) was found to be the only *hsp* which showed direct interaction with the antioxidative enzyme *sod* which in turn had direct interaction with *catalase* (Fig.5). *Catalase* was found to have non-specific interaction with all the *hsps*, *nrf-2*, and *keap1*. *gpx* was found to have no direct interaction with any of the genes studied in the present investigation and, therefore, appeared as a stand-alone molecule (Fig.5).

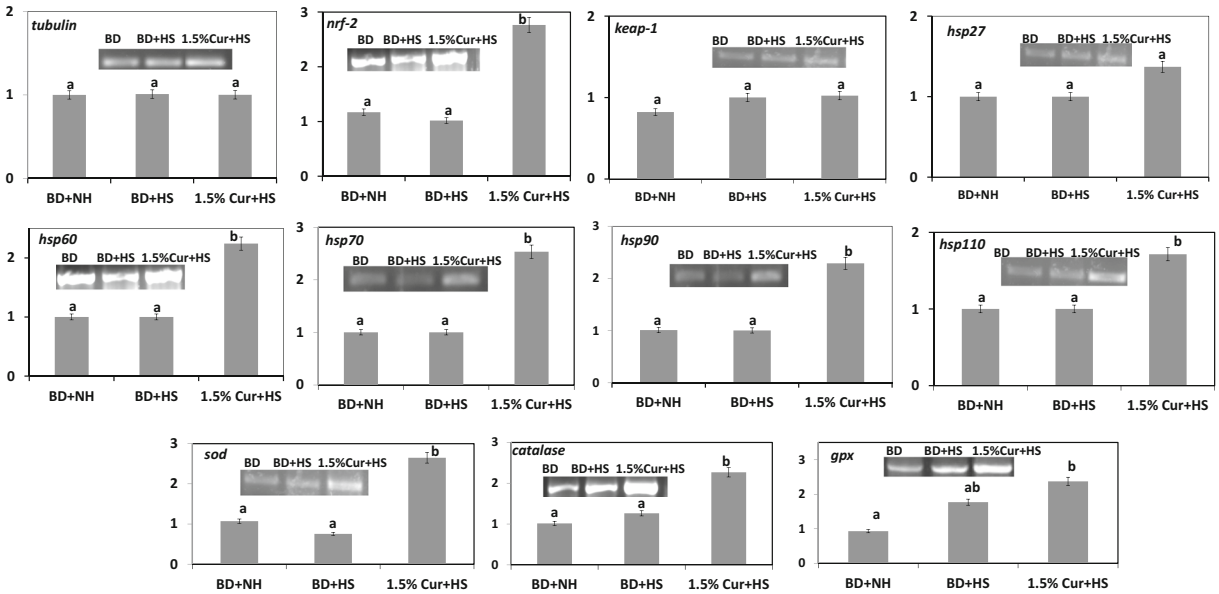

Fig. 3 Fold change in expression of *nrf-2*, *keap-1*, *hsp*, and antioxidative enzymes genes in the gill of *Puntius sophore* fed with 1.5% curcumin-supplemented feed (1.5%Cur+HS) in comparison to basal diet-fed, heat-stressed (BD+HS) and non-heat-stressed groups (control: BD). Comparison of target gene expression between individuals was adjusted with the internal controls (*tubulin*) and the fold change values are calculated as mean ± standard deviation as detailed in "Materials and methods" ($n = 10$ for each group). *Different letters* (*a*, *b*) above the bars indicate significant difference between the values ($p < 0.05$) in different groups. All values are given as relative fold change in comparison to control

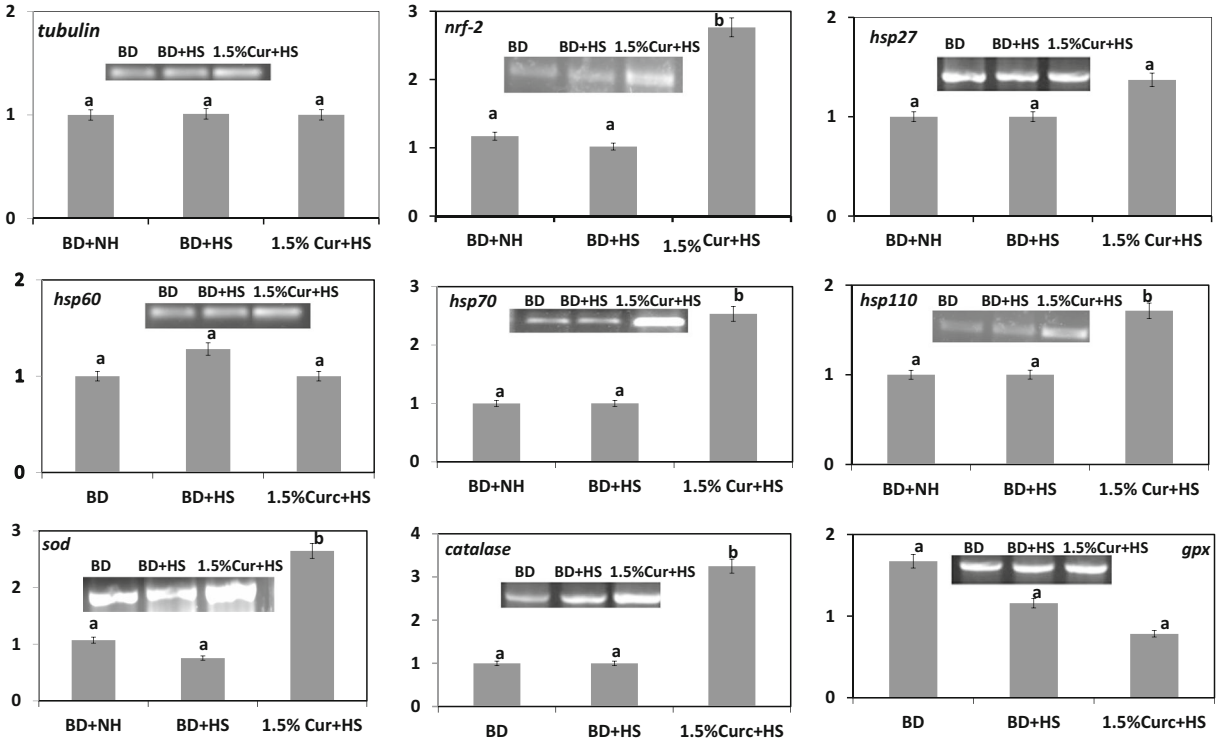

Fig. 4 Fold change in expression of *nrf-2*, *keap-1*, *hsp*, and antioxidative enzymes genes in the liver of *Puntius sophore* fed with 1.5% curcumin-supplemented feed (1.5%Cur+HS) in comparison to basal diet-fed, heat-stressed (BD+HS) and non-heat-stressed groups (control: BD). Comparison of target gene expression between individuals was adjusted with the internal controls (*tubulin*) and the fold change values are calculated as mean ± standard deviation as detailed in "Materials and methods" (n = 10 for each group). *Different letters* (*a*, *b*) above the bars indicate significant difference between the values ($p < 0.05$) in different groups. All values are given as relative fold change in comparison to control

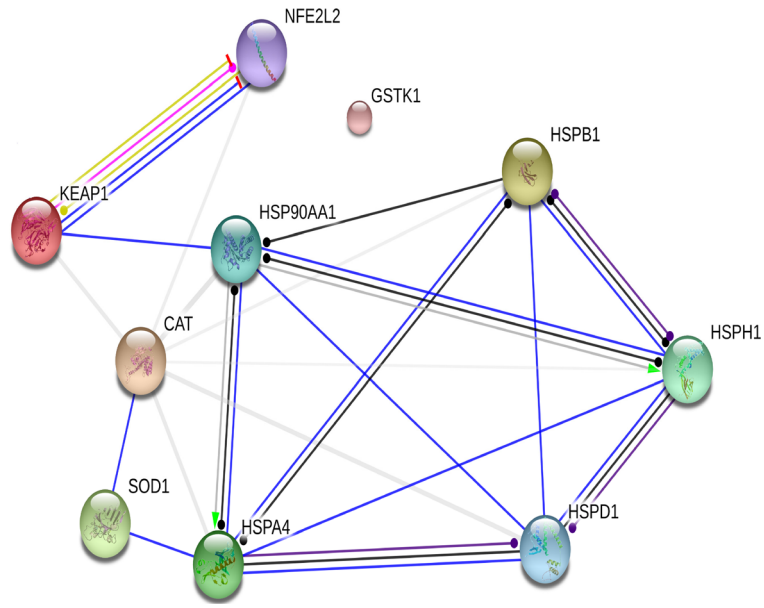

Fig. 5 Network analysis of *nrf-2*, keap-1, antioxidative enzymes, and *hsps* by String 10.0. Interaction were showed between nine genes. These genes were mapped to their respective human homologs as follows: *nrf-2:nfe2l2*, *keap-1:keap1*, *sod:sod1*, *catalase:cat*, *gpx:gstk1*, *hsp27:hspb1*, *hsp60:hspd1*, *hsp70:hspa4*, *hsp90:hsp90aa1*, and *hsp110:hsph1*. *Different colored lines* coded different kinds of interactions: (*black*) reaction, (*blue*) binding, (*yellow*) transcriptional regulations, (*purple*) catalysis, and (*green*) activation

Discussion

Heat stress is one of the most important abiotic factors that influence the physiology of organisms and it has become the major cause of concern in the global climate change regime. As per the IPCC forecast, 20–30% of species assessed so far are likely to be at increased risk of extinction if the increase in global average warming exceeds 1.5–2.5 °C (IPCC 2007). The harmful effects of high temperature are also evident from the number of mass mortalities of fish and other organisms that have occurred in the recent times (Kibria 2014; Mohanty et al. 2010; Mohanty and Mohanty 2009). To ameliorate the effects of heat stress, mitigation mechanisms are being searched for and feed-based mitigation strategies could be one of the best feasible and economic strategies. In this context, we have evaluated the potential of curcumin, a well-known antioxidant, in ameliorating thermal stress in *P. sophore*.

Critical thermal maxima

The CTmax value of the 1.5% curcumin-supplemented feed group was 41.4 ± 0.3 °C which was significantly higher than the CTmax value of the control group (39.43 ± 0.75 °C). This indicates that supplementation of curcumin through feed can alleviate temperature stress in fish *P. sophore* and could aid in survival at higher temperatures. Similar to the present study, Gupta et al. (2010) have reported increase in CTmax values of *L. rohita* after administration of dietary microbial levan.

Gene expression and network analysis

Gene expression analysis showed upregulation in expression of *nrf-2* and most of the *hsps* and antioxidative enzyme genes studied in the 1.5% curcumin-supplemented group. Nrf-2 is a transcription factor that binds at the antioxidant response element (ARE) in the upstream promoter region of antioxidative genes and the expression of antioxidative proteins/enzymes, chaperones, and other metabolizing enzymes involved in protein repair. In an unstressed cell, *nrf-2* remains in the cytoplasm and is readily degraded by other proteins like keap-1 and Cul-3 through ubiquitination (Jain et al. 2015). But under stressed condition, the electrophiles disrupt the keap-1-Cul-3 ubiquitination system and the accumulated Nrf-2 translocates into the nucleus and binds to the ARE in the upstream promoter region of cytoprotective proteins like SOD, GST, and ferritin. Therefore, upregulation of *nrf-2* is mostly accompanied by upregulation of the antioxidative enzymes (Zhang et al. 2015; Zhu et al. 2005).

Considering the important role of *nrf-2* in alleviating the various patho-physiological conditions originating due to oxidative stresses, in recent times, it has been identified as a target for treatment of diseases like cancer (Jiang et al. 2015; Jung and Kwak 2010). Administration of plant-derived flavonoid compounds like curcumin, resveretol which target and activate *nrf-2* has been found to be having beneficial effects in reducing toxicity of chemicals, inhibition of proliferation of cancer cell, and protection from focal ischemia (Yang et al. 2009; González-Reyes et al. 2013; Chen et al. 2014). Curcumin has also been found to have ameliorative effects in heat stress in broilers and quail (Zhang et al. 2015; Sahin et al. 2012). However, reports on its ameliorative effect in heat stressed fish are scanty.

Along with *nrf-2* and the antioxidative enzymes, curcumin has been found to be inducing the expression of some of the *hsps*. Khan and Heikkila (2011) reported acquisition of thermotolerance through induction of *hsp70* following curcumin administration in *Xenopus laevis* A6 cells. Similarly, Sarkar et al. (2014) have reported that curcumin augments the efficacy of antitumor drugs through upregulation of *hsps*: *hsp70*, *hsp90*, and *hsp27*. However, very few reports are available on its effects on the expression of other *hsps* like *hsp90*, *hsp110*, and *hsp60*. Again, the response of *hsps* can vary according to species, tissue, and *hsp* family (Iwama et al. 2004). Thus, we carried out expression analysis of a number of *hsp* genes along with *nrf-2*, *keap-1*, and the antioxidative enzyme genes.

hsp70 family is one of the primary genes that is upregulated in response to external stressor (DuBeau et al. 1998; Washburn et al. 2002; Purohit et al. 2014) and includes two major forms: a constitutively expressed 73-kDa protein (*hsc70*) and a stress-inducible *hsp70* (Barnes et al. 2001). The sequence used in the present study is most likely to be of the inducible paralog (*hsp70*) as the primers used for the expression analysis were designed from the conserved sequences of the inducible form of *hsp70* of other related fish species, and BLAST search of the submitted sequence of *P. sophore* (NCBI Accession no. JX401427) showed identity with the inducible form of *hsp70* (Mahanty et al. 2016b). Although the inducible form of *hsp70* is one of the primary genes that are upregulated in response to external stressor, we did not find any significant change in its expression in basal diet-fed, heat-shocked group (BD+

HS) but their expression increased in the curcumin-fed, heat-stressed group (1.5%Cur+Hs). Similarly, all other *hsp* genes were found to be unaltered in the BD+HS group but along with *hsp70*, *hsp90*, *hsp110*, and *hsp60* were found to be upregulated in gill tissues of the 1.5%Cur+HS group. This could be possibly because we used a short time span (6 h of heat shock treatment) for gene expression analysis. While the basal diet-fed, heat-stressed group could not upregulate *hsp70* expression and possibly requires a longer duration of heat shock treatment for the same to happen, supplementation of curcumin could enhance the expression of *hsp70* within this time span. Similar to the present study, Gupta et al. (2010) have also reported no significant change in the abundance of *hsp70* when *L. rohita* juveniles fed with 0.25–0.75% dietary microbial levan were heat shocked for 6 h but *hsp70* abundance increased in fishes fed with 1 and 1.5% microbial levan and heat shocked for 6 h (Gupta et al. 2010).

In the present study, most of the significant changes in the expression of *hsp* and antioxidative enzyme genes were limited to two- to three fold in the 1.5%Cur+HS group whereas there was no significant alteration in the BD+HS group. In an earlier study also, no significant change in expression of *hsps* except *hsp90* and *hsp47* was observed in *P. sophore* collected from a Atri hot spring runoff (36–38 °C). When *P. sophore* from the hot spring runoff area were heat shocked at further higher temperature, two- to five fold upregulation in expression of *hsp* genes (*hsp60*, *hsp70*, *hsp90*, *hsp110*) was observed. The present study along with our previous study suggests that this kind of response of different *hsp* genes is typical of *P. sophore*. So the *hsp* gene expression in response to heat shock treatment cannot be generalized as unlike the present study; upregulation in *hsp70* expression following high temperature exposure has been reported in a number of fish species (DuBeau et al. 1998; Washburn et al. 2002; Purohit et al. 2014). Similar to the present study, upregulation of *hsp70* has been found to be completely absent in antarctic fish *Trematomus bernacchii* (Buckley et al. 2004) following exposure to heat stress.

Upregulation of *hsp* genes—*hsp70*, *hsp90* and *hsp47*—following curcumin administration has also been reported by others (Sarkar et al. 2014) and the present study also corroborates with these results as we also observed upregulation of *hsp70* and *hsp90* in the curcumin-administered fishes. *hsp27* was also upregulated but not to a significant extent. In an earlier report, we have reported that *hsp90* plays an important role in survival of *P. sophore* in heat-stressed environment of a hot spring runoff, and in the present study also, we observed upregulation in expression of *hsp90* in the curcumin-administered heat-stressed fish which further affirms that *hsp90* plays important role in survival of *P. sophore* in heat-stressed condition (Mahanty et al. 2016b).

In an earlier study, pathway analysis using ingenuity pathway analysis (IPA) had shown that *nrf-2* is a common regulator of both antioxidative enzymes and *hsps* and several study have also shown simultaneous upregulation of *nrf-2*, *sod*, and *hsp70* (Mahanty et al. 2016a). However, the relationship between the downstream molecules of *nrf-2*, i.e., Hsps and the antioxidative enzymes is not clearly known. Therefore, we carried out network analysis using String 10.0 software to know whether any direct interaction between the Hsps and antioxidative enzymes occurs or not and if it occurs, which Hsps interact with antioxidative enzyme. Network analysis showed that there can be direct interaction between *hsp70* and *sod*. However, further protein-protein interaction studies will be necessary to elucidate such interactions.

Conclusion

The present study showed that curcumin could augment thermotolerance in *P. sophore* through *nrf-2*-induced expression of *hsps* particularly *hsp70*, *hsp110*, and *hsp90* and the antioxidative enzymes *sod*, *catalase*, and *gpx*. It has been earlier reported that curcumin stimulates the expression of *hsp70* and *hsp27* (Kato et al. 1998). The present study showed that it can also induce the expression of *hsp90* and *hsp110*. Curcumin is a bioactive compound present in turmeric, a common food ingredient, and the present study suggests that it could be used in aquaculture feeds to enhance thermotolerance in fishes. It could perhaps be an effective agent to control the death of fishes due to sudden heat shock, especially during the peak summers.

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