

Gene Expression Profiles of HSP70 and HSP90 Genes and Biochemical Responses in Juvenile Abalone, *Haliotis diversicolor squamata* in Response to Total Suspended Solids

Ngurah Sedana Yasa¹, Sugeng Joko Purnomo^{1*}, Lutfi Anshory², Murwantoko³, Alim Isnansetyo³, Niken Satuti Nur Handayani⁴

¹Research Center for Fishery, National Research and Innovation Agency (BRIN), Jl. Raya Bogor 16912, Cibinong, Bogor, Jawa Barat, Indonesia

²Research Center for Microbiology, National Research and Innovation Agency (BRIN), Jl. Raya Bogor 16912, Cibinong, Bogor, Jawa Barat, Indonesia

³Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora, 55281, Bulaksumur, Yogyakarta, Indonesia

⁴Department of Genetics, Faculty of Biology, Gadjah Mada University, Jl. Flora, 55281, Bulaksumur, Yogyakarta, Indonesia

Abstract. Prokaryotic and eukaryotic organisms contain heat shock proteins (HSPs), crucial for rapid response to environmental stress. However, their specific roles in different stress conditions are not fully understood. This study investigated HSP70 and HSP90 expression in *H. diversicolor squamata*, using qRT-PCR. Data analysis employed SPSS, including t-tests and ANOVA, with significance set at $P < 0.05$. Results showed distinct expression patterns of HSP genes under varying TSS levels. Both HSP70 and HSP90 mRNA levels significantly increased in response to TSS stressors, with HSP70 exhibiting the highest sensitivity to TSS changes. The duration and amount of TSS exposure influenced gene transcripts, particularly notable at 12 hours and 150 mgL⁻¹ concentration. These findings suggest HSP genes play a role in cellular stress responses to environmental stimuli. HSP70 and HSP90, sensitive to TSS stress, can serve as biomarkers for assessing stress levels from TSS exposure and detecting TSS contamination in abalone farming.

1 Introduction

Intensification of shrimp farming system in Indonesia in addition to increasing production inputs and crops are also produces large quantities of waste that contain solids (e.g. feces and un eaten feed) and nutrients (e.g. nitrogen and phosphorus) and waste biofloc which can be detrimental to the environment, if managed improperly. These solids and nutrients originate from uneaten feed, feces, and animal urea/ammonia [1,2]. If release directly to the

* Corresponding author: sugengjokopurnomo70@gmail.com

environment, these solids and nutrients can be pollutants resulting in environmental issues such as eutrophication [3] or could be directly toxic to aquatic fauna [4,5].

Biofloc technology (BFT) has been widely applied worldwide to reduce environmental impact and the production cost through well manage heterogeneous mixture of heterotrophic bacteria, macroalgae, food, faecal, remnants, exoskeletons, invertebrates into grown flocs and its ability to maintain good water quality [6,7] and disease resistance as a whole can increase the production of various aquaculture species such as shrimp (*Litopenaeus vannamei*) and fish (*Tilapia nilotica*) [8,9]. Organic wastes from bioflocs of shrimp (*L. vannamei*) farming that are truncated to the surrounding waters after the harvesting periods may slightly affect the survival and physiological conditions of various gastropod species [10] as well as abalone cultivated in the coastal waters surrounding shrimp farming area by increasing turbidity that can affect water work. As a marine species which loves media clarity, Abalone has its own sensitivity to various organic wastes and biofloc waste runoff, therefore research is needed regarding these problems.

Research on the impact of TSS on aquatic organisms has been carried out on various marine species such as green grouper *E. coioides* [11], abalone *H. diversicolor* [12], and the bivalve *Sinonovacula constricta* [13]. Optimal TSS concentration will have a good impact on the growth and survival of aquatic organisms, conversely, excessive TSS will be a stressor that can clog and abrasive to the gills, increase variability in feeding rates, physiological stress, changes in behavior, and increase their susceptibility to disease [14,15]. In this research, we will study the adaptability of the abalone *H. diversicolor squamata*, to various concentrations of TSS from the effluent of shrimp ponds in terms of HSP70 and HSP90 genes expression and antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT), and Phenoloxidase (PO).

2 Materials and methods

2.1 Experimental animal sampling and maintenance

A juvenile abalone, *H. diversicolor squamata*, eight months old (240 days after hatching), measuring a total of 32.97 ± 1.83 mm in length and 5.13 ± 0.83 g in weight, was obtained from the Abalone hatchery in dusun Tigarón, Karangasem Regency, Bali and was acclimated to laboratory conditions for one week before trial. 30 abalone in duplicate were used for both the test and control groups in the experiment. In a 100 L glass aquarium, eight repeat test groups with one abalone each underwent the tests for the evaluation.

2.2 TSS source

The total suspended solids used in this study were collected from intensive waste from *L. vannamei* farms that utilize BFT in the Kubu area, Karangasem, Bali. Water waste from shrimp pond containing TSS was taken and poured into the 2m hoff cone and the suspended solid was allowed to settle for 20 minutes to determine the floc volume. The water sample was stored in a sterile bottle and transported (preserved in an ice cool box) to the laboratory for further processing for the TSS test.

2.3 TSS challenge experiment

Three hundred and sixty abalones were distributed into four 100 L aquariums for the experiment. After acclimation of seawater, six abalones were randomly selected as 0 h samples prior to TSS treatment for hemolymph collection. The concentration of TSS treatment was 0, 50, 100 and 150 mgL⁻¹. During bioflock waste supplementation, all

treatments observed from stress response, falling, and surviving juveniles were counted until the end of the test. Abalone hemolymph was collected randomly by 26 gauge microsyringe at 0, 12, 24, 48,72 and 96 hours separately into a different 1500 μ l microtube. The hemolymph of six abalones at each time of sampling is separated and immediately used for RNA extraction and antioxidant enzyme assay.

2.4 RNA extraction and cDNA synthesis

Following the manufacturer's instructions, total RNA was isolated from the hemolymph of abalone from all of the chosen animals using the Quick-RNATM MiniPrepPlus Kit (R1058) from Zymo Research. By using electrophoresis on a 1.2% agarose gel, the quality of the RNA was evaluated. Each sample was created from the same amount of RNA (500 ng) using ReverTra Ace® qPCR RT Master Mix with gDNA removal (FSQ-301) (Toyobo, Japan) in accordance with the manufacturer's protocol using oligo-dT and 6 random primers. RNA purity was assessed by measuring absorbances at 260 and 280 nm with NDD 2000 (Nano Drop Technologies, USA) and cDNA spectrophotometers.

2.5 Quantification of mRNA expression of HSP genes by real-time PCR

HSP gene expression in hemolymph after TSS treatment was measured by real-time reverse PCR transcription in Applied Biosystem (ABI, AS). Generally, total RNA is treated with DNaseI (Toyobo) to remove genomic DNA before reverse transcription. The cDNA obtained as described above is stored at -20 °C until used as a template for PCR reactions. The stress genes HSP70 and HSP90 are adopted based on GeneBank Access numbers AM283515 and AM283516. The primary sequence is shown in Table 3. The reference genes β -actin (access number GenBank AM236595) are used as internal controls to calibrate the cDNA template, and the primary pair (β -actin F and β -actin R) for a 142 bp product (Table 1) are identical as in previous studies [16].

The real-time PCR reaction was performed on a 20 μ L reaction system with a mixture of 2 μ L Thunderbird SYBR® qPCR, 2 μ L forward primary (10 μ M), 2 μ L reverse primer (10 μ M), 2 μ L cDNA template equivalent to total RNA total 50 ng, and 4 μ L free water nuclease. Each reaction is executed in triplicate. The thermal cycling condition was 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. Melt curve analysis was added (65 °C to 95 °C, with 0.5 °C/s addition) to show PCR product specimens, as indicated by a single peak. The nuclease-free water (Promega, USA) is used instead of the cDNA template as the PCR negative control. Meanwhile, the NRT (non-reverse transcription) control reaction using the template treated with gDNA removal RNA was performed to ensure there was no genomic DNA contamination in the reaction.

2.6 SOD, CAT, and PO enzyme activities

Ten abalones were collected from the experimental aquarium at 0, 12, 24, and 48 hours after TSS exposure and hemolymph was collected to measure antioxidant enzyme activity. Superoxide dismutase (SOD) activity is determined by measuring the ability to inhibit the reduction of photochemical nitroblue tetrazolium chloride (NBT), as described previously [17]. SOD activity in this experiment was measured at 450 nm with SOD Kit-WST (water-soluble tetrazolium salt) (www.dojindo.com). The rate of the reduction of WST-1 with O₂ is linearly related to the xanthine oxidase (XO) activity, and this reduction is inhibited by SOD. A unit of SOD activity is defined as the amount of enzyme needed to induce 50% inhibition

at the rate of NBT reduction under certain conditions. The result is expressed as unit activity (U) per mg protein extract (U/mg).

Table 1. Primers used in the present study

Primers	Sequence (5'-3')	Gene bank Accession number	Reference
HSP70 F HSP70 R	CCGCTCTAGAACTAGTGGAT CCGCCAAGTGGGTGTCT	AM283516	[16]
β -actin F β -actin R	GGGTGTGATGGTCGGTAT AGCGAGGGCAGTGATTTC	AM236595	[16]
HSP90 F HSP90 R	CCAGGAAGAATATGCCGAGT CACGGAAGTCCAAGTACC	AM283515	[16]

Phenoloxidase (PO) activity was measured using the indirect method at 20°C according to [18]. Briefly, 100 μ L of 2 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in 0.2 M Tris-HCl-1% SDS (v/v) pH 8, was added to 100 μ L of total hemolymph and the absorbance at 490 nm was read continuously for 30 min. The activity was given in the PO unit corresponding to the oxidation of 1 μ mol of L-DOPA. According to the manufacturer's instructions, catalase activity was measured colorimetrically by CAT activity Assay Kit GeneWay, Biotech, Inc (techine@genwaybio.com). The level of H₂O₂ loss (measured at 492 nm) is used to measure CAT activity of the hemolymph sample. The assay was measured by Microplate reader Heales® MB-580, Shenzhen Huisong Technology Development Co. Ltd. China.

2.7 Data analysis

In accordance with the 2^{- $\Delta\Delta$ CT} method [19], untreated samples were used as calibrators. For each biological sample, the threshold value (CT) obtained from three technical PCR real-time imitations is first averaged and then converted to a 2^{- $\Delta\Delta$ CT} formula, where $\Delta\Delta$ CT = (CT, Target-CT, Actin) Time x - (CT, Target-CT, Actin) Time 0. All data is given in terms of relative expression of mRNA as mean \pm SD (standard deviation). The data were analysed statistically by t-test or one-way ANOVA analysis followed by Duncan trials using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). *P* value of less than 0.05 was considered statistically significant.

3 Results

3.1 Responses of HSP70 and HSP90 genes to different TSS exposure

The expression profiles of the two HSP genes in abalone hemolymph after TSS treatment are shown in Figures 1A and 1B. Although both genes are induced under the same TSS stress, different expression patterns can be observed. Relative expression of HSP70 increased rapidly in the first 12 h, peaked (control 16-fold) after TSS exposure, and then gradually decreased to pre-treatment levels after 24–72 h (Figure 1A). However, the level of HSP70 expression increased again at 96 hours. The level of HSP90 transcription appeared to increase with TSS exposure starting at 12 hours and reached a peak at 24 hours. Furthermore, it decreased at 48-96 hours after treatment, where the value reached (2-10 times under control)

After that, the expression level decreased gradually, but was still higher than control (Figure 1B).

HSP70 and HSP90 are a multigene protein family whose expression is caused by various stress factors [16]. After 12 hours, HSP70 expression was induced by all TSS treatments (Figure 1A). It has been shown that HSP70 expression can be influenced by exposure to aquaculture waste in a TSS concentration-dependent manner. Induction of HSP70 can modulate cytosolic redox status in cells for protection against oxidative stress [20]. HSP90 influences the folding of newly synthesized polypeptides by providing environmental chaperones coupled to translation [21]. Up-regulation of HSP70 is caused by the denaturation of many proteins caused by the accumulation of toxic ROS, and is then used to fold and reassemble these proteins [22].

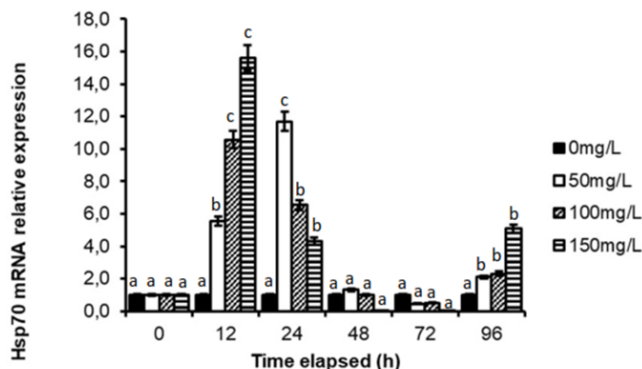
However, in TSS treatment, the abalone HSP90 gene was downregulated from 48 to 96 h of exposure compared with controls (Figure 1B). The defence mechanism can be weakly active oxidative stress. Thus, up-regulation of HSP70 may be associated with a long-term protective mechanism [23]. Upon TSS exposure, the mRNA level of the HSP70 transcript decreased starting at 24 h and reached a lower level than the control after 48 h, whereas the expression of the HSP90 gene decreased after 48 h (Figure 1B). This shows that HSP70 has a faster response to reach a level equivalent to the control when compared to HSP90. When HSPs reach their highest point of abundance, heat shock transcription factors will bind to HSPs and loss of DNA binding activity to heat shock elements can reduce HSP expression levels [24, 25].

3.2 Biochemical responses of *H. diversicolor squamata*

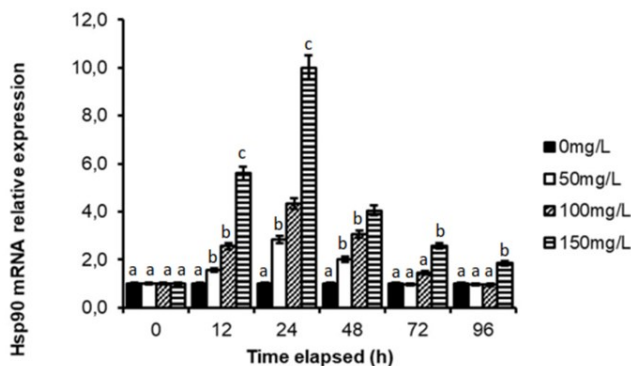
The activities of the antioxidant enzymes SOD, CAT, and PO were investigated to identify changes in biochemical activity that occurred in response to biofloc exposure for 0, 12, 24, 48, 72 and 96 hours (Figures 2A, B, C). The results showed that SOD activity in abalone exposed to TSS increased from the 12th hour above and was significantly different ($P < 0.05$) compared to the 0mgL^{-1} control after 24 hours (Figure 2A).

The most significant decrease in SOD activity was observed in abalone in the 150mgL^{-1} treatment after 24 hours. After 48 hours, SOD activity increased significantly in all treatments until 96 hours. The pattern of SOD enzyme activity was influenced by time and TSS concentration in the *H. diversicolor squamata* culture medium.

CAT was observed to increase significantly ($P < 0.05$) after exposure to a TSS gradient for concentrations of 50 to 150mgL^{-1} (Figure 2B). CAT enzyme activity was significantly induced by 50 to 150mgL^{-1} treatment after 48 hours ($P < 0.05$). After 48 hours, there was a tendency for CAT activity to increase again in the three TSS 50- 150mgL^{-1} treatments. Induction of the CAT enzyme is influenced by time and depends on the TSS concentration. The PO response also experienced a significant increase at 24 to 96 hours in the 100mgL^{-1} and 150mgL^{-1} treatments (Figure 2C).



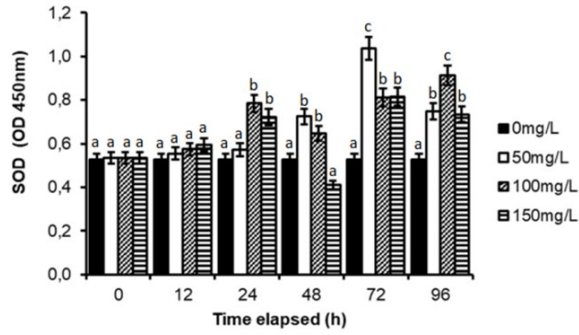
(A)



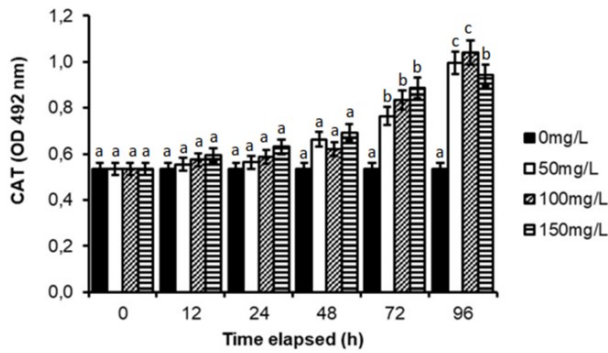
(B)

Fig. 1. HSP70(A) and HSP90 (B) relative expression levels after TSS challenge in abalone. The 0, 12, 24, 48, 72 and 96 h denote 0 (untreated), exposed to the biofloc 0,50,100 and 150mgL⁻¹ exposure condition. The untreated group was used as the control group, and the ratio of which was considered as 1. Data are presented as means±SD (standard deviation) from independent samples with three technical replicates each (n=30). Different lowercase letters indicate statistically significant differences ($P<0.05$) in relative expression.

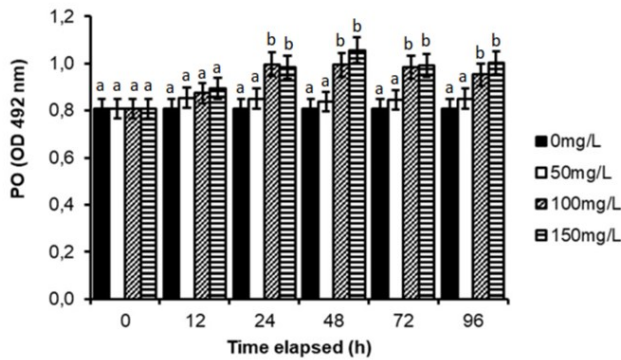
In the aquatic environment, almost all animals are constantly exposed to various environmental stresses that can cause biochemical, physiological and histological changes. One of the factors causing this change is the TSS that comes from aquaculture waste that applies the biofloc system [8,26]. Besides the benefits provided by the biofloc system, it is also capable of producing excess TSS so that the concentration needs to be maintained so that it reaches optimal limits for the health of aquatic organisms [27]. Excess TSS can cause problems for fish health, especially can cause clogging of the gills [28,29,30,31].



(A)



(B)



(C)

Fig. 2. Antioxidant enzyme activity in the abalone *H. diversicolor squamata* exposed to TSS gradient for 0,12, 24, 48, 72, and 96h. The experiments for SOD (A), CAT (B), and PO (C) were conducted in triplicate, and the data represent the mean \pm SD. Differences between treatments and the 0 h control were considered significant at $P < 0.05$

One protective mechanism known to be developed by aquatic animals in response to stress is the induction of HSP. Hemolymph is the main circulating fluid in mollusks that plays an important role in immune defense. Hemocytes are the main defense cells of mollusks, they are capable of recognition, chemotaxis, attachment followed by agglutination and phagocytosis and exocytosis of antimicrobial factors, scavenging of reactive oxygen species, detoxification of xenobiotics, [32,33]. Due to its non-shelf recognition ability, hemolymph may be sensitive to environmental changes.

The involvement of the HSP gene in the heat shock response is well documented in many aquatic animals. It has been shown that the expression levels of HSP70 and HSP90 in various aquatic organisms including abalone significantly induce various organs after heat exposure [34,35,36,37,38]. However, in other cases HSP70 and HSP90 were also studied in response to various biotic and abiotic exposures such as heavy metals in Asian clam (*Corbicula fluminea*) [39]. The above results demonstrate the important role of HSP genes in overcoming heat stress in several organisms. In this study, we compared the expression patterns of HSP70 and HSP90 in response to different TSS exposures using similar stocking density conditions in abalone experiments. Our results show that both genes show clear regulation at the mRNA level, but different expression profiles are obtained.

The maximum expression of HSP70 was observed at 24 h after TSS exposure, and it was 10 times that of the control. After abalone was exposed for 48 hours, HSP70 expression decreased very quickly and became the same level as the control. In contrast, HSP90 expression decreased from 24 h, and values approached normal conditions at 48 h, with a decrease (20–40% of control) found in the 12h TSS exposure treatment, and a down-regulation to pre-treatment levels observed at 48 h after treatment. The above results show that the transcription level of HSP70 is higher than HSP90 upon TSS exposure in both genes. Increasing TSS concentrations dramatically increased HSP70 expression, as well as HSP90 gene expression. Based on the present data, we suspect that, the two known HSP genes in *H. diversicolor squamata* HSP70 and HSP90 play an important role in protecting cells from damage caused by acute TSS stress, and these may be the best gene candidates to be used as a biomarker to assess TSS exposure in abalone farming.

As with other biotic and abiotic factors such as temperature, salinity, heavy metals, and various pathogens, organic matter within TSS can cause an imbalance of reactive oxygen species (ROS) in the body which further may cause oxidative stress (OS). Several antioxidant enzymes play a role in preventing and controlling oxidative stress, namely superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase (SOD) is the enzyme responsible for removing superoxide which catalyzes the dismutation of two O₂ molecules - into oxygen and hydrogen peroxide (H₂O₂). In contrast to non-radical ROS, hydrogen peroxide (H₂O₂) is produced in large quantities in the mitochondrial matrix during the O₂-reduction process. Hydrogen peroxide can be partially removed by several enzymes such as catalase (CAT) [17]. In this study, the increase in SOD expression in hemocytes occurred from 12 to 96 hours. This is the same as research on *R. philippinarum* which was injected with poly I: C which showed increased expression at 48 hours.

TSS residues, caused by shrimp pond waste runoff, and human activities are a common phenomenon in waters with poor waste management. It has been proven that excess TSS exposure can increase intracellular ROS (reactive oxygen species) which ultimately causes oxidative stress in aquatic organisms [40,22]. As mentioned previously, HSP is an important component of various detoxification, antioxidant and stress tolerance pathways [41,42]. We hypothesized that HSPs may also play an important role for cells to overcome stress caused by media contaminated by Vibriosis bacteria [42]. In this study, four TSS gradients (0 mgL⁻¹, 50 mgL⁻¹, 100 mgL⁻¹ and 150 mgL⁻¹) were designed using seawater in a 100 L aquarium to examine the gene expression profiles of two HSP types under TSS stress from low to high doses.

Our results show that exposure to TSS can induce the expression of two HSP genes, suggesting the involvement of HSP genes in mitigating oxidative stress caused by TSS contamination from shrimp aquaculture waste. Likewise, induction of HSP70 and HSP90 was quite high in abalone, *H. rufescens* on day 1 post-exposure to heptachlor (0.7 ppm) and recovery after 4 days [43], and increased accumulation of heat shock proteins in scallops *Chamelea gallina* in polychloro biphenyl, cadmium chloride and organochlorine chemicals was reported in previous studies [44,45,46]. Meanwhile, our study also revealed that the gene expression profiles of both HSPs under TSS stress had the same pattern. However, between the two genes, HSP70 shows a stronger expression response and is most sensitive to TSS fluctuations. In TSS exposure, the relative expression of HSP70 mRNA gradually increased and peaked (3-fold the original rate) in post 12 h treatment, whereas the maximum expression (5 times the original level) of HSP70 was observed at 24 h treatment, and a decreasing trend was found during subsequent treatment.

TSS pollution has become a serious problem in aquatic ecosystems for aquatic organisms. The presence of TSS in marine or estuarine environments is of ecotoxicological concern, because suspended solid particles can cause blockages in the gills of various marine biota which results in death [9]. In the larval stage, the presence of suspended particles can reduce the rate of attachment and survival of abalone *H. iris* [47] and *H. diversicolor supertexta* [48].

4 Conclusion and suggestion

In conclusion, if we compare the expression profiles of HSP70 and HSP90 in abalone hemolymph under TSS exposure in this study, the two genes induced under TSS treatment, HSP70, showed a faster expression response to TSS than HSP90. The activity profiles of antioxidant enzymes such as SOD, CAT and PO are different from the HSP gene with different concentrations of TSS exposure. Our results presented here provide useful insights for investigating cellular stress-related responses and for identifying potential stress-causing environmental biomarkers in TSS-exposed abalone, *H. diversicolor squamata* in shrimp culture effluent. More detailed investigations using other tissues and stages of development should be carried out for a better understanding of the function of this gene. Meanwhile, because the functional molecules used are only a few antioxidant enzymes and 2 HSP genes, information at the mRNA level is limited. Protein quantity as well as activity, with the same specificity and quantitative accuracy as mRNA quantity, should ideally be detected in future studies. In addition, it is also necessary to know how abalone respond when exposed to several stress factors simultaneously in an environmental context, where many stress factors are regulatory rather than exceptional.

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