Analysis of stress-related gene expression in lobster seeds (*Panulirus sp.*) post formalin stress test

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Abstract. Indonesia is the potential international-scale lobster economic activity due to the abundant supply of tropical lobster seeds *Panulirus* sp., however the lobster cultivation sector have still resulted in problems of low survival during the rearing period. This study aimed to analyze the expression levels of the HSP70 gene related to stress response in lobster seeds exposed to formalin dose of LC70 for 1 h. Seeds were obtained from catching lobsters at Ujung Genteng Beach, Sukabumi City, West Java Province, with an average weight of 0.0254±0.004 g and a length of 2.3314±0.1549 cm. The observation parameters tested were the gene expression and survival rates of the post-soaking lobsters. The results showed that formalin immersion at a dose of 220 ppm on juvenile-stage lobster seeds resulted in the death of 70% of the population and the ability to express the stress gene HSP70 at different fitness levels were different with the highest expression level found in fit condition lobster seeds was 2.949±0.420.

1 Introduction

Lobster is a high-value fishery commodity with continuously increasing demand [1]. The global market determines lobster prices based on size and weight, where lobsters weighing over 1 kg each are priced at approximately 100 USD per kg, whereas those in the range of 300-900 grams per lobster are priced at 50 USD per kg [2]. According to FAO data [3], the demand for this commodity reached 159,765 tons in 2013 and subsequently increased to 170,000 tons in 2014. This demand is projected to increase further by 10.1% in the period 2021-2026 [4]. Indonesia, endowed with an abundant tropical lobster seed supply, such as pearl lobster (*Panulirus ornatus*) and sand lobster (*P. homarus*), has the potential to engage in the international lobster economy [5].

To date, lobster production in Indonesia has been dominated by natural capture processes, posing a threat to the sustainability of lobster populations. Consequently, government regulations regarding lobster capture have emerged. According to the Minister of Marine Affairs and Fisheries Regulation No. 1/PERMEN-KP/2015 and Circular Letter (SE) of the Minister of Marine Affairs and Fisheries No. 18/2015 regarding lobster capture policies,

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lobster (*Panulirus* spp.), crab (*Scylla* spp.), and blue swimmer crabs (*Portunus pelagicus*, spp.) can be captured if the carapace size is >8 cm or weighs 200 g/lobster, is not in an egg-bearing condition, and proper documentation is maintained for each captured lobster. Subsequently, this regulation was reinforced by the 2015 SE, specifying that lobsters eligible for capture must weigh more than 200 g. Further clarification was made in 2016 with an additional SE setting the minimum weight for capture at over 300 g. These policies have led to an increase in lobster aquaculture [1].

Active research and development have been conducted to foster the lobster aquaculture sector. However, current cultivation efforts face challenges, particularly the high mortality rates of lobster seeds [6]. The mortality of the puerulus to juvenile lobster phase during cultivation ranges from 50-80% [7]. Disease infections, including opportunistic bacterial, viral, parasitic, and fungal infections, contribute significantly to the mortality rate [8].

Chemical screening processes have been widely applied in aquaculture to control the spread of diseases. Screening involves selecting seeds based on their resistance to specific unfavorable conditions before the cultivation process [9]. Introducing stress before seed stocking improves resistance during cultivation [10]. Various methods and substances, including the use of chemicals such as formalin, can be employed for stress induction. Formalin serves as an antimicrobial agent, killing various microorganisms through protein denaturation and disruption of normal biological functions [11]. Additionally, formalin is prevalent in controlling fungal infections [12], virus inactivation [13], bacteria [14], and parasites [15]. Formalin exposure during screening typically involves immersion for 30–60 minutes. Information on chemical dosage tolerance specific to each species in terms of size, age, and treatment conditions is crucial before administering treatment, as excessive dosages can lead to direct or indirect mortality 1-2 days after treatment [16]. The tolerance level of chemically treated lobster seeds serves as an indicator of seed quality, as high-quality seeds can withstand formalin content during immersion [17].

Differences in seed tolerance to chemical stress can be measured by observing stress responses. The stress response is a reaction to stress that produces measurable alterations in biochemical and physiological parameters due to changes in the condition of certain organs, such as the gills, liver, and kidneys [18]. Stress due to exposure, such as the addition of formalin, can be observed through three stages of response: primary, secondary, and tertiary [10]. One type of primary response observed is an increase in steroid cortisol levels. Secondary responses can be observed from changes in metabolic products, cell shape, and the presence or amount of minerals in the body and body structure. The tertiary response disrupts swimming ability and decreases growth rate.

Formalin disrupts homeostasis, indirectly causing a cellular response in the form of heat shock protein (HSP) synthesis, aimed at maintaining body condition balance during stress [19]. Homeostasis through gene expression can be observed at the HSP 70 gene expression level, measured using quantitative real-time polymerase chain reaction (qRT-PCR) [20]. The measurement of HSP 70 gene expression levels in this study aimed to depict the cellular response of *Panulirus* sp. lobsters to chemical stress based on the number of expressed genes at different fitness levels.

2 Material and methods

2.1 Preparation of tanks and test fish

The containers used in this study were five units of containers measuring 83 cm × 59 cm × 45.5 cm each. Before use, containers were washed with soap and rinsed thoroughly to ensure cleanliness. The containers were allowed to dry for 3 days before being filled with water.

After drying, the containers were filled with seawater to a height of 40.5 cm, with a total volume of 200 L. The final step in tank preparation involved adding shelters as a refuge for seedlings during the maintenance process.

The test fish were lobster seedlings obtained from lobster catches at Ujung Genteng Beach, Sukabumi City, West Java Province, with an average weight of 0.0254±0.004 g and an average length of 2.3314±0.1549 cm. The lobster seedlings were acclimated for seven days. During the acclimatization process, the seedlings were fed with commercial shrimp feed with a protein content of 41%, and the feeding frequency was twice a day at satiation.

2.2 Completeness of organs and seed uniformity

Organ completeness was assessed by directly assessing lobster performance. Lobsters with incomplete body organs, tissue necrosis, and release of outer organs (autotomy) were categorized as defective lobster seedlings. In contrast, lobsters with normal conditions and no tissue damage were categorized as healthy lobster seedlings. The classification data for the seedlings were converted into quantitative data using a numbering system. Length and weight were measured using calipers and a digital scale (0.01 g). Seed uniformity was assessed based on the standard deviation of each replicate. Thirty seedlings were used in this process.

2.3 Determination of lethal concentration 70 for formalin exposure

The determination of LC70 involved setting a dose suspected to cause death in 70% of the population within 1 h. LC70 in this study was used as an initial approach in species with unknown survivability. The formalin doses used in this study were 150, 200, 210, 220, 230, 240, and 250 mg L^{-1}. The use of these doses was based on an arithmetic series summing up to ten and referring to the optimal formalin dose for tiger shrimp commodities [13]. The exposure duration was based on a previous study [21]. LC70 dose testing was conducted in nine containers, each containing 10 lobster seedlings. The containers measured 42 cm × 28 cm ×19.5 cm with a volume of 15 L. LC70 exposure was conducted based on the optimal dose capable of killing 70% of the population within 1 h. Three containers, each filled with 10 lobster seedlings, were used to test the effectiveness of LC70. During treatment, there was no water exchange or feeding. Seedlings were sampled under specific conditions to determine gene expression levels.

2.4 RNA isolation

Total RNA extraction was performed using the GENEzol^{tm} Reagent kit (Geneaid, Taiwan), following the manufacturer's instructions. The samples used for RNA extraction were the lobster hepatopancreas under formalin-stress and healthy conditions. Healthy lobsters displayed responsiveness to movement and straight and closed tail conditions, while stressed lobsters exhibited slow responses, clustering behavior, and a folded tail. Thirty lobsters were sampled under stressed and healthy conditions to observe gene expression levels based on fitness. The concentration and purity of the total RNA from the isolated samples were analyzed using spectrophotometry and confirmed for quality using 1% agarose gel electrophoresis.

2.5 cDNA synthesis

cDNA was synthesized from 50 ng µL-1 total RNA using the RevertraAce® qPCR RT Master mix with a gDNA remover kit (Toyobo, Japan) following the manual instructions from 50 ng μL^{-1} total RNA. The synthesized cDNA was verified by amplification using polymerase chain reaction (PCR). The cDNA samples were stored at -20 °C until analysis.

2.6 Gene expression analysis using real-time PCR

Gene expression in each sample was analyzed by quantitative real-time PCR (qRT-PCR) on a Rotor Gene Machine (Corbett Research, USA). The PCR premix composition included 10 µL SensiFast SYBR NOROX (Bioline, UK), 0.8 µL forward and reverse primers (10 µM), 4.4 nuclease-free water, and 4 µL cDNA (5 ng μL^{-1}). The gene expression levels obtained from the amplification process were processed using the Livak and Schmittgen (2001) method. The gene expression levels were normalized using ELF-1α as an internal control for RNA loading during cDNA synthesis. Primers used in this analysis are listed in **Table 1**.

Table 1. The primers utilized in the RT-PCR analysis.

Treatment	Forward (5'-3')	Reverse (5'-3')
HSP-70 [22]	ACATGAAACACTGGCCCTTC	CTCCTCTGGGTTGAAGGTCT
ELF-1 [23]	CTGTGGTCTGGTTGGTGTTG	TCATTGCCCATGGTGATGACC

Note: heat-shock protein (HSP) 70; immune-related gene, ELF-1.

3 Results

The water quality measurements are presented in **Table 2**. The addition of formalin to the immersion medium resulted in changes in several water quality parameters such as pH, dissolved oxygen (DO), and salinity. The pH values obtained during the immersion period ranged from 8.30 to 8.52, indicating alkaline conditions, with a pH above 7. The temperatures of the maintenance container and treatment ranged from 26 °C to 27 °C. The salinity of the medium was the range of 29-31 ppt. Salinity levels increased after the addition of formalin.

Table 2. Water parameters.

Parameter	Control	0 Hours	1 Hours	Standard	Reference
Temperature (°C)	27.27±0.153	26.70±0.30	26.70±0.25	25-30 [a]	[24]
pH	8.30±0.06	8.42±0.13	8.52±0.05	6.6-8.7 [b]	[25]
Salinity (ppt)	29	31	30	29-34 [c]	[26]
DO (ppm)	5.33±0.06	5.26±0.29	4.85±0.06	>5 [d]	[27]

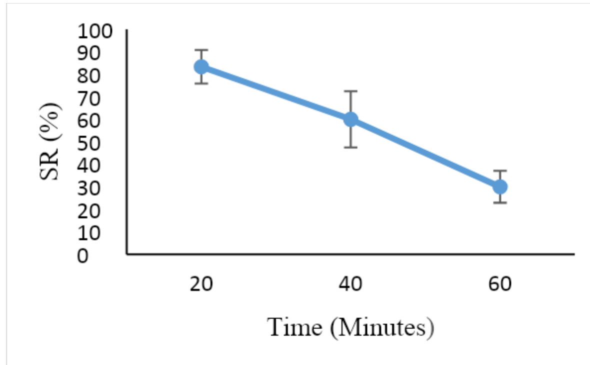

Fig. 1. Survival rate (SR) of lobster seeds after formalin stress exposure by time.

The survival rates of the lobsters during the challenge test using formalin are shown in **Fig. 1**. Observations of the survival rate indicated a gradual decrease in values. The number of deaths occurring at the twenty-minute mark of the test resulted in a decrease in the survival rate decrease to 83.33±7.45%. Subsequently, at the forty-minute mark, additional deaths led to a remaining seed stock of 60.00±12.47%. After the challenge test reached the sixty-minute mark, the remaining survival rate of lobster seedlings was 30.00±7.071%. Lobster seedlings that were still alive after one hour of the challenge test were categorized as healthy seedlings, resulting in three groups of lobster seed fitness: control, weakened, and strong.

The immersion of seedlings in formalin for one hour indicated changes in both the average weight and length. The most significant decrease in the average values before and after treatment was observed for the average weight. The magnitude of the change in average weight reached 0.024 g. Meanwhile, the decrease in the average length before and after immersion reached 0.02 cm. Data on changes in weight and length are presented in **Table 3**.

Table 3. Weight and Length of the body after formalin stress.

Growth	Before stress test	After stress test
Weight (gram)	0.200±0.004	0.176±0.009
Length (cm)	2.21±0.14	2.19±0.15

The pattern of relative gene expression of HSP70/EF1α based on fitness levels is presented in **Fig. 2**. A significant increase in expression levels was observed in different fitness groups of lobster seedlings ($p<0.05$). Elevation in expression levels occurred in fit organisms after one-hour immersion in formalin. The lowest expression of HSP70 was observed in the control condition. Differences in stress tolerance are suspected to be one of the factors triggering variations in gene expression.

The coefficient of variation (CV) determines the extent of variation within the measured population. A higher CV value indicates a population with broad or heterogeneous diversity, whereas a low CV suggests that the utilized sample has low or homogenous variation. The determination of the coefficient of variation for a particular trait was categorized into three groups: low criteria (0.1-25%), moderate (25.1-50%), and high (>50.1%) [28]. The results obtained from the experiment indicated CV values of 15.24% for weight and 6.64% for length. This suggests that the sample exhibits uniformity in size, based on both length and weight.

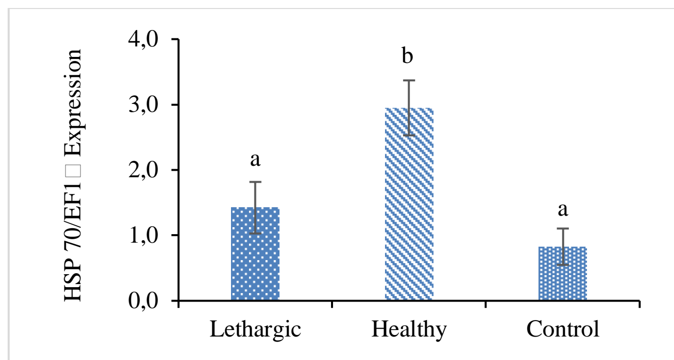

Fig. 2. HSP 70/EF1α gene expression levels.

4 Discussion

Formalin, an aldehyde compound, is extensively utilized in the fisheries industry as a therapeutic and chemotherapeutic agent. A 37-38% concentration of formalin solution is widely employed in aquaculture for preservation, disinfection, and sterilization processes. Its carcinogenic and toxic properties play a crucial role in preventing disease spread and alleviating symptoms of infection [29]. The negative impact of formalin on aquatic organisms can be mitigated by determining its optimal or lethal concentration (LC). The optimal dose for LC70 obtained in this study was 220 ppm, and formalin usage exceeding this concentration led to the death of the entire population within less than 60 min. Lower doses require more than 60 min to reduce survival rates. The correlation between dose and immersion time aligns [30], asserting that formalin toxicity depends on both dosage and duration. Significant differences in dosage can result from species variation, size, age, and treatment conditions [31]. Chemosensory effects or disruptions to the nervous system due to chemical exposure emerge as a cause of death after formalin use. Changes in exposed organ structures, especially the gill lamellae, hinder respiratory rates. Low formalin tolerance can damage other organs such as the liver, kidneys, and spleen [31]. In lobsters, chemosensory effects are observable in organs, such as the antennae, mouth, and legs. Antennae, as external organs, transmit chemical information directly to the frontal brain and detect chemicals through the smell. Lobsters exhibited periodic rubbing movements, particularly on their antennae and eyes, during the initial five minutes of the study. Subsequently, the movements involved rubbing the mouth and walking the legs. This movement is indicative of the transmission of chemical signals from the sensilla to the olfactory receptor neurons (ORNs) in the brain lobe [32].

Formalin toxicity induces behavioral and physiological changes in lobsters, including clustering, hyperventilation, and changes in body color. These observations align with research on *Cyprinus carpio* exposed to formalin [12]. Furthermore, formalin toxicity leads to irregular swimming patterns, inverted body positions, and loss of balance, indicating disruptions in the nervous and receptor systems [33]. The study employed lobster seedlings with an average length of 2.33 cm and an average weight of 0.025 g, considered yuwana phase [34]. Yuwana lobsters, which mark the transition phase after the transparent phase and indicate the first molting, play a crucial role in lobster cultivation [35]. However, the complex behaviors and transportation impact at this stage facilitate stress, affecting seed health and resilience [36, 37]. Sample variations, measured based on the coefficient of variation, indicated low variability for both length (6.64%) and weight (15.24%). Low variability suggests a homogenous population [28]. The chronic effects of formalin use, as stated by [38], resulted in decreased weight and length [39]. Excessive formalin exposure in seeds is

associated with slower growth rates [11], affecting metabolism, nutrient absorption, and increased energy reserve utilization, causing decreased growth [31]. Chronic and acute effects of formalin use induce stress-resistant genes known as Heat Shock Proteins (HSP) at the cellular level. HSP act as chaperone proteins in all cellular compartments, protect against protein denaturation, ensure functional protein folding, and prevent cell death [40]. Chronic exposure to formalin induces the expression of HSP70, a crucial protein category aiding damaged protein disposal, controlling protein quality, directing the degradation of abnormal proteins, and preventing cell apoptosis [41]. HSP70 expression, regulated by the interaction between Heat Shock Factor (HSF1) and Heat Shock Element (HSE) in the promoter region [42], is observed at low levels under normal conditions and increases during stress, playing a role in protein folding, intracellular transport, and maintaining protein shape [43]. Information on HSP70 expression levels can be used to enhance early production processes and improve growth, development, feed efficiency, resistance, and phenotype selection [44]. HSP70 gene identification involves isolating total RNA from the hepatopancreas of lobster seeds. The use of hepatopancreas in gene expression analysis has been conducted in various crustacean studies [45, 46, 47, 48] because of its vital role in metabolic processes [49] RNA concentration in lethargic seed conditions is 40,987±9,068 ng μL^{-1} with a ratio of 1.823±0.063; healthy seed samples had RNA concentrations of 133,570±14,515 ng μL^{-1} with a ratio of 2.063±0.037; and control lobsters exhibited RNA concentrations of 143,640±43,009 ng μL^{-1} with a ratio of 1.799±0.119. The obtained ratios indicated good fitness levels, with purity suggested in the range of 1.8-2.0, as recommended by [50]. Variations in RNA concentrations result from different responses to worsening environmental conditions [51]. HSP70 gene expression analysis using RT-PCR indicated significant differences ($P < 0.05$) in expression levels based on different fitness levels. Lobsters under healthy conditions exhibited higher HSP70 gene expression levels (2.949±0.4205) than lethargic lobsters (1.423±0.394) and control lobsters (0.827±0.278). This aligns with the claim that increased HSP70 gene expression enhances resilience under unfavorable conditions, supporting cell proliferation and reducing apoptosis induction [40]. Chronic and acute impacts of formalin use can induce stress-resistant genes known as Heat Shock Proteins (HSP) at the cellular level. HSP act as chaperone proteins in all cellular compartments, protect against protein denaturation, ensure functional protein folding, and prevent cell death [40]. Chronic exposure to formalin induces the expression of HSP70, a crucial protein category aiding damaged protein disposal, controlling protein quality, directing the degradation of abnormal proteins, and preventing cell apoptosis [41]. HSP70 expression, regulated by the interaction between Heat Shock Factor (HSF1) and the Heat Shock Element (HSE) in the promoter region [42], is observed at low levels under normal conditions and increases during stress, playing a role in protein folding, intracellular transport, and maintaining protein shape [43].

5 Conclusion

HSP70 analysis of lobsters under the fit condition after 220 ppm immersion showed the highest expression level of 2.949 ± 0.420. The survival rate obtained with 220 ppm formalin was 30%. The seeds used in the study were classified as uniform with a large coefficient of variation, measured based on body length (6.64%) and weight (15.24%). The decrease in growth parameters caused a reduction in weight of 0.024 g and a decrease in body length of up to 0.02 cm.

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