Comparative Assessment of Plasma Cortisol and Heat Shock Protein 70 Expression as Indicators of Temperature Stress in Nile Tilapia (*Oreochromis niloticus* Linn.)

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ABSTRACT

Comparative assessment of plasma cortisol and expression of Heat Shock Protein 70 gene (HSP70) from gill tissue as indicators of temperature stress in Nile tilapia (*Oreochromis niloticus*) was investigated. In this study, water temperature at 22, 27 and 32°C was the stressor. Results revealed that the concentration of plasma cortisol responded to temperature stressors at irregular patterns as compared to its response to ambient water temperature. In contrast, fish reared at 22 and 32°C showed significantly higher levels of HSP70 expression than those exposed at control temperature (27°C), at 3 and 6 hours after stress induction ($P<0.05$). However, during 12-168 hours, a significant difference in HSP70 expression was observed only in fish exposed at 32°C water. Studies on correlation between temperature and the severity of Streptococcosis infection in Nile tilapia revealed that fish challenged with *Streptococcus agalactiae* through immersion at 32°C for 21 days had significantly higher mortality than fish challenged at lower temperatures ($P<0.05$). Accumulative mortality rate of 31.67% was observed in 32°C fish while only 8.33±1.53 % and 8.33±0.58% were observed in fish reared in 22 and 27°C water, respectively. This study indicated that the expression of HSP70 gene may be an indicator of temperature stress in Nile tilapia. Since the HSP70 is an important molecule of fish that can respond to various stressors in order to regain the homeostasis of the body, results from this study provide valuable information for further research to investigate whether HSP70 expression can be used specifically as a reliable indicator for other stressors.

INTRODUCTION

Nile tilapia, *Oreochromis niloticus*, native to north African rivers, was introduced into Thailand decades ago, and is now topping the production of freshwater fish in the country. In 2010, Thailand produced 155,500 tons of tilapia (Department of Fisheries, Thailand 2012). Despite the high production, there has been a severe epidemic disease which caused massive mortalities of tilapia during the hot season in the past five years. Disease investigation revealed that *Streptococcus agalactiae* was the major

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cause of these massive tilapia mortalities. Furthermore, the epidemic always occurred in fish reared in earthen ponds during the hottest temperatures in Thailand. There have been several attempts to solve this problem but it is still far from success. Apart from bacterial infection, it is also believed that the cause of the massive mortality may be related to stress caused by high temperature.

Understanding the factors that cause stress is thought to be a key concept for the control of this disastrous fish mortality. Pickering (1981) defined stress in fish as any kind of activity in the body of the fish to respond to stimulus which would affect the stability and balance in the fish. In general, stress in fish may be considered as a complicated topic which needs a lot of knowledge to explain. Stress in teleosts caused by high temperature would make it easier for the fish to be infected by pathogenic bacteria than those in optimal temperature. Fish would physiologically respond to stressors by releasing catecholamine and corticosteroid (also known as cortisol hormone) (Mazeaud *et al*., 1977). Normally, most vertebrates produce heat shock protein (HSP) in response to high ambient temperatures. Concentration of HSP always increases at higher ambient temperatures. There are several forms of HSP according to their molecular weights but the most common is HSP70 (Forreiter and Nover, 1998). Many indicators have been proposed to monitor stress conditions in fish such as plasma glucose, plasma cortisol, plasma chloride and HSP gene expression. In this study, we monitored the effect of high temperature stressors in tilapia by using plasma cortisol concentration and heat shock protein gene expression. A number of research work have been conducted pertaining to levels of cortisol and heat shock protein in teleosts but not much have been studied on Nile tilapia.

High temperatures affect fish susceptibility to harmful bacteria. However, the mechanism on how bacteria especially *Streptococcus agalactiae* would infect the stressed Nile tilapia is not clear. In this study, apart from monitoring cortisol and HSP levels in the blood plasma of Nile tilapia kept at various ambient temperatures, the reliability of these two parameters as indicators of heat stress will be compared to assess usefulness in future research. In addition, inoculating with culture water with stressed fish with *S. agalactiae* was done to enable us to gain a better understanding on the mechanisms of infection. This study was aimed to find the best way to cope with epidemic diseases in Nile tilapia.

MATERIALS AND METHODS

Experimental fish and sample collection

Juvenile tilapia (*O. niloticus*) with an average weight of 14.3 g were acclimated for 1 month in dechlorinated water (27^{o}C) with constant aeration at Kasetsart University. All fish were fed until satiation twice daily. Food was withheld for 48 h prior to sampling periods.

To determine the mortality rate of tilapia at different temperatures after inoculation with *Streptococcus agalactiae* strain AQSA001, 20 fish were exposed to the following treatments with three replications:

(1) control (no stress: 27°C); (2) 22°C (reduce by 5°C from control), and (3) 32°C (add by 5°C from control). *Streptococcus agalactiae* (in $1x10^8$ colony forming unit/ml) were added into *S. agalactiae*-free water in all treatments. Tilapia were fed until satiation twice daily. The mortality of fish exposed to different temperature levels for 168 hours and subsequently immersed in water with *S. agalactiae* for 21 days was determined.

To determine the effects of temperature on cortisol and heat shock protein, fish were exposed to the following treatments with three replications: (1) control (no stress: 27°C); (2) 22°C (reduce by 5°C from control), and (3) 32°C (add by 5°C from control). Three tilapia per treatment were sampled at 1, 3, 6, 12, 24, 48, 72 and 168 h post stress. Fish were removed from their tanks and weighed. Approximately 0.5 ml of blood was drawn from caudal vein using a heparinized syringe and spun at 3,000 rpm for 10 min at 25°C to obtain plasma. Plasma cortisol was measured using Radioimmunoassay (RIA) (Sufi *et al*., 1986) by Adatis Italia S.p.A. Cortisol was reported as mean±SD. Treatment comparisons were tested using one-way analysis of variance (ANOVA) by Completely Randomized Design. A Duncan's New Multiple Range Test was applied for those treatments that had significant differences as P value 0.05. Four gill arches were removed quickly from all fish and submerged in TRITM reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and immediately frozen at -80°C until analysis. Heat Shock Protein 70 Genes Expression in gill was measured using Reversed Transcription Polymerase Chain Reaction (RT-PCR).

RNA isolation and cDNA synthesis

Total RNA was isolated using TRITM reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) following manufacturer's instructions. Briefly, collected tissue samples in TRITM reagent were homogenized using an MP Automatic Tissue Extractor (FastPrep_-24, MP Biochemicals, Santa Ana, CA, USA). Homogenates were mixed with chloroform and incubated at room temperature for 5 min. After centrifugation at 12,000 x g for 15 min at 4°C, clear upper lysates were transferred to a new tube and isopropanol was added to precipitate the RNA. RNA pellets were collected by centrifugation at 12,000 x g for 10 min at 4°C and followed by washing with 75% ethanol in DEPC water. Total RNA pellets were then resuspended in DEPC water and treated with RNase-free DNaseI (Fermentas, Hanover, MD, USA). After treatment, RNA samples were purified again with TRITM reagent as described above. The quantity and purity of total RNA samples were estimated from the absorbance at 260 nm (A260) and the ratio of absorbance A260/A280. Total RNAs were reversely transcribed with RevertAid first-strand cDNA synthesis kit (Fermentas).

Reversed Transcription Polymerase Chain Reaction (RT-PCR analysis)

Relative expression levels of Heat Shock Protein 70 (HSP) gene in the fish gill were analyzed by RT-PCR. The reaction was performed using Heat Shock Protein 70 (HSP)-specific primers: HSP70-F 5′ GA GTCCTACGCCTTCAACATGA 3′/HSPS70-R 5′ ATCTTCAGGGCCTCTTTAGTCC 3′. The Beta-actin gene was used as an internal

control, using Beta-actin-F 5′ GGTCATCA CCATTGGCAATG 3′ and Beta-actin-R 5′ ACTGAAGCCATGCCAATGAG 3′ primers. The reactions were performed in a volume of 30.0 µL containing 1.0 µL cDNA templates, 10X *Taq* Buffer 2.0 µL, 10 µM Forward Primer 1.5 µL, 10 µM Reverse Primer 1.5 µL, 2.5 mM dNTP 2.0 µL, *Taq* DNA Polymerase 0.1 µL and Distilled water 21.9 µL. The cycle conditions were as follows: initial denaturation step at 95^{o}C for 5 min. Secondly, 40 cycles at 95^{o}C for 30 s, at 55^{o}C for 30 s and at 72^{o}C for 1 min and 30 s. Thirdly, at 72^{o}C for 5 min. The relative expressions of Heat Shock Protein 70 (HSP) gene and Beta-actin gene were analyzed using Agarose gel electrophoresis. Relative expression ratio of HSP70 was reported as mean±SD. Treatment comparisons were tested using one-way analysis of variance (ANOVA) by Completely Randomized Design. A Duncan's New Multiple Range Test was applied for treatments which had significant differences at P value < 0.05.

RESULTS

Stress indicators in response to temperature stressor in Nile tilapia

The concentration of cortisol in blood serum of the Nile tilapia kept in three different ambient temperatures of 22, 27 and 32^{o}C were monitored at 1, 3, 6, 12, 24, 48, 72 and 168 hours. Results are shown in Table 1 and Figure 1.

At 22^{o}C, the concentrations of the cortisol in blood serum of Nile tilapia for each monitoring period were 5.63±5.08, 34.80 ±26.41, 105.87±25.14, 38.23±42.32, 14.80 ±17.17, 71.53±24.90, 3.47±4.61 and 2.67 ±1.69 µg/L, respectively. The highest peak of cortisol concentration was found after approaching this temperature for 6 hours (105.87±25.14 µg/L), while the second peak was at 48 hours (71.53±24.90 µg/L). Meanwhile the lowest was found in 168 hours after approaching (2.67±1.69 µg/L).

Table 1. Concentration of cortisol (µg/L) in blood serum of the Nile tilapia after exposure to three levels of temperature for 168 hours

Hour	Temperature		
	22^{o}C	27^{o}C	32^{o}C
1	5.63±5.08^{a}	50.20±44.54^{a}	9.53±8.11^{a}
3	34.80±26.41^{ab}	52.23±18.88^{b}	7.07±9.75^{a}
6	105.87±25.14^{b}	3.24±3.41^{a}	5.30±3.29^{a}
12	38.23±42.32^{ab}	93.22±48.80^{b}	2.74±3.66^{a}
24	14.80±17.17^{a}	7.87±7.70^{a}	38.60±24.81^{a}
48	71.53±24.90^{b}	30.47±20.97^{ab}	17.34±19.40^{a}
72	3.47±4.61^{a}	0.28±0.26^{a}	0.26±0.21^{a}
168	2.67±1.69^{a}	3.03±1.42^{a}	18.80±13.69^{a}

Remarks: Concentration of cortisol shown as mean ±SD. Different superscripts at various times indicate significant statistical difference (P<0.05) .

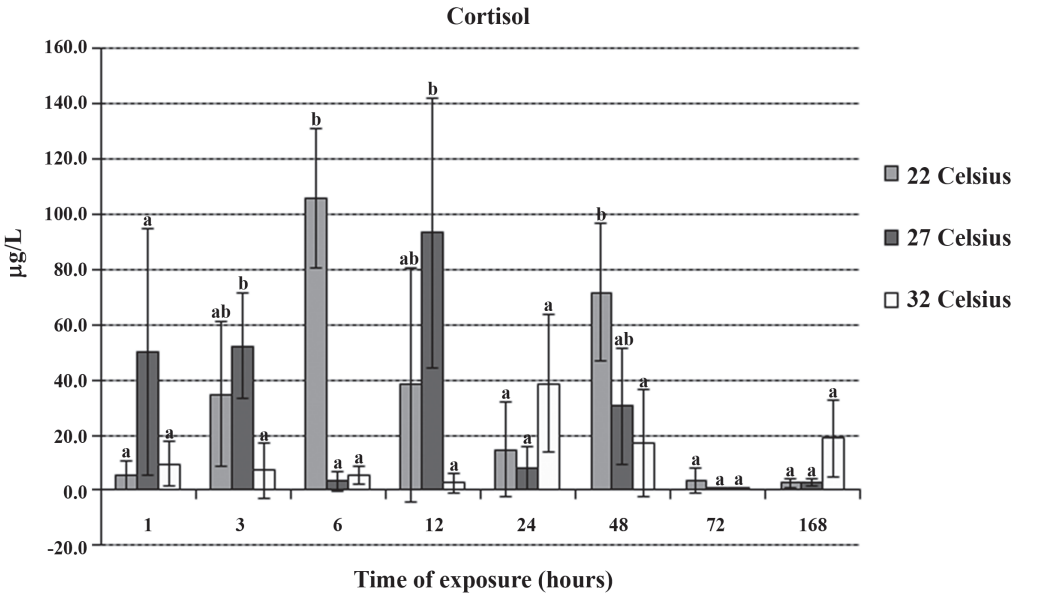

Figure 1. Concentration of cortisol in blood serum of Nile tilapia exposed to three levels of temperature for 168 hours

At 27°C, the concentrations of the cortisol in blood serum of Nile tilapia for each monitoring period were 50.20±44.54, 52.23±18.88, 3.24±3.41, 93.22±48.80, 7.87 ±7.70, 30.47±20.97, 0.28±0.26 and 3.03± 1.42 µg/L, respectively. The highest peak of cortisol concentration was found after approaching this temperature for 12 hours (93.22±48.80 µg/L), and the second peak was found in 3 hours (52.23±18.88 µg/L). The lowest concentration was found 72 hours after approaching (0.28±0.26 µg/L)

At 32°C, the concentrations of the cortisol in blood serum of Nile tilapia for each monitoring period were 9.53±8.11, 7.07±9.75, 5.30±3.29, 2.74±3.66, 38.60± 24.81, 17.34±19.40, 0.26±0.21 and 18.80± 13.69 µg/L, respectively. The highest peak of concentration was found after approaching this temperature for 24 hours (38.60±24.81 µg/L), with the second peak found in 168 hours (18.80±13.69 µg/L). The lowest concentration was found 72 hours after approaching (0.26±0.21 µg/L).

The changing patterns of cortisol concentration in tilapia blood serum as various temperature levels were reached were similar. In general, cortisol concentration would gradually increase until reaching the highest peak and then gradually decrease till it was lower than the initial concentration. However, the highest peak, retention time, and recovery time for each temperature level did not show the same pattern. From the results, we found that cortisol concentration at 22°C stressor showed a rapid change with the highest concentration which was significantly different from the peak concentration of 27°C (control) and 32°C (P<0.05). The highest peak and duration of

peak in concentration of cortisol were also different for each temperature level, with 105.87±25.14 µg/L at 22°C after approaching for 6 hours, while at 27°C the highest peak was 93.22±48.80 µg/L after approaching for 12 hours, and at 32°C was 38.60±24.81 µg/L after approaching for 24 hours.

The expression of Heat Shock Protein 70 (HSP) gene in Nile tilapia

The expression of the Heat Shock Protein 70 (HSP70), indicated as relative expression ratio of the Nile tilapia kept at three different ambient temperatures (22, 27 and 32°C) was monitored after approaching each temperature level at 1, 3, 6, 12, 24, 48, 72 and 168 hours. Results are shown in Table 2 and Fig.2.

At 22°C, the relative expression ratios from gill tissue of Nile tilapia for each monitoring period were 0.46±0.09, 6.69±0.36, 5.59±1.56, 1.19±0.48, 1.25±0.19, 2.30±0.64, 1.11±0.20 and 1.10±0.30, respectively. The highest value was found at 3 hours after approaching this temperature while the lowest was found in the first hour.

At 27°C, the relative expression ratios from gill tissue of the Nile tilapia for each monitoring period were 0.45±0.03, 0.66± 0.13, 1.95±0.57, 1.06±0.13, 1.56±0.18, 1.46 ±0.51, 1.22±0.29 and 0.70±0.53, respectively. The first peak was found at 6 hours after approaching this temperature, the second peak was found at 24 hours, while the lowest was found in the first hour.

At 32°C, the relative expression ratios from gill tissue of the Nile tilapia for each monitoring period were 0.45±0.07, 4.85±0.61, 12.84±3.12, 6.79±1.10, 2.60±0.54, 1.71±0.66, 5.51±0.81 and 2.21±0.51, respectively. The highest peak was found at 6 hours after approaching this temperature, the second highest was found at 12 hours while the lowest was found in the first hour.

Table 2. The relative expression ratio of Heat Shock Protein 70 (HSP70) from gill tissues of Nile tilapia exposed to three levels of temperature for 168 hours

Hour	Temperature		
	22°C	27°C	32°C
1	0.46±0.09^{a}	0.45±0.03^{a}	0.45±0.07^{a}
3	6.69±0.63^{a}	0.66±0.13^{c}	4.85±0.61^{b}
6	5.59±1.56^{a}	1.95±0.57^{c}	12.84±3.12^{b}
12	1.19±0.48^{a}	1.06±0.13^{a}	6.79±1.10^{b}
24	1.25±0.19^{a}	1.56±0.18^{a}	2.60±0.54^{b}
48	2.30±0.64^{a}	1.46±0.51^{a}	1.71±0.66^{a}
72	1.11±0.20^{a}	1.22±0.29^{a}	5.51±0.81^{b}
168	1.10±0.30^{a}	0.70±0.53^{a}	2.21±0.51^{b}

Remarks: The relative expression ratios of HSP70 are shown as mean±SD. Different superscripts at various times indicate significant statistical difference (P<0.05) .

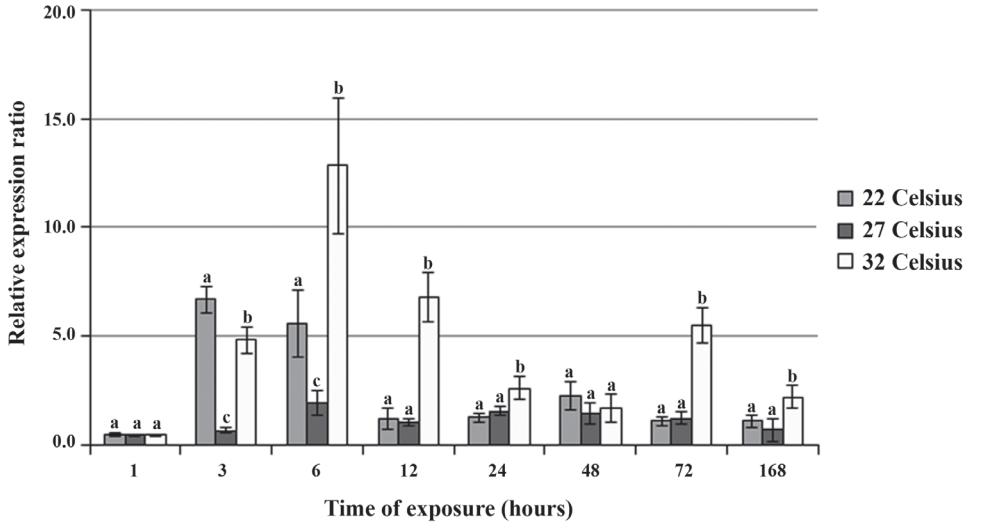

Figure 2. The relative expression ratio of Heat Shock Protein 70 (HSP70) from gill tissues of Nile tilapia exposed to three levels of temperature for 168 hours

The changing pattern of serum cortisol and the relative expression ratio of HSP70 from gill tissue of Nile tilapia showed the same pattern. After approaching the temperature stressor, fish would respond by secreting heat shock protein. Then, the level of HSP70 would rapidly increase within 3-6 hours according to the severity of temperature. Overall, it appeared that the HSP70 response to 27°C was lower than at other temperatures while the expression was highest at 32°C which was significantly different from the other 2 lower temperatures (P>0.05). The HSP 70 expression in fish kept at 22°C showed moderately upregulated during the first 6 hour period with the highest expression at 3 hours after approaching this level of temperature. It remained high until the 6th hour and significantly decreased afterwards. Apart from highest expression of HSP70, fish kept at 32°C also showed a tendency for highest retention time and recovery time.

Mortality rate of Nile tilapia in different temperatures after immersed in water with Streptococcus agalactiae

Accumulated mortality and average of accumulated mortality rates of Nile tilapia exposed for 168 hours at different temperatures and subsequently immersed in water inoculated with *Streptococcus agalactiae* for another 21 days are shown in Table 3 and Figure 3.

At 22°C, 5 fish were found dead after the experimental challenge with *S. agalactiae* for 21 days. Dead fish were first found on the 6th day. No fish were found dead after 17 days of experimentation. The average of accumulative mortality rate was 8.33±1.53%. This temperature had the lowest accumulation of mortalilty rate.

At 27°C, 5 fish were found dead after rearing. Dead fish were first found

on the 5th day, and no fish were found dead after 17 days of experimentation. The average of accumulated mortality rate was 8.33±0.58%. The mortality rate was the same with that of the 22°C treatment (P>0.05).

At 32°C, 19 fish were found dead after rearing. Dead fish were first found on the 4th day, and no fish were found dead after 13 days of experiment. The average accumulated mortality rate was 31.67± 3.51%. This treatment showed the highest accumulative mortality rate and this was significantly different from the other two lower temperatures (P<0.05).

The results from this study clearly showed that temperature could affect the

Table 3. Accumulated mortality and average accumulated mortality rates of Nile tilapia at different temperatures after exposure to three levels of temperature for 168 hours and challenged by *Streptococcus agalactiae* immersion for 21 days

DAY	22°C		27°C		32°C	
	Accumulated mortality	Average of accumulated mortality rate (%)	Accumulated mortality	Average of accumulated mortality rate (%)	Accumulated mortality	Average of accumulated mortality rate (%)
1	0	0±0^{a}	0	0±0^{a}	0	0±0^{a}
2	0	0±0^{a}	0	0±0^{a}	0	0±0^{a}
3	0	0±0^{a}	0	0±0^{a}	0	0±0^{a}
4	0	0±0^{a}	0	0±0^{a}	4	6.67±0.58^{b}
5	0	0±0^{a}	3	5.00±1.00^{a}	13	21.67±1.15^{b}
6	1	1.67±0.58^{a}	4	6.67±1.15^{a}	14	23.33±1.53^{b}
7	1	1.67±0.58^{a}	4	6.67±1.15^{a}	14	23.33±1.53^{b}
8	1	1.67±0.58^{a}	4	6.67±1.15^{a}	14	23.33±1.53^{b}
9	1	1.67±0.58^{a}	4	6.67±1.15^{a}	14	23.33±1.53^{b}
10	2	3.33±0.58^{a}	4	6.67±1.15^{a}	14	23.33±1.53^{b}
11	2	3.33±0.58^{a}	4	6.67±1.15^{a}	17	28.33±3.06^{b}
12	2	3.33±0.58^{a}	4	6.67±1.15^{a}	17	28.33±3.06^{b}
13	2	3.33±0.58^{a}	4	6.67±1.15^{a}	19	31.67±3.51^{b}
14	2	3.33±0.58^{a}	4	6.67±1.15^{a}	19	31.67±3.51^{b}
15	2	3.33±0.58^{a}	4	6.67±1.15^{a}	19	31.67±3.51^{b}
16	2	3.33±0.58^{a}	4	6.67±1.15^{a}	19	31.67±3.51^{b}
17	5	8.33±1.53^{a}	5	8.33±0.58^{a}	19	31.67±3.51^{b}
18	5	8.33±1.53^{a}	5	8.33±0.58^{a}	19	31.67±3.51^{b}
19	5	8.33±1.53^{a}	5	8.33±0.58^{a}	19	31.67±3.51^{b}
20	5	8.33±1.53^{a}	5	8.33±0.58^{a}	19	31.67±3.51^{b}
21	5	8.33±1.53^{a}	5	8.33±0.58^{a}	19	31.67±3.51^{b}

Remarks: Average of accumulated mortality rate is shown as mean±SD. Different superscripts at various times indicate significant statistical difference (P<0.05) .

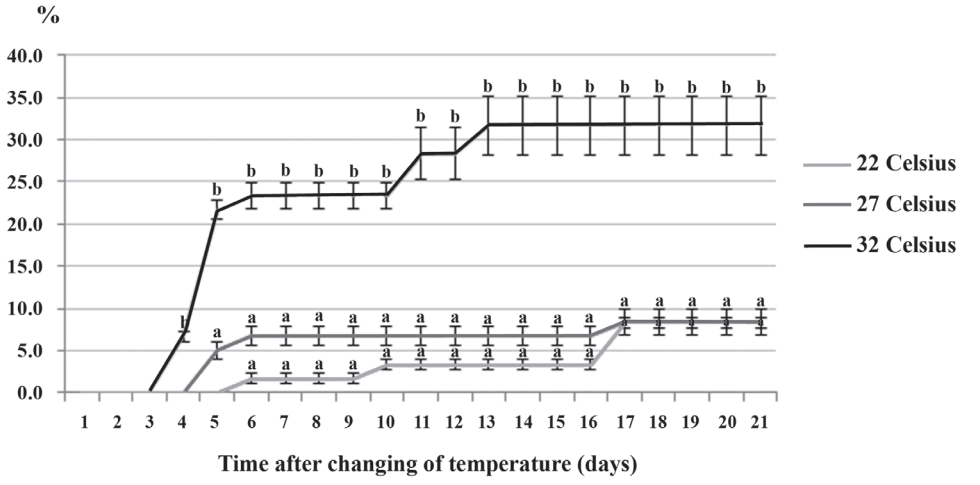

Figure 3. Average of accumulated mortality of Nile tilapia in different temperature after challenged by *Streptococcus agalactiae* immersion for 21 days

severity of *S. agalactiae* infection in Nile tilapia. Nile tilapia kept in higher temperatures showed a higher mortality rate. Mortality could occur earlier than those in the lower temperature (4 days at 32°C, 5 days at 27°C, and 6 days at 22°C). At the higher temperature (32°C), mortality of Nile tilapia could occur rapidly, during the first 10 days than those in lower temperatures. Mortality in lower temperature treatments of 22 and 27°C was also observed and were not significantly different at the end of the trial (P>0.05). However, mortality of infected fish reared at 27°C occurred more rapidly, on the 5th day, compared with that at 22°C which occurred on the 6th day.

DISCUSSION

Effect of temperature on serum cortisol concentration and relative expression ratio of Heat Shock Protein 70 (HSP70) on Nile tilapia

The cortisol

From our results, it was clearly demonstrated that temperature was a major factor to induce stress in fish, especially in Nile tilapia, and in the results from both parameters, serum cortisol and the relative expression ratio of the HSP70 from the gill tissue. However, the pattern of response of these two parameters appeared to be quite different.

After vertebrates are exposed to stress, in general, the cortisol hormone would be released in response to cope with stress. Consequently, the level of glucose in blood stream will be elevated via gluconeogenesis. However, the responding cycle of cortisol to stress by each particular vertebrate was totally different. Iwama *et al*. (2006) concluded that the changing pattern of cortisol concentration in blood plasma of fish should be at least 6 hours for full recovery. But the recovery time was dependent on the species and on

the level of ambient temperature such as 48 hours in the red drum (Roberson *et al*. 1988), 8 hours in common dentex, *Dentex dentex* (Morales *et al*. 2005), and 4 hours in common carp, *Cyprinus carpio* (Pottinger, 1998). Our results also agreed with those studies but we also found that the recovery time for each specific temperature exposure was different. At 22°C, cortisol concentration reached the peak at 6 hours after approaching. After reaching the peak, cortisol tended to decrease but another peak occurred at the 48th hour. The changing pattern of cortisol concentration at 27°C and 32°C was similar. Even cortisol was released to alleviate stress but retaining high concentrations in the body for longer periods would damage body tissue such as in salmon (Wendelaar Bonga, 1997). This is a good reason to explain why fish has to eradicate cortisol from the blood serum. But eradication ability varied among species. For example, the metabolic clearance rate (MCR) of American eel, *Anguilla rostrata* was 45 mg/Kg/hr, while the Sockeye Salmon was 270 mg/Kg/hr and the sea ravern, *Hemitripterus americanus* was 449 mg/Kg /hr (Mommsen *et al*. 1999). In this study, we found that the highest peak for each experiment was different at 6, 12 and 24 hours. When compared to the Mozambique Tilapia (*Oreochromis mossambicus*), concentration of cortisol in blood serum was not significantly different at temperatures of 20°C and 35°C (Fiess *et al*. 2007).

Another finding from this experiment was the presence of the second peak of cortisol concentration in the 22°C and 27°C treatments, while in 32°C, a delayed single peak was observed. This is may explain that apart from temperature, there could be other factors which could enhance cortisol concentrations in the blood serum of Nile tilapia. However, in this article we considered only the effect from temperature. But this finding may imply that using cortisol concentration in blood serum alone might not be a good stress indicator in Nile tilapia.

HSP70

Like cortisol, after being exposed to stress stimulus by changing ambient temperature, fish would secrete the HSP70 for stress alleviation. In general, fish can produce HSP from every organ (Mazur, 1996) but the most sensitive and more reliable organ is the gill tissue, owing to the fact that this is one of the very first organs to get into contact with the stimulus. Tendency of HSP70 cycle in fish tissue was similar to the cycle of cortisol. However, from our results, the relative expression ratio of the HSP70 at the 27°C and 32°C was very similar compared to the HSP70 ratio of the 22°C treatment. Furthermore, our results also showed that the highest peak of the relative expression ratio occurred at 3 hours after encounter with 22°C while the peak of 27°C and 32°C occurred at 6 hours after experiment. Therefore this should indicate that the most preferable temperature for Nile tilapia should be below 32°C and not lower than 27°C. In this study, fish in 27°C treatment showed the lowest HSP70 expression during sampling (P<0.05). Although Likongwe *et. al*. (1996) pointed out that Nile tilapia was a tolerant fish which can easily adjust to a wide range of temperature (24-32°C), other researchers such as Philippart and Ruwet (1982), Sifa *et al*. (2002), Atwood *et al*. (2003) and Charo-Karisa *et al*. (2005) even reported Nile tilapia tolerance to a much wider range (8°C-42°C).

The role of HSP in fish and other vertebrates pertains to folding and packing the incomplete polypeptides and proteins resulting from stress. Furthermore, HSP also helps in the transfer process of these newly complete proteins passing cell membrane into various targeting organelles inside the cell (Morimoto *et al*., 1994 and Cooper, 2000).

It seems that the changing pattern of the relative expression ratio of HSP70 was more regular and more predictable than the cortisol concentration. In every temperature experiments, there were only one highest peak available and then the ratio would gradually decrease. This shows that the production of HSP70 in the gill tissue has less interference factor than the production of cortisol. There could be other factors which can affect HSP expression, such as type of tissue being studied (Rabergh *et al*., 2000), toxic substances such as heavy metal (Airaksinen *et al*., 2003), environmental condition and species of fish (Basu *et al*., 2001 and Nakano and Iwama, 2002), age of fish (Santacruz *et al*., 1997 and Lele *et al*., 1997) and season (Fader *et al*, 1994).

When comparing the pattern of response of serum cortisol concentration and HSP70 expression at the same temperature experiment (as shown in Table 4), it was clearly shown that the trend or changing pattern of both parameters was similar. However, changing pattern of the HSP70 expression seemed to be more regular and consistent than the cortisol concentration. At the same level of temperature (22^{o}C), the highest peak of HSP70 occurred at 3 hours while the peak of the cortisol experiment occurred at 6 hours. Range of changing of HSP70 in the 27^{o}C treatment was narrower than the shifting trend of cortisol concentration. The fluctuation in cortisol was obvious than the changing of expression on HSP70.

Table 4. Cortisol concentration in blood serum and the relative expression ratio of the Heat Shock Protein 70 (HSP70) from gill tissue of the Nile tilapia at different levels of temperature

Hour	Temperature					
	22^{o}C		27^{o}C		32^{o}C	
	Cortisol (µg/L)	HSP70	Cortisol (µg/L)	HSP70	Cortisol (µg/L)	HSP70
1	5.63 ± 5.08	0.46 ± 0.09	50.20 ± 44.54	0.45 ± 0.03	9.53 ± 8.11	0.45 ± 0.07
3	34.80 ± 26.41	6.69 ± 0.63	52.23 ± 18.88	0.66 ± 0.13	7.07 ± 9.75	4.85 ± 0.61
6	105.87 ± 25.14	5.59 ± 1.56	3.24 ± 3.41	1.95 ± 0.57	5.30 ± 3.29	12.84 ± 3.12
12	38.23 ± 42.32	1.19 ± 0.48	93.22 ± 48.80	1.06 ± 0.13	2.74 ± 3.66	6.79 ± 1.10
24	14.80 ± 17.17	1.25 ± 0.19	7.87 ± 7.70	1.56 ± 0.18	38.60 ± 24.81	2.60 ± 0.54
48	71.53 ± 24.90	2.30 ± 0.64	30.47 ± 20.97	1.46 ± 0.51	17.34 ± 19.40	1.71 ± 0.66
72	3.47 ± 4.61	1.11 ± 0.20	0.28 ± 0.26	1.22 ± 0.29	0.26 ± 0.21	5.51 ± 0.81
168	2.67 ± 1.69	1.10 ± 0.30	3.03 ± 1.42	0.70 ± 0.53	18.80 ± 13.69	2.21 ± 0.51

Remarks: Values shown as mean±SD.

From our results, it was obvious that the relative expression ratio of HSP70 could be a better indicator than the cortisol concentration, since it took less time to detect and changing pattern in response to level of stressors as more regular. Cortisol used to be one of the most acceptable indicators in the past, but interference from many other parameters could easily influence the changing of cortisol concentration (Martinez-Porchas *et al*., 2009). The use of cortisol concentration should be applied to the acute effects than the chronic effect. Moreover, the presence of multiple peaks after exposure to stressors made using cortisol less reliable. Although, the expression of HSP70 might have more advantages as a stress indicator, the study was limited to the relative expression ratio only, not the whole strand of protein. Thus, the acquired results were explained as trends in changing of protein only. For more precise in study, Iwama *et al*. (2004) noted that we should have information on specific stress induction for each particular species, in this case, the Nile tilapia. The HSP70 expression was also better for chronic stress induction.

Effect of Temperature on Streptococcosis infection in Nile tilapia

It was assumed that the epidemic of Streptococococsis in Nile tilapia was caused by the combination of temperature stress and more susceptibility to *Streptococcus agalactiae*. Results from the experiment indicated that temperature played an important role on the degree of severity of *S. agalactiae* infection in association with the stress condition of Nile tilapia. After raising tilapia at three levels of temperature for 168 hours before being challenged with *S. agalactiae* by immersion, tilapia raised at high temperature (32^{o}C) showed a significantly higher rate of mortality than those raised in lower temperatures (22^{o}C and 27^{o}C) at the end of the 21-day trial.

It was clearly shown that high temperature would cause Nile Tilapia to die faster and mortality rates would be higher after *S. agalactiae* immersion challenge. This was supported by the results that the accumulative mortality rates in the 22^{o}C and 27^{o}C treatment were significantly lower than that in 32^{o}C treatment (P<0.05). Furthermore, the fish exposed to 32^{o}C started to die on the 4th day after the experiment compared with those in the other two treatments, which were on the 5th and 6th days, respectively. Our results confirmed the report by Rodkhum *et al*. (2011) wherein they mentioned that Nile Tilapia kept at temperatures higher than 30^{o}C would be more susceptible to infection of *S. agalactiae* than those kept at lower temperatures. More recent work by Kayansamruaj *et al*. (2014) has also confirmed this. They then suggested that the increase of *S. agalactiae* (Group B streptococci, GBS) pathogenicity to Nile tilapia could be induced by elevated temperatures (comparison between 28 and 35^{o}C) and associated with massive inflammatory responses which might lead to acute mortality.

Higher mortality rates found in the high temperature treatment (32^{o}C) in this study might be explained according to two points of view, based on cortisol and HSP70 expression.

Based on the cortisol concept, high temperatures can cause stress that leads to the release of more cortisol. Normally, the fish would respond to stress in three phases. The first phase involves secretion of cortisol and catecholamines into the bloodstream. The second phase relates to the response of endocrine glands to the secreted substances, and the third phase is the response from the first and second phases by changing body functions such as metabolic activities and certain behaviors (Barton, 2002). Persistence of high cortisol levels for a long time would lead to fish suffering in various ways, such as deterioration of muscle tissues, enhancing metabolic rate and increase in blood glucose, and collapse of some metabolic pathways. Although high metabolism for certain periods might increase growth rate in fish, long exposure to high temperatures would have a negative influence on some metabolic pathways, including suppression of the immune system leading to weakness and eventually death. Fluctuation of cortisol levels in blood serum in our experiment may be caused by sampling method and individual variation (Cleary *et al*., 2002 and Tsunoda *et al*., 1999).

Based on the HSP70 expression concept, it was known that HSP in the cell would assist the completion of protein assemblage and translocation pathways, and synergy of hormones and their receptors (Cooper, 2000). Even if this protein is named "heat shock", it is not only high temperature which stimulates cells to produce HSP but also other chemical substances such as heavy metals and some physical stimuli which are also classified as heat shock protein stimulus. Iwama *et al*. (1998) stated that understanding the function and mechanism of HSP in fish was just at the initiation stage compared with other organisms, and that the complete understanding of the whole production cycle, including the study of recovery process and time, is still not achieved, and continues to remain a puzzle. Our results on shifting of HSP70 cycle after temperature stimulus would be the very first report on live Nile tilapia study. The previous works were about comparing levels of HSP70 from different tissues while the study on changing cycle in fish was scarce.

From our study, it was also confirmed that high temperature was the key factor for stress stimulus in Nile tilapia. In order to respond to this stress, synthesis of cortisol into the blood serum and launching of HSP70 in tissue were the obvious indicators. Normally, fish kept at optimal temperatures should be less stressed than fish kept at lower or higher temperature levels. Our results clearly showed that temperatures above optimal could induce stress and result in weakness in fish but the effect of lower temperature was unclear. According to the overall results of cortisol concentration and HSP70 expression, lower temperature caused higher stress than the medium temperature, however, this low temperature stress did not have any significant impact on tilapia immunity. This could indicate that research on the effect of low temperature on tilapia physiology and immunity may be needed. According to Shoemaker *et al*. (2001), inoculated Mozambique tilapia kept at temperatures of 19^{o}C and 35^{o}C had exhibited higher mortality rates than those exposed to temperatures between these two levels.

A weakness in our study was the limitation in selected research techniques. The relative expression ratio was measured for level of heat shock gene expression only and not for protein structure. Hence, in the future, if the technique for measuring the amount of heat shock protein from particular tissue is available, this would provide a better explanation of fish response to stress. Using HSP70 gene expression ratio would be superior than using the traditional method of cortisol hormone analysis. However, there should be more research on specific induction for each species both *in situ* and *in vitro* or cell line study. In addition, the variation of responses within species should be also considered. As suggested by Iwama *et al*. (2004), the study of HSP would be suitable in monitoring of chronic effects than acute effect, and then the combination effects of every parameter should also be studied.

CONCLUSION

Temperatures at both above or below the optimal level for Nile tilapia would induce stress leading to physiological process within the body to produce cortisol and released into the bloodstream and HSP into many organs. However, certain characteristics of the HPS70 such as the sensitivity and consistent response pattern might lead to a superior indicator for monitoring stress in fish compared to cortisol.

The HSP70 expression should be a reliable stress indicator; however, there were some limitations that should be taken into consideration such as the concise and precision of detection techniques, and limitation for quantitative analysis. Future research on developing techniques and also variations caused by age, size, sex and health of experimental fish should be considered.

Stress from temperature has been claimed and scientifically proven as a main cofactor that induces the Nile tilapia to be more susceptible to streptococcosis infection. This was also observed in this study, wherein higher accumulative mortality rates and faster pace of infection development occurred in the 32^{o}C experiment compared to those in the 27^{o}C and 22^{o}C treatment. Application -wise, the findings from this study also suggested that the optimal temperature for culturing Nile tilapia should be between 27 to 32^{o}C.

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