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The Effect of Heat Stress on the Oxidative Status of Red Hybrid Tilapia (*Oreochromis* sp.) Infected With *Streptococcus Agalactiae*

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ABSTRACT

A commercial red hybrid tilapia was experimented with *S. agalactiae* infection under influences of heat stress which indicated by plasma malondialdehyde (MDA) and erythrocyte superoxide dismutase (SOD) as biomarkers of stress. To achieve these objectives, 110 red hybrid tilapia in good health were divided into five groups of 22 fish each. Group A was challenged with 2.3×10^9 CFU of *S. agalactiae* and exposed to heat stress at 33 ± 0.5°C on day 1. Group B was challenged on day 1 as in Group A but heat stress was introduced on day 7 post challenge (pc). Group C was exposed to heat stress on day 1 and challenged on day 7 pc while groups D and E served as a positive and negative controls respectively. Blood samples were collected at days 0, 3, 7, 10 and 14 for MDA and SOD analysis. Groups A and B recorded high mortality following exposure to heat stress and bacteria inoculation, with group A reaching 100% mortality at day 7 post inoculation. Overall, Groups A, B, C and Group D showed pattern of increase in MDA level as early as day 3 and decrease pattern for SOD activity. Group E did not show any significant difference in MDA level throughout the study period. Clinical signs such as erratic swimming, exopthalmia, skin haemorrhage and cloudy eye were predominantly observed in group A 24 h post inoculation. Based on the findings of this study, it was concluded that heat stress plays crucial role in the pathogenesis of *S. agalactiae*, via alteration of the oxidant defence system.

KEYWORDS: *red hybrid tilapia, S. agalactiae, malondialdehyde, superoxide dismutase*

INTRODUCTION

Primary contributing factors to fish diseases and mortality in aquaculture are physiological stress and physical injury [1]. Stress is a physical and chemical factor that may cause body reactions that contribute to disease progression and consequently death. *Streptococcus* spp. is the cause of streptococcosis in many fish and mammal and it has been recognized as a major infectious disease producing significant loss in the aquaculture industry worldwide. One of the fish species that are susceptible to streptococcosis is the Nile tilapia (*Oreochromis niloticus*) [2,3,4]. Two species of *Streptococcus*; *Streptococcus iniae* and *S. agalactiae* have been reported in farmed fish in which the latter species was found to have more than 80% prevalence rate [5].

In the case of warm-water streptococcosis associated with *S. agalactiae*, recent studies in barramundi (*Lates calcarifer*) and Mozambique tilapia (*Oreochromis mossambicus*) revealed that the mortality of the challenged fish increased due to inappropriate water temperature [6,7]. Unfortunately, for *S. agalactiae*, a previous epidemiological investigation about streptococcosis occurrence in Nile tilapia farm in Brazil was the only evidence suggesting that susceptibility to the disease is closely related to dynamic change in water temperature since the disease outbreak is usually found when the water temperature is higher than 26°C [7].

Streptococcus agalactiae is the most important pathogen affecting freshwater fish that cause economic losses to the aquaculture industry in Malaysia [8,9,10] leading to high mortality with several physical symptoms such as corneal opacity, distinctive swollen belly, exophthalmia, eye haemorrhages, enlarged liver and congestion of the kidney and spleen. The need for further studies on the effect of heat stress on the pathogenicity and mortality pattern in Red hybrid tilapia infected with *S. agalactiae* is therefore a necessity.

Oxidative stress occurred when imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants ensues. Living organisms are protected from reactive oxygen species by several defence's mechanisms such as superoxide dismutase (SOD) [11]. Malondialdehyde and SOD are example of biomarker for oxidative stress besides catalase (CAT) enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). Santosh *et al.* (1989) stated that fluctuation in temperature was one of the examples that cause stress to aquatic organisms especially fish. When fish are stress, the level of reactive oxygen species (ROS) also increases. Defensive mechanisms to fight the overload ROS are found in many mammalian species including fish. Thus, fish are largely being used as bio indicator of environmental changes. These systems include various antioxidant defence enzymes such as SOD, which catalyse the dismutation of superoxide radical to hydrogen peroxide [12]. There is paucity of information on the effect of heat stress on the oxidative status of red hybrid tilapia (*Oreochromis* sp.) infected with *Streptococcus agalactiae*. Therefore, this study seeks to determine the effect of *Streptococcus agalactiae* infection and heat stress on oxidative stress in red hybrid tilapia (*Oreochromis* sp.).

Materials and Methods

1. Fish, location and experimental design

One hundred and ten red hybrid tilapia in good health were purchased from Aquaculture Extension Centre (AEC), Bukit Tinggi, Pahang, Malaysia. The fish were divided into five groups, A, B, C, D and E of 22 fishes each. Group A was challenged with inoculumn (2.3×10^9 CFU/mL of *S. agalactiae)* and exposed to heat stress (33 ± 0.5ºC) on day 0 through day 7. Group B was challenged on day 0 while exposed to heat stress on day 7 through day 14 post-challenge (pc). Group C were exposed to heat stress on day 0 through day 7 and challenged with similar dose of the inoculum as in A above on day 7 pc while group D were challenged on day 0 with no heat stress serving as positive control. Group E served as negative control with no while they were kept in normal water temperature at 27 ± 0.5ºC. On days 0, 3, 7, 10 and day 14 pc, blood samples from three fish per group were collected for MDA and SOD analysis and the fish was released after that process without killing them. Plasma was prepared for MDA analysis while haemolysates was prepared for SOD analysis. Bacterial isolation from brain, eye and kidney were collected from any dead fishes. Dip immersion method by immersing the fish for 20 minutes in one litre of solution with 2.3×10^9 CFU/mL of *S. agalactiae*. Clinical signs, mortalities or abnormal behaviour for the period of 14 days pc was recorded. All the dead fish were subjected to post-mortem examination and bacterial isolation.

2. Bacterial culture

Streptococcus agalactiae strain SA2K (Biotype 1) was obtained from National Fish Health and Research Centre (NaFisH), Batu Maung, Pulau Pinang, Malaysia. The isolate originated from a field outbreak of streptococcosis in Red hybrid tilapia kept at Kuala Lipis, Pahang, Malaysia in 2007. The isolate was then subcultured onto goat blood agar plate (Merck, USA) and was identified by Rapid ID 32 Strep API kit system (Biomeriux, Germany) for confirmation of *S. agalactiae.*

3. Preparation of *Streptococcus agalactiae* Inoculum

The selected *S. agalactiae* strain was grown on 5% goat blood agar plates (Merck, USA) for 24 hours at 30°C. Thirty single colonies of *S. agalactiae* were picked and sub-cultured in 1 L of fresh brain heart infusion (BHI) broth (Oxoid, UK) for 24 hours at 30°C by shaking at 110 rpm to obtain exponentially growing cells. One mL of the bacterial culture suspension was serially diluted in 9 mL of sterile peptone water and 0.1 mL of the serial dilutions of the suspension was spread plated onto 5% goat blood agar. Colony forming units CFU/mL were determined after 24 hours of incubation at 30°C.

4. Fish

A total of 120 clinically healthy Red hybrid tilapia (*Oreochromis* sp.) were selected from Aquaculture Extension Centre (AEC), Bukit Tinggi, Pahang, Malaysia. The mean weight of the tilapia was 80 ± 10 g. One week before the beginning of the experiment, 10 of the fish were sacrificed and screened for *S. agalactiae* bacterial isolation to ensure they were free from *S. agalactiae* and parasites. Then, 110 fish were randomly divided into five 200-L tanks that represented five groups; Groups A, B, C, D and Group E. Light cycle was provided constantly at 12 hours. Water quality parameters YSI 556 Multiparameter Instrument (USA) was used to monitor and to ensure that they were within the normal values with 4.97 ± 0.3 mg/L dissolved oxygen, 32.6 ± 0.8 ºC water temperature, 7.47 ± 0.1 pH, 2.37 mg/L ammonia and 0.023 mg/L nitrate concentrations. All the fishes were fed once daily for 14 consecutive days using commercial feed while water was continuously aerated. The feeding of the fish was according to the standard feeding rate i.e. 10% of the total body weight

5. Laboratory sampling

5.1 Serum, plasma and bacterial isolation

During post-mortem examinations, samples of several organs such as brain, eyes and kidney of dead fishes from all groups were collected aseptically for bacterial isolation. Sterile wire loop was used to isolate bacteria from those organs and directly streak onto 5% goat blood agar plate (Merck, USA). The plates were then incubated at 30°C for 24 hours. Growth of *S. agalactiae* was recorded and pure culture was obtained for identification. One to two mL blood sample from each three fishes per group were taken at 0, 3, 7, 10 and 14 days for MDA and SOD analysis without killing the fish. Plasma and haemolysate were prepared from the blood of each fish. The plasma and haemolysates were kept at -20°C until further used.

6. Clinical signs and mortality

During the experiment, all fish were observed for any form of clinical signs, infection and mortality.

7. Laboratory analysis

7.1 Bacterial identification

All the isolates were further identified using techniques such as Gram staining and bioMérieux's API microorganism identification test kits such as API20 NE, API STAPH and API rapid ID32 Strep. If suspected *S. agalactiae* is present, then PCR and gel electrophoresis was performed to confirm the colonies.

7.2 Malondialdehyde (MDA) Analysis

The plasma MDA concentration was assayed according to the method described by Ohkawa *et al*. [9], with minor modifications. Absorbency was measured in visible spectrophotometer (OPTIZEN 1412V, Korea) at 532 nm. The concentration of MDA was expressed as nmol/mL of plasma.

7.3 Superoxide Dismutase (SOD) Analysis

The erythrocyte superoxide dismutase (SOD) was measured according to Marklund and Marklund [11] as described by Beutler [12] with minor modification. The spectrophotometer was zeroed at 420 nm with deionized water and the absorbance of the blank and the samples read in 1 minute.

7.4 Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 14.8.1 (MedCalc Software, Ostend, Belgium) and tested at 5% level of significance. The differences in value of MDA and SOD and other data were analysed using a one-way ANOVA. If significant differences were obtained, a student-Newman-Keuls pairwise comparison post-hoc test was employed to determine the statistical differences between the treatments

Results

Clinical Signs

The clinical signs of fish from all groups were observed starting from day zero of the experiment. The first clinical signs observed from the infected fishes were loss of appetite (Figure 3), lethargy and erratic swimming (Figure 4), predominantly from group A and later group C and with less intensity in the other groups. The affected fish showed clinical signs as early as at 12 hours pc. These were followed by unilateral (Figure 2) and or bilateral exophthalmia, cloudy eyes (Figure 4) and inflammation (Figure 1) of the skin after 24 hours of pc.

Figure 1: Skin haemorrhages were observed on the fish's body (red circle) at day 1 pc in group A.

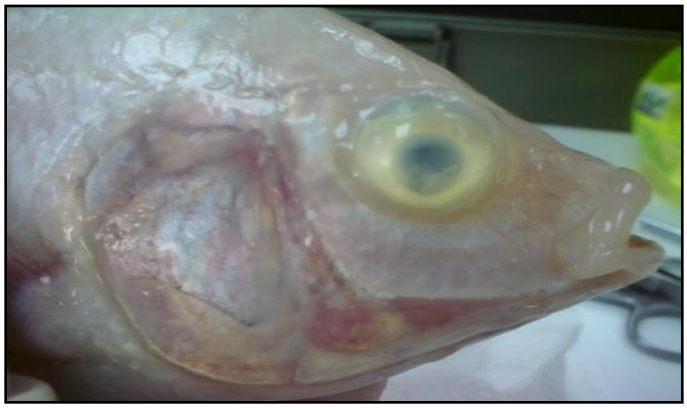

Figure 2: Cloudy eyes observed at day 1 pc in tilapia from group A.

Figure 3: The intestines of the infected fish were empty and reddening

Figure 4: Erratic swimming was observed at day 1 post-challenged in tilapia from group A.

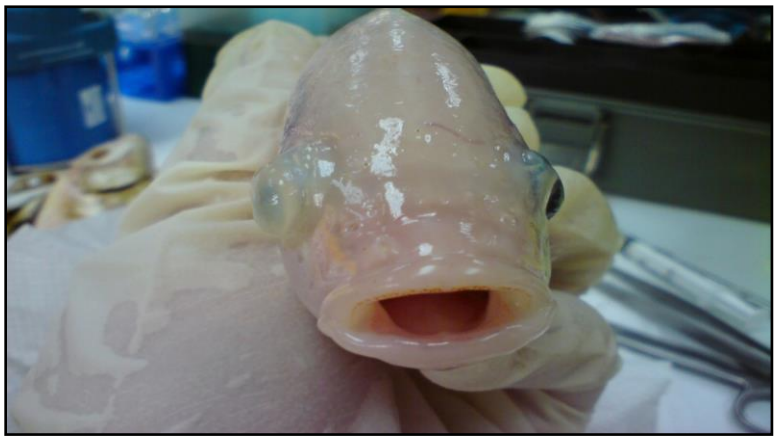

Figure 5: Unilateral exophthalmia and cloudiness of eyes was observed in all infected fish at day 1 post-challenged in group A.

Bacterial isolation

Total of 39 fish died during this experiment out of the 110 red hybrid tilapia used. Samples for bacterial isolation were taken from brain, eye and kidney from all the dead tilapia fish from each group to confirm the presence of *S. agalactiae.* Isolated bacteria were cultured onto blood agar and incubated at 37ºC for 24 hours. Gram stain and RAPID ID 32 Strep system were used to identify *S. agalactiae*. 11 different isolated were obtained but five suspected cultures of *S. agalactiae* were confirmed using PCR technique with the expected band size approximately 1450 base pair (bp) (Figure 6). Bacterial isolates (1, 4, 5, 10 and 11) were confirmed as *S. agalactiae*

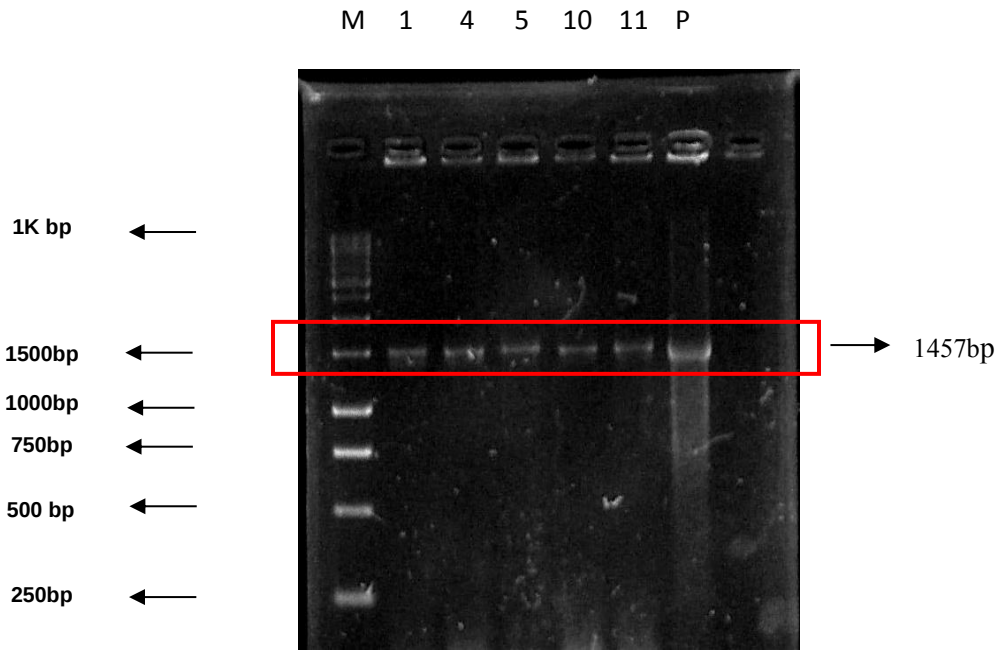

Figure 6: PCR amplification for identification of *S. agalactiae.*
Lane M: Gene Ruler^{TM}1 kb DNA marker (Fermentas, Lithuania), lane 1: isolates 1, lane 4: isolate 4, lane 5: isolate 5, lane 10: isolate 10, lane 11: isolate 11 and lane P: Positive control

Mortality

Streptococcus agalactiae appeared to be highly pathogenic to the red hybrid tilapia challenged with inoculum at the concentration of 2.3×10^9 CFU/mL and subjected to heat stress at 33 ± 0.5°C (Group A). The bacteria were found to induce mortality in the red hybrid tilapia as early as day 2 post-challenge. One hundred percent mortality was recorded before day 7 post challenge in Group A which were exposed to heat stress at 33 ± 0.5°C following challenge with *S. agalactiae.* Within 48 hours pc, mass mortality started to occur in tilapia in Group A and at day seven, all the fish in Group A died. However, low rates of mortality were observed among tilapia of Groups B, C and D. Group C showed the second highest mortality (36.4%) during this experiment which received heat stress at 33 ± 0.5°C on day 0 and challenged of *S. agalactiae* with concentration of 2.3×10^9 CFU/mL on day 7 pc. Group B showed 18.2% mortality during this experiment starting from day 6 through day 8 pc. Mortality of fish in Group C started at day 2 and mass mortality of fish started as from day 10 through day 13. Group D and Group B had 18.2% percentage of mortality. There was no mortality reported in Group E.

Malondialdehyde analysis

The mean levels of MDA for all the groups before and post challenge is presented in figure 7. The mean MDA level for group A was found to increase rapidly following challenge and heat exposure, reaching its peak level at day 3 post challenge. Group B which were challenged on day 0 and started introduced to heat stress on day 7 showed an initial increment in MDA activities on day 3 pc which was maintained through day 10 pc. The group recorded a sharp increase in MDA activities again from day 10 pc to day 14 pc. Group C which were introduced to heat stress on day 0 and challenged on day 7 recorded a significant increase in MDA activities on day 3 and decreased slightly on day 7. Following challenge, it increased again on day 10 and decreased through day 14 post-heat exposure. Group D also recorded an increment in MDA activities on day 3 pc and decreased significantly on day 7 pc. Thereafter, it increased again through day 14 pc. Group E did not show any significant difference in MDA activities throughout the study period.

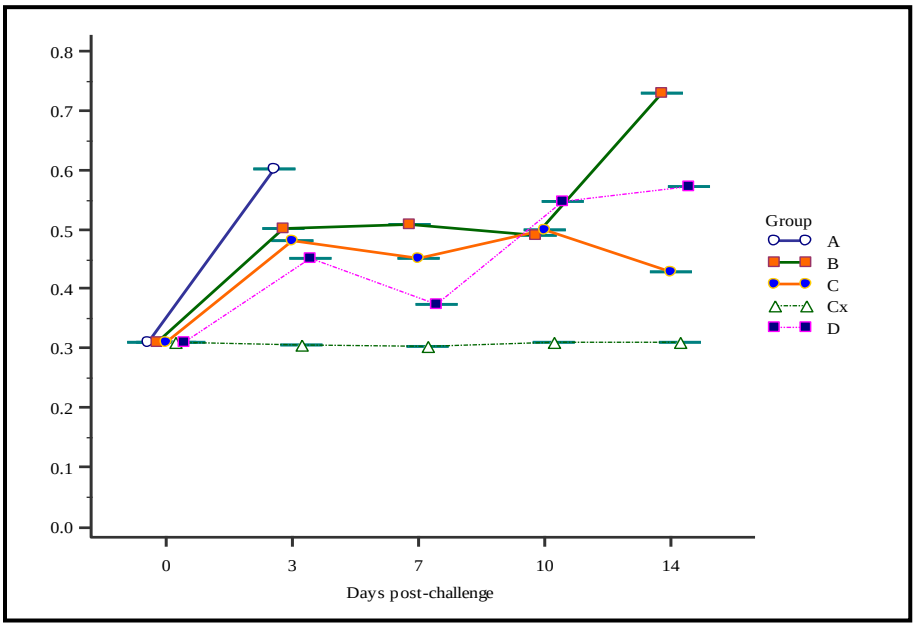

Figure 7: Standard means values of MDA plotted against days post challenge with standard error ± 0.5 of value of MDA

Note :- C_x* = Group E (Negative control)

Superoxide dismutase (SOD) Analysis

In group A, upon bacterial challenge and exposure to heat stress concurrently on day 0, the SOD activities decreased through day 3 pc. Unfortunately all the fish in this group died before the next sampling day (day 7 pc), hence there was no data for mortality after day 3 pc. In group B however, the SOD activity decreased steadily through day 3 until day 7 pc. Upon exposure to heat stress, the SOD activity did not change until day 10 pc then it decreased steadily to its lowest value at day 14 pc. Group C that were exposed to heat stress on day 0 prior to bacterial challenge on day 7 recorded a decreased SOD activity through day 3 post-exposure to heat stress. Following

bacterial challenge on day 7, the SOD activity further decreased from day 7 until day 10 and reached its lowest level of activity at day 14 pc. Group D recorded an unexpected SOD activity as it increased drastically when it should decreased on day 3 pc. This may because of the bacterial challenged was strong enough to give stress and disturb metabolism and physiology of the fish. It then started decreasing through days 7, 10 and 14 pc, recording its lowest activity. Group E as a control group did not record any significant alteration in the SOD activities throughout the study period.

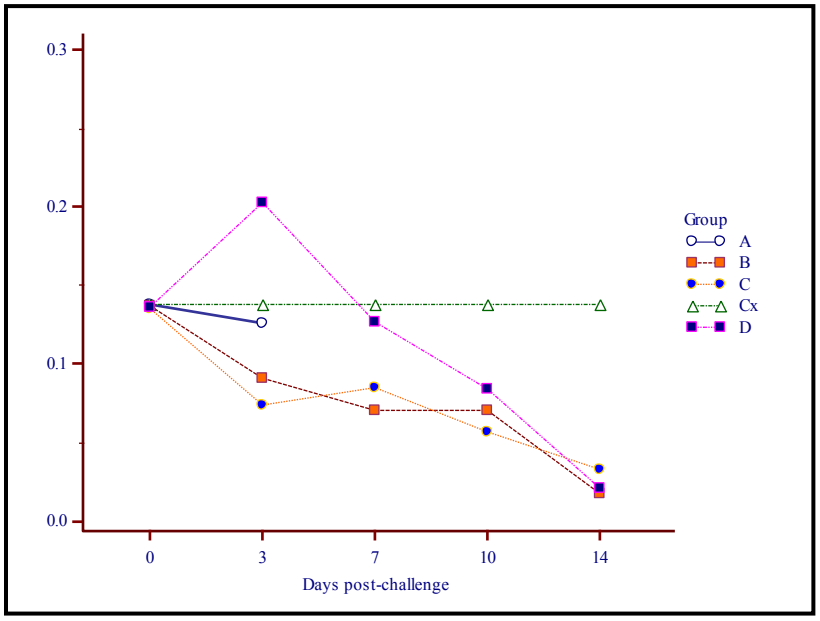

Figure 8: Standard means values of SOD plotted against days post challenge with standard error ± 0.5 of value of SOD

Note :- C_x* = Group E (Negative control)

Discussion

Streptococcus agalactiae is one of the pathogen associated with warm-water *Streptococcus* infections which often lead to massive mortality and loss in the aquaculture industries all over the world. Mortality of up to 30% during a single natural outbreak has been reported in Nile tilapia farms in Thailand and China [14,15] and in Red tilapia farms in Malaysia [16] following Streptococcus infection.

Analysis of the results of this study showed that *S. agalactiae* was very pathogenic to red hybrid tilapia of average size 80 ± 20 g, especially when heat stress was introduced simultaneously with bacterial challenged and these findings agreed with the findings of [17] reported 100% mortality from a group of fish that were experimentally subjected to high water temperature of 33 ± 0.5°C. In this study, bacterial challenged was performed through dip immersion. The temperature of 33ºC was chosen according to [9] who reported that water temperature above 31ºC

may induce heat stress to tilapia and can increased susceptibility to *S. agalactiae* infection. In this study, 100% mortality was recorded when the fish in group A were infected with inoculums at concentration of 2.3×10^9 CFU/mL and temperature at 33 ± 0.5°C in the first seven days of the experiment. The accumulated mortalities of tilapia was highest in group A which showed 100% mortality followed by Group C which had 36.4% mortality where heat stress was introduced on day 0 before challenged with *S. agalactiae* on day 7. Group B and D had same percentage of mortalities which were 18.2% each. Group E with served as negative control without heat stress and inoculation showed zero mortality as normal temperature at 27 ± 0.5°C was used. In Malaysia, the outbreak of streptococcosis has been reported in dry season during which the temperature of the water is known to increase between April to August and these outbreaks are associated with huge economic losses to the farmers [9]. Thus, this result support the previous studies [18] who showed that higher water temperature above 33°C may induce heat stress and reduce the immune response against infection.

High or low water temperature other than optimum temperature range of between 20 - 35°C [20] or around 25 - 30°C [19] was considered as one of the major stress to fish. Heat stress has been shown to be the most important factors that affects the growth, physiology, reproduction and metabolism of tilapia and any increase in heat stress will reduce the rate of dissolved oxygen (DO), thus increasing the rate of respiration and oxygen consumption in tilapia [20]. Increment of water temperature can induce heat stress in the fish and increase the susceptibility of red tilapia to infection by *S. agalactiae*. Fish are generally able to adapt to stress for a limited period of time and when their hormonal imbalance occurs, during which energy reserves are eventually depleted, resulting in suppressed immune system and increasing their susceptibility to diseases [1].

On the other hand, susceptibility to infection was higher when the temperature was more than 30°C as group that was exposed to heat stress 33 ± 0.5°C showed high mortality. Our findings were in conformity with an earlier study [19] where streptococcosis associated to *S. agalactiae* in tilapia farms were found to emerge only when the water temperature was above 26°C. According to previous studies [21], specific and non-specific immune response of teleost are significantly reduced either when the water temperature was over the normal physiological range of the fish.

According to Water Framework Directive (WFD), fish can represent the key elements to evaluate rivers ecological status [22]. When exposed to the stress or unsuitable environment, aerobic organisms will generate reactive oxygen species (ROS) such as hydroxyl radical (•OH), superoxide anion radical (O_2-) and hydrogen peroxide (H_2O_2) as a result of oxidative metabolism. The •OH can initiate lipid peroxidation (LPO) in tissues [23]. Under normal physiological condition, fish have an antioxidant defence (AD) system, which utilizes enzymatic and non-enzymatic mechanisms [24], to mitigate the effects of the reactive Oxygen species, thereby keeping it under control. Oxidative stress may occur when the activity of these antioxidant defence systems decreases or ROS production is increased. Malondialdehyde (MDA) is a metabolite derived from lipid peroxidation which had been widely used as indicator of oxidative stress [13]. Lipid peroxidation is frequently used as an indicator of cell membrane damage by Reactive Oxygen Species (ROS) [25]. For Group A, the MDA activities was increased due to bacteria challenged and heat stress concurrently which lead to imbalance between the production of reactive oxygen metabolite (ROM) and the production of antioxidants that usually break down the ROM into hydrogen peroxide and oxygen. The increment in MDA levels of groups B and C as observed in this study could be due to similar phenomenon as explained above. It was observed that exposing infected fish to heat stress further exacerbated MDA levels of the infected fish, thereby enhancing the pathogenesis of the disease. A previous study [13] has showed that MDA levels only increased at the third week in fish owing to the occurrence of partial or total food deprivation. However, this level was observed to return to normal when the fish was able to re-adapt to the environmental change. The increase in MDA level observed in this study was in relative agreement with the findings in an earlier study [26] who reported that

following 72 hours post exposure to heat, all the organs studied showed a significant increase in lipid peroxidation compared with the control groups. However, in the said study, lipid peroxidation was observed to diminish significantly after 72 hours, indicating that the tilapia defence system was initially overwhelmed. During handling the fish, many precautions step were taken to avoid stress to the fish.

Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide (O_2) into two products which are oxygen and hydrogen peroxide. Superoxide dismutase is known to be essential for biological defences against the superoxide anion [27,28]. Oxidative damage may modify proteins, DNA, lipid and leading to mitochondrial bioenergetics and failure and consequently to cell necrosis or apoptosis. Oxidative stress takes place when pro-oxidant force is higher than antioxidant defences and reactive oxygen species are not adequately removed. Living organisms are protected from reactive oxygen species by several defence mechanism including antioxidant enzymes such as SOD [13]. In biological systems, the induction of antioxidant enzymes is of the first line of defence against oxidative stress [29]. Increase in heat stress could lead to the production of superoxide anions and induction of SOD to convert the superoxide radical to H_2O_2 [30]. In overall, Group A, Group B and Group C showed decline in SOD activity starting from day 0 through day 14 while Group D showed rapid increase on day 3 pc. This could be due to metabolic and physiological changes in fish's body and started decreasing towards day 3 pc. This result agreed with previous study [31], who observed that following acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish, SOD enzymes in the studied organs decreased at 24 hours and 72 hours post inoculation while lipid peroxidation level increased significantly in all the studied organs. The alterations in antioxidant enzymes activity detected were also observed in previous study which tilapia were orally exposed to cyanobacterial cells during 14 and 21 days [24]. The antioxidant defences system in fish play crucial roles in overcoming oxidative stress-induced damages using the antioxidants such as superoxide dismutase (SOD) which acts on the superoxide (O_2).

In this study, the bacteria were easily isolated in large numbers from brains and eyes of infected tilapia. The presence of *S. agalactiae* in these organs is responsible for inflammation [32] leading to the erratic swimming, exopthalmia and cloudy eyes after 24 hours pc. These clinical signs are in accord with the signs observed in an earlier study [17]. Although no statistical analysis was done for bacterial isolation data but from the observations and data recorded, most of the isolate obtained were from the brain of the fish and most of the isolate were from Group A in which 100% mortality was recorded. This agreed with [33] who also reported that the presence of the bacteria in the brain of tilapia are suggestive of higher colonization by *S. agalactiae* compared to other organs when heat stress and bacterial challenged were introduced and this explained the high susceptibility of *S. agalactiae* that leads to higher mortality in dry season. This also agreed with earlier study [17] where it was reported that the percentage of *S. agalactiae* in brain and eyes were much higher compared to the kidney.

The influence of heat stress on the pathogenesis of streptococcosis in tilapia is crucial as it is the major factor that is involved in most outbreaks.

Conclusion

In conclusion, *S. agalactiae* being recognized to be pathogenic to fish especially tilapia causing streptococcosis with clinical signs such as erratic swimming, exophthalmia, inflammation and cloudy eyes. Heat stress was demonstrated to be associated high mortality rate and increase the susceptibility of the fish to *S. agalactiae* to the tilapia fish as water temperature already proved as major stress factor in red tilapia cultured in tropical climates such as Malaysia. The high mortality recorded in this study is shown to be associated with alterations in the oxidant and the antioxidant levels of the fish, specifically increase in lipid peroxidation and decrease in SOD activities. Therefore, since prevention of streptococcosis is a major concern by the farmers for sustainability in the aquaculture industry, there is the need to always minimized chances of heat stress in aquacultures in order to avoid the colossal

loss that could be incurred when and if there is outbreak. Up to this date, there has not been any scientific evidence showing whether the change of virulence of fish *S. agalactiae* corresponds with heat stress. The study of the relationship of heat stress and bacterial infection should be further investigated.

Acknowledgments

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