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ORIGINAL RESEARCH

Prominent vulnerability of red hybrid tilapia (*Oreochromis* spp.) liver to heat stress-induced oxidative damage

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Abstract Many aquatic species are vulnerable to warming water temperatures. Nonetheless, data on the physiological impact of global warming on cultured freshwater fish species are limited compared to marine species. This study investigated the effect of heat stress on oxidative stress response in red hybrid tilapia, *Oreochromis* spp. (total length 37.0 ± 3.2 cm, body weight 500.0 ± 15.0 g). Fish were assigned randomly and exposed to gradual increment of water temperature (1°C. 8 h^{-1}) from 28 to 31°C in aerated and thermoregulated fibreglass tanks for 2 weeks. Oxidative stress response was determined based on malondialdehyde levels and activities of antioxidant enzymes: superoxide dismutase, catalase and gluthathione s-transferase in the muscle, liver and kidney on day 1, 7 and 14. The heat-stressed group showed significantly increased malondialdehyde levels in the muscle, liver and kidney in parallel to the exposure period. The highest malondialdehyde level was observed in the liver tissue. Activity of superoxide dismutase was significantly decreased in the muscle over the course of the exposure period, in contrast to liver and kidney. Catalase activity was significantly higher in the muscle and liver, while gluthathione s-transferase activity was significantly increased in the muscle and liver but decreased in the kidney. The level of malondialdehyde strongly correlated with superoxide dismutase, catalase and gluthathione s-transferase activities in the liver compared with the muscle and kidney. Microscopic examination of liver showed congestion and hepatocytes with karyorrhexis indicating progress of necrosis. Our results suggest that liver is more susceptible to heat stress-induced oxidative damage compared with muscle and kidney. Red hybrid tilapia showed narrow upper thermal tolerance, implicating high vulnerability to the rise in water temperature.

Keywords Global warming . Tilapia farming . Temperature tolerance . Oxidative stress . Lipid peroxidation

Introduction

Global warmig has been one of the major environmental issues facing the world for decades and will remain as one of the biggest threats to human existence for many years to come. Water temperature acts as a major driver for metabolic responses that affect the performance of aquatic animals (Mjoun et al. 2010). The adverse impacts of global warming are inevitable particularly on the marine ecosystem (Jones et al. 2013; Wernberg et al. 2016; Henson et al. 2017). In the freshwater ecosystems, the thermal responses of temperate species are unimodal, in other words, no great variations among different taxonomic groups

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and species within a group (Kärcher et al. 2019). Comparatively, the tropical species have received less attention and sparsely explored for the negative impact of global warming.

Quantitative thermal effect evaluation model based on physiological response is yet to be established for aquatic animals, thus making it difficult to predict their resilience to global warming impacts. Teleosts of temperate regions possess a wider physiological tolerance to changes of water temperature through modulation of metabolism towards homeostasis (Angilletta 2009; Gardiner et al. 2010; Payne et al. 2016). On the contrary, although the physiological impacts of thermal changes on several tropical freshwater fish species have been studied over the past decade (Patra et al. 2007; McDonnell and Lauren 2016), the physiological tolerances of some commercially cultured tropical fish species are arguable. In addition, species sensitivity to global warming is closely related to species's thermal range, thermal distribution and preferred temperature (Markovic et al. 2017). This may imply that the species with a small thermal range and low critical temperature are prone to rising temperatures.

Tilapias are native to Africa and the Middle East. Their adaptability to a wide variety of conditions have resulted in them being introduced in many parts of the world such as Southeast Asia, including Malaysia (Jayaprasad et al. 2011), where they may encounter a wide range of temperatures during warm (28 to 34°C) and rainy or cold (22 to 26°C) seasons. We have previously found that red hybrid tilapia exposed to water temperature of 31°C displayed higher cortisol levels compared to non-exposed group (Nadirah et al. 2017), implicating thermal stressed condition.

In the context of world food security amid global warming, it is important to determine how the world's major food fish will be affected by climate crisis. This study was therefore, designed to investigate the effect of heat-related oxidative stress response onred hybrid tilapia *Oreochromis* spp, one of the most cultured food fish in the world, based on lipid peroxidation (malondialdehyde, MDA) and activities of antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST), as well as histopathological changes. The baseline findings will help address tilapia farming problem resulting from rising water temperature due to global warming, through improvement of fish husbandry practices.

Materials and methods

Fish and experimental protocol

This study was performed in accordance with the international guidelines for protection of animals used for experimental purposes (Directive 2010/63/EU). Healthy male red hybrid tilapia (total length 37.0 ± 3.2 cm, body weight 500.0 ± 15.0 g) purchased from a local farm were acclimated in 1000-L recirculating fibreglass tanks thermoregulated at 28 °C, prior to commencement of trials. During the acclimation period and trials, the fish were fed at 3 % of their body weight with a commercial tilapia diet (Star Feedmills Sdn Bhd).

Experimental Design

The thermal tolerance limits (24 h) of red hybrid tilapia in terms of lethal temperature (LT_{50}) and loss of equilibrium temperature, also known as critical thermal maximum (CTmax) were previously determined to be 33.6 °C and 31.6 °C (Hazza et al. 2014) based on Souchon and Tissot (2012) and Beitinger et al. (2000) , Ospina and Mora (2004), respectively. For the oxidative stress response trials, fish (n = 15) were exposed to heat stress up to 31 °C by gradually increasing the water temperature from 28 °C at a constant increment rate of 1°C 8 h^{-1} (Beitinger et al. 2000) in 750 L water in 1000-L aerated fibreglass tank, and maintained for 2 weeks (Meeuwig et al. 2004). Control group (n = 15) was kept in similar condition as per the treatment group except that the water temperature was maintained at 28 °C. The pH and oxygen saturation levels were maintained within 6.9 to 7.2 and 64.9 to 70.9% for both groups, respectively, with no significant difference ($P > 0.05$) among different temperature treatments.

Five fish were randomly sampled from each group on day 1, 7 and 14 to obtain abdominal muscle, liver and kidney and stored in ice-cold condition . Tissue samples (200 mg) were assayed for protein content estimation, MDA and enzymes activities (SOD, CAT and GST) following Bradford (1976), Ohkawa (1979), McCord and Fridovich (1969), Habig et al. (1974), and Beers and Sizer (1952), respectively. All assay kits (Sigma-Aldrich, USA) were used as per manufacturer's instructions.

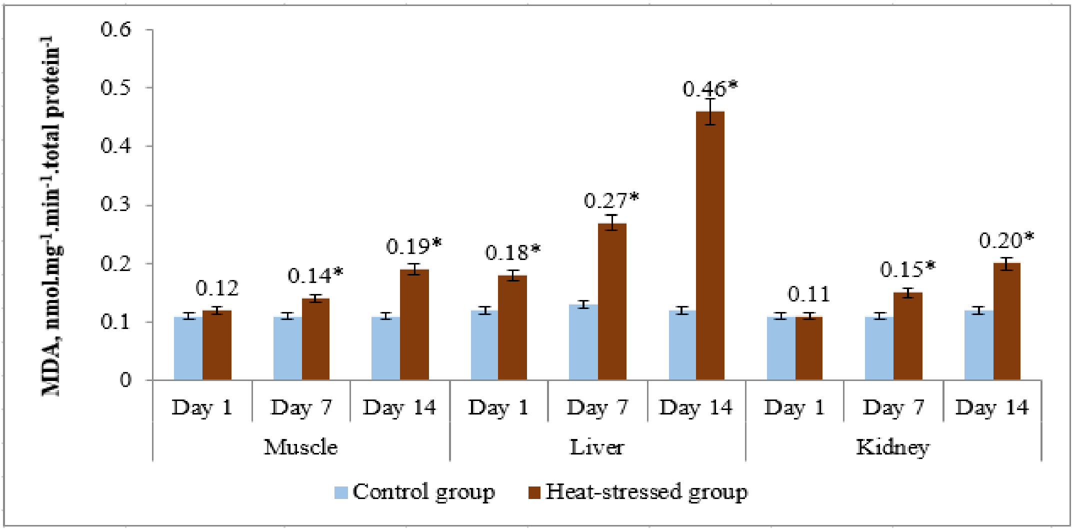

Fig. 1 Levels of MDA in muscle, liver and kidney of *Oreochromis* spp. exposed to heat stress; * denotes P < 0.05 compared with control

For histopathological analysis, abdominal muscle, liver and kidney tissues were cut into small pieces, rinsed in physiological saline and fixed in 10% formalin for 24 h. The fixed tissues were dehydrated in ethyl alcohol series of ascending concentrations, embedded in paraffin and sectioned at 5 mm thick. The tissue sections were stained with hematoxylin and eosin (H&E) following Al Darwesh et al. (2014).

Statistical analyses

T-test was performed to compare the means between control and heat-stressed groups across day 1, 7 and 14 for each parameter. Data were reported as mean ± standard deviation (SD). A one-way between subjects ANOVA (single factor) was conducted to compare the effect of thermal stress on oxidative stress response on day 1, 7 and 14. The data were examined for normality and variance homogeneity prior to ANOVA test. Post hoc test was conducted based on Bonferroni correction. Correlation between heat-stress treatment in association with lipid peroxidation and antioxidant enzyme activities in different tissues were analysed through Pearson correlation test. Statistical analyses were performed using IBM SPSS Statistics version 20.0 and the level of significance for all tests were set at P < 0.05.

Results

On day 14, the cumulative lipid peroxidation in heat-stressed group was highest in the liver as indicated by MDA level (0.46 $nmol.mg^{-1}.min^{-1}.total\ protein^{-1}$), followed by kidney (0.20) and muscle (0.19) (Fig. 1). T-test showed significantly higher (P < 0.05) MDA level in the liver of heat-stressed group compared with control right from day 1, and was consistently higher until day 14. In comparison, significantly higher (P < 0.05) MDA levels were observed in the muscle and kidney on day 7 and 14.

The SOD activities varied among normal tissues with the highest value observed in muscle, followed by liver and kidney (Fig. 2). The SOD activities in control group were between 57.0 - 59.0, 31.5 - 32.0 and 13.4 - 14.3 $U.mg^{-1}.min^{-1}.total\ protein^{-1}$ in muscle, liver and kidney, respectively. T-test showed significantly higher (P < 0.05) SOD activity in muscle of heat-stressed group on day 1 but significantly reduced (P < 0.05) on day 14 compared with control. The SOD activities in liver and kidney were significantly higher (P < 0.05) than control on day 7 and 14.

Similarly, CAT activities also varied among normal tissues (control group) with the highest level found in muscle (73 - 74.3), followed by liver (33.3 - 33.7) and kidney (12.6 - 13.5). T-test showed significantly higher (P < 0.05) activity in the muscle of heat-stressed group, but significantly lower (P < 0.05) in kidney at each duration point. In contrast, day 1 and day 7 recorded significantly lower CAT activities in liver but significantly higher on day 14 (Fig. 3).

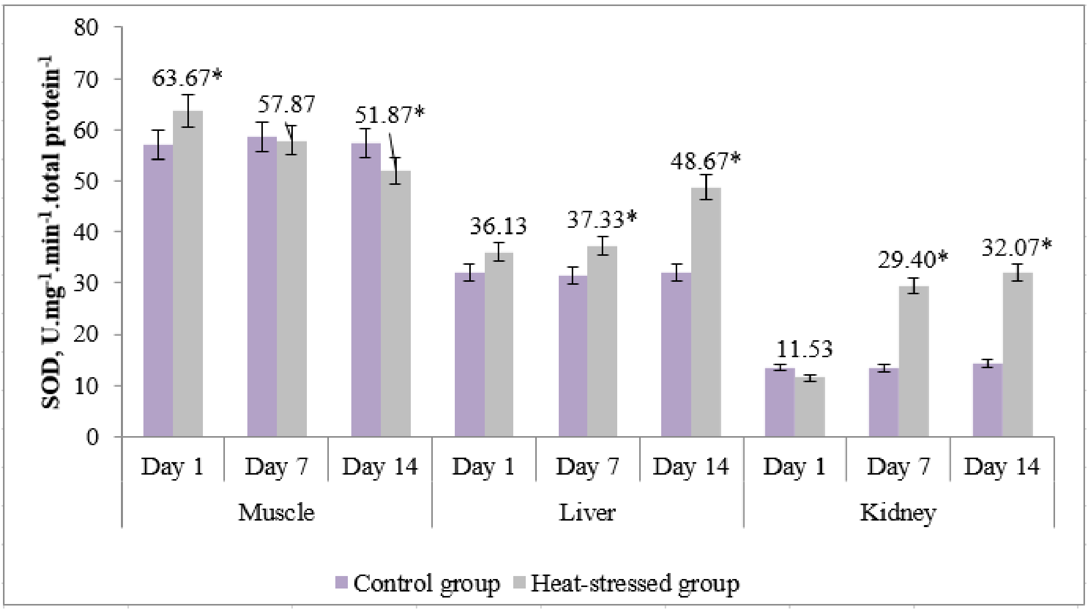

Fig. 2 Activity levels of SOD in muscle, liver and kidney of *Oreochromis* spp. exposed to heat stress; * denotes P < 0.05 compared with control

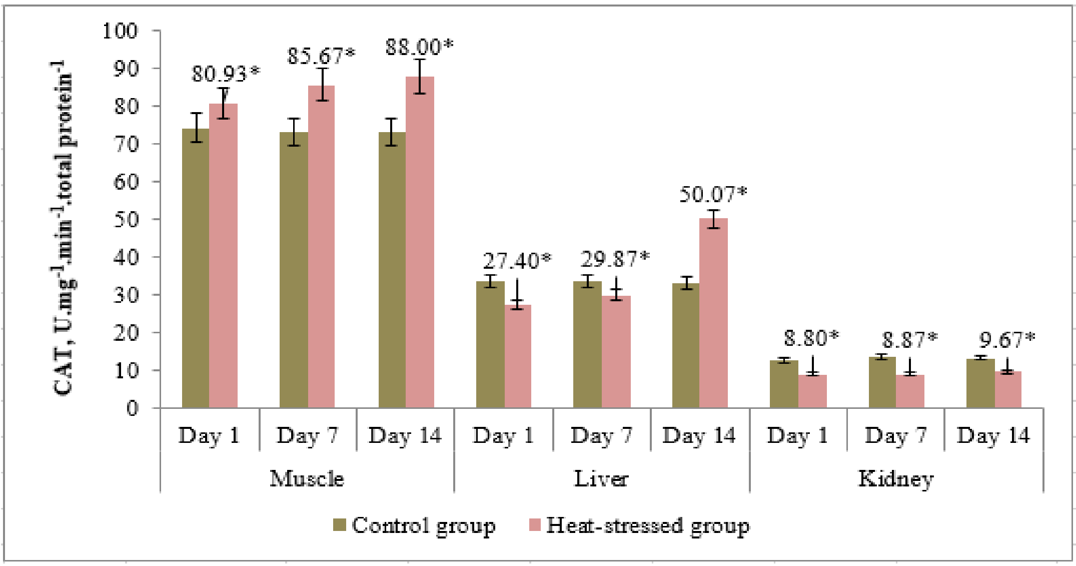

Fig. 3 Levels of CAT activity in muscle, liver and kidney of *Oreochromis* spp. exposed to heat stress; * denotes P < 0.05 compared with control

The levels of GST activity in muscle (24.4 - 25.7), liver (24.5 - 26.57) and kidney (28.7 - 30.4) of control group were pretty much the same (Fig. 4). Day 7 and 14 recorded significantly higher ($P < 0.05$) activities in the muscle and liver of heat-stressed group, while GST activities were significantly higher ($P < 0.05$) in kidney on day 1 and 7.

There was a significant positive-correlation ($P < 0.05$) between MDA level and thermal exposure duration for muscle ($F(2,42) = 178.71$, $P = 0.000$), liver ($F(2,42) = 1009.2$, $P = 0.000$) and kidney ($F(2,42) = 156.02$, $P = 0.000$) due to the accumulation of MDA with the progress of time (duration of thermal exposure). Post hoc comparisons indicated that the mean MDA in each tissue on day 1 was significantly different from day 7 and day 14. The SOD level in muscle was however significantly reduced as time went by ($F(2,42) = 22.362$, $P = 0.000$), contrary to those in liver and kidney, which increased with the progress of thermal exposure. The CAT levels in muscle and liver were also positively

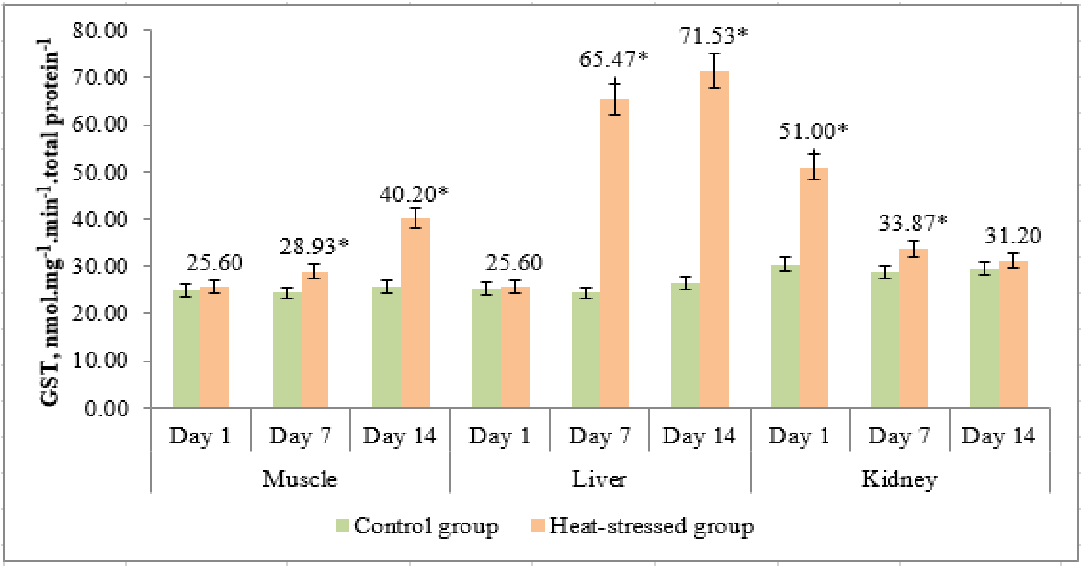

Fig. 4 Levels of GST activity in muscle, liver and kidney of *Oreochromis* spp. exposed to heat stress; * denotes $P < 0.05$ compared with control

correlated with time ($F(2,42) = 12.959$, $P = 0.000$ and $F(2,42) = 111.697$, $P = 0.000$, respectively). The CAT level in kidney was however, not affected by exposure duration. Activity of GST was increased as thermal duration increased as found in muscle ($F(2,42) = 46.042$, $P = 0.000$) and kidney ($F(2,42) = 273.355$, $P = 0.000$) but decreased in kidney as thermal duration increased ($F(2,42) = 155.37$, $P = 0.000$). A strong correlation ($P < 0.05$) exist between SOD and GST with r coefficient of - 0.621 and 0.762, respectively, in relation to MDA levels in muscle. The level of MDA in liver also strongly correlated ($P < 0.05$) with SOD, CAT and GST levels with r coefficient of 0.889, 0.886 and 0.792, respectively. On the other hand, in the kidney, SOD and GST levels were highly correlated with MDA level with a correlation coefficient (r) of 0.811 and - 0.817, respectively.

However, histopathologically, the abdominal muscle and kidney samples did not show any morphological alteration compared to liver. Thus, only the pathological changes of liver were shown. In general, the liver parenchyma of the control group was primarily composed of polyhedral shaped hepatocytes with distinct cytoplasmic lipid droplets (Fig. 5). In the heat stress treatment group, alterations were observed in the liver on day 14, with generally blurred liver parenchyma, sinusoidal congestion and dilatation, venous congestion (Fig. 6), and hepatocytes undergoing karyorrhexis (Fig. 7).

Discussion

Global warming and climate change are inevitable and foreseen to impact the global food security, making food production and supply more challenging than before. Therefore, the knowledge on thermal tolerance of aquatic animals is particularly important in order to understand the impacts of global warming and climate change on the population dynamics of aquatic animals (Kimball et al. 2004; Kimura 2004), and to extrapolate the species of aquatic animals that are vulnerable to environmental changes (Jiguet et al. 2007; Schulte 2013). The increase in atmospheric carbon dioxide (CO_2) contributes to the rise in global temperature, thus affecting the water temperature of the aquatic ecosystems. Increased uptake of atmospheric CO_2 by oceans also leads to ocean acidification (Huertas et al. 2017; Merlivat et al. 2017). Exposures of aquatic animals to both rising water temperature and ongoing decrease in pH accelerate certain biochemical and metabolism pathways, and augment the production of reactive oxygen species (ROS), also known as free radicals. Excess production of ROS results causes oxidation of essential cellular biomolecules (including lipids, proteins and nucleic acids). When ROS degrades polyunsaturated lipids through lipid peroxidation (Parihar et al. 1996), MDA is released, which therefore serves as a prominent biomarker for oxidative stress level in organisms (Davey et al. 2005; Del Rio et al. 2005). The current analysis observed a significant increase

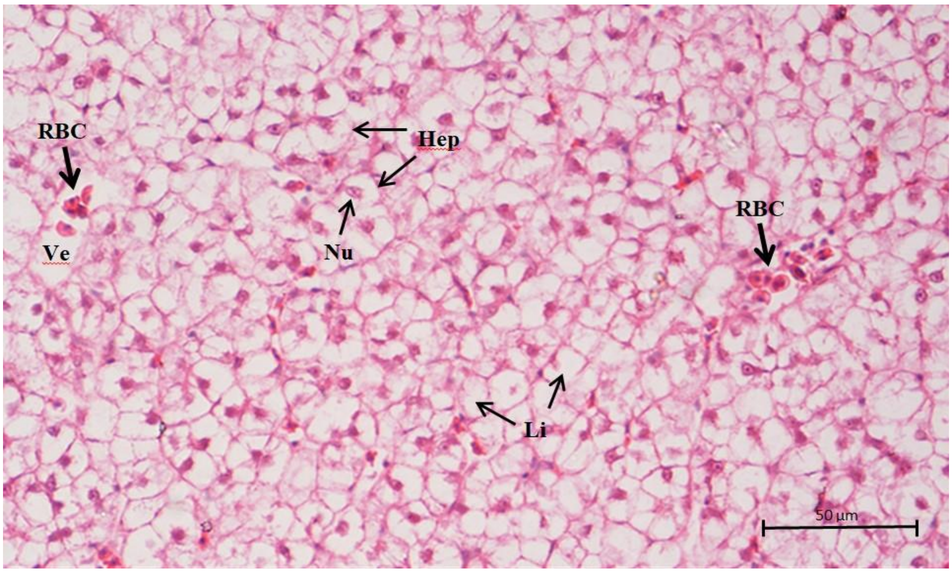

Fig. 5 Photomicrograph of liver parenchyma of control fish showing distinct cytoplasmic lipid droplets (Li) in polyhedral shaped hepatocytes (Hep), red blood cells (RBC) in hepatic venule (Ve); Nu; nucleus. H&E, 40 ×

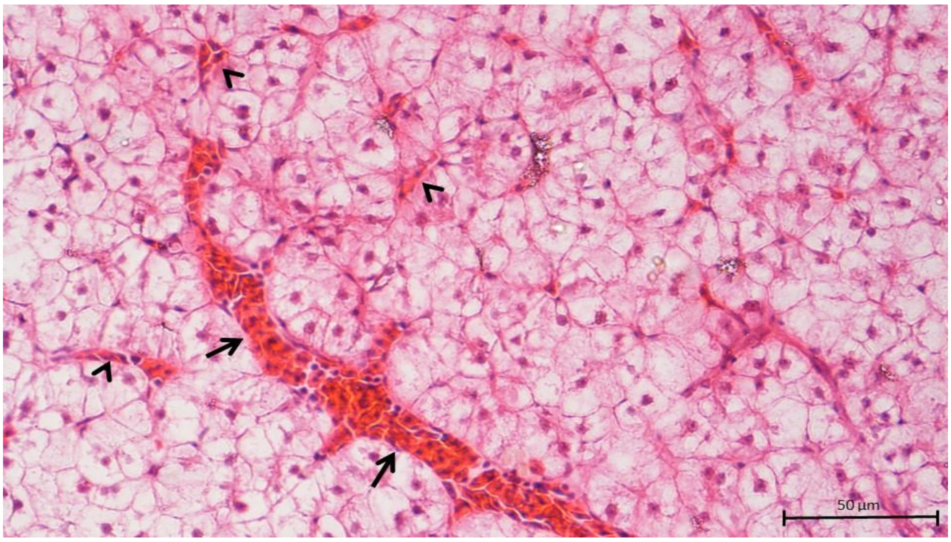

Fig. 6 Day-14 liver parenchyma of fish exposed to continuous heat stress showing sinusoidal congestion and dilatation (arrowhead) as well as venous congestion (arrow) due to accumulation of RBC. Liver parenchyma is largely indistinct. H&E, 40×

of MDA level in liver, kidney and muscle tissues of tilapia. The most significant increase of MDA in liver inferred higher sensitivity and vulnerability of this organ to heat stress. As a central immunological organ linked to gut-associated lymphoid tissue, the importance of liver is attributed to its distinct local immune environment that contributes to immune surveillance (Bode et al. 2012; Trivedi and Adams 2016; Wu et al. 2016). Innate immune cells predominate in liver are continuously exposed to antigens and endotoxins in circulating blood, in which activation of innate immune responses is signified by the increase in innate immune transcripts in liver (Wu et al. 2016).

High water temperatures have been associated with occurrence of certain aquatic animal disease such as warm water streptococcosis. For instances, high water temperatures (31 - 32 °C) in Kenyir Lake, Malaysia have been linked to increased susceptibility of cage cultured red hybrid tilapia to streptococcal infections that caused massive mortality (Siti-Zahrah et al. 2008; Najiah et al. 2012). Thus, the current findings

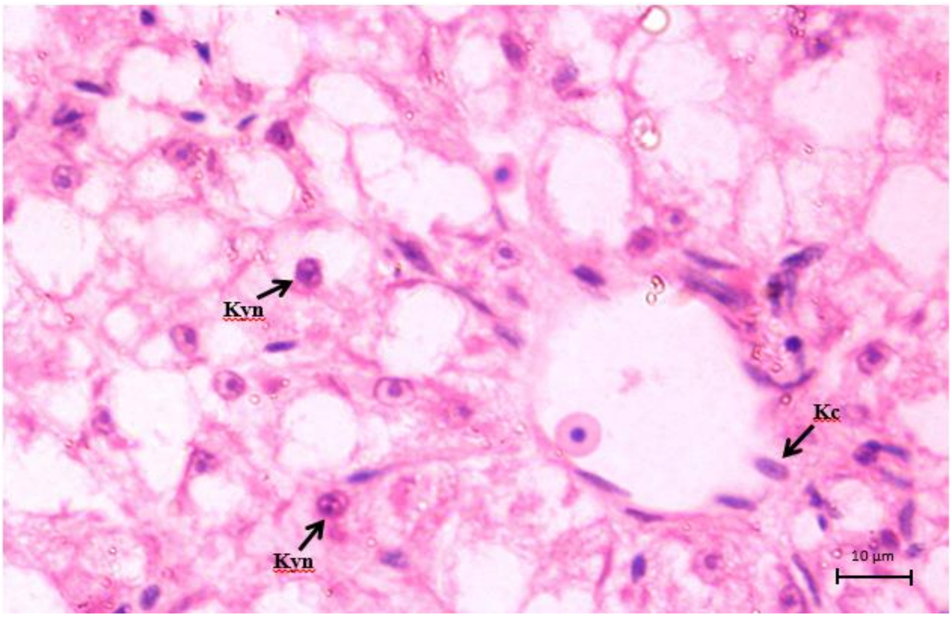

Fig. 7 Day-14 liver parenchyma of continuously heat-stressed fish. Nucleus undergoing karyorrhexis (Kyn); Kupffer cell (Kc). H&E, 100 ×

postulate that increased water temperature leads to oxidative damage of liver through lipid peroxidation, which may in turn overwhelm the innate immune cells such as resident macrophages (Kupffer cells) with removal of necrotic or apoptotic cells. Moreover, MDA, a reactive electrophile species released during lipid peroxidation also causes toxic stress in cells, and impairs innate immune cell functions, which consequently increases the host susceptibility to infections. High water temperatures have also been reported to affect osmoregulatory system of many fish species (Staurnes et al. 2001; Metz et al. 2003; Vargas-Chacoff et al. 2009), and induce sterility in fish (Ito et al. 2003). Elevated water temperatures lower availability of dissolved oxygen, disrupt metabolism and accelerate oxygen demands of fishes. Heat stress can significantly increase the generation of ROS as indicated by increased level of thiobarbituric acid-reactive substances as reported in eelpout (Heise et al. 2006), salmon (Nakano et al. 2014) and koi carps (Dasgupta 2020). Antioxidant enzymes are the main defense components against oxidative stress. Antioxidant enzymes can be activated to counteract the adverse effects of ROS to cope with oxidative stress (Cui et al. 2014). Among the antioxidant enzymes in fish that act in physiological defense mechanisms against oxidative stress are SOD, CAT and GST (Lushchak and Bagnyukova 2006; Madeira et al. 2016). Insufficient production of antioxidant enzymes to counter the production of pro-oxidants will result in disturbance and oxidative damage of tissues or organs (Poljšak and Fink 2014). Under severe oxidative stress, the damages of enzymes further result in loss in the compensatory mechanisms (Zhang et al. 2004).

Our current analysis also revealed that although SOD seems to be a critical antioxidant enzyme in oxidative stress response (Ibtisham et al. 2018), its activity in liver tissues appeared to change simultaneously with CAT and GST activities. However, the opposite was observed in muscle tissues where SOD activity decreased while CAT and GST activities increased. Nevertheless, it has been reported that alterations of SOD and GST activities are tissue-specific (Lushchak and Bagnyukova 2006). Interestingly, the control group in the present study exhibited constantly higher SOD and CAT activities in muscle tissue throughout the course of experiment, suggesting SOD and CAT as the main antioxidant enzymes in muscle tissues under normal physiological conditions compared with liver and kidney. The constant GST activity recorded in all tissue samples of control group, inferring that GST activity is tissue-independent under normal physiological conditions.

Being the vital organ for accumulation, storage and biotransformation of many metabolic compounds, the alterations in liver structure can be significant towards evaluation of fish health (Myers et al. 1998). Liver also plays a central role in degradation of metabolic products in vertebrates, thus susceptible to many free radicals. Nonetheless, the degree of heat stress damage is found to be dependent on water temperature and exposure time. In the present study, day-14 liver sample showed apparent high-temperature induced damages. The results of this study are consistent with the previous findings reported in Wuchang bream *Megalobrama amblycephala* with structural alterations in hepatocytes and nuclei (Liu et al. 2016). In amphibian, Liu et al. (2018) observed that upon prolonged exposure to heat stress, the liver sinusoids in giant spiny frog appeared dilated while the percentage of melanomacrophage centres (MMCs) also changed markedly. Thus, our histopathological examination further supports that liver is more vulnerable to heat stress-induced oxidative damage compared to abdominal muscle and kidney.

Conclusion

The present study demonstrated varied heat stress induced oxidation responses in the muscle, liver and kidney of red hybrid tilapia, with the most notable lipid peroxidation observed in the liver, which, together with the pathological changes of hepatic structure, could consequently impair its physiological function and performance. These also indicated higher vulnerability of liver to prolonged heat stress. This as such postulates that red hybrid tilapia has a narrow upper thermal limit. The baseline findings from this study will help mitigate tilapia farming problems resulting from global warming, in particular, the cage culture system, through advancement of fish husbandry approach.

Competing Interests The authors declare that they have no conflict of interest.

Authors' contributions Nadirah Musa participated in the design of the study, performed the statistical analysis and drafted the manuscript. Hazza Roshada Ramli and Mohammad Tajuddin Abdul Manaf carried out the experimental studies, and participated in the analysis. Chen-Fei Low , Najiah Musa and Alia Syafiqah Aznan participated in the analysis. Chen- Fei Low, Kok-Leong Lee and Shau-Hwai Aileen Tan helped to revise the manuscript. All authors read and approved the final manuscript.

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Data Availability Statement The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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