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Stress, immune and growth responses of bluegill sunfish (*Lepomis macrochirus*) to different environmental temperatures as referred by related gene expression

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Global temperature climate changes affect stress, immune function and growth of aquatic animals. The aim of this study was to investigate the effect of different temperatures on oxidative stress, thermal stress, immune response and growth in bluegill sunfish (*Lepomis macrochirus)*. The experiment included four groups that were exposed to different water temperatures: 10ºC (10.2 ± 0.05), 20ºC (20 ± 0.06), 30ºC (29.7 ± 0.09), and 35ºC (34.7 ± 0.05) for six weeks. Fish were sampled at day 0, 3 weeks, and 6 weeks. Significantly higher (P < 0.05) erythrocyte lysate superoxide dismutase (SOD) wasobserved in the 10ºC, 30ºC and 35ºC groups at day 0 and 3 week, but more pronounced at the 6th week. Heat Shock protein90 (HSP-90) was significantly up-regulated at higher temperatures (20, 30, and 35ºC), while down-regulated at 10ºC. Similarly, immune response biomarker α-2-macroglobulin (A2M) was also up-regulated in 20 and 30ºC groups whereas down-regulated in 10ºC group. All groups exhibited 100% survival except the 35ºC group (86% survival). The highest growth performance was observed for the 30ºC group and the lowest for the 10ºC group. Conclusively, findings support that SOD, HSP-90 and A2M could be considered as biomarkers for climate change and environmental monitoring as referred in fish species.

Key words: Bluegill, growth, Immune response, molecular biomarkers, oxidative stress, temperature, thermal stress

INTRODUCTION

Global climate changes are continuous and can almost affect all living organisms and can be direct, through changing water temperatures or through current ongoing ocean acidification conditions (Diffenbaugh and Field, 2013; Pörtner HO and Peck MA, 2010).

Climatic changes in temperature also affect metabolism, immune function, osmoregulation, growth and reproduction, causing negative effects on physiological functions and adverse effects on growth and reproduction performance in aquatic animals (Ackerman *et al.*, 2000; An *et al.*, 2010).

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Fishes are particularly susceptible to several anthropogenic and natural stressors such as variations in the temperature, salinity, and other caused pollution (Lushchak, 2011; Matoo *et al*., 2013). Increased environmental temperature was found to induce oxidative stress in fish (Bagnyukova *et al*., 2007; Lushchak and Bagnyukova, 2006) through stimulating all metabolic processes, therefore, this may increase ROS production resulting in oxidative stress because fish are unable to detoxify the ROS active species (Ahmed, 2005; An et al., 2010; Halliwell, 1994; Madeira *et al*., 2013; Paital and Chainy, 2010). The measurement of markers of oxidative stress can be applied to any species with aerobic metabolism and even to anaerobic organisms exposed to oxygen (Costantini, 2014). In order to overcome injuries resulting from ROS formation, cells have antioxidant defenses which consist mainly of antioxidant enzymes (AOX), such as superoxide dismutase (SOD) and catalase (CAT), glutathione-dependent enzymes (GSH), and non-enzymatic defenses such as amino acids, tocopherol, and vitamins E, K, and C (Grim *et al*., 2010; Martínez-Álvarez *et al*., 2005). These antioxidants quench singlet oxygen and reduce the flux of reduced oxygen intermediates (e.g. O2- and H2O2) to prevent the production of HO•, which is the most damaging oxygen species (Halliwell, 1994).

It is well-known that noxious ROS are known to increase heat shock factors (heat shock factor 1 - HSF1 in animals) (Lesser, 2011) and increases the expression of heat shock protein70 (HSP70) (Heise *et al*., 2006; Kregel, 2002; Madeira *et al*., 2013; Scarpeci *et al*., 2008; Snoeckx *et al*., 2001). It is a matter of fact that HSPs act as sensors of cellular redox changes to activate ROS scavengers such as SOD, and catalase CAT (Currie *et al*., 1988; Kalmar and Greensmith, 2009; Madeira et al., 2013; Mocanu *et al*., 1993; Snoeckx *et al*., 2001). The HSP90 (90-kDa heat shock protein) plays an important role in folding newly synthesized and refolding denatured proteins under stressful environmental conditions (Fu *et al*., 2011). HSP90 is also involved in the immune response and participates in the cyto-protection of eukaryotic cells (Wei et al., 2013).Alpha 2-macroglobulins (A2M) are proteinase inhibitors function as a binding protein to numerous growth factors, cytokines and hormones and is an important non-specific humoral factor in the defense system of the animals (Chuang *et al.*, 2013; Gollas-Galván *et al.*, 2003; Ma *et al.*, 2005).They are required to control the proteinase activity and to avoid tissue damage as several proteinases are involved in the activation of the coagulation process (Muta *et al.*, 1995) and the prophenoloxidase activation system (Wang *et al*., 2001).

The interplay between oxidative stress, antioxidants, immune response, and HSPs is thus extremely important for the survival of critical temperature stress for fish (Heise *et al.*, 2006; Madeira *et al.*, 2013). Therefore, our present study was to examine the stress, immune and growth responses to different temperatures in bluegill sunfish (*Lepomis macrochirus*), which is an important sport as well as food freshwater fish species in the North American. This species is naturally available in the United States and has been presently introduced in most of the parts of world(Schultz, 2004). This warm water species is sensitive to temperature, and can even reverse sex when temperature changes (Wang *et al*., 2014).

Objective

The main objective of the present integrated study was to investigate the effects of temperature on SOD activity (biomarker for oxidative stress), HSP90 gene expression (molecular biomarker for thermal stress) and A2M gene expression (molecular biomarker for immune response) and growth in bluegill sunfish, which are all expected to vary accordingly to changes in temperature and exposure to different temperatures.

MATERIALS AND METHODS

Experimental Fish and System

Bluegill juveniles with an average body weight of 18.0 ± 0.40g were obtained from Jones Fish Hatcheries, Ohio, USA, were transferred to the Ohio State University South Centers, Ohio, USA and kept in round fiberglass tanks (2.1 m in diameter) for two weeks, where temperature was 15ºC -16 ºC and Dissolved Oxygen (DO) 5 mg/L. After an acclimatization period of two weeks, 360 fish were randomly distributed in a flow-through system (rectangle fiberglass tank, 240 × 60 × 30 cm) into four groups with three replicates; each replicate contained 30 fish. Fish were acclimated for one week under this condition. Thereafter, water temperatures were adjusted gradually at the rate of 2ºC per dayto reach the targeted water temperatures (10, 20, 30, and 35ºC). Those temperatures were chosen to represent a range of temperatures below (10, 20ºC) and above (35ºC) the optimum level (30ºC) for bluegill, which can reflect possible temperature climatic changes that bluegill may be exposed to. Fish were acclimatized for one additional week after reaching targeted temperatures and maintained after reaching those temperatures for 6 weeks, which is the experimental period.

Fish were fed to satiation with commercial basal diet *Aquamax® Fingerling starter 300,PMI Nutritional International, LLC., Brentwood, MO, USA; Lot # 5D03*(Crude Protein Minimum 50.0%, Crude Fat Minimum 16.0%, Crude Fiber Maximum 3.0%, Calcium Minimum 2.0%, Phosphorous (P) Minimum 1.3%, Sodium (NA) Minimum 0.1% and Ash Maximum 12.0%) two times daily. All groups received the basal diet for period of six weeks. Temperature and DO were measured in the morning and afternoon daily and recorded for each tank/treatment. All chambers were siphoned daily to remove excess feed and fecal matter to keep good water

Table 1 Primers sequences for the expression study of selected genes in Bluegill *Lepomismacrochirus*

Gene of interest	Primer sequence(5'-3')	Accession No.
18S rRNA	F: AGGAATTGACGGAAGGGCAC R:GGTGAGGTTTCCCGTGTTGA	[GenBank:JQ896298.1]
Heat-shock protein 90 (HSP90)	F:ATGATGAGCAGTACGCCTGG R: GTACTCCGTCTGGTCCTCCT	[GenBank:AB296302.1]
Alpha2-macroglobulin (A2M)	F:AGTGAGAATGTGATCGCGCA R:AACAGCACTCACACCACACA	[GenBank:AB300222.1]

quality. Water flow was adjusted across all tanks to maintain the targeted temperature for each tank and number of dead juveniles was recorded daily in all groups during the experimental period. Throughout the experiment, all the water parameters were in the optimum range (temperature 10ºC (10.2 ± 0.05), 20ºC (20 ± 0.06), 30ºC (29.7 ± 0.09) and 35ºC (34.7 ± 0.05).

Sampling

At day 0, and the end of 3 and 6 weeks, three fish were taken from each group at each time interval and were anaesthetized by tricainemethane sulfonate (MS222) at 250-350 mg/L in water. These three fish were taken from each group for blood and tissue sampling. Blood was drawn near caudal peduncle from three fish from each treatment and transferred into a heparinized tube. Blood was centrifuged at 1500×*g* for 5 min at (4°C), plasma and buffy coat layers were discarded, and sedimented RBCs were lysed in four times its volume of ice-cold HPLC grade water (Sigma, USA), centrifuged at 5000 g for 15 min. at 4ºC, then the supernatant (erythrocyte lysate) was collected and stored at -80ºC for determination of SOD activity. The same fish were carefully dissected to isolate the liver and stored in RNA later (Ambion, USA) and kept at –80°C for determination of gene expression. At each sampling time, all fish from each group were weighed for growth monitoring.

This study and all experimental procedures involving animals were performed according to the protocol approved by the Ohio State University Institutional Animal Care and Use Committee.

Superoxide Dismutase (SOD) Activity

Superoxide Dismutase activity was measured in eryrthrocyte lysate according to the Superoxide Dismutase Assay Kit manufacture's protocol (Cayman Chemical, USA). Absorbance was measured at 440-460 nm using an absorbance micro plate reader (Biotex EIx800, USA). SOD activity was measured according to the following formula:
SOD (U/ µL) = [((sample LR – y- intercept)/slope) × (0.23 ml) / (0.01 ml)] × Sample dilution.

Gene Expression

RNA Extraction and Real Time RT-PCR

Total RNA was isolated from liver samples using the Trizol method (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The extracted RNA samples were subjected to DNA-free (DNase) treatment to avoid genomic DNA contamination. The quantity of the RNA was evaluated by using Nano-Drop spectrophotometry (Thermo Scientific, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and purity by OD_{260}/OD_{280} nm absorption ratio 1.80: 2.00.

Reverse transcription was performed using a high capacity cDNA reverse transcription kit (Invitrogen, USA) following manufacturer's instructions for 20-µL total volume of cDNA. Briefly, the total volume of 10 RT µL master mix was prepared per reaction on ice by adding 2 µL (10✕ RT Buffer), 0.8 µL (25✕dNTP Mix (100 mM)), 2 µL (10✕ RT Random Primers), 1 µL (RNase Inhibitor), 3.2 µL (Nuclease free H2O) and 1 µL (Multi Scribe™ Reverse Transcriptase) into a microcentrifuge tube. Then, 10 µL of 2✕ RT master mix was added to 10 µL the of RNA sample in each tube and mixed by pipetting, centrifugation and the thermal cycler (Biorad, USA) was adjusted following manufacturer's instructions the resulting cDNA stored at -20°C.

Primer Design

Primers for A2M, HSP90 and 18S rRNA genes were designed using Primer-BLAST from NCBI, as no primers were available through the literature for those genesfor bluegill sunfish. All primers were designed so that amplification covered at least 1 intron, which ensures that cDNA products would be distinct from any potential genomic DNA contamination. Primer sequences and GenBank accession numbers are presented in Table 1. For each gene, two or more primer pairs were evaluated and primer pairs with the best performance were selected for use. Primers were manufactured by IDT (Coralville, IA, USA).

Real-Time PCR

PCR amplification was performed with Light Cycler® 96 System (Roche, USA) using 2 µL of cDNA and 18 µL of SYBR select Master Mix (Applied Biosystems, USA), which was prepared by adding 10 µL of 2x SYBR green Master Mix, 6 µL of ddH_2O, and 1 µL each of forward and reverse primer. The real-time analysis program consisted of 1 cycle of 95ºC for 15 min, and 45 cycles of 95ºC for 15s, 56ºC for 15s, and 72ºC for 10s. On each plate, for every sample, the target gene (gene of interest) and endo-

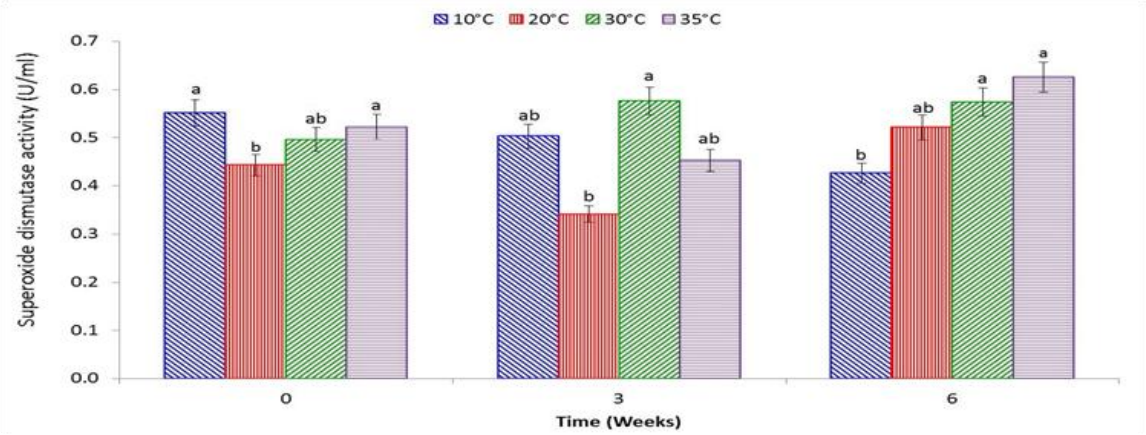

Fig. 1. The effects of different temperatures (10, 20, 30 and 35ºC) on Superoxide Dismutase activity (U/ mL) of bluegill at 3 and 6 weeks, One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Values are mean (n = 3) ± standard error. Mean values with different superscript are differ significantly (P< 0.05).

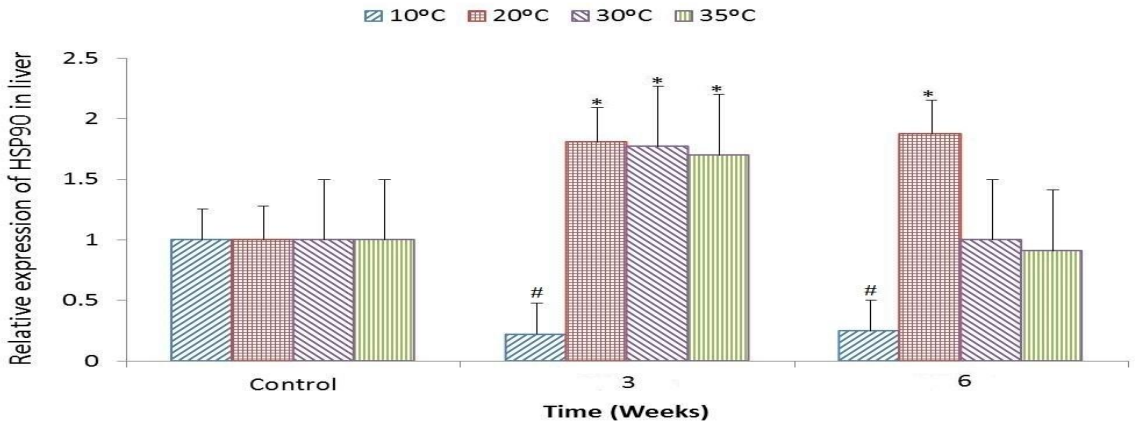

Figure 2: The effects of different temperatures (10, 20, 30 and 35ºC) on Heat Shock Protein 90 (HSP90) gene expression in bluegill at 3 and 6 weeks. Values are mean (n = 3) ± standard error. Mean values with asterisk (*) and hash (#) are significantly (P < 0.05) different.

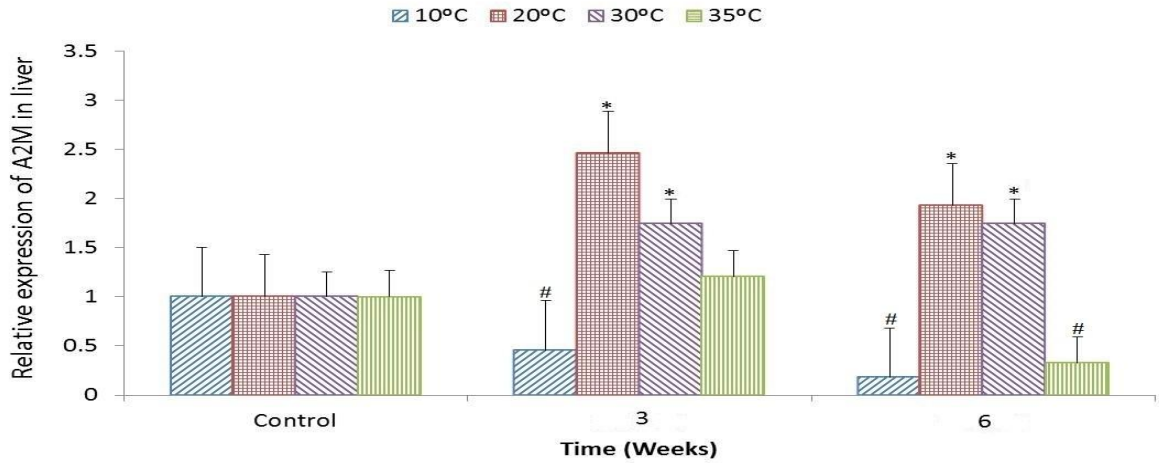

Fig. 3. The effects of different temperatures (10, 20, 30 and 35ºC) on α-2-macroglobulin (A2M) gene expression in bluegill at 3 and 6 weeks. Values are mean (n = 3) ± standard error. Mean values with asterisk (*) and hash (#) are significantly (P < 0.05) different.

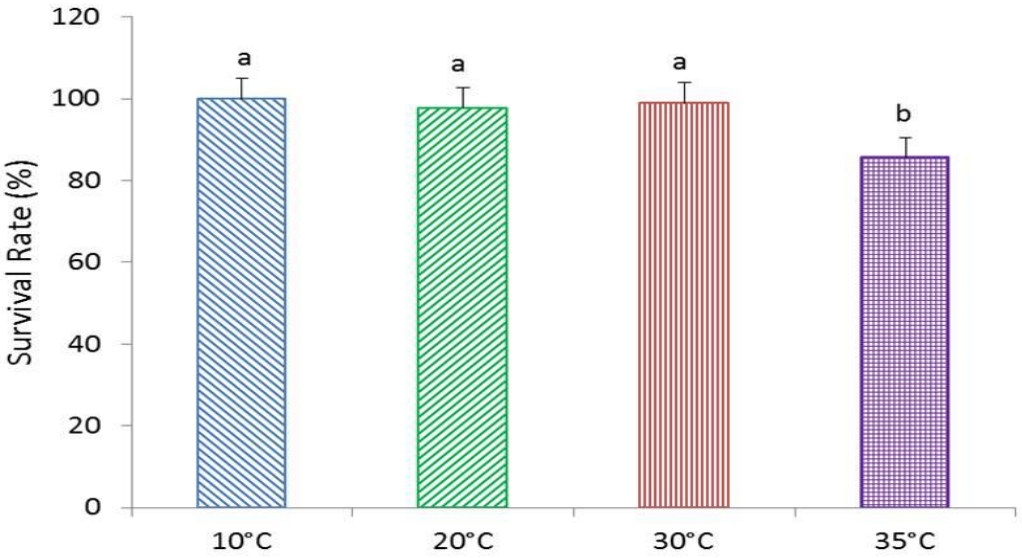

Figure 4: The effects of different temperatures (10, 20, 30 and 35ºC) on Survival rate (%) of bluegill after period of 6 weeks. Values are mean ± standard error. Mean values with different superscript are differ significantly (P< 0.05).

genous control (normalize gene: 18S rRNA) (Filby A and Tyler C 2007)were tested in triplicate. Then expression levels of the genes of interest were normalized to 18S rRNA, and the fluorescence threshold cycle (CT) determined and the relative expression of each gene was calculated.

Relative expression level of the target gene in the test sample relative to the calibrator sample was carried out following protocol described by (Livak and Schmittgen, 2001).

Determination of Growth Parameters

Body mass gain (BMG, %), specific growth rate (SGR, %/day), condition factor (K), length gain rate (LGR, %) and survival rate were calculated as follows:

BMG (%) = 100 ×[final body mass (g) - initial body mass (g)]/ initial body mass (g)]

SGR (% day^{-1}) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100

Length gain rate (%) =100× [Average terminal body length (cm) –Average initial body length (cm)]/Average initial body length (cm)

CF = 100× [Weight, g) / (Length, cm)^3]

Survival rate (%) =100 × (final number of juveniles / initial number of juveniles at end of experiment).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests to determine significance of the differences between groups using Statistical Package for the Social Sciences (SPSS) software (version 16.0). A value of ($P < 0.05$) was considered significant. The data were analyzed by Values are expressed as means ± standard error.

RESULTS

Oxidative Stress and Molecular Biomarkers

Temperature significantly affected the SOD activity, thermal stress molecular biomarker (HSP-90) and immune response biomarker (A2M) gene expression (Figures 1-3). Significantly higher ($P < 0.05$) superoxide dismutase (SOD) in erythrocyte lysate was observed in the 10ºC, 30ºC and 35ºC groups at day 0 and 3 week, but more pronounced at the 6th week(Figure 1).

For gene expression, the day 0 sampling point including the four temperature groups (10, 20, 30 and 35ºC) was considered as the control. HSP-90 (thermal stress molecular biomarker) was significantly up-regulated at higher temperatures (20, 30, and 35ºC), while down-regulated at 10ºC (Figure 2). Similarly, immune response biomarker α-2-macroglobulin (A2M) gene was also up-regulated ($P < 0.05$) in 20 and 30ºC groups whereas it was down-regulated in 10ºC groups (Figure 3).

Survival Rate and Growth Performance

Survival rate, growth performance (SGR, BMG, and LGR), and condition factor were significantly affected ($P < 0.05$) by changes in water temperature and are presented in figures 4-6. The survival rate of fish was observed throughout the experiment (six weeks), and found that the lowest ($P < 0.05$) survival rate was observed in the 35ºC group (86% survival) whereas 100% survival was observed in 10ºC, 20ºC, and 30ºC groups(Figure 4). Highest ($P < 0.05$) biomass and length growth and condition factors were observed for the 30ºC group and

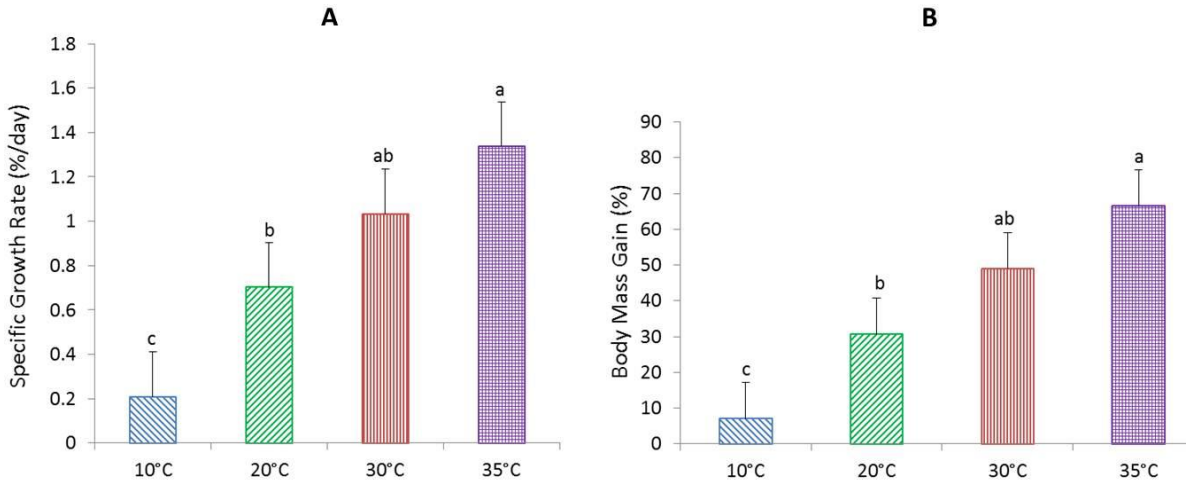

Figure 5: The effects of different temperatures (10, 20, 30 and 35ºC) on (A) Specific Growth Rate (%/day) and (B) Body Mass Gain (%) of bluegill after period of 6 weeks. Values are mean (n = 9) ± standard error. Mean values with different superscript are differ significantly (P < 0.05).

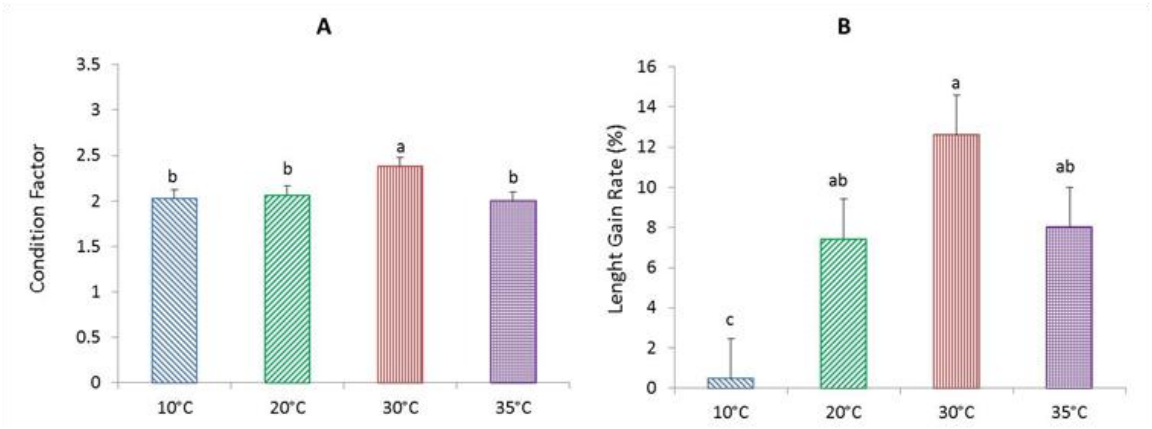

Figure 6: The effects of different temperatures (10, 20, 30 and 35ºC) on (A) Condition Factor and (B) Length Gain Rate (%) of bluegill after period of 6 weeks. Values are mean (n = 9) ± standard error. Mean values with different superscript are differ significantly (P< 0.05).

lowest (P < 0.05) value was observed for the 10ºC group(Figures 5 and 6).

DISCUSSION

Water temperature is one of the most important factors affecting physiological performances in aquatic ectotherms (Dong *et al*., 2008). The increase of water temperature influences parameters such as metabolic rate, and frequently promotes stressful conditions in the fish e.g. oxidative stress which results from the production of ROS (Ahmed, 2005; Halliwell, 1994; Madeira *et al*., 2013). This kind of stress occurs when there is an imbalance between the production of ROS (superoxide (O2–), hydrogen peroxide (H2O2) and the hydroxyl radical (OH) and the activity of antioxidants, which protect biological macromolecules from oxidation (Halliwell, 1994; Kammer *et al*., 2011). However, fish are unable to detoxify the ROS active species (Ahmed, 2005; Halliwell, 1994; Madeira et al., 2013). Thus the induction of antioxidant defenses is an important component of the stress response against oxidative stress in biological systems (Madeira *et al*., 2013; Parihar *et al*., 1997).One of those antioxidant defenses is SOD, which is an important antioxidant enzyme (Fridovich, 1995; Kurtz *et al*., 2006; Somogyi *et al*., 2007).At the beginning of the experiment (week 0 and 3) SOD activity was lower (P < 0.05) in 20oC group than other groups, which reveals lower production of ROS in this group, indicating that fish are likely to be lesser stressedin this group than other higher and lower temperature groups, and that both high and low temperature cause stress. At the end of the experiment (6 weeks), SOD activity increased as water

temperature increased. This could be attributed to that the ectothermic metabolism is influenced by relatively long-term exposure in higher water temperature, resulting increased ROS production in fish (Bagnyukova *et al*., 2007), because temperature is also positively correlated to metabolism and is often associated with an increase in ROS production (Hemmer-Brepson *et al*., 2013). Similarly, high SOD activity with increased levels of lipid peroxides was observed at higher temperature than lower temperature in gold fish, Carassius auratus (Lushchak and Bagnyukova, 2006), medaka, Oryziaslatipes (Hemmer-Brepson *et al*., 2013), fathead minnows Pimephalespromelas (Clotfelter *et al*., 2013).This result suggested that SOD could be considered as one of the biomarkers for oxidative stress in bluegill fish.

Heat shock proteins protect other proteins from unfolding, assist refolding denatured protein, or target them for degradation and their functions are viewed as critical for thermo-tolerance(Dong *et al*., 2008; Frydman and Höhfeld, 1997; R. John, 1999). The expression of HSPs is often regarded as a molecular biomarker of thermal stress in fish (Dong *et al*., 2008; Feder and Hofmann, 1999). In the present study, HSP90 was down-regulated (more than 3 fold decreased) in 10°C group at 3rdand 6thweeks. This down-regulation of HSP90 can be attributed to continued synthesis of HSPs requiring a great deal of energy and has an impact on the synthesis of other proteins which led to poor growth performance of fish in this group (Krebs and Feder, 1997; Viant *et al*., 2003).The higher ($P < 0.05$)HSP-90 gene expression was observed in higher temperature group. Although increase of HSP90 could result in an energetic cost (Feder and Hofmann, 1999; Lindquist, 1986), it appeared that growth ability at the temperature of ~30ºC could override the cost. Our results are in consistent with other researchers, wherein they have found that expression of HSP-90 gene was shown to be heat inducible in chinook salmon (Oncorhynchustshawytscha) (Palmisano *et al*., 2000), gobies (Gobiidae) (Thomas J. Dietz, 1993) and sea cucumber (Apostichopus japonicas) (Zhao *et al*., 2011).

Alpha-2-macroglobulin is an important non-specific humoral factor in the defense system of the animals and it is also known as binding proteins for many growth factors and cytokines, including growth hormone (Chuang et al., 2013). In the present study, A2M was significantly up-regulated at 20 and 30ºC whereas it was down-regulated ($P < 0.05$) in 10ºC group (at 3rd and 6th weeks). At the end of experiment (6th week) a down-regulation at 35°C compared to the control (0 week) showed this can be correlated to the ability of A2M to inactivate a variety of proteinases in fish, which is believed to play a significant role in host resistance to infection (Enghild *et al*., 1990) and are required to control the proteinase activity and to avoid tissue damage(Muta *et al*., 1995). The down regulation can be attributed to the time dependent expression of stress proteins which can be correlated to the HSP90 gene expression pattern in our study (Martínez-Álvarez et al., 2005; Zhao *et al*., 2011). At the end of the experiment, it was observed that high temperature (35ºC) down regulated the A2M gene in bluegill, which reveals that high temperature (35ºC) suppress the immunity in bluegill therefore this group exhibited highest mortality among the groups.

Generally, fish require optimum temperature for growth and survival and these factors may change with age and size, as juveniles of many fish species prefer higher temperatures than adults require (Brett, 1979; Handeland *et al*., 2008; McCAULEY and Huggins, 1979; Pedersen and Jobling, 1989). High growth of juvenile bluegill occurred over a broad range from a few degrees below the ultimate incipient upper lethal (35.6 to 37.3°C) (Arnold Banner, 1973) to temperatures approaching 20°C (Beitinger and Magnuson, 1979). In the present study it was found that the effects of thermal stress on growth of bluegill were significant. The measurements test showed that the effect of temperature treatments on growth was found to be positively correlated with water temperature in a certain range. Higher growth performance was observed in bluegill juvenile in warmer water (30 and 35°C) than colder water (10 and 20°C). Our results are similar to other researchers (Beitinger and Magnuson, 1979; Carlander, 1977; Lemke, 1977; Wang *et al*., 2014) wherein they have observed that growth rate of bluegill increased with temperature to approximately 30°C. The reason that fish had the similar growth performance at 35°Cas 30°C could be lower density due to lower survival (86% survival) compared to other groups (100% survival). Previous studies on other freshwater fish species attributed the increase of growth under higher temperatures to enhanced food intake (Diana, 1984), change in the pattern of energy allocation (Cox and Coutant, 1981; Pilditch and Grant, 1999; Tian and Dong, 2006) and that high temperature results in increasing the activity of digestive enzymes, which may accelerate the digestion of the nutrients, thus resulting in better growth (Imsland et al., 2006). Similarly, Atlantic salmon (Salmosala) and Rohu (Labeorohita) were subjected to different temperatures, and found that increased temperature enhances fish growth because of high feed intake and feed utilization(Imsland *et al*., 2006; SALIM, 2006).This trend was supported with our results which showed that water temperature and growth of bluegill were positively correlated in a certain range. In the present study, the lowest survival was observed in warm water (35°C) compared to other cold water groups (100% survival). This result was concurred with (Pepin, 1991) wherein it was found that mortality rates of juvenile fish increased with increasing temperature in a certain range. Based on fish survival and growth performance in the present study, the optimum water temperature for bluegill culture is about 30°C. This optimum water temperature for bluegill was also reported by (Lemke, 1977) and (Wang *et al*., 2014).

CONCLUSION AND RECOMMENDATIONS

The present study revealed that different water temperature altered oxidative stress biomarker (SOD)

anmolecular biomarkers for thermal stress (HSP90) and immune response (alpha-2-macroglobulin), which is of great relevance to climate change effect, environmental monitoring, the fish farming and fisheries industry. Our results support that SOD, HSP-90 and A2M could be considered as biomarkers for climate change and environmental monitoring as referred in fish species. Overall, these findings may have important significances for optimization of recreational and aquacutural production of bluegill sunfish, environmental monitoring and understanding of climate change effect on aquatic animals. Trials investigating the interactions between temperature, growth, sexual maturity and survivability will be advantageous for further research. It remains for further work, studying the consequences of increased water temperature for fitness.

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Conflicts of Interest

The authors declare no conflict of interest.

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