

THERMAL STRESS OF AMBIENT TEMPERATURE MODULATE EXPRESSION OF STRESS AND IMMUNE-RELATED GENES AND DNA FRAGMENTATION IN NILE TILAPIA (*OREOCHROMIS NILOTICUS* (LINNAEUS, 1758))

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Abstract. Owing to its cold-blooded nature, water temperature is a driving force in fish life. The aim of this study is to explore the effect of the thermal stress of ambient temperature during summer and winter in Nile tilapia (*Oreochromis niloticus*) on heat shock proteins (HSP70, HSP27), oxidative stress genes (MT and GST) and some immune response genes (IL-1, IL-8, and TLR7). As well as to compare the DNA fragmentation at both variant temperatures using RAPD-PCR and micronucleus assay. The fish were collected twice, once at the end of December (winter sample, temperature 14°C), and the other at August (summer sample, temperature 36°C). qRT-PCR analysis of mRNA expression for the studied genes were done. HSP70 expression increased equally in gills and liver at both summer and winter, suggesting its advantageous role in fish survival in the labile environment. Its expression exceeds HSP27 level, whose expression increased in summer. GST expression increased in summer in liver than gills and expressed equally at both organs in winter. No differences were observed in MT and immune response related gene expression in both seasons. The very close pattern of RAPD-DNA fragmentation and micronuclei were recorded at both thermal stresses. We conclude that, thermal stress of cold and hot extremes of ambient temperature in Nile tilapia have a nearby role in expression of HSP70, MT, liver GST and immune response related genes. As well as the incidence of micronucleus and DNA fragmentation.

Keywords: HSP, GST, MT, immune related genes, micronucleus, RAPD-PCR

Introduction

The major species of tilapia farmed worldwide was Nile tilapia (*Oreochromis niloticus*), with 71% of total world production in 2016 reported by Engle et al. (2016). Nile tilapia is capable of tolerance of a wide range of environmental conditions, fast growth, successful reproductive strategies, and ability to feed at different trophic levels; make it as a successful species in subtropical and temperate environments (Peterson et al., 2005).

All levels of an organism from genes to behavior are influenced by temperature; over day, seasonal periods, inducing numerous metabolic and hormonal adaptations (Hochachka and Somero, 2002). Fish response varies considerably according to whether it is cold, temperate or warm-water species (Manning and Nakanishi, 1996).

The optimal temperature for growth of most tipline species is between 25-28 °C and these fish do not grow well at a temperature below 16 °C, when the water temperature drops down to about 13 °C, they will go into a stress induced dormant state, death occurs from 10-12 °C. Concurrently, similar sequences of events occur at higher temperatures (Wolfforth and Hulata, 1981; Donaldson et al., 2008; Pundit and Nakamura, 2010).

Among genes affected by temperature are Heat shock proteins (HSPs), oxidative stress genes, as well as modulators of both innate and acquired immunity especially cytokines. HSPs have been found to repair and prevent damage from cellular stress associated with protein denaturation at high and low temperatures (Nakano and Iwama, 2002; Werner et al., 2005). However, HSPs expression is fluctuating at both stresses according to fish species (Werner et al., 2005; Wilde, 1988). Glutathione S-transferases (GST), one of the oxidative stress-related genes, its expression associated with different kinds of stressors (Almroth et al., 2015; Garcia et al., 2015). Also, Metallothioneins (MTs) are a family of stress proteins. One of their functions is the protection from oxidative stress (Nordberg, 1998; Viarengo et al., 2000). Variation in transcription of cytokines (IL-1 β and IL-8) and Tol-like Receptors (TLRs) in fish following exposure to variable temperatures were recorded (Basu et al., 2015; Polinski et al., 2013).

The enhanced environmental temperature induced oxidative stress in fish, associated with single and double DNA strand break and chromosomal fragments (Parihar and Dubey, 1995; Lushchak, 2011). RAPD and micronuclei assay are a reliable, sensitive and reproducible assays have the potential to detect a wide range of DNA damage and can be applied to genotoxicity studies in fish (El-Nahas, 2015; Cáceres-Vélez et al., 2016).

The aim of this study is to explore the effect of thermal stress of ambient temperature extremes during summer and winter in Nile tilapia on heat shock proteins (HSP70, HSP27), oxidative stress genes; Glutathione S-transferases (GST) and Metallothioneins (MT) and some immune response genes (IL-1, IL-8 and TLR7). Also to compare the DNA fragmentation at both variant temperatures using RAPD and micronucleus assay.

Material and methods

Sample collection and DNA extraction

Nile tilapia fish (*Oreochromis niloticus*) were collected from private fish farm in Kaferelsheik province, Egypt. The collection of samples were twice, once at the end of December, 2015 (winter sample, temperature 14 °C) the other at August, 2016 (summer sample, temperature 36 °C). Blood samples (0.5 ml/fish) from five fish were collected from the caudal vein then transferred to vacuum plastic tube containing EDTA to be used for micronucleus (MN) assay and RAPD technique. Liver and gill samples were taken from three fish and frozen in liquid nitrogen and maintained at -80 °C till subsequent use in RNA extraction. DNA extraction was performed from blood using G-spin total DNA extraction kits and quantified using the Nanodrop (Uv-Vis spectrophotometer Q5000/USA).

cDNA Synthesis and Quantitative real-time PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA expression for heat shock proteins (HSP70, HSP27) with designed primer using primer 3 program (www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi), oxidative stress genes (Metallothionein (MT) and glutathione S-transferase- α 1 (GST- α 1)) and some immune response genes (interleukin-1 β (IL-1 β), interleukin-8 β (IL-8 β), and toll-like receptor 7 (TLR7) and β -actin (as a reference gene) was performed using the primers shown in *Table 1*.

Table 1. Primers sequence of quantities expression qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Target size (Bb)	Ref.
β-actin	CAGCAAGCAGGAGTACGATGAG	TGTGTGGTGTGTGGTTGTTTTG	62	(Pang et al., 2013)
GST	TAATGGGAGAGGGAAGATGG	CTCTGCGATGTAATTCAGGA	220	(Puerto et al., 2011)
MT	TTTTGTTTTCAAGGTGGAACC	AGAGGTGGTGAACTTTGTGG	300	(Alhama et al., 2006)
IL-1β	TGCACTGTCACTGACAGCCAA	ATGTTCAAGGTGCACTATGCGG	113	(Choi et al., 2007)
IL-8	GCACTGCCGCTGCATTAAG	GCAGTGGGAGTTGGGAAGAA	85	(Ming et al., 2013)
TLR7	TCAGCAGGGTGAGAGCATAC	ACATATCCCAGCCGTAGAGG	143	(Abo-Al-Ela et. al., 2017)
Hsp70	CTCCACCCGAATCCCCAAAA	TCGATACCCAGGGACAGAGG	195	Primer3
Hsp27	CTGAGGAGCTGGTGGTGAAG	GATCAAAGGAGCCTCCACGG	194	Primer3

Table.2. Oligonucleotides primers used in RAPD-PCR assay.

Primer name	Sequence	Annealing temperature
OPA07	GAAACGGGTG	32°C
OPA08	GTGACGTAGG	32°C
OPA10	GTGATCGTAGG	32°C
OPA17	GACCGCTTGT	32°C
OPA19	CAAACGTCGG	32°C

The total RNA was extracted from the samples using Easy-Red (iNtRON Biotechnology, Inc cat. no. 17063). The quality of the extracted RNA was confirmed using Nanodrop (Uv-Vis spectrophotometer Q 5000/USA). 2 μ g of total RNA was reverse transcribed to first-strand cDNA kits Smart scribe reverse transcriptase

(protocol-at-a-glance PT4080-2 (clontech) according to the manufacturer's instructions. The cDNAs were used as the template for RT-PCR, SensiFast™ SYBR Lo-Rox kit (Bioline, United Kingdom) was used in Mx3005P Real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). Δ ct was calculated for each expressed gene using threshold cycle (CT) values that were normalized to those of the β -actin housekeeping (Livak and Schmittgen, 2001). Lower Δ CT indicates increased expression. All samples were performed and analyzed in triplicate.

Random amplified polymorphic DNA – Polymerase chain reaction (RAPD-PCR)

A set of five random 5-mer primers (Table 2) were used for screening of DNA polymorphism among summer and winter sample of Nile Tilapia. The PCR amplifications were performed using primer described in Abd El Naby et al. (2015). The reaction (25 μ l) consists of 12.5 μ l Thermo PCR master mix, 5 μ l primer (10 pmole) and 6.5 ddH₂O and 1 μ l genomic DNA (20 ng/ μ l). The reactions were carried out in a thermal cycler (Veriti, Applied Biosystem) and thermal cycling program denaturing at 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, annealing temperature as shown in table 1 for 1 min and extension at 72 °C for 1 min, final extension step at 72 °C for 10 min. PCR product of each sample and 100 bp DNA ladder (TIANGEN) were loaded in 3% agarose gel staining with ethidium bromide. The electrophoresis was carried out for 30 min at 120 volts. The bands were visualized on and photographed by Gel Documentation system (Gel Doc. Alpha-chem. imager, USA). The presence of a band was scored as 1 and the absence of it as 0 in the RAPD profile of the six sample of Nile tilapia (three sample in each winter and summer). The cluster analysis and dendrogram construction were performed with Statistica 5 (Statistica, 1995). The similarity matrix was analyzed by the unweight pair group methods with Euclidian distance (UPGMA).

Micronucleus assay

Blood smears were done on clean microscopic slides, left for complete dryness and fixed in pure methanol for 10 min. Slides were immersed in 10% Giemsa (w/v) staining for 10 min. The slides were examined under the light microscope using 1000 $\times$ magnification to determine the frequencies of micronucleus and nuclear abnormalities (Al-Sabti and Metcalfe, 1995). At each assessment, 1000 cells/fish were analyzed. The characteristics used for the identification of the micronucleus were circular or oval bodies having no connection with the main nucleus, smaller than one-third of the main nucleus and showing the same staining and focusing pattern as the main nucleus. Micronucleus frequency was calculated from the formula:

$$MN\% = \frac{\text{number of cells containing micronucleus}}{\text{total number of cell counted}} \times 100$$

Results

Expression of HSPs, antioxidant and immune response related genes in winter and summer

Expression analysis of Heat shock proteins (HSP70 and HSP27) revealed that mRNA transcript level in liver and gill tissues of the two genes was altered at both winter and

summer. The level of expression of HSP70 exceeds HSP27. However, a significant difference was present between the mRNA transcripts of HSP27 in liver and gills tissues in both summer and winter. Meanwhile, HSP70 expression is almost equal in both organs in summer and winter (Fig. 1A, B).

GST in the liver at summer significantly exceed the winter and their levels are almost equal in gills at both seasons (Fig. 1C). No differences at MT transcripts in winter and summer fishes (Fig. 1D).

No significant differences were observed between summer and winter levels of mRNA transcripts of pro-inflammatory cytokines (IL1- β , IL8- β) or TLR7 (Fig. 1E). TLR7 showed a high expression level in both summer and winter (Fig. 1E).

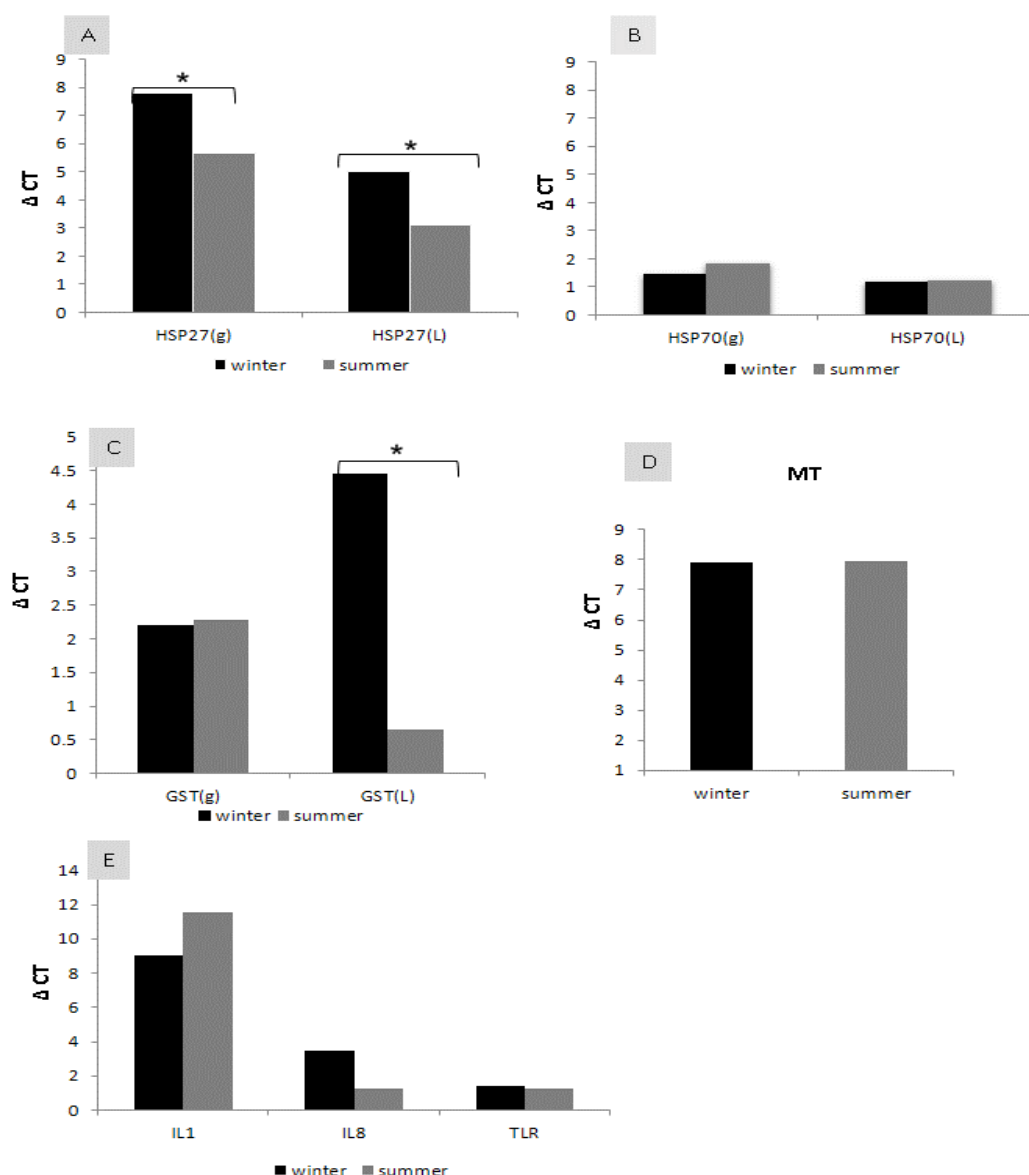


Figure 1. Relative gene expression of (A) Hsp27 in gill tissue (g) and liver (L). (B) Hsp70 in gill (g) and liver (L), (C) GST in gill (g) and liver (L). (D) MT in liver tissues and (E) immune related genes (IL1, IL8 and TLR) in liver tissue of *O. niloticus* in winter and summer. The β -actin gene was used as a reference gene to normalize the data and shown as $\Delta CT \pm SE$ ($n=3$). Lower ΔCT indicate increased expression

RAPD-PCR assay

The pattern of DNA fragmentation of summer and winter samples of *O. niloticus* did not show a distinct difference between both samples as shown in Fig. 2 A, B, C, D, E. Phylogenetic tree based on 46 bands generated from five RAPD primers indicate similarity between summer and winter pattern of DNA fragmentation. No definite cluster for summer or winter samples was obtained (Fig. 2F).

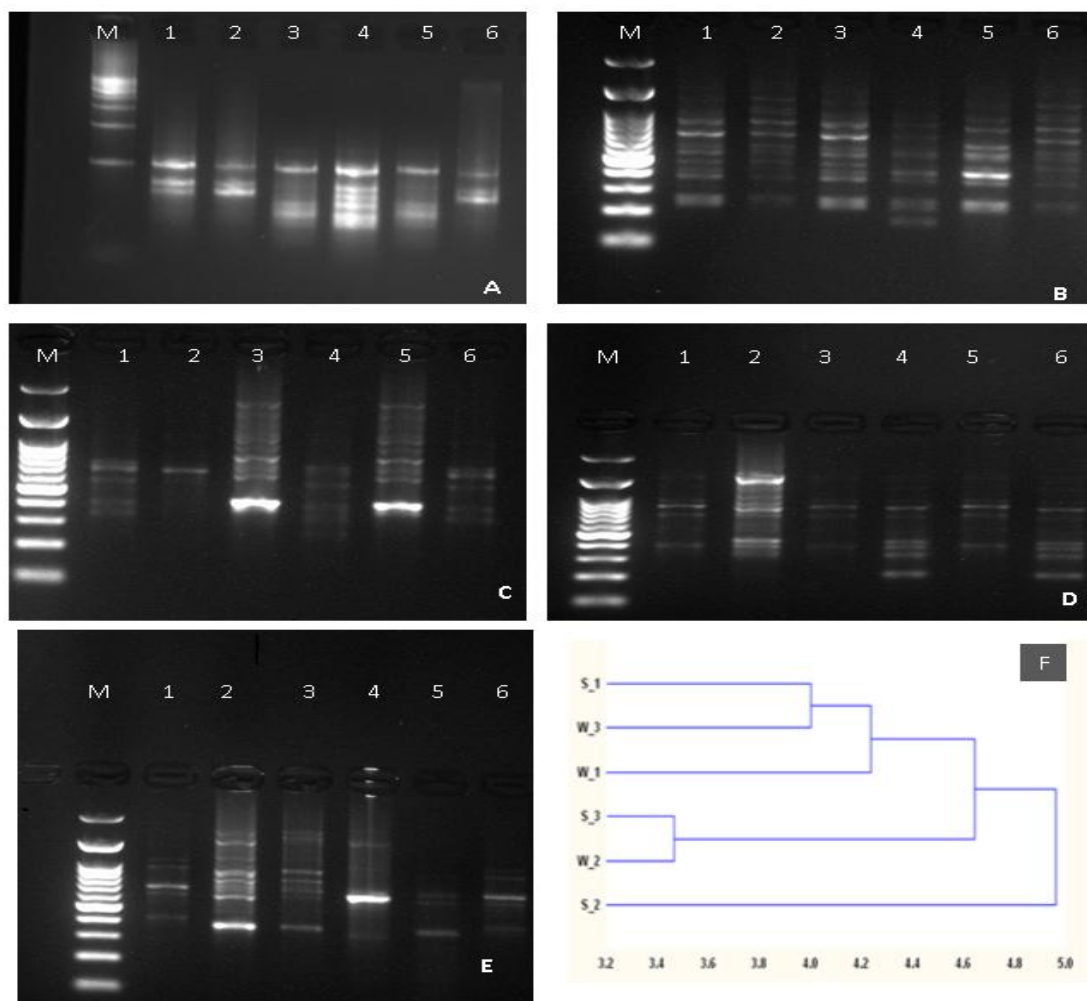


Figure 2. Ethidium bromide stained 3% agarose gel of PCR product of (A) OPA7, (B) OPA8, (C) OPA10, (D) OPA17, (E) OPA19 and (F) phylogenetic tree based on RAPD primers in Nile tilapia during winter and summer with similarity matrix based on unweight pair group methods with Euclidian distance (UPGMA). M: ladder, number 1, 2, 3, (summer samples); 4, 5, 6, (winter samples). S: summer; W: winter

Micronucleus assay

The thermal difference between winter and summer did not induce a significant difference in the number of micronucleated erythrocyte (Fig. 3).

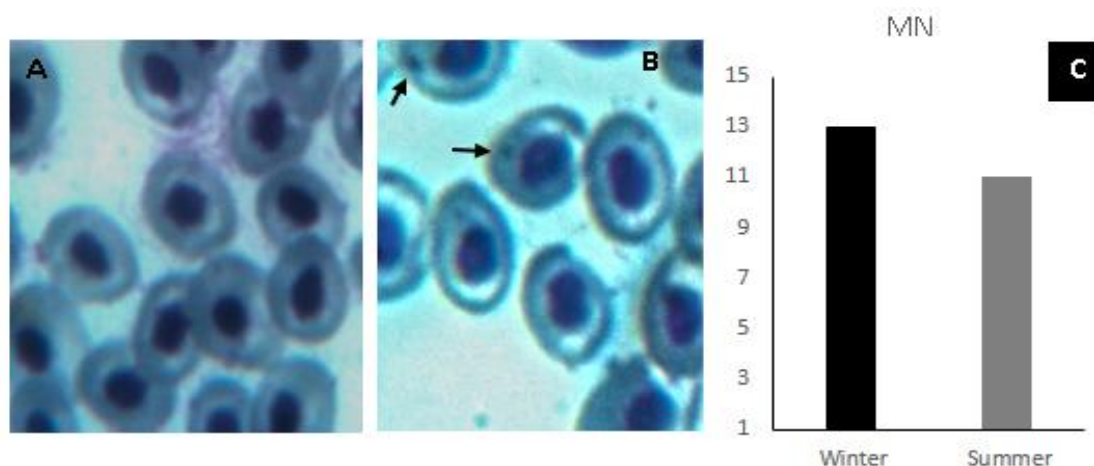


Figure 3. A, B: Photomicrographs of *O. Niloticus* erythrocytes A: normal erythrocyte. B: micronucleated erythrocyte. C: Number of micronucleated erythrocytes in winter and summer fish

Discussion

Owing to its cold-blooded nature, water temperature is a driving force in fish life. Livability and growth in fish are optimum within a defined temperature range (Godowsky and Caddell, 2015). Daily weather conditions, as well as seasonal variation, have different influences on fish (Buckley et al., 2006; Place et al., 2004; Podrabsky and Somero, 2004). Extremes of both ambient temperatures at different seasons may induce fish mortalities (Rijnsdorp et al., 2009). Although both high and low temperature may have different routes as a stressor on fish, they may share some effects on gene expression (Werner et al., 2006; Basu et al., 2015).

Heat-shock proteins are chaperones that allow biological processes to work well in a stressful environment. The usefulness of HSP70 expression to identify anthropogenic stress under field conditions was evaluated (Lund et al., 2006; Boone and Vijavan, 2002; Hamer et al., 2004). Expression analysis of HSP70 in our data indicates high expression levels in liver and gill tissues and no specific difference was found between winter and summer stresses. Mladineo and Block (2009) studied HSP70 in splenic tissue which showed upregulation at temperature 14 °C. Furthermore, Fader et al. (1994) demonstrated tissue levels of constitutive HSP70 in four species of the stream were varied according to seasons. Also, Hermes et al. (2001) demonstrated that the level of HSP70 mRNA was increased after a 1 h exposure to the elevated temperature. The observed expression levels of HSP70 in our study in both liver and gills are almost equal. Iwata et al. (2007) detected steady expression of *Lepomis* HSP70 mRNA regardless of sex and seasonality, they also suggest that certain level of HSP expression might be advantageous to survive in the labile environment.

The expression of the small heat shock protein27 (HSP27) is increased in response to diverse stress conditions (Arrigo, 2001). Wang et al. (2007) observed its up-regulation among HSPs expressed in summer in goldfish and its expression was higher in gills than liver. We also observed that its summer level of expression is higher than winter but liver level exceed gills which may be attributed to species difference in regulatory roles of HSPs (Liang et al., 2016).

For Metallothioneine in this study we did not found significant difference between mRNA transcript of liver tissue between winter and summer in Nile tilapia, it remain

constant in both seasons in comparison with Hermes et al. (2001) who found that cold stress resulted in a significantly higher induction of MT-1 than of MT-2 in carp. Additionally, Lee and Nam (2001) observed up-regulation of MT by elevation of water temperature. Amiard et al.; Dragun et al.; Lee and Nam (2006; 2009; 2001) proposed that expression of MT could be greatly modulated by various abiotic as temperature, salinity, and seasons. We suggest that both cold and heat stress would have similar effect on MT expression.

In this study mRNA transcript level of the toll-like receptor was up-regulated in winter and summer by the same level. Basu et al. (2015) observed induction of TLR2, TLR4 gene expression in the majority of the tested tissues at different thermal stresses temperatures in fish as compared to the control.

Amado et al. (2006) in their study about biomarkers in croakers *Micropogonias furnieri* (Sciaenidae) from southern Brazil; indicated a clear seasonal variation pattern for GST activity. The results suggest that the seasonal adjustments in the antioxidant defense of fish GST from this subtropical area with a wide water temperature range (up to 32 °C in summer and below 9 °C in some winter) was observed (Peel et al., 2007). and this agree with our study as GST showed up-regulation in both winter and summer and was sharply increased in liver tissue in summer. This match the study of Carvalho-Neta (2013) who had found that GST activity showed a similar pattern in *S. herzbergii* from both rainy (June) and dry (November) periods.

In this study mRNA transcript level of liver tissue of interleukin1 and interleukin8 was up-regulated in winter and summer, but interleukin-8 show higher mRNA transcript level in winter than summer. in contrast with Pérez-Casanova et al. (2008) who evaluated the blood expression of specific immune-related genes as interleukin-1 β (IL-1 β) in Atlantic cod by exposing them to a thermal challenge. Interleukin-1 (IL-1) gene was up-regulated at 19 °C (the highest tested temperature that is near to the cod's upper thermal limit). Feder and Hofmann (1999) proposed that, there is an important difference in the heat shock responsiveness exhibited by the classical HSP genes and IL-8. While exposure to heat shock alone is sufficient to activate HSP genes, heat shock exposure alone has no effect on IL-8 expression, but it enhances IL-8 gene activation when accompanied by a co-stimulus (e.g., TNF- α or IL-1 β).

Genotoxicity in fish induced by heat stress has been described in several studies. Exposure of fish to heat shock at various levels produced DNA single-strand break, micronuclei, inhibited cell proliferation and chromosome abnormalities (Cáceres-Vélez et al., 2016; Li et al., 1990; Ila et al., 2015). However, scarce data are available on the effect of cold stress on DNA fragmentation or chromosomal aberrations. From our data, we suggest that both heat and cold stress has a nearby effect on DNA fragmentation and micronucleus induction. Exposure to cold temperature (5 °C) for 4 h induced the fragmentation of thymocyte DNA in rats (Morishita et al., 1993).

We conclude that, thermal stress of cold and hot extremes of ambient temperature in Nile tilapia have a nearby role on expression of HSP70, MT, liver GST and immune response genes. As well as induction of micronucleus and DNA fragmentation.

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