Evaluation of physiological responses in selected tissues of a freshwater fish (*Cyprinus carpio* var *koi*) maintained in hard waters: A report on glucose, oxidative stress and antioxidant biomarkers

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Abstract

Fishes endemic to freshwaters are strongly influenced by water hardness because of low ionic compositions in the surrounding aquatic media. This compels it to change tissue physiology especially gill and muscle, majorly responsible for calcium metabolism and ionic regulation. Therefore, to understand the interplay between hardness and physiology, the present study evaluated biomarkers in selected tissues (gills and muscle) of a freshwater fish, Koi carp (*Cyprinus carpio* var *koi*) exposed to a sequential increase in water hardness. Glucose, Oxidative stress (Malondialdehyde) and Antioxidant markers (Catalase, Glutathione-S-Transferase and Glutathione) were quantified after 14 days of exposure to a four-fold increase from 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) to 300 (Very Hard - TV) mg $CaCO_3$/L. Although the fish showed appreciable endurance to all exposures, soft and moderate waters proved harmful to both tissues. Successive increase was observed in the glucose content especially in gills ($p < 0.05$). Hardness of 75 and 150 mg $CaCO_3$/L was detrimental to both gill and muscle because of elevated MDA concentrations ($p < 0.05$) than corresponding higher hardness indices. Elevated CAT, GST activity and GSH levels indicated well-coordinated antioxidant enforcement. Overall, the results revealed assorted tissue-specific biomarker responses and more importantly, concentrations < 225 mg $CaCO_3$/L elicited strong oxidative impairment in both gill and muscle.

Keywords – Calcium; Carps; Gill; Hardness; Muscle; Peroxidation; Physiology; Tissue

Introduction

Hardness is among the most significant physicochemical properties of water because it directly or indirectly influences physiology. Aquatic environments manifest varying concentrations of hardness constituted majorly by the divalent cations Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) (Baldisserotto 2011; Romano et al. 2020). Based on the concentration of these cations, water is classified into soft (< 75 mg $CaCO_3$/L) and hard (> 75 mg $CaCO_3$/L) (Portz et al. 2006). Both types exert their own physiological effects. Soft water poses challenges to the survival of fish, although various methods of adaptation have been reported, like compensation of calcium deficit environment through the consumed feed (Baldisserotto 2011). Similarly, hard waters can help balance ammonia, pH, and reduce mortality in animals (Baldisserotto et al. 2014). However, excessive hardness can also cause hypercalcemia, as reported in Mozambique tilapia (*Sarotherodon mossambicus*) (Wendelaar Bonga et al. 1983). Whatever the adjustment might be, these investigations are unequivocal that hardness ultimately targets tissues that lead to physiological changes including cellular rearrangements (Gonzalez et al. 1998; Gundersen and Curtis 1995).

Primarily, gills and muscle are favoured over other major tissues to assess hard water effects. While gills are the epicentre of Ca^{2+} homeostasis and osmoregulation (Evans et al. 2005; Wendelaar Bonga et al. 1983), muscle is susceptible to changes in constituent amino acids and ionic shifts due to water hardness (Buentello and Gatlin 2002). Abundant literature involving these tissues to understand consequences of water hardness at ionic-level and associated cellular adaptations in freshwater species such as Pinfish (*Lagodon rhomboides*) and Mozambique tilapia is accessible (Carrier and Evans 1976; Flik and Verbost 1995; Wendelaar Bonga et al. 1983). The fact that these tissues localise diverse redox reactions and biomarker changes, examination

of which gives worthy information about environmentally induced physiological alterations also, make them ideal to study effects of hard waters (Lushchak 2011).

The prior literature indicates that biomarkers are instrumental in understanding the effects of water hardness in fish. Investigations in Pacu (*Piaractus mesopotamicus*) (Copatti et al. 2019a; Neves et al. 2022), Silver Catfish (*Rhamdia quelen*) (Neves et al. 2017) and Kutum fingerlings (*Rutilus frisii kutum*) (Taghizadeh et al. 2013), justify the importance of glucose as as marker for effects of water hardness. Even redox status also provides substantial insights into the physiological effects of water hardness in fish. An evidence of this is the study of oxidative stress by Copatti et al. (2019b) and Neves et al. (2022) in different tissues of Pacu exposed to a range of hard waters. The consequences of oxidative stress are prevented by antioxidants (Betteridge, 2000), either enzymatic (Catalase, Glutathione-S-Transferase) or non-enzymatic (Glutathione) which attempt to restore the correct prooxidant/antioxidant ratio (Lushchak, 2016).

Despite a distinct precision offered by glucose and redox markers, these have not been judiciously explored to understand the interplay of hardness with physiology and fish health. As evident it is, investigations involving its usage have gained momentum recently (Copatti et al. 2019b; Michelotti et al. 2018). Biomarker examinations seem promising to understand the extent and efficiency of physiological adaptations due to external hardness (Limbaugh et al. 2021). Considering such proposition, the present study attempts to evaluate the effects of water hardness (75, 150, 225 and 300 mg $CaCO_3$/L) on biomarkers (glucose, oxidative stress and antioxidant profile) in selected tissues (gill and muscle) of Koi carps (*Cyprinus carpio* var *koi*), an economically important freshwater ornamental fish.

Methods

Acclimatisation of fish

Juveniles (6.70 ± 0.15 g; 5.90 ± 0.12 cm) were purchased from the Ornamental Fish Research Center, Bengaluru, Karnataka. Fish were randomly distributed in 50 L glass tanks filled with tap water. The tanks were maintained under natural photoperiod (≈12 Light/12 Dark) with continuous aeration (Venus Aqua AP-608A, China). Fish were fed twice a day (09:00 and 18:00) at 2% body weight with commercial feed pellets (Taiyo Grow, India).

Experimental setup

The two week study consisted of four levels of hardness: 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH), and 300 (Very Hard - TV) mg $CaCO_3$/L, selected on the basis of its occurrence in natural systems (Stumm and Morgan 1996). Every treatment (hardness) was maintained in triplicate (three tanks per treatment × two fishes per tank = six fishes per treatment). All concentrations were prepared by the addition of Calcium carbonate ($CaCO_3$) to water and calibrated by complexometric EDTA titration (APHA 2005). Faecal matter accumulated in the tank was drained out by suction pipe with subsequent renewal of the specific hardness. The tanks were covered with a mesh net to prevent the fish from escaping. Water parameters were monitored for temperature (25.1 ± 1°C), pH (7.04 ± 0.1), dissolved oxygen (6.5 ± 0.08 mg/L) and alkalinity (213 ± 0.02 mg/L) (APHA 2005).

Sampling

A total of six fish (n = 6) per treatment were sacrificed for biomarker evaluation. Individual fish were dissected for white muscle and gill. Tissues were washed with an ice-cold phosphate buffer solution (0.1M; pH 7.4) and homogenised in Potter-Elvehjem grinder. The homogenate (10%

w/v) was then centrifuged at 5000 × *g* following which supernatant (stored at -20°C) was retained for all assays except Glutathione (homogenate precipitated with TCA before centrifugation). The absorbance values were measured on a spectrophotometer (Systronics UV-VIS 118, India).

Biochemical analyses

Glucose

Glucose was assayed according to Nelson (1944) and Somogyi (1952). The deproteinizing agent ($Ba(OH)_2$ and $ZnSO_4$) was added to the supernatant and centrifuged at 5000 × *g* for 10 minutes. Alkaline copper reagent (potassium-sodium tartrate; Na_2CO_3; $NaHCO_3$ and Na_2SO_4 in distilled water) was added to the supernatant. The mixture was heated, followed by the addition of an arseno-molybdate reagent and the optical density of the solution was recorded at 540 nm.

Malondialdehyde (MDA)

Lipid peroxidation was estimated by using the Niehaus and Samuelsson (1968) protocol. The supernatant was mixed with this TCA-TBA-HCl reagent (15% Trichloroacetic acid, 0.38% Thiobarbituric acid and 0.25N Hydrochloric acid) in the ratio of 1:2. This reaction mixture was heated in a boiling water bath for 15 minutes, cooled and centrifuged at 1100 × *g* for 5 minutes. The optical density of the solution was recorded at 535 nm.

Catalase (CAT)

Catalase activity was measured by using the Aebi (1984) protocol. The reaction was started by adding the supernatant to an equimolar solution of H_2O_2 and phosphate buffer (50 mM; pH 7.1). A decrease in absorbance was continuously recorded at 240 nm (UV) for 3 min.

Glutathione-S-Transferase (GST)

GST activity was estimated by using the Habig et al. (1974) protocol. The reaction mixture contained the supernatant, phosphate buffer (0.1M; pH 6.5) and 2,4-Dinitrochlorobenzene (30mM). The volume was adjusted with distilled water after which the reaction was initiated by adding Glutathione (0.1 M). The optical density of the solution was recorded at 340 nm.

Glutathione (GSH)

GSH was estimated by using the Moron et al. (1979) protocol. Homogenate was precipitated with TCA (5%) and centrifuged at 3000 × *g* for 10 minutes. The supernatant collected after centrifugation was then added to the phosphate buffer (pH 6.5) and Ellman's reagent. The optical density of the solution was recorded at 420 nm.

Protein

Total protein content of the tissues was estimated according to Lowry et al. (1951) protocol. Bovine serum albumin was used as standard. The optical density of the supernatant-reagents mixture was recorded at 660 nm.

Statistical analysis

Mean values (± SE) were tabulated for box plot representations. Normality and homoscedasticity were evaluated using the Shapiro–Wilk and Levene tests, respectively. Group means were

analysed by One-way ANOVA followed by a post-hoc test (Tukey). Significant differences were fixed at 95 % ($p < 0.05$). GraphPad Prism (Version 5.0, USA) and JASP (Version 0.16.2, Netherlands) were used for statistical computation and visual presentations.

Results

Biomarkers in Gill

The glucose concentration increased progressively from TS to TV. Significant differences ($F = 10.91$; $p < 0.05$) were found between TV and the remaining treatments (Figure 1A). Soft water TS elevated the concentration of MDA, followed by a spike in TV. Only TH differed significantly ($F = 21.27$; $p < 0.001$) from the remaining treatments (Figure 2A). Highest antioxidant Catalase activity was observed for TH, which differed significantly ($F = 50.26$; $p < 0.001$) from TS, TM and TV. Also, TV differed significantly from TM and TS ($F = 50.26$; $p < 0.001$) (Figure 3A). GST activity for TH and TV was comparatively higher than for TS and TM. While no significant differences ($F = 26.45$; $p > 0.05$) were found between the low (TS and TM) and high hardness groups (TH and TV), differences were observed between treatment pairs (Figure 4A). The highest concentration of GSH was recorded for TV. Except TS that did not show significant differences ($F = 42.78$; $p > 0.05$) with TM and TH, all treatments recorded intergroup differences (Figure 5A).

Biomarkers in White Muscle

The glucose concentration progressively increased from TS to TH but reduced at TV. Pairwise, similar concentrations and no significant differences ($F = 22.98$; $p > 0.05$) were observed for TS and TV and for TM and TH (Figure 1B). The highest level of MDA was observed for TM which

differed significantly (F = 37.89; $p < 0.001$) from TS, TH and TV (Figure 2B). The highest CAT activity was observed in TM followed by a gradual reduction for TH and TV. Catalase was the only biomarker that varied significantly (F = 49.38; $p < 0.05$) among all treatments for white muscles (Figure 3B). No significant differences (F = 57.13; $p > 0.05$) were recorded for GST between TM and TH. The highest GST activity was recorded for TH, which differed significantly with TS and TV (F = 57.13; $p < 0.001$) (Figure 4B). Glutathione (GSH) initially increased from TS to TM, thereafter, reducing gradually for the remaining treatments. Only significant difference (F = 8.87; $p < 0.05$) was observed between TM and the remaining groups (Figure 5B).

Discussion

Effect of hardness on glucose

Glucose is the primary energy source for metabolism due to its involvement in glycolysis. Therefore, the assessment of glucose concentration would provide insight into the energy expenditure for metabolic adaptations to hardness. Flik and Verbost (1995) have mentioned that freshwater fishes take up Ca^{2+} through the gills, and this transcellular movement is dependent on the surrounding Ca^{2+} concentrations, which affect the branchial permeability of the gills. Therefore, external hardness strongly influences the energy reserves such as glucose to regulate fish metabolism. An environment with high hardness reduces gill permeability and loss of ions to water, thereby conserving energy (Golombieski et al. 2013). This study upholds the same rationale in the fact that glucose increased subsequently in gills of Koi carp from soft to hard waters. Contrarily, muscle showed reductions in glucose at the highest hardness level (TV), an observation also reported by Michelotti et al. (2018) in juvenile Common Snook (*Centropomus*

undecimalis) exposed to 1000 mg $CaCO_3$/L. They concluded that increased energy demands for survival and growth lowered muscle glucose levels. It would not be incorrect to assume that whereas gill is conserving energy, muscles on the other hand strike balance to the metabolic needs. This assumption however would need more justification.

Oxidative stress due to hardness

Secondary lipid peroxidation products such as Malondialdehyde (MDA) are used as biomarkers of oxidative stress (Ayala 2014). In this study, elevated MDA in the Koi carp gills exposed to soft and moderate waters indicated improper osmoregulatory functioning. Therefore, soft and moderate water hardness has detrimental effects on gills as also reported in Pacu gills (Copatti et al. 2019b). An increase in MDA of gills exposed to TV was also observed. As a deviation from the expected outcome, an explanation of this seems possible only after further investigations. MDA concentration also surged in muscles of Koi carps exposed to moderate hardness (TM), indicating oxidative damage to cell membrane fluidity and possible permeability of muscle tissues. Previous literature suggests that fishes are predisposed to peroxidation due to large amounts of Polyunsaturated Fatty Acids (PUFAs), ultimately changing lipid membrane properties (Lushchak 2011). Therefore, the present study cannot negate the probable oxidative damage in muscles exposed to moderately hard water.

Antioxidant response

Catalase is an important antioxidant enzyme that protects cells and tissues from oxidative damage because it reduces harmful hydrogen peroxide (H_2O_2) to water (H_2O) (Betteridge 2000). The level of CAT activity in gills underwent reduction from soft to moderate waters of Koi carps which indicated oxidative damage due to such exposure. Since freshwater species already have

less ionic presence in the surrounding environment, it might burden the gills to reduce loss of ions to water. Probably this must have decreased CAT activity, indicating that fish were inept in adjusting to concentration changes. Whereas in muscle, progressive decrease in Catalase activity from TM to TV, indicates subsided oxidative damage which might account for the protective effects of high-water hardness as suggested by (Copatti et al. 2019a).

Antioxidant activity of GST is specific to detoxification of xenobiotics. It conjugates GSH to various electrophiles, thereby preventing oxidative damage. After that, GSH indirectly or directly scavenges free radicals to defend the tissues from injury due to oxidative stress (Srikanth 2013). Elevated GST activity was noted in Koi carp gills at TH and TV, indicating that some degree of oxidative stress might have affected this tissue. But it is not feasible to generalise that the fish was under stress because antioxidant defence is always coordinated (Bagnyukova et al. 2006). Therefore, CAT and GSH might also have a role to play in protecting Koi carps from high hardness. Biomarker evaluation is majorly dependent on many factors including experimental conditions (Bagnyukova et al. 2006). Muscle also exhibited an elevated GST activity for all the exposures except soft waters (TS). This signifies that concentrations < 75 mg $CaCO_3$/L would be detrimental for freshwater fishes, with unrecoverable losses to osmoregulatory functioning. Given the intensive association between Glutathione antioxidant system, assorted expression of both GST and GSH in the investigated tissues indicated a well-coordinated response to all hardness exposures. Lower GST levels were complemented by a higher concentration of GSH for both the gills and muscle. Such type of tissue-specific antioxidant responses for the Glutathione antioxidant system has also been reported in Nile Tilapia, Sharp Tooth Catfish (*Clarias lazera*) and Common carp (*Cyprinus carpio*) (Hamed et al. 2004).

Conclusions

The results show that sequential increase in water hardness reshuffled the physiological adjustments in both the examined tissues. Glucose proved to be an excellent marker for hard waters especially in case of gills. It can also be deduced that soft and moderately hard waters can prove detrimental to freshwater fishes due to tissue oxidation induced elevated MDA concentrations. Since the antioxidant markers revealed multitudinous and assorted responses, an extension of this study warrants an Integrated Biomarker Response (IBR) due to sequential water hardness.

List of abbreviations -

Malondialdehyde - MDA; Catalase - CAT; Glutathione-S-Transferase - GST; Glutathione GSH; Glucose - GLU; Soft water - TS; Moderately hard water - TM; Hard water - TH; Very hard water - TV

Declaration -

Ethics approval and consent to participate – Animal use within this study was approved by the welfare committee of Bangalore University, India, in accordance with the national legislation.

Competing interests – The author declares that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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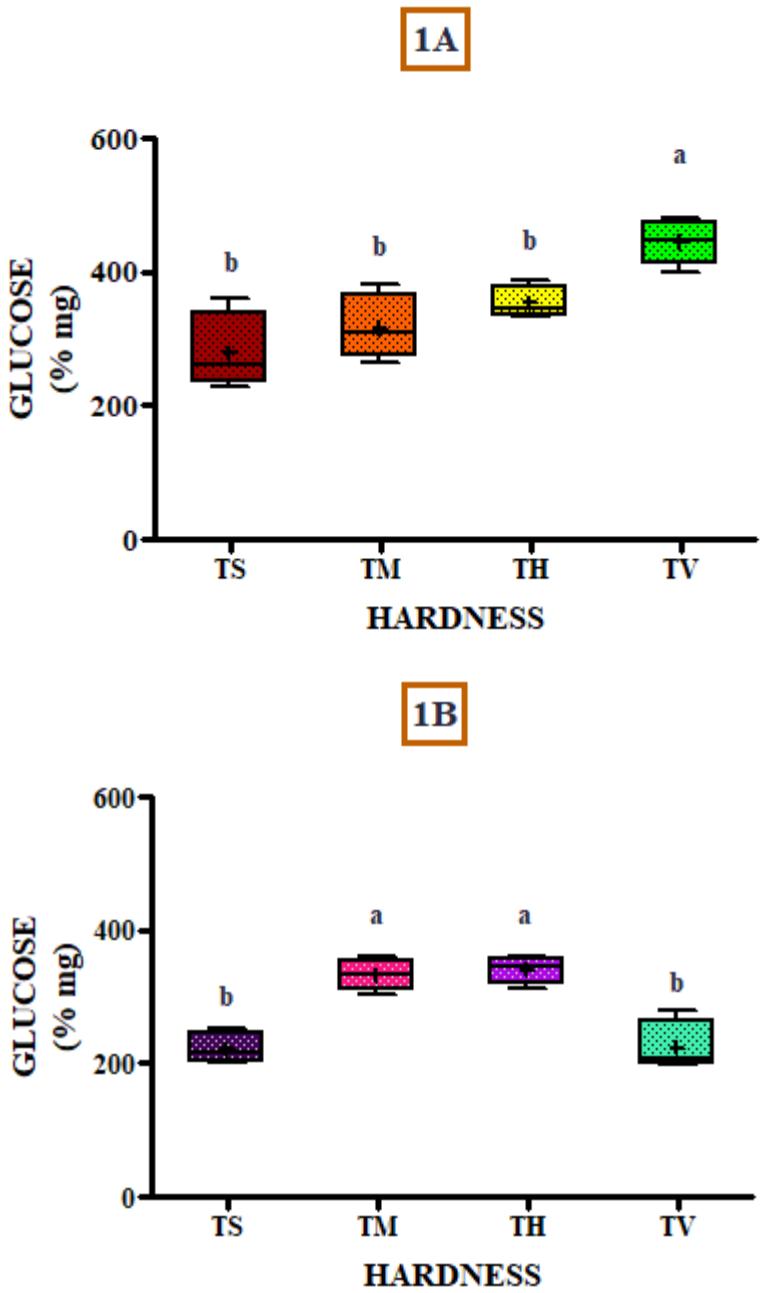

Figure 1 - Box plot presentations for Glucose concentration in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Superscripts among exposure groups indicate statistical significance ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

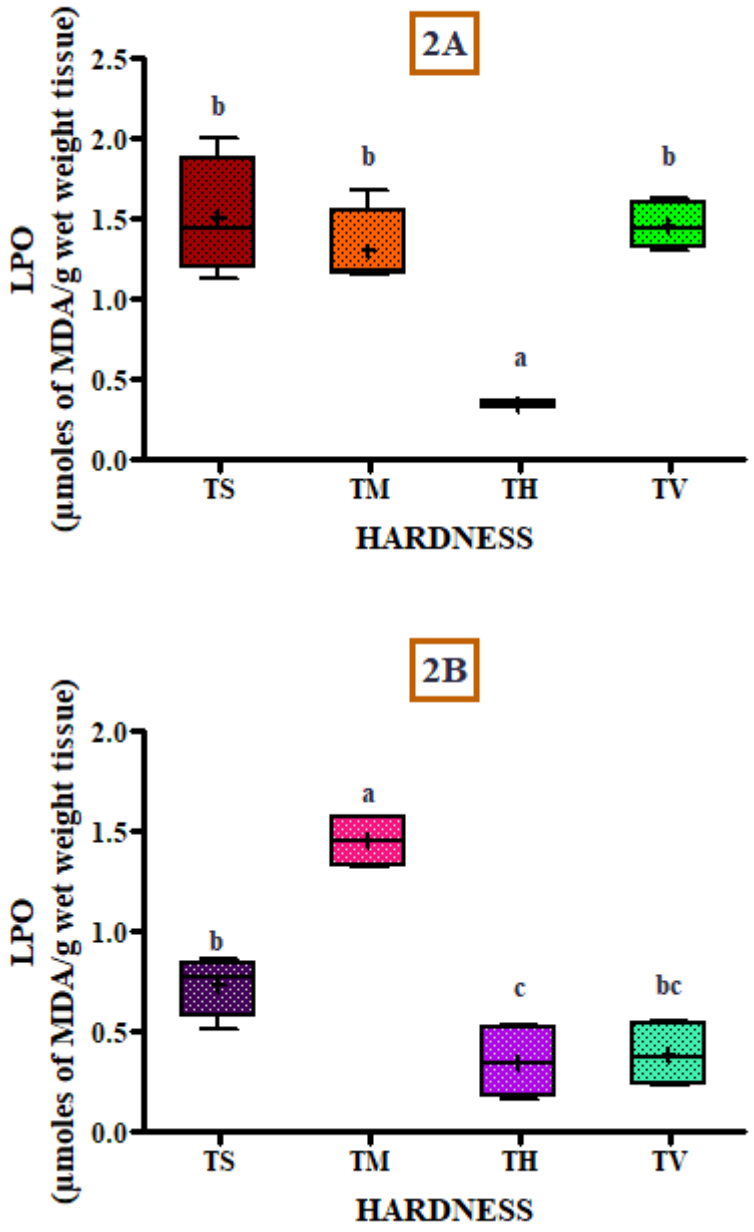

Figure 2 - Box plot presentations for MDA concentration in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

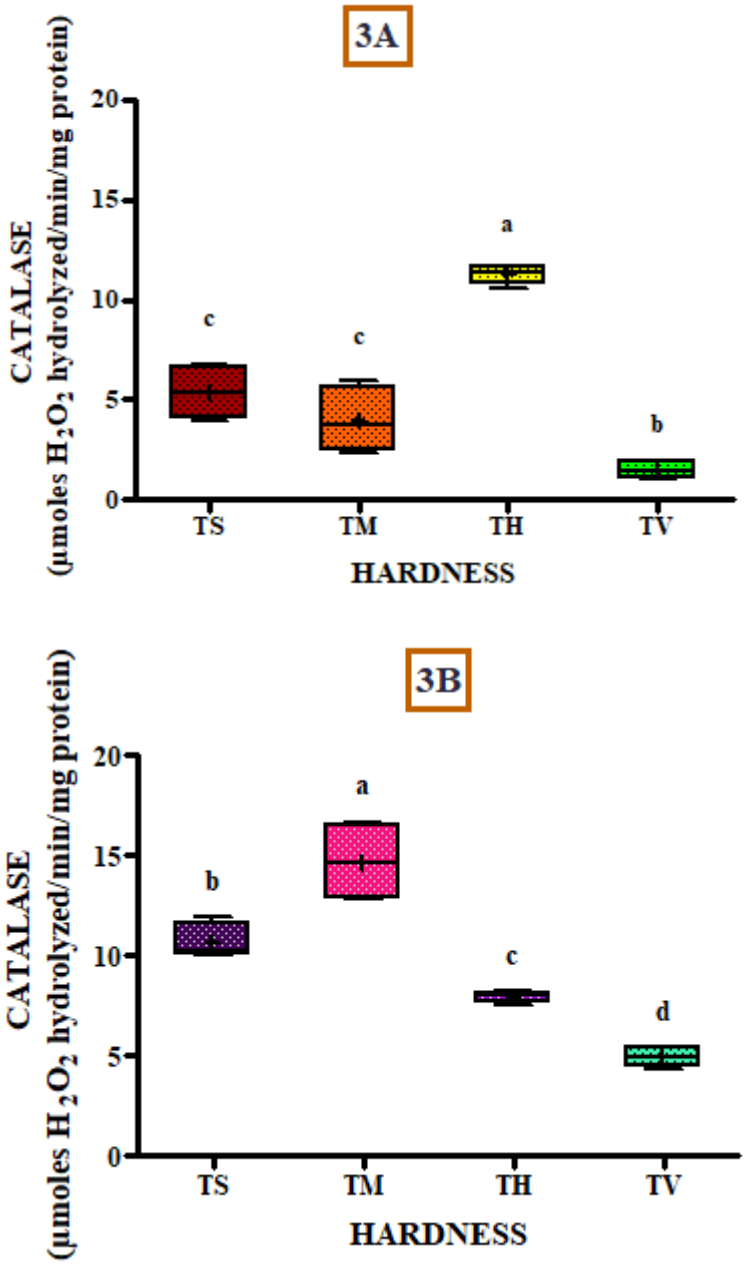

Figure 3 - Box plot presentations for Catalase activity in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

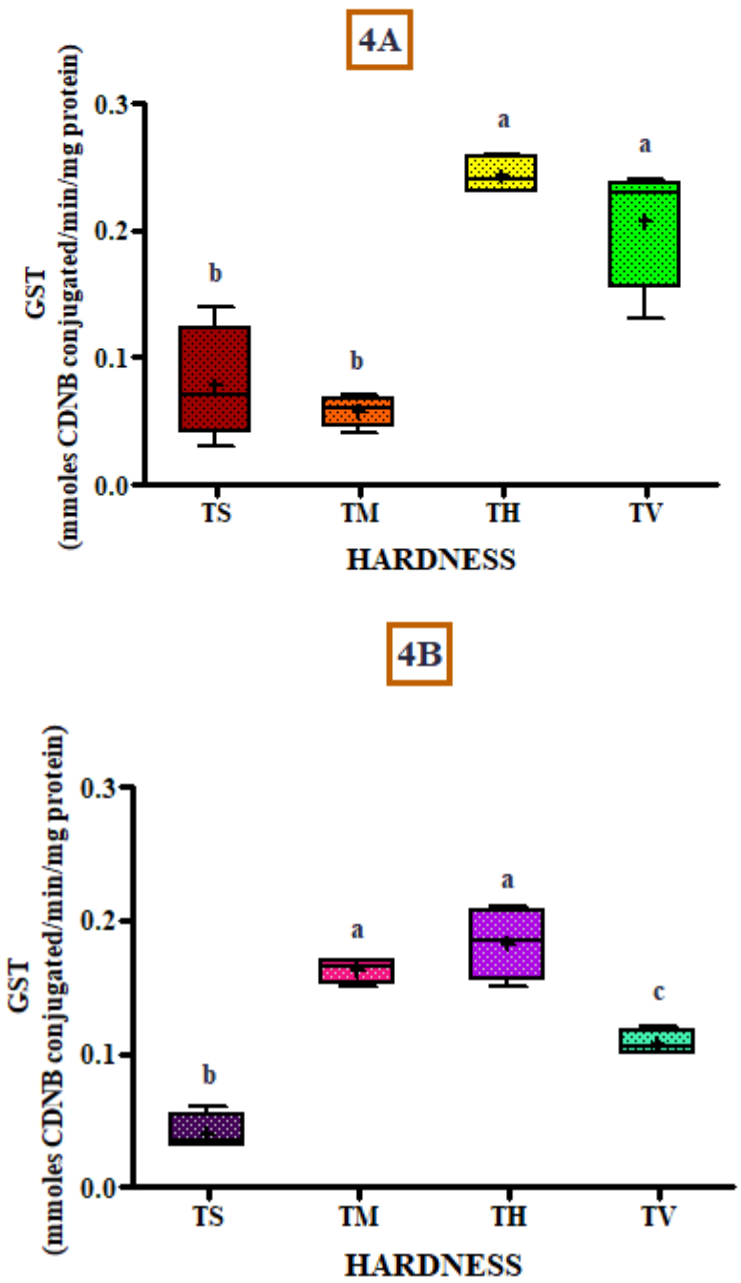

Figure 4 - Box plot presentations for Glutathione-S-transferase (GST) activity in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

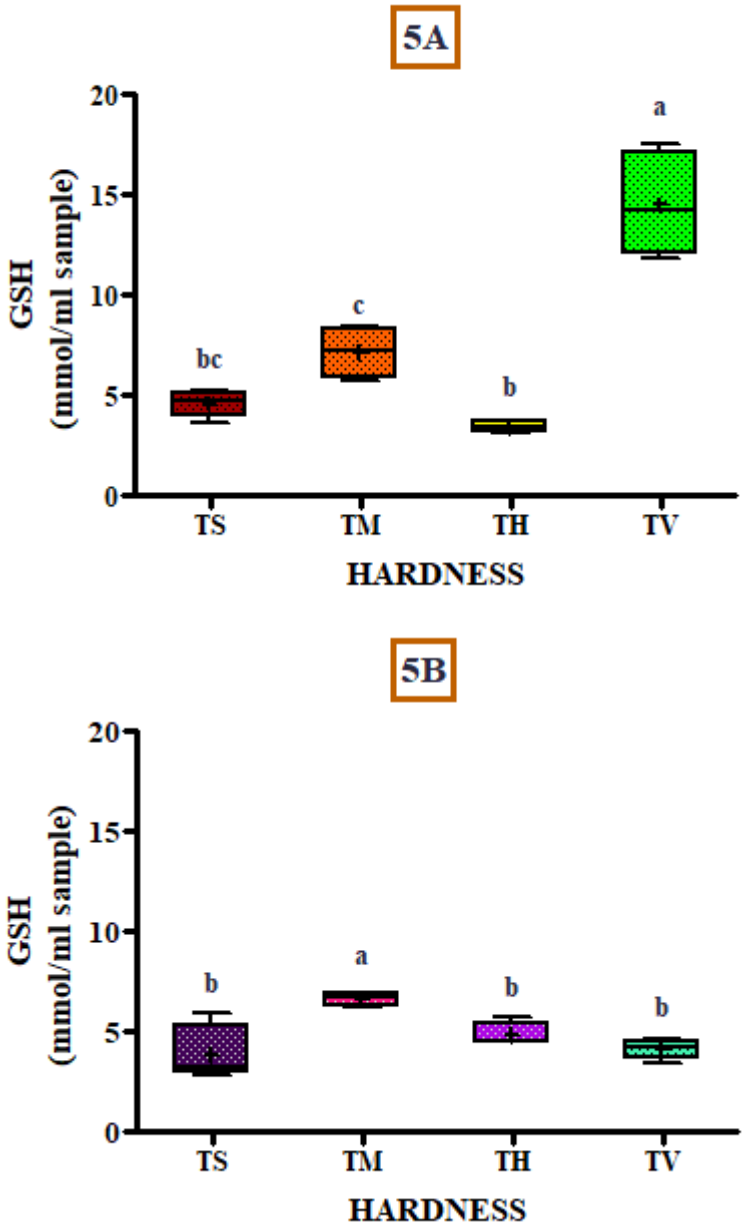

Figure 5 - Box plot representations for Glutathione (GSH) in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.