RESEARCH ARTICLE

Influence of Water Hardness on Tissue Physiology of Freshwater Fish *Cyprinus carpio* var *koi*: Report on Glucose, Oxidative Stress and Antioxidant Biomarkers

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ABSTRACT

Fishes endemic to freshwater habitat are strongly influenced by water hardness, initiating physiological changes. The present study aimed to understand the effects of a four-fold sequential increase from soft to hard waters on selected tissues of Koi carp, a commercially valued ornamental freshwater fish. Secondary stress markers, Glucose, Oxidative Stress (Malondialdehyde (MDA)/Lipid Peroxidation (LPO) and Antioxidants (Catalase (CAT)), Glutathione-S-Transferase (GST), and Glutathione (GSH) were quantified in gill and white muscle (hereafter referred as muscle) after 14 days of exposure to soft waters of 75 mg $CaCO_3$/L (TS), moderately hard waters of 150 mg $CaCO_3$/L (TM), hard waters of 225 mg $CaCO_3$/L (TH), and very hard waters of 225 mg $CaCO_3$/L (TV). Both the examined tissues were distinctly affected by soft and moderate waters. Glucose in gills ($p < 0.05$) was proportional to the rise in hardness levels. Soft, moderate, and very hard waters (75, 150, and 300 mg $CaCO_3$/L) affected gills and muscle due to elevated MDA ($p < 0.05$). CAT and GST provided considerable antioxidant protection to the tissues. Conclusively, results revealed tissue-specific differential responses and suitability of holding water hardness approximating 225 mg $CaCO_3$/L.

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1. INTRODUCTION

Hardness is perhaps the most significant physicochemical property of water because it directly or indirectly influences the osmoregulatory efficiency of fishes (Copatti and Baldisserotto 2021). Aquatic environments are constituted by varying hardness, mainly due to Calcium (Ca^{2+}) and Magnesium (Mg^{2+}), along with some trace cations (Zn^{2+}, Mn^{2+}, etc.) (Baldisserotto 2011; Romano et al. 2020). Based on the quantity of major cations, water is classified into soft water (< 75 mg $CaCO_3$/L) and hard water (> 75 mg $CaCO_3$/L) (Portz et al. 2006), both of which exert biological challenges. Soft water poses challenges to the survival of fish, causing efflux across ion channels and destabilising ionic balance, while excessive hardness can cause hypercalcemia (Wendelaar Bonga et al. 1983), leading to bone ossification (Blanksma et al. 2009; Copatti and Baldisserotto 2021). It is, therefore, quite clear that water hardness leads to physiological changes that can potentially alter the biochemistry of fishes. Without doubt, such changes can be studied through tissues that are largely impacted due to their contact with hardness (Gonzalez et al. 1998; Gundersen and Curtis 1995).

Gill and muscle have provided substantial information about adaptation to water hardness in freshwater species such as Pinfish (*Lagodon rhomboides*) and Mozambique Tilapia (*Oreochromis mossambicus*) (Carrier and Evans 1976; Flik and Verbost 1995; Wendelaar Bonga et al. 1983). 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). The tendency of muscle mitochondria to take up Ca^{2+} from external

sources and compensate for ionic stress has been reported in Mozambique tilapia (Sulochana et al. 1977). Both tissues localize various redox reactions, and such biomarker examinations provide information about physiological adjustments due to hard waters (Lushchak 2011).

Secondary stress biomarkers are instrumental in assessing the effects of hard and soft waters. Reports by Copatti et al. (2019a) and Neves et al. (2017) highlight glucose usage to evaluate the effects of water hardness. Malondialdehyde (MDA) is a marker of Lipid Peroxidation (LPO), the consequences of which are prevented by enzymatic antioxidants (Catalase, Glutathione-S-Transferase) or non-enzymatic (Glutathione), thereby rectifying the prooxidant/antioxidant ratio (Betteridge 2000; Lushchak 2016). 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 activity of GST is specific to the detoxification of xenobiotics. It conjugates GSH to various electrophiles, thereby preventing oxidative damage, although GSH can also independently scavenge free radicals to defend the tissues from stress (Srikanth et al. 2013). Despite broad insights offered by all the above, it is noteworthy that investigations involving its usage have gained momentum recently (Copatti et al. 2019b; Michelotti et al. 2018) to understand the extent and efficiency of physiological adaptations due to external hardness. Relevant information about the evaluation of secondary markers can make way for further molecular insights.

Koi carp, a commercially valued ornamental freshwater carp species, is popular among aquarists for its aesthetic features. The species has a distinct barbel, a major identifying feature that differentiates it from the closely related Goldfish (Supplementary Material S1) (Thomas 2021; Balon 2004; Kailola et al. 1993). Popularly seen in almost all domesticated ponds, aquariums, reservoirs, streams, and lakes, it is physiologically a very sturdy species even under captive conditions (Balon 2004; Liu et al. 2024a; Liu et al. 2024b; Fife-Cook and Franks 2021; Maître-Allain and Piednoir 1995). Data involving oxidative stress response and glucose fluctuations due to water hardness will boost the culture of Koi carps and can be applicable in general to the carp family. Therefore, this report aims to evaluate the effects of different levels of water hardness (75, 150, 225, and 300 mg $CaCO_3$/L) on biomarkers (glucose, oxidative stress, and antioxidant profile) in the gill and muscle of Koi carp.

2. MATERIALS AND METHODS

2.1 Acclimation and pre-exposure maintenance

Juveniles (6.70 ± 0.15 g; 5.90 ± 0.12 cm) were procured from the Ornamental Fish Research Centre (Bengaluru, Karnataka). They were randomly distributed in separate glass tanks marked as stocking tanks (8 tanks; 50 L each; 5 fish/tank). Fish were acclimated for two weeks under natural photoperiod (≈ 12 Light/12 Dark) with continuous aeration (Venus Aqua AP-608A, China) and thermostat (RS Electrical RS008A, China). They were fed twice a day (09:00 and 18:00) at 2% body weight with commercial feed pellets (Taiyo Grow, India).

2.2 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, based upon occurrence in natural systems (Stumm and Morgan 1996; Portz et al. 2006; Boyd et al. 2016; Pinheiro et al. 2021). The hardness of TS and TM was maintained by reverse osmosis (RO) treated water. The remaining levels were adjusted with Calcium carbonate ($CaCO_3$ dilutions in HCl), eventually calibrated by complexometric EDTA titration (American Public Health Association 2005). Prepared concentrations concluded with the following range: TS (74 - 77); TM (148 - 152); TH (223 - 238), and TV (298 - 304). Experimental tanks were maintained in triplicate under a static-renewal system, with randomly assigned acclimated fish. Toxicity due to accumulated faeces was prevented by drainage and renewal on alternate days (≈ 10% replacement). Tanks were covered with a mesh net to prevent the escape of fish. Water parameters were monitored every 48 hours 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) (American Public Health Association 2005).

2.3 Sampling

A total of 24 individuals (2 fish × 3 tanks × 4 hardness levels) were euthanized in clove oil solution (50 μl/L) (American Veterinary Medical Association 2020; CPCSEA 2021) and dissected for muscle and gill. Tissues were washed with ice-cold phosphate buffer (0.1 M; pH 7.4) and homogenised in a Potter-Elvehjem grinder. The homogenate (10% w/v) was centrifuged at 5000 × *g*, following which the supernatant (stored at -20°C) was retained for all

assays except Glutathione (homogenate precipitated with TCA before centrifugation). Absorbance values were measured with a spectrophotometer (Systronics UV-VIS 118, India).

2.4 Biochemical analyses

2.4.1 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. The optical density of the solution was recorded at 540 nm. Standard glucose concentration (1 mg/ml) was correlated with the sample.

2.4.2 Malondialdehyde (MDA)

Secondary product of Lipid peroxidation (LPO) - Malondialdehyde was estimated by the protocol of Niehaus and Samuelsson (1968). 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. MDA was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm.

2.4.3 Catalase (CAT)

Catalase activity was measured according to the protocol of Aebi (1984). The reaction was started by adding 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 an incubation time of 3 minutes. The difference in absorbance between the initial and final points was computed for the activity.

2.4.4 Glutathione-S-Transferase (GST)

GST activity was estimated by using the protocol by Habig et al. (1974). Reaction mixture contained supernatant, phosphate buffer (0.1 M; pH 6.5), and 2,4-Dinitrochlorobenzene (30 mM). Volume was adjusted with distilled water, after which the reaction was initiated by adding Glutathione (0.1 M). Optical density of the solution was recorded at 340 nm using a molar extinction coefficient of $9.6 \times 10^3 M^{-1}$ cm^{-1}.

2.4.5 Glutathione (GSH)

GSH was estimated according to the protocol by 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.

2.4.6 Total protein

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

3. DATA ANALYSIS

Data was summated as Mean ± SE. Normality and homoscedasticity were evaluated using the Shapiro–Wilk and Levene tests, respectively. Inter-group comparisons were performed using 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.

4. RESULTS

4.1 Mortality

There was no mortality throughout the exposure period of 14 days. However, physical exhaustion was apparent at the time of sampling in the TS- and TM-exposed fish.

4.2 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; Table 1). Soft waters showed elevated MDA, followed by a spike in TV. Only TH differed significantly ($F = 21.27$; $p < 0.001$) from the remaining treatments (Figure 2A;

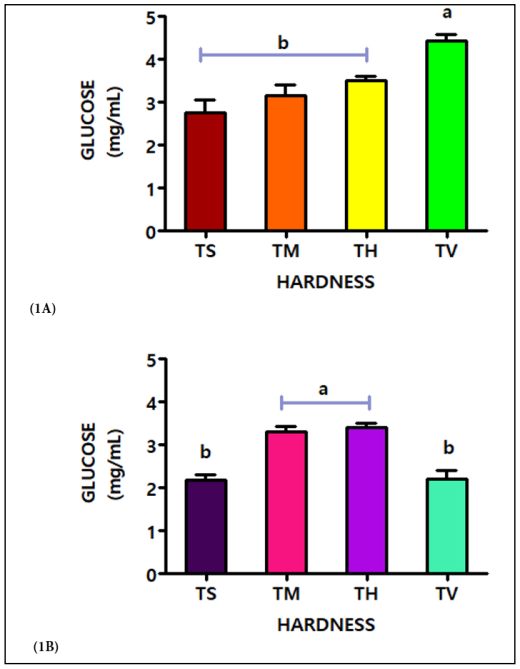

Figure 1 – Effect of 75 (TS), 150 (TM), 225 (TH), and 300 (TV) mg $CaCO_3$/L water hardness on Glucose concentration in **(A)** Gill and **(B)** Muscle of Koi carps. ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

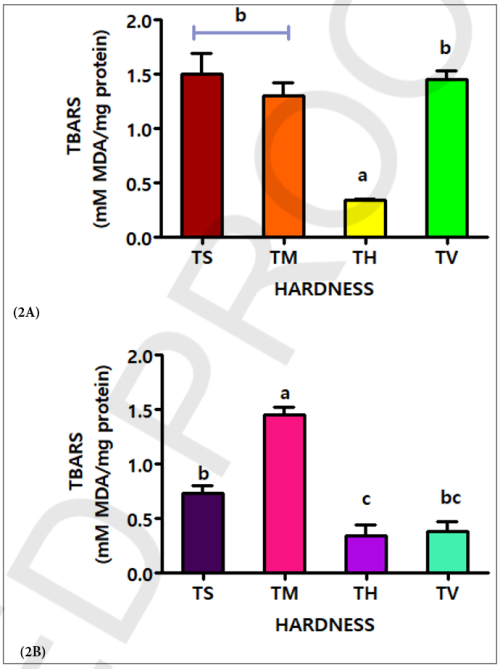

Figure 2 – Effect of 75 (TS), 150 (TM), 225 (TH), and 300 (TV) mg $CaCO_3$/L water hardness on MDA in **(A)** Gill and **(B)** Muscle of Koi carps. ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

Table 1). The 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; Table 1). 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; Table 1). The highest concentration of GSH was recorded for TV. Except for TS, which was not significant ($F = 42.78$; $p > 0.05$) with TM and TH, the remaining treatments recorded intergroup differences (Figure 5A; Table 1).

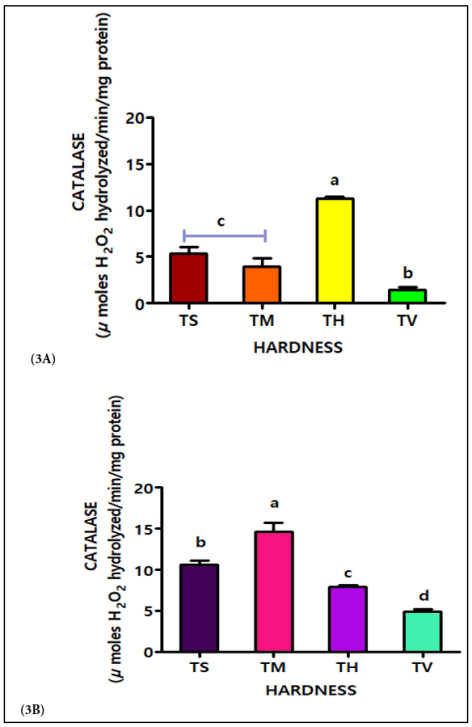

Figure 3 – Effect of 75 (TS), 150 (TM), 225 (TH), and 300 (TV) mg $CaCO_3$/L water hardness on Catalase activity in **(A)** Gill and **(B)** Muscle of Koi carps. ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

5. DISCUSSION

5.1 Effect of hardness on glucose concentration

In the present study, glucose increased sequentially in gills, indicating that it was more conserved at higher levels of hardness. Progressive hardness led to an increase in glucose, probably adding to the energy reserves. Since glucose serves as a primary energy for metabolism (Carragher and Rees 1994; da Santa Lopes et al. 2023), its estimation

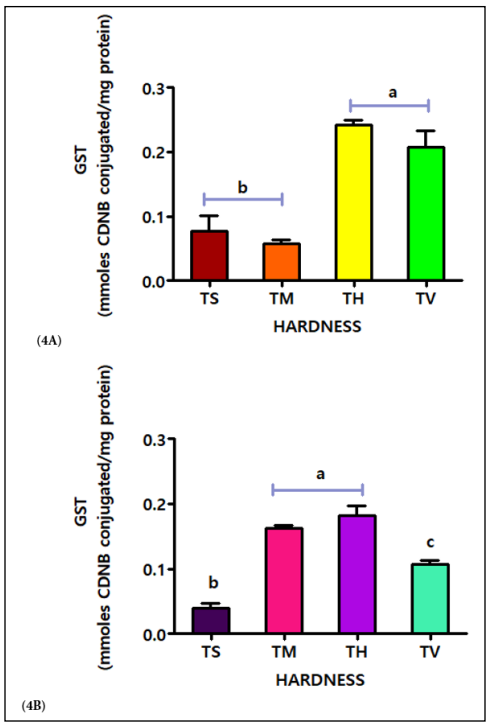

Figure 4 – Effect of 75 (TS), 150 (TM), 225 (TH), and 300 (TV) mg CaCO3/L water hardness on activity of GST in (A) Gill and (B) Muscle of Koi carps. ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

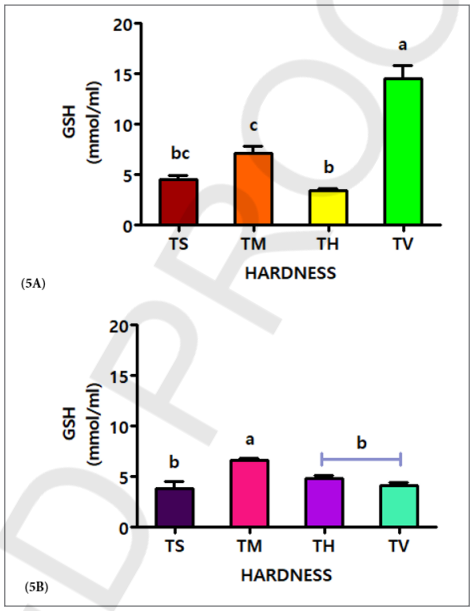

Figure 5 – Effect of 75 (TS), 150 (TM), 225 (TH), and 300 (TV) mg $CaCO_3$/L water hardness on Glutathione concentration in **(A)** Gill and **(B)** Muscle of Koi carps. ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

Table 1 – Data set (Mean ± SE) of Glucose (*GLU), Oxidative stress (†MDA) and Antioxidant profile (‡CAT - Catalase, §GST - Glutathione-S-Transferase, ¶GSH - Glutathione) in Gill and Muscle of Koi carps exposed to 75 (TS), 150 (TM), 225 (TH) and 300 (TV) mg $CaCO_3$/L of water hardness. Units expressed as: Glucose (mg/ml), MDA (mM MDA/mg protein), Catalase (μ moles H_2O_2 hydrolyzed/min/mg protein), GST (mmoles CDNB conjugated/mg protein), and GSH (mmol/ml). ANOVA descriptions: Non-identical superscript indicates statistical significance between groups ($p < 0.05$); At least one identical superscript indicates non-significance ($p > 0.05$) between groups.

	TS	TM	TH	TV
		GILL		
***GLU**	2.75 ± 0.30 b	3.15 ± 0.25 b	3.50 ± 0.10 b	4.42 ± 0.16 a
†MDA	1.50 ± 0.18 b	1.29 ± 0.12 b	0.34 ± 0.01a	1.45 ± 0.07 b
‡CAT	5.35 ± 0.71c	3.93 ± 0.86 c	11.25 ± 0.24 a	1.47 ± 0.22 b
§GST	0.07 ± 0.02 b	0.05 ± 0.006 b	0.24 ± 0.007 a	0.20 ± 0.02 a
¶GSH	4.55 ± 0.33 b c	7.11 ± 0.65 c	3.42 ± 0.15 b	14.47 ± 1.32 a
		MUSCLE		
***GLU**	2.17 ± 0.11 b	3.30 ± 0.12 a	3.40 ± 0.10 a	2.20 ± 0.20 b
†MDA	0.72 ± 0.07 b	1.45 ± 0.06 a	0.34 ± 0.09 c	0.38 ± 0.08 b c
‡CAT	10.62 ± 0.45 b	14.65 ± 1.04 a	7.94 ± 0.15 c	4.89 ± 0.26 d
§GST	0.04 ± 0.007 b	0.16 ± 0.004 a	0.18 ± 0.01 a	0.10 ± 0.004 c
¶GSH	3.80 ± 0.72 b	6.60 ± 0.17 a	4.80 ± 0.30 b	4.11 ± 0.25 b

can provide insights into the energy consumption for adaptations to hardness. Freshwater fish 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 (Flik and Verbost 1995). Generally, an environment with high hardness reduces gill permeability (through tightening cellular junctions) and subsequent loss of ions to water, ultimately conserving energy (Golombieski et al. 2013). This is clear through the results of the present study. Contrarily, muscle showed reductions in glucose at TV (300 mg $CaCO_3$/L), which was also observed in juvenile Common Snook (*Centropomus undecimalis*) exposed to elevated hardness (Michelotti et al. 2018). Probably, increased energy demands lowered muscle glucose levels and upregulated glycolysis.

5.2 Effect of hardness on lipid peroxidation

The presence of enormous amounts of Polyunsaturated Fatty Acids (PUFAs) predisposes fishes to peroxidation, ultimately damaging the cell membrane (Lushchak 2011), which is proportional to MDA. In the present study, excluding 225 mg $CaCO_3$/L, MDA for the remaining exposures was elevated in the gills. Soft (75), moderate (150), and very hard (300) waters quite possibly led to damage to the gills. In environments with low ionic concentration, certain membranes (such as the apical membrane of the gill) mechanize the uptake of divalent cations from hard waters through Ca^{2+} channels embedded in them to meet the demand for necessary biological processes (Limbaugh et al. 2021). Presumably, this leads to a burden on the tissues, causing membrane damage and cell injury as indicated by elevated MDA. Further, interaction of pH and hardness might provoke peroxidation (Copatti et al. 2019b; Diggs and Parker 2009; McWilliams and Potts 1978; Parker et al. 1985). Hard waters dynamically affect pH due to higher cationic levels, cascading buffering action. Contrarily, soft waters (lower cations) favour acidification; therefore, pH in soft water adversely affects fishes (Boyd 1998; Townsend et al. 2003; Townsend and Baldisserotto 2001). This plausibly impacts tissue physiology and aggravates oxidative stress, as evidenced by elevated MDA in Koi exposed to TS and TM.

5.3 Effect of hardness on antioxidant response

Low Catalase activity in gills exposed to soft (75) and moderate (150) waters indicated the failure to prevent oxidative damage. As already known, low ionic composition of freshwater environments is osmotically taxing for gills due to loss of ions to water (Hunn 1985; McDonald and Robinson 1993). The efflux of ions to the external environment in hardness < 150 mg $CaCO_3$/L might have led to oxidative stress in Koi carps. A noteworthy observation in gill is the sharp increase in Catalase activity at hard waters (225), which indicates adaptive efficiency of Koi carps at this concentration. Contrarily, muscle showed a sequential decrease in antioxidant activity with exposures above 150 mg $CaCO_3$/L, largely remaining unaffected. Given the elevated expression of Catalase at moderate exposures (TM), it proves that antioxidative activity was more robust in muscle than in gill.

Increase in GST activity was observed in gills exposed to hard (225) and very hard waters (300). On the contrary, muscle showed elevated GST activity for all the exposures except soft waters (75). Though higher GST activity clearly indicated greater antioxidant capacity in both the tissues, by far, the antioxidative response was greater in muscle than in gills. Also, compared to Catalase, GST activity was much higher in both the tissues for exposures above 225 mg $CaCO_3$/L, indicating better antioxidant activity at higher levels of water hardness.

Antioxidant GSH was relatively lower in muscle than in gills, indicating better muscle antioxidant capacity. In gills, a spike was observed for TH, which is conclusive that anything beyond 300 mg $CaCO_3$/L is harmful. GSH can scavenge free radicals independently or in conjunction with GST to provide antioxidant defence (Srikanth et al. 2013). The present study showed variance in GSH for both tissues, conforming to its specificity or tissue-specific antioxidant response. This has previously been reported in other popular freshwater species such as Nile Tilapia (*Oreochromis niloticus*), Sharp Tooth Catfish (*Clarias lazera*) and Common carp (*Cyprinus carpio*) (Hamed et al. 2004).

6. CONCLUSION

In conclusion, hardness of 75 and 150 mg $CaCO_3$/L can lower glucose reserves and cause oxidative stress in the tissues of Koi carps. On the contrary, the fish can efficiently adapt to 225, rather than 300 mg $CaCO_3$/L. At such hardness, glucose was also found to be conserved, a feature that metabolically benefits the carps and might be useful for aquaculture.

CONFLICT OF INTEREST

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

ETHICS STATEMENT

All methods and animals used within this study were in accordance with the Good Scientific Practice guidelines (GSP) and national legislation (CPCSEA Guidelines of 2021).

AUTHOR CONTRIBUTIONS

The author confirms the sole responsibility for the conception of the study, presented results and manuscript preparation.

AVAILABILITY OF DATA AND MATERIAL

All the relevant data is tabulated and presented as graphics within the article. Please contact the corresponding author for any further queries.

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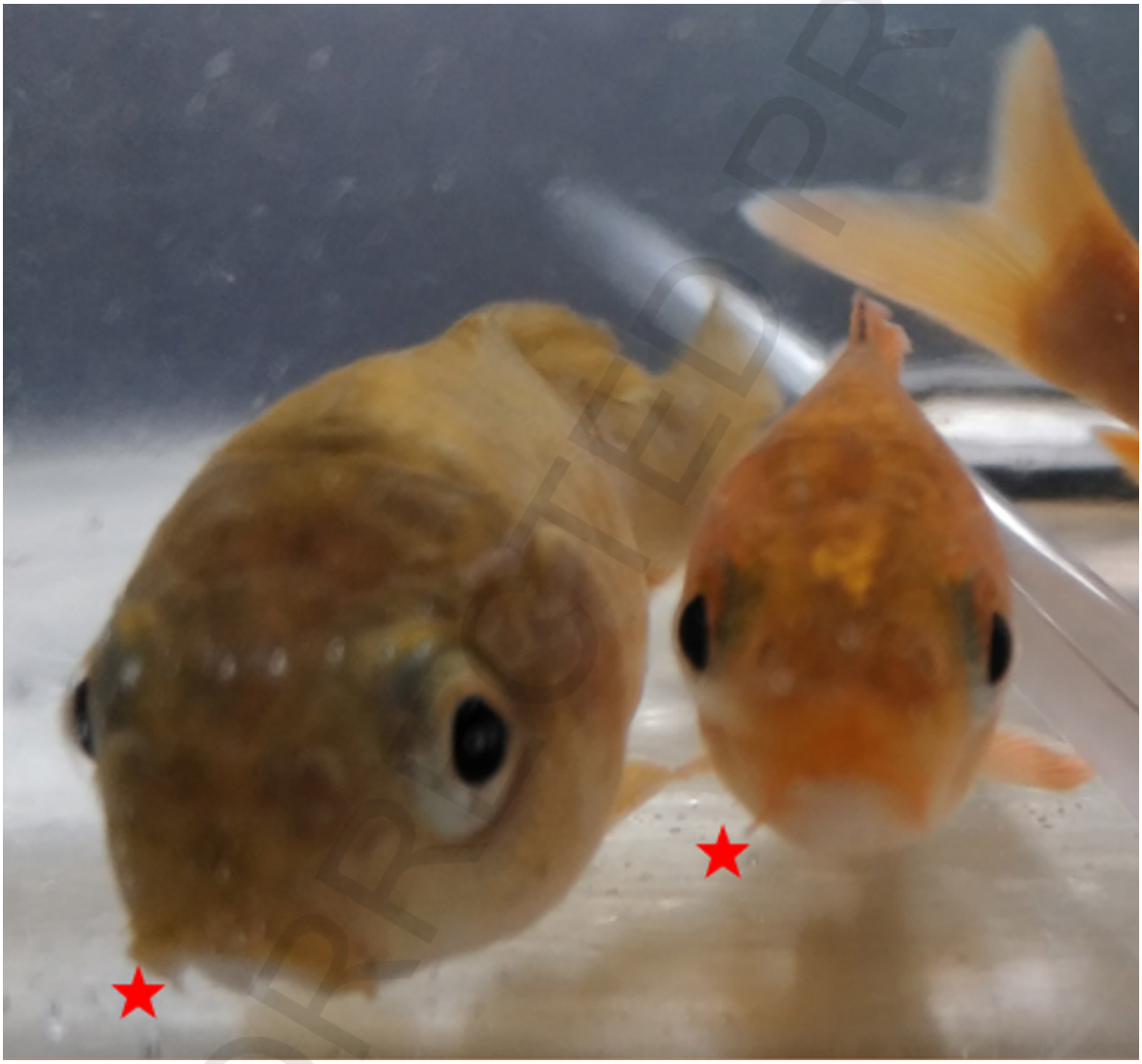

Supplementary Material S1. Test species - *Cyprinus carpio* var *koi*.
★ indicate barbel, a distinct morphological feature