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Impact of transient temperature disturbance on the oxidative stress indices and glucose levels of juvenile Koi carps (*Cyprinus carpio* var *koi*)

Bela Zutshi, Aradhana Singh and Proteek Dasgupta*

Abstract

Background: The present study reports the changes in the redox state and glucose activity in gills and muscles of Koi carps, exposed to a short duration of acute thermal stress. The variation in levels of lipid peroxidation (LPO) and glutathione (GSH), catalase (CAT) and glutathione-S-transferase (GST) activity, in addition to glucose levels, were analysed after exposing the fishes to 15 °C (low), 25 °C (control) and 35 °C (high) for a duration of 3 and 6 h. The upper and lower temperatures regimes were decided by the Critical Thermal Limit (CT_{MAX} and CT_{MIN}).

Results: The CAT activity was high in both the tissues at 35 °C within 3 h, while LPO activity decreased throughout the study when compared with the control group indicating immoderate cellular disturbance and surplus oxidative stress. At 15 °C (3 h), tissues reported more glucose compared with the upper thermal slab. GSH increased in both the tissues at 15 °C compared with the control for 3 and 6 h. GST activity, however, flourished at 35 °C in muscles and gills for the 3-h regime.

Conclusion: The current study demonstrated disturbing impacts of temperature on the survivability of the Koi carps in a domesticated environment.

Keywords: Transient temperature, Oxidative stress, Glucose, Koi carps, Juveniles, Gills, Muscles

Background

Temperature is an important physiological regulator and primarily affects the body metabolism of aquatic organisms. Temperature disruption might affect a variety of activities ranging from lifestyle (Freitas et al., 2017), behaviour and swimming patterns (Campos, Val, & Almeida-Val, 2018) to breeding and reproduction (Madeira, Vinagre, & Diniz 2016a), predator-prey dynamics (Allan, Paolo, Munday, & McCormick, 2015) and most importantly, longevity (Madeira, Madeira, Diniz, Cabral, & Vinagre 2016b; Hsu & Chiu, 2009; Dong, Dong, & Ji, 2008). Acute temperature level has been closely associated with the generation of reactive oxygen/nitrogen species or the free radicals through mitochondria (Lushchak, 2016), thereby inducing oxidative stress (Abele & Puntarulo, 2004) and fishes are no exception to the same.

Lipid peroxidation products are among the best biomarkers for monitoring and analysing the cellular damage due to the generation of free radicals in aquatic organisms. This is substantiated by the fact that the fishes have high quantity polyunsaturated fatty acid residues that form an excellent substrate for the oxidation of fatty acids (Lushchak, 2011). The architecture of lipid peroxides is very balanced at normal physiological temperatures; however, they are prone to lose their stability once they are subjected to altered levels outside the ambient range (Gutteridge, 1995).

Superoxide radical ($O_2^{\bullet -}$) is reduced to hydrogen peroxide (H_2O_2) and water through the first line of the antioxidant enzymes, the superoxide dismutase. Further, H_2O_2 is converted into water and oxygen by catalase (Di Giulio, Washburn, Wenning, Winston, & Jewell, 1989;

* Correspondence: proteek.dasgupta@gmail.com
Department of Zoology, Aquaculture Laboratory, Bangalore University, Bengaluru, Karnataka 560056, India

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Lushchak, 2016). In case, catalase is not able to comprehend the reaction then, H_2O_2 eventually converts to highly reactive hydroxyl radical ($OH^{\bullet}$) by the Fenton reaction. Any minute change in the metabolic balance due to stress might disrupt the enzymatic action of catalase.

The glutathione family of non-enzymatic antioxidants works on similar lines of catalase. Through, the multi-reactional approach, both GST and GSH along with other members remove H_2O_2 and are largely instrumental in quenching free radicals (Di Giulio et al., 1989), thereby alleviating oxidative stress. A 5 °C temperature variance in fishes might elevate the glutathione level and it would become more oxidised, thereby harmonizing the intracellular redox system (Kaur, Atif, Ali, Rehman, & Raisuddin, 2005; Lushchak & Bagnyukova, 2006; Parihar & Dubey, 1995). Glucose, as a stress indicator has majorly been associated with a variety of stress factors (Kumar, Krishnani, & Singh, 2018; Wells & Pankhurst, 1999). Glycogenolysis and glucogenesis pathways elevate the production of glucose, which in mutual terms facilitates the cortisol level to counter the stressful conditions (Helly Jr., 1976).

Ornamental fish industry is a rapidly growing innovative economic venture and a major source of global livelihood. *Cyprinus carpio* var *koi*, an ornamental strain of the common carp is popular for its aesthetic values and coloration among aquarists. It is an endemic species of Japan, however, grown worldwide (Mabuchi, Seno, Suzuki, & Nishida, 2005; Tripathi, Latimer, Lewis, & Burnley, 2003). Like any ornamental fish, oxidative stress and associated survivability will undoubtedly influence the commercial importance of Koi carps in any habitat as there is no “zero stress” environment. Koi carps, known for displaying flashy colours has also been a model organism for the study of pigmentation pathways. Therefore, the present study was conducted to assess the role of temperature as a stress factor and evaluate the response through the quantification of glucose and antioxidant enzymes.

Materials and methods

Fish acclimatisation

Healthy juvenile Koi carps (5.00 ± 0.34 g) were procured from Ornamental Fish Research Centre, Bengaluru, Karnataka. The fishes were acclimatised to laboratory condition for 14 days in 30 L glass water tanks filled with normal tap water, installed with aerator and thermostat. They were kept under natural daylight and darkness (12 L: 12D) and fed with commercial feed pellets at ad libitum. The pH, temperature and dissolved oxygen level of water were recorded to be 7.04 ± 0.1, 25 ± 1 °C (control temperature) and 6.2 mL/L respectively. Hardness was found to be negligible. The physico-chemical standards of water were maintained according to APHA, AWWA, WPCF (2005).

Critical temperature (CT_{MIN} and CT_{MAX})

Critical temperature (CT) is the degree at which the locomotor activity of the fish is seized which eventually leads to death called temperature of lethality (LT) (Moyano et al., 2017). The CT_{MIN} and CT_{MAX} was recorded by increasing and decreasing the temperature of 2 separate tanks by a magnitude of 1 ° every 1 h starting from 25 °C (control temperature). This was finally found to be 15 °C (CT_{MIN}) and 35 °C (CT_{MAX}).

Experimental design

Fishes were maintained at three different temperature levels for experimentation viz., 15 °C ± 1 °C (low), 25 °C ± 1 °C (control) and 35 °C ± 1 °C (high). Two replicates with 5 fishes in every tank were maintained for each temperature level. The setup was maintained for two time periods viz, 3 and 6 h. At the end of each time slot, the fishes were sacrificed by transferring them into clove oil solution (1 mL/L) till complete operculum arrest.

Tissue homogenate preparation

The gills and dorsal white muscles were carefully dissected and washed in ice-cold buffer solution (phosphate buffer, 0.1 M, pH 7.4). Tissues were quashed, and 10% homogenate was made in a glass/Teflon Potter-Elvejhem tissue grinder. The samples were centrifuged at 5000×*g* and the supernatants were stored immediately at −20 °C, until biochemical analyses. All the absorbance values were recorded using visible spectrophotometer (Systronics).

Biochemical analyses

Lipid peroxidation assay

LPO was estimated by the the method of Niehaus and Samuelsson (1968). A mixture of trichloroacetic acid (15%), thiobarbituric acid (0.38%) and hydrochloric acid (0.25 N) was made in equal proportions. A sample of 0.5 mL was mixed with 1 mL of the TCA-TBA-HCl reagent. The reaction mixture was heated in a boiling water bath for 15 min, cooled and centrifuged at 1100×*g*. The absorbance of the supernatant was read at 535 nm using a spectrophotometer. The rate of peroxidised lipid in each sample was measured as mM malondialdehyde (MDA) per mg protein.

Catalase

Catalase activity was measured by the method of Sinha (1972). Briefly, 0.9 mL of phosphate buffer (0.01 M, pH 7.4), 0.4 mL of H_2O_2 (0.2 M) was mixed properly. The reaction was initiated by adding 0.4 mL of tissue homogenate and after 30 s, 2 mL of the dichromate-acetic acid reagent (5% potassium dichromate in glacial acetic

acid) was added. The reaction mixture was kept for a boiling water bath for 10 min and then cooled. The absorbance of the colour was read at 610 nm using a spectrophotometer. Catalase activity was recorded as μmole H_2O_2 decomposed/min/mg protein.

Glutathione-S-transferase

GST activity was measured spectrophotometrically at 340 nm by the protocol of Habig, Pabst, and Jakoby (1974). The reaction mixture contained sample, phosphate buffer (0.1 M; pH 6.5), distilled water and 30 mM of 2,4-Dinitrochlorobenzene (CDNB) amounting to 2.5 mL. The activity was started by the addition of 0.1 M GSH. The activity was expressed as mmoles CDNB conjugated/mg protein.

Glutathione (reduced)

GSH activity was measured according to the method described by Moron, Depierre, and Mannervik (1979). The reaction mixture consisted of 3 mL phosphate buffer, 0.1 mL of the tissue homogenate and 0.5 mL Ellman's reagent. The solution was read spectrophotometrically at 420 nm and expressed as mmol/mL sample.

Glucose

Glucose level was assayed according to Nelson and Smogyi (Nelson, 1944; Smogyi, 1952). A 4 mL of reaction mixture (sample and deproteinizing agent $Ba(OH)_2$; $ZnSO_4$) was centrifuged at 5000×*g* for 10 min. A 1 mL of this supernatant was added to 1 mL alkaline copper reagent (potassium-sodium tartrate; Na_2CO_3; $NaHCO_3$ and Na_2SO_4 in distilled water). This mixture was heated in a boiling water bath for 20 min and cooled after which arseno-molybdate reagent and distilled water was added. The colour developed was read at 540 nm using a spectrophotometer and the concentration expressed as % mg glucose.

Protein

Protein was estimated according to Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard at 660 nm.

Statistical analyses

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Inc. CA, USA). Data was represented as mean ± SE. The mean difference was determined by two-way analysis of variance (ANOVA), followed by Bonferroni post-test. Statistical threshold was fixed at 95% probability value ($p < 0.05$).

Results

Lipid peroxidation

In the gills, MDA level ranged from 0.45 ± 0.04 mM MDA/mg protein (15 °C; 3 h) to 4.01 ± 0.54 mM MDA/mg protein (35 °C; 6 h) (Fig. 1a). In the muscles, the level ranged from 0.57 ± 0.10 (15 °C; 3 h) to 1.81 ± 0.14 mM MDA/mg protein (15 °C; 6 h) (Fig. 1b). Compared with the control, the levels of MDA were low in both the tissues, throughout the study. Temperature exerted a greater effect than time for LPO levels on the gills, while, time as an individual entity did not have any significant impact on the muscles (Table 1).

Catalase

The catalase activity ranged from 3.66 ± 0.52 (15 °C; 3 h) to 8.825 ± 0.34 μmole H_2O_2 decomposed/min/mg protein (35 °C; 3 h) in gills (Fig. 2a). This was higher compared with the muscles that ranged from 2.65 ± 0.23 (15 °C; 3 h) to 4.32 ± 0.62 μmole H_2O_2 decomposed/min/mg protein (35 °C; 3 h) (Fig. 2b). Both tissues reported the maximum catalase activity at 35 °C (3 h period) (8.82 ± 0.69 (gills) and 4.32 ± 1.24 (muscles)). Further, the catalase activity in the gills was elevated throughout the study at all temperatures as opposed to the muscles, when compared with the controls (Table 1).

Glutathione S-transferase

The GST activity ranged from 0.33 ± 0.01 mmoles CDNB conjugated/mg protein (35 °C; 6 h) to 12.68 ± 0.54 mmoles CDNB conjugated/mg protein (35 °C; 3 h) for the gills (Fig. 3a). In the muscles, it was from 0.34 ± 0.01 mmoles CDNB conjugated/mg protein (15 °C; 6 h) to 14.25 ± 0.06 mmoles CDNB conjugated/mg protein (35 °C; 3 h) (Fig. 3b). A significant spike in the GST activity was noted at 35 °C (3 h) which recorded 12.68 ± 0.54 mmoles CDNB conjugated/mg protein for the gills and 14.25 ± 0.06 mmoles CDNB conjugated/mg protein for the muscles (Table 1).

Glutathione reduced

GSH level was elevated throughout the study in both the tissues at all the experimental temperature-time levels, compared with the control. The value in the gills ranged from 3.14 ± 0.54 mmol/mL sample (35 °C; 3 h to 12.89 ± 0.40 mmol/mL sample (15 °C; 3 h) (Fig. 4a). In the muscles, it ranged from 2.21 ± 0.26 mmol/mL sample (35 °C; 6 h) to 5.78 ± 0.13 mmol/mL sample (35 °C; 3 h) (Fig. 4b). Significant differences were observed in the experimental temperature regimes when compared to the controls for both 3 and 6 h (Table 1).

Glucose

Barring 15 °C, the gills reported a nearly half fold glucose level when compared with the 25 °C (control group). The highest value for glucose was found at 15 °C for the 3-h regime for both the tissues (610 ± 170.9% mg glucose (gills) and 830 ± 132.2% mg glucose (muscles)) (Fig. 5a). While in the muscles, the range was observed as 199 ± 1.43% mg

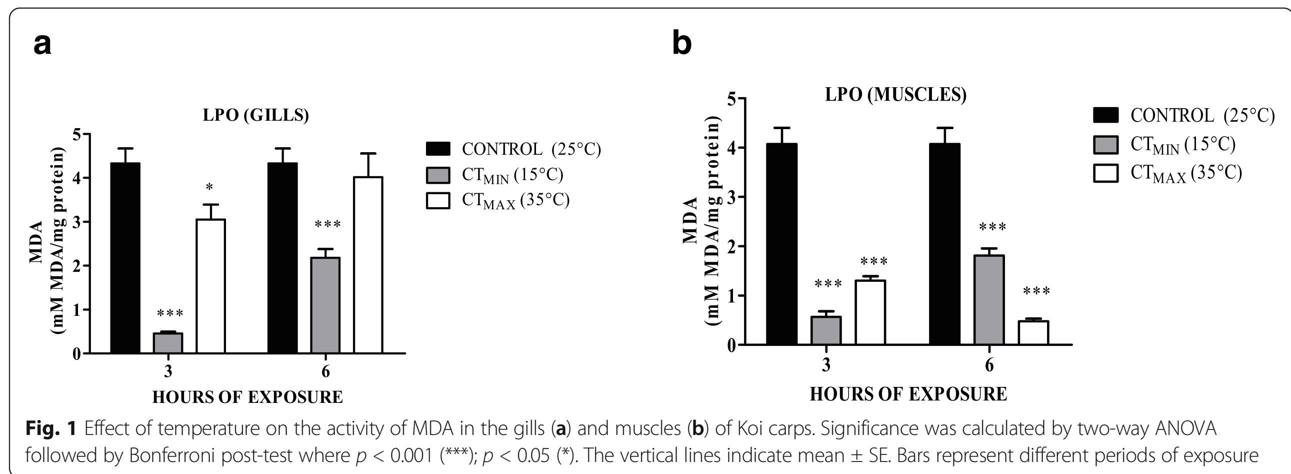

Fig. 1 Effect of temperature on the activity of MDA in the gills (**a**) and muscles (**b**) of Koi carps. Significance was calculated by two-way ANOVA followed by Bonferroni post-test where $p < 0.001$ (***); $p < 0.05$ (*). The vertical lines indicate mean ± SE. Bars represent different periods of exposure

glucose (35 °C; 6 h) to 830 ± 66.08% mg glucose (15 °C; 3 h) (Fig. 5b). Time had more significant effects (44.89%) rather than the temperature for the glucose activity in the muscles (Table 1). However, in the case of the gills, the temperature had a greater (47.90%) impact on the tissue (Table 1).

Behavioural observations

We observed signs of body fatigue prompted by the exposed temperature levels. The swerved swimming pattern was mainly noted at 35 °C, while at 15 °C, they became lazy and spent time majorly at the bottom of the tank. Laziness, slow secretion of mucous, change in the coloration of the gills as well as body and shedding of scales were majorly observed at both the temperature levels.

Discussion

The present study focuses on the sustainability of the Koi carps in domesticated environments at two experimental

Table 1 Significance levels of the two-way ANOVA testing effects of temperature (temp: 15 °C, 25 °C and 35 °C) and time (time: 3 and 6 h) on organs (gills and muscles) of *Cyprinus carpio* var *koi* for MDA, catalase, GST, GSH and glucose

Enzyme	Tissue	Source of variation	Interaction(%) (temp vs time)	df	SS	MSQ	*F*	*P* value
MDA	Gill	Temp	70.79	2	38.89	19.45	42.48	<0.0001
		Time	8.76	1	4.815	4.815	10.52	0.0045
	Muscle	Temp	86.8	2	49.43	24.71	145.2	<0.0001
		Time	0.21	1	0.119	0.119	0.699	0.4141
Catalase	Gill	Temp	85.2	2	113.4	56.72	73.13	<0.0001
		Time	0.23	1	0.3038	0.3038	0.3916	0.5393
	Muscle	Temp	61.82	2	20.66	10.33	22.14	<0.0001
		Time	0.74	1	0.246	0.246	0.5272	0.4771
GST	Gill	Temp	32.13	2	146.4	73.18	284	<0.0001
		Time	22.62	1	103	103	399.9	<0.0001
	Muscle	Temp	36.89	2	224.8	112.4	9356	<0.0001
		Time	21.14	1	128.9	128.9	10720	<0.0001
GSH	Gill	Temp	84.46	2	294.7	147.4	364.8	<0.0001
		Time	2.5	1	8.712	8.712	21.57	0.0002
	Muscle	Temp	37.51	2	18.15	9.073	38.61	<0.0001
		Time	13.48	1	6.521	6.521	27.75	<0.0001
Glucose	Gill	Temp	47.9	2	305800	152900	26.68	<0.0001
		Time	11.06	1	70630	70630	12.32	0.0025
	Muscle	Temp	8.3	2	112800	56390	4.169	0.0325
		Time	44.89	1	609600	609600	45.07	<0.0001

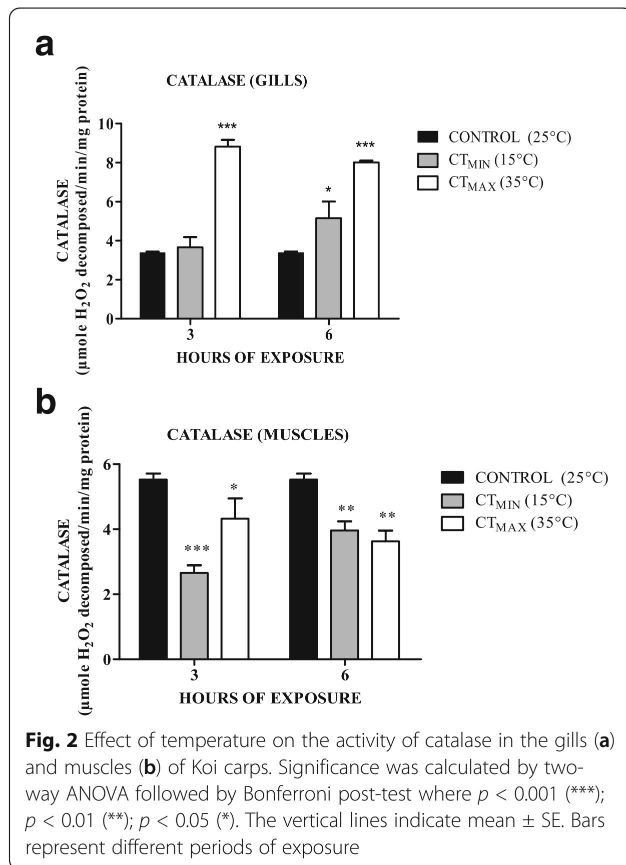

Fig. 2 Effect of temperature on the activity of catalase in the gills (**a**) and muscles (**b**) of Koi carps. Significance was calculated by two-way ANOVA followed by Bonferroni post-test where $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*). The vertical lines indicate mean ± SE. Bars represent different periods of exposure

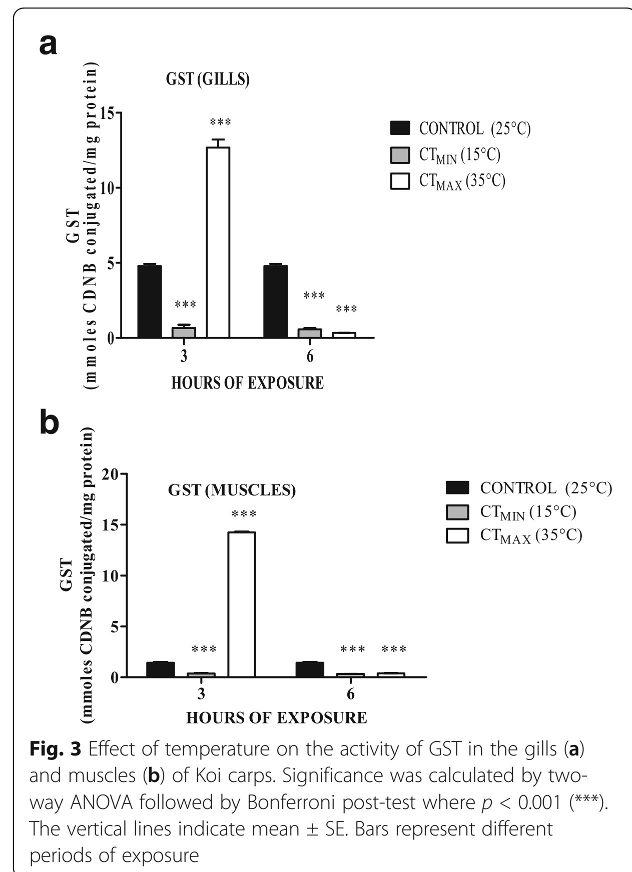

Fig. 3 Effect of temperature on the activity of GST in the gills (**a**) and muscles (**b**) of Koi carps. Significance was calculated by two-way ANOVA followed by Bonferroni post-test where $p < 0.001$ (***). The vertical lines indicate mean ± SE. Bars represent different periods of exposure

sub-lethal temperatures amounting to short-term stress. For any aquatic organism, the temperature has closely been associated with several metabolic processes such as oxygen consumption (Das et al., 2005; Koopman, Collas, van der Velde, & Verberk, 2016), biochemical composition, growth and reproduction (Tropea, Stumpf, & López Greco, 2015) and behaviour (Padilla-Ramírez et al., 2015).

The present study shows a significant impact of temperature on the MDA levels of the gills and muscles, which was dependent on the source of variation between time and temperature. An increasing trend for MDA was observed in our study initiating from 15 °C to 35 °C, likewise, reported in *Dicentrarchus labrax* (Vinagre, Madeira, Narciso, Cabral, & Diniz, 2012) and *Sparus aurata* (Madeira, Madeira, et al., 2016a). Perhaps, the temperature gradient is proportional to the level of MDA, which has also been reported in *Heteropneustes fossilis* by Parihar and Dubey (1995). Generally, as the time increases, high temperatures disrupt the lipid stability and therefore decomposition of the fatty acids might produce free radicals (Joy et al., 2017). Hence, we believe that the increase in the temperature at the short duration has the same analogy as that of longer duration.

Akhtar, Pal, Sahu, Ciji, and Mahanta (2013) and Vinagre et al. (2012) have accounted for the activity of catalase as an antioxidant enzyme and its modulation under temperature stress. Our results showed an increasing trend of catalase activity in the gills for 3- and 6-hour exposure period (15 °C and 35 °C) as compared with the muscle (6 h), indicating that the temperature stress has induced a more stubborn defence in the gills than the muscles. Such a trend was also observed in *Paralichthys orbignyanus* from a transition of 23 °C to 28.8 °C (Garcia et al., 2015). In our study, catalase antioxidant activity outweighed the LPO levels of the muscles at both the experimental temperature-time levels indicating the surprising capacity of the fish to overcome stress in a short period.

Leggatt, Brauner, Schulte, and Iwama (2007) and Bagnyukova, Lushchak, Storey, and Lushchak (2007) have reported the importance of glutathione family and their coordinated responses to counteract oxidative stress. The present study demonstrated higher GSH level than that of control temperature for both the thermal levels and tissues. Nakano et al. (2014), also reported the elevated GSH levels in the liver of *Oncorhynchus kisutch*. The initial 3-h exposure (15 °C and 35 °C) recorded a consistent increase in the GSH levels of the muscles, therefore, leading to a significant impact of both temperature and time, on the expression of GSH levels. This was similar in case of two species analysis of *Notothenia* (*Notothenia coriiceps* and *Notothenia rossii*) (Machado et al., 2014).

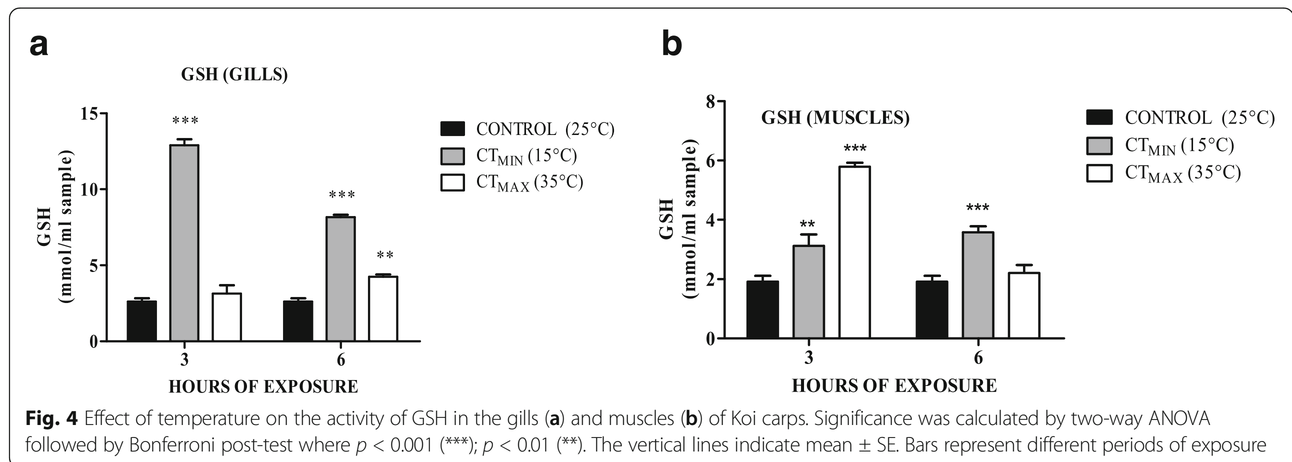

Fig. 4 Effect of temperature on the activity of GSH in the gills (**a**) and muscles (**b**) of Koi carps. Significance was calculated by two-way ANOVA followed by Bonferroni post-test where $p < 0.001$ (***); $p < 0.01$ (**). The vertical lines indicate mean ± SE. Bars represent different periods of exposure

Glutathione metabolism is affected by various temperature fluctuations irrespective of the time (Lushchak & Bagnyukova, 2006; Nakano et al., 2014). Initially, for a 3-h duration (35 °C), both the tissues reported GST increase along with the temperature. However, contrary to our expectations, the level reduced drastically as the duration proceeded to 6 h for 35 °C. Rossi, Bacchetta, and Cazenave (2017) have observed a similar shift in the gills of *Hoplosternum littorale* from 10 °C to 33 °C. It might be considered that antioxidant enzyme responses might depend upon the conditions, the organism is exposed towards. For example, Madeira, Narciso, Cabral, Vinagre, and Diniz (2013) reported a species-specific experimental analysis of temperature affected GST in *Diplodus vulgaris* and found that its proliferation is facilitated as the CT_{MAX} is approached.

Glucose is majorly involved in necessity-based transformation of chemical to mechanical energy. Being a primary energy molecule, it is extremely sensitive to varying levels of stress and its regulation is done according to the feedback mechanism (Jiang, Wu, Huang, Ren, & Wang, 2017). Wells and Pankhurst (1999) have also reported a positive correlation of stress with glucose. We observed an initial elevation of glucose in the gills and muscles at 15 °C (3-h and 6-h exposure), which further reduced as the temperature was raised. A similar condition has been studied in *Sebastes mystinus* at 5 °C, which suggested a reduced need for ATP and its associated metabolites and metabolism at low temperature, concluding with an evident reduction in glycolysis. In *S. mystinus* and *S. serranoides*, it was observed that low-temperature adaptations were useful in orienting glucose to hexose monophosphate (HMP) shunt (Helly Jr., 1976).

Conclusion

Our study attempts to draw an inference between glucose content and antioxidative profiling in the gills and muscles. We noted a fluctuation in the enzyme levels with the influence of thermal stress on the gills and muscles of Koi carps, in relation to the tolerance capacities. However, the work might vary upon the laboratory conditions and the consequences of these fluctuations might be dictated by such experimental conditions. Our work corroborates the fact that an abiotic factor like

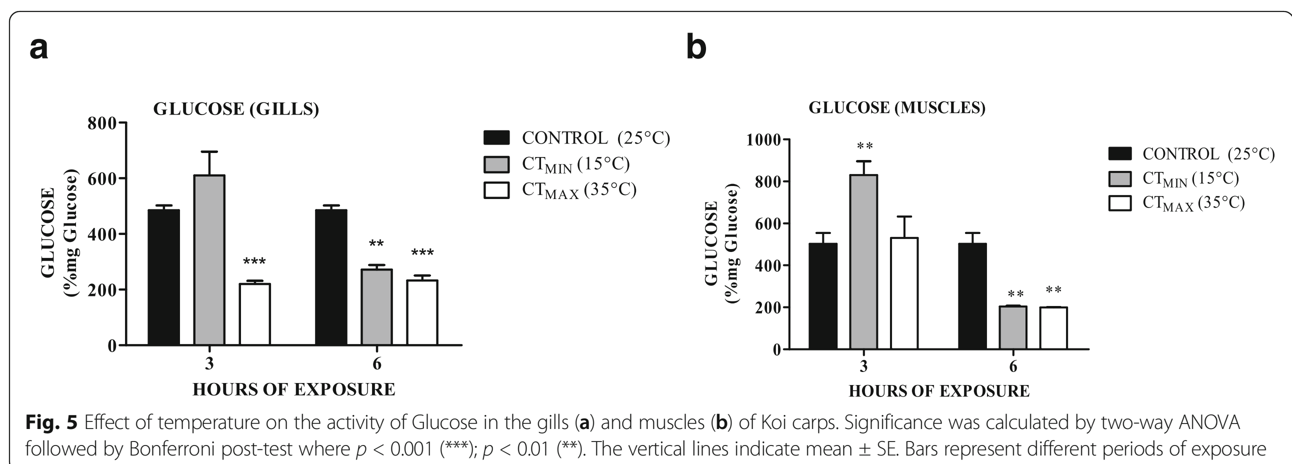

Fig. 5 Effect of temperature on the activity of Glucose in the gills (**a**) and muscles (**b**) of Koi carps. Significance was calculated by two-way ANOVA followed by Bonferroni post-test where $p < 0.001$ (***); $p < 0.01$ (**). The vertical lines indicate mean ± SE. Bars represent different periods of exposure

temperature has a great calibre to disrupt the metabolism and the energy disbursement system of the fish even in a domesticated environment as that of an aquarium. Owing to the commercial aspects, one cannot deny the importance of sustainable propagation of these fishes in the aquaculture industry. Since the rearing and caring of any aquatic organism are dependent on the abiotic factors as they are always in direct contact with water, the temperature is a major contributor to their life span in the domesticated environments.

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Authors' contributions
PD designed the research idea, over-all work and wrote the manuscript. AS helped with the experimental protocols. PD and AS worked on calculations and statistical analysis. BZ revised the drafted manuscript and made necessary corrections. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Indian researchers do not require ethical approval for research on fishes unless they belong to subclass Elasmobranchii (Rays and Sharks) (University Grants Commission (UGC) (Letter–No. F. 14-6/2014 (CPP-II) (https://www.ugc.ac.in/pdfnews/6819407_ugcletterzoology.pdf). Moreover, the present study was carried out in compliance with institutional and national guidelines for handling the experimental animals.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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