Effects of a gradual increase in temperature on the antioxidant defense system and plasma metabolic parameters in Antarctic fish Notothenia rossii

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Abstract

Antarctica is considered a thermally stable ecosystem; however, climate studies point to increases in air and surface water temperatures in this region. These thermal changes may affect the biological processes of animals inhabiting such regions because they are stress factors and may promote metabolic changes, rendering the animals more vulnerable to oxidative damage. Plasma parameters are also good indicators of stress and allow analysis of the metabolic status of fish under temperature increases. The present study assessed the effect of acclimation temperature on the levels of plasma, osmoregulatory and oxidative metabolism parameters and antioxidant defenses in kidney, gill, liver and brain tissues of *Notothenia rossii* subjected to gradual temperature changes of 0.5°C/day until reaching temperatures of 2, 4, 6 and 8°C. Under the effect of the 0.5°C/day acclimation rate, gill tissue showed increased glutathione-S-transferase (GST) activity, and kidney tissue showed increased H⁺-ATPase at 9 days of the experiment (2°C). In the liver, consistent increases in the MDA concentration as an indicator of lipid peroxidation (9 (2°C),13 (4°C),17 (6°C) and 21 (6°C) days) were noted, as well as an increase in GSH at 9 days (2°C). In plasma, gradual decreases in the concentrations of total proteins and globulins were observed. These responses indicate the presence of thermal plasticity and an attempt at regulation to mitigate thermal stress. The changes showed that a gradual increase in temperature may cause opposite responses to the thermal shock model in *N. rossii.*

1 Introduction

Despite being considered thermally stable ecosystems, Antarctica and the Austral ocean have shown an increase in temperature over the last decades. Climatic studies in the Antarctic Peninsula, the site of this study, shows increases in temperatures of air and surface water in this region (TURNER et al., 2005; STASTNA, 2010; KEJNA *et al.*, 2013, TURNER et al., 2014), which are higher compared to those in the rest of the Antarctic continent (CONVEY et al., 2009).

These temperature increases can lead to changes in the food chains of these ecosystems (ATKINSON et al., 2004) and affect the rates of biological processes and the performance of animals (HUEY; STEVENSON, 1979; JOHNSON; BENNETT, 1995; PÖRTNER; PECK, 2010). Despite the vulnerability of these organisms to global climate change, some show greater plasticity than others (PECK, 2002). Studies on Antarctic fish have reported less acute heat tolerance in these organisms compared to non-Antarctic species (PODRABSKY; SOMERO, 2006; BILYK; DE VRIES, 2011; BEERS; JAYASUNDARA, 2015).

This group of teleosts, the nototheniids, is believed to have evolved and developed mechanisms of adaptation to extremely low and stable temperatures, which ensure their survival in this environment (BUCKLEY et al., 2004; PLACE *et al.*, 2005; DE VRIES and CHENG, 2005; HOFMANN et al., 2000). Among their adaptive characteristics, nototheniids have developed a high concentration of solutes in their cytosol, exhibiting high plasma osmolarity and an increase in mitochondrial surface area and density (SIDEL; HAZEL, 1987; SIDELL, 1998; GUDERLEY; ST-PIERRE, 2002; ABELE; PUNTARULO, 2004).

These characteristics help provide tolerance to the high oxygen solubility in cold ocean waters by generating a higher oxygen concentration in the tissues of Antarctic fish and consequently, higher levels of reactive oxygen species (ROS) (ANSALDO et al., 2000; ABELE; PUNTARULO, 2004).

Increases in temperature are stress factors for Antarctic organisms because they can promote changes in electron transport from the inner mitochondrial membrane, thus generating even more ROS that render them more vulnerable to oxidative damage (PÖRTNER, 2002; ABELE; PUNTARULO, 2004; MUELLER *et al.*, 2011;). ROS can be neutralized by the antioxidant defense system. However, when an imbalance exists between that system and ROS generation, oxidation of biomolecules and irreversible damage to cells, as well as an increase in the energy costs for basal cellular maintenance, may occur (ABELE; PUNTARULO, 2004; HALLIWELL; GUTTERIDGE, 2007).

Plasma parameters are indicators of stress (BEGG; PANKHURST, 2004; LOWE; DAVIDSON, 2005; JIN; DEVRIES, 2006; RODRIGUES JR et al., 2013; KREISS et al., 2015; KANDALSKI et al., 2018) and the metabolic state of fish (BEGG; PANKHURST, 2004). Parameters such as glucose and cortisol are well-known markers of stress in fish (BEGG; PANKHURST, 2004). Increased plasma levels of corticosteroids and catecholamines are considered the primary response to stress (BARTON, 2002). Furthermore, cortisol plays an important role in osmoregulation through its effects on stimulation of Na^+/K^+-ATPase activity and reduction of serum osmolality resulting from the loss of Na^+ and Cl^- (GONZALEZ-CABRERA et al., 1995; GUYNN et al., 2002; PETZEL, 2005).

Although studies on thermal tolerance and acclimation have shown that Antarctic fish can acclimate to a known temperature increase (SOMERO; DE VRIES, 1967; WILSON et al., 2002; THORNE et al., 2010; BILYK; DE VRIES, 2011; STROBEL et al., 2012), little is known about the acclimation costs and mechanisms involved.

Considering that gradual changes in temperature may allow plasticity adjustments, the present study was developed to investigate the effects of a gradual increase in temperature (0.5°C/day) to temperatures of 2°C, 4°C, 6°C and 8°C in the Antarctic fish *Notothenia rossii* through analysis of antioxidant defense markers, cell damage and plasma markers, and plasma levels of osmo-ionic, protein and non-protein metabolites, cortisol, lactate dehydrogenase (LDH) and osmoregulation markers.

2 Materials And Methods

2.1 Statement of ethics

This work was performed in accordance to the ethical principles and rules of the Brazilian laws and the Antarctic Treaty. The Ministry of the Environment granted the environmental permit, and the study was approved by the Ethics Committee on the Use of Animals of the Federal University of Paraná (CEUA - UFPR) under No. 496/2010 and 840/2015.

2.2 Collection and maintenance of animals

Fish were captured with line and hook at a depth of 10 to 25 meters in Admiralty Bay (*Punta Plaza* – 62° 05'64.5" S; 58° 24'31.7" W and Arctowski – 62° 10'.65" S; 58° 26.5' W), King George Island, Antarctic Peninsula. They were then transported from the collection sites to the Brazilian Antarctic Research Station 'Comandante Ferraz' (EACF) (62º 05'0" S/58º 23'28" W) and maintained (n = 7 fish per 1000-liter tank) under controlled temperature (0˚C + 0.5), salinity (35 psu + 0.5), and photoperiods (12-h light/12-h dark) and constant aeration for 5 days (DONATTI; FANTA, 2002). The fish were identified with colored rows and randomly assigned to the experimental and control groups for the experimental times of 9, 13, 17 and 21 days.

2.3 Gradual temperature increase

N. rossii specimens (n = 56; length = 34 ± 3 cm, weight = 443 ± 134 g) were subjected to a gradual increase in temperature, i.e., every 24 hours, the water temperature was increased 0.5°C. On the first day of the experiment, the water temperature in the tanks was 0°C. Aquaterm 09-01T-11457 (Full Cauge) thermostats coupled to water heaters (Altman) were used until temperatures of 2, 4, 6 and 8°C were reached. The fish were then kept at the respective temperatures for 96 hours (4 days), totaling 9 (2°C), 13 (4°C), 17 (6°C) and 21 (8°C) days of experiments.

Every two days, the tanks were cleaned, and 50% of the water was exchanged with the temperature controlled according to the experiment. The fish were individually fed (JAYASUNDARA et al., 2013; ALMROTH et al., 2015; RODRIGUES JR et al., 2015) every 48 hours with fish epaxial muscle (1% of each fish's body mass) on the days between tank water exchange. Fish that did not feed were excluded from the experiments. For all groups, the first day of the experiment was the first day of feeding. The experimental groups and their respective control groups were fed at the same time (FORGATI et al., 2017; SOUZA et al., 2018).

At the end of each experiment, blood was collected with a heparinized syringe by puncture of the caudal vein. Plasma was obtained after centrifugation of whole blood at 2000 rpm for 10 minutes. Then, the fish were anesthetized with 20 $mg.L^{-1}$ of 1% benzocaine (from a 0.1% (bw^{-1}) stock solution in 95% ethanol) until responses to any stimulus ceased (stage 4 according to WOODY et al., 2002) and euthanized by severing the spinal cord. Plasma, gill, kidney (median posterior), liver and brain samples were frozen in liquid nitrogen. For each experimental situation, fish samples kept at 0°C were collected and used as controls.

2.4 Analytical methods

2.4.1. Determination of activity levels of antioxidant enzymes and the levels of non-protein thiols

Spectrophotometric analyses were performed in a microplate reader (EPOCH Microplate Spectrophotometer, BioTek, Winooski, VT, USA).

Gill, kidney, liver and brain samples were weighed and homogenized in 50 mM Tris-HCl buffer (pH 7.4) at a 1:5 ratio (bw^{-1}) with the aid of the Potter-Elvehjem tissue grinder. The homogenate was centrifuged at

14,000 g and 4°C for 10 min, and the supernatant was used for determination of enzymatic activity at 20°C (JAYASUDARA *et al*., 2013; RODRIGUES JR et al., 2015; FORGATI et al., 2017, SOUZA et al., 2018).

Enzymatic activity was expressed in nmol or µmol of substrate converted into product per minute (nmol.min^{-1}, mU; and µmol.min^{-1}, U; respectively). The specific activities of the enzymes were expressed according to the protein concentration in the samples (mU.mg^{-1} or U.mg^{-1}) after normalization to a protein concentration of 1 mg.ml^{-1}. The protein concentration was determined by the method described by Bradford (1976), with bovine serum albumin as the standard.

The activity levels of superoxide dismutase (SOD, EC 1.15.1.1) were determined based on the inhibition of Nitro blue tetrazolium chloride (NBT) by the superoxide anion in the presence of hydroxylamine (CROUCH et al., 1981). Absorbance variation was measured at 560 nm. The activity levels of catalase (CAT, EC1.11.1.6) were measured based on the consumption of hydrogen peroxide (H_2O_2). The absorbance variation was measured at 240 nm (BEUTLER, 1975). The activity levels of glutathione peroxidase (GPx, EC 1.11.1.9) were measured based on the reduction of organic peroxide by glutathione (GSH), resulting in glutathione disulfide (GSSG). GSSG was then reduced by glutathione reductase (GR) through the reducing power of NADPH (WENDEL, 1981), and the absorbance variation was measured at 340 nm. GR activity levels (EC 1.8.1.7) were measured based on NADPH oxidation and concomitant GSSG reduction, and the absorbance variation was monitored at 340 nm (CARLBERG; MANNERVIK, 1975). Glutathione-S-transferase (GST) activity levels (EC 2.5.1.18) were measured based on the method described by Keen *et al.* (1976), where the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) forms the thiolate anion (TBT), and the absorbance variation was monitored at 340 nm. The concentrations of GSH and other non-protein thiols were determined using the method described by Sedilak and Lindsay (1968) based on protein precipitation and the subsequent reaction of non-protein thiols with DTNB to generate a product that absorbs light at 415 nm.

2.4.2. Determination of oxidative damage marker levels

The lipid peroxidation (LPO) index was evaluated using the thiobarbituric acid reactive substances (TBARS) method adapted from Federici *et al.* (2007) in which the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) produces a chromophore that can be measured at 535 nm. The protein carbonylation (PCO) index was determined using the method described by Levine *et al.* (1994), which is based on the reaction of carbonylated proteins with 2,4-dinitrophenylhydrazine (DNPH) generating dinitrophenylhydrazones, whose concentration was measured at 370 nm. The PCO analysis was not performed for the brain samples due to the limited volume of the samples obtained.

2.4.3 Determination of plasma cortisol levels

Plasma cortisol levels were quantified using the enzyme immunoassay principle and the Cortisol ELISA kit (Cayman Chemical, *Item 500360*) according to the manufacturer's instructions.

2.4.4 Determination of plasma metabolic parameters

Metabolites (albumin, cholesterol, glucose, total protein and triglycerides) were measured in the plasma of *N. rossii* using reagent kits from Labtest Diagnóstica SA, Brazil. The total protein content was determined using the Biuret method (545 nm). The albumin fraction was quantified by the bromocresol green method (630 nm). Plasma glucose was measured using Trinder's glucose oxidase method (505 nm). Total cholesterol was measured enzymatically in the presence of esterases and cholesterol oxidase (500 nm). Triglycerides were determined by the enzymatic method using glycerol-3-phosphate oxidase (505 nm). Plasma lactate was determined through the formation of a complex between lactate and copper sulfate ($CuSO_4$) (570 nm) (HARROWER; BROWN, 1972). LDH activity was monitored by NADH oxidation at 340 nm (THUESEN et al., 2005). The concentration of plasma globulins was determined by calculating the difference between the total protein and albumin concentrations.

2.4.5 Determination of plasma osmo-ionic parameters

Calcium, chloride, magnesium and phosphate ions were determined using commercial reagent kits (Labtest Diagnóstica SA, Brazil) according to the manufacturer's instructions. The following colorimetric methodologies were employed: calcium was evaluated by the cresolphthalein method (570 nm), chloride was evaluated by the thiocyanate method (470 nm), magnesium was evaluated by the Magon sulfonate method (505 nm), and phosphate was evaluated by the phosphomolybdate method (340 nm).

2.4.6 Determination of gill and kidney osmoregulation parameters

Median-posterior kidney and gill tissues were homogenized in 0.5% SEID buffer (150 mM sucrose, 10 mM EDTA disodium, 50 mM imidazole, 0.1% sodium deoxycholate). Na^+/K^+-ATPase (NKA) and H^+-ATPase activities were measured in a reaction medium composed of 30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2*6H_2O$, 0.4 mM KCN, 1.0 mM ATP, 0.2 mM NADH, 3 lU/ml PK, 2 lU/ml LDH, 0.1 mM fructose-1,6-diphosphate and 2 mM PEP. The analyses were performed in 96-well microplates in quadruplicate (GIBBS; SOMERO, 1989 modified by KÜLTZ; SOMERO, 1995). In the first 4 replicates, only the reaction medium was added without inhibitors. In the next 4 replicates, a reaction medium containing 2 mM of ouabain (Na^+/K^+-ATPase inhibitor) was added. In the last 4 replicates, NEM (H^+-ATPase inhibitor) was added. The readings were performed at 340-nm absorbance and a temperature of 20°C.

2.5 Statistical analyses

First, a univariate analysis of variance (ANOVA) was applied to the responses of the various biomarkers with a parametric distribution to determine differences between the groups subjected to temperature and time variations (ANDERSON, 2001). Accordingly, the sampling units were grouped into two factors (time factor: 9, 13, 17 and 21 days of the experiment; temperature factor: control (0°C) and experimental (2, 4, 6 and 8°C)). For the biomarkers with a non-parametric distribution, Kruskal-Wallis analysis was applied with similar groupings to those of the ANOVA. These analyses were performed in the vega package of the R platform (DEVELOPMENT CORE TEAM, 2013). Significant differences were considered when $p \leq 0.05$.

3 Results

All animals survived for 21 days at all temperatures (0, 2, 4, 6 and 8°C). The analyses of variance showed that among the biomarkers tested, some showed significant differences and were influenced by the factors temperature and time as well as the interaction between the two factors (Table 1).

3.1 ANOVA of oxidative metabolism biomarkers, non-protein thiol levels and oxidative damage markers

In *N. rossii* gills, the enzyme biomarkers CAT (F = 10.94, $p = 0.012$), GR (F = 13.09, $p = 0.0044$), GPx (F = 11.52, $p = 0.0092$), GST (F = 21.91, $p = 1.0^{-4}$) and SOD (F = 10.67, $p = 0.0136$) were affected by time, while temperature affected only GPx (F = 4.2, $p = 0.04$). The interaction of time and temperature influenced only the biomarker GST (F = 8.37, $p = 0.038$). Non-protein thiols (F = 9.07, $p = 0.0283$) were altered by the time factor, as well as the damage markers LPO (F = 6.48, $p = 0.0014$) and PCP (F = 5.97, $p = 0.0022$) (Table 1).

In the *N. rossii* kidney, the GR, GST, LPO and PCO biomarkers were not influenced by time, temperature or the interaction between the two. Time influenced the biomarkers CAT (F = 26.87, $p = 0$), GPx (F = 3.9, $p = 0.0145$) and SOD (F = 27.67, $p = 0$). GPx (F = 4.29, $p = 0.043$) was also influenced by temperature, whereas the interaction between time and temperature only influenced the non-protein thiols (F = 8.52, $p = 0.0363$) (Table 1).

In the liver of *N. rossii*, the enzymatic biomarkers CAT (F = 12.28, $p = 0.0065$), GPx (F = 18.07, $p = 4^{-4}$), GST (F = 37.56, $p = 6.0^{-4}$) and SOD (F = 12.96, $p = 0.0047^{-4}$) were influenced by time, while GR did not change due to time, temperature or their interaction. For the oxidative damage markers, temperature influenced LPO (F = 13.83, $p = 2.0^{-4}$) and PCO (F = 13.71, $p = 2.0^{-4}$). Time alone also influenced PCO (F = 8.29, $p = 0.0402$), while the interaction between time and temperature only influenced the non-protein thiols (F = 19.26, $p = 2.0^{-4}$) (Table 1).

In the *N. rossii* brain, the biomarkers CAT (F = 38.63, $p = 0$), GPx (F = 38.65, $p = 0$), GR (F = 38.92, $p = 0$), GST (F = 37.56, $p = 0$) and SOD (F = 36, $p = 0$) were influenced by time. The temperature factor alone did not affect any of the analyzed biomarkers. The damage marker LPO was not influenced by time, temperature or their interaction. The interaction between time and temperature only influenced the non-protein thiols (F = 10.14, $p = 0.0174$) (Table 1).

3.2 Antioxidant enzyme activity levels

In *N. rossii* gills, no change in the activity levels of CAT (Fig. 1a) and SOD (Fig. 1e) were found compared to those in the respective control groups at each experimental temperature and between the different experimental times. The experimental and control groups at 13 days showed a decrease compared to those in the experimental and control groups at 21 days. The experimental and control groups at 13 and 17 days showed a decrease compared to those in the experimental and control groups at 21 days. The GPx activity level (Fig. 1b) increased in the control group at 9 days (0°C) compared to those in the other control groups at 13 and 21 days.

The gill GR activity level (Fig. 1c) increased in the control group at 21 days (0°C) compared to those in the other control groups (9, 13 and 17 days) and in the experimental groups. The GST activity level (Fig. 1d)

increased in the experimental group at 9 days (2°C) compared to those in the respective control group (0°C) and the experimental groups at 13 and 17 days (4 and 6°C, respectively). The control group (0°C) showed increased GST activity levels at 21 days compared to those in the other control groups for the other experimental times (9, 13 and 17 days).

No changes in SOD (Fig. 2e), GR (Fig. 2c) and GST (Fig. 2d) activity levels were observed in the *N. rossii* kidneys compared with those in the respective control groups at all tested temperatures (2°C, 4°C, 6°C and 8°C) or between experimental times (9, 13, 17 and 21 days). The CAT activity level (Fig. 2a) increased in the control group at 17 days (6°C) compared to those in the other control groups at 9, 13 and 21 days (2°C, 4°C and 8°C). The GPx activity level (Fig. 2b) increased in the control group at 17 days (6°C) compared to that in the control group at 13 days (4°C).

In the liver of *N. rossii*, no change in the GR activity level (Fig. 3c) was observed compared to those in the respective control groups (2°C, 4°C, 6°C and 8°C) or between experimental times (9, 13, 17 and 21 days). The CAT activity level (Fig. 3a) was higher at 9 days (0°C) than those at the experimental times of 17 and 21 days.

The liver GPx activity level (Fig. 3b) was higher at 17 days (6°C) than those at 9, 13 and 21 days (2°C, 4°C and 8°C).

The liver GST activity levels (Fig. 3d) were lower in the control and experimental groups at 21 days than those in the control and experimental groups at the other experimental times (9, 13, 17 and 21 days). The SOD activity levels (Fig. 3e) were higher in the control and experimental groups at 9 days than those in the control and experimental groups at 13 and 17 days.

In the brain of *N. rossii*, the GR activity level (Fig. 4c) decreased at 21 days compared to those in the control and experimental groups at the other experimental times (9, 13 and 17 days). The CAT activity level (Fig. 4a) was higher at 9 days (0°C) than those in the control groups at 13, 17 and 21 days.

The brain GPx activity level (Fig. 4b) decreased in the control and experimental groups at 21 days (0°C) compared to those in the control and experimental groups at 9 days. SOD activity levels (Fig. 4e) increased in the control and experimental groups at 9 days (0°C) compared to those in the other control and experimental groups at 13 and 17 days. At 21 days, in the control and experimental groups, the SOD activity levels decreased compared to those in the control and experimental groups at other experimental times (9, 13 and 17 days).

The brain GST activity levels (Fig. 4d) decreased in the control and experimental groups at 21 days compared to those in the other control and experimental groups at 9 and 17 days.

3.3 Concentrations of oxidative damage markers and non-protein thiols

In *N. rossii* gills, LPO (Fig. 5b) increased in the control and experimental groups at 21 days compared to that in the other experimental groups at the other experimental times (9, 13 and 17 days). No difference in LPO was found between the experimental and control groups. PCO (Fig. 5a) decreased in the control

group at 13 days (0°C) compared to that in the control groups at 9 and 21 days. No significant difference in PCO was found between the experimental and control groups.

In the gills, the concentrations of glutathione and other non-protein thiols (Fig. 5c) increased in the control and experimental groups at 21 days compared to those in the control and experimental groups at 13 days. No significant variation in non-protein thiols was observed between the experimental and control groups.

In *N. rossii* kidneys, the levels of LPO (Fig. 6a) and PCO (Fig. 6b) did not change compared to those in the respective control groups (2°C, 4°C, 6°C and 8°C) or between the different experimental times (9, 13, 17 and 21 days).

In the kidneys, the concentrations of glutathione and other non-protein thiols (Fig. 6c) increased in the experimental group at 17 days (6°C) compared with those in the experimental groups at 13 and 21 days (4°C and 8°C). No significant changes in glutathione and other non-protein thiols were observed between the control and experimental groups.

In the liver of *N. rossii*, LPO levels (Fig. 7a) increased at all temperatures (2, 4, 6 and 8°C) after 9, 13, 17 and 21 days compared to that in the control group (0°C). No change in PCO levels was found between the control and experimental groups (Fig. 7b) or between the different experimental times (9, 13, 17 and 21 days).

In the liver, the concentrations of glutathione and other non-protein thiols (Fig. 7c) increased in the experimental group at 9 days (2°C) compared to those in the control group. The experimental group at 21 days (8°C) showed lower concentrations compared with those in the experimental groups at 9 and 17 days (2°C and 6°C). The control group at 17 days (0°C) had a higher concentration of non-protein thiols compared to that in the control group at 9 days.

In the brain, no differences in LPO (Fig. 8a) or glutathione and other non-protein thiol levels (Fig. 8b) were noted between the control and experimental groups or between the different experimental times (9, 13, 17 and 21 days).

3.4 ANOVA of plasma biomarkers

In the *N. rossii* plasma, the osmo-ionic biomarkers calcium ($F = 17.07$, $p = 7.0^{-4}$), chloride ($F = 7.45$, $p = 4.0^{-4}$), phosphorus ($F = 4.01$, $p = 0.013$) and magnesium ($F = 16.86$, $p = 9.0^{-4}$) were influenced by the time factor (Table 2). For the plasma metabolic parameters, time influenced albumin ($F = 14.77$, $p = 0.002$), cholesterol ($F = 23.89$, $p = 0$) (Table 2), lactate ($F = 11.67$, $p = 0.0086$), glucose ($F = 7.96$, $p = 0.0467$) and LDH ($F = 12.24$, $p = 0.0066$) (Table 3). The biomarkers total protein ($F = 9.17$, $p = 0.0025$), globulin ($F = 10.43$, $p = 0.0012$) and LDH ($F = 6.52$, $p = 0.0106$) were influenced by temperature (Table 3). Triglycerides and cortisol were not influenced by time, temperature or their interaction (Table 3). The interaction between time and temperature did not influence any biomarker (Tables 2 and 3).

3.5 Concentration of plasma cortisol

In *N. rossii* plasma, the cortisol concentration (Fig. 9c) did not change in any experimental group compared to those in the respective control groups (2, 4, 6 and 8°C) or between experimental times (9, 13, 17 and 21 days).

3.6 Concentrations of plasma metabolic parameters

In *N. rossii* plasma, the albumin concentrations (Fig. 10d) decreased in the control and experimental groups at 13 days (4°C) compared with those in the control and experimental groups at 17 and 21 days (6°C and 8°C). No change in the albumin concentration was found in any experimental group compared with those in the respective control groups (2, 4, 6 and 8°C) or between experimental times (9, 13, 17 and 21 days). Cholesterol concentrations (Fig. 10a) increased in the control groups at 17 and 21 days (6°C and 8°C) compared with those in the control groups at 9 and 13 days (2°C and 4°C). No change in the cholesterol concentration was noted in any experimental group compared to those in the respective control groups (2, 4, 6 and 8°C) or between experimental times (9, 13, 17 and 21 days).

The plasma concentrations of glucose (Fig. 10f), lactate (Fig. 9a), and triglycerides (Fig. 10b) did not differ between the experimental groups compared to those in the respective control groups (2, 4, 6 and 8°C) or between experimental times (9, 13, 17 and 21 days).

The plasma globulin (Fig. 10e) and total protein (Fig. 10c) concentrations decreased in all experimental groups (2, 4, 6 and 8°C) compared with those in the respective control groups. No differences in plasma globulin and total protein concentrations were noted when the groups were compared between experimental times (9, 13, 17 and 21 days).

Plasma LDH activity levels (Fig. 9b) increased in the experimental group at 9 days (2°C) compared to those in the other experimental groups at 13, 17 and 21 days (4, 6 and 8°C). No difference in LDH was observed between the experimental groups and their respective control groups (2, 4, 6 and 8°C).

3.7 Concentrations of plasma osmo-ionic parameters

In *N. rossii*, the plasma calcium concentration (Fig. 11a) increased in the control and experimental groups at 13 days (4°C) compared with those in the control and experimental groups at 21 days (8°C). The concentrations in the control and experimental groups at 17 days decreased compared to those in the control and experimental groups at 9 (2°C) and 13 days (4°C). No difference in the calcium concentration was found between the experimental groups and their respective control groups (2, 4, 6 and 8°C). The plasma chloride concentration (Fig. 11b) decreased in the control and experimental groups at 21 days (8°C) compared to those in the control and experimental groups at 9 and 13 days (2°C and 4°C). No difference in the chloride concentration was observed between the experimental groups and their respective control groups (2, 4, 6 and 8°C).

The plasma phosphorus concentration (Fig. 11c) increased in the control and experimental groups at 21 days compared to those in the other control and experimental groups at 9, 13 and 17 days (2, 4 and 6°C). No difference in the phosphorus concentration was noted between the experimental groups and their

respective control groups (2, 4, 6 and 8°C). The plasma magnesium concentration (Fig. 11d) increased in the control and experimental groups at 17 days (6°C) compared to those in the control and experimental groups at 9 and 13 days (2°C and 4°C). No difference in the magnesium concentration was found between the experimental groups and their respective control groups (2, 4, 6 and 8°C).

3.8 ANOVAs of the enzymes Na⁺/K⁺ ATPase and H⁺-ATPase in kidney and gill tissues

In *N. rossii* gills, the activity levels of Na⁺/K⁺ ATPase ($F = 7.9701$, $p = 2.0^{-4}$) and H⁺-ATPase ($F = 3.8672$, $p = 0.0148$) were influenced by the time factor (Table 4). In the kidney, Na⁺/K⁺ ATPase ($F = 13.4066$, $p = 0.0038$) and H⁺-ATPase ($F = 14.4552$, $p = 0.0023$) were influenced by the interaction between the time and temperature factors (Table 4). The time factor alone did not influence the activity levels of Na⁺/K⁺ ATPase and H⁺-ATPase (Table 4)

3.9 Na⁺/ K⁺ ATPase and H⁺-ATPase enzyme activity levels in the gills

In the gills of *N. rossii*, Na⁺/ K⁺ ATPase activity levels at 17 days in the control (0°C) and experimental (6°C) groups were lower than those at the other experimental times and temperatures tested (Fig. 12a). H⁺-ATPase activity levels at day 9 in the experimental (2°C) and control (0°C) groups were also lower than those at the other experimental times and temperatures tested (Fig. 12b).

3.10 Na⁺/ K⁺ ATPase and H⁺-ATPase enzyme activity levels in the kidney

In the kidney of *N. rossii*, Na⁺/ K⁺ ATPase activity levels were lower in the control group at 9 days than those in the control groups at 17 and 21 days (Fig. 13a). H⁺-ATPase activity levels in the experimental group (2°C) at 9 days were higher than those in the respective control group and the control and experimental groups at 13 days (4 and 0°C, respectively) (Fig. 13b).

4 Discussion

Although Antarctic fish can live in extremely cold and stable environments, due to higher tissue concentrations of oxygen and ROS (ANSALDO et al., 2000; ABELE; PUNTARULO, 2004), temperature increases may render such fish more vulnerable to oxidative damage because they can generate even more ROS due to increased oxygen consumption and routine metabolic rates (ABELE et al., 1998; HEISE et al., 2003; KELLER et al., 2004; MUELLER *et al.*, 2011; PÖRTNER, 2002; STROBEL *et al.*, 2013).

When ROS production increases, the body must respond with compensatory mechanisms such that the resulting metabolic processes occur in a balanced manner to preserve a good physiological state among cells (FRANKLIN, 2010).

A response of the gills to heat stress is expected because the gills are in close contact with water and are directly exposed to changes in the surrounding aquatic environment, such as temperature changes (PERRY; LAURENT, 1993; SABER, 2011). In their normal physiological state, the gills already have a high metabolic rate mainly due to the process of ionic regulation (JOHANSE; PETTERSON, 1981; MOMMSEN, 1984).

In the gills at 9 days of the experiment, when the temperature reached 2°C, an increase in GST activity was observed (Fig. 14). This enzyme is responsible for the conjugation of xenobiotics to GSH, facilitating their elimination. Other GST isoforms with diverse functions have been identified, such as intracellular protein transport and regulation of apoptosis, a process that may be increased under stressful circumstances (HALLIWELL; GUTTERIDGE., 2007, LABORDE, 2010).

Exposure to environmental stress factors can regulate several genes, stimulating or inhibiting the gene expression of proteins related to cell protection functions (RIZWAN-UL-HAQ *et al.*, 2012).

In studies of acute stress, i.e., abrupt changes in temperature, no increase in gill GST activity was observed (FORGATI et al., 2017, KLEIN *et al.*, 2017), and only a transient increase in GSH was noted (FORGATI et al., 2017). In the present study, no change in the GSH concentration with increased GST activity was observed, possibly due to the rapid consumption and replacement of GSH as this tripeptide is rapidly oxidized when free radical production is increased (SK; BHATTACHARYA, 2006; TAPIERO; TEW 2003).

The reactions catalyzed by GST produce oxidized GSH or GSSG (HERMES-LIMA, 2004); thus, the cell needs to replenish GSH stocks through the use of NADPH. Replacement of NADPH through the pentose pathways diverts glucose from energy production, which could contribute to heat stress and the need for an organism to trigger compensatory responses as observed in *N. rossii* gills subjected to the same experimental model (GUILLEN *et al.*,2019)

At 9 days of the experiment, when the temperature reached 2°C, an increase in the concentration of non-protein thiols was found in the liver of *N. rossii*, with the most pronounced increase observed in GSH (Fig. 14). GSH is considered the main intracellular antioxidant and can act as a cofactor for GSH-dependent enzymes (SK; BHATTACHARYA, 2006; TAPIERO, 2003).

A similar response was observed in a study of acute stress in *N. rossii* maintained at 8°C for 6 days, where GSH levels increased significantly over time (MACHADO et al., 2014).

Increased availability of GSH can increase the availability of substrate, allowing higher turnover of enzymes that require GSH and thus conferring cells with a greater ability to neutralize ROS. In this study, however, no changes in the activity levels of GSH-dependent enzymes were observed in the liver, possibly because the constitutive concentrations of these enzymes were sufficient, which was also observed by Machado *et al.*, 2014 in *N. rossii*.

During the acclimation period, the liver MDA levels remained high, reflecting a continuous lipid peroxidation response (Fig. 14). A high LPO index is expected for Antarctic fish under oxidative stress given the high levels of mono- and polyunsaturated fatty acids in these animals (SIDELL, 1998).

The products generated by LPO are detoxified through their conjugation with GSH by GST, which may justify the observed increase in thiols at 9 days (2°C) as an attempt to maintain redox balance. However, the permanent increase in LPO concentrations indicates that equilibrium was not achieved.

In *N. rossii*, increases in liver LPO did not occur in acute stress models (MACHADO et al., 2014; KLEIN *et al.*, 2017). Increased levels of liver LPO were also observed in other Antarctic fish, such as *Pagothenia borchgrevinki* (ALMROTH et al., 2015) and *Pachycara brachycephalum* (HEISE et al., 2007), exposed to high temperatures in the long term, which may suggest that for LPO, the exposure time to a stressor agent may be more relevant than its magnitude.

The transient effects of the gradual temperature increase observed in this study can be explained by the mechanisms of adaptation to the deleterious actions of oxygen in nototheniids. The levels of antioxidant enzymes present in each tissue may be sufficient to combat the effects of oxidative stress generated by a temperature increase (ENZOR; PLACE, 2014).

N. rossii also has other enzymatic antioxidant mechanisms, such as thioredoxins and peroxiredoxins, and non-enzymatic mechanisms, such as vitamins C and E, to consider in the redox balance as a whole (GIESEG et al., 2000; ABELE; PUNTARULO, 2004).

In *N. rossii* plasma, a decrease in the concentration of total proteins (albumin and globulins) (Fig. 14) accompanied by a decrease in the globulin concentration was observed, indicating that the former decrease occurred at the expense of globulin consumption (Fig. 14). This result is similar to that found in a long-term stress model (90 days), where a decrease in the plasma globulin concentration was also observed (KANDALSKI *et al.*, 2019).

Thorne *et al.* (2010) exposed the notothenioid *Harpagifer antarcticus* to acute thermal stress (6°C for 48 h), and the response observed was inflammation in hepatic tissue; when the inflammatory process is chronic, hypoglobulinemia may occur. The thermal stress analyzed in the liver of *P. brachycephalum* caused a decrease in the protein content in this tissue (BRODTE et al., 2008).

The liver LPO levels observed at all experimental times of this study may also have hindered the synthesis of proteins, including globulins, because the liver is responsible for 85–90% of the circulating protein volume (TREFTS et al., 2017). The albumin concentration may have been maintained due to albumin's relevant role in maintaining oncotic pressure in plasma and the transport of several molecules such as hormones and lipids. Moreover, albumin's long half-life may have masked reductions in its concentration (ANDREEVA, 2010; PERES et al., 2015; TREFTS et al., 2017).

The increase in H^+-ATPase in the kidneys at 9 days and 2°C may indicate that plasma acidification was already occurring (Fig. 15). Renal intercalated cells are one of the types of specialized cells that contain H^+-ATPase in the plasma membrane (NISCH; FORGAC, 2002), whose function is important for the systemic acid-base balance through the secretion of acid in the urine (BROWN; BRETON, 2000; PERRY et al., 2000; DUARTE et al., 2013). H^+-ATPase activity entails ATP phosphorylation, which may aggravate the imbalance in the energy demand in this organ (GUILLEN et al., 2019) leading to activation of the gluconeogenic pathway.

In hypertensive and heat shock models, a hyperglycemic effect was observed in response to heat stress in *N. rossii* (KANDALSKI et al., 2018) and other Antarctic species (LOWE; DAVIDSON, 2015; KANDALSKI et al., 2018), while in the present study, no variation in glucose levels was observed. The maintained cortisol levels may explain the lack of hyperglycemia because glucose and cortisol are related (MOMMSEN et al., 1999; BARTON, 2002; CHENG et al., 2017).

The concentrations of the osmo-ionic parameters calcium, chloride, phosphorus and magnesium also did not change with the gradual increase in temperature. However, the time of the inter-renal response to adrenocorticotrophic hormone (ACTH) for cortisol production, as well as changes in the plasma levels of ions, has a latency from minutes to hours (PANKHURST, 2011).

Due to the experimental design of our study, where the first samples were collected 9 days after exposure to the stressor agent, we may have missed plasma variations for some markers. When subjected to heat shock, *N. rossii* presented changes only in the Mg ion until up to 12 hours of exposure to a temperature of 8°C (KANDALSKI et al., 2018).

In *Ramdhia quelen*, a subtropical fish, a cortisol peak occurred rapidly in juveniles between 5 and 30 minutes and in adults at 60 minutes. After 240 minutes, the cortisol levels had already returned to the basal level (KOAKOSKI *et al.*, 2012). Furthermore, Hudson *et al*. (2008) observed no variation in the cortisol levels of *Trematomus bernachii* when exposed to a temperature increase over time, including at 24 and 48 hours and 1, 2 and 4 weeks.

No increase in the plasma markers lactate and the enzyme LDH were observed, which is similar to other results found in *N. rossii* after heat shock (STROBEL et al., 2012; KANDALSKI et al., 2018). The lack of changes in plasma lactate and LDH levels is consistent with the lack of increase in these metabolic intermediates in tissues, except for the specific increase in the liver as described by Guillen *et al.* (2019).

Although the results suggest an increased demand for ATP as discussed in another study with the same experimental model (GUILLEN et al., 2019), no increase was noted in the plasma concentration of triglycerides, which act as a rapid source of energy during stress conditions (LOWE; DAVIDSON, 2005) and can be transported bound to albumin via the bloodstream (ADAMU; KORI–SIAKPERE, 2011). An increase in the concentration of triglycerides may have occurred before the first collection time. The cholesterol concentration also did not change, indicating that gradually increasing heat stress did not compromise the cellular structure or the production of steroid hormones as previously observed in *N. rossii* under heat shock conditions (KANDALSKI et al., 2018).

5 Conclusion

The gradual increase in temperature triggered plasma and oxidative metabolic responses unlike those reported in studies of thermal shock. Such responses include increased gill GST activity, indicating an increase in ROS production and a persistent increase in liver LPO, which suggests that for LPO, the exposure time to a stressor agent seems to be more relevant than its magnitude.

The data obtained for liver LPO may be related to increased GSH, and liver impairment seems to have influenced the plasma protein composition because the synthesis of globulins may have been altered by the gradual increase in temperature.

The increased H^+-ATPase activity in kidneys may aggravate the imbalance in the energy demand in this organ leading to activation of the gluconeogenic pathway.

In cases of thermal acclimation, ionic parameters, cortisol and glucose are not considered good markers possibly due to their rapid release in the plasma.

Declarations

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DECLARATIONS

All authors have participated in conception and design, or analysis and interpretation of the data; drafting the article or revising it critically for important intellectual content; and approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

All authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

This work was performed in accordance to the ethical principles and rules of the Brazilian laws and the Antarctic Treaty. The Ministry of the Environment granted the environmental permit, and the study was approved by the Ethics Committee on the Use of Animals of the Federal University of Paraná (CEUA - UFPR) under No. 496/2010 and 840/2015.

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Tables

		CATALASE		G6PDH		GPx		GR		GST		LPO		Non protein thiols		SOD		PCO	
		F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
GILLS	Temperature	2.5517	0.1102	0.0571	0.8111	4.2033	0.0403*	2.00E-04	0.99	2.00E-04	0.99	0.9771	0.3299	0.0078	0.9298	0.0837	0.7724	0.6243	0.4349
	Time	10.9426	0.012*	22.686	0*	11.5291	0.0092*	13.0989	0.0044*	21.9181	1.00E-04*	6.4838	0.0014*	9.0723	0.0283*	10.676	0.0136*	5.9755	0.0022*
	Time*Temp	0.5012	0.9186	0.5669	0.904	0.9523	0.8128	0.9113	0.8227	8.3779	0.0388*	1.1674	0.3365	0.4381	0.9323	5.5983	0.1329	2.5107	0.0751
BRAIN	Temperature	0.5908	0.4421	0	1	0.0218	0.8828	0.5	0.4795	0.2924	0.5887	0.0108	0.9173	0.4647	0.4954	1.4705	0.2253	-	-
	Time	38.6377	0*	11.9542	0.0075*	38.6574	0*	38.9289	0*	37.5634	0*	4.655	0.1989	4.0875	0.2522	36.0027	0*	-	-
	Time*Temp	4.8482	0.1833	2.8748	0.4113	4.0115	0.2602	5.3697	0.1466	5.8832	0.1174	2.2301	0.526	10.1476	0.0174*	5.0532	0.1679	-	-
LIVER	Temperature	0.0596	0.8071	7.1204	0.0104	0.0963	0.7563	2.7534	0.104	2.3472	0.1255	13.8375	2.00E-04*	0.092	0.7617	0.0097	0.9217	13.7154	2.00E-04*
	Time	12.2812	0.0065*	18.3164	0*	18.0768	4.00E-04*	0.2	0.8959	17.3018	6.00E-04*	2.2046	0.5311	2.0403	0.5641	12.964	0.0047*	8.2991	0.0402*
	Time*Temp	1.8769	0.5984	0.3504	0.789	1.2115	0.7503	0.9839	0.4088	3.7848	0.2857	5.7624	0.1238	19.2668	2.00E-04*	3.5516	0.3141	4.1187	0.2489
KIDNEYS	Temperature	1.3107	0.2523	1.2012	0.2731	4.293	0.0439*	0.8574	0.3545	0.2185	0.6402	0.0897	0.7659	0.1995	0.6551	0.8118	0.3676	0.4786	0.4891
	Time	26.8713	0*	3.6171	0.3059	3.9001	0.0145*	5.5552	0.1354	4.5472	0.2081	0.3875	0.7625	1.1769	0.7586	27.6708	0*	2.8935	0.4083
	Time*Temp	0.4236	0.9353	13.5323	0.0036*	0.898	0.4494	2.1055	0.5508	3.381	0.3365	0.075	0.9731	8.525	0.0363*	2.1138	0.5491	0.6091	0.8944

Table 1 - *N. rossii* when subjected to a gradual temperature increase (0.5°C/day until reaching temperatures of 2°C, 4°C, 6°C and 8°C) over time (T9, T13, T17, and T21) and the effects of the temperature×time interaction on the responses of oxidative metabolism and antioxidant defense biomarkers. GPx - Glutathione peroxidase; GR - Glutathione reductase; GST – Glutathione-S-transferase; LPO - lipoperoxidation; Non protein thiols; SOD - Superoxide dismutase and PCO - Protein carbonylation in the gill, brain, liver and kidney tissues of *N. rossii*. * indicates statistically significant variation, p=0.05.

		CALCIUM		CHLORIDE		PHOSPHORUS		MAGNESIUM		CORTISOL		ALBUMIN		CHOLESTEROL	
		F	p	F	p	F	p	F	p	F	p	F	p	F	p
PLASMA	Temperature	0.1408	0.7075	1.7934	0.1874	0.2435	0.6242	1.5947	0.2067	1.0999	0.9033	0.343	0.5581	1.6412	0.2002
	Time	17.0775	7.00E-04*	7.4512	4.00E-04*	4.0186	0.013*	16.8669	8.00E-04*	0.8926	0.8272	14.7784	0.002*	23.895	0*
	Temp*Time	2.8091	0.422	1.1079	0.3561	1.3265	0.2779	5.501	0.1386	7.7299	0.0519	4.6219	0.2017	4.0081	0.2606

Table 2- *N. rossii* under the effects of a gradual temperature increase (0.5°C/day until reaching temperatures of 2°C, 4°C, 6°C and 8°C) over time (T9, T13, T17, and T21) and the effects of the temperature×time interaction on the responses of the plasma parameters calcium, chloride, phosphorus, magnesium, cortisol, albumin and cholesterol. * indicates statistically significant variation, p=0.05.

		TOTAL PROTEINS		TRIGLYCERIDES		LACTATE		LDH		GLOBULINS		GLUCOSE	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
PLASMA	Temperature	9.1743	0.0025*	1.8095	0.1786	0.5345	0.4647	6.5275	0.0106*	10.4344	0.0012*	0.6193	0.4313
	Time	3.0824	0.3791	1.7689	0.6217	11.6749	0.0086*	12.2455	0.0066*	0.6735	0.8794	7.9649	0.0467*
	Temp*Time	1.5934	0.6609	0.4698	0.9255	2.8336	0.418	5.5085	0.1381	3.7948	0.2845	5.1348	0.1622

Table 3- *N. rossii* under the effects of a gradual temperature increase (0.5°C/day until reaching temperatures of 2°C, 4°C, 6°C and 8°C) over time (T9, T13, T17, T21) and the effects of the temperature×time interaction on the responses of the plasma parameters total protein, triglycerides, lactate,lactate dehydrogenase (LDH), globulins and glucose in *N. rossii*. * indicates statistically significant variation, p=0.05.

		Na^+/K^+ ATPase		H^+-ATPase	
		F	*p*	F	*P*
GILLS	Temperature	0.0019	0.3757	0.2741	0.6031
	Time	7.9701	2.00E-04*	3.8672	0.0148*
	Time*Temp	2.5853	0.064	0.2248	0.8787
KIDNEY	Temperature	0.3104	0.5774	0.6985	0.4033
	Time	4.0994	0.2509	5.8575	0.1188
	Time*Temp	13.4066	0.0038*	14.4552	0.0023*

Table 4- *N. rossii* under the effects of a gradual temperature increase (0.5°C/day until reaching temperatures of 2°C, 4°C, 6°C and 8°C) over time (T9, T13, T17, and T21) and the effects of the temperature×time interaction on the responses of the enzymes Na^+/K^+ ATPase and H^+-ATPase in the gill and kidney tissues of *N. rossii*. * indicates statistically significant variation, $p \leq 0.05$.

Figures

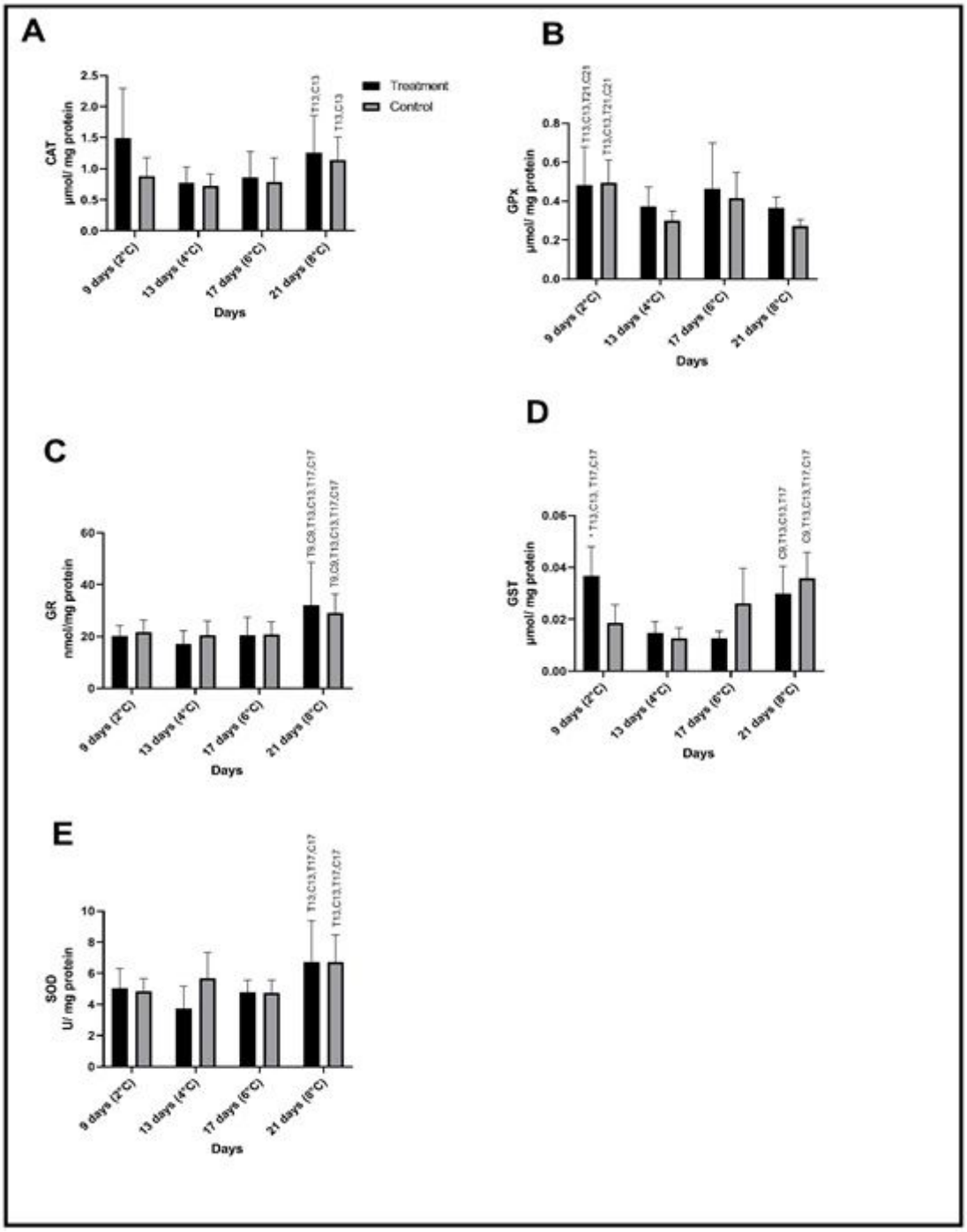

Figure 1

N. rossii's gills when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. Catalase - CAT; Glutathione peroxidase - GPx; Glutathione reductase - GR; Glutathione S-transferase - GST; and Superoxide dismutase - SOD. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

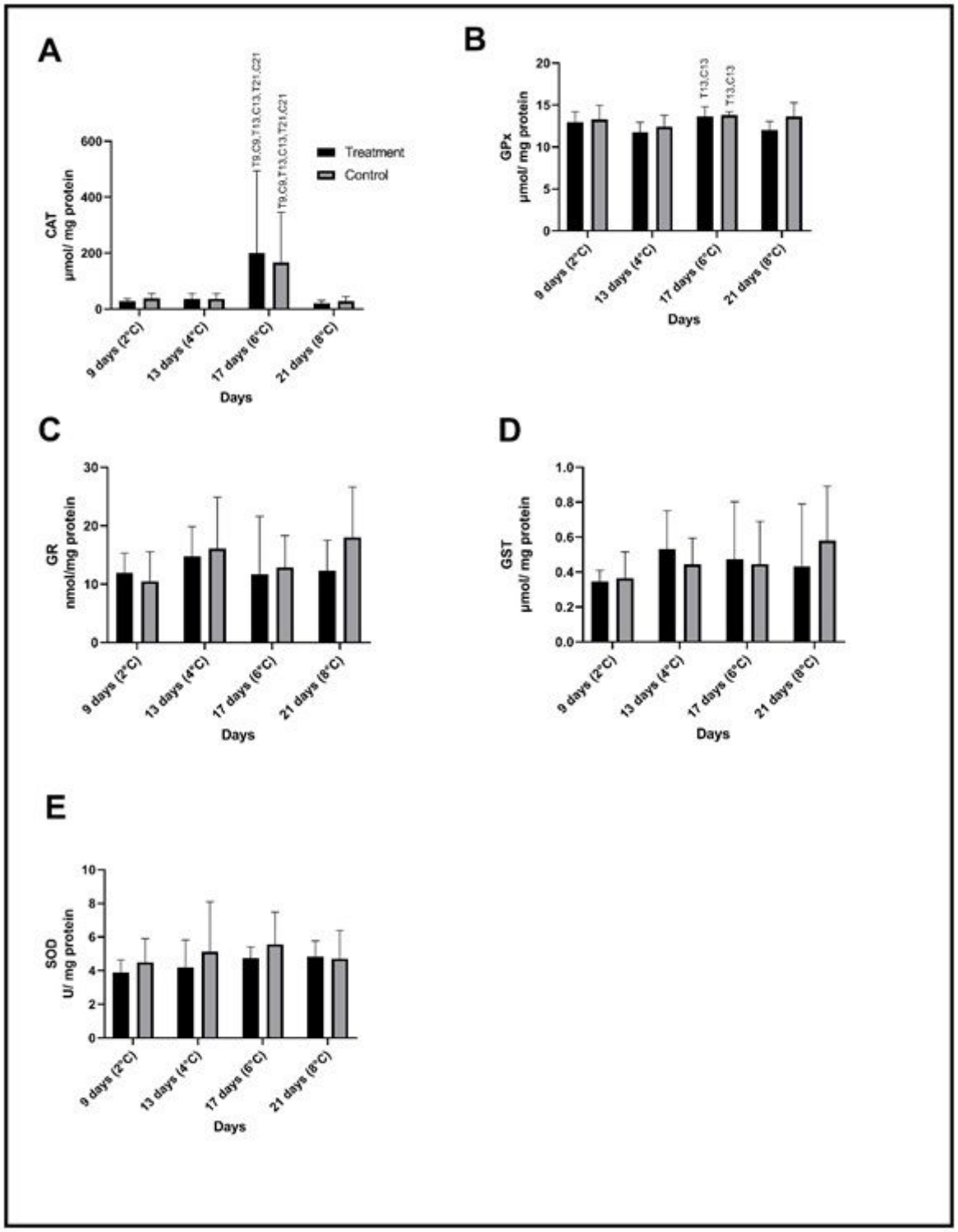

Figure 2

N. rossii's kidneys when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. Catalase - CAT; Glutathione peroxidase - GPx; Glutathione reductase - GR; Glutathione S-transferase - GST; and Superoxide dismutase - SOD. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

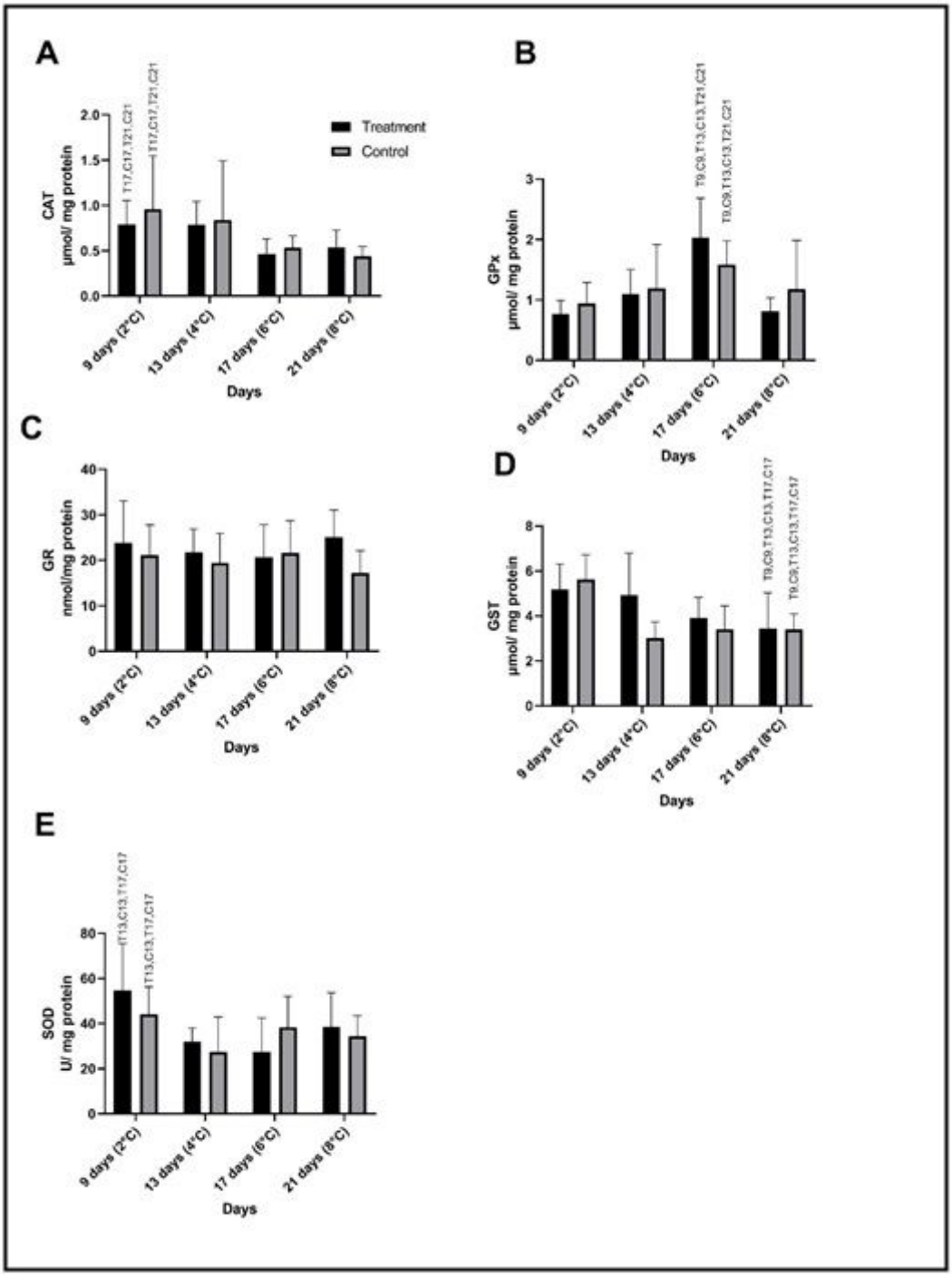

Figure 3

N. rossii's liver when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. Catalase - CAT; Glutathione peroxidase - GPx; Glutathione reductase - GR; Glutathione S-transferase - GST; and Superoxide dismutase - SOD. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

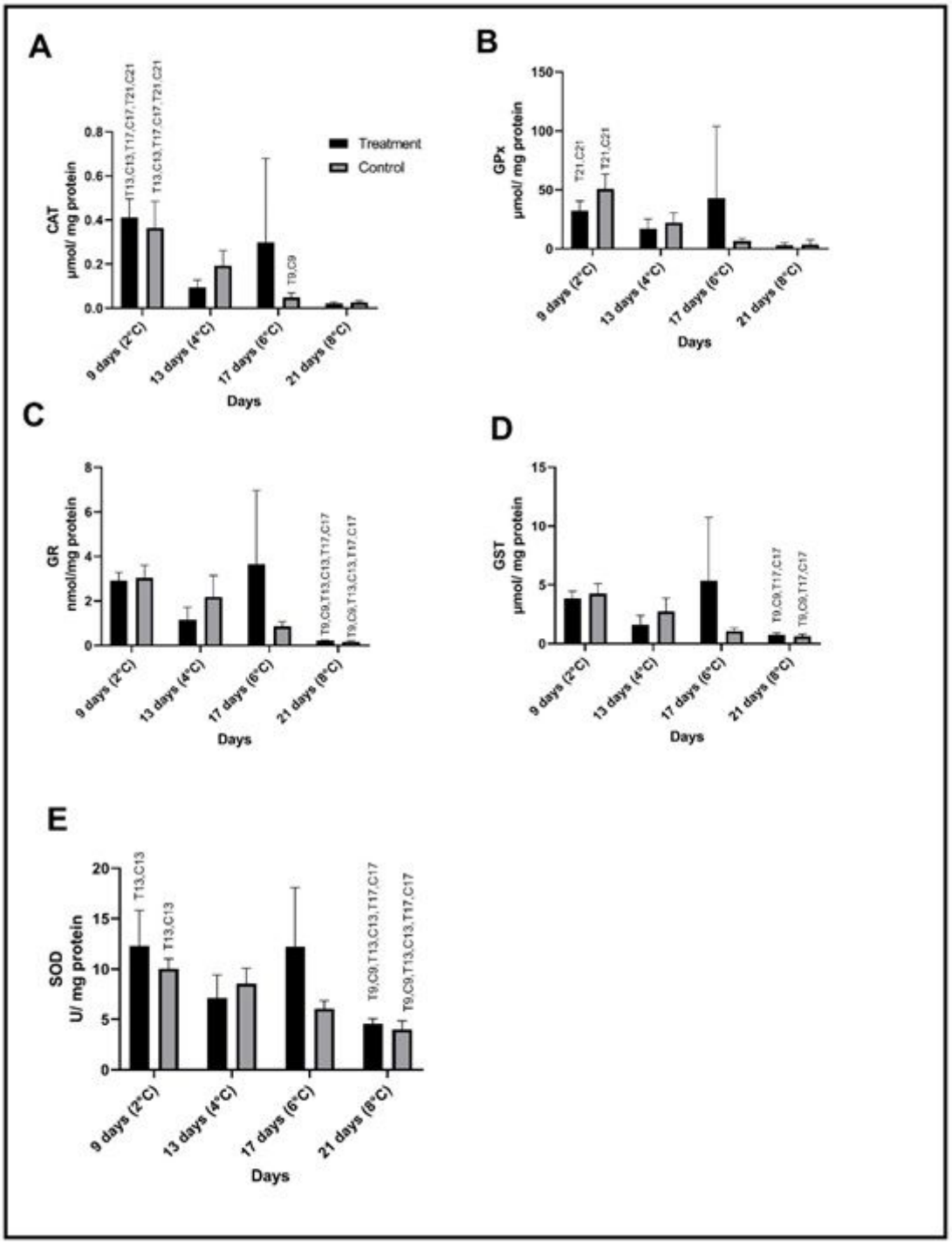

Figure 4

N. rossii's brain when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. Catalase - CAT; Glutathione peroxidase - GPx; Glutathione reductase - GR; Glutathione S-transferase - GST; and Superoxide dismutase - SOD. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

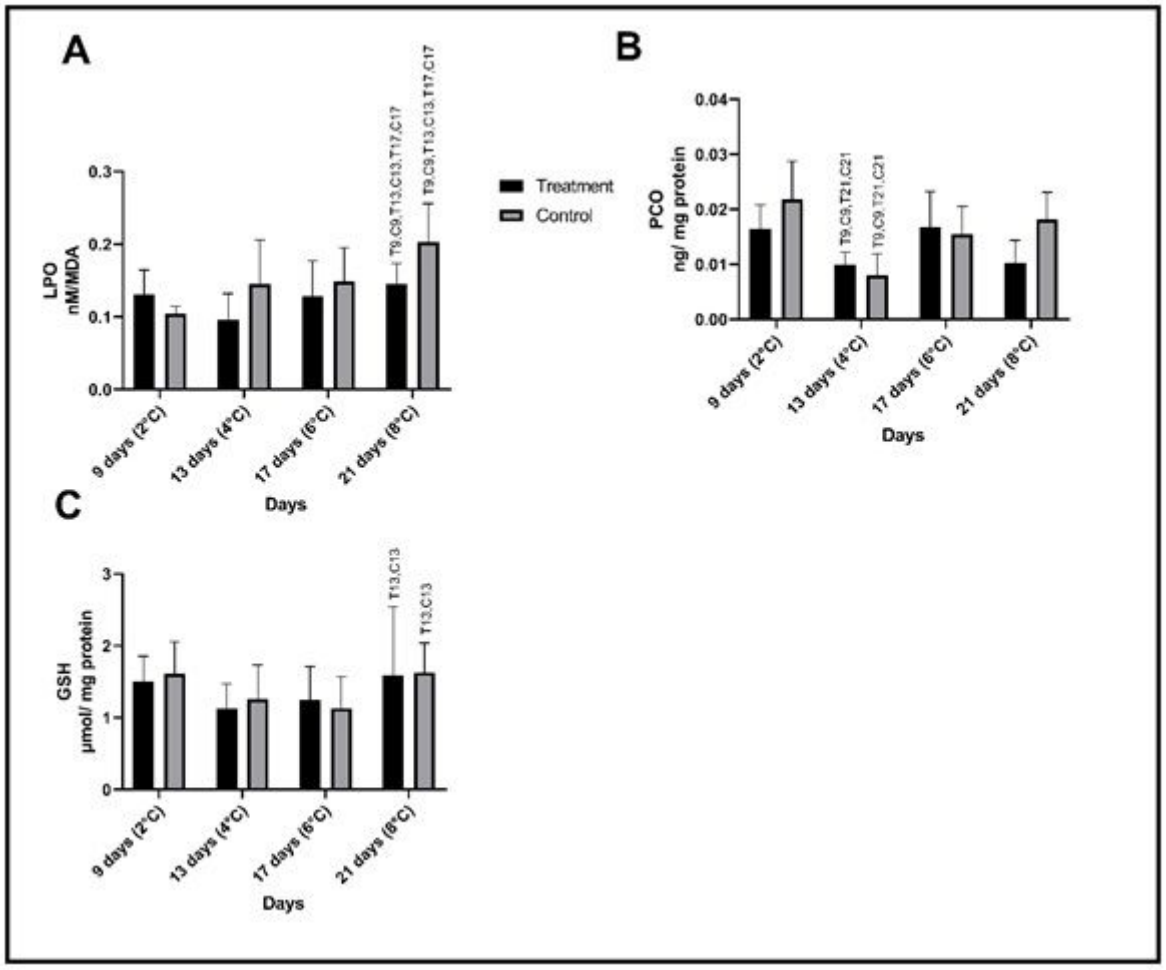

Figure 5

N. rossii's gills when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. PCO - Protein carbonylation; LPO - Lipid peroxidation and GSH - glutathione and other non-protein thiols. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

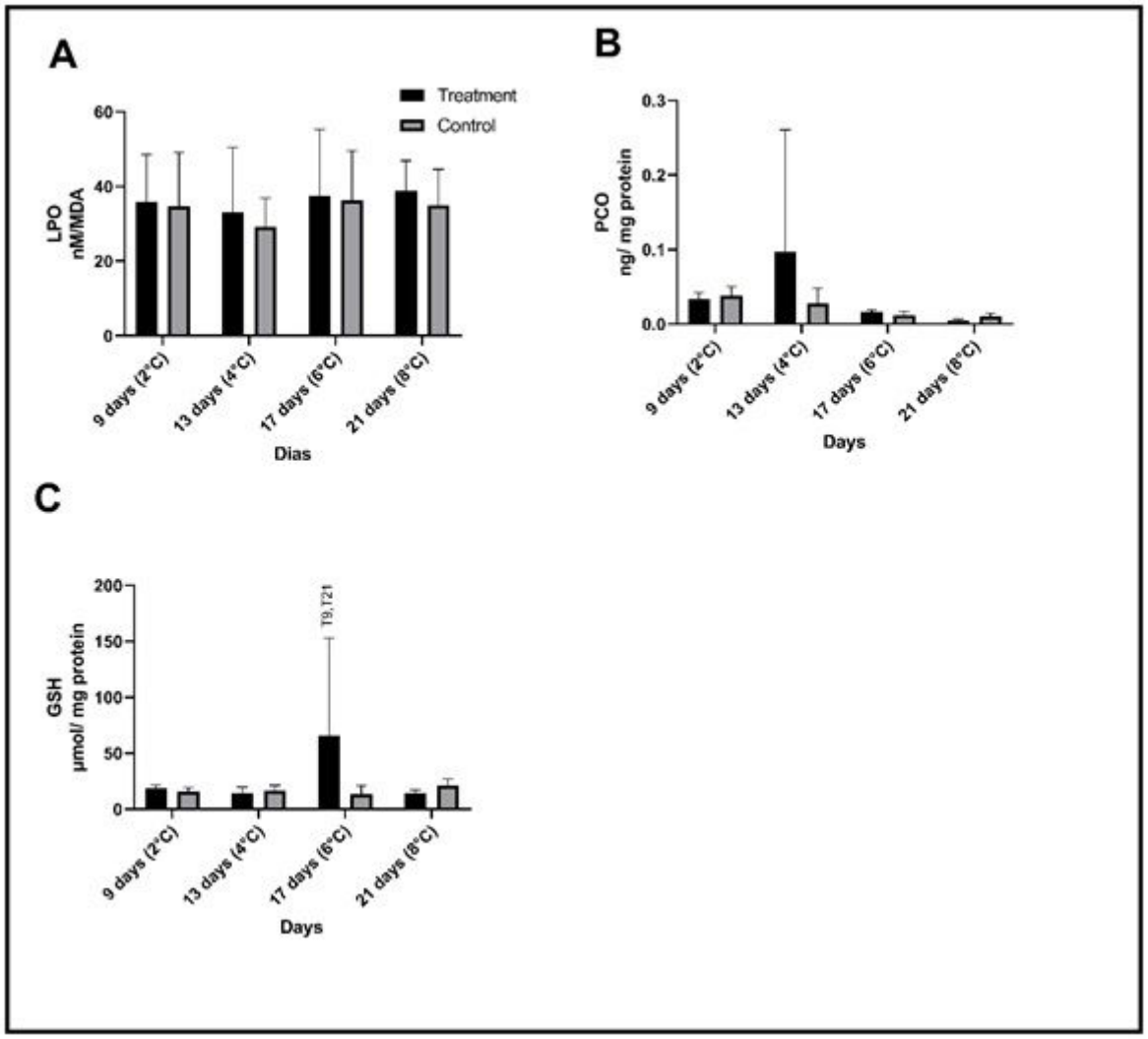

Figure 6

N. rossii's kidneys when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. PCO - Protein carbonylation; LPO - Lipid peroxidation and GSH - glutathione and other non-protein thiols. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

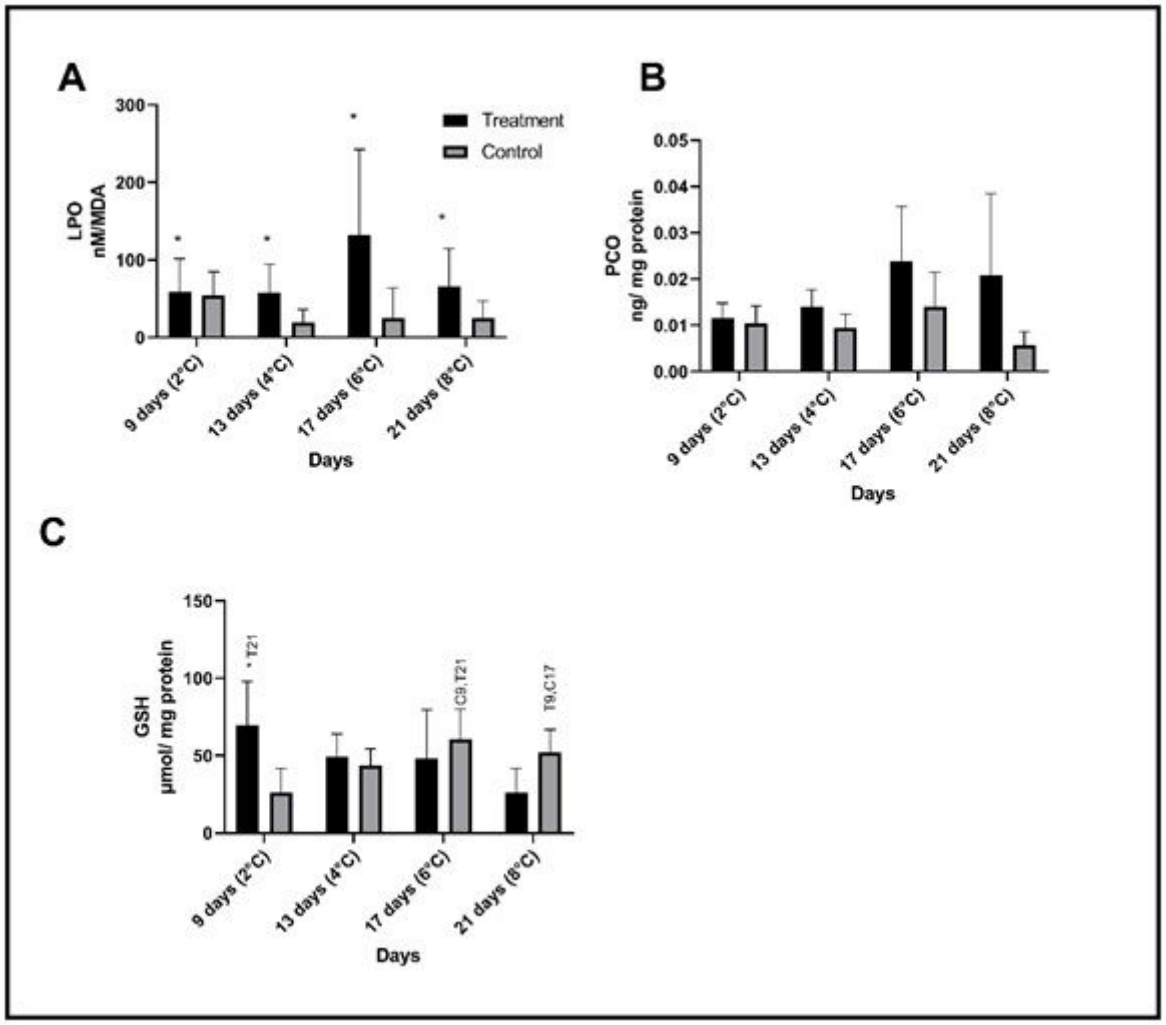

Figure 7

N. rossii's liver when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. PCO - Protein carbonylation; LPO - Lipid peroxidation and GSH - glutathione and other non-protein thiols. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

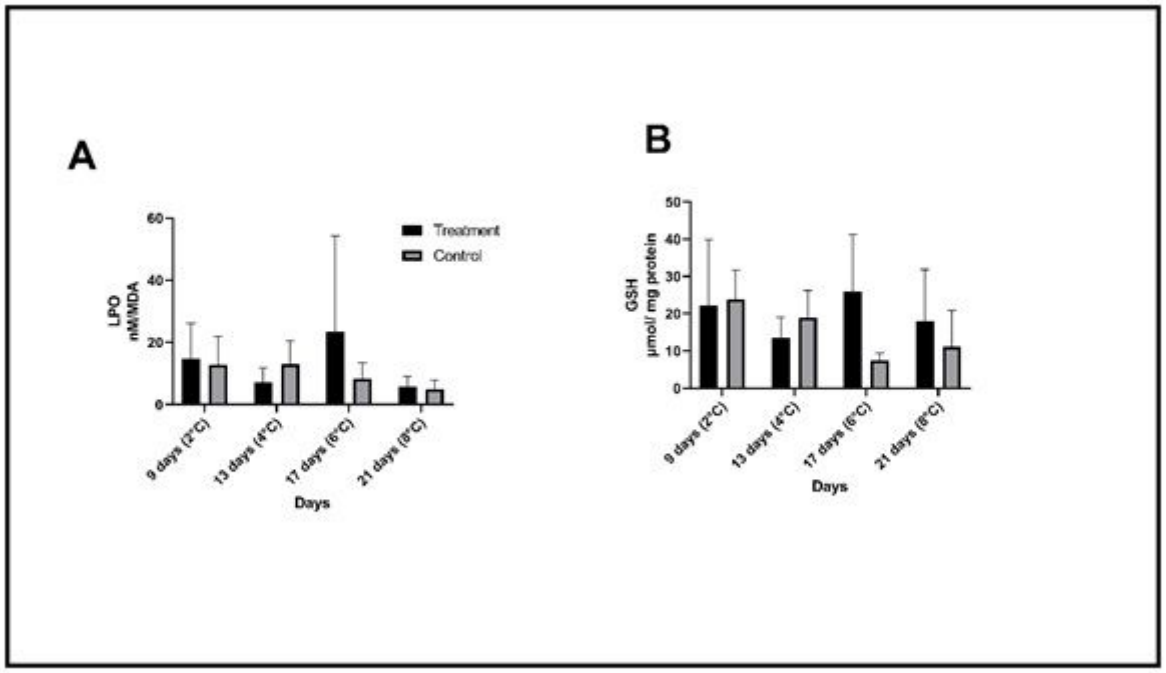

Figure 8

N. rossii's brain when subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached LPO - Lipid peroxidation and GSH - glutathione and other non-protein thiols. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

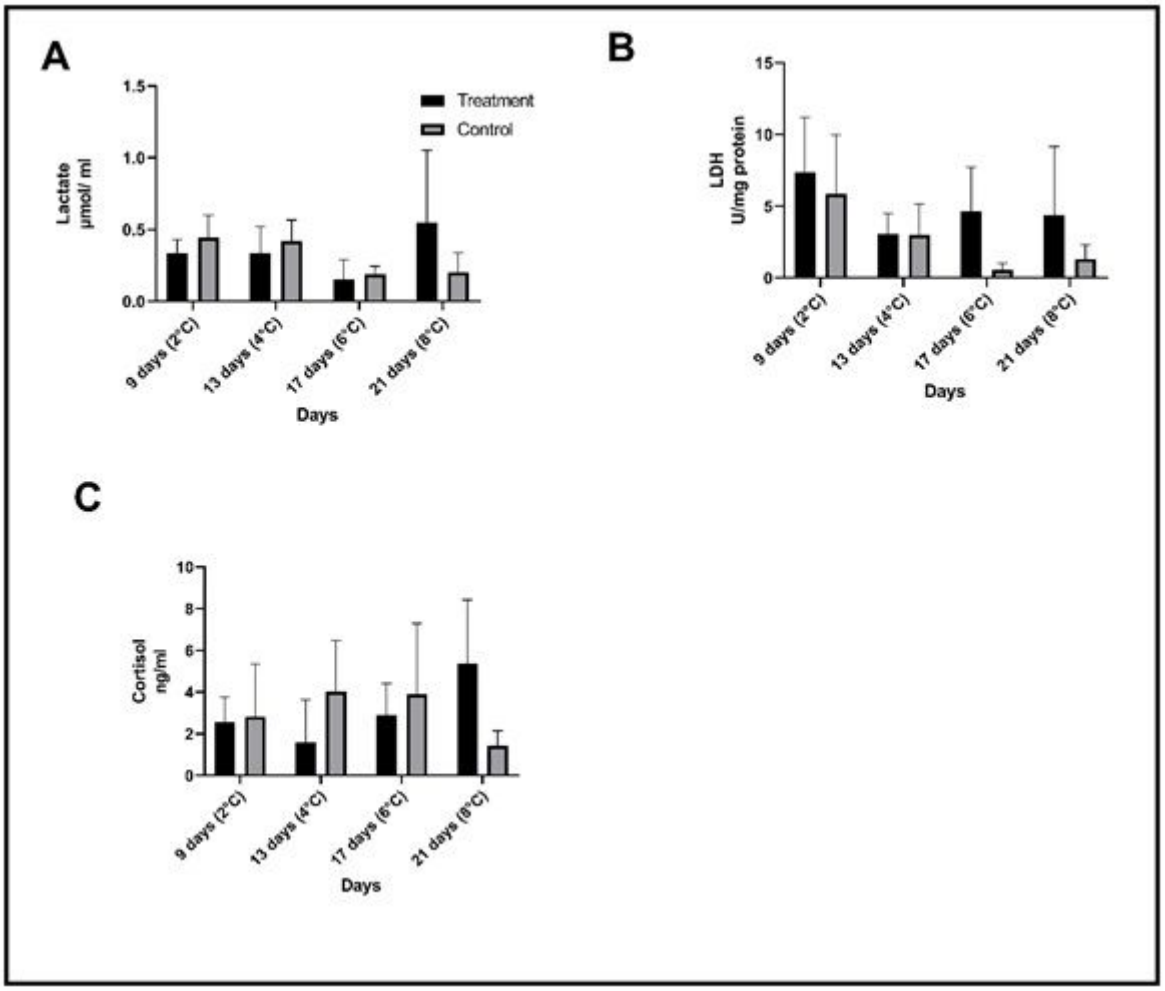

Figure 9

Concentrations of the plasma markers lactate, cortisol and lactate dehydrogenase (LDH) in N. rossii subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

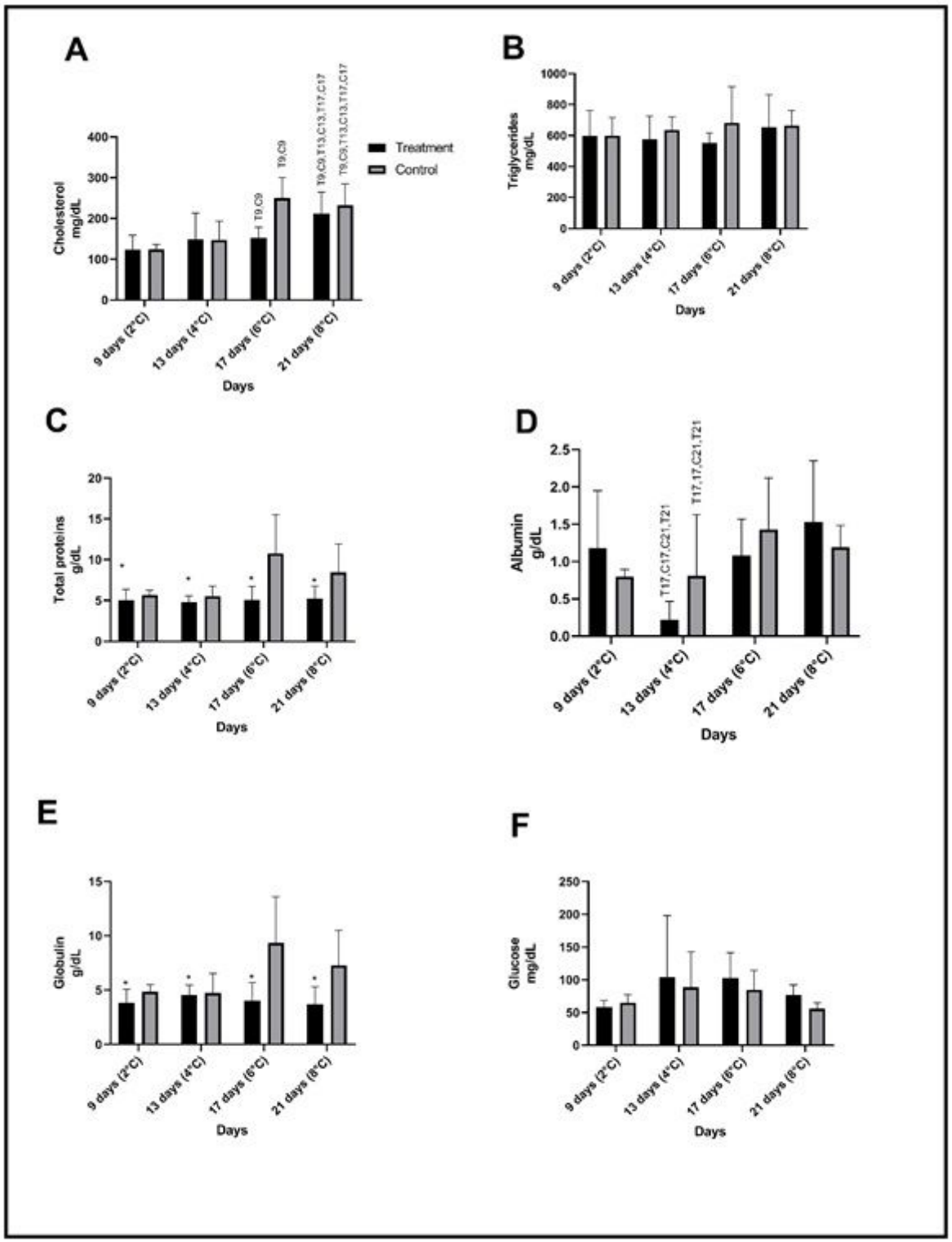

Figure 10

Concentrations of the plasma markers cholesterol, triglycerides, total protein, albumin, globulins and glucose in N. rossii subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

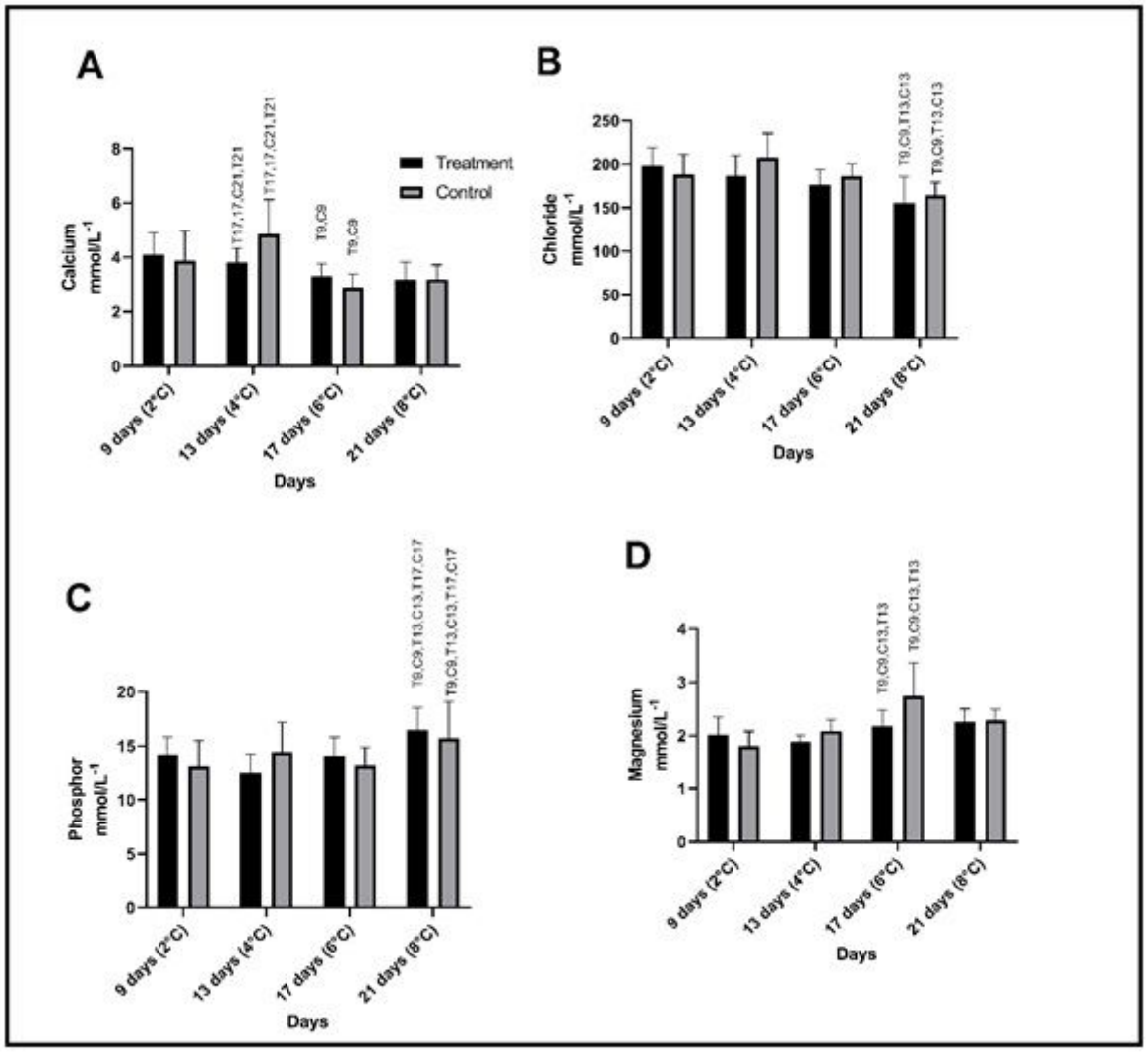

Figure 11

Concentrations of the osmo-ionic parameters calcium, phosphorus, and magnesium in N. rossii subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

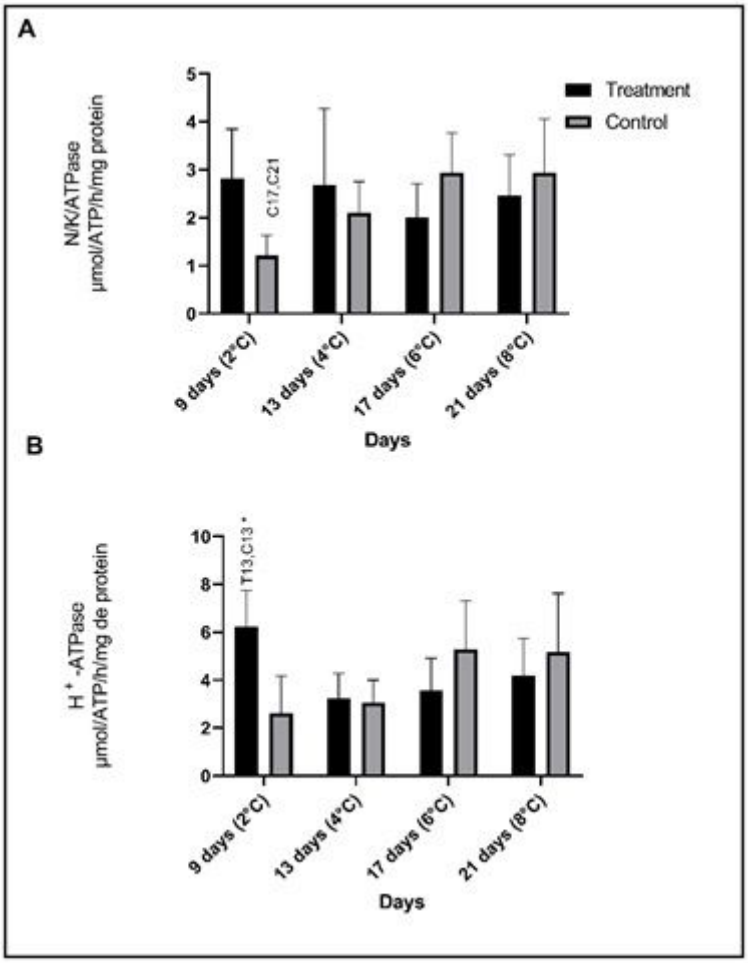

Figure 12

Concentrations of the enzymes Na⁺/ K⁺ ATPase and H⁺-ATPase in the gill tissue of N. rossii. subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

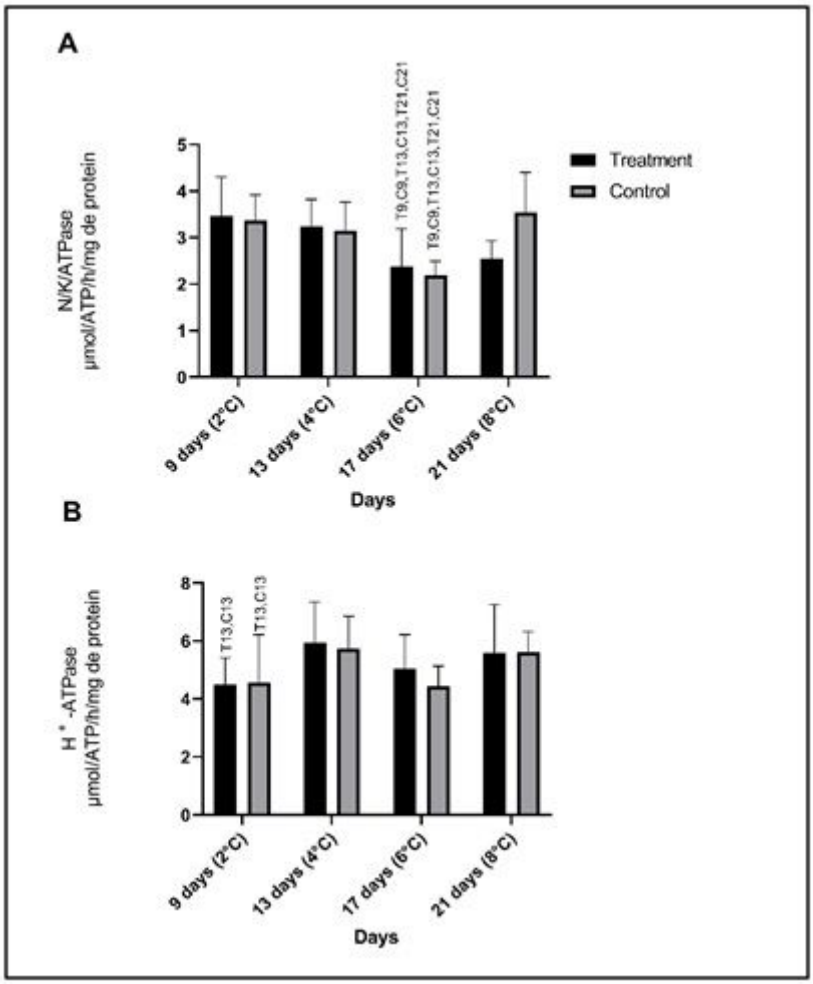

Figure 13

Concentrations of the enzymes Na⁺/ K⁺ ATPase and H⁺-ATPase in the kidney tissue of N. rossii. subjected to a gradual temperature increase (0.5°C/day) until temperatures of 2, 4, 6 and 8°C were reached. * indicates statistically significant variation, $p \leq 0.05$ between the experimental group and respective control group. The numbers above the bars indicate significant differences between the values obtained at different experimental times. **C=control, T=treatment

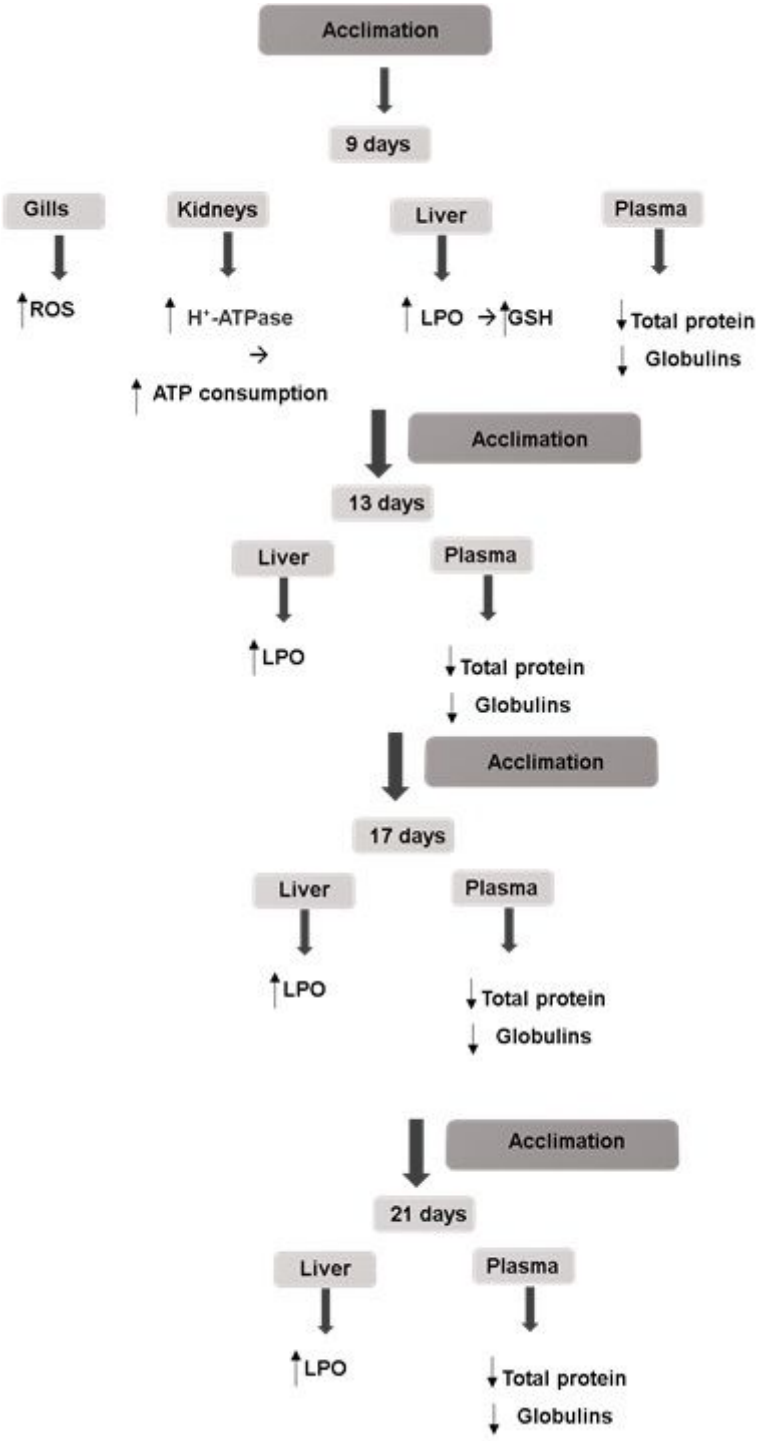

Figure 14

Biomarkers of oxidative metabolism and antioxidant defenses in plasma and H⁺-ATPase enzyme biomarkers over time in N. rossii subjected to a gradual temperature increase (0.5°C/day). ATP - Adenosine triphosphate; GST - Glutathione-S-transferase; ROS - Reactive oxygen species; LPO- Lipid peroxidation and GSH - Reduced glutathione.

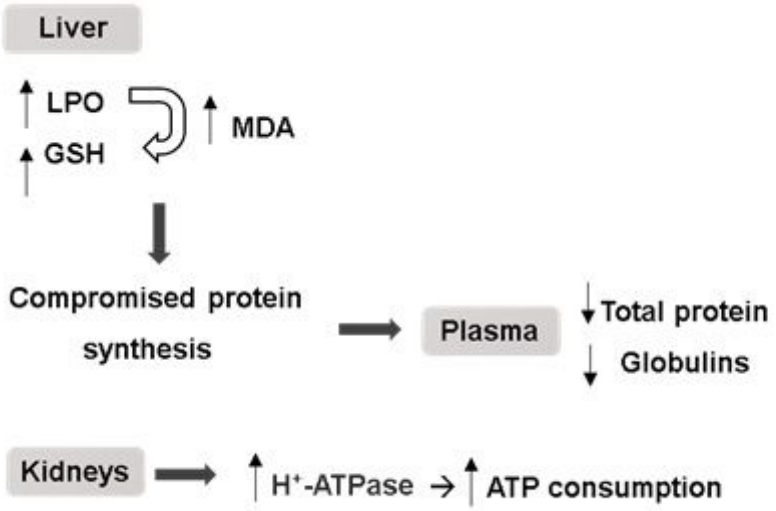

Figure 15

Diagram of changes in biomarkers over time in N. rossii subjected to a gradual temperature increase (0.5°C/day). MDA - Malondialdehyde; ATP - Adenosine triphosphate; GSH - Glutathione; LPO - Lipid peroxidation.