Thermal plasticity of the cardiorespiratory system provides cross-tolerance protection to fish exposed to elevated nitrate

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ABSTRACT

Exposure to nitrate is toxic to aquatic animals due to the formation of methaemoglobin and a subsequent loss of blood-oxygen carrying capacity. Yet, nitrate toxicity can be modulated by other stressors in the environment, such as elevated temperatures. Acclimation to elevated temperatures has been shown to offset the negative effects of nitrate on whole animal performance in fish, but the mechanisms underlying this cross-tolerance interaction remain unclear. In this study, juvenile silver perch (*Bidyanus bidyanus*) were exposed to a factorial combination of temperature (28ºC or 32ºC) and nitrate concentrations (0, 50 or 100 mg NO_3^- L^{-1}) treatments to test the hypothesis that thermal acclimation offsets the effects of nitrate via compensatory changes to the cardiorespiratory system (gills, ventricle and blood oxygen carrying capacity). Following 21 weeks of thermal acclimation, we found that fish acclimated to 32ºC experienced an expansion of gill surface area and an increase in ventricular thickness regardless of nitrate exposure concentration. Exposure to nitrate (both 50 and 100 mg NO_3^- L^{-1}) reduced the blood oxygen carrying capacity of silver perch due to increases in methaemoglobin concentration and a right shift in oxygen-haemoglobin binding curves in fish from both thermal acclimation treatment. These results indicate that plasticity of the gills and ventricle of warm acclimated fish are potential mechanisms which may provide cross-tolerance protection to elevated nitrate concentrations despite nitrate induced reductions to oxygen transport.

Key words: Blood oxygen affinity, Multiple stressors, Temperature, Thermal acclimation, Oxygen equilibrium curves.

INTRODUCTION

Anthropogenic activities have caused rampant increases in nitrate (NO_3^-) concentration, rendering nitrate a ubiquitous pollutant in freshwater ecosystems (Camargo et al., 2005). Nitrate is often discharged at high levels from various anthropogenic sources, including from fertilisers, intensive aquaculture, urban runoff, and atmospheric deposition (Camargo and Alonso, 2006; Camargo et al., 2005). Nitrate pollution is particularly prominent in areas of high fertiliser use where agricultural land practices (crop and livestock) have increased nitrate concentrations in surrounding waters (Camargo et al., 2005; Glibert, 2017). For example, after heavy rainfall, the use of a nitrate-based fertiliser (ammonium nitrate) caused the concentration of nitrate in surface-water to spike from 2 mg NO_3^- L^{-1} to 100 mg NO_3^- L^{-1} in sites adjacent to agriculture plantations (Jaynes et al., 2001). Highly urbanised areas face a similar problem; a positive correlation between nitrate concentration and human population density has been documented for many river systems worldwide (Gu et al., 2013; Mayo et al., 2019; Ouedraogo and Vanclooster, 2016), where surface- and ground-water nitrate concentrations have been recorded at persistently high levels of ~ 25 mg NO_3^- L^{-1} and up to 100 mg NO_3^- L^{-1}, respectively (reviewed by Galloway et al., 2004; Vitousek et al., 1997). Elevated nitrate concentrations can severely impact entire ecosystems, causing eutrophication, algal blooms, anoxic dead zones, altered food webs, and nitrate toxicity to residing species (Camargo et al., 2005; Glibert, 2017).

High levels of nitrate pollution are toxic to aquatic animals (Camargo et al., 2005; Gomez Isaza et al., 2020a). In waters high in nitrate, passive uptake of nitrate ions occurs across the gill epithelium where nitrate is transferred and dissolves in the plasma (Cheng et al., 2002; Jensen, 1996; Stormer et al., 1996). The main toxic action of nitrate is attributable to the oxidation of haemoglobin to methaemoglobin- a molecule which is unable to reversibly bind oxygen, resulting in inherent loss in blood oxygen transport capacity (Jensen, 1996, 2003; Monsees et al., 2017; Yang et al., 2019). The oxygen transport capacity of nitrate exposed fish is further impaired due to a right-shift in blood-oxygen binding curves (Gomez Isaza et al., 2020b), which reduces oxygen uptake at the gills. Nitrate toxicity, however, can be mediated by other stressors in the environment (Gomez Isaza et al., 2020a), such as low pH (Gomez Isaza et al., 2018, 2020b), water hardness (Baker et al., 2017), or elevated temperatures (Egea-Serrano and Van Buskirk, 2016; Opinion et al., 2020).

Elevated temperatures typically increase the toxicity of pollutants (Little and Seebacher, 2015; Patra et al., 2015; Philippe et al., 2018). The underlying increase in toxicity is likely due to an increased uptake rate, higher metabolic rates, or loss of respiration efficiency

(Noyes et al., 2009). Yet, chronic exposure to elevated temperatures has been shown to mask the effects of nitrate (Gomez Isaza et al., 2020c). Silver perch (*Bidyanus bidyanus*) acclimated to 32ºC were able to offset the negative effects of nitrate on aerobic scope, swimming performance, and upper thermal tolerance (i.e. CT_{MAX}) when compared to nitrate-exposed fish acclimated to the cooler temperature of 28ºC. Similar effects were shown for nitrate-exposed European grayling (*Thymallus thymallus*), whereby fish acclimated to a warmer temperature (22ºC) showed an increased, rather than decrease, in aerobic scope (Opinion et al., 2020). The capacity for performance to be maintained in nitrate-exposed fish exposed to a warmer temperature is suggestive of a cross-tolerance interaction between these two stressors, but the physiological mechanisms underlying this cross-tolerance interaction are unclear.

Physiological remodelling of the cardio-respiratory system that occurs during thermal acclimation are potential mechanisms facilitating cross-tolerance between elevated temperatures and elevated nitrate concentrations. Acclimation to elevated temperatures can be achieved via physiological remodelling of the cardio-respiratory system at all levels of the oxygen transport cascade (gills – blood – heart – etc.). Some adjustments include haematological (e.g. increase haemoglobin concentration, blood oxygen affinity; Akhtar et al., 2013; Kaufman et al., 2013), cardiac (e.g. changes to the proportion of ventricle muscle types; Anttila et al., 2015; Nyboer and Chapman, 2018), or remodelling of the gills (e.g. increased surface area; Bowden et al., 2014; Sollid and Nilsson, 2006), all of which can facilitate blood delivery to working tissues. As such, physiological adjustments that act to increase oxygen delivery in response to high temperature acclimation may provide cross-tolerance protection to fish experiencing nitrate-induced anaemia.

This study aimed to understand the mechanism/s underlying the cross-tolerance interaction between thermal acclimation and elevated nitrate concentrations which allowed warm acclimated silver perch to override the detrimental effect of elevated nitrate concentrations and maintain performance across a range of water temperatures (28 – 36ºC; Gomez Isaza et al., 2020c). We hypothesised that warm-acclimated silver perch would show plasticity of the cardiorespiratory system (heart, gills, blood), and that this plasticity provided protection against elevated nitrate concentrations. Specifically, we examined potential changes to the heart, which is known to be remarkably plastic (Franklin and Davie, 1992; Gamperl and Farrell, 2004; Keen et al., 2017) and hypothesised that warm acclimation would cause a reduction in ventricular mass but an increase the thickness of the compact layer. We also assessed changes to the gills. Because the gills are the primary site of oxygen uptake in fishes

(Evans et al., 2005), increases in the gill surface area were expected (e.g. reduced interlamellar cell mass, reduced lamellar thickness) to act as potential mechanisms allowing warm-acclimated fish to override the effects of nitrate (Gomez Isaza et al., 2020c). Lastly, we examined changes in blood-oxygen carrying capacity (haematocrit, haemoglobin concentration, methaemoglobin concentrations) and oxygen-haemoglobin binding affinity to test the hypothesis that warm-acclimated fish would increase oxygen carrying capacity and affinity to compensate for temperature changes.

MATERIAL AND METHODS

Animal Maintenance

Juvenile silver perch (*Bidyanus bidyanus*; n = 366) were sourced from a commercial hatchery (Ausyfish Pty. Ltd.) and transported to The University of Queensland in oxygenated transport bags. Fish were distributed among twenty-four, 40L glass tanks (60 × 25 × 30 cm; $l \times w \times h$) at a density of 15 – 16 fish per tank. Tanks were filled with filtered tap water and each equipped with a sponge filter for filtration and an air-stone for additional aeration. Fish were fed once daily with sinking pellets (2 mm pellets; Ridley Aqua-feeds TM, Narangba, Queensland, Australia). Fish were maintained under a constant 12:12 h light: dark cycle and allowed to adjust to laboratory conditions for one week. After this adjustment period, all fish were tagged using visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Shaw Island, USA) to allow for the tracking of individual fish. Fish were lightly anaesthetised (Aqui-S TM, Aqui-S Pty LTD, Lower Hutt, New Zealand) and tags (2 – 3 mm) were implanted below the skin, parallel to the dorsal fin. Fish were allowed one week to recover from tagging prior to the commencement of the experiment. During this time, all fish resumed eating and post-tag survival was 100%. All experiments were conducted in accordance with the Australian Animal Care guidelines and approved by The University of Queensland animal ethics committee (Ethics Approval No. SBS/249/17).

Experimental treatments

We employed a full 2 × 3 factorial design with two thermal acclimation treatments (28 and 32°C) and three nitrate concentrations (0, 50 and 100 mg NO_3^- L^{-1}). Thermal acclimation treatments were chosen to reflect (i) current day summer temperatures (28°C) along the northern Murray-Darling Basin where this fish occurs naturally and (ii) a high rate of climate warming (32°C) forecasted under a high degree of radiative forcing (high emissions - representative concentration pathway (RCP) 8.5) (CSIRO and Bureau of Meteorology, 2015).

Nitrate concentrations were chosen to reflect moderate (i.e. 50 mg NO_3^- L^{-1}– current recommended maximum level) and high levels (100 mg NO_3^- L^{-1}) of nitrate pollution (Environment Australia, 2002). Temperatures were adjusted and maintained using 300 W submersible heaters (Aqua Zonic Eco aquarium heaters). Temperature loggers (iButtons, Maxim Integrated, San Jose, USA) were submerged in each tank to record water temperature every hour. Water temperatures did not fluctuate by more than 1°C from target temperatures. Nitrate concentrations were prepared using reagent-grade sodium nitrate (ThermoFisher Scientific, Scoresby, Australia) and measured once daily using a nitrate meter (LAQUAtwin-NO3-11 meter, Horiba Scientific). Nitrate levels did not deviate from nominal concentrations by more than 10%. Fish were exposed to experimental treatments for 21 weeks prior to blood and tissue sampling.

Heart and Gill Histology

Fish (n = 6 – 9) were euthanased with an overdose of anaesthetic (250 mg L^{-1}; Aqui-S TM), weighed, and gills, ventricle, and spleen were removed. Spleen and ventricles were weighed fresh and relative spleen mass (RSM = spleen mass/fish body mass) and relative ventricle mass (RVM = ventricle mass/fish body mass) were calculated. Ventricle and gill samples were stored in zinc buffered formalin fixative (Z-fix, Anatech, MI, USA) for 24 h, then transferred to 70% ethanol and stored at room temperature prior to embedding. Organ samples were dehydrated through an ascending ethanol series (70% EtOH 1 h, 94% EtOH 1 h, 100% EtOH 3×1 h), cleared in xylene and embedded in paraffin wax (Histoplast Paraffin, ThermoFisher Scientific, Sydney, Australia). Serial sections (6 µm-thick) cut with a microtome (Leica RM2245, Leica Microsystems, NSW, Australia) were mounted on glass slides, de-waxed with UltraClear and rehydrated with an alcohol series and distilled water. Samples were stained with hematoxylin and eosin stain before being photographed (NIS-Elements software; v. 4.10, Nikon Instruments Inc., Tokyo, Japan). Organ samples were measured following published protocols (Anttila et al., 2015; Chapman et al., 2008; Nyboer and Chapman, 2018). Briefly, ventricle thickness was determined by measuring the distance from the distal edge of compact layer to the spongy myocardium. The area of compact myocardium was divided by the total area (compact + spongy) of the heart section to calculate the percent of compact myocardium (%CM). A series of four gill traits were selected and measured including: lamellar length, lamellar width, interlamellar distance and interlamellar cell mass (ILCM).

Blood carrying capacity

A small blood sample (~0.2 mL) was taken from fish (n = 6 per treatment) via caudal puncture using a 0.5 mL needle and 29-gauge syringe. Blood was immediately transferred to a 1.5 mL Eppendorf tube and stored on ice until analysis. A subsample of blood was transferred into two haematocrit tubes, centrifuged (3 min at 5000 *g*, micro-haematocrit centrifuge; Hawksley, Sussex, UK) and haematocrit (H_{CT}) was measured as the proportion of red blood cells in whole blood. The remaining blood was aliquoted and used to determine haemoglobin and methaemoglobin concentrations. Haemoglobin concentration ($[H_B]$) was determined spectrophotometrically at 405 nm and quantified against a standard curve of known $[H_B]$ using a Sigma-Aldrich haemoglobin assay kit (MAK115; St Louis, USA). Mean corpuscular haemoglobin concentration (MCHC) was calculated as $[H_B] \times 10/H_{CT}$. Methaemoglobin concentration ($[MetH_B]$) was determined by diluting 20 µL of whole blood in 1 mL of 34 mM phosphate buffer at pH 7.3. The haemolysate was centrifuged (microfuge®18 centrifuge, Beckman Coulter, Brea, United States) for 3 min at 12 000 *g,* and the absorbance (DU800 spectrophotometer, Beckman Coulter, Brea, United States) was measured at 560, 576 and 630 nm, following published protocols (Benesch et al., 1973). All assays were run in duplicate.

Oxygen binding affinity

Blood-oxygen binding affinities were determined using a Hemox-Analyser (Model B, TCS Scientific Corp., USA) and assayed at three acute test temperatures (28, 32, and 36ºC; n = 6 per treatment, per temperature). A sample of 50 µL of blood was suspended in 5 mL of buffered saline (Hemox™-Solution, pH 7.4), 20 µL of bovine serum albumin (BSA, additive A, Hemox™) and 10 µL of an anti-foaming agent (additive B, Hemox™). Nitrogen gas (compressed nitrogen pure, gas code 032, BOC, North Ryde, Australia) was used to achieve zero percent saturation of haemoglobin oxygen and then air (i.e. 20.9% oxygen, compressed air gas code 054) was used to obtain full saturation.

The effect of nitrate and acclimation temperature on the affinity constant, P_{50}, was obtained from the Hemox Analyser software (Hemox analytical software version 2, TCS Scientific Corp.). The temperature sensitivity of oxygen binding affinity was expressed using the van't Hoff equation:

$$\Delta H^o = 2.303 \times R \times ((\Delta \log P_{50})/(\Delta 1/T)) \text{ kJ mol}^{-1}$$

where R is the universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and T is the measurement temperature in K. Hill plots were also constructed by the Hemox software based on the equation (Laursen et al., 1985):

$$\log Y / (100 - Y) = n\log(PO_2) - n\log P_{50}$$

where Y is the percent oxygen saturation of Hb. Hill coefficients (n_H) were then obtained by calculating the slopes of these plots.

Statistical analyses

All data were analysed in the *R* programming environment (R Core Team, 2018) using the RStudio interface (version 1.0.153). Parametric assumptions of normality and equal variances were tested using the Shapiro-Wilk and Levene tests, respectively. Linear mixed effects models were used to test for statistical differences between nitrate and thermal acclimation treatments for all analyses, using the *lme* function of the *nlme* package (Pinheiro et al., 2017). Significant differences were accepted as $P < 0.05$. Data are presented as mean ± standard error unless otherwise stated.

RESULTS

Histology

Fish acclimated to 32°C had significantly larger ventricles relative to body size (RVM; Table 1) than those acclimated to 28°C ($t = 5.39$, $df = 19$, $P < 0.0001$). RVM was, however, unaffected by nitrate concentration (50 mg NO_3^- L^{-1}: $t = 0.17$, $df = 19$, $P = 0.86$; 100 mg NO_3^- L^{-1}: $t = 1.38$, $df = 19$, $P = 0.18$) or the interaction between nitrate concentration and acclimation temperature (50 mg NO_3^- L^{-1}: $t = -0.23$, $df = 17$, $P = 0.82$; mg NO_3^- L^{-1}: $t = -0.86$, $df = 17$, $P = 0.39$). An increase in RVM was accompanied by increases in compact myocardium thickness ($t = 5.07$, $df = 25$, $P < 0.0001$; Fig. 1A, B) among 32°C-acclimated fish, but myocardium thickness did not change with exposure to elevated nitrate concentrations (50 mg NO_3^- L^{-1}: $t = -0.25$, $df = 17$, $P = 0.81$; 100 mg NO_3^- L^{-1}: $t = -0.81$, $df = 17$, $P = 0.43$).

Thermal acclimation and nitrate exposure treatments influenced the respiratory surface area of the gills (Fig. 2). Exposure to elevated nitrate concentrations affected the lamellar length of silver perch, but the effect was dependent on thermal acclimation treatment ($F_{2, 12} = 9.62$, $P = 0.003$). In 28°C-acclimated fish, exposure to elevated nitrate concentrations caused a mean decrease in lamellar length of 1.5 μm and 9.5 μm in fish exposed to 50 and 100 mg NO_3^- L^{-1}, respectively (Fig. 2C), while exposure to nitrate did not influence the lamellar length of 32°C-acclimated fish. Instead, the lamellar length of 32°C-acclimated fish was increased by approximately 23% to 46.7 ± 0.6 μm regardless of nitrate-exposure treatment compared to the lamellae of 28°C-acclimated fish (37.9 ± 1.5 μm). Increases in lamellar length were largely due to a 3.5 – 31.2% reduction in the height of the interlamellar cell mass (ILCM; Fig. 2F) among 32°C-acclimated fish ($F_{1, 14} = 6.54$, $P = 0.02$), but ILCM was unaffected by nitrate exposure

($F_{2, 14}$ = 1.80, P = 0.20). Fish exposed to nitrate (from both concentrations) tended to have significantly wider lamellae ($F_{2, 12}$ = 5.10, P = 0.02), as did fish acclimated to 32ºC ($F_{1, 12}$ = 11.89, P = 0.044); however, lamellar width was not affected by their interaction ($F_{2, 12}$ = 2.70, P = 0.11; Fig. 2D). The interlamellar distance of 32ºC-acclimated fish was also decreased compared to 28ºC-acclimated fish ($F_{1, 14}$ = 6.03, P = 0.03; Fig. 2E), but interlamellar distance was unaffected by nitrate exposure at either acclimation temperature ($F_{2, 14}$ = 1.99, P = 0.17).

Blood carrying capacity

Exposure to nitrate (50 and 100 mg NO_3^- L^{-1}) caused a stepwise decrease in haemoglobin concentration ([H_B]: 50 mg NO_3^- L^{-1}: t = -5.86, df = 19, P < 0.0001; 100 mg NO_3^- L^{-1}: t = -10.01, df = 19, P < 0.0001), haematocrit (H_{CT}: 50 mg NO_3^- L^{-1}: t = -5.49, df = 19, P < 0.0001; 100 mg NO_3^- L^{-1}: t = -6.51, df = 19, P < 0.0001), and mean corpuscular haemoglobin concentration (MCHC: 50 mg NO_3^- L^{-1}: t = -1.59, df = 19, P = 0.12; 100 mg NO_3^- L^{-1}: t = -4.46, df = 19, P < 0.001) of silver perch from both thermal acclimation treatments (Table 1), but the interaction between nitrate and acclimation treatment on these parameters was not significant ([H_B]: 50 mg NO_3^- L^{-1}= t = -0.33, df = 17, P = 0.75; 100 mg NO_3^- L^{-1} = t = -0.22, df = 17, P = 0.83; H_{CT}: 50 mg NO_3^- L^{-1}= t = -1.09, df = 17, P = 0.29; 100 mg NO_3^- L^{-1} = t = 0.07, df = 17, P = 0.94). Methaemoglobin concentrations ($MetH_B$) were significantly affected by the interaction between nitrate and thermal acclimation treatments (50 mg NO_3^- L^{-1} = t = -4.17, df = 17, P < 0.001; 100 mg NO_3^- L^{-1}= t = -4.61, df = 17, P < 0.001). $MetH_B$ levels increased linearly with increasing nitrate concentration in fish from both acclimation treatments, but 28ºC-acclimated fish tended to have higher levels of $MetH_B$ when exposed to the same nitrate concentration (Table 1). As such, $MetH_B$ of 28ºC-acclimated fish were 33% and 24% higher in fish exposed to 50 and 100 mg NO_3^- L^{-1}, respectively, than fish acclimated to 32ºC.

Oxygen Equilibrium Curves

Test temperature caused a linear increase in the oxygen binding affinity constant, P_{50}, of silver perch (32ºC: t = 2.84, df = 81, P = 0.006; 36ºC: t = 6.83, df = 81, P < 0.0001) and P_{50} increased by about 22 – 29% over an 8ºC temperature range in fish from all treatments (Fig. 3A, B). Both nitrate and thermal acclimation treatment had significant effects on the P_{50} of fish (50 mg NO_3^- L^{-1}: t = 2.63, df = 81, P = 0.01; 100 mg NO_3^- L^{-1}: t = 2.99, df = 81, P = 0.003; acclimation temperature: t = 2.61, df = 21, P = 0.02), whereas the interaction between these two factors was not significant (50 mg NO_3^- L^{-1}: t = 0.62, df = 19, P = 0.54; 100 mg NO_3^- L^{-1}: t = 0.77, df = 19, P = 0.45). Fish acclimated to 32ºC experienced higher P_{50} values than

28ºC fish but the ΔP_{50} was similar between fish from both acclimation temperatures. Moreover, the ΔP_{50} of control fish (i.e. unexposed) tended to increase more than nitrate exposed fish (Table 6.2) across an 8ºC temperature range (28 – 36ºC). Hill coefficients (n_H) ranged between 0.75 – 1.99 in silver perch from all treatments. This value remained relatively constant among 28ºC-acclimated fish across test temperatures ($\Delta n_H > 0.12$) but tended to increase in 32ºC-acclimated fish (Table 2). The temperature effect on blood oxygen affinity tended to increase with test temperature (Table 2), as shown by increases in the van't Hoff value, $\Delta H°$. Moreover, the temperature sensitivity of oxygen binding ($\Delta H°$) was less pronounced among nitrate exposed fish than in control (unexposed) fish.

DISCUSSION

Thermal acclimation can provide protective effects against nitrate pollution (Gomez Isaza et al., 2020c), but the mechanism underlying this cross-tolerance interaction is unknown. In support of our first two hypotheses, we found that silver perch remodelled critical aspects of their cardiorespiratory system (i.e. increase ventricular thickness, decreased lamellar thickness, reduced interlamellar cell mass) in response to elevated temperatures and the plasticity of these was independent of nitrate exposure concentration. The blood-oxygen carrying capacity of fish was, however, unchanged following 21 weeks of thermal acclimation, which rejects our third hypothesis, indicating that thermal plasticity of the blood-oxygen carrying capacity is absent in this species. Plastic responses to the cardiorespiratory system of silver perch likely underlie the cross-tolerance interaction experienced by warm-acclimated silver perch despite being exposed to high levels of nitrate (Gomez Isaza et al., 2020c).

Plasticity of the cardiorespiratory system

Silver perch acclimated to 32ºC increased the morphological capacity of the gills for gas exchange. This increase in gill surface area was primarily a result of reductions in the interlamellar cell mass (ILCM) that increased the length of the exposed lamellae. ILCM remodelling is a highly plastic trait that allows fish to quickly respond to environmental stressors that increase the demand for oxygen uptake (Evans et al., 2005; Gilmore and Perry, 2018; Nilsson, 2007) and numerous studies have documented such changes following warm temperature acclimation (Anttila et al., 2015; Bowden et al., 2014; Nyboer and Chapman, 2018; Sollid and Nilsson, 2006). These plastic changes facilitated oxygen uptake at the gills of 32ºC-acclimated silver perch but may come at the cost of increased ion-regulatory demands and increased uptake of pollutants (Evans, 1987; Gilmore and Perry, 2018).

Our results suggest that nitrate exposure does not trigger the remodelling of the gills to cope with an increased oxygen demand. Instead, there is some evidence of lamellar shortening and widening, which may be indicative of histopathological changes to the gills. These morphological changes, however, only occurred among 28ºC-acclimated fish. Histopathological changes to the gills of nitrate exposed fish have been widely studied because of the direct contact between the gills and the nitrate ions in the water and because the gills are thought to be the main site of nitrate uptake (Cheng et al., 2002; Stormer et al., 1996). These studies have revealed a number of changes following nitrate exposure, including hyperplasia and hypertrophy of the secondary lamellae, haemorrhages, hyperaemia and necrosis (Davidson et al., 2014; Monsees et al., 2017; Pereira et al., 2017; Rodrigues et al., 2011). These changes are likely non-specific responses to the presence of toxicants, aiming to protect the animal from toxicity (Rodrigues et al., 2011). These histopathological changes, however, can compromise the functionality of the gills and may partially explain the poor aerobic performance of nitrate exposed fish (Gomez Isaza et al., 2020b, c)

The cardiorespiratory system of various fishes has been shown to be remarkably plastic to cope with long term changes in temperature (Farrell et al., 2009; Gamperl and Farrell, 2004; Keen et al., 2017). Typically, warm temperature acclimation causes a reduction in the ventricle mass, but an increase in the thickness of the compact layer (Anttila et al., 2015; Gräns et al., 2014; Nyboer and Chapman, 2018). Consistent with these previous findings, silver perch acclimated to the warmer temperature of 32ºC increased the thickness of the compact layer but also had larger ventricles (RVM) relative to conspecifics acclimated to 28ºC. These morphological changes likely allow for an increase in cardiac output to meet the increased oxygen demand of the tissues at elevated temperatures. The degree of cardiac remodelling seen in silver perch was quite remarkable. The 32ºC acclimated fish increased the thickness of compact layer by approximately 30 – 40% compared to 28ºC-acclimated fish. This indicates a high degree of plasticity of the ventricle compared to other fishes. For example, the compact layer of 29ºC-acclimated Nile perch (*Lates niloticus*) was ~30% thicker than conspecific acclimated to 25ºC (Nyboer and Chapman, 2018), while rainbow trout (*Oncorhynchus mykiss*) experienced a ~22% increase in the compact layer following acclimation to 17ºC compared to their cold-temperature acclimated (12ºC) conspecifics (Klaiman et al., 2011). Increases in ventricular characteristics were independent of nitrate concentration such that all fish acclimated to 32ºC had larger ventricles than conspecifics acclimated to 28ºC. Such changes to the ventricle architecture among 32ºC-acclimated fish may have contributed to the

maintenance of cardiorespiratory performance of silver perch across an 8ºC temperature range (Gomez Isaza et al., 2020c) in spite of nitrate-induced reductions to oxygen transport. Indeed, cardiorespiratory changes in fish have been linked to higher temperature tolerance in various fishes. Cardiorespiratory capacity was thought to underlie resilience to warming temperatures in pink salmon (*Oncorhynchus gorbuscha*; Clark et al., 2011) and ventricle size has been positively correlated with higher temperature tolerance in populations of Atlantic Salmon (Anttila et al., 2013) and landlocked salmon (*Salmo salar* m. sebago; Anttila et al., 2015) indicating that cardiac plasticity may enable fish to cope with elevated temperatures. It is interesting, however, that nitrate-exposed fish acclimated to 28ºC were unable to responsively increase RVM or ventricular thickness as ventricular plasticity has been documented in response to declines in blood oxygen supply (McClelland et al., 2005; Simonot and Farrell, 2007). For example, rainbow trout compensated for experimentally induced anaemia (induced by injections of phenylhydrazine hydrochloride) by increasing their ventricular mass. More importantly, warm-acclimated (17.6ºC) fish were better able to compensate for anaemic conditions, with ventricular mass increasing by 28% relative to control (not injected) fish versus only a 15% increase in cold-acclimated (6.4ºC) fish (Simonot and Farrell, 2007). Nitrate exposure, however, does not induce extreme reductions in haemoglobin, H_B (~15 – 30% decrease in H_B), or haematocrit (H_{CT}) levels (~ 9 – 15% decrease in H_{CT}) so it is likely that more extreme reductions, as seen in fish injected with phenylhydrazine hydrochloride (~40 – 83% decrease in H_B, ~60 – 84% decrease in H_{CT}; Simonot and Farrell, 2007), are required before physiological changes to the RVM are triggered.

Blood carrying capacity and oxygen affinity

The blood-oxygen carrying capacity of silver perch was reduced by chronic exposure to elevated nitrate concentrations. H_B concentrations were reduced and methaemoglobin ($MetH_B$) levels were increased in fish exposed to both 50 and 100 mg NO_3^- L^{-1}; however, levels of $MetH_B$ tended to be lower in fish acclimated to 32ºC than 28ºC-acclimated animals. These results reflect the main toxic action of nitrate (oxidation of H_B to $MetH_B$; Grabda et al., 1974; Monsees et al., 2017; Yang et al., 2019) and explain the decrements in aerobic scope and performance seen in nitrate-exposed silver perch (Gomez Isaza et al., 2020c). Similar effects have been documented for other fish; for example, $MetH_B$ of nitrite-exposed striped catfish (*Pangasianodon hypophthalmus*) were approximately 20% lower in warm-acclimated (33ºC) fish than fish acclimated to the cooler temperature of 27ºC (Ha et al., 2019). This result is suggestive of an increased efficiency of methaemoglobin reductase activity (the enzyme

responsible for converting $MetH_B$ back to functional H_B inside erythrocytes; Jensen, 2003) at elevated temperatures. Indeed, erythrocyte methaemoglobin reduction has been shown to be highly thermally sensitive with a thermal sensitivity quotient (Q_{10}) of 2.8 in rainbow trout between 15ºC and 25ºC (Jensen and Nielsen, 2018), and methaemoglobin reductase activity levels were about 2-fold greater in warm- verses cold-acclimated striped catfish (Ha et al., 2019). This high thermal sensitivity of fish methaemoglobin reductase has likely been selected for to counter H_B autoxidation which is accelerated at elevated temperatures (Jensen, 2001) and is a likely mechanism underlying the improved performance of nitrate-exposed fish at acclimated to elevated temperatures (Gomez Isaza et al., 2020c; Opinion et al., 2020)

Nitrate exposure also decreased blood-oxygen affinity (measured as P_{50}, the PO_2 at which blood is 50% oxygen saturated**)**. This decrease in oxygen binding affinity is likely caused by an increase in nucleoside triphosphates (NTP) levels in the red-blood cells of nitrate-exposed fish (Jensen et al., 1987), which increases the ratio of NTP to functional haemoglobin (NTP/H_B), thereby lowering oxygen binding affinity (Val, 2000). Similar right-shifts have been documented in nitrate-exposed spangled perch (Gomez Isaza et al., 2020b) and nitrite exposed carp (*Cyprinus carpio*; Jensen, 1990; Jensen et al., 1987; Williams et al., 1993) and rainbow trout (*Oncorhynchus mykiss*; Nikinmaa and Jensen, 1992) and indicate a reduced oxygen binding at the gills. Interestingly, however, there were no differences among thermal acclimation treatments on the oxygen binding affinity indicating a limited degree of plasticity in oxygen binding capacity of silver perch.

Fish respond to changes in temperature through a combination of mechanisms aimed at increasing blood-oxygen carrying capacity or by changing haemoglobin-oxygen affinity (Akhtar et al., 2013; Kaufman et al., 2013). Most commonly, fish can increase the proportion of red-blood cells (i.e. H_{CT}) or increase H_B to cope with a higher oxygen demand (Ahmed et al., 2020). Fish may also express multiple haemoglobin isoforms with different oxygen binding and affinity properties (Andersen et al., 2009) or modify haemoglobin-oxygen affinity via allosteric modifiers (ATP and GTP; Albers et al., 1983; Damsgaard et al., 2013). Such changes to the blood carrying capacity following thermal acclimation were hypothesised to underlie the cross-tolerance protection to elevated nitrate concentrations seen in silver perch (Gomez Isaza et al., 2020c). However, there was no indication that silver perch increased $[H_B]$ or H_{CT} levels following 21 weeks of thermal acclimation, suggesting that plasticity of the blood-oxygen carrying capacity is absent or minimal in silver perch. The H_{CT} and H_B levels of silver perch are on the high end of the normocythemic ranges of freshwater fishes (H_{CT} range 17 – 44%;

Ahmed et al., 2020; Gallaugher et al., 1995), including various Australian temperate species (Gomez Isaza et al., 2020b; Wells et al., 1997), and may indicate that H_{CT} levels are already maximised at a level that does not compromise cardiac performance due to elevated blood viscosity (Gallaugher et al., 1995). There were also no changes to blood-oxygen affinity following thermal acclimation. The P_{50} of silver perch averaged at 33.2 mmHg at a test temperature of 28ºC, which indicates a low oxygen affinity relative to other temperate fishes (Du et al., 2018; Gomez Isaza et al., 2020b; Kaufman et al., 2013). P_{50} was however, thermally sensitive and increased by 38% to 45.8 mmHg across an 8ºC temperature range indicating a reduced affinity with increases in temperature. Temperature has been recognised as a key regulator of haemoglobin function because oxygen binding by the heme group is exothermic (i.e. the oxygenation enthalpy, ΔH^{O}, is negative) and underlies the commonly observed reduction in Hb-oxygen affinity with rising temperatures (Albers et al., 1983; Cech et al., 1994; Powers, 1980). Most fishes typically have ΔH^{O} values of -15 – -50 kJ mol^{-1} (Kaufman et al., 2013; Soldatov, 2003; Verheyen et al., 1986). Silver perch red-blood cells showed this typical exothermic oxygenation reaction, with a negative ΔH^{O} value of -38.3 (-6.1 – -67.6, range) over an 8ºC temperature range. This temperature effect is thought to facilitate the unloading of oxygen at elevated temperatures, but temperature-induced increases in P_{50} compromises oxygen binding at the gills (Soldatov, 2003), especially when coupled with other stressors like nitrate exposure or environmental hypoxia. Together, these data suggest that thermal plasticity of blood-oxygen carrying capacity is absent in silver perch and did not contribute to the cross-tolerance protection of elevated nitrate concentrations seen in silver perch (Gomez Isaza et al., 2020c).

CONCLUSION

Nitrate exposure reduced the blood-oxygen carrying capacity of fishes by increasing the formation of methaemoglobin, thereby lowering the concentrations of functional haemoglobin and reducing oxygen binding affinity of haemoglobin. These changes can increase susceptibility to other stressors, such as elevated temperatures. Yet, here we show that when fish are acclimated to a warmer temperature (32ºC), they undergo remodelling of the cardiorespiratory systems (increase ventricular thickness, decreased lamellar thickness, reduced interlamellar cell mass). Plasticity of the heart and gills are potential mechanisms induced by thermal acclimation to 32ºC which provided cross-tolerance protection to elevated nitrate concentrations and likely contribute to the maintenance of aerobic scope and performance at elevated temperatures (Gomez Isaza et al., 2020c) in spite of nitrate induced

reductions to oxygen transport. These results highlight the unpredictability of stressor interactions which can, in some instances, provide cross-over protection to other stressors.

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COMPETING INTEREST

The authors declare no competing interests.

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686 **Tables**

687 **Table 1.** Blood delivery parameters of silver perch (*Bidyanus bidyanus*) acclimated to one of
688 two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100
689 mg NO_3^- L^{-1}).

	28°C-acclimated			32°C-acclimated		
Nitrate (mg L^{-1})	**0**	**50**	**100**	**0**	**50**	**100**
Haematocrit (%)	32.7 (± 0.5)	29.7 (± 0.4)	28.3 (± 0.6)	32.2 (± 0.5)	28.1 (± 0.4)	27.5 (± 0.4)
H_B (g dL^{-1})	8.7 (± 0.2)	7.4 (± 0.3)	6.4 (± 0.3)	8.7 (± 0.1)	7.2 (± 0.2)	6.2 (± 0.3)
Functional H_B (%)	95.7 (0.7)	79.4 (± 0.7)	66.2 (± 1.2)	95.8 (± 0.6)	86.3 (± 0.6)	74.3 (± 0.8)
MetHb (%)	4.3 (± 0.7)	20.6 (± 0.7)	33.8 (± 1.2)	4.2 (± 0.6)	13.7 (± 0.6)	25.7 (± 0.8)
MCHC (g dL^{-1})	2.7 (± 0.1)	2.5 (± 0.1)	2.3 (± 0.1)	2.7 (± 0.1)	2.5 (± 0.1)	2.2 (± 0.1)
RVM (%)	0.05 (± 0.002)	0.05 (± 0.003)	0.06 (± 0.004)	0.07 (± 0.004)	0.07 (± 0.004)	0.08 (±0.003)
RSM (%)	0.04 (± 0.001)	0.04 (± 0.002)	0.04 (± 0.002)	0.04 (± 0.003)	0.04 (± 0.002)	0.04 (± 0.002)

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Table 2. Thermodynamic effect of blood-oxygen affinity data from silver perch (*Bidyanus bidyanus*) acclimated to one of two temperatures (28 or 32ºC) and exposed to one of three nitrate concentrations (0, 50 or 100 mg NO_3^- L^{-1}), where $\Delta H°$ is the apparent heat of oxygenation, and Δn_H is the temperature dependence of Hill's cooperativity coefficient.

	28ºC-acclimated			**32ºC-acclimated**		
Nitrate (mg L^{-1})	**0**	**50**	**100**	**0**	**50**	**100**
28 – 32						
$\Delta H°$ (KJ mol^{-1})	-28.9 (± 4.3)	-32.7 (± 8.7)	-28.1 (± 7.1)	-45.8 (± 14.7)	-26.5 (± 10.5)	-28.4 (± 13.1)
ΔP_{50}	8.4 (± 1.7)	6.2 (± 1.6)	5.4 (± 1.2)	8.2 (± 2.7)	6.0 (± 2.4)	5.8 (± 2.4)
Δn_H	-0.12 (± 0.09)	-0.08 (± 0.13)	-0.02 (± 0.13)	0.37 (± 0.16)	0.32 (± 0.13)	0.22(± 0.15)
32 – 36						
$\Delta H°$ (KJ mol^{-1})	-72.9 (± 18.5)	-40.4 (± 13.9)	-42.7 (± 12.5)	-50.5 (± 13.7)	-31.4 (± 13.6)	-32.1 (± 7.4)
ΔP_{50}	8.1 (± 2.9)	9.4 (± 3.3)	9.7 (± 2.8)	11.2 (± 3.0)	8.4 (± 3.4)	8.4 (± 2.2)
Δn_H	0.11 (± 0.17)	0.11 (± 0.16)	0.01 (± 0.11)	0.10 (± 0.06)	0.06 (± 0.07)	0.18 (± 0.05)
28 – 36						
$\Delta H°$ (KJ mol^{-1})	-50.7 (± 8.5)	-36.5 (± 6.2)	-35.3 (± 7.7)	-48.1 (± 10.4)	-28.9 (± 8.8)	-30.2 (± 8.9)
ΔP_{50}	16.5 (± 2.9)	15.6 (± 2.9)	15.1 (± 3.0)	19.5 (± 4.3)	14.4 (± 4.0)	14.2 (± 3.9)
Δn_H	-0.09 (± 0.19)	0.03 (± 0.07)	-0.01 (± 0.11)	0.47 (± 0.13)	0.37 (± 0.15)	0.40 (± 0.13)

Figures:

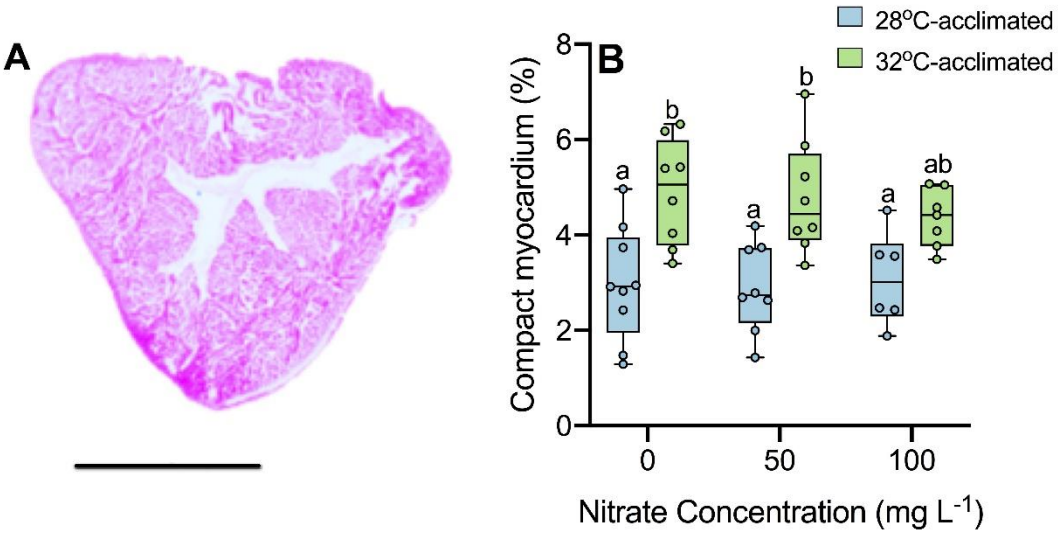

Figure 1. Histological sections and measurements of the ventricle of silver perch (*Bidyanus bidyanus*) exposed to a factorial combination of nitrate and temperature acclimation treatments. (A) Representative image of a ventricle of 28°C-acclimated fish exposed to 0 mg NO_3^- L^{-1}, with scale bar representing 100 µm. (B) Compact myocardium thickness (%) expressed as a percentage of ventricle mass. Data are presented as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum], and dots represent individual data points (n = 6 – 9).

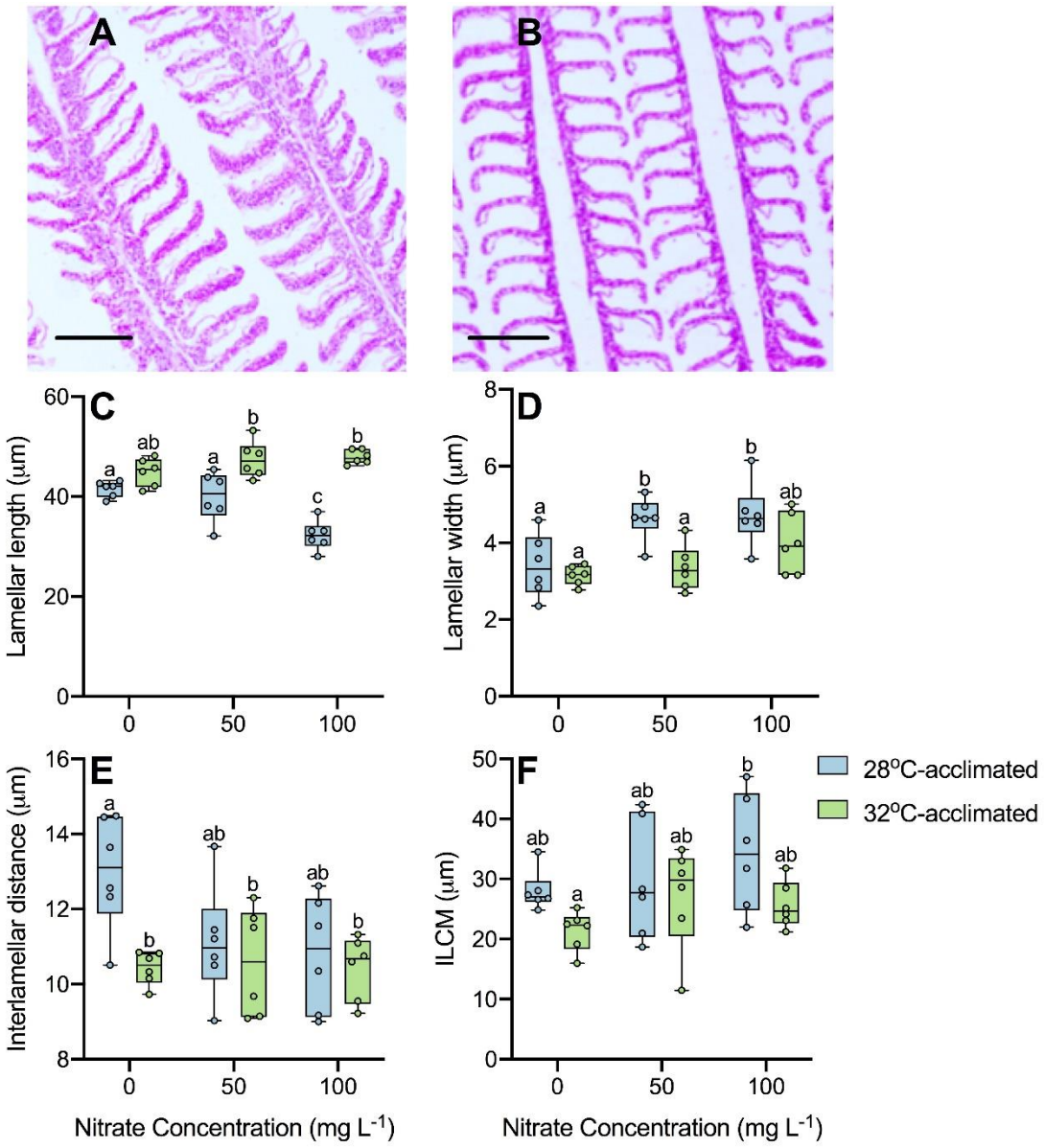

Figure 2. Histological sections and measurements of the gill filaments of silver perch (*Bidyanus bidyanus*) exposed to a factorial combination of nitrate and temperature acclimation treatments. Representative images of (A) 28°C-acclimated fish exposed to 50 mg NO_3^- L^{-1} and (B) 32°C-acclimated fish exposed to 50 mg NO_3^- L^{-1}. Scale bar represents 50 µm. (C) Lamella length (µm), (D) lamellar width (µm), (E) interlamellar distance (µm) and (F) interlamellar cell mass (ILCM; µm). Data are presented as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum], and dots represent individual data points (n = 6).

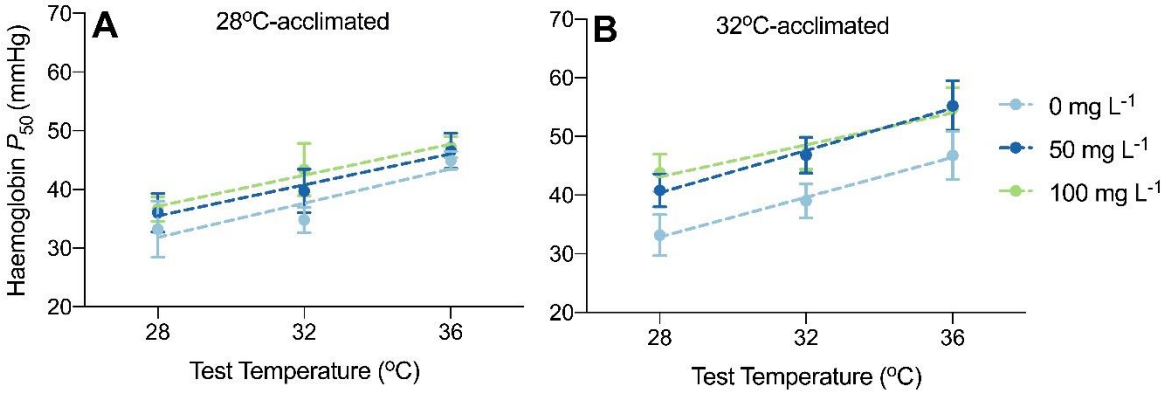

Figure 3. Oxygen affinity (P_{50}) of silver perch (*Bidyanus bidyanus*) exposed to a factorial combination of nitrate and temperature acclimation treatments. Data are represented as mean ± standard error.