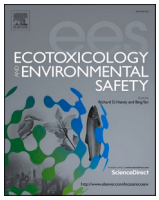
Ecotoxicology and Environmental Safety 225 (2021) 112777

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Better together: Cross-tolerance induced by warm acclimation and nitrate exposure improved the aerobic capacity and stress tolerance of common carp *Cyprinus carpio*

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ARTICLE INFO

Edited by: Paul Sibley

Keywords:
Climate change
Eutrophication
Hypoxia
Aerobic capacity
Cross-tolerance
Multiple stressors

ABSTRACT

Climate warming is a threat of imminent concern that may exacerbate the impact of nitrate pollution on fish fitness. These stressors can individually affect the aerobic capacity and stress tolerance of fish. In combination, they may interact in unexpected ways where exposure to one stressor may heighten or reduce the resilience to another stressor and their interactive effects may not be uniform across species. Here, we examined how nitrate pollution under a warming scenario affects the aerobic scope (AS), and the hypoxia and heat stress susceptibility of a generally tolerant fish species, common carp *Cyprinus carpio*. We used a 3 × 2 factorial design, where fish were exposed to one of three ecologically relevant levels of nitrate (0, 50, or 200 mg NO_3^- L^{-1}) and one of two temperatures (18 °C or 26 °C) for 5 weeks. Warm acclimation increased the AS by 11% due to the maintained standard metabolic rate and increased maximum metabolic rate at higher temperature, and the AS improvement seemed greater at higher nitrate concentration. Warm-acclimated fish exposed to 200 mg NO_3^- L^{-1} were less susceptible to acute hypoxia, and fish acclimated at higher temperature exhibited improved heat tolerance (critical thermal maxima, CTMax) by 5 °C. This cross-tolerance can be attributed to the hematological results including maintained haemoglobin and increased haematocrit levels that may have compensated for the initial surge in methaemoglobin at higher nitrate exposure.

1. Introduction

Aquatic ecosystems are afflicted by "cocktails" of environmental stressors which put tremendous pressure on the organisms living therein (Jackson, et al., 2015; Reid et al., 2018). As such, protection of aquatic biota hinges on how well we understand the compounding effects of stressors. However, acquiring comprehensive knowledge on the cumulative effects of stressor combinations is complicated by the diversity of ecological adaptation among species and the tendency of stressors to interact non-additively. This renders single stressor research with little predictive power, and thus necessitates multi-stressor experiments on a vast number of species (Todgham and Stillman, 2013; Jackson et al., 2015; Côté et al., 2016; Reid et al., 2018).

Climate warming is one of the environmental stressors that poses immense threat on the persistence of aquatic biota. Global average temperature has been rising since 1970 (IPCC, 2018) and models predict up to 7 °C rise in global air temperature over the next century (Ficke et al., 2007) with similar increases in water temperature (Reiger and Meisner, 1990). The continuous temperature rise, together with the more frequent and intense heat waves that are forecasted to occur as climate warming progresses (Ficke et al., 2007), are particularly precarious for ectothermic organisms, including fishes. Physiological functioning of ectotherms is dictated by their thermal environment (Guderley and Blier, 1988) making them at risk of performance reductions when temperatures exceeds their thermal limits (Pörtner et al., 2001; 2017). As conceptualized by the oxygen- and capacity-limited thermal tolerance (OCLTT) concept, the reduction in organismal performance induced by high temperature is assumed to arise from the diminished aerobic scope (AS, Pörtner et al., 2017). AS is the difference between the standard (SMR) and maximum metabolic rates (MMR), and it represents the capacity of organisms to supply oxygen needed beyond maintenance requirements to support fitness-related activities (Clark et al., 2011; Pörtner et al., 2001). The OCLTT concept presents that AS is optimized within a given temperature range but it declines as temperature approaches both lower and upper thermal limits (Pörtner et al., 2001; 2017). Concurring with this concept, several studies reported that

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https://doi.org/10.1016/j.ecoenv.2021.112777
Received 2 August 2021; Received in revised form 8 September 2021; Accepted 10 September 2021
Available online 14 September 2021

AS declines at temperatures beyond the upper thermal optima due to the inefficiency of the cardiorespiratory systems (manifested by the plateauing or declining MMR) to support the increased cellular oxygen demands (reflected by the increasing SMR) induced by warming (Eliason et al., 2013; Healy and Schulte, 2012; Muñoz et al., 2018; Sandblom et al., 2016). This warming-induced narrowing of AS may eventually reduce fitness-related performance such as growth, locomotion, digestion, stress tolerance, and reproduction (Clark et al., 2011; Healy and Schulte, 2012; Pörtner et al., 2017). However, the generality of this concept was brought into question as it is not applicable across species (Gräns et al., 2014; Lefevre, 2016; Norin et al., 2013; Poletto et al., 2017).

The menace posed by rising global temperature is further complicated by its probable interaction with existing environmental threats including eutrophication. Eutrophication is characterized by the development of nuisance algal blooms stimulated by excess concentration of nutrients such as nitrogen (ammonia, nitrite and nitrate) and phosphorus, which eventually results in nightly or prolonged hypoxia events (Camargo and Alonso, 2006; Díaz-Alvarez et al., 2018). As climate warming proceeds, more eutrophic events are expected as temperature elevation may boost nutrient cycling rates (Dokulil and Teubner, 2011; Ficke et al., 2007) and accelerate algal growth (Paerl and Huisman, 2008). Furthermore, warming is foreseen to intensify hypoxic events by lowering the oxygen solubility of water and by boosting the metabolism of microorganisms that drive eutrophication-induced hypoxia (Ficke et al., 2007; Paerl and Huisman, 2008).

Of the nutrients that trigger eutrophication, nitrate is the most prevalent and abundant (Camargo et al., 2005; Camargo and Alonso, 2006; Durand et al., 2011) with concentrations approaching 10–100 folds higher than background levels (0–2 mg NO_3^- L^{-1}) for prolonged periods in areas with high agricultural and urban runoff (Galloway et al., 2004; Goeller et al., 2019). Prolonged exposure to nitrate can be detrimental to aquatic fauna (Gomez Isaza et al., 2020a). Nitrate is taken up by freshwater fishes passively through the gills (via the HCO_3^-/Cl^- exchanger) and accumulates in the plasma (Stormer et al., 1996; Camargo and Alonso, 2006). Nitrate that is taken-up is endogenously transformed to nitrite (Camargo et al., 2005; Monsees et al., 2017) which converts blood oxygen-carrying protein, such as haemoglobin (Hb) in fish, to a non-oxygen carrier form – methaemoglobin (MetHb, Gomez Isaza et al., 2020b). The formation of MetHB, together with the reduction of total haemoglobin content and haematocrit (Hct), may lower blood oxygen carrying capacity and consequently reduce MMR (Gomez Isaza et al., 2020b). At the same time, stimulation of energy demanding coping mechanisms (such as the MetHB reductase system: Huey et al., 1984; Jensen et al., 1987) in response to nitrate exposure may also increase the SMR. Just like warming, these nitrate-induced metabolic changes can result in the narrowing of AS which can cause detrimental downstream effects on functional performance. Indeed, a recent meta-analysis showed that nitrate exposure generally reduces survival by 62%, curbs growth by 29%, lessens activity by 79%, and increases incidence of developmental deformity by 184% in freshwater fauna (Gomez Isaza et al., 2020a).

Despite the negative isolated effects of warming and nitrate, some species seem to thrive in warm nutrient-rich waters. For instance, common carp (*Cyprinus carpio*), a widely distributed fish species with great ecological and economic importance, tends to live in warmer rivers (Korwin-Kossakowski, 2008; Oyugi et al., 2012). Moreover, their natural habitats are characterized by turbid, nitrate-rich (thus frequently hypoxic) waters (Huser et al., 2016), partly due to the their tendency to perform bioturbation (Bajer and Sorensen, 2015). Despite this, the mechanism underlying their resilience under this multi-stress condition is not completely understood. Stressors often interact non-additively, where the effects of combined stressors can be greater (synergistic) or lesser (antagonistic) than the summed effects of individual stressors (Crain et al., 2008; Piggott et al., 2015; Todgham and Stillman, 2013). Organismal responses after exposure to one stressor may heighten an organism's susceptibility to other stressors – a phenomenon called cross-susceptibility. Alternatively, exposure to a stressor may stimulate protective mechanism enabling the development of increased tolerance to other stressors; termed cross-tolerance (Sinclair et al., 2013; Kampinga et al., 1995). For instance, cross-tolerance between warm acclimation (+4 °C) and nitrate (50 mg NO_3^- $L^{-1}$and 200 mg NO_3^- L^{-1}) has been noted when compensatory cardiorespiratory responses induced by both stressors resulted in a synergistic improvement in aerobic scope of European grayling, *Thymallus thymallus* (Opinion et al., 2020). Moreover, it was documented that plasticity of the gills and ventricle of warm-acclimated silver perch *Bidyanus bidyanus* neutralized the negative effects of nitrate on the aerobic scope, swimming performance and thermal tolerance (Gomez Isaza et al., 2020c, 2021). However, *T. thymallus* exposed to nitrate (50 mg NO_3^- L^{-1} and 200 mg NO_3^- L^{-1}) were found to be more susceptible to acute hypoxia (Rodgers et al., 2021) indicating cross-susceptibility between these stressors and the inefficiency of the current EU maximum nitrate limit (50 mg NO_3^- L^{-1}, Nitrates Directive 91/676/EEC) to protect this species against hypoxia.

In this study, we determined the interactive effects of nitrate and warm acclimation on haematological parameters (Hb, MetHb, HCT, and mean corpuscular haemoglobin concentrations- MCHC), aerobic performance (AS, SMR, MMR) and acute stress tolerance (heat and hypoxia stress) using a hardy eurythermal fish species, *C. carpio*, as model species. We employed a 3 × 2 factorial experimental design (total of 6 treatments) where we exposed juvenile *C. carpio* to one of the three nitrate concentrations, 0 mg L^{-1} (control), 50 mg L^{-1} (EU Nitrates Directive 1991: nitrate target of 50 mg L^{-1} for the protection of freshwaters systems from nitrate pollution) and 200 mg L^{-1} (realistic nitrate concentration in natural and artificial freshwater systems, Hrubec et al., 1996; Grizzetti et al., 2011; Shukla and Sexena, 2018) and one of the two temperature levels, 18 °C (control: no heat wave scenario, mean summer water temperatures in Europe) and 26 °C (intense heat wave scenario, Hardenbicker et al., 2016; Woolway et al., 2021) for 5 weeks. Given the resilient nature of the model species, we hypothesized that exposure to nitrate and warm acclimation would induce *C. carpio* to exhibit protective responses enabling it to at least maintain its aerobic performance and its tolerance to acute hypoxia and heat stress. Furthermore, we hypothesized that alterations to maintain oxygen transport capacity, as reflected by sustained haematological parameters, would underly the resistance of *C. carpio* under nitrate and warming exposure.

2. Methodology

2.1. Acclimatization and experimental design

All experimental methods complied with the Federation of European Laboratory Animal Science Associations' regulations and were approved by the University of Antwerp's institutional ethics committee (Permit Number: 2019–13). Juvenile common carp (*C. carpio*) were sourced from Wageningen University, The Netherlands and were transported to the facilities of University of Antwerp, Belgium where they were acclimatized to laboratory conditions for seven months prior to the experiment. Fish were maintained in four 210 L tanks (52 cm width × 72 cm length × 56 cm height; 100 fish/tank) filled with ~175 L EPA medium hard water (USEPA, 2002; water hardness 84.6 mg L^{-1} $CaCO_3$) reconstituted from demineralized (Eurowater DPRO B1–1/1) by the addition of 4 mg L^{-1} KCl, 123 mg L^{-1} $MgSO_4$ *H_2O, 96 mg L^{-1} $NaHCO_3$, and 60 mg L^{-1} $CaSO_4$ *H_2O (AnalaR NORMAPUR® analytical reagent by VWR Chemicals). The fish were fed daily with commercial pellets of 1 mm (Alltech Coppens) at a rate of 1.5% body mass per day. The tanks were equipped with submersible water heaters (300 W, Digital Smart Heater, Superfish), airlines and water filtration systems flowing at an average rate of 5.0 L min^{-1} through a three-layer biofiltration system (cotton--gravel-cotton filter layers, 39 cm width, 53 cm length, and 12 cm height for each layer). The water temperature in the tanks was gradually

changed from 20 °C to 18 °C and 26 °C at a rate of 1 °C every two days, and the fish were acclimatized at such temperature for 2 weeks before the start of the experiment. Temperature was maintained at treatment levels (± 1 °C) by water heaters. Nitrate levels were kept below 15 mg L^{-1} by partial water change (~50%) every two days.

After acclimatization, 390 fish (5.7 ± 1.8 g) were randomly distributed to thirty rectangular tanks (28 cm width × 60 cm length × 25 cm height) at a density of 13 fish per tank. Each tank was filled with 42 L EPA medium hard water, and equipped with fish shelters (PVC tube, 17 cm height x 5 cm diameter) and airlift filters (8 × 8 × 10.5 cm, BioAir Filter, SuperFish). Tanks were randomly assigned to one of the six treatments using a random number generator: two acclimation temperatures (18 ± 1 °C and 26 ± 1 °C) × three nitrate levels (0 ± 5 mg NO_3^- L^{-1}, 50 ± 5 mg NO_3^- L^{-1}, 200 ± 20 mg NO_3^- L^{-1}), producing four replicate tanks for each acclimation temperature × nitrate level combination. Fish were fed commercial pellets (1 mm, Skretting ME Presta) to satiation daily. Temperature was maintained at appropriate treatment levels (± 1 °C) by water heaters (300 W, Digital Smart Heater, Superfish) installed in each tank. Nitrate concentrations were kept within 10% of nominal concentration by diluting reagent grade $NaNO_3$ (GPR RECTAPUR by VWR Chemicals) as well as daily partial water changes (~50%) and siphoning of residual wastes.

Throughout the acclimatization and experimental period, fish were subjected to 12-h light and 12-h dark photoperiod. Temperature and nitrate levels were monitored and recorded daily by portable meters WTW ProfiLine 3310 and HORIBA LAQUAtwin NO_3^-11, respectively. The pH and conductivity, measured by a HQ30D Portable Multi-meter (Hach, USA), was respectively 7.9 ± 0.1 and 313 ± 4 μS cm^{-1}. Ammonia and nitrite were also measured by Tetra test kits daily and were maintained below 0.25 mg NH_3/NH_4^+ L^{-1} and 0.3 mg NO_2^- L^{-1}, respectively, whereas DO was checked by WTW ProfiLine 3310 and maintained at levels above 80%.

2.2. Heat tolerance

After four weeks of treatment exposure, heat tolerance was assessed by measuring the critical thermal maxima (CTMax), a repeatable method to determine maximum thermal limits of organisms (Beitinger et al., 2000). Similar to the method described by Rodgers et al. (2021), eight randomly selected, fasted (24 h) and weighed fish from each treatment were individually placed in cylindrical glass chambers (9 cm diameter × 17 cm height) filled with 900 ml water at corresponding treatment condition (± 1 °C; ± 10% mg NO_3^- L^{-1}). Glass chambers were situated in a water bath filled up to maximum level (GFL 1003 model), and were provided with airlines (to ensure DO saturation remain above 80% during trials) and WTW ProfiLine 3310 portable meter (to monitor temperature and DO levels). Fish were allowed to recover from handling stress for 30 min. Thereafter, water temperature within each chamber was increased at a rate of 0.3 °C min^{-1}. Fish were closely observed throughout the trials, and trials were immediately terminated once the endpoint was reached indicated by the loss of equilibrium (LOE). LOE was defined as the inability to maintain an upright position in the water column for 10 s and is typically used as endpoint for CTmax tests (Beitinger et al., 2000). The temperature at which fish lost equilibrium was recorded, and fish were removed, placed in recovery tanks for 2 h and euthanized with tricaine methanesulfunate (MS-222) overdose.

2.3. Hypoxia tolerance

Hypoxia tolerance of eight randomly selected fish from each treatment was measured after four weeks of exposure by closed respirometry similar to the method described by Rodgers et al. (2021). Fasted (24 h) and pre-weighed fish were individually placed in a cylindrical glass chamber (7 cm diameter × 13 cm height;) fully filled with water at corresponding treatment condition (± 1 °C; ± 10% mg NO_3^- L^{-1}). Each chamber was provided with aeration by running airlines and a magnetic stir bar (0.8 cm length; 0.3 cm diameter). Four glass chambers were positioned on a magnetic stirrer (Snijder Magnetic Stirrer 34532) set at maximum stirring capacity (2800 rpm) to facilitate water mixing. After a 30-minute adjustment period, aeration was removed and the glass chambers were sealed with rubber stoppers holed to hermetically fit a calibrated oxygen meter probe (WTW ProfiLine 3310) which was set to measure DO every 5 s until LOE. The DO level at LOE, regarded as most ecologically relevant measure of hypoxia tolerance (Rodgers et al., 2021), was recorded, and fish were immediately transferred to an aerated recovery tank for 2 h and euthanized with overdose of MS-222.

2.4. Respirometry

After five weeks of treatment exposure, aerobic scope (AS) of eight randomly selected, fasted (24 h) and pre-weighed fish from each treatment was measured following the method described by Opinion et al. (2020). AS was calculated by subtracting the standard metabolic rate (SMR) from the maximum metabolic rate (MMR), and these metabolic rates (MO_2, mg O_2 h^{-1}) were calculated using Eq. (1):

$$MO_2 = -1 \times \left[\frac{(m_f - m_b)}{100}\right] \times V \times \beta O_2 \quad (1)$$

where *mf* is the rate of change of oxygen saturation during measurement period of a respirometer containing a fish (Δ% air saturation per hour), *mb* is the background respiration rate measured as the rate of change of oxygen saturation of a respirometer without a fish, V is the volume of the respirometer minus the volume of the fish (assuming 1 g displaces 1 ml of water), and βO_2 is the oxygen capacitance at the appropriate water temperature (Cameron, 1986).

SMR measurements were done by intermittent-flow respirometry. Fish were individually placed in Blazka-type respirometers (4.2 ± 0.03 L volume, motor: 3E–12NRY Little Giant Pump Company) positioned within reservoir baths (70 cm length × 60 cm width × 39 cm height) filled with water at corresponding treatment condition and equipped with airlines and heaters. Fish were maintained in the respirometers overnight (at least 14 h). Water velocity in the respirometers was set at 10 cm s^{-1} (~1.3 BL s^{-1}), to enable water mixing in the respirometers. Each respirometer was attached to a flush pump (EHEIM universal 300 submersible pump), which is regulated by a mechanical timer (Chacon Timer M100) set to flush for 15 min every 30 min to prevent oxygen depletion below 80% saturation and build-up of toxic metabolic waste. A calibrated oxygen probe (WTW ProfiLine 3310) was set to record the DO level in the respirometer every minute overnight. The oxygen decline rate (during a 20 min period in between flushing cycles) belonging to the lower 10th percentile was considered for the computation of SMR. Background respiration rates was determined by a blank run after SMR determinations for an hour and were < 1.2% of the animal respiration rates (mg O_2 kg^{-1} h^{-1}).

After the SMR measurement, MMR of fish subjected to SMR trials was determined using the standard chase protocol. Each fish was transferred in circular chasing chambers (32 cm diameter × 38 cm height) containing aerated water at corresponding treatment condition, and allowed to recover for 15 min. After the recovery period, fish were chased with a net for 3 min to ensure maximal exhausted. After chasing, fish were immediately placed in a Brett-type respirometer (5.5 L, SWIM-5, Loligo® Systems) submerged in a water bath (75 cm length × 30 cm width × 17 cm height; filled up to 12 cm) with corresponding treatment condition. The respirometer was set at a speed similar to the SMR protocol and was equipped with a calibrated oxygen meter probe set to record DO level every 5 s for 15 min. MMR represents the maximum capacity of the fish to take up oxygen, therefore highest decline rate of DO during a 2-minute interval (occurred within the first 4 min) was used to calculate MMR. Fish were transferred back to their respective tanks after the trial and background respiration was again determined by a blank run.

2.5. Haematological analyses

Blood collection and analyses were done weekly for four weeks. For each timepoint, eight fasted fish (24 h) from each treatment were netted out of the tanks, euthanized by an overdose of MS-222 and weighed. Blood samples were immediately obtained by severing the caudal peduncle. The blood was collected by heparinised capillary tubes (60 μL capacity; 0.75 cm length, Hirschmann® Laborgerate) and was transferred to 1.5 ml Eppendorf tubes buried in ice until analyses.

The measurements of Hb and MetHb were done using colorimetric assay kits (Methaemoglobin Colorimetric Assay Kit, Catalogue no. MBS2540547, MyBioSource). The optical density (OD) values for Hb content were measured with a spectrophotometer (GENESYS™ 20 Visible Spectrophotometer, Thermo Fisher Scientific) at 540 nm wavelength with 1 cm diameter cuvettes while OD values for MetHb content were measured at 630 and 602 nm wavelength. To determine the HCT, heparinized capillary tubes were filled with freshly collected blood sample, sealed with plasticine clay, and centrifuged for 3 min at 10,000 rpm in a micro HCT centrifuge (Heraeus Christ GmbH Mikro-Hämatokrit 00912). HCT was estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube. Haematological parameters were measured within one hour after the blood collection. MCHC, a measure of the average concentration of Hb in a given volume of erythrocytes, was calculated using standard formula.

2.6. Statistical analyses

Data analyses were performed in R (version 4.0.3; R studio version 1.3.1093). Linear mixed effects models were run to determine the main and interactive effects of nitrate exposure (three-level, fixed factor), acclimation temperature (two-level, fixed factor), and experimental week (four-level, fixed factor) on haematological parameters (HCT, Hb, MetHb, and MCHC). Effect of temperature, nitrate and their interaction on metabolic (SMR, MMR, and AS), and stress tolerance parameters (CTmax and hypoxia tolerance) were also analyzed by linear mixed effects models. SMR and MetHb datasets were log transformed while MCHC was square-root transformed to fit the assumption of normality. Tank ID and body mass were included as a random effect and as a covariate in all models, respectively. Respirometer ID was included as additional random effect in the models of metabolic parameters, while chamber ID was included as random effect in the models of stress tolerance parameters. Tukey's post hoc test (Tukey-adjusted least square means) was run to determine statistical differences among treatment groups and fixed effect factors. P-values for multiple comparisons were adjusted using the Tukey method. Statistical significance was accepted at $p < 0.05$. Data are presented as mean ± standard error.

3. Results

3.1. Metabolic rate

No treatment-induced mortalities occurred during the exposure period. Warm acclimation had no significant main effect on SMR ($p = 0.20$, $F_{1,39} = 1.70$), nor did nitrate exposure ($p = 0.37$, $F_{2,39} = 1.01$, Fig. 1A), even though SMR seemed to decrease with nitrate exposure at both acclimation temperatures. Acclimation at higher temperature significantly increased the MMR of *C. carpio* ($p < 0.001$, $F_{1,39} = 12.80$, Fig. 1B) where 26 °C-acclimated fish (1276.8 ± 52.9 mg O_2 kg^{-1} h^{-1}, average over nitrate levels) had 16% higher MMR than 18 °C-acclimated fish (1105.4 ± 34.7 mg O_2 kg^{-1} h^{-1}, average over nitrate levels). Nitrate, on the other hand, did not have a significant effect on MMR ($p = 0.72$, $F_{2,29} = 0.32$). Nitrate and temperature treatment had non-significant interactive effects on MMR ($p = 0.18$, $F_{2,29} = 1.83$) and SMR ($p = 0.50$, $F_{2,36} = 0.70$). Similar to MMR, acclimation temperature affected fish AS significantly ($p < 0.001$, $F_{1,39} = 9.67$, Fig. 1C), where 26 °C-acclimated fish (1073.9 ± 52.9 mg O_2 kg^{-1} h^{-1}, average over nitrate levels) had 11% higher AS than 18 °C-acclimated fish (965.7 ± 34.7 mg O_2 kg^{-1} h^{-1}, average over nitrate levels), while nitrate exposure had no significant effect on AS ($p = 0.53$, $F_{2,29} = 0.65$). The stressors exhibited a non-significant interaction on AS ($p = 0.12$, $F_{2,29} = 2.27$).

3.2. Haematological parameters

Nitrate and experimental week had significant main effects on the HCT of *C. carpio*, and interacted significantly to affect HCT (Table 1, Fig. 2A–D). HCT of 26 °C-acclimated fish was significantly elevated after one-week exposure to nitrate (50 mg NO_3^- L^{-1} and 200 mg NO_3^- L^{-1}), but returned to control levels on the second week. On the other hand, 18 °C- acclimated fish exposed to 200 mg NO_3^- L^{-1}nitrate treatment exhibited significantly higher HCT on the fourth week compared to the second and third week. Temperature did not have a significant main effect on HCT, but it interacted with experimental week. Regardless of nitrate exposure, HCT of 26 °C-acclimated fish on the first week is higher compared to HCT of 18 °C-acclimated fish in the same week but returned to control level on the second week until the end of the experiment.

Hb was significantly affected by temperature, experimental week, and their interaction (Table 1, Fig. 2E-H). Regardless of nitrate treatment, Hb was unaffected by acclimation temperature on the first week, but was 11% and 18% lower during the second and third week relative to levels at control temperature, respectively. On the fourth week, Hb of 26 °C-acclimated fish returned to levels comparable to control temperature. Nitrate exposure did not affect Hb of *C. carpio* and did not interact with other tested variables to affect Hb.

Temperature, experimental week, and their interaction significantly influenced the MetHb of *C. carpio* (Table 1, Fig. 2 I–L). MetHb levels generally decreased with exposure time, but the duration at which MetHb was elevated differed between the tested temperature. Acclimation at 26 °C caused MetHb to rise until the second week. Despite having lower MetHb compared to 26 °C acclimated fish during the first two weeks, fish acclimated at 18 °C generally showed elevated MetHb until the third week of exposure. Nitrate exposure had a significant main effect on MetHb where MetHb of fish exposed to 200 mg NO_3^- L^{-1} treatment was 55% and 44% higher compared to 0 mg NO_3^- L^{-1} and 50 mg NO_3^- L^{-1} nitrate treatment, respectively. Nitrate did not interact with experimental week to affect MetHb, but MetHb levels of fish exposed to nitrate seemed to generally decrease as the exposure proceeded.

MCHC was affected by temperature, experimental week and their interaction (Table 1, Fig. 2M–P). MCHC of fish acclimated at 26 °C was lower compared to control temperature regardless of nitrate treatment, but returned to levels comparable to those at control temperature on the fourth week. Despite a non-significant main effect, nitrate interacted with experimental week and temperature. When combined with warm acclimation, exposure to nitrate treatment caused reduction in MCHC on the first week but levels improved over the succeeding weeks. MCHC increased significantly over the weeks in 26 °C-acclimated fish exposed to 50 mg NO_3^- L^{-1} by 52% between the first (150.17 ± 21.5 g L^{-1}) and the fourth week (228.56 ± 23.5 g L^{-1}). MCHC also increased significantly in 26 °C-acclimated fish exposed 200 NO_3^- mg L^{-1} nitrate by 21% between the first (162.14 ± 7.39 g L^{-1}) and the fourth week (195.94 ± 7.39 g L^{-1}).

3.3. Thermal tolerance

Warm acclimation increased the CTmax of *C. carpio* ($p < 0.001$, $F_{1,14} = 723.5$., Fig. 3), where 26 °C-acclimated fish (39.3 ± 0.15 °C, average over nitrate levels) had 14% higher CTmax than 18 °C-acclimated fish (34.4 ± 0.15 °C, average over nitrate levels). Nitrate treatment did not significantly affect CTmax ($p = 0.35$, $F_{2,14} = 1.14$). No significant

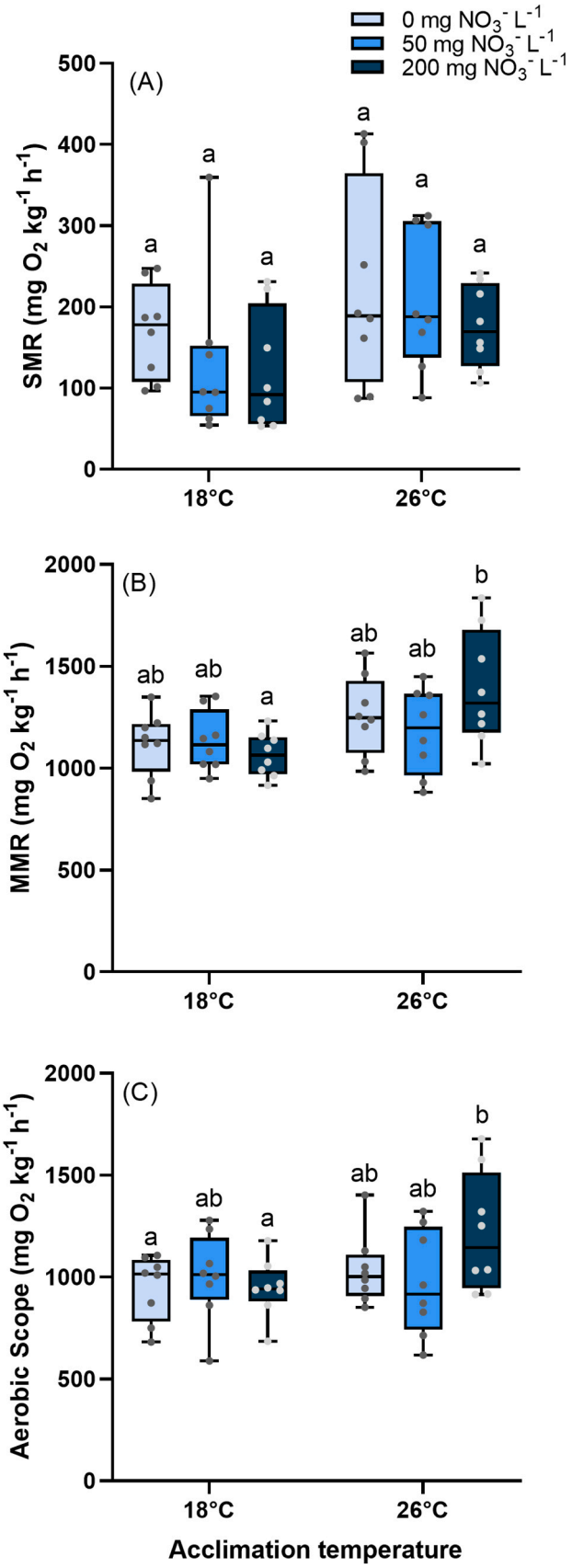

Fig. 1. Interactive effects of acclimation temperature and nitrate on the (A) standard metabolic rate (SMR), (B) maximum metabolic rate (MMR), and (C) aerobic scope (AS) of common carp (*Cyprinus carpio*) exposed to a factorial combination with two levels of temperature (18 °C or 26 °C) and nitrate levels (0, 50, or 200 mg NO_3^- L^{-1}) for 5 weeks. Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups. Data are presented as boxplots where whiskers represent the minimum and maximum value of all data. Dots represent individual data points (n = 8 fish per treatment group).

Table 1
Interactive effects of temperature, nitrate and experimental week on the haematological parameters (HCT- haematocrit; Hb – haemoglobin; MetHb – methaemoglobin; and MCHC- mean corpuscular haemoglobin concencration) of common carp *Cyprinus carpio*. Full linear mixed models are shown with temperature, nitrate and experimental week as fixed effects, bodymass as covariate and tanks as random effects.

	Source of variation	Sum of squares	Mean squares	NumDF	DenDF	*F*-value	*p*-value
HCT	Temperature	4.55	4.55	1	153	0.72	0.40
	Nitrate	103.88	51.94	2	153	8.21	**<0.001**
	Time	212.80	70.93	3	153	11.21	**<0.001**
	Bodymass	39.04	39.04	1	153	6.17	**<0.05**
	Temperature: Nitrate	5.84	2.92	3	153	0.46	0.63
	Temperature: Time	147.27	49.09	6	153	7.76	**<0.001**
	Nitrate: Time	161.62	26.94	6	153	4.26	**<0.001**
	Temperature: Nitrate: Time	66.98	11.16	6	153	1.76	0.11
Hb	Temperature	610.54	610.54	1	55	11.82	**<0.01**
	Nitrate	208.83	104.42	2	23	2.02	0.16
	Time	594.03	198.01	3	152	3.83	**<0.05**
	Bodymass	497.30	497.30	1	161	9.63	**<0.01**
	Temperature: Nitrate	115.93	57.96	2	23	1.12	0.34
	Temperature: Time	1138.25	379.42	3	153	7.35	**<0.001**
	Nitrate: Time	632.17	105.36	6	152	2.04	0.06
	Temperature: Nitrate: Time	460.1	76.68	6	152	1.48	0.19
MetHb	Temperature	0.37	0.40	1	51	4.60	**<0.05**
	Nitrate	4.80	2.40	2	23	2.87	**<0.001**
	Time	3.13	1.04	3	150	12.96	**<0.001**
	Bodymass	0.01	0.01	1	166	0.13	0.72
	Temperature: Nitrate	0.35	0.17	2	23	2.17	0.14
	Temperature: Time	3.99	1.33	3	150	16.52	**<0.001**
	Nitrate: Time	0.82	0.14	6	150	1.70	0.13
	Temperature: Nitrate: Time	0.60	0.10	6	150	1.24	0.29
MCHC	Temperature	11.161	11.1610	1	48	12.75	**<0.001**
	Nitrate	4.177	2.0884	2	22	2.39	0.12
	Time	17.905	5.9684	3	140	6.82	**<0.001**
	Bodymass	1.757	1.7568	1	149	2.01	0.16
	Temperature: Nitrate	4.401	2.2006	2	22	2.51	0.10
	Temperature: Time	40.124	13.3745	3	140	15.28	**<0.001**
	Nitrate: Time	20.440	3.4067	6	140	3.89	**<0.01**
	Temperature: Nitrate: Time	12.489	2.0815	6	139	2.38	**<0.05**

interaction between acclimation temperature and nitrate treatment was observed ($p > 0.33$, $F_{2,13} = 1.21$).

3.4. Hypoxia tolerance

Hypoxia tolerance of *C. carpio* was significantly affected by warm acclimation ($p < 0.0001$, $F_{1,37} = 24.13$, Fig. 4): 18 °C-acclimated fish ($3.98 \pm 0.23\%$, average over nitrate levels) lost equilibrium at DO saturation 36% higher than 26 °C-acclimated fish ($2.92 \pm 0.16\%$, average over nitrate levels). Nitrate exposure also significantly affected the fish hypoxia tolerance ($p < 0.05$, $F_{2,36} = 3.68$). *C. carpio* exposed to 200 mg NO_3^- L^{-1} treatment ($3.00 \pm 0.27\%$, average over temperature levels) lost equilibrium at DO saturation 23% lower than fish exposed to 50 mg NO_3^- L^{-1} treatment ($3.89 \pm 0.26\%$, average over temperature levels). Nevertheless, the hypoxia tolerance of *C. carpio* exposed to 0 NO_3^- mg L^{-1} treatment ($3.45 \pm 0.27\%$, average over temperature levels) is not significantly different from *C. carpio* exposed to higher nitrate levels. Nitrate treatment and acclimation temperature interacted non-significantly ($p = 0.95$, $F_{2,34} = 0.03$).

4. Discussion

4.1. Aerobic scope associated with stressor-induced haematological fluctuations

Temperature has an important impact on the performance of aquatic ectotherms through its effects on metabolism (Dillon et al., 2010; Lefevre, 2016). Temperature elevation generally leads to an increase in cellular respiration rate and aerobic energy demand, followed by a rise in the oxygen consumption rate (i.e. SMR, Clarke and Johnston, 1999; Ferreira et al., 2014; Chen et al., 2015). However, many species have the capacity to adjust their metabolic processes when given sufficient exposure time and can therefore maintain basal energy requirements (SMR) over wide range of temperatures (Sandblom et al., 2014; Lefevre, 2016). In this study, SMR of *C. carpio* was not statistically different between tested temperatures after 5 weeks, indicating downregulation of basal energy requirements resulting in a complete thermal compensation of SMR (Sandblom et al., 2014). Downregulation of basal oxygen demand is suggested to be possible by adjustment of mitochondrial densities and their functional properties to maintain AS (Pörtner, 2001; Fangue et al., 2009). Cyprinid species have been observed to exhibit a flexible SMR (Fu et al., 2018) which is attributed to their morphological and physiological adaptability (Brauner et al., 2011). For instance, some cyprinids can restructure their gills and adjust enzyme activities under energy demanding situations such as temperature elevation and hypoxia (Brauner et al., 2011; Fu et al., 2014; He et al., 2014). Sun et al. (2019) have found that genes involved in metabolic processes were differentially expressed in the liver of *C. carpio*, a major metabolic organ that is suspected to play crucial role in temperature adaptation in eurythermal fish exposed to warmer temperature. Though metabolic adjustments are probable, it is also important to note there was a large individual variation in the SMR data which may mask the thermal effects statistically.

Warm acclimation significantly increased the MMR of *C. carpio* in this study, resulting in an increase in AS. It is hypothesized that sub- and supra-optimal temperatures reduce the MMR due to the limited biochemical mechanisms that sets the aerobic capacity of cells and tissues of the circulatory and ventilatory systems (Pörtner, 2017). Given this, elevation of MMR in 26 °C-acclimated fish seems logical as *C. carpio* has been documented to prefer living in warmer rivers with temperature reaching 28 °C (Oyugi et al., 2012), and the species can tolerate temperature approaching 35 °C (Eaton and Scheller, 1996). Hence, it is likely that 18 °C is suboptimal for AS relative to 26 °C, and that 26 °C is not high enough to reduce the AS of *C. carpio*. Nevertheless, some species, including a cyprinid species *Carassius auratus*, show expansion of

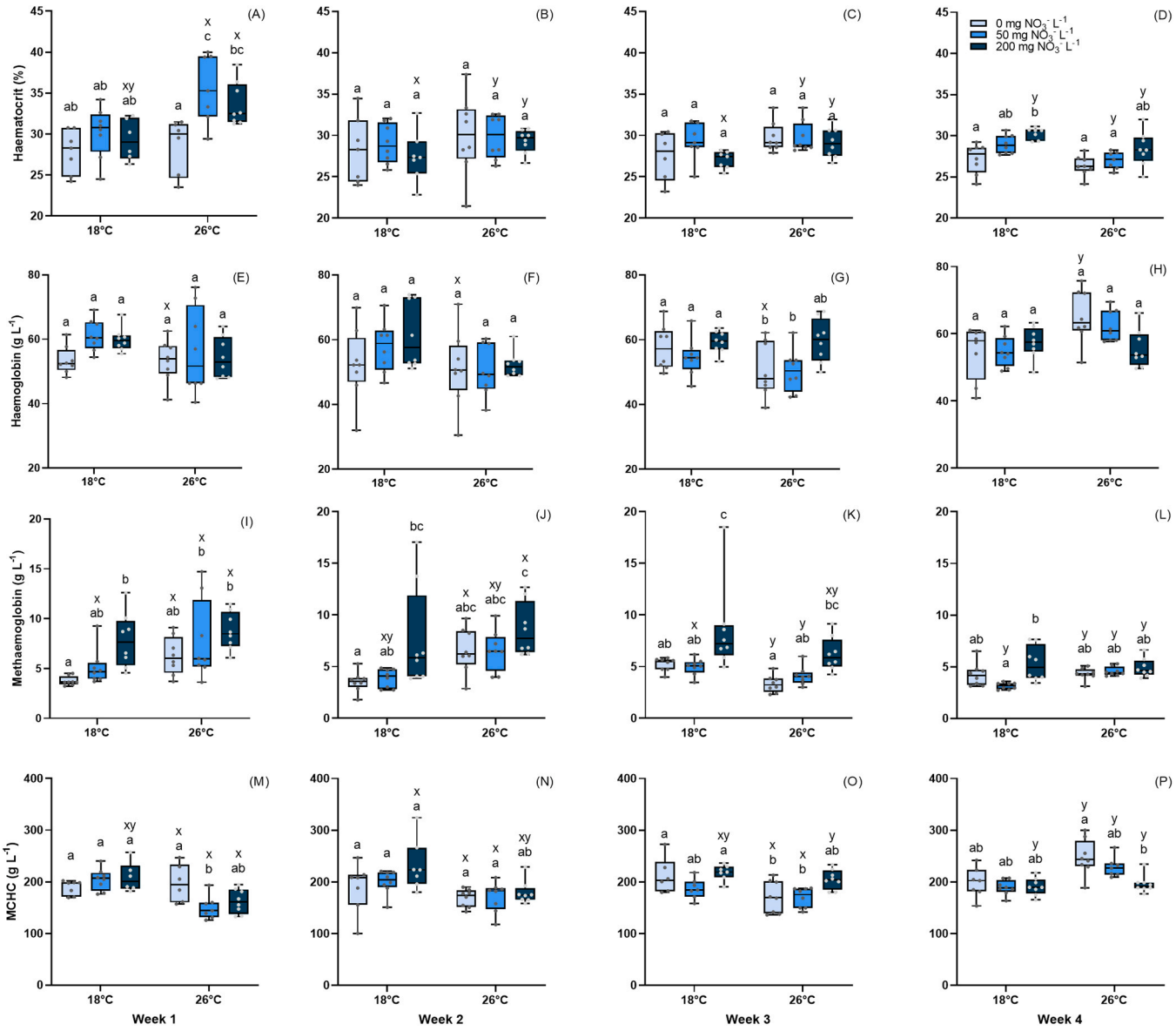

Fig. 2. Interactive effects of acclimation temperature, nitrate and experimental on the haematological parameters including haematocrit (%: A-D), haemoglobin (g L^{-1}: E-H), methaemoglobin (g L^{-1}: I-L); and mean corpuscular haemoglobin concencration (MCHC, gL^{-1}: M-P) of common carp (*Cyprinus carpio*) exposed to a factorial combination with two levels of temperature (18 °C or 26 °C) and nitrate levels (0, 50, or 200 mg NO_3^- L^{-1}) for 5 weeks. Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups (a,b) and weekly sampling periods (x,y). Data are presented as boxplots where whiskers represent the minimum and maximum value of all data. Dots represent individual data points (n = 8 fish per treatment group).

AS and MMR at temperature approaching upper thermal limits (Lefevre, 2016). MMR expansion is possible by adjustments in the circulatory and respiratory systems to maintain oxygen transport capacity after long-term exposure to higher temperature (Gräns et al., 2014). For example, cardiorespiratory responses (e.g. heart remodeling and blood reserve expansion in the spleen, Opinion et al., 2020) to enhance and maintain haematological parameters (i.e. HCT and Hb) have been associated with improvement of AS following acclimation to higher temperature (Gräns et al., 2014; Opinion et al., 2020). Indeed, haematological parameters were generally maintained during exposure to warmer temperature: HCT and Hb returned to control levels on the second and fourth week, respectively.

Sokolova et al. (2012) presented in the Energy-Limited Stress Tolerance concept that aquatic pollutants, such as nitrate, can disturb the energy homeostasis and compromise fish fitness by reducing the AS. High concentration of nitrate may reduce the MMR and increase the SMR which may ultimately diminish the AS (Gomez Isaza et al., 2020b). In this experiment, nitrate did not pose disruption on the SMR, MMR, and AS of *C. carpio*. The lack of nitrate-induced change in SMR coincided with the results of nitrate-exposure studies on blueclaw crayfish (*Cherax destructor*, Ellis and Morris, 1995; Gomez Isaza et al., 2018), redclaw crayfish (*Cherax quadricarinatus*, Meade and Watts, 1995), and spangled perch (*Leiopotherapon unicolor*, Gomez Isaza et al., 2020b). On the contrary, SMR of European grayling (*Thymallus thymallus*) was influenced by nitrate exposure where a doubling of SMR was observed after exposure to 200 mg NO_3^- L^{-1} relative to lower nitrate levels (0 mg NO_3^- L^{-1} and 50 mg NO_3^- L^{-1}, Opinion et al., 2020). Discrepancies between results may be attributed to species specific differences in nitrate tolerance (Camargo and Alonso, 2006; Gomez Isaza et al., 2020a). Salmonid species (such as *T. thymallus*) were noted to be most sensitive to nitrate exposure, and exhibited lower LC_{50} values (96 h) compared to cyprinid species when exposed to nitrite (Camargo and Alonso, 2006).

The observed non-significant effect of nitrate on MMR and AS does not coincide with related studies on *C. destructor* (Gomez Isaza et al.,

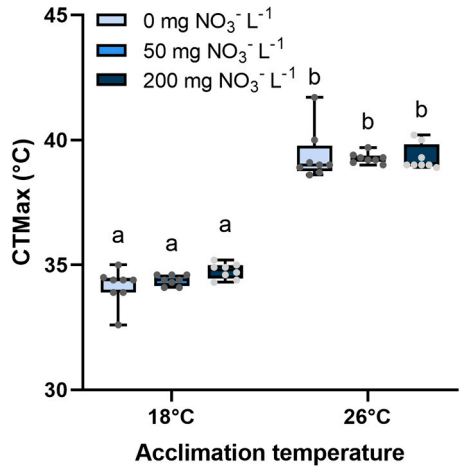

Fig. 3. Interactive effects of acclimation temperature and nitrate on the thermal tolerance (CTMax) of common carp (*Cyprinus carpio*) exposed to a factorial combination with two levels of temperature (18 °C or 26 °C) and nitrate levels (0, 50, or 200 mg NO_3^- L^{-1}) for 4 weeks. Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups. Data are presented as boxplots where whiskers represent the minimum and maximum value of all data. Dots represent individual data points (n = 8 fish per treatment group).

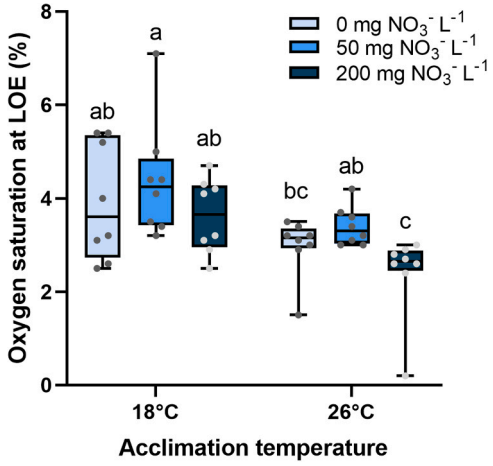

Fig. 4. Interactive effects of acclimation temperature and nitrate on the hypoxia tolerance (oxygen saturation at loss of equilibrium, %) of common carp (*Cyprinus carpio*) exposed to a factorial combination with two levels of temperature (18 °C or 26 °C) and nitrate levels (0, 50, or 200 mg NO_3^- L^{-1}) for 4 weeks. Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups. Data are presented as boxplots where whiskers represent the minimum and maximum value of all data. Dots represent individual data points (n = 8 fish per treatment group).

2018) and L. *unicolor* where chronic nitrate exposure caused reduction in AS and MMR due to the limiting effects of nitrate on blood oxygen-carrying capacity as demonstrated by reduced Hb and HCT, and elevated MetHb (Gomez Isaza et al., 2020b). However, haematological fluctuations in this study indicate that *C. carpio* were able to develop mechanisms to cope with methaemoglobinemia and its limiting effects: nitrate did not influence Hb and MCHC, and caused initial rise in HCT which may be a response to the nitrate-induced MetHb elevation that eventually decreased to control levels. In nitrate-exposed *T. thymallus*, expansion of spleen volume, which indicates higher blood reserve to maintain blood oxygen transport capacity during exercise (e.g. fatiguing exercise during MMR measurement) and hypoxia (e.g. tissue hypoxia caused by nitrate-induced hypoxia), has been associated with maintenance of MMR (Opinion et al., 2020). Other studies have also reported non-significant influence of chronic nitrate exposure on the blood carrying capacity of fish despite nitrate/nitrite accumulation in the plasma (Schram et al., 2014; van Bussel et al., 2012). As such, it was suggested that species-specific differences in nitrate toxicity seems more related to the capacity of organisms to handle nitrate (elimination, storage or detoxification) within the body rather than their capacity to maintain low plasma-nitrate concentration (Gomez Isaza et al., 2020b). Confirming this scenario requires data on the internal build-up of nitrate/-nitrite which were not collected due to insufficiency of blood samples. In future experiments, it is recommended to investigate nitrate (and/or nitrite) accumulation to ascertain that the physiological alterations observed were induced by nitrate, and to better understand its mechanistic effect in *C. carpio*.

4.2. Thermal tolerance linked to aerobic scope improvement

More frequent and intense climatic events such as heat waves causing acute elevation of water temperature are forecasted alongside with climate warming (Ficke et al., 2007). Thus, it is vital to understand how warming affects thermal tolerance of organisms in combination with existing stressor, like nutrient pollution. Acclimation at higher temperature increased the CTMax of *C. carpio* and this is consistent with previous studies on the same species reporting positive correlation between acclimation temperature and upper thermal tolerance (Chatterjee et al., 2004; Golovanov and Smirnov, 2007). Improved CTMax in warm-acclimated ectotherms is proposed to result from the ability of organism to make metabolic adjustments to maintain the AS at higher temperature (Pörtner, 2001). CTMax could be increased through AS in two ways which were observed in this study: (1) MMR could be expanded (e.g. by cardiorespiratory adjustments) at temperatures approaching upper critical limits, and/or (2) SMR could be maintained or reduced (e.g. by mitochondrial function downregulation) at temperatures approaching upper critical limits (Pörtner, 2001; Fangue et al., 2009; McArley et al., 2017).

In a related study, nitrite-exposed *C. carpio* exhibited significant decrease in CTMax which is mainly attributed to nitrite-induced reduction in oxygen transport capacity (Rodgers and De Boeck, 2019). Similar to nitrite, nitrate is expected to have a reducing effect on blood oxygen transport capacity and consequently on the thermal tolerance of fish. Contrary to this, nitrate exposure in this study did not influence the CTMax of *C. carpio* in both acclimation temperatures. This result, however, is supported by the minimal effect of nitrate on haematology. Contradicting results may be further explained by the differences in toxicity between nitrate and nitrite (Camargo and Alonso, 2006), and the distinction between experimental designs. *C. carpio* in the previous study was only exposed to nitrite for 7 days before CTMax trials (Rodgers and De Boeck, 2019), unlike the 4-week exposure in this study which possibly provided the species sufficient time to develop protective mechanisms (e.g. HSPs upregulation, Sun et al., 2014; Jensen et al., 2015). Moreover, acclimation temperatures between studies are different (nitrite exposed *C. carpio* was acclimated at 23 °C, Rodgers and De Boeck, 2019) and temperature dependent effects of nitrate on thermal tolerance of fish has been documented (Gomez Isaza et al., 2020c; Rodgers et al., 2021). For instance, 18 °C-acclimated *T. thymallus* exposed to nitrate considerably decreased the upper thermal tolerance while the opposite effect was observed in 22 °C-acclimated (Rodgers et al., 2021). Comparably, nitrate exposure reduced the CTMax of 28 °C-acclimated *B. bidyanus* while CTMax of 32 °C-acclimated conspecifics did not change after nitrate exposure (Gomez Isaza et al., 2020c).

4.3. Cross-tolerance among warming, nitrate and hypoxia

Hypoxia events often occur as a consequence of excessive nutrient levels, and these events are expected to intensify as climate warming progresses (Smith, 2003; Diaz and Rosenberg, 2008; Paerl and Huisman,

2008). Given this, understanding the combined effects of these stressors on hypoxia tolerance will provide an important indication of the fate of species under warm eutrophic waters. Hypoxia tolerance of *C. carpio* increased significantly after warm acclimation, regardless of nitrate concentration. Acclimation temperature and hypoxia tolerance tend to exhibit an inverse relationship in many aquatic species (Nilsson et al., 2010; Remen et al., 2013; He et al., 2015; McDonnell and Chapman, 2015; Rodgers et al., 2021), as a consequence of increased metabolic demand (SMR) and the decreased oxygen solubility and content in warmer water (Zhou et al., 2019). Meeting the increased demand for oxygen thus becomes more difficult for fish exposed to warm and hypoxic conditions, thereby causing a weakening in the capacity of the fish to tolerate hypoxia (Nilsson et al., 2010).

Nevertheless, opposite effects have been observed in other fish species, where acclimation to warmer temperatures improved hypoxia tolerance (Burleson and Silva, 2011; Fu et al., 2014; Anttila et al., 2015; McBryan et al., 2016), because high temperature and hypoxia are observed to independently trigger the same responses to extract more oxygen from ambient water (Sollid et al., 2005). Acclimation to higher temperatures induces wide physiological adjustments related to oxygen supply and demand, including metabolic, cardiorespiratory, and haematological adjustments (Aguiar et al., 2002; Steyck and Farrell, 2002), which consequently improve fish tolerance to hypoxic conditions. Downregulation of metabolic demand, as reflected by the maintained SMR in this study, has been noted as one of the thermal responses which plays a role in improving hypoxia tolerance (He et al., 2015). *C. carpio* has also been observed to exhibit gill remodeling to increase the functional surface area of the gills (i.e. by shrinking their interlamellar cell mass) to improve oxygen uptake efficiency in response to warming and/or hypoxic conditions (Dhillon et al., 2013). Remodeling-stimulated improvement of oxygen uptake efficiency in 26 °C-acclimated *C. carpio* may be indicated by warming-induced elevation in MMR observed in this study. Besides oxygen supply and demand adjustments, greater capacity for anaerobic respiration, including recruitment of tissue glycogen for anaerobic production of cellular fuel and liver lactate clearance capacity, has been associated with maintained hypoxia tolerance of cyprinids acclimated at higher temperature (He et al., 2015).

Given the well-established toxic effect of nitrate – formation of MetHb – it is assumed that fish exposed to nitrate are confronted with a decreased oxygen carrying capacity and tissue hypoxia, and are therefore expected to exhibit reduced hypoxia tolerance. Indeed, reduction in hypoxia tolerance proportional to nitrate exposure levels has been reported in a salmonid species, and histopathological changes in the gills of nitrate-exposed fish (i.e. hypertrophy and hyperplasia) have been noted to further impair oxygen uptake (Rodgers et al., 2021). In this study, however, *C. carpio* exposed to 200 mg NO_3^- L^{-1} nitrate had statistically higher hypoxia tolerance than *C. carpio* exposed to 50 mg NO_3^- L^{-1} treatment. This result is counterintuitive as MetHb levels, hence magnitude of oxygen carrying capacity reduction, is directly proportional with nitrate level. Nevertheless, pre-conditioning to hypoxia has been shown to increase survival time during exposure to lethal hypoxia (Rees et al., 2001) through physiological adjustments ensued from pre-conditioning (Burleson and Silva, 2011), and the magnitude of pre-treatment (level and duration) appears to be critical for development of cross-tolerance (Todgham et al., 2005; Burleson and Silva, 2011). For instance, the positive effect of moderate hypoxia exposure (50% air saturation) on thermal tolerance and cardiorespiratory functioning was not large when compared to results of other studies that used more severe hypoxia (i.e. 10% saturation) for longer period (i.e. 28 days, Burleson and Silva, 2011). This may explain our results: severity of tissue hypoxia was proportional with nitrate levels and the more severe hypoxia ensued from pre-treatment to higher nitrate concentration (200 mg NO_3^- L^{-1}) possibly urged the organism to make superior adjustments causing the improved hypoxia tolerance.

Stressor interactions can be classified into three categories: (i) additive, when there is no significant interaction between stressors and the combined effect is not significantly different to the sum of the isolated effects of the stressors, (ii) antagonistic, when the interactive effect is weaker than the sum of individual effects of the stressors, and (iii) synergistic, when the interactive effect is stronger than the sum of individual effects of the stressors (Piggott et al., 2015). In this study, nitrate and warm acclimation individually improved hypoxia tolerance but their interactive effect was not significantly different from the sum of isolated effects indicating an additive interaction- fish exposed to 200 mg NO_3^- L^{-1} at higher temperature showed less susceptibility to acute hypoxia. The observed additive improvement on hypoxia tolerance indicates cross-tolerance which may be attributed to related compensatory responses induced by priming stressors (Todgham et al., 2005; Burleson and Silva, 2011). In this case, warm acclimation and nitrate treatment, enhanced the blood oxygen carrying capacity causing improved tolerance under acute hypoxic conditions. This cross-tolerance may partially explain the tendency of *C. carpio* to thrive in warm habitats frequently affected by nitrate accumulation and consequent hypoxic conditions. Indeed, cross-tolerance is normally noted among stressors that co-occur in natural habitats (Sinclair et al., 2013).

5. Conclusion

Our findings demonstrated the resilience of *C. carpio* under warm eutrophic habitats with frequent acute thermal and oxygen fluctuations. Exceeding our expectations, we found that the aerobic performance and stress tolerance of *C. carpio* were not merely maintained, but were generally enhanced after warm acclimation and nitrate exposure. Warm acclimation independently enhanced the aerobic capacity and upper thermal tolerance of the species, and together with nitrate exposure (200 mg NO_3^- L^{-1}), additively improved the hypoxia tolerance of *C. carpio*. Additive improvement in hypoxia tolerance indicates cross-tolerance among nitrate, warming, and hypoxia, which can be attributed to the enhanced oxygen transport capacity as reflected by the hematological results including preserved Hb and increased HCT levels that may have offset the effects of the initial surge in MetHb at higher nitrate exposure. This cross-tolerance may explain the capacity of *C. carpio* to survive in warm habitats affected by nitrate accumulation and hypoxic conditions, and it suggests the propensity of this species to cope or even widen its fairly ubiquitous distribution in a warmer and more eutrophic future.

CRediT authorship contribution statement

April Grace R. Opinion: Writing – original draft, Conceptualization, Investigation, Data collection, Data curation, Visualization, Formal analysis. **Rümeysa Çakir:** Writing – original draft, Investigation, Data collection, Data curation, Visualization, Formal analysis. **Gudrun de Boeck:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition, Resources.

Declaration of Competing Interest

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

Acknowledgements

We would like to thank Dr. Essie M. Rodgers for her expert input during the study conceptualization and conduct of pilot test. This study was supported by the University Research Fund (BOF) of the University of Antwerp Research Council, Belgium under the research grants "Predicting Organismal responses To Eutrophication and Climate warming in Tandem (PROTECT)" (Grant number 40485), and "Freshwater

Ecosystems With A Burn-out: Extra Stress Caused By Heatwaves?" (Grant number: 42190) awarded to G.D.B.

Data sharing

Datasets have been deposited in Dryad: https://doi.org/10.5061/dryad.s4mw6m96v.

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