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Thermal acclimation offsets the negative effects of nitrate on aerobic scope and performance

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High temperatures raise resilience to nitrate

Summary statement
Nitrate exposure increases the susceptibility of fish to acute changes in temperature by lowering aerobic scope and performance, but thermal phenotypic plasticity can override these potential detrimental effects.

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ABSTRACT

Rising temperatures are set to imperil freshwater fishes as climate change ensues unless compensatory strategies are employed. However, the presence of additional stressors, such as elevated nitrate concentrations, may affect the efficacy of compensatory responses. Here, juvenile silver perch (*Bidyanus bidyanus*) were exposed to current-day summer temperatures (28°C) or a future climate-warming scenario (32°C) and simultaneously exposed to one of three ecologically relevant nitrate concentrations (0, 50 or 100 mg L\(^{-1}\)). We measured indicators of fish performance (growth, swimming), aerobic scope (AS) and upper thermal tolerance (*CT_{MAX}*) to test the hypothesis that nitrate exposure would increase susceptibility to elevated temperatures and limit thermal compensatory responses. After 8 weeks of acclimation, the thermal sensitivity and plasticity of AS and swimming performance were tested at three test temperatures (28, 32, 36°C). The AS of 28°C-acclimated fish declined with increasing temperature, and the effect was more pronounced in nitrate exposed individuals. In these fish, declines in AS corresponded with poorer swimming performance and a 0.8°C decrease in *CT_{MAX}* compared to unexposed fish. In contrast, acclimation to 32°C masked the effects of nitrate; fish acclimated to 32°C displayed a thermally insensitive phenotype whereby locomotor performance remained unchanged, AS was maintained and *CT_{MAX}* was increased by ~1°C irrespective of nitrate treatment compared to fish acclimated to 28°C. Growth was however markedly reduced in 32°C-acclimated compared to 28°C-acclimated fish. Our results indicate that nitrate exposure increases the susceptibility of fish to acute high temperatures, but thermal compensation can override some of these potential detrimental effects.

Key words: climate change, cross tolerance, eutrophication, multiple stressors, plasticity, swimming performance.
INTRODUCTION

The cumulative effects of co-occurring environmental stressors are placing immense pressure on the world’s biota. To survive, species must now contend with a myriad of environmental challenges simultaneously (e.g. habitat degradation, pollution and climate change) which can often have a combined effect that is unpredictable and multifaceted (Gunderson et al., 2016; Reid et al., 2019). Climate warming is one threat of imminent concern. The world has undergone a considerable amount of warming since the 1970s (Hansen et al., 2006) and temperatures are projected to continue to rise over the next century (IPCC, 2014). The frequency, intensity and severity of extreme thermal events are predicted to increase as the climate warms, exposing life to elevated, and often physiologically stressful temperatures (Meehl and Tebaldi, 2004; Seneviratne et al., 2014; Stillman, 2019). The threat of climate warming is particularly salient to ectothermic species, such as fishes, whose body temperatures are dictated by their surrounding environment (Huey and Kingsolver, 1989).

For ectotherms, the thermal environment governs the rate of all biochemical functions and when temperatures exceed specific thermal thresholds (i.e. thermal optima, TOPT) they face reductions in performance at all levels of biological organisation (Pörtner, 2010; Pörtner and Knust, 2007). To survive, ectothermic species can behaviourally seek suitable habitat or physiologically compensate to thermal changes via genetic alterations and/or environmentally induced phenotypic plasticity (Seebacher et al., 2015b).

Thermal phenotypic plasticity may be pivotal in determining a species’ capacity to cope with climate change (Seebacher et al., 2015b; Somero, 2010). Long-term exposure to elevated temperatures can induce physiological alterations at all levels of organisation, spanning from biochemical changes to behavioural responses (Franklin et al., 2007; Nyboer and Chapman, 2018; Szabo et al., 2008), which together improve performance under a new thermal regime. Thermal acclimation can shift physiological performance up to a new optimum temperature (Seebacher et al., 2005; Seebacher et al., 2015a), reduce the thermal sensitivity of physiological functions (Franklin et al., 2007; Rodgers et al., 2018) and/or alter thermal limits (i.e. upper thermal tolerance, CTMAX) (Anttila et al., 2015; Morgan et al., 2019; Peck et al., 2014) thereby allowing for performance to be maintained over a wider range of environmental temperatures. However, in spite of the considerable number of studies examining the efficacy of thermal acclimation responses, few studies have examined how these responses may change when species are exposed to an additional stressor.
Eutrophic events may render fish unable to cope with thermal extremes. Eutrophic events are triggered by the oversaturation of nutrients, particularly nitrogen (ammonia, nitrite and nitrate) and phosphorous, dissolved in water. Of these nutrients, nitrate ($\text{NO}_3^-$) is the most abundant and widespread due to the overexploitation of nitrogen-based fertilisers, the combustion of fossil fuels and accumulation of urban waste (Camargo and Alonso, 2006). Nitrate concentrations are increasing among disturbed waterways and can remain elevated for prolonged periods of time (Fowler et al., 2013; Mitchell et al., 2009; Sudduth et al., 2013). This is particularly true for areas of high agricultural and urban runoff (Goeller et al., 2019). Prolonged elevations in nitrate concentrations pose significant threats to aquatic taxa, as the impacts of nitrate on aquatic organisms increase with longer exposures (Gomez Isaza et al., 2020a). When water-nitrate concentrations are elevated, nitrate enters the body of aquatic animals passively through the gill epithelium and accumulates in plasma (Stormer et al., 1996). Inside the body, nitrate can lower concentrations of functional haemoglobin, via the oxidation of the central iron ion ($\text{Fe}^{2+}$ to $\text{Fe}^{3+}$), to a non-oxygen binding form, methaemoglobin (Monsees et al., 2017; Yang et al., 2019). Elevated concentrations of methaemoglobin cause an inherent loss of oxygen transport capacity (i.e. functional anaemia) resulting in tissue hypoxia (Avilez et al., 2004). High levels of nitrate within the blood also decreases levels of total haemoglobin and haematocrit (Monsees et al., 2017; Yang et al., 2019), and lowers the oxygen-haemoglobin binding affinity (Gomez Isaza et al., 2020b). Reductions in blood-oxygen carrying capacity manifest by altering whole animal energy expenditure (i.e. aerobic scope; Gomez Isaza et al., 2018; Gomez Isaza et al., 2020b) and causes cascading, negative effects on whole animal performance (e.g. growth, locomotion; Davidson et al., 2014; Gomez Isaza et al., 2020a; Monsees et al., 2017). As such, nitrate induced reductions to oxygen delivery are hypothesised to lower species’ tolerance of elevated temperatures.

Tolerance of thermal extremes in ectotherms has been experimentally and theoretically linked to an organism’s aerobic capacity. The oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis suggests that at both low and high temperatures, performance is limited by the inability of the cardio-respiratory system to supply oxygen to respiring mitochondria – leading to oxygen limitation at critical temperatures (Eliason et al., 2013; Frederich and Pörtner, 2000; Pörtner, 2010; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Pörtner et al., 2004; Pörtner and Peck, 2010). Oxygen limitation is hypothesised to be reflected in an animal’s aerobic scope, which is an integrative measure of the capacity
of the cardiorespiratory systems to provide oxygen for essential activities (e.g. growth, locomotion, reproduction) beyond basal metabolic processes (Claireaux and Lefrançois, 2007; Fry, 1971; Schulte, 2015). Typically, aerobic scope is assessed by determining the difference between maximum ($\dot{M}O_{2\text{MAX}}$) and standard ($\dot{M}O_{2\text{STANDARD}}$) oxygen uptake rates. $\dot{M}O_{2\text{STANDARD}}$ refers to the minimum metabolic costs required to sustain regular physiological functions of a post-absorptive, resting ectotherm (Norin and Malte, 2011). On the other hand, $\dot{M}O_{2\text{MAX}}$ defines the upper boundary for aerobic metabolism, and is usually measured in fish during or immediately following sustained exercise (Norin and Clark, 2016). The OCLTT predicts that aerobic scope narrows as temperature rise due to an exponential increase in $\dot{M}O_{2\text{STANDARD}}$ from the acceleration of all biochemical processes, while $\dot{M}O_{2\text{MAX}}$ reaches a plateau or decreases at elevated temperatures as dictated by the maximum capacity of the cardiorespiratory system (Eliason and Farrell, 2016; Pörtner, 2010). A narrowing of aerobic scope at elevated temperature is expected to cause declines in fitness-related traits such as growth and locomotion (Clark et al., 2011; Healy and Schulte, 2012; Nilsson et al., 2009; Runmer et al., 2015). Evidence for this hypothesis has shown that for some species oxygen delivery mechanisms are unable to meet oxygen demands at high temperatures due to physiological constraints on cardiac, respiratory and blood-oxygen delivery mechanisms (Adamczewska and Morris, 1994; Anttila et al., 2013; Beers and Sidell, 2011; Ekström et al., 2019; Eliason et al., 2013; Muñoz et al., 2018; Pörtner and Knust, 2007; Sandblom et al., 2016). However, the generality of this concept has been brought into question (Jutfelt et al., 2018) as it is not broadly applicable across species (Gomez Isaza et al., 2019; Gräns et al., 2014; Norin et al., 2014; Poletto et al., 2017) suggesting that mechanisms other than a mismatch in oxygen supply may be at play. Indeed, causal evidence to show that a reduction in oxygen transport (i.e. a reduction in haemoglobin levels) corresponds with a reduction in thermal tolerance remain scarce with only two studies having experimentally manipulated oxygen carrying capacity (Brijs et al., 2015; Wang et al., 2014) and the two studies produced contrasting results making it difficult to draw conclusions about the role that oxygen supply has in determining upper thermal limits.

Here we aimed to investigate how elevated temperatures and nitrate pollution, two common stressors in freshwater ecosystems, interact to affect the thermal sensitivity and plasticity of aerobic scope, swimming performance, growth and upper thermal limits of a freshwater fish, silver perch ($Bidyamus bidyanus$), following eight-weeks of acclimation. We chose to focus on how critical swim speeds ($U_{\text{CRIT}}$) are affected at stressfully high
temperatures, as $U_{\text{crit}}$ is hypothetically bound within an fish’s aerobic scope (Hvas et al., 2017). $U_{\text{crit}}$ refers to the highest swim speed that a fish can sustain using (primarily) aerobic metabolism (Plaut, 2001), and has been positively correlated with metabolism and body size in some fishes suggesting that it has some ecological relevance (Marras et al., 2013; Peake, 2004). Moreover, we assessed the interactive effects of thermal acclimation and nitrate pollution on the critical thermal maximum (CT$_{\text{max}}$) of silver perch. CT$_{\text{max}}$ was used as it is a repeatable measure of an animal’s upper thermal tolerance limit and the capacity to tolerate high temperature may be a key determinant of a species vulnerability to climate change (Grinder et al., 2020; Morgan et al., 2018).

As the primary determinant of blood oxygen carrying capacity in fishes (Gallaugher and Farrell, 1998), changes to haemoglobin concentrations caused by nitrate exposure (Gomez Isaza et al., 2020b) are predicted to cause a narrowing of aerobic scope at elevated temperatures and lower organismal tolerance of thermal extremes following the predictions of the OCLTT hypothesis (Pörtner, 2010; Pörtner et al., 2017). Specifically, we hypothesised that exposure to nitrate would: 1. lower aerobic scope, growth and swimming performance of fish at elevated temperatures; 2. lower whole-animal upper thermal tolerance; 3. inhibit or restrict thermal acclimation responses—thereby negating this potential compensatory mechanism.

**MATERIAL AND METHODS**

**Animal Maintenance**

Juvenile silver perch (*Bidyanus bidyanus*; n = 366; mass = 9.20 ± 4.92, mean ± SD, range 3.04 – 21.4) 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. During this period, the temperature in all tanks was kept at 26°C to match the thermal conditions at the hatchery (Ausyfish). Fish were fed once daily to satiety on a commercial, pelleted diet (average size of the pellets = 2 mm; 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 with a visible implant elastomer (VIE) tag (Northwest Marine Technology, Inc., Shaw Island, USA) to allow the tracking of individual fish. Fish were lightly anaesthetised (Aquit-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 No. SBS/249/17).

Experimental Design

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 L−1 NO₃⁻). Each treatment combination was replicated four times at the tank level. Thermal acclimation treatments were reflective of (i) current day summer temperatures (28°C) along the northern Murray-Darling Basin 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). The RCP8.5 represents a future warming scenario with little curbing of emission and carbon dioxide concentrations reaching 940 ppm by 2100 (CSIRO and Bureau of Meteorology, 2015). Nitrate concentrations were chosen to reflect control (0 mg L−1 NO₃⁻), moderate (50 mg L−1NO₃⁻ – current recommended maximum level) and high levels (100 mg L−1 NO₃⁻) 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 and did not fluctuate by more than 1°C (Table S1) from target temperatures. Nitrate concentrations were manipulated by dissolving sodium nitrate salt (ThermoFisher Scientific, Scoresby, Australia) in a 20 L bucket of filtered water, which was then added to each tank. Nitrate concentrations were 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% (Table S1). Fish were acclimated to experimental treatments for eight weeks. Experimental traits were measured in the following 8 – 15 weeks after this acclimation period (see Table 1 for specific timelines). Food was withheld for 24 h prior to all experiments.

Mass, growth and condition

The body mass (M; wet mass, g) and total length (LT; cm) was measured at four time points during the experiment (0, 7, 14, 21 weeks post exposure), during which fish remained within in their experimental treatments for the entire 21 weeks. Fish were individually
weighed using an electronic balance (Kern KB1200-2N, Balingen, Germany), $L_T$ was measured and returned to their holding tanks. Tank averages were used to calculate growth rates as the specific growth rate (SGR (% d$^{-1}$) = $\frac{[\log(M_F) – \log(M_I)]/t} \times 100$), where $M_F$ and $M_I$ are the final and initial mass, respectively, and $t$ is time (days) (Lugert et al., 2014). Fish body condition factor ($K$) was calculated as $K = \frac{M}{L_T^3} \times 100$.

**Thermal Sensitivity of Aerobic Scope**

The thermal sensitivity of standard and maximal oxygen uptake rates ($\dot{M}O_2^{\text{STANDARD}}$ and $\dot{M}O_2^{\text{MAX}}$, respectively) were assessed in fish from all six treatments at three acute test temperatures (28, 32 and 36°C). Six fish per treatment were tested at each test temperature, and fish were randomly taken from different replicate tanks. Oxygen uptake rates ($\dot{M}O_2$) were measured using intermittent-flow through respirometry (Clark et al., 2013). The respirometry set-up consisted of three acrylic respirometers, each of which was submerged in separate black, 96 L tanks. Two sizes of respirometers were used to accommodate fish of varying sizes; one large (1.96 L total volume, including tubing) and two small (0.69 L total volume including tubing) respirometers were used. Each respirometer was fitted with two circulation loops. The first loop was fitted with a continuously operating water pump (Eheim 1048-219, Germany) which circulated the water within the respirometer and past an oxygen flow-through cell (Presens, Regensburg, Germany). A fibre-optic cable connected to a Fibox 3 reader (Presens, Regensburg, Germany) was fitted to the oxygen flow-through cell and measured oxygen concentrations within the respirometers every second. A second circulation loop flushed the respirometers with oxygenated water from the surrounding water bath. An automated timer was connected to the pump and was set on a 15 min on/off cycle to ensure that oxygen saturation did not drop below 75% during trials. Water baths were continuously aerated using air-stones. Water temperature was adjusted to test temperature using a TK1000 Chiller/Heater (Teco, Ravenna, Italy) and maintained within ± 1°C of the target temperature. Test temperature was randomised to minimise any potential confounding effects. Nitrate concentrations were adjusted prior to fish introduction to reflect fish treatment group.

Individual silver perch from each treatment were randomly selected from their holding tanks and placed inside respirometer chambers. Fish were introduced to respirometry chambers a few minutes prior to the first $\dot{M}O_2$ recording at approximately 17:00 and remained inside the respirometers until the following morning (total duration ~14 h). Fish $\dot{M}O_2$ (mg O$_2$ kg$^{-1}$ h$^{-1}$) was calculated as the slope of the decline in oxygen
concentration inside the respirometers during the closed phase of the respirometry cycles. Specifically, $\dot{M}O_2$ was calculated as:

$$\dot{M}O_2 = \Delta O_2 / \Delta t \times V$$

where $\Delta O_2$ is the rate of change of oxygen concentration inside the respirometer containing a fish, $\Delta t$ is the change in time over which the $\Delta O_2$ was measured, and $V$ is the volume of the respirometer minus the volume of the fish (assuming 1 g displaces 1 ml of water). To measure $\dot{M}O_2_{MAX}$, fish were removed from respirometers, chased for 5 min in a circular container (65 cm diameter, 10cm height), then returned to their respirometers. Background respiration was measured for ~ 2 h after fish were removed from respirometry chambers. This period was sufficient to produce a decline in oxygen content and gave accurate ($r^2 > 0.92$) measurements (Svendsen et al., 2015). Because we only measured background respiration at the end of each respirometry trial, background respiration was assumed to be constant. $\dot{M}O_2$ were extracted and calculated using the *calc_rate* function of the *respR* package (Harianto et al., 2019) in R (R Core Team, 2018) following best practices (Clark et al., 2013). $\dot{M}O_2_{STANDARD}$ was determined as the lowest 10% of $\dot{M}O_2$ values during overnight measurements. $\dot{M}O_2_{MAX}$ was defined as the greatest decline in oxygen measured over a 1 min period. We corrected for background respiration by subtracting background $\dot{M}O_2$ from an animal’s $\dot{M}O_2$. Absolute aerobic scope (AAS = $\dot{M}O_2_{MAX} - \dot{M}O_2_{STANDARD}$) and factorial aerobic scope (FAS = $\dot{M}O_2_{MAX} / \dot{M}O_2_{STANDARD}$) were also calculated.

**Thermal Sensitivity of Swimming Performance**

Swimming performance trials were conducted in a 10 L, flow-controlled hydraulic flume (Loligo, Tjele, Denmark; swimming-chamber dimensions = $40 \times 10 \times 10$ cm; $l \times w \times h$). Water speeds generated by the propeller were calibrated using a Prandtl-pitot tube, as describe by Kern et al. (2018). Water temperature was adjusted to test temperature using a TK1000 Chiller/Heater and maintained within ± 0.5°C of the target temperature. Test temperature order was randomised to minimise any potential confounding effects. Nitrate concentrations were adjusted prior to fish introduction to reflect fish treatment group. The thermal sensitivity of prolonged swimming performance ($U_{CRIT}$) was assessed on a separate subset of fish from all six treatments at three test temperatures (28, 32 and 36°C; n = 6 per treatment, per temperature). Individual fish were placed in the swimming chamber of the flume and allowed to habituate to continuous flow conditions (0.05 m s⁻¹) for 1 h prior to $U_{CRIT}$ measurement. The anterior portion of the flume was covered with black plastic to encourage the fish to stay in the anterior part of the swim chamber. After the habituation
period, water velocity in the flume was increased to 0.2 m s\(^{-1}\) (approximately 1.5 – 2 body lengths per second; BL s\(^{-1}\)) and then increased incrementally every five minutes at a rate of 0.03 m s\(^{-1}\) until the fish fatigued. Fatigue was defined as the time when fish were unable to move off the rear screen of the swimming chamber for 10 s. Total swimming time and water velocity at fatigue were recorded to calculate \(U_{CRIT}\) using Brett’s (1964) equation:

\[
U_{CRIT} = U_F + \left( \frac{T_F}{T_I} \right) U_I
\]

where \(U_F\) is the highest water velocity maintained for the entire five-minute interval (m s\(^{-1}\)), \(U_I\) is the water velocity increment (0.03 m s\(^{-1}\)), \(T_F\) is the time swum during the final increment (s) and \(T_I\) is an entire velocity interval (300 s). Swimming performance was standardised for body length and expressed as BL s\(^{-1}\). The cross-sectional body-area of the fish did not exceed 10% of the cross-sectional area of the swimming chamber, therefore corrections for solid blocking effects were not necessary (Bell and Terhune, 1970).

**Upper thermal tolerance**

The upper thermal tolerance of silver perch was assessed as the critical thermal maximum (CT\(_{MAX}\)) (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997). CT\(_{MAX}\) determination were conducted in a waterbath (Clayson Microprocessor Temperature Controller = 50 × 35 × 19 cm, \(l \times w \times h\)) which was used to manipulate water temperature. Six cylindrical, glass chambers (\(ID \times h = 8.0 \times 16.0\) cm) were set up within the waterbath and allowed for the determination of CT\(_{MAX}\) on six fish simultaneously, with minimal interaction. Fasted fish (\(n = 12\) per treatment, separate subset from those used in \(\dot{M}O_2\) and \(U_{CRIT}\) measurements) were randomly selected from their holding tanks and individually placed within the glass chambers, which were filled with 1000 ml of water matching their respective acclimation temperature (either 28 or 32°C) and nitrate concentration (0, 50 or 100 mg L\(^{-1}\)). Water within the chambers was constantly aerated by running airlines into each chamber and water temperature was monitored using a YSI 85 dissolved oxygen and conductivity meter (Yellow Springs, OH, USA). Fish were allowed 1 h to habituate to chamber conditions, after which, water temperature was increased at a rate of 0.2°C min\(^{-1}\). Fish were continually observed during CT\(_{MAX}\) trials. Loss of equilibrium (LOE), defined as the failure to maintain dorsal-ventral orientation for more than 10 s, was used as the CT\(_{MAX}\) endpoint (Lutterschmidt and Hutchison, 1997). Following LOE, fish were placed in an aerated recovery tank matching their treatment conditions (nitrate and temperature) and left to recover for 1 h. Fish were then
weighed, measured (TL) and returned to their holding tanks. Post-CT\textsubscript{MAX} survival was monitored for 24 h and was high across all treatments ($\geq 92\%$).

**Statistical Analyses**

Statistical analyses were performed 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. Data that failed these assumptions ($\dot{M}O\text{\textsubscript{2STANDARD}}, \dot{M}O\text{\textsubscript{2MAX}},$ AAS, and FAS) were log transformed before parametric analyses were undertaken. Linear mixed effects models were used to test for statistical differences between nitrate and thermal acclimation treatments for each of the response variables: growth, condition, $\dot{M}O\text{\textsubscript{2STANDARD}}, \dot{M}O\text{\textsubscript{2MAX}},$ AAS, FAS and $U\text{\textsubscript{CRIT}}$. The effect of test temperature on $U\text{\textsubscript{CRIT}}$ was modelled as a second-degree polynomial to account for the curvilinear shape of the thermal performances (Angilletta, 2006) and set as a continuous variable. Nitrate concentration and acclimation temperature were included as fixed effects in all models, with tank ID (24 levels) and trial number as random effects. Body mass was included as a covariate in all analyses, except for the $U\text{\textsubscript{CRIT}}$ data where total length was included as a covariate. Minimal adequate model were determined using maximum likelihood (ML) simplification. The `lme` function of the `nlme` package (Pinheiro et al., 2017) was used for all the aforementioned analyses. Post hoc pairwise comparisons were performed using the `lsmeans` function of the `lsmeans` package (Russel, 2015). Significant differences were accepted as $P < 0.05$. Data are presented as mean ± standard error unless otherwise stated.

**RESULTS**

**Body size and condition**

Silver perch acclimated to 32\degree C experienced significantly lower growth rates ($t = -2.53, df = 17, P = 0.02$) and were in significantly poorer condition ($t = -2.22, df = 19, P = 0.04$) than their counterparts acclimated to 28\degree C (Table 2). In fact, 28\degree C-acclimated fish were 53\% heavier than fish acclimated to 32\degree C following 21 weeks of exposure to temperature treatments (Fig. 1). Nitrate exposure did not affect fish growth (50 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = 0.70, df = 19, P = 0.49$; 100 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = -0.30, df = 19, P = 0.77$) nor condition factor (K; 50 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = 0.86, df = 19, P = 0.40$; 100 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = 0.003, df = 19, P = 0.99$) and did not interact with acclimation temperature to affect body size metrics at either nitrate concentration (SGR = 50 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = -1.15, df = 17, P = 0.27$; 100 mg L\textsuperscript{-1}NO\textsubscript{3}\textsuperscript{-}: $t = -1.33,$
 Thermal Sensitivity of Aerobic Scope

Standard oxygen uptake (\(\dot{M}O_{2\text{STANDARD}}\)) rates were significantly affected by thermal acclimation, nitrate and test temperature treatments (Table 3). Warming from 28 – 36°C resulted in an approximate 2 – 3-fold increase in \(\dot{M}O_{2\text{STANDARD}}\) across all nitrate and thermal acclimation treatments (Fig. 2A, B; Table 4). However, the \(\dot{M}O_{2\text{STANDARD}}\) of 32°C-acclimated fish was, on average, significantly lower than that of fish acclimated to 28°C (regardless of nitrate treatment). For control fish acclimated to 28°C, \(\dot{M}O_{2\text{STANDARD}}\) increased exponentially with temperature from 174.5 ± 11.8 to 445.1 ± 39.0 mg O₂ kg⁻¹ h⁻¹, corresponding to a thermal sensitivity quotient (Q₁₀) of 3.2 over an 8°C temperature range (Table 4).

Acclimation to 32°C significantly lowered the thermal sensitivity of \(\dot{M}O_{2\text{STANDARD}}\) over the same temperature range, resulting in a Q₁₀ of 1.9 (Table 4). Nitrate exposure increased \(\dot{M}O_{2\text{STANDARD}}\), but only in 28°C-acclimated fish (Fig. 2A, B; Table S2). In these fish, exposure to 50 mg L⁻¹ NO₃⁻, but not 100 mg L⁻¹ NO₃⁻, raised metabolic costs by 40.0 and 14.5% above control (unexposed) fish at test temperatures of 28°C and 32°C, respectively (Fig. 2A; \(P > 0.05\), lsmeans; Table S2). There was no effect of nitrate treatment at the highest test temperature. The thermal sensitivity (Q₁₀ values) of \(\dot{M}O_{2\text{STANDARD}}\) was therefore higher among control (unexposed) fish than nitrate-exposed fish acclimated 28°C, especially between the test temperatures of 28 – 32°C (Table 4). Conversely, Q₁₀ values were similar for all fish acclimated to 32°C, regardless of nitrate treatment.

Maximum oxygen uptake (\(\dot{M}O_{2\text{MAX}}\)) rates were significantly affected by thermal acclimation, nitrate and test temperature treatments (Table 3). In fish from all treatments, \(\dot{M}O_{2\text{MAX}}\) rose with rising temperatures (Fig. 2C, D). 32°C-acclimated fish tended to have higher \(\dot{M}O_{2\text{MAX}}\) than 28°C-acclimated fish (Table 3), however, posthoc pairwise comparisons (lsmeans) tests suggest that these differences were marginal (Fig. 2C, D; Table S3). Q₁₀ values (Table 4) were similar across treatments; \(\dot{M}O_{2\text{MAX}}\) was more thermally sensitive between 28 – 32°C and reached a plateau between 32 – 36°C as indicated by the relatively low Q₁₀ values 1.02 – 1.18. \(\dot{M}O_{2\text{MAX}}\) of fish was lowered by nitrate exposure, but only when exposed to 100 mg L⁻¹ NO₃⁻ (\(t = -3.01, df = 20, P = 0.007\)).

Absolute aerobic scope (AAS) was significantly affected by thermal acclimation, nitrate and test temperature treatments (Table 3). AAS was maintained between the test
temperatures of 28 and 32°C in fish from both acclimation treatments but was reduced by acute exposure to 36°C (Fig. 3A, B; Table S4). Fish acclimated to 32°C retained 92% of their AAS at 36°C, whilst the AAS of fish kept at 28°C was reduced by 42% at this elevated temperature (280.9 ± 67.7 mg O₂ kg⁻¹ h⁻¹ down from 411.1 ± 32.8 mg O₂ kg⁻¹ h⁻¹ at 28°C). Overall, 28°C-acclimated fish tended to have a lower AAS than 32°C-acclimated animals across all three test temperatures (Fig. 3A, B). Nitrate exposure, however, did not influence AAS (Table 3). In contrast to AAS, factorial aerobic scope (FAS) saw a stepwise declined with increasing temperature in fish from all treatments (Table 3; Fig.32C, D). Differences among thermal acclimation treatments were also observed for FAS. FAS was significantly higher for 32°C-acclimated fish compared with fish acclimated to 28°C. Moreover, exposure to 50 and 100 mg L⁻¹NO₃⁻ had a significant effect on FAS, with nitrate exposed fish tending to have lower FAS than control animals (Fig. 3C, D; Table S5).

**Thermal Sensitivity of Swimming performance**

Exposure to nitrate lowered the prolonged swimming performance (U_CRIT) of silver perch, but only in 28°C-acclimated fish (Fig. 4A, B; \( t = -2.51, df = 19, P = 0.02 \)). In these fish, exposure to either 50 or 100 mg L⁻¹ NO₃⁻ resulted in poorer swimming performance (50 mg L⁻¹: \( t = -2.21, df = 19, P = 0.04 \); 100 mg L⁻¹: \( t = -4.13, df = 19, P < 0.001 \)). In contrast, nitrate exposure did not impact on the swimming performance of 32°C-acclimated fish (Fig. 4B). Test temperature had a significant influence on U_CRIT (\( t = -2.99, df = 83, P = 0.004 \)). The U_CRIT of 28-acclimated fish was optimised at 32°C irrespective of nitrate treatment but declined steeply at 36°C in all 28-acclimated animals (Table 4). Conversely, the swimming performance of 32°C-acclimated fish was thermally insensitive over an eight-degree temperature range (28 – 36°C; Table 4), but maximal performance was compromised. Fish acclimated to 32°C achieved maximal swim speeds of 4.5 ± 0.08 BL s⁻¹, while maximal performance of 28°C-acclimated fish was considerably higher at 5.3 ± 0.16 BL s⁻¹. Fish body length had marginal effects on U_CRIT (\( t = 1.89, df = 83, P = 0.06 \)).

**Upper thermal tolerance**

The impact of nitrate on upper thermal tolerance (CT_MAX) differed between acclimation treatments (nitrate × acclimation treatment: \( t = 5.72, df = 17, P < 0.0001 \); Fig. 5). In 28°C-acclimated fish, exposure to 100 mg L⁻¹NO₃⁻ (\( t = 6.82, df = 17, P < 0.0001 \)), but not 50 mg L⁻¹NO₃⁻ (\( t = 1.23, df = 17, P = 0.24 \)), reduced CT_MAX by 0.8°C (37.47 ± 0.06°C) relative to unexposed fish (38.31 ± 0.10°C). Conversely, CT_MAX was unaffected by nitrate exposure in 32°C-acclimated animals. CT_MAX was significantly affected by thermal
acclimation treatment (t = 6.96, df = 17, P < 0.0001) such that fish acclimated to 32°C (39.25 ± 0.08°C) lost equilibrium at ~1°C higher than 28°C-acclimated fish. Fish body mass did not influence CTMAX (t = -2.23, df = 2, P = 0.15).

**DISCUSSION**

Thermal plasticity may play a key role in buffering organisms against the effects of climate warming, yet few studies have investigated whether plastic responses are altered under the presence of multiple, co-occurring stressors. In accordance with our first two hypotheses, juvenile silver perch exposed to nitrate and acclimated to the cooler temperature of 28°C showed marked reductions in aerobic scope, swimming performance and upper thermal tolerance as compared to 28°C-acclimated fish. In contrast to our hypothesis, acclimation to 32°C masked the effects of nitrate. Fish acclimated to 32°C displayed a thermally insensitive phenotype whereby locomotor performance remained unchanged across an 8°C temperature range, aerobic scope was maintained, and the upper thermal tolerance limit was increased independent of nitrate exposure treatment. Together, we found that thermal acclimation capabilities were not hindered by concurrent nitrate exposure suggestive of a cross-tolerance interaction among these two stressors.

**Nitrate exposure causes a narrowing of aerobic scope**

Exposure to elevated nitrate concentrations (both 50 and 100 mg L⁻¹) lowered the aerobic scope (AS) of fish acclimated to 28°C. Reductions in AS were driven by both increases in standard (ṀO₂STANDARD) and decreases in maximum oxygen uptake (ṀO₂MAX) of nitrate-exposed fish. This is in agreement with other studies that have found elevated resting metabolic costs in response to nitrate pollution due to detoxification and cellular maintenance costs (de Campos et al., 2014), as well as decreases in ṀO₂MAX caused by the oxidation of haemoglobin to methaemoglobin (Gomez Isaza et al., 2020b). Moreover, the AS of 28°C-acclimated fish declined with increasing test temperature due to the thermal dependence of ṀO₂STANDARD increasing at a greater rate than that of ṀO₂MAX. A narrowing of AS has been reported for various fish exposed acutely to elevated temperatures (Clark et al., 2011; Healy and Schulte, 2012; Nilsson et al., 2009; Rummer et al., 2015), although it is recognised that this pattern does not hold true for all species (Gomez Isaza et al., 2019; Gräns et al., 2014; Poletto et al., 2017).

Declines in AS are hypothesised to stem from the limited ability of the cardio-ventilatory system to match oxygen demands at elevated temperatures (i.e. the OCLTT
hypothesis; Pörtner and Knust, 2007; Pörtner et al., 2004). In support of the OCLTT, we found that nitrate exposure led to greater declines in AS. Such constraints on AS corresponded with declines in the sustained swimming performance of silver perch at elevated temperatures—indicating a compromised capacity to support aerobic functions (Claireaux and Lefrançois, 2007; Pörtner and Knust, 2007). Our results are however, in contrast with previous studies that have manipulated blood-oxygen carrying capacity of fish. Wang et al. (2014) injected fish (European sea bass; Dicentrarchus labrax) with phenylhydrazine, a haemolytic agent, to lower blood-oxygen carrying capacity. They found that anaemic fish were able to compensate for a reduced blood-oxygen carrying capacity and maintain $\dot{M}O_2$ at elevated temperatures via a significant increase in cardiac output. Similarly, simulated anaemia (induced via the removal of 40% of blood volume) caused a small, but non-significant effect on the AS of European perch (Perca fluviatilis) during an acute thermal ramp (Brijs et al., 2015). However, these studies are representative of short-term anaemia (hours – days) and different responses may be seen in fish with chronic anaemia. Although we did not measure blood-oxygen carrying capacity in this study, chronic exposure to nitrate has been shown to reduce the blood-oxygen carrying capacity of fish via a reduction in haemoglobin concentration (Monseser et al., 2017; Yang et al., 2019) and a reduced blood-oxygen binding affinity (Gomez Isaza et al., 2020b). Indeed, carp (Cyprinus carpio) were shown to make compensatory cardiorespiratory adjustments (increased ventilation rate) for short periods of time following nitrite exposure but were unable to maintain these compensatory adjustments past 24 h of exposure (Williams et al., 1997). Taken together, previous and current findings suggest that reductions to oxygen transport capacity plays some role in governing changes in AS at elevated temperatures but compensatory changes along the oxygen transport cascade may be able to offset these effects.

**Thermal acclimation offsets the effects of nitrate on aerobic scope and performance**

Unlike in 28°C-acclimated fish, fish acclimated to 32°C and exposed to nitrate (either 50 or 100 mg L$^{-1}$NO$_3^-$) did not experience reductions in AS. Instead, acclimation to 32°C increased AS regardless of nitrate treatment which was facilitated by positive plastic responses to $\dot{M}O_{2\text{MAX}}$ across all three test temperatures. This result refutes our hypothesis that thermal acclimation responses would be inhibited by nitrate exposure and is instead suggestive of a cross-tolerance interaction between nitrate and high temperature acclimation. Cross-tolerance interactions can occur as exposure to one stressor confers tolerance to a second stressor (Sinclair et al., 2013). Studies on fish have shown that a thermal shock treatment can increase
tolerance of subsequent chemical (Brown et al., 1992), osmotic (Todgham et al., 2005), and hypoxic challenges (Burleson and Silva, 2011). Similarly, prior exposure to zinc for example, increases tolerance to subsequent exposure to other heavy metal mixtures (Brinkman and Woodling, 2014; Harper et al., 2008). Selection for cross-tolerance between two environmental stressors can arise in species co-adapted to both stressors or from long-term acclimation to one stressors if they influence similar behavioural or physiological mechanisms (Todgham et al., 2005). It is possible that physiological changes evoked by high temperature acclimation, including cardiac (Gamperl and Farrell, 2004; Nyboer and Chapman, 2018), respiratory (Anttila et al., 2015) or haematological (Ekström et al., 2016; Valenzuela et al., 2008) adjustments, may have provided overlapping protection to elevated nitrate concentrations but further research is warranted to uncover the exact mechanism behind this cross-tolerance interaction.

The maintenance of AS across test temperatures may have allowed for the swimming performance of 32°C-acclimated fish to be thermally insensitive over an 8°C temperature range. As for AS, nitrate treatment had negligible impacts on the swimming performance of 32°C-acclimated fish which provides further indication of a cross-tolerance/protective interaction between these two stressors. However, this thermal insensitivity of swimming performance came at the cost of lower maximum performance. Maximum swimming performance of 32°C-acclimated fish was ~4.5 BL s⁻¹, which is considerably lower than the maximum swim performance of 28°C-acclimated individuals at 32°C (5.3 BL s⁻¹). Thermal acclimation can favour a widening of performance at the cost of lower maximum performance, as has been observed in various fishes (Hvas et al., 2017; Rodgers et al., 2018; Seebacher et al., 2015a), in a phenomenon termed the generalist-specialist trade-off (Huey and Hertz, 1984; Schulte et al., 2011). The mechanisms which allow for these plastic responses are not well understood, but changes in the relative proportions of muscle fibre types (Hammil et al., 2004), enhancement of biochemical reaction rates (Franklin, 1998), changes to muscle contractile properties (e.g. myofibrillar ATPase activity; Johnston et al., 1990) and cardiac remodelling (Keen and Farrell, 1994) have been documented in fish following thermal acclimation. It is likely that the simultaneous remodelling of various organ systems contribute to the maintenance of locomotor performance over a wide thermal range. Such plasticity of sustained swimming performance likely influences the capacity of silver perch to perform a myriad of behaviours across a wide range of temperatures such as feeding,
predator avoidance, and avoiding unfavourable conditions (Plaut, 2001; Wolter and Arlinghaus, 2003) and would be beneficial as the climate warms.

Despite plasticity of key physiological traits ($\text{MO}_{2\text{MAX}}$ and swimming performance), the growth performance of 32°C-acclimated silver perch was compromised. Indeed, fish at 32°C experience marginal growth rates and were in significantly poorer condition than their 28°C-acclimated counterparts which may be indicative of energy allocation trade-offs.

Thermal acclimation is an energy-intensive process that can result in physiological trade-offs (Relyea, 2002) such that the energy used for the acclimation of one trait can be traded off against another (Angilletta et al., 2003). Similar trade-offs have been reported for other temperate fishes; for example, Atlantic halibut ($\text{Hippoglossus hippocampus}$) acclimated to various elevated temperature treatments experienced plasticity in AS but such changes did not align with improvements in growth performance (Gräns et al., 2014). Similarly, the AS of killifish ($\text{Fundulus heteroclitus}$; northern population) was optimised at 25-30°C but their growth performance was compromised at these temperatures (Healy and Schulte, 2012). Poor growth performance may also be related to the inability of this species to lower rates of maintenance metabolism or raise food consumption at elevated temperatures such that the energy available for growth was diminished (Present and Conover, 1992). Overall, our results indicate that although the potential for plasticity exists for certain traits, other critical traits (i.e. growth) of silver perch will likely be compromised under future climate change scenarios which can limit their long-term persistence.

**Critical thermal maximum is affected by nitrate and thermal acclimation**

Critical thermal limits provide essential baseline information on the relative ability of species to cope with acute thermal spikes which are set to increase in frequency and intensity in the coming decades (Hansen et al., 2006; IPCC, 2014). However, our understanding of how upper thermal limits are affected by the presence of additional environmental stressors is lacking, potentially leading to under- or over-estimates of species’ capacity to cope with thermal extremes. Contrary to our hypothesis, fish acclimated to 32°C and exposed to nitrate did not experience reductions in whole animal thermal tolerance ($\text{CT}_{\text{MAX}}$). This result is again indicates that high temperature acclimation may provide overlapping protection to elevated nitrate concentrations. However, in 28°C-acclimated fish, nitrate exposure (100 mg L$^{-1}$) reduced $\text{CT}_{\text{MAX}}$ by approximately 0.8°C. This reduction in $\text{CT}_{\text{MAX}}$ is attributed to the oxidation of haemoglobin and a decrease in red-blood cell numbers caused by exposure to nitrate (Gomez Isaza et al., 2020b). Similar effects have been shown in other fishes;
experimentally-induced anaemia in the European sea bass lowered CTMAX by 0.7°C (Wang et al., 2014) and, across different families of Chinook salmon (Oncorhynchus tshawytscha), thermal tolerance was positively associated with the oxygen-carrying capacity of blood (Muñoz et al., 2018). Taken together, these results are in keeping with the prediction of the OCLTT, which proposes that upper thermal limits are associated with oxygen supply (Pörtner, 2010; Pörtner et al., 2017). However, a decrease of this magnitude is modest and suggests that other mechanisms (e.g. protein or enzymes limitations, ion channel and neural function, mitochondrial function or effects on membrane fluidity; Iftikar and Hickey, 2013; Overgaard et al., 2012; Vornanen et al., 2014) on top of oxygen limitation combine to define whole animal thermal limits. Overall, our CTMAX results are in keeping with those measured in response to other nitrogenous waste products (e.g. nitrite; Rodgers and De Boeck, 2019) and environmental pollutants (e.g. organic chemicals; Patra et al., 2007), indicating that the presence of co-occurring stressors is likely to hamper species’ capacity to cope with heatwaves.

Juvenile silver perch demonstrated some degree of thermal plasticity in relation to their upper thermal limit. In 32°C-acclimated fish, CTMAX was increased by ~1°C relative to 28°C-acclimated individuals. This change in CTMAX reflects a comparatively low degree of thermal plasticity of upper thermal limits when compared to other species. Various temperate fishes acclimated to +3 – 5°C above summer temperatures have shown increases of 1.4 – 2.5°C in CTMAX (Akhtar et al., 2013; Das et al., 2004; Fangue et al., 2006). It is possible that longer term acclimation may further shift the CTMAX of silver perch, as was shown for Nile perch (Lates niloticus) (Nyboer and Chapman, 2017). However, our measures of CTMAX were made following ~7 – 8 weeks of exposure to acclimation treatments which is considerably longer than many other thermal acclimation trials (e.g. 4 weeks: Akhtar et al., 2013; Das et al., 2004; Fangue et al., 2006) indicating that further shifts in CTMAX are unlikely in this species.

Ecological implications

The presence of various environmental stressors, including nitrate, can impact on the capacity of fish to respond to warming waters and estimates ignoring these environmental complexities are likely to over- or under-estimate species susceptibility to climate change. This study showed the importance of considering the short- and long-term impacts of warming on fish. The negative effects of elevated temperatures on organismal traits are compounded by simultaneous exposure to elevated nitrate concentrations when considered on
a short-time scale and may prove fatal for many fishes living along inland rivers in Australia which often consist of a series of pools or billabongs where temperatures can reach up to 40°C in summer (CSIRO and Bureau of Meteorology, 2015). However, in the long term, silver perch displayed a remarkable capacity for thermal compensation, which provided overlapping protection of elevated nitrate concentrations. The mechanisms that allow for this overlapping protection may prove invaluable in predicting how fish may respond to the combined impacts of climate warming and other stressors and should be the focus of future studies. This capacity for thermal compensation was traded off against growth performance which may impact on the ecological success of this species as growth performance is inversely related to predation risk (Ribeiro and Qin, 2015) and provides a competitive advantage for resources (Cutts et al., 1999) to juvenile fish. Collectively, this work provides valuable information impact of nitrate exposure on the acclimation capacity of silver perch and highlights the potential for a cross-tolerance interaction between these two stressors.

COMPETING INTEREST
The authors declare no competing interests.

FUNDING
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Brinkman, S. F. and Woodling, J. D. (2014). Acclimation and Deacclimation of Brown Trout (Salmo trutta) to Zinc and Copper Singly and in Combination with Cadmium or Copper. Arch. Environ. Contam. Toxicol. 67, 214-223.


Table 1. Experiment timeline (weeks) and body mass (g) of silver perch (*Bidyanus bidyanus*) used for each trait. Fish were acclimated to one of two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100 mg L⁻¹) for eight weeks prior to testing. Experimental traits were measured between 8 – 15 weeks after the beginning of the acclimation period, with some overlap between traits. For respirometry and swimming performance (*U_{CRIT}*), six fish per treatment were tested at three test temperatures (28, 32 or 36°C), totalling 18 fish. Data are presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Timeline (weeks)</th>
<th>Trait</th>
<th>Sample size (n)</th>
<th>28°C-acclimated</th>
<th>32°C-acclimated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 mg L⁻¹</td>
<td>50 mg L⁻¹</td>
</tr>
<tr>
<td>10 – 15</td>
<td>Respirometry</td>
<td>18</td>
<td>18.6 ± 9.9</td>
<td>19.0 ± 8.3</td>
</tr>
<tr>
<td>8 – 12</td>
<td><em>U_{CRIT}</em></td>
<td>18</td>
<td>12.4 ± 3.4</td>
<td>13.7 ± 3.6</td>
</tr>
<tr>
<td>13 – 14</td>
<td>CT_{MAX}</td>
<td>12</td>
<td>13.6 ± 4.4</td>
<td>13.9 ± 3.3</td>
</tr>
</tbody>
</table>
Table 2. Mass and condition factor (K) of silver perch (*Bidyanus bidyanus*) used in the experiment. Juvenile silver perch acclimated to one of two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100 mg L⁻¹). Abbreviations = Condition factor, K; Specific growth rate, SGR (% d⁻¹). See main text for calculations of K and SGR.

<table>
<thead>
<tr>
<th>Nitrate (mg L⁻¹)</th>
<th>28°C -acclimated</th>
<th>32°C- acclimated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Initial mass (g)</td>
<td>9.3 (± 0.2)</td>
<td>9.1 (± 0.2)</td>
</tr>
<tr>
<td>Initial K</td>
<td>1.26 (± 0.01)</td>
<td>1.24 (± 0.01)</td>
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<tr>
<td>Final mass (g)</td>
<td>28.7 (± 4.4)</td>
<td>29.9 (± 1.2)</td>
</tr>
<tr>
<td>Final K</td>
<td>1.19 (± 0.02)</td>
<td>1.17 (± 0.02)</td>
</tr>
<tr>
<td>SGR (% d⁻¹)</td>
<td>0.3 (± 0.04)</td>
<td>0.3 (± 0.01)</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>98.0 (± 0.02)</td>
<td>100 (± 0.00)</td>
</tr>
</tbody>
</table>
Table 3. Results of linear mixed effects (LME) models testing for differences between nitrate concentration, acclimation temperature, and test temperature on metabolic attributes. Results of minimum adequate models presented, with significant values in bold. Fish were acclimated to one of two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100 mg L⁻¹). Fish were tested at three acute test temperatures (28, 32 and 36°C) treatments. Abbreviations = standard oxygen uptake, $\dot{M}O_{2\text{STANDARD}}$; maximal oxygen uptake, $\dot{M}O_{2\text{MAX}}$; absolute aerobic scope, AAS; factorial aerobic scope FAS.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>s.e.</th>
<th>df</th>
<th>t-value</th>
<th>P-value</th>
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<tr>
<td>$\dot{M}O_{2\text{STANDARD}}$</td>
<td></td>
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<tr>
<td>Acclimation temperature</td>
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<td>0.021</td>
<td>20</td>
<td>-2.587</td>
<td>0.017</td>
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<tr>
<td>Nitrate (50 mg L⁻¹ NO₃⁻)</td>
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<td>0.025</td>
<td>20</td>
<td>2.442</td>
<td>0.024</td>
</tr>
<tr>
<td>Nitrate (100 mg L⁻¹ NO₃⁻)</td>
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<td>0.025</td>
<td>20</td>
<td>0.818</td>
<td>0.423</td>
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<td>Test temperature (32°C)</td>
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<td>0.025</td>
<td>35</td>
<td>5.312</td>
<td>&lt;0.0001</td>
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<tr>
<td>Test temperature (36°C)</td>
<td>0.283</td>
<td>0.025</td>
<td>47</td>
<td>11.179</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\dot{M}O_{2\text{MAX}}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Acclimation temperature</td>
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<td>0.010</td>
<td>20</td>
<td>4.780</td>
<td>0.0001</td>
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<tr>
<td>Nitrate (50 mg L⁻¹ NO₃⁻)</td>
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<td>0.012</td>
<td>20</td>
<td>0.163</td>
<td>0.873</td>
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<tr>
<td>Nitrate (100 mg L⁻¹ NO₃⁻)</td>
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<td>-2.921</td>
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<td>Test temperature (32°C)</td>
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<td>0.013</td>
<td>35</td>
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<td>0.001</td>
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<tr>
<td>Test temperature (36°C)</td>
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<td>0.013</td>
<td>47</td>
<td>4.871</td>
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<tr>
<td>AAS</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Acclimation temperature</td>
<td>0.170</td>
<td>0.035</td>
<td>20</td>
<td>4.448</td>
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<tr>
<td>Nitrate (50 mg L⁻¹ NO₃⁻)</td>
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<td>20</td>
<td>-1.419</td>
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<td>0.042</td>
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<td>0.925</td>
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<tr>
<td>FAS</td>
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<td></td>
</tr>
<tr>
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<td>0.022</td>
<td>20</td>
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<td>0.0002</td>
</tr>
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<td>-2.204</td>
<td>0.039</td>
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<td>-3.207</td>
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<td>Test temperature (36°C)</td>
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<td>0.027</td>
<td>47</td>
<td>-8.084</td>
<td>&lt;0.0001</td>
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Table 4. Thermal sensitivity ($Q_{10}$) of critical swimming performance ($U_{CRIT}$), standard oxygen uptake ($\dot{M}O_{2\text{STANDARD}}$) and maximal oxygen uptake ($\dot{M}O_{2\text{MAX}}$) of silver perch (*Bidyanus bidyanus*). Fish were acclimated to one of two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100 mg L$^{-1}$). Fish were tested at three acute test temperatures (28, 32 and 36°C) and $Q_{10}$ values were calculated over the entire test temperature range (28 and 36°C), as well as the upper (32 and 36°C) and lower (28 and 32°C) test temperatures.

<table>
<thead>
<tr>
<th>Nitrate (mg L$^{-1}$)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>28°C-acclimated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$U_{CRIT}$</td>
<td>1.1</td>
<td>1.4</td>
<td>1.1</td>
<td>0.9</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>$\dot{M}O_{2\text{STANDARD}}$</td>
<td>3.31</td>
<td>1.37</td>
<td>1.50</td>
<td>1.83</td>
<td>2.17</td>
<td>2.95</td>
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<tr>
<td>$\dot{M}O_{2\text{MAX}}$</td>
<td>1.45</td>
<td>1.26</td>
<td>1.21</td>
<td>1.27</td>
<td>1.19</td>
<td>1.56</td>
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<tr>
<td><strong>32°C-acclimated</strong></td>
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<td></td>
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<td>$U_{CRIT}$</td>
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<td>$\dot{M}O_{2\text{STANDARD}}$</td>
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<tr>
<td>$\dot{M}O_{2\text{MAX}}$</td>
<td>1.18</td>
<td>1.03</td>
<td>1.09</td>
<td>1.13</td>
<td>1.05</td>
<td>1.02</td>
</tr>
<tr>
<td><strong>28 – 36</strong></td>
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<td></td>
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<tr>
<td>$U_{CRIT}$</td>
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<tr>
<td>$\dot{M}O_{2\text{STANDARD}}$</td>
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<td>1.91</td>
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<tr>
<td>$\dot{M}O_{2\text{MAX}}$</td>
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<td>1.14</td>
<td>1.15</td>
<td>1.20</td>
<td>1.12</td>
<td>1.26</td>
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Figure 1. Change in mass (g) over 21-weeks of exposure to experimental treatments.

Silver perch (*Bidyanus bidyanus*) were acclimated to either (A) 28 or (B) 32°C and exposed to one of three nitrate concentration (0, 50 or 100 mg L$^{-1}$). Data are presented as mean (dots) and shaded regions represent standard deviations.
Figure 2. Metabolic responses to temperature in silver perch (*Bidyanus bidyanus*) exposed to factorial combination of nitrate and temperature acclimation treatments. Standard (\(\dot{M}O_2\)\text{\_standard}) and maximum oxygen uptake (\(\dot{M}O_2\)\text{\_max}) of (A, C) 28°C and (B, D) 32°C-acclimated fish, respectively. Data are presented as mean ± s.e.m. and n = 6 fish treatment\(^{-1}\) temperature\(^{-1}\).
Figure 3. Aerobic scope of silver perch (Bidyanus bidyanus) acclimated either 28 or 32°C and exposed to one of three nitrate treatments (0, 50 or 100 mg L\(^{-1}\)). (A) Absolute aerobic scope (AAS; mg O\(_2\) kg\(^{-1}\) h\(^{-1}\)) and (B) factorial aerobic scope (FAS; fold change) are presented. See main text for calculations. Data are presented as mean ± s.e.m. and n = 6 fish treatment\(^{-1}\) temperature\(^{-1}\).
Figure 4. Thermal sensitivity of swimming performance ($U_{\text{CRIT}}$ BL s$^{-1}$) in silver perch (*Bidyanus bidyanus*) acclimated either 28 (A) or 32°C (B) and exposed to one of three nitrate treatments (0, 50 or 100 mg L$^{-1}$). Data are presented as mean ± s.e.m. and n = 6 fish treatment$^{-1}$ temperature$^{-1}$. 
Figure 5. Effect of thermal acclimation and nitrate exposure on the critical thermal maxima ($C_{\text{MAX}}$, °C) of silver perch. Data are presented as boxplots [minimum, first quartile (Q1), median, third quartile (Q3) and maximum], and dots represent individual data points (n = 12). Uppercase letters represent statistical difference ($P < 0.05$, lme) between treatment groups.