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1	Thermal acclimation offsets the negative effects of nitrate on
2	aerobic scope and performance
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4	Daniel F. Gomez Isaza <sup>1*</sup> , Rebecca L. Cramp <sup>1</sup> and Craig E. Franklin <sup>1</sup>
5	<sup>1</sup> School of Biological Science, The University of Queensland, Brisbane, QLD 4072,
6	Australia.
7	
8	* Corresponding author: Daniel F. Gomez Isaza
9	School of Biological Science, The University of Queensland, Brisbane, Queensland 4072,
10	Email: <u>daniel.gomezisaza@uq.net.au</u> .
11	
12	Orcid
13	D.F.G.I: 0000-0003-3112-8683
14	R.L.C: 0000-0001-9798-2271
15	C.E.F: 0000-0003-1315-3797
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18	High temperatures raise resilience to nitrate
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20	Summary statement
21	Nitrate exposure increases the susceptibility of fish to acute changes in temperature by
22	lowering aerobic scope and performance, but thermal phenotypic plasticity can override these
23	potential detrimental effects.
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#### 33 ABSTRACT

34 Rising temperatures are set to imperil freshwater fishes as climate change ensues unless 35 compensatory strategies are employed. However, the presence of additional stressors, such as 36 elevated nitrate concentrations, may affect the efficacy of compensatory responses. Here, 37 juvenile silver perch (*Bidvanus*) were exposed to current-day summer temperatures 38 (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 39 40 indicators of fish performance (growth, swimming), aerobic scope (AS) and upper thermal 41 tolerance (CT<sub>MAX</sub>) to test the hypothesis that nitrate exposure would increase susceptibility to 42 elevated temperatures and limit thermal compensatory responses. After 8 weeks of 43 acclimation, the thermal sensitivity and plasticity of AS and swimming performance were 44 tested at three test temperatures (28, 32, 36°C). The AS of 28°C-acclimated fish declined with 45 increasing temperature, and the effect was more pronounced in nitrate exposed individuals. In 46 these fish, declines in AS corresponded with poorer swimming performance and a 0.8°C 47 decrease in CT<sub>MAX</sub> compared to unexposed fish. In contrast, acclimation to 32°C masked the 48 effects of nitrate; fish acclimated to 32°C displayed a thermally insensitive phenotype 49 whereby locomotor performance remained unchanged, AS was maintained and CT<sub>MAX</sub> was 50 increased by ~1°C irrespective of nitrate treatment compared to fish acclimated to 28°C. 51 Growth was however markedly reduced in 32°C-acclimated compared to 28°C-acclimated 52 fish. Our results indicate that nitrate exposure increases the susceptibility of fish to acute high 53 temperatures, but thermal compensation can override some of these potential detrimental 54 effects. 55 56 Key words: climate change, cross tolerance, eutrophication, multiple stressors, plasticity, 57 58 swimming performance. 59 60 61 62 63 64 65 66

#### 67 INTRODUCTION

68 The cumulative effects of co-occurring environmental stressors are placing immense pressure 69 on the world's biota. To survive, species must now contend with a myriad of environmental 70 challenges simultaneously (e.g. habitat degradation, pollution and climate change) which can 71 often have a combined effect that is unpredictable and multifaceted (Gunderson et al., 2016; 72 Reid et al., 2019). Climate warming is one threat of imminent concern. The world has 73 undergone a considerable amount of warming since the 1970s (Hansen et al., 2006) and 74 temperatures are projected to continue to rise over the next century (IPCC, 2014). The 75 frequency, intensity and severity of extreme thermal events are predicted to increase as the 76 climate warms, exposing life to elevated, and often physiologically stressful temperatures 77 (Meehl and Tebaldi, 2004; Seneviratne et al., 2014; Stillman, 2019). The threat of climate 78 warming is particularly salient to ectothermic species, such as fishes, whose body 79 temperatures are dictated by their surrounding environment (Huey and Kingsolver, 1989). 80 For ectotherms, the thermal environment governs the rate of all biochemical functions and 81 when temperatures exceed specific thermal thresholds (i.e. thermal optima, TOPT) they face 82 reductions in performance at all levels of biological organisation (Pörtner, 2010; Pörtner and 83 Knust, 2007). To survive, ectothermic species can behaviourally seek suitable habitat or 84 physiologically compensate to thermal changes via genetic alterations and/or environmentally 85 induced phenotypic plasticity (Seebacher et al., 2015b).

86 Thermal phenotypic plasticity may be pivotal in determining a species' capacity to 87 cope with climate change (Seebacher et al., 2015b; Somero, 2010). Long-term exposure to 88 elevated temperatures can induce physiological alterations at all levels of organisation, 89 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 90 91 thermal regime. Thermal acclimation can shift physiological performance up to a new 92 optimum temperature (Seebacher et al., 2005; Seebacher et al., 2015a), reduce the thermal 93 sensitivity of physiological functions (Franklin et al., 2007; Rodgers et al., 2018) and/or alter 94 thermal limits (i.e. upper thermal tolerance, CT<sub>MAX</sub>) (Anttila et al., 2015; Morgan et al., 2019; 95 Peck et al., 2014) thereby allowing for performance to be maintained over a wider range of 96 environmental temperatures. However, in spite of the considerable number of studies 97 examining the efficacy of thermal acclimation responses, few studies have examined how 98 these responses may change when species are exposed to an additional stressor.

99 Eutrophic events may render fish unable to cope with thermal extremes. Eutrophic 100 events are triggered by the oversaturation of nutrients, particularly nitrogen (ammonia, nitrite 101 and nitrate) and phosphorous, dissolved in water. Of these nutrients, nitrate  $(NO_3^-)$  is the most 102 abundant and widespread due to the overexploitation of nitrogen-based fertilisers, the 103 combustion of fossil fuels and accumulation of urban waste (Camargo and Alonso, 2006). 104 Nitrate concentrations are increasing among disturbed waterways and can remain elevated for 105 prolonged periods of time (Fowler et al., 2013; Mitchell et al., 2009; Sudduth et al., 2013). 106 This is particularly true for areas of high agricultural and urban runoff (Goeller et al., 2019). 107 Prolonged elevations in nitrate concentrations pose significant threats to aquatic taxa, as the 108 impacts of nitrate on aquatic organisms increase with longer exposures (Gomez Isaza et al., 109 2020a). When water-nitrate concentrations are elevated, nitrate enters the body of aquatic 110 animals passively through the gill epithelium and accumulates in plasma (Stormer et al., 111 1996). Inside the body, nitrate can lower concentrations of functional haemoglobin, via the oxidation of the central iron ion ( $Fe^{2+}$  to  $Fe^{3+}$ ), to a non-oxygen binding form, 112 113 methaemoglobin (Monsees et al., 2017; Yang et al., 2019). Elevated concentrations of 114 methaemoglobin cause an inherent loss of oxygen transport capacity (i.e. functional anaemia) 115 resulting in tissue hypoxia (Avilez et al., 2004). High levels of nitrate within the blood also 116 decreases levels of total haemoglobin and haematocrit (Monsees et al., 2017; Yang et al., 117 2019), and lowers the oxygen-haemoglobin binding affinity (Gomez Isaza et al., 2020b). 118 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 119 120 causes cascading, negative effects on whole animal performance (e.g. growth, locomotion; 121 Davidson et al., 2014; Gomez Isaza et al., 2020a; Monsees et al., 2017). As such, nitrate 122 induced reductions to oxygen delivery are hypothesised to lower species' tolerance of 123 elevated temperatures.

124 Tolerance of thermal extremes in ectotherms has been experimentally and 125 theoretically linked to an organism's aerobic capacity. The oxygen and capacity-limited 126 thermal tolerance (OCLTT) hypothesis suggests that at both low and high temperatures, 127 performance is limited by the inability of the cardio-respiratory system to supply oxygen to 128 respiring mitochondria – leading to oxygen limitation at critical temperatures (Eliason et al., 129 2013; Frederich and Pörtner, 2000; Pörtner, 2010; Pörtner and Farrell, 2008; Pörtner and 130 Knust, 2007; Pörtner et al., 2004; Pörtner and Peck, 2010). Oxygen limitation is hypothesised 131 to be reflected in an animal's aerobic scope, which is an integrative measure of the capacity

132 of the cardiorespiratory systems to provide oxygen for essential activities (e.g. growth, 133 locomotion, reproduction) beyond basal metabolic processes (Claireaux and Lefrançois, 134 2007; Fry, 1971; Schulte, 2015). Typically, aerobic scope is assessed by determining the 135 difference between maximum (MO<sub>2MAX</sub>) and standard (MO<sub>2STANDARD</sub>) oxygen uptake rates. 136 MO<sub>2STANDARD</sub> refers to the minimum metabolic costs required to sustain regular physiological 137 functions of a post-absorptive, resting ectotherm (Norin and Malte, 2011). On the other hand, 138 MO<sub>2MAX</sub> defines the upper boundary for aerobic metabolism, and is usually measured in fish 139 during or immediately following sustained exercise (Norin and Clark, 2016). The OCLTT 140 predicts that aerobic scope narrows as temperature rise due to an exponential increase in 141 MO2STANDARD from the acceleration of all biochemical processes, while MO2MAX reaches a 142 plateau or decreases at elevated temperatures as dictated by the maximum capacity of the 143 cardiorespiratory system (Eliason and Farrell, 2016; Pörtner, 2010). A narrowing of aerobic 144 scope at elevated temperature is expected to cause declines in fitness-related traits such as 145 growth and locomotion (Clark et al., 2011; Healy and Schulte, 2012; Nilsson et al., 2009; 146 Rummer et al., 2015). Evidence for this hypothesis has shown that for some species oxygen 147 delivery mechanisms are unable to meet oxygen demands at high temperatures due to 148 physiological constraints on cardiac, respiratory and blood-oxygen delivery mechanisms 149 (Adamczewska and Morris, 1994; Anttila et al., 2013; Beers and Sidell, 2011; Ekström et al., 150 2019; Eliason et al., 2013; Muñoz et al., 2018; Pörtner and Knust, 2007; Sandblom et al., 151 2016). However, the generality of this concept has been brought into question (Jutfelt et al., 152 2018) as it is not broadly applicable across species (Gomez Isaza et al., 2019; Gräns et al., 153 2014; Norin et al., 2014; Poletto et al., 2017) suggesting that mechanisms other than a 154 mismatch in oxygen supply may be at play. Indeed, causal evidence to show that a reduction 155 in oxygen transport (i.e. a reduction in haemoglobin levels) corresponds with a reduction in 156 thermal tolerance remain scarce with only two studies having experimentally manipulated 157 oxygen carrying capacity (Brijs et al., 2015; Wang et al., 2014) and the two studies produced 158 contrasting results making it difficult to draw conclusions about the role that oxygen supply 159 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 (*Bidyanus bidyanus*), following eight-weeks of acclimation. We chose to focus on how critical swim speeds (*U*<sub>CRIT</sub>) are affected at stressfully high

165 temperatures, as UCRIT is hypothetically bound within an fish's aerobic scope (Hvas et al.,

- 166 2017). UCRIT refers to the highest swim speed that a fish can sustain using (primarily) aerobic
- 167 metabolism (Plaut, 2001), and has been positively correlated with metabolism and body size
- 168 in some fishes suggesting that it has some ecological relevance (Marras et al., 2013; Peake,
- 169 2004). Moreover, we assessed the interactive effects of thermal acclimation and nitrate
- 170 pollution on the critical thermal maximum (CT<sub>MAX</sub>) of silver perch. CT<sub>MAX</sub> was used as it is a
- 171 repeatable measure of an animal's upper thermal tolerance limit and the capacity to tolerate
- 172 high temperature may be a key determinant of a species vulnerability to climate change
- 173 (Grinder et al., 2020; Morgan et al., 2018).

174 As the primary determinant of blood oxygen carrying capacity in fishes (Gallaugher 175 and Farrell, 1998), changes to haemoglobin concentrations caused by nitrate exposure 176 (Gomez Isaza et al., 2020b) are predicted to cause a narrowing of aerobic scope at elevated 177 temperatures and lower organismal tolerance of thermal extremes following the predictions 178 of the OCLTT hypothesis (Pörtner, 2010; Pörtner et al., 2017). Specifically, we hypothesised 179 that exposure to nitrate would: 1. lower aerobic scope, growth and swimming performance of 180 fish at elevated temperatures; 2. lower whole-animal upper thermal tolerance; 3. inhibit or 181 restrict thermal acclimation responses- thereby negating this potential compensatory

182 mechanism.

## 183 MATERIAL AND METHODS

184 Animal Maintenance

185 Juvenile silver perch (*Bidyanus bidyanus*; n = 366; mass =  $9.20 \pm 4.92$ , mean  $\pm$  SD, 186 range 3.04 - 21.4) were sourced from a commercial hatchery (Ausyfish Pty. Ltd.) and 187 transported to The University of Queensland in oxygenated transport bags. Fish were distributed among twenty-four, 40L glass tanks ( $60 \times 25 \times 30$  cm;  $l \times w \times h$ ) at a density of 188 189 15 - 16 fish per tank. Tanks were filled with filtered tap water and each equipped with a 190 sponge filter for filtration and an air-stone for additional aeration. During this period, the 191 temperature in all tanks was kept at 26°C to match the thermal conditions at the hatchery 192 (Ausyfish). Fish were fed once daily to satiety on a commercial, pelleted diet (average size of 193 the pellets = 2 mm; Ridley Aqua-feeds TM, Narangba, Queensland, Australia). Fish were 194 maintained under a constant 12:12 h light: dark cycle and allowed to adjust to laboratory 195 conditions for one week. After this adjustment period, all fish were tagged with a visible 196 implant elastomer (VIE) tag (Northwest Marine Technology, Inc., Shaw Island, USA) to 197 allow the tracking of individual fish. Fish were lightly anaesthetised (Aqui-S TM, Aqui-S Pty

LTD, Lower Hutt, New Zealand) and tags (2 – 3 mm) were implanted below the skin, parallel
to the dorsal fin. Fish were allowed one week to recover from tagging prior to the
commencement of the experiment. During this time all fish resumed eating and post-tag
survival was 100%. All experiments were conducted in accordance with the Australian
Animal Care guidelines and approved by The University of Queensland animal ethics
committee (Ethics No. SBS/249/17).

204 Experimental Design

205 We employed a full  $2 \times 3$  factorial design with two thermal acclimation treatments (28 and 32°C) and three nitrate concentrations (0, 50 and 100 mg  $L^{-1} NO_3^{-1}$ ). Each treatment 206 207 combination was replicated four times at the tank level. Thermal acclimation treatments were 208 reflective of (i) current day summer temperatures (28°C) along the northern Murray-Darling 209 Basin and (ii) a high rate of climate warming (32°C) forecasted under a high degree of 210 radiative forcing (high emissions - representative concentration pathway (RCP) 8.5). The 211 RCP8.5 represents a future warming scenario with little curbing of emission and carbon 212 dioxide concentrations reaching 940 ppm by 2100 (CSIRO and Bureau of Meteorology, 2015). Nitrate concentrations were chosen to reflect control (0 mg  $L^{-1} NO_3^{-1}$ ), moderate (50 213 mg  $L^{-1}NO_3^-$  – current recommended maximum level) and high levels (100 mg  $L^{-1}NO_3^-$ ) of 214 nitrate pollution (Environment Australia, 2002). Temperatures were adjusted and maintained 215 216 using 300 W submersible heaters (Aqua Zonic Eco aquarium heaters). Temperature loggers 217 (iButtons, Maxim Integrated, San Jose, USA) were submerged in each tank to record water 218 temperature every hour and did not fluctuate by more than 1°C (Table S1) from target 219 temperatures. Nitrate concentrations were manipulated by dissolving sodium nitrate salt 220 (ThermoFisher Scientific, Scoresby, Australia) in a 20 L bucket of filtered water, which was 221 then added to each tank. Nitrate concentrations were measured once daily using a nitrate 222 meter (LAQUAtwin-NO3-11 meter, Horiba Scientific). Nitrate levels did not deviate from 223 nominal concentrations by more than 10% (Table S1). Fish were acclimated to experimental 224 treatments for eight weeks. Experimental traits were measured in the following 8 - 15 weeks 225 after this acclimation period (see Table 1 for specific timelines). Food was withheld for 24 h 226 prior to all experiments.

227 Mass, growth and condition

The body mass (M; wet mass, g) and total length ( $L_T$ ; 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

- 231 weighed using an electronic balance (Kern KB1200-2N, Balingen, Germany), L<sub>T</sub> was
- 232 measured and returned to their holding tanks. Tank averages were used to calculate growth
- rates as the specific growth rate (SGR (%  $d^{-1}$ ) = [(log(M<sub>F</sub>) log(M<sub>I</sub>))/t] × 100), where M<sub>F</sub> and
- 234 M<sub>I</sub> are the final and initial mass, respectively, and *t* is time (days) (Lugert et al., 2014). Fish
- body condition factor (K) was calculated as  $K = (M/LT^3) \times 100$ .
- 236 Thermal Sensitivity of Aerobic Scope

237 The thermal sensitivity of standard and maximal oxygen uptake rates (MO2STANDARD 238 and MO<sub>2MAX</sub>, respectively) were assessed in fish from all six treatments at three acute test 239 temperatures (28, 32 and 36°C). Six fish per treatment were tested at each test temperature, 240 and fish were randomly taken from different replicate tanks. Oxygen uptake rates (MO<sub>2</sub>) were 241 measured using intermittent-flow through respirometry (Clark et al., 2013). The respirometry 242 set-up consisted of three acrylic respirometers, each of which was submerged in separate 243 black, 96 L tanks. Two sizes of respirometers were used to accommodate fish of varying 244 sizes; one large (1.96 L total volume, including tubing) and two small (0.69 L total volume 245 including tubing) respirometers were used. Each respirometer was fitted with two circulation 246 loops. The first loop was fitted with a continuously operating water pump (Eheim 1048-219, 247 Germany) which circulated the water within the respirometer and past an oxygen flow-248 through cell (Presens, Regensburg, Germany). A fibre-optic cable connected to a Fibox 3 249 reader (Presens, Regensburg, Germany) was fitted to the oxygen flow-through cell and 250 measured oxygen concentrations within the respirometers every second. A second circulation 251 loop flushed the respirometers with oxygenated water from the surrounding water bath. An 252 automated timer was connected to the pump and was set on a 15 min on/off cycle to ensure 253 that oxygen saturation did not drop below 75% during trials. Water baths were continuously 254 aerated using air-stones. Water temperature was adjusted to test temperature using a TK1000 255 Chiller/Heater (Teco, Ravenna, Italy) and maintained within  $\pm 1^{\circ}$ C of the target temperature. 256 Test temperature was randomised to minimise any potential confounding effects. Nitrate 257 concentrations were adjusted prior to fish introduction to reflect fish treatment group. 258 Individual silver perch from each treatment were randomly selected from their 259 holding tanks and placed inside respirometer chambers. Fish were introduced to 260 respirometery chambers a few minutes prior to the first  $\dot{M}O_2$  recording at approximately 261 17:00 and remained inside the respirometers until the following morning (total duration ~14 h). Fish  $\dot{M}O_2$  (mg  $O_2$  kg<sup>-1</sup> h<sup>-1</sup>) was calculated as the slope of the decline in oxygen 262

263 concentration inside the respirometers during the closed phase of the respirometry cycles. Specifically,  $\dot{M}O_2$  was calculated as: 264

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

265 266 where  $\Delta O_2$  is the rate of change of oxygen concentration inside the respirometer containing a 267 fish,  $\Delta t$  is the change in time over which the  $\Delta O_2$  was measured, and V is the volume of the 268 respirometer minus the volume of the fish (assuming 1 g displaces 1 ml of water). To 269 measure MO<sub>2MAX</sub>, fish were removed from respirometers, chased for 5 min in a circular 270 container (65 cm diameter, 10cm height), then returned to their respirometers. Background 271 respiration was measured for  $\sim 2$  h after fish were removed from respirometry chambers. This period was sufficient to produce a decline in oxygen content and gave accurate ( $r^2 > 0.92$ ) 272 273 measurements (Svendsen et al., 2015). Because we only measured background respiration at 274 the end of each respirometry trial, background respiration was assumed to be constant.  $\dot{M}O_2$ 275 were extracted and calculated using the *calc* rate function of the respR package (Harianto et 276 al., 2019) in R (R Core Team, 2018) following best practices (Clark et al., 2013). 277 MO<sub>2STANDARD</sub> was determined as the lowest 10% of MO<sub>2</sub> values during overnight 278 measurements. MO<sub>2MAX</sub> was defined as the greatest decline in oxygen measured over a 1 min 279 period. We corrected for background respiration by subtracting background MO<sub>2</sub> from an 280 animal's  $\dot{M}O_2$ . Absolute aerobic scope (AAS =  $\dot{M}O_{2MAX} - \dot{M}O_{2STANDARD}$ ) and factorial aerobic scope (FAS =  $\dot{M}O_{2MAX}/\dot{M}O_{2STANDARD}$ ) were also calculated. 281

#### 282 Thermal Sensitivity of Swimming Performance

283 Swimming performance trials were conducted in a 10 L, flow-controlled hydraulic 284 flume (Loligo, Tjele, Denmark; swimming-chamber dimensions =  $40 \times 10 \times 10$  cm;  $l \times w \times 10^{-10}$  cm;  $l \to 10^{-10}$  cm; 285 *h*). Water speeds generated by the propeller were calibrated using a Prandtl-pitot tube, as 286 describe by Kern et al. (2018). Water temperature was adjusted to test temperature using a 287 TK1000 Chiller/Heater and maintained within  $\pm 0.5^{\circ}$ C of the target temperature. Test 288 temperature order was randomised to minimise any potential confounding effects. Nitrate 289 concentrations were adjusted prior to fish introduction to reflect fish treatment group. The 290 thermal sensitivity of prolonged swimming performance  $(U_{CRIT})$  was assessed on a separate 291 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 292 293 flume and allowed to habituate to continuous flow conditions  $(0.05 \text{ m s}^{-1})$  for 1 h prior to 294 UCRIT measurement. The anterior portion of the flume was covered with black plastic to 295 encourage the fish to stay in the anterior part of the swim chamber. After the habituation

296 period, water velocity in the flume was increased to  $0.2 \text{ m s}^{-1}$  (approximately 1.5 - 2 body

lengths per second; BL s<sup>-1</sup>) and then increased incrementally every five minutes at a rate of

 $298 \quad 0.03 \text{ m s}^{-1}$  until the fish fatigued. Fatigue was defined as the time when fish were unable to

299 move off the rear screen of the swimming chamber for 10 s. Total swimming time and water

300 velocity at fatigue were recorded to calculate UCRIT using Brett's (1964) equation:

301 
$$U_{\text{CRIT}} = U_{\text{F}} + \left(\frac{1_{\text{F}}}{T_{\text{I}}}\right) U_{\text{I}}$$

where  $U_F$  is the highest water velocity maintained for the entire five-minute interval (m s<sup>-1</sup>),  $U_I$  is the water velocity increment (0.03 m s<sup>-1</sup>),  $T_F$  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<sup>-1</sup>. 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

307 blocking effects were not necessary (Bell and Terhune, 1970).

# 308 Upper thermal tolerance

309 The upper thermal tolerance of silver perch was assessed as the critical thermal 310 maximum (CT<sub>MAX</sub>) (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997). 311 CT<sub>MAX</sub> determination were conducted in a waterbath (Clayson Microprocessor Temperature 312 Controller =  $50 \times 35 \times 19$  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 313 314 allowed for the determination of CT<sub>MAX</sub> on six fish simultaneously, with minimal interaction. 315 Fasted fish (n = 12 per treatment, separate subset from those used in  $\dot{M}O_2$  and  $U_{CRIT}$ 316 measurements) were randomly selected from their holding tanks and individually placed 317 within the glass chambers, which were filled with 1000 ml of water matching their respective acclimation temperature (either 28 or  $32^{\circ}$ C) and nitrate concentration (0, 50 or 100 mg L<sup>-1</sup>). 318 319 Water within the chambers was constantly aerated by running airlines into each chamber and 320 water temperature was monitored using a YSI 85 dissolved oxygen and conductivity meter 321 (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<sup>-1</sup>. Fish were continually 322 323 observed during CT<sub>MAX</sub> trials. Loss of equilibrium (LOE), defined as the failure to maintain 324 dorsal-ventral orientation for more than 10 s, was used as the CT<sub>MAX</sub> endpoint (Lutterschmidt 325 and Hutchison, 1997). Following LOE, fish were placed in an aerated recovery tank matching 326 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<sub>MAX</sub> survival was monitored for 24 h and was high across all treatments ( $\geq$  92%).
- 329 Statistical Analyses

330 Statistical analyses were performed in the R programming environment (R Core Team, 2018) using the RStudio interface (version 1.0.153). Parametric assumptions of 331 332 normality and equal variances were tested using the Shapiro-Wilk and Levene tests, 333 respectively. Data that failed these assumptions (MO<sub>2STANDARD</sub>, MO<sub>2MAX</sub>, AAS, and FAS) 334 were log transformed before parametric analyses were undertaken. Linear mixed effects 335 models were used to test for statistical differences between nitrate and thermal acclimation 336 treatments for each of the response variables: growth, condition, MO<sub>2STANDARD</sub>, MO<sub>2MAX</sub>, 337 AAS, FAS and  $U_{CRIT}$ . The effect of test temperature on  $U_{CRIT}$  was modelled as a second-338 degree polynomial to account for the curvilinear shape of the thermal performances 339 (Angilletta, 2006) and set as a continuous variable. Nitrate concentration and acclimation 340 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 341 342 the  $U_{CRIT}$  data where total length was included as a covariate. Minimal adequate model were 343 determined using maximum likelihood (ML) simplification. The *lme* function of the *nlme* 344 package (Pinheiro et al., 2017) was used for all the aforementioned analyses. Post hoc 345 pairwise comparisons were performed using the *lsmeans* function of the *lsmeans* package 346 (Russel, 2015). Significant differences were accepted as P < 0.05. Data are presented as 347 mean  $\pm$  standard error unless otherwise stated.

#### 348 **RESULTS**

349 Body size and condition

350 Silver perch acclimated to  $32^{\circ}$ 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 = -2.22, df = 19, P = -2.22, df = 19, P = -2.22, df = -2.22351 0.04) than their counterparts acclimated to 28°C (Table 2). In fact, 28°C-acclimated fish were 352 353 53% heavier than fish acclimated to 32°C following 21 weeks of exposure to temperature treatments (Fig. 1). Nitrate exposure did not affect fish growth (50 mg L<sup>-1</sup>NO<sub>3</sub><sup>-</sup>: t = 0.70, df =354 19, P = 0.49; 100 mg L<sup>-1</sup>NO<sub>3</sub><sup>-</sup>: t = -0.30, df = 19, P = 0.77) nor condition factor (K; 50 mg L<sup>-1</sup>) 355  ${}^{1}NO_{3}^{-}$ : t = 0.86, df = 19, P = 0.40; 100 mg L ${}^{-1}NO_{3}^{-}$ : t = 0.003, df = 19, P = 0.99) and did not 356 357 interact with acclimation temperature to affect body size metrics at either nitrate concentration (SGR = 50 mg L<sup>-1</sup>NO<sub>3</sub><sup>-</sup>: t = -1.15, df = 17, P = 0.27; 100 mg L<sup>-1</sup>NO<sub>3</sub><sup>-</sup>: t = -1.33, 358

359  $df = 17, P = 0.20; K = 50 \text{ mg } L^{-1}NO_3^-: t = 1.57, df = 17, P = 0.13; 100 \text{ mg } L^{-1}NO_3^-: t = -0.82,$ 360 df = 17, P = 0.42; Table 2).

### 361 Thermal Sensitivity of Aerobic Scope

Standard oxygen uptake (MO<sub>2STANDARD</sub>) rates were significantly affected by thermal 362 363 acclimation, nitrate and test temperature treatments (Table 3). Warming from 28 – 36°C 364 resulted in an approximate 2 - 3-fold increase in  $\dot{M}O_{2STANDARD}$  across all nitrate and thermal 365 acclimation treatments (Fig. 2A, B; Table 4). However, the MO<sub>2STANDARD</sub> of 32°C-acclimated fish was, on average, significantly lower than that of fish acclimated to 28°C (regardless of 366 nitrate treatment). For control fish acclimated to 28°C, MO2STANDARD increased exponentially 367 with temperature from  $174.5 \pm 11.8$  to  $445.1 \pm 39.0$  mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>, corresponding to a 368 369 thermal sensitivity quotient (Q<sub>10</sub>) of 3.2 over an 8°C temperature range (Table 4). 370 Acclimation to 32°C significantly lowered the thermal sensitivity of MO<sub>2STANDARD</sub> over the 371 same temperature range, resulting in a Q10 of 1.9 (Table 4). Nitrate exposure increased 372 MO2STANDARD, but only in 28°C-acclimated fish (Fig. 2A, B; Table S2). In these fish, exposure to 50 mg  $L^{-1}$  NO<sub>3</sub>, but not 100 mg  $L^{-1}$  NO<sub>3</sub>, raised metabolic costs by 40.0 and 373 14.5% above control (unexposed) fish at test temperatures of 28°C and 32°C, respectively 374 375 (Fig. 2A; P > 0.05, *lsmeans*; Table S2). There was no effect of nitrate treatment at the highest 376 test temperature. The thermal sensitivity (Q10 values) of MO2STANDARD was therefore higher 377 among control (unexposed) fish than nitrate-exposed fish acclimated 28°C, especially 378 between the test temperatures of  $28 - 32^{\circ}$ C (Table 4). Conversely, Q<sub>10</sub> values were similar for 379 all fish acclimated to 32°C, regardless of nitrate treatment.

380 Maximum oxygen uptake ( $\dot{M}O_{2MAX}$ ) rates were significantly affected by thermal 381 acclimation, nitrate and test temperature treatments (Table 3). In fish from all treatments, 382 MO<sub>2MAX</sub> rose with rising temperatures (Fig. 2C, D). 32°C-acclimated fish tended to have 383 higher MO<sub>2MAX</sub> than 28°C-acclimated fish (Table 3), however, *posthoc* pairwise comparisons 384 (lsmeans) tests suggest that these differences were marginal (Fig. 2C, D; Table S3). Q10 values (Table 4) were similar across treatments; MO<sub>2MAX</sub> was more thermally sensitive 385 between  $28 - 32^{\circ}$ C and reached a plateau between  $32 - 36^{\circ}$ C as indicated by the relatively 386 low  $Q_{10}$  values 1.02 - 1.18.  $\dot{M}O_{2MAX}$  of fish was lowered by nitrate exposure, but only when 387 exposed to 100 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup> (t = -3.01, df = 20, P = 0.007). 388 389 Absolute aerobic scope (AAS) was significantly affected by thermal acclimation,

390 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
- 394 temperature  $(280.9 \pm 67.7 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1} \text{ down from } 411.1 \pm 32.8 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1} \text{ at } 28^{\circ}\text{C}).$
- 395 Overall, 28°C-acclimated fish tended to have a lower AAS than 32°C-acclimated animals
- 396 across all three test temperatures (Fig. 3A, B). Nitrate exposure, however, did not influence
- 397 AAS (Table 3). In contrast to AAS, factorial aerobic scope (FAS) saw a stepwise declined
- 398 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
- 400 higher for 32°C-acclimated fish compared with fish acclimated to 28°C. Moreover, exposure
- 401 to 50 and 100 mg  $L^{-1}NO_3^{-1}$  had a significant effect on FAS, with nitrate exposed fish tending
- 402 to have lower FAS than control animals (Fig. 3C, D; Table S5).

## 403 Thermal Sensitivity of Swimming performance

404 Exposure to nitrate lowered the prolonged swimming performance (UCRIT) of silver 405 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  $100 \text{mg L}^{-1} \text{ NO}_3^-$  resulted in poorer swimming performance (50 406 mg L<sup>-1</sup>: t = -2.21, df = 19, P = 0.04; 100 mg L<sup>-1</sup>: t = -4.13, df = 19, P < 0.001). In contrast, 407 nitrate exposure did not impact on the swimming performance of 32°C-acclimated fish (Fig. 408 4B). Test temperature had a significant influence on  $U_{CRIT}$  (t = -2.99, df = 83, P = 0.004). The 409 410 U<sub>CRIT</sub> of 28-acclimated fish was optimised at 32°C irrespective of nitrate treatment but 411 declined steeply at 36°C in all 28-acclimated animals (Table 4). Conversely, the swimming 412 performance of 32°C-acclimated fish was thermally insensitive over an eight-degree 413 temperature range  $(28 - 36^{\circ}C; Table 4)$ , but maximal performance was compromised. Fish acclimated to 32°C achieved maximal swim speeds of  $4.5 \pm 0.08$  BL s<sup>-1</sup>, while maximal 414 performance of 28°C-acclimated fish was considerably higher at  $5.3 \pm 0.16$  BL s<sup>-1</sup>. Fish body 415 416 length had marginal effects on  $U_{CRIT}$  (t = 1.89, df = 83, P = 0.06).

#### 417 *Upper thermal tolerance*

The impact of nitrate on upper thermal tolerance (CT<sub>MAX</sub>) 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<sup>-1</sup>NO<sub>3</sub><sup>-</sup> (t = 6.82, df = 17, P < 0.0001), but not 50 mg L<sup>-1</sup>NO<sub>3</sub><sup>-</sup> (t = 1.23, df = 17, P = 0.24), reduced CT<sub>MAX</sub> by 0.8°C (37.47 ± 0.06°C) relative to unexposed fish (38.31 ± 0.10°C). Conversely, CT<sub>MAX</sub> was unaffected by nitrate exposure in 32°C-acclimated animals. CT<sub>MAX</sub> was significantly affected by thermal 424 acclimation treatment (t = 6.96, df = 17, P < 0.0001) such that fish acclimated to 32°C (39.25 425  $\pm 0.08$ °C) lost equilibrium at ~1°C higher than 28°C-acclimated fish. Fish body mass did not 426 influence CT<sub>MAX</sub> (t = -2.23, df = 2, P = 0.15).

### 427 **DISCUSSION**

428 Thermal plasticity may play a key role in buffering organisms against the effects of climate 429 warming, yet, few studies have investigated whether plastic responses are altered under the 430 presence of multiple, co-occurring stressors. In accordance with our first two hypotheses, 431 juvenile silver perch exposed to nitrate and acclimated to the cooler temperature of 28°C 432 showed marked reductions in aerobic scope, swimming performance and upper thermal 433 tolerance as compared to 28°C-acclimated fish. In contrast to our hypothesis, acclimation to 434 32°C masked the effects of nitrate. Fish acclimated to 32°C displayed a thermally insensitive 435 phenotype whereby locomotor performance remained unchanged across an 8°C temperature 436 range, aerobic scope was maintained, and the upper thermal tolerance limit was increased 437 independent of nitrate exposure treatment. Together, we found that thermal acclimation 438 capabilities were not hindered by concurrent nitrate exposure suggestive of a cross-tolerance 439 interaction among these two stressors.

#### 440 Nitrate exposure causes a narrowing of aerobic scope

Exposure to elevated nitrate concentrations (both 50 and 100 mg L<sup>-1</sup>) lowered the aerobic 441 442 scope (AS) of fish acclimated to 28°C. Reductions in AS were driven by both increases in standard (MO<sub>2STANDARD</sub>) and decreases in maximum oxygen uptake (MO<sub>2MAX</sub>) of nitrate-443 444 exposed fish. This is in agreement with other studies that have found elevated resting 445 metabolic costs in response to nitrate pollution due to detoxification and cellular maintenance 446 costs (de Campos et al., 2014), as well as decreases in MO<sub>2MAX</sub> caused by the oxidation of 447 haemoglobin to methaemoglobin (Gomez Isaza et al., 2020b). Moreover, the AS of 28°C-448 acclimated fish declined with increasing test temperature due to the thermal dependence of 449 MO<sub>2STANDARD</sub> increasing at a greater rate than that of MO<sub>2MAX</sub>. A narrowing of AS has been 450 reported for various fish exposed acutely to elevated temperatures (Clark et al., 2011; Healy 451 and Schulte, 2012; Nilsson et al., 2009; Rummer et al., 2015), although it is recognised that 452 this pattern does not hold true for all species (Gomez Isaza et al., 2019; Gräns et al., 2014; 453 Poletto et al., 2017).

454 Declines in AS are hypothesised to stem from the limited ability of the cardio-455 ventilatory system to match oxygen demands at elevated temperatures (i.e. the OCLTT

456 hypothesis; Pörtner and Knust, 2007; Pörtner et al., 2004). In support of the OCLTT, we 457 found that nitrate exposure led to greater declines in AS. Such constraints on AS 458 corresponded with declines in the sustained swimming performance of silver perch at 459 elevated temperatures- indicating a compromised capacity to support aerobic functions 460 (Claireaux and Lefrançois, 2007; Pörtner and Knust, 2007). Our results are however, in 461 contrast with previous studies that have manipulated blood-oxygen carrying capacity of fish. 462 Wang et al. (2014) injected fish (European sea bass; Dicentrarchus labrax) with 463 phenylhydrazine, a haemolytic agent, to lower blood-oxygen carrying capacity. They found 464 that anaemic fish were able to compensate for a reduced blood-oxygen carrying capacity and 465 maintain  $MO_2$  at elevated temperatures via a significant increase in cardiac output. Similarly, 466 simulated anaemia (induced via the removal of 40% of blood volume) caused a small, but 467 non-significant effect on the AS of European perch (Perca fluviatilis) during an acute thermal 468 ramp (Brijs et al., 2015). However, these studies are representative of short-term anaemia 469 (hours – days) and different responses may be seen in fish with chronic anaemia. Although 470 we did not measure blood-oxygen carrying capacity in this study, chronic exposure to nitrate 471 has been shown to reduce the blood-oxygen carrying capacity of fish via a reduction in 472 haemoglobin concentration (Monsees et al., 2017; Yang et al., 2019) and a reduced blood-473 oxygen binding affinity (Gomez Isaza et al., 2020b). Indeed, carp (Cyprinus carpio) were 474 shown to make compensatory cardiorespiratory adjustments (increased ventilation rate) for 475 short periods of time following nitrite exposure but were unable to maintain these 476 compensatory adjustments past 24 h of exposure (Williams et al., 1997). Taken together, 477 previous and current findings suggest that reductions to oxygen transport capacity plays some 478 role in governing changes in AS at elevated temperatures but compensatory changes along 479 the oxygen transport cascade may be able to offset these effects.

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

481 Unlike in 28°C-acclimated fish, fish acclimated to 32°C and exposed to nitrate (either 50 or 482 100 mg L<sup>-1</sup>NO<sub>3</sub>) did not experience reductions in AS. Instead, acclimation to 32°C increased 483 AS regardless of nitrate treatment which was facilitated by positive plastic responses to 484  $\dot{M}O_{2MAX}$  across all three test temperatures. This result refutes our hypothesis that thermal 485 acclimation responses would be inhibited by nitrate exposure and is instead suggestive of a 486 cross-tolerance interaction between nitrate and high temperature acclimation. Cross-tolerance 487 interactions can occur as exposure to one stressor confers tolerance to a second stressor 488 (Sinclair et al., 2013). Studies on fish have shown that a thermal shock treatment can increase

489 tolerance of subsequent chemical (Brown et al., 1992), osmotic (Todgham et al., 2005), and 490 hypoxic challenges (Burleson and Silva, 2011). Similarly, prior exposure to zinc for example, 491 increases tolerance to subsequent exposure to other heavy metal mixtures (Brinkman and 492 Woodling, 2014; Harper et al., 2008). Selection for cross-tolerance between two 493 environmental stressors can arise in species co-adapted to both stressors or from long-term 494 acclimation to one stressors if they influence similar behavioural or physiological 495 mechanisms (Todgham et al., 2005). It is possible that physiological changes evoked by high 496 temperature acclimation, including cardiac (Gamperl and Farrell, 2004; Nyboer and 497 Chapman, 2018), respiratory (Anttila et al., 2015) or haematological (Ekström et al., 2016; 498 Valenzuela et al., 2008) adjustments, may have provided overlapping protection to elevated 499 nitrate concentrations but further research is warranted to uncover the exact mechanism 500 behind this cross-tolerance interaction.

501 The maintenance of AS across test temperatures may have allowed for the swimming 502 performance of 32°C-acclimated fish to be thermally insensitive over an 8°C temperature 503 range. As for AS, nitrate treatment had negligible impacts on the swimming performance of 504 32°C-acclimated fish which provides further indication of a cross-tolerance/protective 505 interaction between these two stressors. However, this thermal insensitivity of swimming 506 performance came at the cost of lower maximum performance. Maximum swimming performance of 32°C-acclimated fish was ~4.5 BL s<sup>-1</sup>, which is considerably lower than the 507 508 maximum swim performance of 28°C-acclimated individuals at 32°C (5.3 BL s<sup>-1</sup>). Thermal 509 acclimation can favour a widening of performance at the cost of lower maximum 510 performance, as has been observed in various fishes (Hvas et al., 2017; Rodgers et al., 2018; 511 Seebacher et al., 2015a), in a phenomenon termed the generalist-specialist trade-off (Huey 512 and Hertz, 1984; Schulte et al., 2011). The mechanisms which allow for these plastic 513 responses are not well understood, but changes in the relative proportions of muscle fibre 514 types (Hammil et al., 2004), enhancement of biochemical reaction rates (Franklin, 1998), 515 changes to muscle contractile properties (e.g. myofibrillar ATPase activity; Johnston et al., 516 1990) and cardiac remodelling (Keen and Farrell, 1994) have been documented in fish 517 following thermal acclimation. It is likely that the simultaneous remodelling of various organ 518 systems contribute to the maintenance of locomotor performance over a wide thermal range. 519 Such plasticity of sustained swimming performance likely influences the capacity of silver 520 perch to perform a myriad of behaviours across a wide range of temperatures such as feeding, 521 predator avoidance, and avoiding unfavourable conditions (Plaut, 2001; Wolter and
522 Arlinghaus, 2003) and would be beneficial as the climate warms.

523 Despite plasticity of key physiological traits (MO<sub>2MAX</sub> and swimming performance), 524 the growth performance of 32°C-acclimated silver perch was compromised. Indeed, fish at 525 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. 526 527 Thermal acclimation is an energy-intensive process that can result in physiological trade-offs 528 (Relyea, 2002) such that the energy used for the acclimation of one trait can be traded off 529 against another (Angilletta et al., 2003). Similar trade-offs have been reported for other 530 temperate fishes; for example, Atlantic halibut (*Hippoglossus hippoglossus*) acclimated to 531 various elevated temperature treatments experienced plasticity in AS but such changes did 532 not align with improvements in growth performance (Gräns et al., 2014). Similarly, the AS of 533 killifish (Fundulus heteroclitus; northern population) was optimised at 25-30°C but their 534 growth performance was compromised at these temperatures (Healy and Schulte, 2012). Poor 535 growth performance may also be related to the inability of this species to lower rates of 536 maintenance metabolism or raise food consumption at elevated temperatures such that the 537 energy available for growth was diminished (Present and Conover, 1992). Overall, our results 538 indicate that although the potential for plasticity exists for certain traits, other critical traits 539 (i.e. growth) of silver perch will likely be compromised under future climate change 540 scenarios which can limit their long-term persistence.

#### 541 Critical thermal maximum is affected by nitrate and thermal acclimation

542 Critical thermal limits provide essential baseline information on the relative ability of species 543 to cope with acute thermal spikes which are set to increase in frequency and intensity in the 544 coming decades (Hansen et al., 2006; IPCC, 2014). However, our understanding of how 545 upper thermal limits are affected by the presence of additional environmental stressors is 546 lacking, potentially leading to under- or over-estimates of species' capacity to cope with 547 thermal extremes. Contrary to our hypothesis, fish acclimated to 32°C and exposed to nitrate 548 did not experience reductions in whole animal thermal tolerance (CT<sub>MAX</sub>). This result is again 549 indicates that high temperature acclimation may provide overlapping protection to elevated 550 nitrate concentrations. However, in  $28^{\circ}$ C-acclimated fish, nitrate exposure (100 mg L<sup>-1</sup>) 551 reduced CT<sub>MAX</sub> by approximately 0.8°C. This reduction in CT<sub>MAX</sub> is attributed to the 552 oxidation of haemoglobin and a decrease in red-blood cell numbers caused by exposure to 553 nitrate (Gomez Isaza et al., 2020b). Similar effects have been shown in other fishes;

554 experimentally-induced anaemia in the European sea bass lowered CT<sub>MAX</sub> by 0.7°C (Wang et 555 al., 2014) and, across different families of Chinook salmon (Oncorhynchus tshawytscha), 556 thermal tolerance was positively associated with the oxygen-carrying capacity of blood 557 (Muñoz et al., 2018). Taken together, these results are in keeping with the prediction of the 558 OCLTT, which proposes that upper thermal limits are associated with oxygen supply 559 (Pörtner, 2010; Pörtner et al., 2017). However, a decrease of this magnitude is modest and 560 suggests that other mechanisms (e.g. protein or enzymes limitations, ion channel and neural 561 function, mitochondrial function or effects on membrane fluidity; Iftikar and Hickey, 2013; 562 Overgaard et al., 2012; Vornanen et al., 2014) on top of oxygen limitation combine to define 563 whole animal thermal limits. Overall, our CT<sub>MAX</sub> results are in keeping with those measured 564 in response to other nitrogenous waste products (e.g. nitrite; Rodgers and De Boeck, 2019) 565 and environmental pollutants (e.g. organic chemicals; Patra et al., 2007), indicating that the 566 presence of co-occurring stressors is likely to hamper species' capacity to cope with 567 heatwaves.

568 Juvenile silver perch demonstrated some degree of thermal plasticity in relation to 569 their upper thermal limit. In 32°C-acclimated fish, CT<sub>MAX</sub> was increased by ~1°C relative to 570 28°C-acclimated individuals. This change in CT<sub>MAX</sub> reflects a comparatively low degree of 571 thermal plasticity of upper thermal limits when compared to other species. Various temperate 572 fishes acclimated to  $+3 - 5^{\circ}$ C above summer temperatures have shown increases of 1.4 – 573 2.5°C in CT<sub>MAX</sub> (Akhtar et al., 2013; Das et al., 2004; Fangue et al., 2006). It is possible that 574 longer term acclimation may further shift the CT<sub>MAX</sub> of silver perch, as was shown for Nile 575 perch (Lates niloticus) (Nyboer and Chapman, 2017). However, our measures of CT<sub>MAX</sub> were 576 made following  $\sim 7-8$  weeks of exposure to acclimation treatments which is considerably 577 longer than many other thermal acclimation trials (e.g. 4 weeks: Akhtar et al., 2013; Das et 578 al., 2004; Fangue et al., 2006) indicating that further shifts in CT<sub>MAX</sub> are unlikely in this 579 species.

### 580 Ecological implications

581 The presence of various environmental stressors, including nitrate, can impact on the capacity

582 of fish to respond to warming waters and estimates ignoring these environmental

- 583 complexities are likely to over- or under-estimate species susceptibility to climate change.
- 584 This study showed the importance of considering the short- and long-term impacts of
- 585 warming on fish. The negative effects of elevated temperatures on organismal traits are
- 586 compounded by simultaneous exposure to elevated nitrate concentrations when considered on

587 a short-time scale and may prove fatal for many fishes living along inland rivers in Australia 588 which often consist of a series of pools or billabongs where temperatures can reach up to 589 40°C in summer (CSIRO and Bureau of Meteorology, 2015). However, in the long term, 590 silver perch displayed a remarkable capacity for thermal compensation, which provided 591 overlapping protection of elevated nitrate concentrations. The mechanisms that allow for this 592 overlapping protection may prove invaluable in predicting how fish may respond to the 593 combined impacts of climate warming and other stressors and should be the focus of future 594 studies. This capacity for thermal compensation was traded off against growth performance 595 which may impact on the ecological success of this species as growth performance is 596 inversely related to predation risk (Ribeiro and Qin, 2015) and provides a competitive 597 advantage for resources (Cutts et al., 1999) to juvenile fish. Collectively, this work provides 598 valuable information impact of nitrate exposure on the acclimation capacity of silver perch 599 and highlights the potential for a cross-tolerance interaction between these two stressors. 600 601 **COMPETING INTEREST** 602 The authors declare no competing interests. 603 604 **FUNDING** 605 This research received support from the Australian Government's National Environmental 606 Science Program through the Threatened Species Recovery Hub (Project 3.3.7) and a NESP 607 TSR Hub PhD support fund awarded to DFGI. DFGI was supported by an Australian 608 Government Research Training Program (RTP) Scholarship. 609 610 REFERENCES 611 Adamczewska, A. and Morris, S. (1994). Exercise in the terrestrial Christmas Island 612 red crab Gecarcoidea natalis - energetics of locomotion. J. Exp. Biol. 188, 257-274. 613 Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A. and Mahanta, P. C. (2013). 614 Thermal tolerance, oxygen consumption and haemato-biochemical variables of Tor putitora 615 juveniles acclimated to five temperatures. Fish Physiol. Biochem. 39, 1387-1398. 616 Angilletta, M. J. (2006). Estimating and comparing thermal performance curves. J. 617 Therm. Biol. 31, 541–545. Angilletta, M. J., Wilson, R. S., Navas, C. A. and James, R. S. (2003). Tradeoffs 618 619 and the evolution of thermal reaction norms. Trends Ecol. Evol. 18.

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## 948 Table 1. Experiment timeline (weeks) and body mass (g) of silver perch (*Bidyanus*

949 *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  $(0, 50 \text{ or } 100 \text{ mg L}^{-1})$  for eight weeks prior

951 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

953 performance (UCRIT), six fish per treatment were tested at three test temperatures (28, 32 or

954 36°C), totalling 18 fish. Data are presented as mean  $\pm$  standard deviation.

			28ºC-acclimated		32°C-acclimated			
Timeline (weeks)	Trait	Sample size (n)	0 mg L-1	50 mg L <sup>-1</sup>	100 mg L <sup>-1</sup>	0 mg L-1	50 mg L <sup>-1</sup>	100 mg L <sup>-1</sup>
10 - 15	Respirometry	18	$18.6\pm9.9$	$19.0\pm8.3$	$18.9\pm9.2$	$16.6\pm9.5$	$18.3\pm11.7$	$20.0\pm10.6$
8-12	$U_{\rm CRIT}$	18	$12.4\pm3.4$	$13.7\pm3.6$	$14.1\pm3.8$	$12.3\pm2.7$	$10.9\pm2.1$	$10.4\pm2.3$
$\frac{13-14}{255}$	CT <sub>MAX</sub>	12	$13.6\pm4.4$	$13.9\pm3.3$	$13.5 \pm 3.6$	$11.2 \pm 3.2$	$11.3 \pm 3.4$	$14.1 \pm 5.7$
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976Table 2. Mass and condition factor (K) of silver perch (*Bidyanus bidyanus*) used in the977experiment. Juvenile silver perch acclimated to one of two temperatures (28 or  $32^{\circ}$ C) and978exposed to one of three nitrate concentrations (0, 50 or 100 mg L<sup>-1</sup>). Abbreviations =979Condition factor, K; Specific growth rate, SGR (% d<sup>-1</sup>). See main text for calculations of K980and SGR.981

		28°C –acclimate	ed	32°C- acclimated			
Nitrate (mg L <sup>-1</sup> )	0	50	100	0	50	100	
Initial mass (g)	9.3 (± 0.2)	9.1 (± 0.2)	8.8 (± 0.5)	9.0 (± 0.4)	9.3 (± 0.2)	9.6 (± 0.2)	
Initial K	1.26 (± 0.01)	$1.24 \ (\pm \ 0.01)$	$1.24 \ (\pm \ 0.01)$	1.26 (± 0.02)	$1.29 (\pm 0.02)$	1.30 (± 0.01)	
Final mass (g)	28.7 (± 4.4)	29.9 (± 1.2)	23.1 (± 3.4)	13.4 (± 0.9)	14.5 (± 2.2)	14.5 (± 0.6)	
Final K	1.19 (± 0.02)	1.17 (± 0.02)	1.21 (± 0.02)	1.11 (± 0.02)	1.18 (± 0.03)	1.11 (± 0.05)	
SGR (% d <sup>-1</sup> )	0.3 (± 0.04)	0.3 (± 0.01)	0.3 (± 0.03)	0.2 (± 0.01)	$0.2 (\pm 0.03)$	$0.1 (\pm 0.02)$	
Survival (%)	98.0 (± 0.02)	$100 (\pm 0.00)$	$100 (\pm 0.00)$	78.4 (± 0.20)	95.3 (± 0.05)	85.1 (± 0.11)	
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# 1003 Table 3. Results of linear mixed effects (LME) models testing for differences between

# 1004 nitrate concentration, acclimation temperature, and test temperature on metabolic

1005 attributes. Results of minimum adequate models presented, with significant values in bold.

1006 Fish were acclimated to one of two temperatures (28 or 32°C) and exposed to one of three

1007 nitrate concentrations (0, 50 or 100 mg  $L^{-1}$ ). Fish were tested at three acute test temperatures

1008 (28, 32 and 36°C) treatments. Abbreviations = standard oxygen uptake,  $\dot{M}O_{2STANDARD}$ ;

1009 maximal oxygen uptake,  $\dot{M}O_{2MAX}$ ; absolute aerobic scope, AAS; factorial aerobic scope FAS.

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		Estimate	s.e.	df	<i>t</i> -value	<b>P-value</b>
MO <sub>2</sub> standard	Acclimation temperature	-0.053	0.021	20	-2.587	0.017
	Nitrate (50 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	0.062	0.025	20	2.442	0.024
	Nitrate (100 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	0.021	0.025	20	0.818	0.423
	Test temperature (32°C)	0.134	0.025	35	5.312	< 0.0001
	Test temperature (36°C)	0.283	0.025	47	11.179	< 0.0001
MO <sub>2MAX</sub>	Acclimation temperature	0.049	0.010	20	4.780	0.0001
	Nitrate (50 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	0.002	0.012	20	0.163	0.873
	Nitrate (100 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	-0.037	0.013	20	-2.921	0.008
	Test temperature (32°C)	0.047	0.013	35	3.665	0.001
	Test temperature (36°C)	0.062	0.013	47	4.871	< 0.0001
AAS	Acclimation temperature	0.170	0.035	20	4.448	0.0001
	Nitrate (50 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	-0.060	0.043	20	-1.419	0.171
	Nitrate (100 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	-0.078	0.043	20	-1.825	0.083
	Test temperature (32°C)	0.004	0.042	35	0.095	0.925
	Test temperature (36°C)	-0.016	0.042	47	-3.695	0.0006
FAS	Acclimation temperature	0.103	0.022	20	4.634	0.0002
	Nitrate (50 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	-0.060	0.027	20	-2.204	0.039
	Nitrate (100 mg L <sup>-1</sup> NO <sub>3</sub> <sup>-</sup> )	-0.058	0.027	20	-2.118	0.047
	Test temperature (32°C)	-0.087	0.027	35	-3.207	0.003
	Test temperature (36°C)	-0.221	0.027	47	-8.084	< 0.0001

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1015Table 4. Thermal sensitivity ( $Q_{10}$ ) of critical swimming performance ( $U_{CRIT}$ ), standard1016oxygen uptake ( $\dot{M}O_{2STANDARD}$ ) and maximal oxygen uptake ( $\dot{M}O_{2MAX}$ ) of silver perch1017(*Bidyanus bidyanus*). Fish were acclimated to one of two temperatures (28 or 32°C) and1018exposed to one of three nitrate concentrations (0, 50 or 100 mg L<sup>-1</sup>). Fish were tested at three1019acute test temperatures (28, 32 and 36°C) and Q<sub>10</sub> values were calculated over the entire test1020temperature range (28 and 36°C), as well as the upper (32 and 36°C) and lower (28 and102132°C) test temperatures.

	28°C-acclimated			32°C-acclimated		
Nitrate (mg L <sup>-1</sup> )	0	50	100	0	50	100
	28 - 32					
Ucrit	1.1	1.4	1.1	0.9	1.1	1.0
MO2standard	3.31	1.37	1.50	1.83	2.17	2.95
МО <sub>2MAX</sub>	1.45	1.26	1.21	1.27	1.19	1.56
	32 - 36					
Ucrit	0.6	0.5	0.5	1.0	0.7	0.9
MO2standard	3.14	2.03	2.87	2.00	2.45	1.76
МО <sub>2MAX</sub>	1.18	1.03	1.09	1.13	1.05	1.02
	28-36					
Ucrit	0.8	0.9	0.8	1.0	0.9	0.9
MO2standard	3.22	1.67	2.08	1.91	2.30	2.28
МО <sub>2MAX</sub>	1.31	1.14	1.15	1.20	1.12	1.26

1034 Figures





1036 Figure 1. Change in mass (g) over 21-weeks of exposure to experimental treatments.

1037 Silver perch (*Bidyanus bidyanus*) were acclimated to either (A) 28 or (B) 32°C and exposed

1038 to one of three nitrate concentration (0, 50 or 100 mg L<sup>-1</sup>). Data are presented as mean (dots)

1039 and shaded regions represent standard deviations.





1042 Figure 2. Metabolic responses to temperature in silver perch (*Bidyanus bidyanus*)

# 1043 exposed to factorial combination of nitrate and temperature acclimation treatments.

1044 Standard (MO<sub>2STANDARD</sub>) and maximum oxygen uptake (MO<sub>2MAX</sub>) of (A, C) 28°C and (B, D)

1045 32°C-acclimated fish, respectively. Data are presented as mean  $\pm$  s.e.m. and n = 6 fish

1046 treatment<sup>-1</sup> temperature<sup>-1</sup>.





1049 Figure 3. Aerobic scope of silver perch (*Bidyanus bidyanus*) acclimated either 28 or 32°C

1050 and exposed to one of three nitrate treatments (0, 50 or 100 mg L<sup>-1</sup>). (A) Absolute

1051 aerobic scope (AAS; mg  $O_2$  kg<sup>-1</sup> h<sup>-1</sup>) and (B) factorial aerobic scope (FAS; fold change) are

1052 presented. See main text for calculations. Data are presented as mean  $\pm$  s.e.m. and n = 6 fish

1053 treatment<sup>-1</sup> temperature<sup>-1</sup>.





1056 Figure 4. Thermal sensitivity of swimming performance ( $U_{CRIT}$  BL s<sup>-1</sup>) in silver perch

- 1057 (*Bidyanus bidyanus*) acclimated either 28 (A) or 32°C (B) and exposed to one of three
- 1058 **nitrate treatments (0, 50 or 100 mg**  $L^{-1}$ ). Data are presented as mean  $\pm$  s.e.m. and n = 6 fish
- 1059 treatment<sup>-1</sup> temperature<sup>-1</sup>.





1062 Figure 5. Effect of thermal acclimation and nitrate exposure on the critical thermal

1063 maxima (CT<sub>MAX</sub>, °C) of silver perch. Data are presented as boxplots [minimum, first

1064 quartile (Q1), median, third quartile (Q3) and maximum], and dots represent individual data

- 1065 points (n = 12). Uppercase letters represent statistical difference (P < 0.05, *lme*) between
- 1066 treatment groups.