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

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33 **ABSTRACT**

34 Exposure to nitrate is toxic to aquatic animals due to the formation of methaemoglobin and a
35 subsequent loss of blood-oxygen carrying capacity. Yet, nitrate toxicity can be modulated by
36 other stressors in the environment, such as elevated temperatures. Acclimation to elevated
37 temperatures has been shown to offset the negative effects of nitrate on whole animal
38 performance in fish, but the mechanisms underlying this cross-tolerance interaction remain
39 unclear. In this study, juvenile silver perch (*Bidyanus bidyanus*) were exposed to a factorial
40 combination of temperature (28°C or 32°C) and nitrate concentrations (0, 50 or 100 mg NO₃⁻
41 L⁻¹) treatments to test the hypothesis that thermal acclimation offsets the effects of nitrate via
42 compensatory changes to the cardiorespiratory system (gills, ventricle and blood oxygen
43 carrying capacity). Following 21 weeks of thermal acclimation, we found that fish acclimated
44 to 32°C experienced an expansion of gill surface area and an increase in ventricular thickness
45 regardless of nitrate exposure concentration. Exposure to nitrate (both 50 and 100 mg NO₃⁻ L⁻¹)
46 reduced the blood oxygen carrying capacity of silver perch due to increases in
47 methaemoglobin concentration and a right shift in oxygen-haemoglobin binding curves in fish
48 from both thermal acclimation treatment. These results indicate that plasticity of the gills and
49 ventricle of warm acclimated fish are potential mechanisms which may provide cross-tolerance
50 protection to elevated nitrate concentrations despite nitrate induced reductions to oxygen
51 transport.

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

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66 INTRODUCTION

67 Anthropogenic activities have caused rampant increases in nitrate (NO_3^-) concentration,
68 rendering nitrate a ubiquitous pollutant in freshwater ecosystems (Camargo et al., 2005).
69 Nitrate is often discharged at high levels from various anthropogenic sources, including from
70 fertilisers, intensive aquaculture, urban runoff, and atmospheric deposition (Camargo and
71 Alonso, 2006; Camargo et al., 2005). Nitrate pollution is particularly prominent in areas of
72 high fertiliser use where agricultural land practices (crop and livestock) have increased nitrate
73 concentrations in surrounding waters (Camargo et al., 2005; Glibert, 2017). For example, after
74 heavy rainfall, the use of a nitrate-based fertiliser (ammonium nitrate) caused the concentration
75 of nitrate in surface-water to spike from $2 \text{ mg NO}_3^- \text{ L}^{-1}$ to $100 \text{ mg NO}_3^- \text{ L}^{-1}$ in sites adjacent to
76 agriculture plantations (Jaynes et al., 2001). Highly urbanised areas face a similar problem; a
77 positive correlation between nitrate concentration and human population density has been
78 documented for many river systems worldwide (Gu et al., 2013; Mayo et al., 2019; Ouedraogo
79 and Vanclooster, 2016), where surface- and ground-water nitrate concentrations have been
80 recorded at persistently high levels of $\sim 25 \text{ mg NO}_3^- \text{ L}^{-1}$ and up to $100 \text{ mg NO}_3^- \text{ L}^{-1}$, respectively
81 (reviewed by Galloway et al., 2004; Vitousek et al., 1997). Elevated nitrate concentrations can
82 severely impact entire ecosystems, causing eutrophication, algal blooms, anoxic dead zones,
83 altered food webs, and nitrate toxicity to residing species (Camargo et al., 2005; Glibert, 2017).

84 High levels of nitrate pollution are toxic to aquatic animals (Camargo et al., 2005;
85 Gomez Isaza et al., 2020a). In waters high in nitrate, passive uptake of nitrate ions occurs across
86 the gill epithelium where nitrate is transferred and dissolves in the plasma (Cheng et al., 2002;
87 Jensen, 1996; Stormer et al., 1996). The main toxic action of nitrate is attributable to the
88 oxidation of haemoglobin to methaemoglobin- a molecule which is unable to reversibly bind
89 oxygen, resulting in inherent loss in blood oxygen transport capacity (Jensen, 1996, 2003;
90 Monsees et al., 2017; Yang et al., 2019). The oxygen transport capacity of nitrate exposed fish
91 is further impaired due to a right-shift in blood-oxygen binding curves (Gomez Isaza et al.,
92 2020b), which reduces oxygen uptake at the gills. Nitrate toxicity, however, can be mediated
93 by other stressors in the environment (Gomez Isaza et al., 2020a), such as low pH (Gomez
94 Isaza et al., 2018, 2020b), water hardness (Baker et al., 2017), or elevated temperatures (Egea-
95 Serrano and Van Buskirk, 2016; Opinion et al., 2020).

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

99 (Noyes et al., 2009). Yet, chronic exposure to elevated temperatures has been shown to mask
100 the effects of nitrate (Gomez Isaza et al., 2020c). Silver perch (*Bidyanus bidyanus*) acclimated
101 to 32°C were able to offset the negative effects of nitrate on aerobic scope, swimming
102 performance, and upper thermal tolerance (i.e. CT_{MAX}) when compared to nitrate-exposed fish
103 acclimated to the cooler temperature of 28°C. Similar effects were shown for nitrate-exposed
104 European grayling (*Thymallus thymallus*), whereby fish acclimated to a warmer temperature
105 (22°C) showed an increased, rather than decrease, in aerobic scope (Opinion et al., 2020). The
106 capacity for performance to be maintained in nitrate-exposed fish exposed to a warmer
107 temperature is suggestive of a cross-tolerance interaction between these two stressors, but the
108 physiological mechanisms underlying this cross-tolerance interaction are unclear.

109 Physiological remodelling of the cardio-respiratory system that occurs during thermal
110 acclimation are potential mechanisms facilitating cross-tolerance between elevated
111 temperatures and elevated nitrate concentrations. Acclimation to elevated temperatures can be
112 achieved via physiological remodelling of the cardio-respiratory system at all levels of the
113 oxygen transport cascade (gills – blood – heart – etc.). Some adjustments include
114 haematological (e.g. increase haemoglobin concentration, blood oxygen affinity; Akhtar et al.,
115 2013; Kaufman et al., 2013), cardiac (e.g. changes to the proportion of ventricle muscle types;
116 Anttila et al., 2015; Nyboer and Chapman, 2018), or remodelling of the gills (e.g. increased
117 surface area; Bowden et al., 2014; Sollid and Nilsson, 2006), all of which can facilitate blood
118 delivery to working tissues. As such, physiological adjustments that act to increase oxygen
119 delivery in response to high temperature acclimation may provide cross-tolerance protection to
120 fish experiencing nitrate-induced anaemia.

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

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

139 MATERIAL AND METHODS

140 *Animal Maintenance*

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

157 *Experimental treatments*

158 We employed a full 2 × 3 factorial design with two thermal acclimation treatments (28
159 and 32°C) and three nitrate concentrations (0, 50 and 100 mg NO₃⁻ L⁻¹). Thermal acclimation
160 treatments were chosen to reflect (i) current day summer temperatures (28°C) along the
161 northern Murray-Darling Basin where this fish occurs naturally and (ii) a high rate of climate
162 warming (32°C) forecasted under a high degree of radiative forcing (high emissions -
163 representative concentration pathway (RCP) 8.5) (CSIRO and Bureau of Meteorology, 2015).

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

175 *Heart and Gill Histology*

176 Fish (n = 6 – 9) were euthanased with an overdose of anaesthetic (250 mg L⁻¹; Aqui-S
177 TM), weighed, and gills, ventricle, and spleen were removed. Spleen and ventricles were
178 weighed fresh and relative spleen mass (RSM = spleen mass/fish body mass) and relative
179 ventricle mass (RVM = ventricle mass/fish body mass) were calculated. Ventricle and gill
180 samples were stored in zinc buffered formalin fixative (Z-fix, Anatech, MI, USA) for 24 h,
181 then transferred to 70% ethanol and stored at room temperature prior to embedding. Organ
182 samples were dehydrated through an ascending ethanol series (70% EtOH 1 h, 94% EtOH 1 h,
183 100% EtOH 3×1 h), cleared in xylene and embedded in paraffin wax (Histoplast Paraffin,
184 ThermoFisher Scientific, Sydney, Australia). Serial sections (6 µm-thick) cut with a microtome
185 (Leica RM2245, Leica Microsystems, NSW, Australia) were mounted on glass slides, de-
186 waxed with UltraClear and rehydrated with an alcohol series and distilled water. Samples were
187 stained with hematoxylin and eosin stain before being photographed (NIS-Elements software;
188 v. 4.10, Nikon Instruments Inc., Tokyo, Japan). Organ samples were measured following
189 published protocols (Anttila et al., 2015; Chapman et al., 2008; Nyboer and Chapman, 2018).
190 Briefly, ventricle thickness was determined by measuring the distance from the distal edge of
191 compact layer to the spongy myocardium. The area of compact myocardium was divided by
192 the total area (compact + spongy) of the heart section to calculate the percent of compact
193 myocardium (%CM). A series of four gill traits were selected and measured including: lamellar
194 length, lamellar width, interlamellar distance and interlamellar cell mass (ILCM).

195 *Blood carrying capacity*

196 A small blood sample (~0.2 mL) was taken from fish (n = 6 per treatment) via caudal
197 puncture using a 0.5 mL needle and 29-gauge syringe. Blood was immediately transferred to a
198 1.5 mL Eppendorf tube and stored on ice until analysis. A subsample of blood was transferred
199 into two haematocrit tubes, centrifuged (3 min at 5000 g, micro-haematocrit centrifuge;
200 Hawksley, Sussex, UK) and haematocrit (H_{CT}) was measured as the proportion of red blood
201 cells in whole blood. The remaining blood was aliquoted and used to determine haemoglobin
202 and methaemoglobin concentrations. Haemoglobin concentration ([H_B]) was determined
203 spectrophotometrically at 405 nm and quantified against a standard curve of known [H_B] using
204 a Sigma-Aldrich haemoglobin assay kit (MAK115; St Louis, USA). Mean corpuscular
205 haemoglobin concentration (MCHC) was calculated as [H_B] × 10/H_{CT}. Methaemoglobin
206 concentration ([MetH_B]) was determined by diluting 20 µL of whole blood in 1 mL of 34 mM
207 phosphate buffer at pH 7.3. The haemolysate was centrifuged (microfuge®18 centrifuge,
208 Beckman Coulter, Brea, United States) for 3 min at 12 000 g, and the absorbance (DU800
209 spectrophotometer, Beckman Coulter, Brea, United States) was measured at 560, 576 and 630
210 nm, following published protocols (Benesch et al., 1973). All assays were run in duplicate.

211 *Oxygen binding affinity*

212 Blood-oxygen binding affinities were determined using a Hemox-Analyser (Model B,
213 TCS Scientific Corp., USA) and assayed at three acute test temperatures (28, 32, and 36°C; n
214 = 6 per treatment, per temperature). A sample of 50 µL of blood was suspended in 5 mL of
215 buffered saline (Hemox™-Solution, pH 7.4), 20 µL of bovine serum albumin (BSA, additive
216 A, Hemox™) and 10 µL of an anti-foaming agent (additive B, Hemox™). Nitrogen gas
217 (compressed nitrogen pure, gas code 032, BOC, North Ryde, Australia) was used to achieve
218 zero percent saturation of haemoglobin oxygen and then air (i.e. 20.9% oxygen, compressed
219 air gas code 054) was used to obtain full saturation.

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

$$224 \quad \Delta H^\circ = 2.303 \times R \times ((\Delta \log P_{50})/(\Delta 1/T)) \text{ kJ mol}^{-1}$$

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

$$228 \quad \log Y / (100 - Y) = n \log(P_{O_2}) - n \log P_{50}$$

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

231 *Statistical analyses*

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

239 **RESULTS**

240 *Histology*

241 Fish acclimated to 32°C had significantly larger ventricles relative to body size (RVM;
242 Table 1) than those acclimated to 28°C ($t = 5.39$, $df = 19$, $P < 0.0001$). RVM was, however,
243 unaffected by nitrate concentration (50 mg NO₃⁻ L⁻¹: $t = 0.17$, $df = 19$, $P = 0.86$; 100 mg NO₃⁻
244 L⁻¹: $t = 1.38$, $df = 19$, $P = 0.18$) or the interaction between nitrate concentration and acclimation
245 temperature (50 mg NO₃⁻ L⁻¹: $t = -0.23$, $df = 17$, $P = 0.82$; mg NO₃⁻ L⁻¹: $t = -0.86$, $df = 17$, $P =$
246 0.39). An increase in RVM was accompanied by increases in compact myocardium thickness
247 ($t = 5.07$, $df = 25$, $P < 0.0001$; Fig. 1A, B) among 32°C-acclimated fish, but myocardium
248 thickness did not change with exposure to elevated nitrate concentrations (50 mg NO₃⁻ L⁻¹: $t =$
249 -0.25 , $df = 17$, $P = 0.81$; 100 mg NO₃⁻ L⁻¹: $t = -0.81$, $df = 17$, $P = 0.43$).

250 Thermal acclimation and nitrate exposure treatments influenced the respiratory surface
251 area of the gills (Fig. 2). Exposure to elevated nitrate concentrations affected the lamellar length
252 of silver perch, but the effect was dependent on thermal acclimation treatment ($F_{2, 12} = 9.62$, P
253 $= 0.003$). In 28°C-acclimated fish, exposure to elevated nitrate concentrations caused a mean
254 decrease in lamellar length of 1.5 μm and 9.5 μm in fish exposed to 50 and 100 mg NO₃⁻ L⁻¹,
255 respectively (Fig. 2C), while exposure to nitrate did not influence the lamellar length of 32°C-
256 acclimated fish. Instead, the lamellar length of 32°C-acclimated fish was increased by
257 approximately 23% to $46.7 \pm 0.6 \mu\text{m}$ regardless of nitrate-exposure treatment compared to the
258 lamellae of 28°C-acclimated fish ($37.9 \pm 1.5 \mu\text{m}$). Increases in lamellar length were largely due
259 to a 3.5 – 31.2% reduction in the height of the interlamellar cell mass (ILCM; Fig. 2F) among
260 32°C-acclimated fish ($F_{1, 14} = 6.54$, $P = 0.02$), but ILCM was unaffected by nitrate exposure

261 ($F_{2, 14} = 1.80, P = 0.20$). Fish exposed to nitrate (from both concentrations) tended to have
262 significantly wider lamellae ($F_{2, 12} = 5.10, P = 0.02$), as did fish acclimated to 32°C ($F_{1, 12} =$
263 $11.89, P = 0.044$); however, lamellar width was not affected by their interaction ($F_{2, 12} = 2.70,$
264 $P = 0.11$; Fig. 2D). The interlamellar distance of 32°C-acclimated fish was also decreased
265 compared to 28°C-acclimated fish ($F_{1, 14} = 6.03, P = 0.03$; Fig. 2E), but interlamellar distance
266 was unaffected by nitrate exposure at either acclimation temperature ($F_{2, 14} = 1.99, P = 0.17$).

267 *Blood carrying capacity*

268 Exposure to nitrate (50 and 100 mg NO₃⁻ L⁻¹) caused a stepwise decrease in
269 haemoglobin concentration ([H_B]: 50 mg NO₃⁻ L⁻¹: $t = -5.86, df = 19, P < 0.0001$; 100 mg NO₃⁻
270 L⁻¹: $t = -10.01, df = 19, P < 0.0001$), haematocrit (H_{CT}: 50 mg NO₃⁻ L⁻¹: $t = -5.49, df = 19, P <$
271 0.0001 ; 100 mg NO₃⁻ L⁻¹: $t = -6.51, df = 19, P < 0.0001$), and mean corpuscular haemoglobin
272 concentration (MCHC: 50 mg NO₃⁻ L⁻¹: $t = -1.59, df = 19, P = 0.12$; 100 mg NO₃⁻ L⁻¹: $t = -4.46,$
273 $df = 19, P < 0.001$) of silver perch from both thermal acclimation treatments (Table 1), but the
274 interaction between nitrate and acclimation treatment on these parameters was not significant
275 ([H_B]: 50 mg NO₃⁻ L⁻¹: $t = -0.33, df = 17, P = 0.75$; 100 mg NO₃⁻ L⁻¹: $t = -0.22, df = 17, P =$
276 0.83 ; H_{CT}: 50 mg NO₃⁻ L⁻¹: $t = -1.09, df = 17, P = 0.29$; 100 mg NO₃⁻ L⁻¹: $t = 0.07, df = 17, P$
277 $= 0.94$). Methaemoglobin concentrations (MetH_B) were significantly affected by the interaction
278 between nitrate and thermal acclimation treatments (50 mg NO₃⁻ L⁻¹: $t = -4.17, df = 17, P <$
279 0.001 ; 100 mg NO₃⁻ L⁻¹: $t = -4.61, df = 17, P < 0.001$). MetH_B levels increased linearly with
280 increasing nitrate concentration in fish from both acclimation treatments, but 28°C-acclimated
281 fish tended to have higher levels of MetH_B when exposed to the same nitrate concentration
282 (Table 1). As such, MetH_B of 28°C-acclimated fish were 33% and 24% higher in fish exposed
283 to 50 and 100 mg NO₃⁻ L⁻¹, respectively, than fish acclimated to 32°C.

284 *Oxygen Equilibrium Curves*

285 Test temperature caused a linear increase in the oxygen binding affinity constant,
286 P_{50} , of silver perch (32°C: $t = 2.84, df = 81, P = 0.006$; 36°C: $t = 6.83, df = 81, P < 0.0001$) and
287 P_{50} increased by about 22 – 29% over an 8°C temperature range in fish from all treatments
288 (Fig. 3A, B). Both nitrate and thermal acclimation treatment had significant effects on the P_{50}
289 of fish (50 mg NO₃⁻ L⁻¹: $t = 2.63, df = 81, P = 0.01$; 100 mg NO₃⁻ L⁻¹: $t = 2.99, df = 81, P =$
290 0.003 ; acclimation temperature: $t = 2.61, df = 21, P = 0.02$), whereas the interaction between
291 these two factors was not significant (50 mg NO₃⁻ L⁻¹: $t = 0.62, df = 19, P = 0.54$; 100 mg NO₃⁻
292 L⁻¹: $t = 0.77, df = 19, P = 0.45$). Fish acclimated to 32°C experienced higher P_{50} values than

293 28°C fish but the ΔP_{50} was similar between fish from both acclimation temperatures. Moreover,
294 the ΔP_{50} of control fish (i.e. unexposed) tended to increase more than nitrate exposed fish
295 (Table 6.2) across an 8°C temperature range (28 – 36°C). Hill coefficients (n_H) ranged between
296 0.75 – 1.99 in silver perch from all treatments. This value remained relatively constant among
297 28°C-acclimated fish across test temperatures ($\Delta n_H > 0.12$) but tended to increase in 32°C-
298 acclimated fish (Table 2). The temperature effect on blood oxygen affinity tended to increase
299 with test temperature (Table 2), as shown by increases in the van't Hoff value, ΔH° . Moreover,
300 the temperature sensitivity of oxygen binding (ΔH°) was less pronounced among nitrate
301 exposed fish than in control (unexposed) fish.

302 **DISCUSSION**

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

314 ***Plasticity of the cardiorespiratory system***

315 Silver perch acclimated to 32°C increased the morphological capacity of the gills for
316 gas exchange. This increase in gill surface area was primarily a result of reductions in the
317 interlamellar cell mass (ILCM) that increased the length of the exposed lamellae. ILCM
318 remodelling is a highly plastic trait that allows fish to quickly respond to environmental
319 stressors that increase the demand for oxygen uptake (Evans et al., 2005; Gilmore and Perry,
320 2018; Nilsson, 2007) and numerous studies have documented such changes following warm
321 temperature acclimation (Anttila et al., 2015; Bowden et al., 2014; Nyboer and Chapman, 2018;
322 Sollid and Nilsson, 2006). These plastic changes facilitated oxygen uptake at the gills of 32°C-
323 acclimated silver perch but may come at the cost of increased ion-regulatory demands and
324 increased uptake of pollutants (Evans, 1987; Gilmore and Perry, 2018).

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

339 The cardiorespiratory system of various fishes has been shown to be remarkably plastic
340 to cope with long term changes in temperature (Farrell et al., 2009; Gamperl and Farrell, 2004;
341 Keen et al., 2017). Typically, warm temperature acclimation causes a reduction in the ventricle
342 mass, but an increase in the thickness of the compact layer (Anttila et al., 2015; Gräns et al.,
343 2014; Nyboer and Chapman, 2018). Consistent with these previous findings, silver perch
344 acclimated to the warmer temperature of 32°C increased the thickness of the compact layer but
345 also had larger ventricles (RVM) relative to conspecifics acclimated to 28°C. These
346 morphological changes likely allow for an increase in cardiac output to meet the increased
347 oxygen demand of the tissues at elevated temperatures. The degree of cardiac remodelling seen
348 in silver perch was quite remarkable. The 32°C acclimated fish increased the thickness of
349 compact layer by approximately 30 – 40% compared to 28°C-acclimated fish. This indicates a
350 high degree of plasticity of the ventricle compared to other fishes. For example, the compact
351 layer of 29°C-acclimated Nile perch (*Lates niloticus*) was ~30% thicker than conspecific
352 acclimated to 25°C (Nyboer and Chapman, 2018), while rainbow trout (*Oncorhynchus mykiss*)
353 experienced a ~22% increase in the compact layer following acclimation to 17°C compared to
354 their cold-temperature acclimated (12°C) conspecifics (Klaiman et al., 2011). Increases in
355 ventricular characteristics were independent of nitrate concentration such that all fish
356 acclimated to 32°C had larger ventricles than conspecifics acclimated to 28°C. Such changes
357 to the ventricle architecture among 32°C-acclimated fish may have contributed to the

358 maintenance of cardiorespiratory performance of silver perch across an 8°C temperature range
359 (Gomez Isaza et al., 2020c) in spite of nitrate-induced reductions to oxygen transport. Indeed,
360 cardiorespiratory changes in fish have been linked to higher temperature tolerance in various
361 fishes. Cardiorespiratory capacity was thought to underlie resilience to warming temperatures
362 in pink salmon (*Oncorhynchus gorbuscha*; Clark et al., 2011) and ventricle size has been
363 positively correlated with higher temperature tolerance in populations of Atlantic Salmon
364 (Anttila et al., 2013) and landlocked salmon (*Salmo salar* m. sebago; Anttila et al., 2015)
365 indicating that cardiac plasticity may enable fish to cope with elevated temperatures. It is
366 interesting, however, that nitrate-exposed fish acclimated to 28°C were unable to responsively
367 increase RVM or ventricular thickness as ventricular plasticity has been documented in
368 response to declines in blood oxygen supply (McClelland et al., 2005; Simonot and Farrell,
369 2007). For example, rainbow trout compensated for experimentally induced anaemia (induced
370 by injections of phenylhydrazine hydrochloride) by increasing their ventricular mass. More
371 importantly, warm-acclimated (17.6°C) fish were better able to compensate for anaemic
372 conditions, with ventricular mass increasing by 28% relative to control (not injected) fish
373 versus only a 15% increase in cold-acclimated (6.4°C) fish (Simonot and Farrell, 2007). Nitrate
374 exposure, however, does not induce extreme reductions in haemoglobin, H_B (~15 – 30%
375 decrease in H_B), or haematocrit (H_{CT}) levels (~ 9 – 15% decrease in H_{CT}) so it is likely that
376 more extreme reductions, as seen in fish injected with phenylhydrazine hydrochloride (~40 –
377 83% decrease in H_B, ~60 – 84% decrease in H_{CT}; Simonot and Farrell, 2007), are required
378 before physiological changes to the RVM are triggered.

379 ***Blood carrying capacity and oxygen affinity***

380 The blood-oxygen carrying capacity of silver perch was reduced by chronic exposure to
381 elevated nitrate concentrations. H_B concentrations were reduced and methaemoglobin (MetH_B)
382 levels were increased in fish exposed to both 50 and 100 mg NO₃⁻ L⁻¹; however, levels of MetH_B
383 tended to be lower in fish acclimated to 32°C than 28°C-acclimated animals. These results
384 reflect the main toxic action of nitrate (oxidation of H_B to MetH_B; Grabda et al., 1974; Monsees
385 et al., 2017; Yang et al., 2019) and explain the decrements in aerobic scope and performance
386 seen in nitrate-exposed silver perch (Gomez Isaza et al., 2020c). Similar effects have been
387 documented for other fish; for example, MetH_B of nitrite-exposed striped catfish
388 (*Pangasianodon hypophthalmus*) were approximately 20% lower in warm-acclimated (33°C)
389 fish than fish acclimated to the cooler temperature of 27°C (Ha et al., 2019). This result is
390 suggestive of an increased efficiency of methaemoglobin reductase activity (the enzyme

391 responsible for converting MetH_B back to functional H_B inside erythrocytes; Jensen, 2003) at
392 elevated temperatures. Indeed, erythrocyte methaemoglobin reduction has been shown to be
393 highly thermally sensitive with a thermal sensitivity quotient (Q_{10}) of 2.8 in rainbow trout
394 between 15°C and 25°C (Jensen and Nielsen, 2018), and methaemoglobin reductase activity
395 levels were about 2-fold greater in warm- versus cold-acclimated striped catfish (Ha et al.,
396 2019). This high thermal sensitivity of fish methaemoglobin reductase has likely been selected
397 for to counter H_B autoxidation which is accelerated at elevated temperatures (Jensen, 2001)
398 and is a likely mechanism underlying the improved performance of nitrate-exposed fish at
399 acclimated to elevated temperatures (Gomez Isaza et al., 2020c; Opinion et al., 2020)

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

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

424 Ahmed et al., 2020; Gallagher et al., 1995), including various Australian temperate species
425 (Gomez Isaza et al., 2020b; Wells et al., 1997), and may indicate that H_{CT} levels are already
426 maximised at a level that does not compromise cardiac performance due to elevated blood
427 viscosity (Gallagher et al., 1995). There were also no changes to blood-oxygen affinity
428 following thermal acclimation. The P_{50} of silver perch averaged at 33.2 mmHg at a test
429 temperature of 28°C, which indicates a low oxygen affinity relative to other temperate fishes
430 (Du et al., 2018; Gomez Isaza et al., 2020b; Kaufman et al., 2013). P_{50} was however, thermally
431 sensitive and increased by 38% to 45.8 mmHg across an 8°C temperature range indicating a
432 reduced affinity with increases in temperature. Temperature has been recognised as a key
433 regulator of haemoglobin function because oxygen binding by the heme group is exothermic
434 (i.e. the oxygenation enthalpy, ΔH^O , is negative) and underlies the commonly observed
435 reduction in Hb-oxygen affinity with rising temperatures (Albers et al., 1983; Cech et al., 1994;
436 Powers, 1980). Most fishes typically have ΔH^O values of -15 – -50 kJ mol⁻¹ (Kaufman et al.,
437 2013; Soldatov, 2003; Verheyen et al., 1986). Silver perch red-blood cells showed this typical
438 exothermic oxygenation reaction, with a negative ΔH^O value of -38.3 (-6.1 – -67.6, range) over
439 an 8°C temperature range. This temperature effect is thought to facilitate the unloading of
440 oxygen at elevated temperatures, but temperature-induced increases in P_{50} compromises
441 oxygen binding at the gills (Soldatov, 2003), especially when coupled with other stressors like
442 nitrate exposure or environmental hypoxia. Together, these data suggest that thermal plasticity
443 of blood-oxygen carrying capacity is absent in silver perch and did not contribute to the cross-
444 tolerance protection of elevated nitrate concentrations seen in silver perch (Gomez Isaza et al.,
445 2020c).

446 **CONCLUSION**

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

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

459

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463

464 **COMPETING INTEREST**

465 The authors declare no competing interests.

466

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686 **Tables**

687 **Table 1.** Blood delivery parameters of silver perch (*Bidyanus bidyanus*) acclimated to one of
 688 two temperatures (28 or 32°C) and exposed to one of three nitrate concentrations (0, 50 or 100
 689 mg NO₃⁻ L⁻¹).

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

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711 **Table 2.** Thermodynamic effect of blood-oxygen affinity data from silver perch (*Bidyanus*
712 *bidyanus*) acclimated to one of two temperatures (28 or 32°C) and exposed to one of three
713 nitrate concentrations (0, 50 or 100 mg NO₃⁻ L⁻¹), where ΔH° is the apparent heat of
714 oxygenation, and Δn_H is the temperature dependence of Hill's cooperativity coefficient.

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

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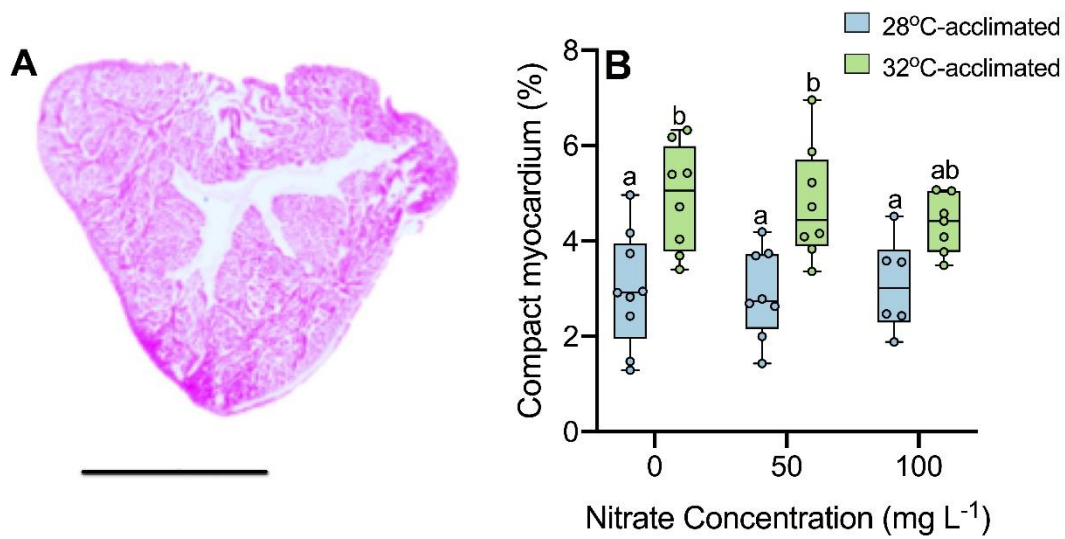
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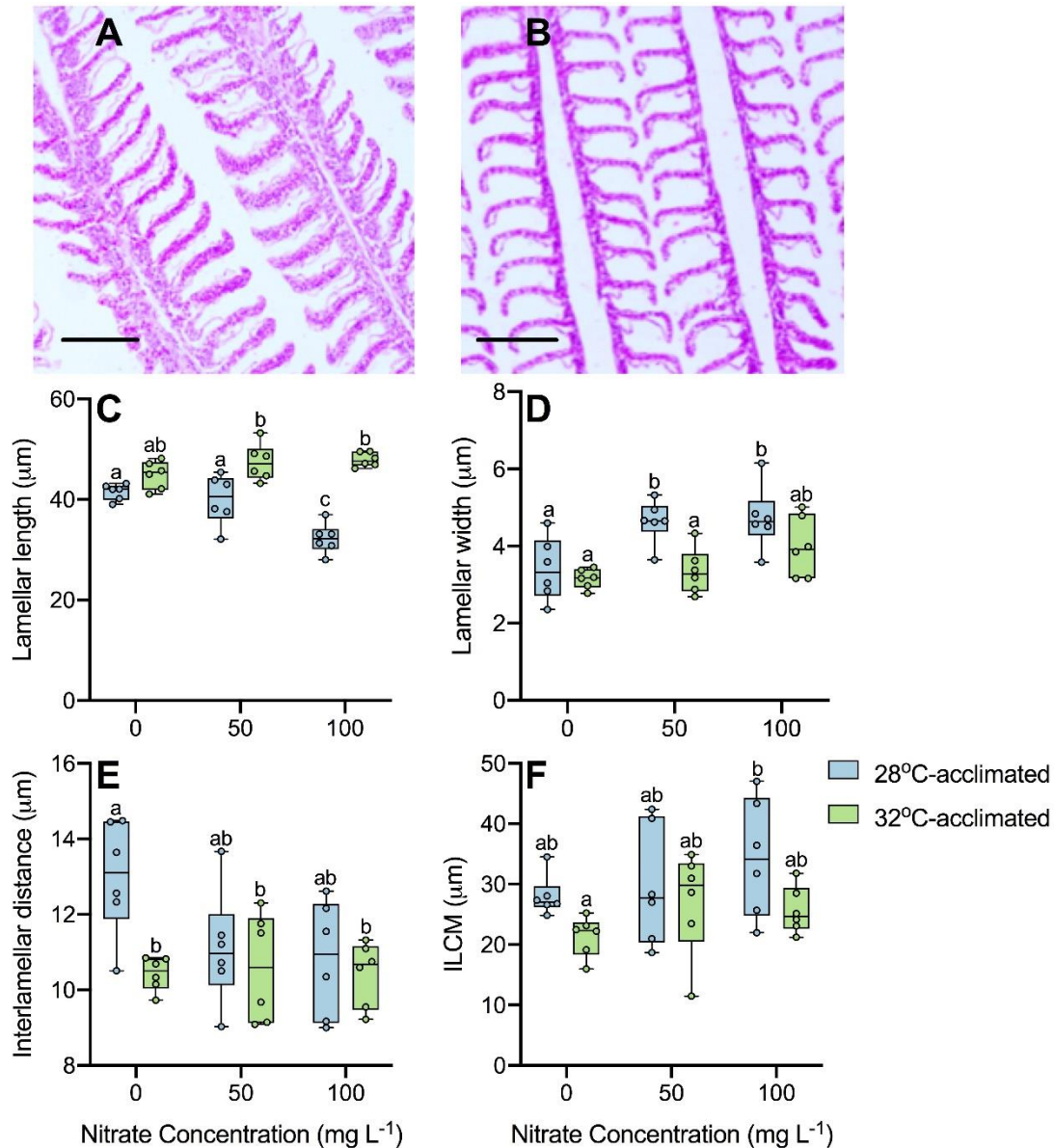
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732 **Figures:**



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734 **Figure 1.** Histological sections and measurements of the ventricle of silver perch (*Bidyanus*
735 *bidyanus*) exposed to a factorial combination of nitrate and temperature acclimation treatments.
736 (A) Representative image of a ventricle of 28°C-acclimated fish exposed to 0 mg NO₃⁻ L⁻¹, with
737 scale bar representing 100 μm. (B) Compact myocardium thickness (%) expressed as a
738 percentage of ventricle mass. Data are presented as boxplots [minimum, first quartile (Q1),
739 median, third quartile (Q3) and maximum], and dots represent individual data points (n = 6 –
740 9).

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757 **Figure 2.** Histological sections and measurements of the gill filaments of silver perch

758 (*Bidyanus bidyanus*) exposed to a factorial combination of nitrate and temperature acclimation

759 treatments. Representative images of (A) 28°C-acclimated fish exposed to 50 mg NO₃⁻ L⁻¹ and

760 (B) 32°C-acclimated fish exposed to 50 mg NO₃⁻ L⁻¹. Scale bar represents 50 μm. (C) Lamella

761 length (μm), (D) lamellar width (μm), (E) interlamellar distance (μm) and (F) interlamellar cell

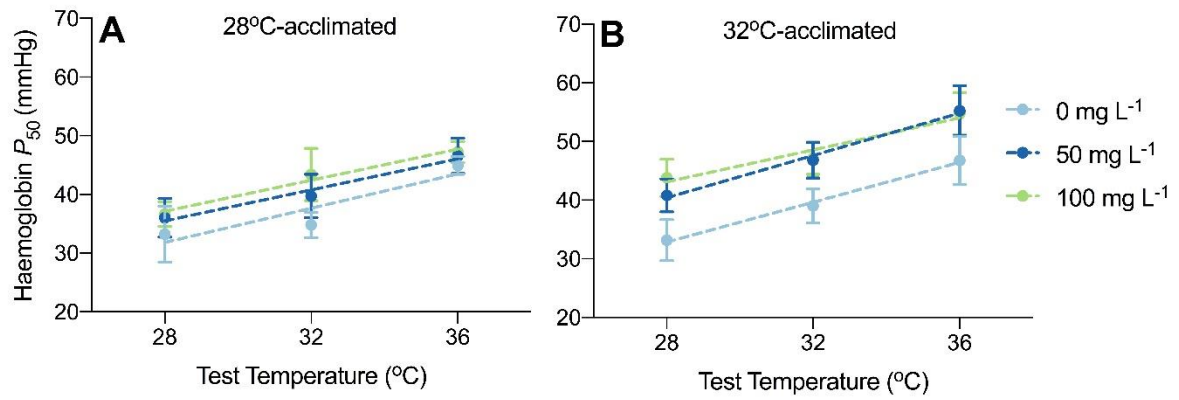
762 mass (ILCM; μm). Data are presented as boxplots [minimum, first quartile (Q1), median, third

763 quartile (Q3) and maximum], and dots represent individual data points (n = 6).

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