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SHORT COMMUNICATION

Nitrite-induced reductions in heat tolerance are independent of aerobic scope in a freshwater teleost

Essie M. Rodgers*,‡ and Gudrun De Boeck

ABSTRACT

Nitrite is a widespread form of pollution that directly lowers the blood oxygen carrying capacity of aquatically respiring species. It is unknown if this impairment of oxygen transport translates into an increased susceptibility to elevated temperatures. We hypothesised that nitrite exposure would lower blood oxygen carrying capacity and decrease both aerobic scope (maximum−standard metabolic rate) and heat tolerance. To test these hypotheses, juvenile European carp (*Cyprinus carpio*) were exposed to two levels of nitrite (0 mmol l^{-1} or 1 mmol l^{-1}) for 7 days and haematological parameters, critical thermal maxima (CT_{max}) and aerobic scope were assessed. Nitrite exposure reduced total haemoglobin by 32.9%. Aerobic scope remained unchanged in fish exposed to nitrite; however, marked declines in CT_{max} (1.2°C reduction) were observed in nitrite-exposed fish. These findings demonstrate that nitrite exposure can significantly impair heat tolerance, even when aerobic capacity is maintained.

KEY WORDS: CT_{max}, Critical thermal maxima, Thermal tolerance, Nitrite pollution, Nutrient pollution, Metabolism

INTRODUCTION

As temperatures in critical habitats continually rise, it is crucial to understand how stressors affect species' heat tolerance (Sokolova and Lannig, 2008; Rohr and Palmer, 2013). Exposure to stressors, such as pollution, can leave species in an energy-deficient state and less able to cope with additional threats like climate warming (Sokolova, 2013). Nutrient pollution is a pervasive, global stressor that is intrinsically linked to declines in the abundance of aquatic species, particularly fishes (Jenkins, 2003). Yet, the impact of nutrient pollution on organismal heat tolerance, and thus capacity to cope with climate warming is poorly understood.

Nitrite is a pervasive form of nutrient pollution that is routinely discharged into aquatic habitats from various sources, including nitrogen-based fertilisers, livestock manure, atmospheric deposits and sewage wastewater (Kroupová et al., 2018). In pristine habitats, nitrite concentrations are very low (<1 µmol l^{-1}), but concentrations in disturbed habitats can be 1000-fold higher (Kroupová et al., 2018). Elevated nitrite concentrations are also of concern in recirculating aquaculture systems, particularly during the establishment of biological filters (Hargreaves, 1998). Elevated nitrite levels in aquaculture systems can lead to compromised fish health and mass mortalities (Svobodová et al., 2005; Kroupová et al., 2018).

Freshwater fishes are particularly susceptible to nitrite pollution because they actively take up nitrite across the gills (Eddy and Williams, 1987; Jensen, 2003). As a result, internal nitrite concentrations (stored in the plasma) often far exceed that of surrounding water and can be up to 60 times higher (Fontenot et al., 1998). The most well-documented toxic action of nitrite is the induction of methaemoglobinaemia, where nitrite crosses the red blood cell membrane and causes the oxidation of functional haemoglobin (from Fe^{2+} to Fe^{3+}) to a non-oxygen-binding form, methaemoglobin (metHb; Kosaka and Tyuma, 1987; Jensen, 2007, 2009). This process induces anaemia, effectively lowering the blood oxygen carrying capacity, and under extreme conditions is expected to lead to respiratory collapse and disrupted physiological function (Jensen et al., 1987; Jensen, 2003). In general, the haematological symptoms induced by nitrite exposure include a decrease in total and functional haemoglobin concentration ([Hb]), reduced haematocrit (Hct; percentage packed red blood cell volume), erythrocyte shrinking or reduced counts, and an increase in mean corpuscular haemoglobin concentration (MCHC; mean erythrocyte [Hb]) (Witeska, 2015).

Although the haematological effects of nitrite are well understood, very little is known regarding the effects of nitrite on heat tolerance in fishes. Heat waves are increasing in frequency, intensity and duration around the world, and as such, fish are being exposed to near-lethal temperatures at unprecedented rates (Stillman, 2019). Therefore, understanding how prominent stressors, such as nitrite pollution, impact heat tolerance is a priority. Critical thermal maxima (CT_{max}) is a commonly used and repeatable method for assessing organismal upper thermal limits and is defined as the as the sublethal thermal endpoint at which the organism loses equilibrium (i.e. locomotory movements become disorganised) and cannot escape local conditions (Beitinger et al., 2000; Morgan et al., 2018). Only a single study has examined the effect of nitrite exposure on CT_{max} in fish; Watenpaugh et al. (1985) found that CT_{max} of channel catfish (*Ictalurus punctatus*) was reduced by 2°C following 24 h of exposure to 1.4 mmol l^{-1} of nitrite. The physiological mechanism underlying this reduction in heat tolerance remains unclear, and more data are urgently required to understand if this finding translates to other fishes because nitrite susceptibility, in terms of accumulation rates and toxic effects, varies greatly among species (Eddy and Williams, 1987).

Reductions in heat tolerance induced by nitrite may arise from diminished aerobic scope [difference between maximum metabolic rate and standard metabolic rate (MMR−SMR)], because thermal limits of aquatic organisms are thought to be caused by oxygen demands exceeding oxygen supply capacities (oxygen and capacity limitation of thermal tolerance hypothesis; Pörtner, 2001, 2002, cf. Jutfelt et al., 2018). Aerobic scope represents an organism's

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capacity to supply oxygen required for protective stress responses and fitness related activities (e.g. locomotion, digestion and growth) beyond maintenance costs (Fry, 1947; Sokolova et al., 2012). Reductions in aerobic scope can leave organisms in an oxygen-deficient state, where their capacity to mount a stress response to heating (or other stressors) is impaired (i.e. energy-limited stress tolerance hypothesis; Sokolova et al., 2012; Sokolova, 2013). Both a reduction in MMR and/or an increase in SMR can result in diminished aerobic scope. Nitrite is known to compromise a key component of the oxygen transport cascade: the blood oxygen carrying capacity (via the oxidation of Hb to MetHb; Kosaka and Tyuma, 1987; Jensen, 2007, 2009), which may in turn lower MMR (Lefevre et al., 2011). Aerobic scope may be further reduced by increases in SMR, because nitrite exposure can stimulate energy-demanding detoxifying mechanisms, such as the methaemoglobin reductase system (Jensen et al., 1987; Gam et al., 2017).

Despite the potentially multi-faceted effects of nitrite on heat tolerance and aerobic scope, this topic remains largely unexplored. We endeavoured to remedy this knowledge deficiency and as such, the aim of this study was to examine the effect of nitrite exposure on SMR, MMR, aerobic scope and heat tolerance in a commercially valuable, aquaculture teleost. Nitrite exposure was predicted to lower aerobic scope via a decrease in MMR and an increase in SMR. Similarly, heat tolerance was predicted to decline in nitrite-exposed fish. To test these predictions, European carp (*Cyprinus carpio*) were exposed to 0 mmol l^{-1} (control) or 1 mmol l^{-1} of nitrite, a concentration representative of a highly polluted waterway or aquaculture system prior to the establishment of a biological filtration system (Hargreaves, 1998; Kroupová et al., 2018). The effect of nitrite exposure on blood oxygen carrying capacity was gauged by measuring [Hb], Hct and calculating MCHC. Heat tolerance was measured as CT_{max}, and SMR and MMR were measured using intermittent aquatic respirometry. Together, these data were used to determine whether nitrite pollution increases the susceptibility of *C. carpio* to climate warming.

MATERIALS AND METHODS

Animal maintenance

All experimental methods complied with the Federation of European Laboratory Animal Science Associations' regulations and were approved by the University of Antwerp's local ethics committee (Permit Number: LA-1100134, Project 2018-68). Juvenile common carp (*Cyprinus carpio* Linneaus 1758; 4 months old, mixed sex) were sourced from the Agricultural University of Wageningen hatchery (The Netherlands) and transported to the University of Antwerp (Antwerp, Belgium) where they were housed in 1000 litre aquaria at 20°C for 10 months prior to experimentation. A 12 h light:12 h dark photoperiod was used, and fish were fed daily to satiety with commercial pellets (Hikari, Staple, Klundert, The Netherlands).

Experimental design

Fish (body mass: 14.05±5.76 g; total length: 9.96±1.42 cm; fork length: 8.74±1.17 cm; means±s.d.) were randomly distributed among nine 200 litre polyethylene tanks (15 fish $tank^{-1}$) and allowed a 2 week habituation period, during which temperatures were increased to 23°C at a rate of 0.5°C day^{-1}. Tanks were supplied with oxygenated, aged tap water (mmol l^{-1}: Cl^{-}=1.8, Ca^{2+}=1.4, K^{+}=0.1, Na^{+}=1.3, Mg^{2+}=0.3, HCO_3^{-}=0.5, pH 8) and temperature was maintained at 23±0.5°C using submersible heaters (200 W, Aquatic Nature, Roeselare, Belgium). Following the habituation period, tanks were randomly assigned, using a random number generator, to one of three nitrite treatments (3 tanks $treatment^{-1}$): control (0 mmol l^{-1} nitrite; 0.002±0.003 mmol l^{-1}, mean±s.d.), acute nitrite exposure (1 mmol l^{-1} for 24 h; 1.02±0.04, mean±s.d.) and chronic nitrite exposure (1 mmol l^{-1} for 7 days; 1.00±0.07 mmol l^{-1}, mean±s.d.). No mortality was observed during nitrite exposures. CT_{max} measurements were taken at two time points (after 1 day and 7 days of nitrite exposure) to test our first hypothesis, and aerobic scope measurements were taken at one time point (after 7 days of nitrite exposure) to test hypothesis 2. Tank water changes (30% replacement) occurred daily to ensure ammonia and nitrate levels were maintained at or below 0 mmol l^{-1} and 0.2 mmol l^{-1}, respectively. Nitrite concentrations were achieved by dissolving sodium nitrite ($NaNO_2$) in tank water and concentrations were checked daily using a colorimetric aquarium nitrite kit (API freshwater test kit, MARS Fishcare, Chalfont, PA, USA) that was modified for use on a 96-well plate (Edwards et al., 2006). The standard curve was prepared by serial dilution of $NaNO_2$ and ranged from 0 to 0.11 mmol l^{-1} NO_2. Tank water samples were diluted 10-fold in distilled water to ensure they fell within the standard curve. For the colorimetric assay, each well contained 200 µl of sample or 200 µl of standard and 10 µl of the test kit solution (final well volume=210 µl). The well plate was incubated at room temperature for 5 min and absorbance was subsequently read on a microplate spectrophotometer (ELx808, Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 450 nm. All tank samples, standard curve samples and inter-assay variance samples were run in triplicate. Tank sample concentrations of NO_2 were calculated using the standard curve (r^2=0.99) and correcting for the dilution factor.

Blood sampling and analyses

Fish were netted from holding tanks and euthanised with an overdose of pH-buffered tricaine methanesulfonate (MS222). Once opercular ventilations ceased, blood was sampled by severing the caudal peduncle and collecting blood into heparinised microcapillary tubes. An aliquot (~5 µl) of whole blood was placed on ice for haemoglobin concentration ([Hb], g l^{-1}) determination, and two microcapillary tubes were centrifuged at 5000 ***g*** for 3 min (micro-haematocrit centrifuge; Heraeus Christ GmbH Mikro-Hämatokrit 00912) to determine haematocrit (Hct, %). Haematocrit was calculated as the proportion of red blood cells in whole blood. A colorimetric assay kit (Sigma-Aldrich; MAK115, St Louis, MO, USA) was used to determine total [Hb] (including both functional and non-functional haemoglobin) spectrophotometrically at 405 nm, where samples were run in triplicate and quantified against a standard curve of known [Hb]. Mean corpuscular haemoglobin concentration (MCHC, g l^{-1}) was calculated as [Hb]/(Hct/100).

Aerobic scope

Intermittent respirometry (30 min measurement–30 min flush cycle) was used to measure standard oxygen uptake rates (i.e. SMR) over 14 h and SMR was calculated as the lowest 10% of recordings (excluding outliers, mean±2 s.d.), as recommended by Clark et al., (2013). Following a 24 h fasting period, individual fish were placed inside custom-built Blazka-type respirometers (volume=4.2 l, inner swim chamber dimensions: 6.0×35.0 cm, depth×length; outer tunnel: 11.0×49.0 cm, depth×length). Respirometers were surrounded by black shade material to minimise disturbance and water temperature and nitrite concentration matched the treatment conditions of each fish (i.e. 0 or 1 mmol l^{-1} nitrite at 23°C).

To obtain oxygen uptake recordings over 14 h, a flush pump attached to an automated timer was used to circulate oxygenated water through each respirometer every 30 min, followed by a 30 min

closed measurement period. Regular flushing ensured that oxygen levels were maintained above 80% saturation throughout all trials. Oxygen saturation of the water inside the respirometer was continuously recorded using a WTW oxygen sensor (Oxi 325, Denver, CO, USA) and data storage system (Oxi 3310, Denver, USA). Water flow was set to a low velocity (0.75 BL s^{-1}) to ensure adequate mixing within respirometers, without stimulating steady or burst swimming in fish.

Background microbial respiration in the absence of the fish was measured for 2 h following each trial and was subtracted from metabolic rate measurements. Metabolic rates ($\dot{M}_{O_2}$; mg O_2 h^{-1}) were calculated using Eqn 1:

$$\dot{M}_{O_2} = -1 \times \left[\frac{(m_a - m_c)}{100}\right] \times V \times \beta_{O_2}, \quad (1)$$

where m_a is the rate of change of oxygen saturation during a closed measurement period of a respirometer containing a fish (Δ% air saturation h^{-1}), m_c is the background respiration rate measured as the rate of change of oxygen saturation of a respirometer containing only water, V is the volume of the respirometer minus the volume of the fish (assuming 1 g displaces 1 ml of water) and β_{O_2} is the oxygen capacitance at the appropriate water temperature (Cameron, 1986).

An exhaustive chase protocol was used to measure MMR, as pilot studies showed that *C. carpio* did not fatigue at the fastest speeds generated by the swim tunnel respirometers. Individual fish were placed inside a cylindrical aquarium (30×60 cm, depth×height) containing well-aerated water (0.1 m deep), with temperature and nitrite levels matching treatment conditions. Fish were then chased continuously with a net for 3 min, throughout which the experimenter would lightly touch the tail of the fish if it stopped swimming. All fish repeatedly burst away from the stimulus, and were immediately transferred into a sealed swim tunnel respirometer (Loligo, Denmark, volume=5.5 liters, race-track style; swim chamber dimensions=30.0×7.5×7.5 cm, length×width×height), where measurements commenced within 10 s and continued for 15 min. Similarly to the SMR protocol, flow was set to a low velocity (0.75 BL s^{-1}) to ensure adequate mixing within the respirometer. MMR was calculated as the highest 1 min of oxygen uptake during this 15 min recording (Clark et al., 2013) and background respiration was subtracted. Absolute aerobic scope (AAS) was calculated for each fish as MMR−SMR, and factorial aerobic scope (FAS) was calculated as MMR/SMR.

Critical thermal maxima

CT_{max} were assessed in fish from all treatments (N=7–9 $treatment^{-1}$) between 07:00 h and 10:00 h. The CT_{max} test chamber was a glass aquarium (dimensions: 35.0×24.5×30.0 cm, length×width×height) where water temperature was manipulated using two submersible heaters (200 W, Aquatic Nature, Roeselare, Belgium) placed below fish chambers. Water mixing was maintained using a 5 W pump (Eheim, Deizisau, Germany) and pre-calibration ensured all fish chambers heated at identical rates (0.3°C min^{-1}). Following a 24 h fasting period, fish were individually placed inside one of three cylindrical, plastic, opaque chambers (dimensions: 11.0×20.0 cm, d×h), filled with 0.8 l of water at 23°C. Water within fish chambers was constantly aerated by running airlines attached to pipette tips (10 µl, Thermo Fisher Scientific, Waltham, USA) into each chamber. Fish were allowed a 1 h adjustment period prior to the commencement of the CT_{max} trial. Fish chamber water temperatures were monitored using a WTW probe (Oxi 3310, Denver, USA), which was calibrated against a standardised temperature probe (FRIO-Temp digital thermometer, HB Instruments, Trappe, MD, USA). Fish were observed throughout trials and loss of equilibrium (LOE) for 10 s was used as an endpoint. Following LOE, fish were immediately placed in an aerated, recovery tank at 23°C, and body mass and total length were recorded once fish had regained equilibrium. Post-trial survival was monitored for 24 h and survival was 100% for all treatments, indicating that CT_{max} were not overestimated (Beitinger et al., 2000).

Statistical analyses

Data analyses were performed in R Studio (version 3.1.3; www.Rproject.org/) using the nlme (linear and non-linear mixed effects models; https://CRAN.R-project.org/package=nlme) and multcomp (simultaneous inference in general parametric models; https://cran.r-project.org/web/packages/multcomp/index.html) packages. A series of linear mixed effects (LME) models were run to examine the effects of nitrite exposure (two-level, fixed factor) on SMR, MMR, AAS, FAS, CT_{max} and haematological parameters. Tank ID was included as a random effect and body mass as a covariate in all models. For metabolic parameters, initial models included respirometer number as a four-level fixed factor, but respirometer number was removed from final, minimal adequate models using maximum likelihood simplification. Similarly, an LME model was run to determine the effect of nitrite on CT_{max}, where body mass was included as a covariate, CT_{max} chamber as a fixed factor and tank ID as a random effect. CT_{max} chamber number was excluded from final adequate model using maximum likelihood simplification. Three LME models were run to determine the effect of nitrite exposure on Hb, Hct and MCHC; tank ID was included as a random effect and body mass as a covariate in all models. Assumptions of homoscedasticity and normality of errors were graphically checked, and response variables were log_{10}-transformed where necessary. Statistical significance was accepted at P<0.05.

RESULTS AND DISCUSSION

Nitrite-induced anaemia is typical in freshwater fishes (Witeska, 2015). We observed a marked decline in the central determinant of blood oxygen carrying capacity; total [Hb] decreased by 32.9% following 7 days of nitrite exposure (Table 1, $F_{3,14}$=4.19, P<0.05, LME). Hct remained unchanged in nitrite-exposed fish (Table 1, $F_{1,12}$=0.01, P=0.91, LME) and this together with a decrease in total [Hb], resulted in substantial reductions (45.4%) in MCHC (Table 1, $F_{3,12}$=4.87, P<0.0.5, LME). Nitrite exposure typically causes increases in MCHC as a result of erythrocyte shrinkage, but our observed decrease reflects lowered [Hb] within erythrocytes. Woo and Chiu (1995) observed similar findings, where 8 days of exposure to 0.2 mmol l^{-1} nitrite caused a 38.9% decrease in MCHC in *Lates calcarifer*. As expected, nitrite exposure caused a marked decline in CT_{max} (1.2°C decline), but lowered blood oxygen carrying capacity did not affect metabolic attributes (SMR, MMR and aerobic scope).

SMR of *C. carpio* remained unchanged in nitrite-exposed fish (Fig. 1A; $F_{1,4}$=0.70, P=0.44, LME), suggesting that detoxifying mechanisms were either not upregulated or their associated costs were negligible. Similar findings have been reported in other fishes, where routine metabolic rates of both striped catfish (*Pangasianodon hypophthalmus*) and zebrafish (*Danio rerio*) were unaffected by nitrite exposure (0.9 mmol l^{-1} and 2.0 mmol l^{-1}), despite 62–71% reductions in functional haemoglobin (Jensen, 2007; Lefevre et al., 2011). MMR was also independent of nitrite exposure in *C. carpio*

Table 1. Haematological parameters of juvenile European carp (*Cyprinus carpio*) following 1 day and 7 days of exposure to two concentrations of nitrite (0 mmol l^{-1} or 1 mmol l^{-1})

	0 mmol l^{-1}		1 mmol l^{-1}	
	1 day	7 days	1 day	7 days
Hct (%)	36.1±0.8 (6)	36.4±2.6 (5)	33.8±0.9 (7)	33.6±0.9 (7)
[Hb] (g l^{-1})	76.8±6.8 (6)	81.8±3.1 (5)	79.4±8.1 (6)	53.3±5.3* (7)
MCHC (g l^{-1})	214.6±21.9 (6)	228.5±15.3 (5)	259.7±30.9 (5)	141.9±8.9* (6)

Values are means±s.e.m. and sample sizes (individual fish) are shown in brackets. Asterisks denote a significant difference between treatment groups of the same exposure duration. Hct, haematocrit; [Hb], total haemoglobin concentration; MCHC, mean corpuscular haemoglobin concentration.

(Fig. 1B; $F_{1,4}$=0.05, P=0.83, lme), and was approximately 4-fold higher than SMR, which is comparable to values in other teleosts at similar temperatures (Malekpouri et al., 2016; Marras et al., 2010; Zeng et al., 2010).

The independence of SMR and MMR of nitrite exposure translated into both absolute and factorial aerobic scope remaining unchanged (Fig. 1C,D; AAS $F_{1,4}$=0.00, P=0.97, lme; FAS $F_{1,4}$=0.00, P=0.96, lme). This was unexpected, particularly in light of the observed reduction in [Hb] and probable increase in MetHb content. Previous studies have manipulated blood oxygen carrying capacity by inducing anaemia in fish via blood withdrawal or intraperitoneal injection of the haemolytic agent phenylhydrazine (Simonot and Farrell, 2007; Brijs et al., 2015). These studies showed similar results, where anaemic fish were able to maintain aerobic scope. For example, European perch (*Perca fluviatilis*) were able to maintain aerobic scope despite 43% and 50% reductions in [Hb] and Hct, respectively (Brijs et al., 2015). There is mounting evidence to show that many teleosts can compensate for lowered blood oxygen carrying capacity by altering other components of the cardiorespiratory system, including ventilation rates, cardiac output and tissue oxygen extraction efficiency (Cameron and Davis, 1970; Simonot and Farrell, 2007; Wang et al., 2014). For example, *C. carpio* exposed to 1 mmol l^{-1} nitrite increased their ventilatory response by 2–3 breaths min^{-1}, which may have been sufficient to maintain resting aerobic capacity (Williams et al., 1997). Similarly, European sea bass (*Dicentrarchus labrax*) were able to compensate for a 52% reduction in Hct by increasing their cardiac output (Wang et al., 2014). Although not measured directly here, *C. carpio* likely compensated for the anaemic effects of nitrite by increasing ventilatory rates and cardiac output, so that both SMR and MMR were maintained, respectively.

Despite aerobic scope remaining unchanged with nitrite exposure, a marked reduction in critical thermal maxima (CT_{max}) was observed in fish exposed to nitrite for 7 days (Fig. 2; $F_{3,21}$=3.67,

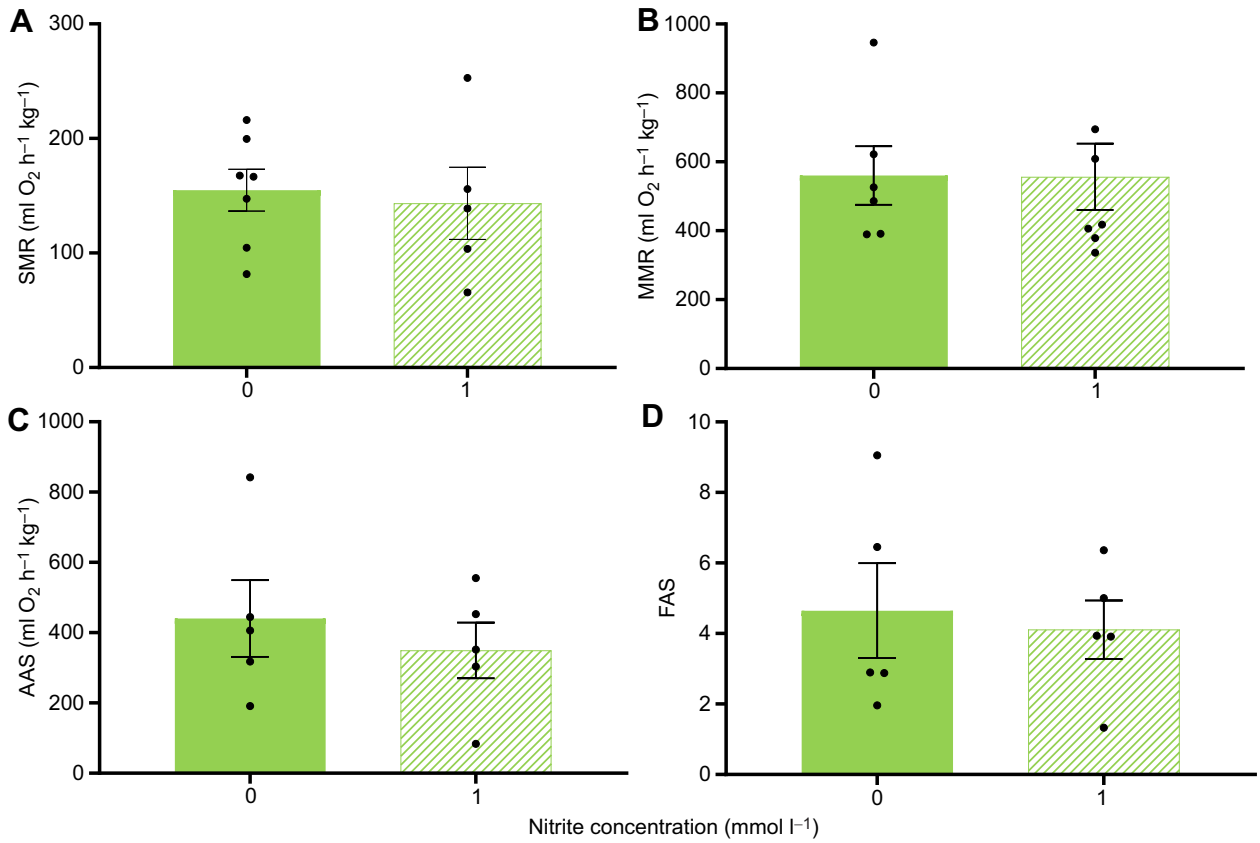

Fig. 1. Effect of 7 days of nitrite exposure on oxygen uptake rates in juvenile European carp (*Cyprinus carpio*). (A) Standard metabolic rate (SMR; 0 mmol l^{-1} NO_2^-, N=7; 1 mmol l^{-1} NO_2^-, N=5). (B) Maximum metabolic rate (MMR; 0 mmol l^{-1} NO_2^-, N=6; 1 mmol l^{-1} NO_2^-, N=7). (C) Absolute aerobic scope (AAS; 0 mmol l^{-1} NO_2^-, N=5; 1 mmol l^{-1} NO_2^-, N=5). (D) Factorial aerobic scope (FAS; 0 mmol l^{-1} NO_2^-, N=5; 1 mmol l^{-1} NO_2^-, N=5). All variables were independent of nitrite exposure (P>0.40, linear mixed effects models). Data are means±s.e.m. and raw data points are overlaid.

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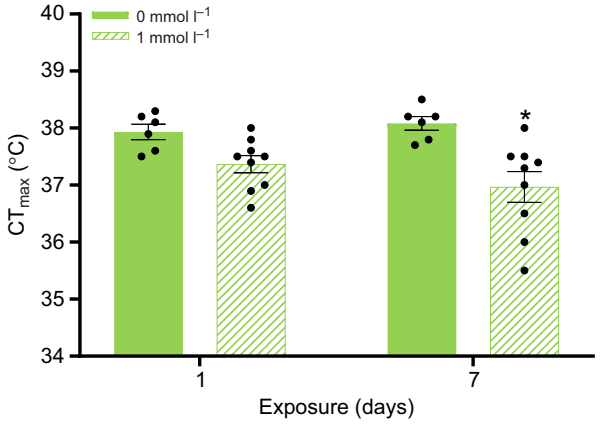

Fig. 2. Effect of 1 and 7 days of nitrite exposure on critical thermal maxima (CT_{max}) in juvenile European carp (*Cyprinus carpio*). CT_{max} remained unchanged following 1 day of nitrite exposure (P=0.11; 0 mmol l^{-1} NO_2^-; N=6; 1 mmol l^{-1} NO_2^-, N=9; linear mixed effects model, LME), but was significantly reduced after 7 days of exposure (*P<0.05, $F_{3,21}$=3.67; 0 mmol l^{-1} NO_2^-, N=6; 1 mmol l^{-1} NO_2^-, N=9; LME). Data are means±s.e.m. and raw data points are overlaid.

P<0.05, lme). CT_{max} was 1.2°C lower in nitrite-exposed fish (36.9± 0.3°C, mean±s.e.m.) compared with control fish (38.1±0.1°C, mean ±s.e.m.), and the effect of nitrite was temporally sensitive, with no effect of nitrite evident following 1 day of exposure (Fig. 2; P=0.11, LME). The temporal sensitivity of this effect aligns closely with prior findings, which showed that *C. carpio* can compensate for the development of methaemoglobinaemia via cardiorespiratory adjustments (increased ventilation rate) for short periods of time but cannot maintain these compensatory adjustments past 24 h of exposure (Williams et al., 1997). Nitrite-induced reductions in CT_{max} have only been shown twice previously; Alcaraz et al. (1997) found that nitrite exposure (1.1–2.2 mmol l^{-1}) reduced CT_{max} of white shrimp (*Penaeus setiferus*) by 2–3°C, and Watenpaugh et al. (1985) found that CT_{max} of channel catfish (*Ictalurus punctatus*) declined by 2°C following exposure to 1.4 mmol l^{-1} nitrite. However, both studies only examined the effects of acute nitrite exposure (24 h), and aerobic scope was not assessed.

Studies examining the relationship between blood oxygen carrying capacity and CT_{max} have returned mixed findings (Beers and Sidell, 2011; Wang et al., 2014; Brijs et al., 2015), but many have reported CT_{max} to be independent of [Hb] and Hct. Our study shows that manipulations of blood oxygen carrying capacity via nitrite exposure reduced CT_{max} but had no effect on aerobic scope. Given that nitrite-exposed fish were able to maintain aerobic scope, it is unlikely that limitation in oxygen supply is the sole, causal mechanism determining acute heat tolerance. This highlights the ongoing demand to identify alternative or co-occurring mechanisms that determine the acute thermal tolerance of ectotherms. For instance, nitrite exposure may accelerate the onset of nervous system dysfunction underlying loss of equilibrium at elevated temperatures, because nitrite is known to degrade organelle membrane integrity and tissue hypoxia is thought to exacerbate this rate of degradation (Inoue, 1978; Mensi et al., 1982).

Our findings contrast to that of Lefevre et al. (2011), where *P. hypophthalmus* exposed to 0.9 mmol l^{-1} for 2 days displayed a 40% decrease in MMR, and a corresponding decline in absolute aerobic scope. Disparities between these findings likely reflect differences between water chloride concentrations ([Cl^-]). Water [Cl^-] in the Lefevre et al. (2011) study was very low (0.3 mmol l^{-1}) compared with the present study (1.8 mmol l^{-1}), and it is well established that nitrite toxicity is inversely related to water [Cl^-] (Lewis and Morris, 1986). Since Cl^- and nitrite compete for the same uptake route in fish, high water [Cl^-] plays a protective role and aerobic scope of *C. carpio* may have been reduced by nitrite if water [Cl^-] was lower. Nonetheless, our water Cl^- concentrations were representative of many freshwater habitats near human development (Todd and Kaltenecker, 2012). Disparities among findings may also partly reflect species-specific differences and age/size effects. For example, although *P. hypophthalmus* is more tolerant of nitrite than cold-water salmonid species, its 96 h LC_{50} is 1.25 mmol l^{-1} lower than that of *C. carpio* (Lewis and Morris, 1986; Lefevre et al., 2011). As a cyprinid, *C. carpio* is more resilient to nitrite exposure than many freshwater fishes, particularly salmonids, which typically have 96 h LC_{50} values ≤0.5 mmol l^{-1} (Lewis and Morris, 1986). Therefore, nitrite-induced reductions in heat tolerance are likely to be more pronounced in less-resistant species like salmonids, particularly in waters with low [Cl^-].

The biological significance of a 1.2°C reduction in heat tolerance cannot be overestimated, particularly in light of rapid climate warming. Reports of mass fish mortalities during heat waves are growing in frequency, but the potential links between nutrient pollution and heat tolerance have been largely overlooked (Stillman, 2019). Nitrite pollution threatens to significantly narrow thermal safety margins (CT_{max}−environmental temperatures) of both wild and commercially reared freshwater fishes. Although our results suggest that transient increases (≤24 h) in nitrite have little to no effect on heat tolerance in *C. carpio,* chronic exposure increases their susceptibility to thermal stress. Ensuring high water quality in freshwater habitats and aquaculture systems should be a priority, particularly as rising temperatures threaten the persistence of both wild and commercial fish stocks.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.M.R., G.D.B.; Methodology: E.M.R., G.D.B.; Software: E.M.R.; Validation: E.M.R.; Formal analysis: E.M.R.; Investigation: E.M.R.; Resources: G.D.B.; Data curation: E.M.R.; Writing - original draft: E.M.R.; Writing - review & editing: E.M.R., G.D.B.; Visualization: E.M.R.; Supervision: G.D.B.; Project administration: E.M.R., G.D.B.; Funding acquisition: E.M.R., G.D.B.

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References
Alcaraz, G., Chiappa-Carrara, X. and Vanegas, C. (1997). Temperature tolerance of *Penaeus setiferus* postlarvae exposed to ammonia and nitrite. *Aquat. Toxicol.* **39**, 345-353. doi:10.1016/S0166-445X(96)00852-1

Beers, J. M. and Sidell, B. D. (2011). Thermal tolerance of Antarctic Notothenioid fishes correlates with level of circulating hemoglobin. *Physiol. Biochem. Zool.* **84**, 353-362. doi:10.1086/660191

Beitinger, T. L., Bennett, W. A. and McCauley, R. W. (2000). Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ. Biol. Fish.* **58**, 237-275. doi:10.1023/A:1007676325825

Brijs, J., Jutfelt, F., Clark, T. D., Grans, A., Ekstrom, A. and Sandblom, E. (2015). Experimental manipulations of tissue oxygen supply do not affect warming tolerance of European perch. *J. Exp. Biol.* **218**, 2448-2454. doi:10.1242/jeb.121889

Cameron, J. N. (1986). *Principles of Physiological Measurement*. London, UK: Academic Press Inc.

Cameron, J. N. and Davis, J. C. (1970). Gas exchange in rainbow trout (*Salmo gairdneri*) with varying blood oxygen capacity. *J. Fish. Res. Board Can.* **27**, 1069-1085. doi:10.1139/f70-123

Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782. doi:10.1242/jeb.084251
Eddy, F. B. and Williams, E. M. (1987). Nitrite and freshwater fish. *Chem. Ecol.* **3**, 1-38. doi:10.1080/02757548708070832
Edwards, T. M., McCoy, K. A., Barbeau, T., McCoy, M. W., Thro, J. M. and Guillette, L. J., Jr. (2006). Environmental context determines nitrate toxicity in Southern toad (*Bufo terrestris*) tadpoles. *Aquat. Toxicol.* **78**, 50-58. doi:10.1016/j.aquatox.2006.02.003
Fontenot, Q. C., Isely, J. J. and Tomasso, J. R. (1998). Acute toxicity of ammonia and nitrite to shortnose sturgeon fingerlings. *Prog. Fish Cult.* **60**, 315-318. doi:10.1577/1548-8640(1998)060<0315:ATOAAN>2.0.CO;2
Fry, F. J. (1947). *Effects of the Environment on Animal Activity. Publications of the Ontario Fisheries Research Laboratory No. 68*, pp. 1-60. Toronto, ON: The University of Toronto Press.
Gam, L. T. H., Jensen, F. B., Damsgaard, C., Huong, D. T. T., Phuong, N. T. and Bayley, M. (2017). Extreme nitrite tolerance in the clown knifefish *Chitala ornata* is linked to up-regulation of methaemoglobin reductase activity. *Aquat. Toxicol.* **187**, 9-17. doi:10.1016/j.aquatox.2017.03.013
Hargreaves, J. A. (1998). Nitrogen biogeochemistry of aquaculture ponds. *Aquaculture.* **166**, 181-212. doi:10.1016/S0044-8486(98)00298-1
Inoue, K. (1978). Effects of sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) on the mitochondrial membrane. *Okayarea Igakkai Zasshi* **90**, 257-263. doi:10.4044/joma1947.90.3-4_257
Jenkins, M. (2003). Prospects of biodiversity. *Science* **302**, 1175-1177. doi:10.1126/science.1088666
Jensen, F. B. (2003). Nitrite disrupts multiple physiological functions in aquatic animals. *Comp. Biochem. Physiol. A* **135**, 9-24. doi:10.1016/S1095-6433(02)00323-9
Jensen, F. B. (2007). Nitric oxide formation from nitrite in zebrafish. *J. Exp. Biol.* **210**, 3387-3394. doi:10.1242/jeb.008748
Jensen, F. B. (2009). The role of nitrite in nitric oxide homeostasis: a comparative perspective. *Biochim. Biophys. Acta. Bioenerget.* **1787**, 841-848. doi:10.1016/j.bbabio.2009.02.010
Jensen, F. B., Andersen, N. A. and Heisler, N. (1987). Effects of nitrite exposure on blood respiratory properties, acid–base and electrolyte regulation in the carp (*Cyprinus carpio*). *J. Comp. Physiol. B* **157**, 533-541. doi:10.1007/BF00700972
Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D. J., Lefevre, S., Nilsson, G. E., Metcalfe, N. B., Hickey, A. J. R. et al. (2018). Oxygen- and capacity-limited thermal tolerance: blurring ecology and physiology. *J. Exp. Biol.* **221**, 169615. doi:10.1242/jeb.169615
Kosaka, H. Tyuma, I. (1987). Mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite. *Environ. Health Persp.* **73**, 147-151. doi:10.1289/ehp.8773147
Kroupová, K. H., Valentová, O., Svobodová, Z., Šauer, P. and Máchová, J. (2018). Toxic effects of nitrite on freshwater organisms: a review. *Revs. Aquacult.* **10**, 525-542. doi:10.1111/raq.12184
Lefevre, S., Jensen, F. B., Huong, D. T. T., Wang, T., Phuong, N. T. and Bayley, M. (2011). Effects of nitrite exposure on functional haemoglobin levels, bimodal respiration, and swimming performance in the facultative air-breathing fish *Pangasianodon hypophthalmus*. *Aquat. Toxicol.* **104**, 86-93. doi:10.1016/j.aquatox.2011.03.019
Lewis, W. M. and Morris, D. P. (1986). Toxicity of nitrite to fish—a review. *Trans. Am. Fish. Soc.* **115**, 183-195. doi:10.1577/1548-8659(1986)115<183:TONTF>2.0.CO;2
Malekpouri, P., Peyghan, R., Mahboobi-Soofiani, N. and Mohammadian, B. (2016). Metabolic capacities of common carp (*Cyprinus carpio*) following combined exposures to copper and environmental hypoxia. *Ecotox. Environ. Saf.* **127**, 1-11. doi:10.1016/j.ecoenv.2016.01.004
Marras, S., Claireaux, G., McKenzie, D. J. and Nelson, J. A. (2010). Individual variation and repeatability in aerobic and anaerobic swimming performance of European sea bass, *Dicentrarchus labrax*. *J. Exp. Biol.* **213**, 26-32. doi:10.1242/jeb.032136
Mensi, P., Arillo, C., Margioccoa, N. D. and Schenone, O. (1982). Lysosomal damage under nitrite intoxication in rainbow trout (*Salmo gairdneri* Rich.). *Comp. Biochem. Physiol. C* **73**, 161-165. doi:10.1016/0306-4492(82)90185-X
Morgan, R., Finnøen, M. H. and Jutfelt, F. (2018). CTmax is repeatable and doesn't reduce growth in zebrafish. *Sci. Rep.* **8**, 7099. doi:10.1038/s41598-018-25593-4
Pörtner, H. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146. doi:10.1007/s001140100216
Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. A* **132**, 739-761. doi:10.1016/S1095-6433(02)00045-4
Rohr, J. R. and Palmer, B. D. (2013). Climate change, multiple stressors, and the decline of ectotherms. *Conserv. Biol.* **27**, 741-751. doi:10.1111/cobi.12086
Simonot, D. L. and Farrell, A. P. (2007). Cardiac remodelling in rainbow trout (Oncorhynchus mykiss Walbaum) in response to phenylhydrazine-induced anaemia. *J. Exp. Biol.* **210**, 2574-2584. doi:10.1242/jeb.004028
Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integr. Comp. Biol.* **53**, 597-608. doi:10.1093/icb/ict028
Sokolova, I. M. and Lannig, G. (2008). Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. *Clim. Res.* **37**, 181-201. doi:10.3354/cr00764
Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar. Environ. Res.* **79**, 1-15. doi:10.1016/j.marenvres.2012.04.003
Stillman, J. H. (2019). Heat waves, the new normal: summertime temperature extremes will impact animals, ecosystems, and human communities. *Physiology* **34**, 86. doi:10.1152/physiol.00040.2018
Svobodová, Z., Máchová, J., Poleszczuk, G., Hůda, J., Hamáčková, J. and Kroupová, H. (2005). Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. *Acta. Vet. Brno.* **74**, 129-137. doi:10.2754/avb200574010129
Todd, A. K. and Kaltenecker, M. G. (2012). Warm season chloride concentrations in stream habitats of freshwater mussel species at risk. *Environ. Pollut.* **171**, 199-206. doi:10.1016/j.envpol.2012.07.040
Wang, T., Lefevre, S., Iversen, N. K., Findorf, I., Buchanan, R. and McKenzie, D. J. (2014). Anaemia only causes a small reduction in the upper critical temperature of sea bass: is oxygen delivery the limiting factor for tolerance of acute warming in fishes? *J. Exp. Biol.* **217**, 4275-4278. doi:10.1242/jeb.104166
Watenpaugh, D. E., Beitinger, T. L. and Huey, D. W. (1985). Temperature tolerance of nitrite-exposed channel catfish. *Trans. Am. Fish. Soc.* **114**, 274-278. doi:10.1577/1548-8659(1985)114<274:TTONCC>2.0.CO;2
Williams, E. M., Nelson, J. A. and Heisler, N. (1997). Cardio-respiratory function in carp exposed to environmental nitrite. *J. Fish Biol.* **50**, 137-149. doi:10.1111/j.1095-8649.1997.tb01346.x
Witeska, M. (2015). Anemia in teleost fishes. *B. Eur. Assoc. Fish Pat.* **35**, 148-160.
Woo, N. Y. S. and Chiu, S. F. (1995). Effect of nitrite exposure on hematological parameters and blood respiratory properties in the sea bass *Lates calcarifer*. *Environ. Toxic. Water* **10**, 259-266. doi:10.1002/tox.2530100405
Zeng, L.-Q., Zhang, Y.-G., Cao, Z.-D. and Fu, S.-J. (2010). Effect of temperature on excess postexercise oxygen consumption in juvenile southern catfish (*Silurus meridionalis* Chen) following exhaustive exercise. *Fish Physiol. Biochem.* **36**, 1243-1252. doi:10.1007/s10695-010-9404-9