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Synergism between elevated temperature and nitrate: Impact on aerobic capacity of European grayling, *Thymallusthymallus* in warm, eutrophic waters

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ABSTRACT

Climate warming and nitrate pollution are pervasive aquatic stressors that endanger the persistence of fishes prevailing in anthropogenically disturbed habitats. Individually, elevated nitrate and temperature can influence fish energy homeostasis by increasing maintenance costs and impairing oxygen transport capacity. However, it remains unknown how fish respond to simultaneous exposure to elevated temperature and nitrate pollution. Hence, we examined the combined effects of nitrate and elevated temperatures on aerobic scope (AS, maximum-standard metabolic rates) and cardiorespiratory attributes (haemoglobin H_B, haematocrit H_{CT}, relative ventricle mass RVM, and somatic spleen index SSI) in a freshwater salmonid, *Thymallus thymallus*. A 3 × 2 factorial design was used, where fish were exposed to one of three ecologically relevant levels of nitrate (0, 50, or 200 mg NO_3^- l^{-1}) and one of two temperatures (18 °C or 22 °C) for 6 weeks. Elevated temperature increased AS by 36 % and the improvement was stronger when coupled with nitrate exposure, indicating a positive synergistic interaction. H_B was reduced by nitrate exposure, while H_{CT} was independent of nitrate pollution and temperature. Stressor exposure induced remodeling of key elements of the cardiorespiratory system. RVM was 39 % higher in fish exposed to 22 °C compared to 18 °C but was independent of nitrate exposure. SSI was independent of temperature but was 85 % and 57 % higher in fish exposed to 50 and 200 mg NO_3^- l^{-1}, respectively. Taken together, these results highlight that simultaneous exposure to elevated temperatures and nitrate pollution offers cross-tolerance benefits, which may be underscored by cardiorespiratory remodeling.

1. Introduction

Anthropogenic impacts on the global environment are now so ubiquitous and multi-faceted that single-factor studies hold little predictive power (Jackson et al., 2015; Reid et al., 2018). To survive, species must navigate a complex milieu of stressors, which often interact in complex ways (Reid et al., 2018; Todgham and Stillman, 2013). Climate warming may exacerbate the negative effects of stressors already threatening species. Alternatively, climate warming may stimulate protective responses, which enable organisms to develop increased resilience to other stressors; a phenomenon termed cross-tolerance (Sinclair et al., 2013). Despite the complexities of stressor interactions, understanding how species respond to co-occurring stressors is one of the largest knowledge deficiencies in ecological conservation (Darling and Côté, 2008).

Nutrient pollution is one of the most pervasive global stressors. Anthropogenic activities have significantly altered the global nitrogen cycle by increasing the availability and mobility of nitrogen on the planet (Galloway et al., 2004; Vitousek et al., 1997). Among the forms of nitrogen in water, nitrate (NO_3^-) occurs at relatively higher concentration in most aquatic systems (Camargo et al., 2005; Camargo and Alonso, 2006; Durand et al., 2011). Accumulation of nitrate at levels beyond the acceptable limit set between 45–50 mg NO_3^- l^{-1} as prescribed by international standards is a global crisis in surface and groundwaters (European Commission, 2018; Rouse et al., 1999). Indeed, nitrate in aquatic systems affected by agricultural and urban activities can reach levels as high as 100–1000 mg NO_3^- l^{-1} (Camargo and Alonso, 2006; Rouse et al., 1999).

Nitrate pollution can have profound impacts on aquatic organisms. High ambient nitrate levels facilitate net nitrate uptake in fish by passive or facilitated diffusion (HCO_3^-/Cl^- channel) through the branchial epithelium (Camargo and Alonso, 2006; Stormer et al., 1996).

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Consequently, elevated internal nitrate can exert toxic effects and disrupt functional performance (Camargo et al., 2005; Camargo and Alonso, 2006). Nitrate exposure can retard developmental and growth rates (Schram et al., 2014; Van Bussel et al., 2012), alter swimming behaviour (Davidson et al., 2011), disrupt reproductive processes (Good and Davidson, 2016; Hamlin et al., 2008) and lower survival (Luo et al., 2016). Nitrate-induced reductions in performance may arise from diminished aerobic scope (AS, Sokolova et al., 2012). Aerobic scope is the difference between maximum and standard metabolic rates (MMR and SMR, respectively), and represents an organism's capacity to supply oxygen beyond basal requirements to support fitness related activities (e.g. growth, locomotion and digestion, Pörtner et al., 2017; Sokolova et al., 2012). Similar to nitrite, organismal uptake of nitrate leads to the conversion of the oxygen carrying protein, haemoglobin (H_B), to a non-oxygen carrier form - methaemoglobin ($MetH_B$, Grabda et al., 1974). The formation of MetHB lowers blood oxygen carrying capacity, which may in turn reduce MMR. Nitrate exposure may also increase SMR by stimulating energy demanding coping mechanisms (i.e. MetHB reductase system: Huey et al., 1984; Jensen et al., 1987). Together, nitrate-induced reductions of MMR and increases of SMR can diminish AS (Fig. 1 A&B, solid lines) and have negative downstream effects on functional performance.

The problems associated with nitrate pollution are foreseen to intensify as climate change progresses. Temperature rise, accompanied by more frequent and extreme heat waves, is anticipated to boost nitrate levels in aquatic systems (Dokulil and Teubner, 2011; Ficke et al., 2007), and is expected to demand changes in the metabolic attributes of aquatic ectotherms which may have fatal consequences for their fitness (Pörtner et al., 2017). As quantified by several studies (Grans et al., 2014; Healy and Schulte, 2012; Norin et al., 2013), SMR increases with temperature, while MMR generally rises initially and then declines beyond optimum temperatures, which ultimately reduces AS at high temperatures (Fig. 1 C&D, solid lines). According to the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, the inability of the cardiorespiratory system to keep pace with the elevated cellular metabolic (oxygen) demands as temperature rises, is the primary culprit for the decline in aerobic performance at high temperatures. Therefore, alterations of the cardiorespiratory system to maintain or enhance oxygen transport efficiency play a vital role in acclimation of fishes to elevated temperature (Pörtner et al., 2017). Indeed, morphological remodeling of some cardiorespiratory and circulatory organs has been observed with exposure to elevated temperatures. For example, adjustment in size, geometry and myocardial proportion (spongy versus compact) of the ventricle to maintain cardiac output has been observed in fish exposed to warmer temperature (Gamperl, 2004; Gamperl et al., 2020; Keen et al., 2016). Spleen, an important erythrocyte reservoir and erythropoietic organ in salmonid fish, also exhibits mass and size fluctuations as a result of erythrocyte release/storage during environmental changes (i.e. hypoxia, Lai, 2006; Wells and Weber, 1990).

Co-occurrence of stressors in freshwater ecosystems is common (Sokolova et al., 2012), and determining how stressors interact to affect organismal performance is essential. Both nitrate pollution and elevated temperatures have the potential to disrupt energy homeostasis and lower functional performance in isolation (Chen et al., 2015; de Campos et al., 2014; Gomez Isaza et al., 2018, 2020; Lefevre, 2016; McArley et al., 2017). Interactions between temperature and chemical toxicity are complex because temperature impacts chemical availability, uptake, internal processing and detoxification processes (Ficke et al., 2007; Huey et al., 1984), while toxicants can alter an organism's lethal thermal limits (Cairns et al., 1975). Responses of organisms to one stressor may deteriorate or boost the negative effects of the other stressor (26). Moreover, development of protective mechanisms against one stressor may be inhibited or accelerated in the presence of another stressor. Hence, simply adding the individual effects of these stressors may lead to inaccurate conclusions (Crain et al., 2008; Piggott et al., 2015; Todgham and Stillman, 2013). Stressor interactions can be classified into three categories: (i) additive, when stressors do not interact and the combined effect is not significantly different to the sum of the isolated effects of the stressors, (ii) antagonistic, when the interactive effect is weaker than the sum of individual effects of the stressors, and (iii) synergistic, when the interactive effect is stronger than the sum of individual effects of the stressors (Piggott et al., 2015).

We aimed to determine the interactive effects of nitrate pollution and elevated temperature on cardiorespiratory parameters (haematocrit H_{CT}, H_B, relative ventricle mass RVM, and somatic spleen index SSI) and metabolic attributes (SMR, MMR and AS) in juvenile European

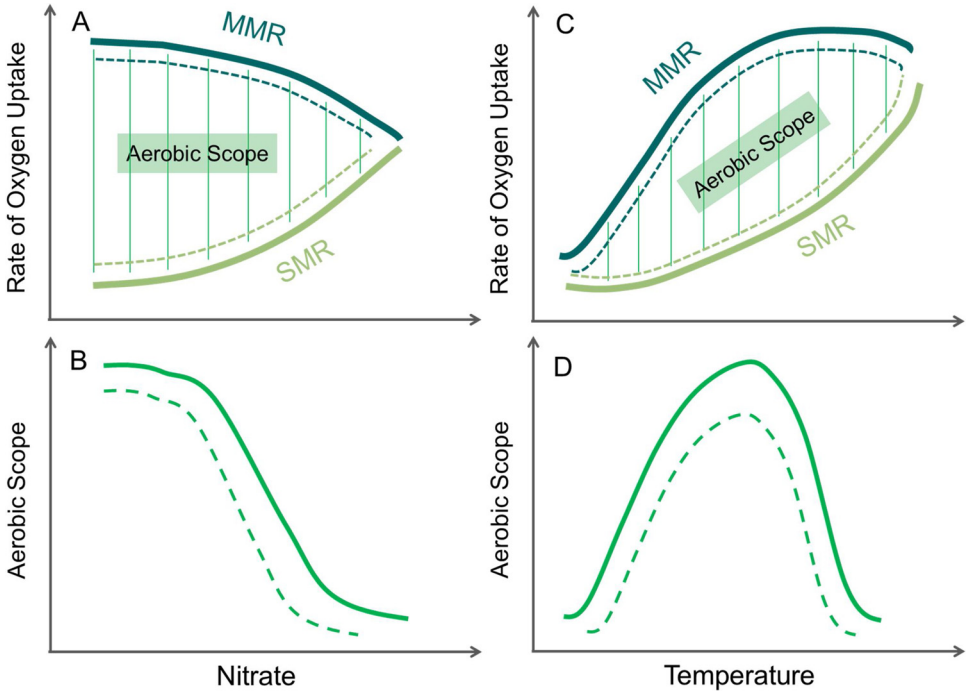

Fig. 1. Conceptualized individual and combined impacts of temperature and nitrate on aerobic performance. Adapted from Sokolova et al., 2012. Solid lines refer to the individual effects of nitrate or temperature, and dashed lines refer to the hypothesized combined effects of both stressors on aerobic scope. Aerobic scope, AS, which is the difference between maximum and standard metabolic rates (MMR and SMR, respectively), represents an organism's capacity to supply oxygen beyond basal requirements to support fitness related activities (e.g. growth, stress tolerance, reproduction). (A) Nitrate may reduce MMR due to the formation of MetHB which lowers blood oxygen carrying capacity, and may increase SMR by stimulating energy demanding coping mechanisms, (B) which ultimately diminish AS. (C) On the other hand, SMR increases with temperature, while MMR generally rises initially and then declines beyond optimum temperatures, (D) which eventually reduces AS at high temperatures. In combination (dashed lines), nitrate and elevated temperature exposure would jointly increase the SMR and diminish the MMR due to compensatory responses and impairment of cardiorespiratory capacity leading to further reduction of AS.

grayling, *Thymallus thymallus*. *T. thymallus* is a freshwater, temperate and stenothermic salmonid (Northcote, 1995), which suffered considerable population losses during past and recent summer heat wave events in the Rhine River (Deutsche Welle, 2018; Jager, 2018). This species continues to be at a high risk of climate warming and heat wave events because freshwater and temperate regions are forecasted to experience larger, more rapid increases in temperature than tropical and marine systems (Ficke et al., 2007). Moreover, *T. thymallus* is vulnerable to the compounding effects of nitrate pollution because nitrate concentrations in European surface waters where *T. thymallus* is native (and abundant) can exceed the set 50 mg NO_3^- l^{-1} standard limit (Durand et al., 2011; European commission, 2018).

We hypothesized that simultaneous nitrate and elevated temperature exposure would increase the SMR and diminish the MMR due to compensatory responses and impairment of cardiorespiratory capacity, which would ultimately reduce the AS of *T. thymallus* (Fig. 1 A–D, dashed lines). Fish were exposed for a minimum of six weeks to one of the three levels of nitrate, 0 mg NO_3^- l^{-1} (control), 50 mg NO_3^- l^{-1} (EU standard limit to identify nitrate polluted water: 10), and 200 mg NO_3^- l^{-1} (realistic level of nitrate in anthropogenically disturbed freshwater systems, Grizzetti et al., 2011; Shukla and Sexena, 2018), and one of two temperatures: 18 °C (average summer temperature), and 22 °C (mild heat wave simulation). Following treatment exposure, aerobic scope (AS, maximum-standard metabolic rates), and cardiorespiratory attributes, including H_B, H_{CT}, RVM and SSI, were assessed to disentangle the interaction between elevated temperature and nitrate pollution on fish performance.

2. Methodology

2.1. Animal maintenance

European grayling (*T. thymallus*) juveniles were obtained from Fischzucht Kauth, Schonecken, Germany, and transferred to the Systemic Physiological and Ecotoxicological Research laboratory, Faculty of Science, University of Antwerp where they were habituated to laboratory conditions for one month prior to the experiment. Fish were maintained in six tanks (210 l capacity; 52 cm width ×72 cm length ×56 cm height; 50 fish $tank^{-1}$) filled with 150 l dechlorinated freshwater (25 ml dechlorinator solution per 200 ml water, API STRESS COAT Water Conditioner), and were fed commercial pellets (0.5 mm, Skretting ME Presta) at a rate of 1% body mass (BM) day^{-1}. Water in the tanks was continuously recirculated at an average flow rate of 5.3 l min^{-1} through a three-layer biofiltration system (cotton-gravel-cotton filter layers, 39 cm width, 53 cm length, and 12 cm height for each layer), and all tanks were subjected to 12-h light and 12-h dark photoperiod. Water temperature was monitored daily by WTW ProfiLine 3310 portable meter and was gradually increased from 8 °C to 18 °C at a rate of 1 °C every two days. Ammonia, nitrite, and nitrate levels were checked daily by test kits (Tetra, Melle, Germany) and kept at levels below 0.25 mg NH_3/NH_4^+ l^{-1}, 0.3 mg NO_2- l^{-1}, and 25 mg NO_3- l^{-1}, respectively. Dissolved oxygen (DO) was determined daily by WTW ProfiLine 3310 portable meter and maintained at levels above 80 %.

2.2. Experimental design

At a stocking density of approximately 1 g l^{-1} (10–11 fish per tank), 246 fish (3.7 ± 0.1 g, mean ± s.e.) were randomly distributed among 24 rectangular tanks (29 cm width, 58 cm length, and 35 cm height) equipped with airlift filters (8 cm length, 8 cm width, and 10.5 cm height: BioAir Filter, SuperFish) and fish shelters (PVC tubes, 17 cm length ×5 cm diameter), and filled with 40.8 l of demineralized (Eurowater DPRO B1−1/1) and enriched (0.4 g KCl: 12.3 g $MgSO_4$*H_2O: 9.6 g $NaHCO_3$: 6.0 g $CaSO_4$*H_2O per 100 l water; all chemicals are AnalaR NORMAPUR® analytical reagent by VWR Chemicals) water maintained at 18 ± 1 °C. After a 7-day adjustment period, tanks were randomly assigned to one of the six treatments using a random number generator: two acclimation temperatures (18 °C and 22 °C: actual values of 18.2 ± 0.4 °C and 22.1 ± 0.5 °C, respectively, average ± SD, n = 792 measurements per temperature level) × three nitrate levels (0 mg NO_3- l^{-1}, 50 mg NO_3- l^{-1}, 200 mg NO_3- l^{-1}: actual values of 5.7 ± 1.0 mg NO_3- l^{-1}, 50.5 ± 3.0 mg NO_3- $l^{-1}$and 200.2 ± 8.0 mg NO_3- l^{-1}, respectively, average ± SD, n = 528 measurements per nitrate level), yielding four replicate tanks for each acclimation temperature × nitrate level combination. Selection of the nitrate treatment levels was based on the standard limit to identify nitrate polluted ground and surface waters in European countries (European commission, 2018) established at 50 mg NO_3- l^{-1}, while realistic levels of nitrate in some freshwater systems can approach or reach beyond 200 mg NO_3- l^{-1} (Grizzetti et al., 2011; Shukla and Sexena (2018)). On the other hand, consideration of 18 °C as control temperature was based on the recorded average summer temperature, while 22 °C is considered as mild heat wave temperature in the Rhine River (Hardenbicker et al., 2016).

The fish were exposed to treatments for a minimum of 6 weeks prior to performance assessments. Fish were fed commercial pellets (0.5 mm, Skretting ME Presta) to satiation daily and were subjected to 12-h light and 12-h dark photoperiod throughout the experiment. Temperature and nitrate levels were recorded daily by portable meters WTW ProfiLine 3310 and HORIBA LAQUAtwin NO_3^-11, respectively, and maintained at appropriate treatment levels (± 1 °C; ± 10 mg NO_3^- l^{-1}) by water heaters (Buyo Digital Aquarium Heater DR-9300) and the addition of sodium nitrate (GPR RECTAPUR by VWR Chemicals), respectively. Ammonia and nitrite were also measured by test kits (Tetra, Melle, Germany) daily and were maintained below 0.25 mg NH_3/NH_4^+ l^{-1} and 0.3 mg NO_2^- l^{-1}, respectively, whereas DO was checked by WTW ProfiLine 3310 and maintained at levels above 80 %. Water changes of up to 50 % depending on the water quality values (generally nitrate concentration as the determinant parameter) were performed daily. Feces and residual feeds were also siphoned out during the daily water change procedure. Nitrate and temperature were checked again after water change to ensure that levels were within the targeted values.

2.3. Intermittent respirometry

Aerobic scope (AS) was measured in a subset of randomly selected fasted (24 h) fish from each treatment (18 °C × 0 mg NO_3^- l^{-1}: 4.97 ± 0.92 g [n = 9]; 18 °C × 50 mg NO_3^- l^{-1}: 4.87 ± 0.70 g [n = 5]; 18 °C × 200 mg NO_3^- l^{-1}: 5.49 ± 0.83 g [n = 5]; 22 °C × 0 mg NO_3^- l^{-1}: 4.44 ± 0.71 g [n = 7]; 22 °C × 50 mg NO_3^- l^{-1}: 4.64 ± 1.13 g [n = 7]; 22 °C × 200 mg NO_3^- l^{-1}: 4.75 ± 0.84 g [n = 5]; values expressed as average BM ± SD [n = sample size]). Following 6 weeks of treatment exposure, AS was measured by subtracting the standard metabolic rate (SMR) from the maximum metabolic rate (MMR). Metabolic rates (MO_2, mg O_2 h^{-1}) were calculated using Eq. 1:

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

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 per hour), 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 βO_2 is the oxygen capacitance at the appropriate water temperature (Cameron, 1986).

SMR was measured by automated intermittent-flow respirometry (Clark et al., 2013; Svendsen et al., 2015). Fish were maintained overnight for a minimum of 14 h in respirometers (4.2 ± 0.03 l volume, motor: 3E-12NRY Little Giant Pump Company) set up within a rectangular reservoir bath (70 cm length; 59.5 cm width; 39 cm height; filled up to 18 cm) containing water at corresponding treatment

conditions and equipped with continuous aeration system. Water flow speed was set at 1.7 BL s^{-1} during each trial to facilitate water mixing within the respirometers, without stimulating steady or burst swimming. Each respirometer was connected to flush pump (average flow rate of 1.8 l min^{-1}, EHEIM universal 300 submersible pump, EHEIM Gmbh & Co. KG, Germany) that was switched on and off by a mechanical timer (Chacon Timer M100) to prevent oxygen depletion and build-up of CO_2 and nitrogenous waste. Flushing occurred for 15 min every 30 min and excess water flowed out of a tube on top of the respirometer chamber that extended above the water level of the reservoir bath. A calibrated portable oxygen meter (WTW ProfiLine 3310) was set to monitor the DO level in the respirometer every minute for a minimum of 14 h. Oxygen level decline rate (during a 20 min period in between flushing cycles) belonging to the lower 10th percentile was considered for the computation of SMR (Clark et al., 2013). Background respiration rates was determined by a blank run after SMR determinations for an hour and were < 2.6 % of the animal respiration rates (mg O_2 kg^{-1} h^{-1}). Some of the SMR trials were discarded due to the technical problems encountered during the SMR measurement, hence the unequal sample sizes among treatments. Some of the respirometer motors either overheated causing water temperature to exceed the targeted range (± 1°C of the test temperature), or stopped working causing inadequate water mixing and consequently irregular DO fluctuation (DO decline was not apparent) during the SMR trials.

After SMR measurement, the MMR of the same fish was determined using the standard chase protocol (Clark et al., 2013). We opted to measure MMR using a chase method because our pilot study showed that *T. thymallus* did not fatigue at the fastest speeds generated by the swim-tunnel respirometers. Each fish was placed into a circular exercise tank (36 cm top diameter; 32 cm base diameter; 38 cm height) containing approximately 10 l of well-aerated water at corresponding treatment conditions. Fish were allowed a 10 min habituation period and were then continuously chased with a net for 3 min. After chasing, fish were immediately placed in a 5.5 l Brett-type respirometer (SWIM-5, Loligo® Systems) submerged in a water bath (75 cm length; 30 cm width; 17 cm height; filled up to 12 cm) with corresponding treatment condition. Maximum oxygen consumption of fish normally occurs within the first few minutes after chasing (Clark et al., 2013), yet this was difficult to measure with the respirometer used for SMR (removal air bubbles and sealing required around 2 − 3 min) during pilot trial, hence the Brett type respirometer (SWIM-5, Loligo® Systems), which can be sealed immediately (in less than 10 s), was used. The respirometer was set at velocity similar to the SMR protocol (1.7BL s^{-1}) to ensure adequate mixing, and was equipped with calibrated portable oxygen meter (WTW ProfiLine 3310) set to measure the DO level every 5 s for 15 min. The highest DO concentration decline rate during a 2 min interval (usually within the first 5 min) was considered for the computation of MMR. The BM of fish was measured after the trial. Background respiration was again determined by a blank run.

2.4. Blood and tissue collection

After 8 weeks of treatment exposure, fish samples (not subjected to respirometry) were collected, euthanized by an overdose of buffered tricaine methanesulfonate (MS-222, Acros Organics), and weighed. Blood samples were obtained by severing the caudal peduncle, and heparinized capillary tubes (60 µl capacity; 0.75 cm length, Hirschmann® Laborgerate) were used to collect blood. Collected blood was then transferred to 1.5 ml microtubes (Eppendorf® microtubes 3810X) buried in ice until H_B and H_{CT} analyses. Spleen and ventricle were then dissected from the fish. Fat and connective tissues were carefully removed from the spleen and ventricle. Ventricle and spleen masses were weighed using an analytical balance (Sartorius 1712 MP8 Dual Range 0–30/160 g) to 0.00001 g. RVM and SSI were calculated by dividing ventricle and spleen mass by total BM, respectively.

2.5. Haematological parameters analyses

H_B concentration in blood samples was determined by a colorimetric commercial assay kit (Sigma-Aldrich MAK 115, Triton®/NaOH method) read by ELX808™ Absorbance Microplate Reader (BioTek® Instruments) under 405 nm wavelength. To determine the H_{CT}, heparinized capillary tubes were filled with freshly collected blood sample, sealed with plasticine clay, and centrifuged for 3 min at 10,000 rpm in a micro H_{CT} centrifuge (Heraeus Christ GmbH Mikro-Hämatokrit 00912 type centrifuge). H_B and H_{CT} analyses were completed in less than 30 min after blood collection. H_{CT} was estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube.

2.6. Statistical analyses

Data analyses were carried out using R Studio (version 3.6.0). Linear mixed effects (lme) models were performed to discern the effect of acclimation temperature (two-level, fixed factor) and nitrate concentration (three-level, fixed factor) and the interaction between these factors on the aerobic performance (SMR, MMR, and AS), H_B, H_{CT}, RVM and SSI. Tank ID was included as a random effect in all mixed effects models, and respirometer ID was included as an additional random effect SMR, MMR and AS models. Body mass is included as a covariate in all models. The lme model assumptions were checked and Tukey's post hoc tests (Tukey adjusted lsmeans) were run to determine statistical differences among treatment groups and fixed factors levels. Statistical significance was accepted at probability level less than or equal to 0.05 ($p \leq 0.05$). Classification of stressor interaction was based on the classification system outlined by Piggott et al. (46). Outliers were defined as values outside the interval two times the standard deviation (SD) from the average value (average value ± 2*SD), and were not included in statistical analyses. Data were presented as average value ± standard error (SE).

3. Results

3.1. Metabolic attributes

No treatment-induced mortalities occurred during the exposure period. Nitrate exposure increased the SMR of graylings ($p < 0.05$, $F_{2,29} = 5.72$, lme, Fig. 2A) but this was only evident in 22 °C-acclimated fish, where SMR of 200 mg l^{-1} exposed fish (129.7 O_2 kg^{-1} h^{-1} ± 8.5) was 69 % and 55 % higher than the SMR of fish exposed to 0 mg l^{-1} (76.5 O_2 kg^{-1} h^{-1} ± 5.9) and 50 mg l^{-1} (83.5 O_2 kg^{-1} h^{-1} ± 7.0), respectively. Acclimation temperature had no effect on SMR ($p = 0.71$, $F_{1,29} = 0.02$, lme). In contrast to SMR, elevated temperature increased the MMR by 32 % ($p < 0.001$, $F_{1,27} = 23.60$, $MMR_{18°C}$ = 1224.0 O_2 kg^{-1} h^{-1} ± 68.5, $MMR_{22°C}$ = 1620.5 O_2 kg^{-1} h^{-1} ± 52.6, averaged over nitrate levels), but no main effect of nitrate treatment was noted ($p = 0.09$, $F_{2,27} = 2.54$, lme, Fig. 2B). AS was influenced to a larger extent by MMR than by SMR (Fig. 2C): AS was affected by acclimation temperature ($p < 0.001$, $F_{1,25} = 25.77$, lme) where 22 °C-acclimated fish (1512.7 O_2 kg^{-1} h^{-1} ± 51.7, averaged over nitrate levels) had a 36 % higher AS than 18 °C-acclimated fish (1114.8 O_2 kg^{-1} h^{-1} ± 65.4, averaged over nitrate levels), but AS was independent of nitrate exposure ($p = 0.23$, $F_{2,25} = 1.55$, lme). Despite the non-significant main effect of nitrate, fish exposed to 200 mg l^{-1} exhibited significantly higher ($p < 0.05$) MMR and AS compared to control nitrate level at 22 °C. A significant synergistic interaction between elevated temperature and nitrate exposure was noted in SMR ($p < 0.05$, $F_{2,29} = 5.67$, lme), MMR ($p < 0.05$, $F_{2,27} = 5.05$, lme), and AS ($p < 0.05$, $F_{2,25} = 4.58$, lme).

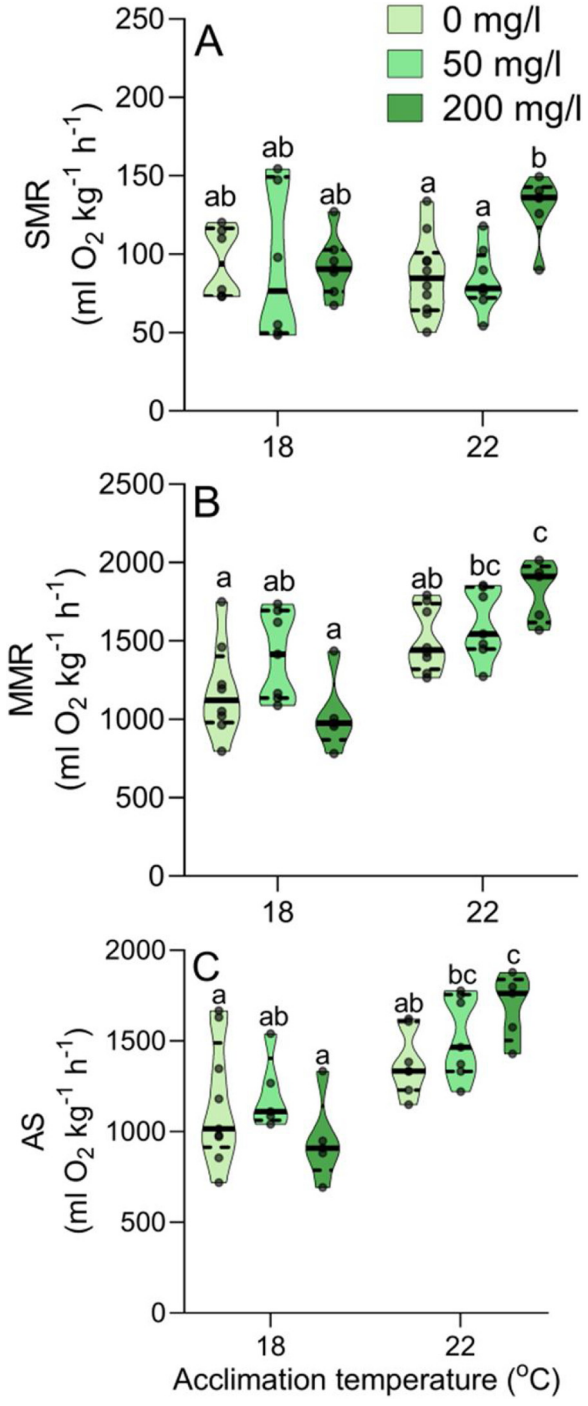

Fig. 2. Interactive effects of nitrate and thermal acclimation treatments on metabolic traits of the European grayling (*Thymallus thymallus*). Fish were acclimated to one of two thermal acclimation treatments (18 or 22 °C) and one of three nitrate concentrations (0, 50 or 200 mg/l) for 6 weeks. The (A) standard metabolic rate (SMR, mg O_2 kg^{-1} h^{-1}) was significantly affected by nitrate treatment ($p < 0.05$, $F_{2,14} = 4.04$), and the interaction between temperature and nitrate ($p < 0.05$, $F_{2,14} = 4.16$). Elevated temperature increased the (B) maximum metabolic rate (MMR, mg O_2 kg^{-1} h^{-1}, $p < 0.001$, $F_{1,13} = 23.60$) and the (C) aerobic scope (AS, mg O_2 kg^{-1} h^{-1}, $p < 0.001$, $F_{1,13} = 25.77$), and interacted with nitrate to further escalate both attributes (MMR: $p < 0.05$, $F_{2,13} = 5.05$; AS: $p < 0.05$, $F_{2,13} = 4.58$). Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups. Data are presented as a violin plot where the lines represent the upper 95 % confidence interval, median and lower 95 % confidence interval, respectively. Dots represent individual data points (n = 5-9 fish/treatment group).

3.2. Cardiorespiratory remodeling

Remodeling of the cardiorespiratory organs, such as ventricle and spleen, was induced by stressor exposure. RVM was 39 % higher in fish exposed to 22 °C (0.09 % ± 0.01, averaged over nitrate levels; $p < 0.01$, $F_{1,18} = 10.70$, lme) compared to 18 °C (0.07 % ± 0.00, averaged over nitrate levels) but was independent of nitrate exposure ($p = 0.98$, lme, $F_{2,18} = 0.02$, ; Fig. 3A). Elevated temperature did not influence the SSI ($p = 0.62$, $F_{1,18} = 0.26$, lme) but nitrate exposure induced an 85 % and 57 % SSI expansion ($p < 0.05$, $F_{2,18} = 3.79$, lme) in fish exposed to 50 mg NO_3^- l^{-1} (0.06 % ± 0.01) and 200 mg NO_3^- l^{-1}(0.05 % ± 0.01), respectively (Fig. 3B). Interaction between elevated temperature and nitrate was non-significant in both measured cardiorespiratory attributes (RVM: $p = 0.64$, $F_{2,16} = 0.45$, lme; SSI: $p = 0.27$, $F_{2,16} = 1.40$, lme).

3.3. Haematological parameters

Haematological parameters were minimally affected by stressor exposure (Table 1). Elevated temperature had no main effect on H_{CT} ($p = 0.23$, $F_{1,34} = 1.47$, lme) and H_B ($p = 0.26$, $F_{1,31} = 1.30$, lme). Nitrate exposure reduced H_B concentration ($p < 0.05$, $F_{1,31} = 4.34$, lme), while H_{CT} was independent of nitrate exposure ($p = 0.62$, $F_{2,34} = 0.50$, lme). Interaction between nitrate and temperature was non-significant on both blood parameters (H_{CT}: $p = 0.29$, $F_{2,34} = 1.2$; H_B: $p = 0.21$, $F_{2,\ 29} = 1.60$, lme). Post-hoc analyses indicated no significant differences among treatment combinations ($p > 0.05$).

4. Discussion

Although it is anticipated that nitrate pollution will worsen under climate change (Dokulil and Teubner, 2011; Ficke et al., 2007), knowledge on the combined effects and the interaction between these prevalent stressors remains very limited. To this end, we assessed the interactive effects of nitrate pollution and elevated temperature on AS and cardiorespiratory attributes in *T. thymallus*. The two stressors interacted synergistically to increase AS, indicating beneficial cross-tolerance. This synergistic interaction was likely underscored by stressor-induced remodeling of cardiorespiratory attributes (i.e. ventricle and spleen mass), which may have provided overlapping protection against both stressors. These data highlight the unpredictability of stressor interactions and may inform both conservation and aquaculture practices.

Temperature influences SMR due to the thermal dependence of chemical and enzymatic processes associated with homeostatic maintenance (Lefevre, 2016). In acute cases (minutes, hours or days at a new temperature), SMR of fish rises with increasing temperature exposure (Chen et al., 2015; Ferreira et al., 2014; Lefevre, 2016). However, SMR response to temperature can differ when the fish is given sufficient time to adjust their metabolic processes and thereby their costs (Lefevre, 2016; Sandblom et al., 2014; Seebacher et al., 2014). For example, Donelson et al. (2010) showed that spiny chromis damselfish, *Acanthochromis polyacanthus*, reared 3 °C higher than the natural temperature in their habitat, partly compensated for the rise in SMR that was displayed by conspecifics acutely exposed to the same temperature. Here, chronic exposure (6 weeks) to elevated temperature did not affect the SMR of *T. thymallus*, suggesting that the fish remodeled their physiology to downregulate basal oxygen demands. For example, it is proposed that mitochondrial function down-regulation enables fish to decrease their resting oxygen demand to preserve the AS during warming (Fangue et al., 2009). However, further testing is required (i.e. determination of SMR after acute exposure at the alternative acclimation temperature) to confirm if a thermal acclimation response was demonstrated here.

SMR was influenced by nitrate treatment but the effect was only noted in 22 °C-acclimated fish indicating an interaction between elevated temperature and nitrate treatment. Chronic nitrate exposure

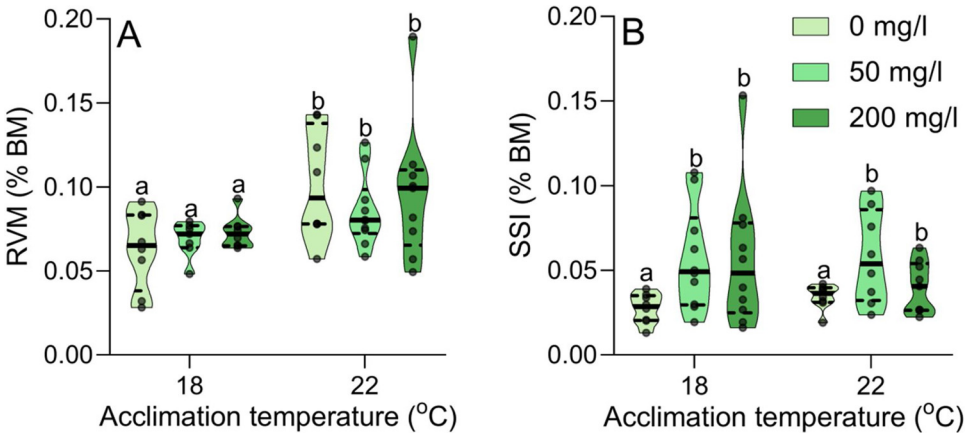

Fig. 3. Cardiorespiratory remodeling in European grayling (*Thymallus thymallus*) exposed to a factorial combination of nitrate and thermal acclimation treatments. Fish were acclimated to one of two thermal acclimation treatments (18 or 22 °C) and one of three nitrate concentrations (0, 50 or 200 mg/l) for 8 weeks. Elevated temperature increased the (A) relative ventricle mass (RVM, %BM $F_{1,18}$ = 10.70, $p < 0.01$, lme), and nitrate exposure increased the (B) spleen somatic index (SSI, %BM, $F_{2,18}$ = 3.79, $p < 0.05$, lme). Different lowercase letters indicates statistical differences ($p \leq 0.05$) among treatment groups. Data are presented as a violin plot where the lines represent the upper 95 % confidence interval, median and lower 95 % confidence interval, respectively. Dots represent individual data points (n = 9-10 fish/treatment group).

Table 1
Haematocrit (H_{CT}) and haemoglobin (H_B) of European grayling (*Thymallus thymallus*) exposed for 8 weeks to different treatment groups: temperature (18 or 22 °C) and nitrate (0, 50, 200 mg l^{-1} NO_3-) combination. Values are shown as mean (± standard error), and sample size range from 5 to 11 fish per treatment group.

Treatment group	H_{CT} (%)	H_B (g l^{-1})
18 °C ; 0 mg l^{-1} NO_3-	34.3 (± 1.9)	84.5 (± 2.4)
18 °C ; 50 mg l^{-1} NO_3-	33.1 (± 2.1)	75.4 (± 2.3)
18 °C ; 200 mg l^{-1} NO_3-	36.8 (± 0.8)	80.0 (± 0.9)
22 °C ; 0 mg l^{-1} NO_3-	38.5 (± 1.4)	84.0 (± 1.1)
22 °C ; 50 mg l^{-1} NO_3-	30.0 (± 3.7)	76.3 (± 4.8)
22 °C ; 200 mg l^{-1} NO_3-	32.9 (± 3.1)	68.8 (± 3.3)

without warm exposure in this study did not pose disruptions to SMR, which coincided with the results of nitrate exposure experiments on other aquatic ectotherms (Gomez Isaza et al., 2018; Gomez Isaza et al., 2020; Meade and Watts, 1995). In contrast, it was observed that 22 °C-acclimation coupled with 200 mg l^{-1} nitrate exposure almost doubled the SMR of fish relative to those exposed to lower nitrate concentrations, which is comparable to the findings of de Campos et al., (2014), wherein 30-day exposure to elevated nitrate (182 mg l^{-1}) at 25 °C significantly increased the SMR of juvenile pink shrimp, *Farfantepenaeus brasiliensis*, relative to lower nitrate (45 mg l^{-1} and 91 mg l^{-1}) levels. It is speculated that activated nitrate response mechanisms during warming may have limited the ability of fish exposed to 200 mg l^{-1} to make physiological changes to down-regulate their basal oxygen demand similar to that of fish exposed to lower nitrate concentrations. Exposure to nitrate may activate energy coping mechanisms such as mobilization of the MetHB reductase system (Huey et al., 1984) and the stimulation of an active excretion mechanism for unbound nitrate and/or nitrite (Eddy et al., 1983). Possibly, maintenance of relatively higher mitochondrial densities to maintain energy production was deemed necessary to fuel these energy demanding nitrate response mechanisms. Alternatively, thermal stress may have also constrained the ability of fish to sustainably cope with nitrate treatment.

AS was largely influenced by MMR rather than SMR, which is similar to the AS findings of Lefevre (2016) on at least 30 fish species. In this study, MMR significantly increased following exposure to elevated temperature (22 °C), resulting in an increase in AS of *T. thymallus*. It is generally assumed that exposure to temperature beyond thermal optima reduces MMR due to the diminished efficiency of the cardiorespiratory system to take up and transport oxygen (McArley et al., 2017; Pörtner et al., 2017). However, negative correlation of MMR with temperature (above optimal) is not universal as some species show expansion of MMR at elevated temperature approaching thermal limits (Donelson et al., 2010). Thermal preference (temperature at which feeding occurs without abnormal behavior) of *T. thymallus* ranges from 4 to 18 °C, with an absolute maximum of 25–27 °C (Cove et al., 2018; Crisp, 1996). Since only two temperatures were tested in this study, it is possible that 22 °C was not high enough to negatively affect the cardiorespiratory functioning of *T. thymallus,* hence a reduction in MMR was not observed.

Increase in MMR is also possible by adjustments in the circulatory and respiratory systems after long-term exposure to elevated temperature (Grans et al., 2014; Lefevre, 2016). For example, morphological remodeling of the heart after exposure to elevated temperature (i.e. developing larger ventricle and/or stronger contractile cardiac muscle) corresponds to higher cardiac output (Gamperl, 2004; Keen et al., 2016), and has been positively correlated with AS (Nyboer and Chapman, 2018). The higher RVM noted in 22 °C exposed fish is therefore one mechanism to explain the improved MMR and AS at elevated temperature in this experiment. This RVM increase at elevated temperature, however, does not coincide with many studies on salmonid fish where RVM did not significantly change (Keen et al., 2016; Klaiman et al., 2011) or decreased (Anttila et al., 2015), and fishes relied on other heart remodeling mechanism to maintain cardiac function at elevated temperature (i.e. increase in the proportion of compact myocardium, Anttila et al., 2015; Nyboer and Chapman, 2018). Nevertheless, the acclimation temperatures in these studies were only between 15 and 19 °C. Similar to our findings, Gemperl et al. (2020) found an increase in the RVM when rainbow trout were exposed to elevated acclimation temperatures (up to 23 °C), suggesting that the response of salmonid heart to elevated temperatures is dependent on the maximum temperature to which it is exposed.

Nitrate exposure can reduce MMR, hence it can also reduce the AS, of fish by the reduction of oxygen transport capacity due to the oxidation of H_B to MetHB (Grabda et al., 1974). However, nitrate did not significantly affect the MMR and the AS of *T. thymallus* in this study, which differs from the results obtained by Gomez Isaza et al. (2018), where nitrate exposure (50 mg l^{-1} and 100 mg l^{-1}) of blueclaw crayfish, *Cherax destructor*, for up to 20 days led to significant reduction in MMR and AS. Inconsistencies between the results may be explained by species-specific differences in nitrate tolerance (Camargo and Alonso, 2006), as well as differences in their respiratory pigments: *T. thymallus* has H_B while *C. destructor* carries haemocyanin.

The lack of nitrate-induced change of MMR and AS indicates that the fish were able to cope with the oxygen transport capacity reduction caused by methaemoglobinemia, which is supported by the nitrate-induced increase in SSI and the minimal haematological variation among fish exposed to different nitrate levels. The spleen is an important erythrocyte storage site in fish, and plays an essential role in maintaining

or increasing blood oxygen-carrying capacity during exercise and hypoxic conditions (Wells and Weber, 1990). Hence, spleen changes become very relevant because MMR trials involve fatiguing exercise and nitrate-exposed fish methaemoglobinemia can induce tissue hypoxia. Splenic contraction, indicated by the reduction of SSI, followed by the subsequent release of (up to 95 %) stored erythrocytes to the circulation have been identified as an immediate response of salmonid fish during exercise and severe, acute hypoxic conditions (Lai, 2006; Wells and Weber, 1990). Nevertheless, a different case is observed during moderate and chronic hypoxia. A lack of splenic contraction and an increase of SSI was observed in rainbow trout, *Oncorhynchus mykiss,* after chronic exposure (2 weeks) to hypoxia (Wells and Weber, 1990). Moreover, it was found that the SSI of *O. mykiss* returned to normal after a few days (1–2 days) of exposure to moderate hypoxia (50 % oxygen saturation), which is attributed to the formation of new blood cells in the circulation and storage in the spleen (Lai, 2006). The same scenario possibly occurred in this experiment: nitrate-induced methaemoglobinemia (indicated by slightly reduced H_B) may have caused moderate tissue hypoxia, and the species responded by forming new blood cells in the circulation and storing excess blood cells in spleen (hence the nonsignificant levels of H_{CT} among treatments), causing the increase of SSI after nitrate exposure. Higher resting SSI indicates a greater blood supply when the spleen contracts in response to exercise induced during MMR trials, and compensates for the oxygen carrying capacity limitation by methaemoglobinemia, hence the undifferentiated MMR in nitrate-exposed fish. Erythropoiesis, however, should result in an increase in H_{CT} levels in circulation beyond control levels to fully offset the effects of hypoxia, which was not the case in this experiment. It is speculated that nitrate may have also caused damage to red blood cells. Previous nitrite exposure studies on sea bass, *Dicentrarchus labrax*, found that methaemoglobin formation caused death of red blood cells due to oxidative hemolysis and/or reduction of erythrocyte life span (Scarano et al., 1984; Scarano and Saroglia, 1984).

Elevated temperature and nitrate treatments interacted synergistically to increase the MMR and AS of *T. thymallus* in this study. The effect of simultaneous exposure to both stressors was greater than the sum of the individual effect and greater than any individual effects (Piggott et al., 2015). Related compensatory mechanisms may be the reason for the synergistic interaction between these two stressors: compensatory responses induced by temperature may have been associated with heightened tolerance to nitrate, or vice versa. Phenomena where protective responses against one stressor enable organisms to transiently increase tolerance to another form of stress are called cross-tolerance (Kampinga et al., 1995) and are known to occur in fish. Initial heat shock exposure (+12 °C) increased the survival of tidepool sculpins, *Oligocottus maculosus,* when subsequently exposed to osmotic and hypoxic stressors (Todgham et al., 2005). Channel catfish, *Ictalurus punctatus,* acclimated to moderate hypoxia (50 % oxygen saturation) exhibited higher thermal tolerance (measured as critical thermal maximum, CTMax) which is associated with differentiated cardiovascular responses (higher resting heart rate and increased systolic blood pressure, Burleson and Silva, 2011). Similarly, each stressor tested in this experiment caused changes in cardiorespiratory and circulatory attributes that possibly heightened the tolerance of fish to the other stressor. Temperature-induced RVM increase may have overcompensated for the nitrate-induced reduction of oxygen transport capacity resulting in increased MMR and AS when exposed to combined stressors. At the same time, the nitrate-induced blood reserve expansion in the spleen to offset the MMR-limiting effects of methaemoglobinemia likely reinforced the positive effects of cardiorespiratory adjustments induced by elevated temperature.

Cross-tolerances between stressors appear to be mediated through several molecular mechanisms, primarily through the induction of heat shock proteins (HSPs) – stress proteins that play crucial roles in cyto-protection and cell repair (Todgham et al., 2005). Thermal shock and nitrate-related stressor (i.e. hypoxia, nitrite exposure) have been already documented to induce over-expression of HSPs in several fish tissues (Burleson and Silva, 2011; Jensen et al., 2015; McBryan et al., 2013), which supports our speculation that cross-tolerance underlies the positive synergistic interaction between elevated temperature and nitrate exposure observed in this study. Cross-tolerance is generally observed between stressors which co-occur in natural habitats (Sinclair et al., 2013). Indeed, European surface waters, where *T. thymallus* proliferate, can have nitrate levels exceeding the 50 mg NO_3^- l^{-1} standard limit (Durand et al., 2011; European Commission, 2018), and experience harsh temperature rises during summer heat waves.

AS represents the capacity of the organism to supply oxygen needed for protective stress responses as well as fitness related activities (Piggott et al., 2015; Sokolova et al., 2012). Several studies found association between AS and fish functional performance such as thermal tolerance, growth, swimming performance, and post-exercise recovery after exposure to stressor, including warm acclimation and nitrate treatment (Gomez Isaza et al., 2020; McArley et al., 2017; Nyboer and Chapman, 2017). Hence, the observed synergistic improvement of AS owed to the stressor-induced cardiorespiratory remodeling indicates sufficient oxygen supply to produce a stress response to warming or toxicants (Sokolova et al., 2012), which may render higher survival chance of the species during eutrophication and heat wave events. Nevertheless, measurement of the combined effects of temperature and nitrate on relevant organismal performance such as CTMax, hypoxia tolerance (PCrit), swimming performance (UCrit) and growth is necessary provide further insights on how the fish will thrive during these events. Moreover, given the seasonality of these events, knowledge on the responses or recovery of the species during stressor removal and repeated exposure reflecting realistic scenarios would support the development of holistic conservation efforts.

This increase in AS and cardiorespiratory remodeling could not justify the mass mortalities of *T. thymallus* during previous summer heat wave events in the Rhine river. Possibly, the magnitude of stressors during these heat wave events exceeded the experiment levels and the organism's limit. In the River Ain of Southern France, for example, water temperature rose up to 27 °C in 2003 causing acute mortalities of graylings (Cove et al., 2018). Temperature and nitrate elevation may also alter other environmental parameters negatively- reduction of dissolved oxygen to hypoxic levels being one of the most common shifts. Moreover, since cross-tolerance is only inducible over a narrow stressor extent (Burleson and Silva, 2011), the stressor levels during those events were possibly disproportionate to induce cross-tolerance. Under severe situations, heat waves and eutrophic conditions (beyond tested levels) may remain threats to *T. thymallus* in natural habitats.

5. Conclusion

Our findings demonstrated that nitrate and temperature interacted synergistically to increase the AS of *T. thymallus*. This positive synergistic interaction was attributed to the stressor-induced cardiorespiratory remodeling, including expansion of RVM and SSI, which may have provided overlapping protection against both stressors. These results cannot explain the mass mortalities of *T. thymallus* during past summer heat wave events in the Rhine river. It is likely that the magnitude of stressors during these heat wave events exceeded the levels tested in this study, and may have unfavorably altered other environmental parameters, including dissolved oxygen availability. Elevated temperature and nitrate can promote hypoxia conditions in aquatic systems, and may reduce hypoxia tolerance of fish. Hence, research on the interactive effects of nitrate, temperature, and hypoxia on organismal performance is currently in progress, and should provide crucial information for the conservation of *T. thymallus*.

Ethics Statement

Ethics approval for the experimental use of study animal (*Thymallus*

thymallus) was granted by the University of Antwerp's local ethics committee (Permit Number: LA-1100134, Project 2018 – 68).

Data Accessibility

Raw datasets have been deposited in Dryad: https://doi.org/10.5061/dryad.gf1vhhmkc.

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CRediT authorship contribution statement

April Grace R. Opinion: Writing - original draft, Investigation, Formal analysis. **Gudrun De Boeck:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Essie M. Rodgers:** Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

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

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References

Anttila, K., Lewis, M., Prokkola, J.M., Kanerva, M., Seppanen, E., Kolari, I., et al., 2015. Warm acclimation and oxygen depletion induce species-specific responses in salmonids. J. Exp. Biol. 218, 1471–1477. https://doi.org/10.1242/jeb.119115.

Burleson, M.L., Silva, P.E., 2011. Cross tolerance to environmental stressors: effects of hypoxic acclimation on cardiovascular responses of channel catfish (*Ictalurus punctatus*) to a thermal challenge. J. Therm. Biol. 6, 250–254. https://doi.org/10.1016/j.jtherbio.2011.03.009).

Cairns, J., Heath, A.G., Parker, B.C., 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia 47, 135–171. https://doi.org/10.1007/BF00036747).

Camargo, J.A., Alonso, Á, 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ. Int. 32, 831–849. https://doi.org/10.1016/j.envint.2006.05.002).

Camargo, J.A., Alonso, A., Salamanca, A., 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58, 1255–1267. https://doi.org/10.1016/j.chemosphere.2004.10.044).

Cameron, J.N., 1986. Principles of Physiological Measurement. Academic Press Inc, London. https://doi.org/10.1016/B978-0-12-156955-6.X5001-4).

Chen, Z., Snow, M., Lawrence, C.S., Church, A.R., Narum, S.R., Devlin, R.H., et al., 2015. Selection for upper thermal tolerance in rainbow trout (*Oncorhynchus mykiss* Walbaum). J. Exp. Biol. 218, 803–812. https://doi.org/10.1242/jeb.113993).

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J. Exp. Biol. 216, 2771–2782. https://doi.org/10.1242/jeb.084251).

Cove, R.J., Taylor, R.J., Gardiner, R., 2018. European Grayling Conservation, Ecology & Management: a Practical Conservation Guide for United Kingdom. Printcentre Wales Limited, North Wales.

Crain, C.M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecol. Lett. 11, 1304–1315. https://doi.org/10.1111/j.1461-0248.2008.01253.x).

Crisp, D.T., 1996. Environmental requirements of common riverine European salmonid species in freshwater with particular reference to physical and chemical aspects. Hydrobiologia 323, 201–221. https://doi.org/10.1007/BF00007847).

Darling, E.S., Côté, I.M., 2008. Quantifying the evidence for ecological synergies. Ecol. Lett. 11, 1278–1286. https://doi.org/10.1111/j.1461-0248.2008.01243.x).

Davidson, J., Good, C., Welsh, C., Summerfelt, S.T., 2011. Abnormal swimming behavior and increased deformities in rainbow trout *Oncorhynchus mykiss* cultured in low exchange water recirculating aquaculture systems. Aquac. Eng. 45, 109–117. https://doi.org/10.1016/j.aquaeng.2011.08.005).

de Campos, B.R., Furtado, P.S., D'Incao, F., Poersch, L.H., Wasielesky, W.J., 2014. The effect of ammonia, nitrite, and nitrate on the oxygen consumption of juvenile pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817). J. Appl. Aquac. 26, 94–101. https://doi.org/10.1080/10454438.2014.882216).

Deutsche Welle, 2018. Heat Kills Thousands of Fish in River Rhine. https://p.dw.com/p/32g7g).

Dokulil, M.T., Teubner, K., 2011. Eutrophication and climate change: present situation and future scenarios. In: Ansari, A.A., Sarvajeet, S.G., Lanza, G.R., Rast, W. (Eds.), Eutrophication: Causes, Consequences and Control. Springer, Dordrecht, Heidelberg, London, New York, pp. 1–16.

Donelson, J.M., Munday, P.L., McCormick, M.I., Nilsson, G.E., 2010. Acclimation to predicted ocean warming through developmental plasticity in a tropical reef fish. Glob. Chang. Biol. 17, 1712–1719. https://doi.org/10.1111/j.1365-2486.2010.02339.x).

Durand, P., Breuer, L., Johnes, P.J., Billen, G., Butturini, A., Billen, P., et al., 2011. Nitrogen processes in aquatic ecosystems. In: Sutton, M.A., Howard, C.M., Erisman, J.W., Billen, G., Bleeker, A., Grennfelt, P., van Grinsven, H., Grizzetti, B. (Eds.), The European Nitrogen. Cambridge University press, Cambridge, pp. 126–146.

Eddy, F.B., Kunzlik, P.A., Bath, R.N., 1983. Uptake and loss of nitrite from the blood of rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, *Salmo salar* L. In fresh water and in dilute sea water. J. Fish Biol. 23, 105–116. https://doi.org/10.1111/j.1095-8649.1983.tb02885.x).

European Commission, 2018. Report From the Commission to the Council and the European Parliament on the Implementation of Council Directive 91/676/EEC Concerning the Protection of Waters Against Pollution Caused by Nitrates From Agricultural Sources Based on Member State Reports for the Period 2012–2015. European Commission, Brussels, Belgium Report No.: COM/2018/257.

Fangue, N.A., Richards, J.G., Schulte, P.M., 2009. Do mitochondrial properties explain intraspecific variation in thermal tolerance? J. Exp. Biol. 212, 514–522. https://doi.org/10.1242/jeb.024034).

Ferreira, E.O., Anttila, K., Farrell, A.P., 2014. Thermal optima and tolerance in the eurythermic goldfish (*Carassius auratus*): relationships between whole-animal aerobic capacity and maximum heart rate. Physiol. Biochem. Zool. 87, 599–611. https://doi.org/10.1086/677317).

Ficke, A.D., Myrick, C.A., Hansen, L.J., 2007. Potential impacts of global climate change on freshwater fisheries. Rev. Fish Biol. Fish. 17, 581–613. https://doi.org/10.1007/s11160-007-9059-5).

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., et al., 2004. Nitrogen cycles: past, present, and future. Biogeochemistry 70, 153–226. https://doi.org/10.1007/s10533-004-0370-0).

Gamperl, A.K., 2004. Cardiac plasticity in fishes: environmental influences and intraspecific differences. J. Exp. Biol. 207, 2539–2550. https://doi.org/10.1242/jeb.01057).

Gamperl, A.K., Ajiboye, O.O., Zanuzzo, F.S., Sandrelli, R.M., Peroni E de, F.C., Beemelmanns, A., 2020. The impacts of increasing temperature and moderate hypoxia on the production characteristics, cardiac morphology and haematology of Atlantic salmon (*Salmo salar*). Aquaculture 519, 734874. https://doi.org/10.1016/j.aquaculture.2019.734874).

Gomez Isaza, D.F., Cramp, R.L., Franklin, C.E., 2018. Negative impacts of elevated nitrate on physiological performance are not exacerbated by low pH. Aquat. Toxicol. 200, 217–225. https://doi.org/10.1016/j.aquatox.2018.05.004).

Gomez Isaza, D.F., Cramp, R.L., Franklin, C.E., 2020. Simultaneous exposure to nitrate and low pH reduces the blood oxygen-carrying capacity and functional performance of a freshwater fish. Conserv. Physiol. 8, 1–15. https://doi.org/10.1093/conphys/coz092).

Good, C., Davidson, J., 2016. A review of factors influencing maturation of Atlantic salmon, *Salmo salar*, with focus on water recirculation aquaculture system environments. J. World Aquac. Soc. 47, 605–632. https://doi.org/10.1111/jwas.12342).

Grabda, E., Einszporn-Orecka, T., Felinska, C., Zbanysek, R., 1974. Experimental methaemoglobinemia in rainbow trout. Acta Ichthyol. Piscat. 4, 43–71.

Grans, A., Jutfelt, F., Sandblom, E., Jonsson, E., Wiklander, K., Seth, H., et al., 2014. Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO2 in Atlantic halibut. J. Exp. Biol. 217, 11–17. https://doi.org/10.1242/jeb.096743).

Grizzetti, B., Bouraoui, F., Billen, G., van Grinsven, H., Cardoso, A.C., Thieu, V., Garnier, J., Curtis, C., Howarth, R., Jones, P., et al., 2011. Nitrogen as a threat to European water quality. In: Sutton, M.A., Howard, C.M., Erisman, J.W., Bleeker, A., Grennfelt, P., van Grinsven, H., Grizzetti, B. (Eds.), The European Nitrogen Assessment. Cambridge University Press, pp. 379–404. https://doi.org/10.1017/CBO9780511976988.020.

Hamlin, H.J., Moore, B.C., Edwards, T.M., Larkin, I.L.V., Boggs, A., High, W.J., et al., 2008. Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. Aquaculture 281, 118–125. https://doi.org/10.1016/j.aquaculture.2008.05.030).

Hardenbicker, P., Viergutz, C., Becker, A., Kirchesch, V., Nilson, E., Fischer, H., 2016. Water temperature increases in the river Rhine in response to climate change. Reg. Environ. Change 17, 299–308. https://doi.org/10.1007/s10113-016-1006-3).

Healy, T.M., Schulte, P.M., 2012. Thermal acclimation is not necessary to maintain a wide thermal breadth of aerobic scope in the common killifish (*Fundulus heteroclitus*). Physiol. Biochem. Zool. 85, 107–119. https://doi.org/10.1086/664584).

Huey, D.W., Beitinger, T.L., Wooten, M.C., 1984. Nitrite-induced methemoglobin formation and recovery in channel catfish (*Ictalurus punctatus*) at three acclimation temperatures. Bull. Environ. Contam. Toxicol. 32, 674–681. https://doi.org/10.1007/bf01607555).

Jackson, M.C., Loewen, C.J.G., Vinebrooke, R.D., Chimimba, C.T., 2015. Net effects of multiple stressors in freshwater ecosystems: a meta-analysis. Glob. Chang. Biol. 22, 180–189. https://doi.org/10.1111/gcb.13028).

Jager, K., 2018. Dying Fish and Drying Rivers-consequences of Europe's Summer Heat Wave. https://p.dw.com/p/32tcy).

Jensen, F.B., Andersen, N.A., 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. https://doi.org/10.1007/BF00700972).

Jensen, F.B., Gerber, L., Hansen, M.N., Madsen, S.S., 2015. Metabolic fates and effects of nitrite in brown trout under normoxic and hypoxic conditions: blood and tissue nitrite metabolism and interactions with branchial NOS, Na+/K+-ATPase and hsp70 expression. J. Exp. Biol. 218, 2015–2022. https://doi.org/10.1242/jeb.120394).

Kampinga, H.H., Brunsting, J.F., Stege, G.J.J., Burgman, P.W.J.J., Konings, A.W.T., 1995. Thermal protein denaturation and protein aggregation in cells made thermotolerant by various chemicals: role of heat shock proteins. Exp. Cell Res. 219, 536–546. https://doi.org/10.1006/excr.1995.1262).

Keen, A.N., Klaiman, J.M., Shiels, H.A., Gillis, T.E., 2016. Temperature-induced cardiac remodelling in fish. J. Exp. Biol. 220, 147–160. https://doi.org/10.1242/jeb.128496).

Klaiman, J.M., Fenna, A.J., Shiels, H.A., Macri, J., Gillis, T.E., 2011. Cardiac remodeling in fish: strategies to maintain heart function during temperature change. PLoS One 6, e24464. https://doi.org/10.1371/journal.pone.0024464).

Lai, J.C.C., 2006. Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. J. Exp. Biol. 209, 2734–2738. https://doi.org/10.1242/jeb.02279).

Lefevre, S., 2016. Are global warming and ocean acidification conspiring against marine ectotherms? A meta-analysis of the respiratory effects of elevated temperature, high CO_2 and their interaction. Conserv. Physiol. 4https://doi.org/10.1093/conphys/cow009). cow009.

Luo, S., Wu, B., Xiong, X., Wang, J., 2016. Short-term toxicity of ammonia, nitrite, and nitrate to early life stages of the rare minnow (*Gobiocypris rarus*). Environ. Toxicol. Chem. 35, 1422–1427. https://doi.org/10.1002/etc.3283).

McArley, T.J., Hickey, A.J.R., Herbert, N.A., 2017. Chronic warm exposure impairs growth performance and reduces thermal safety margins in the common triplefin fish (*Forsterygion lapillum*). J. Exp. Biol. 220, 3527–3535. https://doi.org/10.1242/jeb.162099).

McBryan, T.L., Anttila, K., Healy, T.M., Schulte, P.M., 2013. Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. Integr. Comp. Biol. 53, 648–659. https://doi.org/10.1093/icb/ict066).

Meade, M.E., Watts, S.A., 1995. Toxicity of ammonia, nitrite, and nitrate to juvenile Australian crayfish, *Cherax quadricarinatus*. J. Shellfish Res. 14, 341–346.

Norin, T., Malte, H., Clark, T.D., 2013. Aerobic scope does not predict the performance of a tropical eurythermal fish at elevated temperatures. J. Exp. Biol. 217, 244–251. https://doi.org/10.1242/jeb.089755).

Northcote, T., 1995. Comparative biology and management of Arctic and European grayling (Salmonidae, Thymallus). Rev. Fish Biol. Fish. 5, 141–194. https://doi.org/10.1007/BF00179755).

Nyboer, E.A., Chapman, L.J., 2017. Elevated temperature and acclimation time affect metabolic performance in the heavily exploited Nile perch of Lake Victoria. J. Exp. Biol. 220, 3782–3793. https://doi.org/10.1242/jeb.163022).

Nyboer, E.A., Chapman, L.J., 2018. Cardiac plasticity influences aerobic performance and thermal tolerance in a tropical, freshwater fish at elevated temperatures. J. Exp. Biol. 221https://doi.org/10.1242/jeb.178087). jeb178087.

Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol. 5, 1538–1547. https://doi.org/10.1002/ece3.1465).

Pörtner, H.-O., Bock, C., Mark, F.C., 2017. Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. J. Exp. Biol. 220, 2685–2696. https://doi.org/10.1242/jeb.134585).

Reid, A.J., Carlson, A.K., Creed, I.F., Eliason, E.J., Gell, P.A., Johnson, P.T.J., et al., 2018. Emerging threats and persistent conservation challenges for freshwater biodiversity. Biol. Rev. 94, 849–873. https://doi.org/10.1111/brv.12480).

Rouse, J.D., Bishop, C.A., Struger, J., 1999. Nitrogen pollution: an assessment of its threat to amphibian survival. Environ. Health Perspect. 107, 799–803. https://doi.org/10.1289/ehp.99107799).

Sandblom, E., Gräns, A., Axelsson, M., Seth, H., 2014. Temperature acclimation rate of aerobic scope and feeding metabolism in fishes: implications in a thermally extreme future. Proceedings of the Royal Society B 281https://doi.org/10.1098/rspb.2014.1490). 20141490.

Scarano, G., Saroglia, M.G., 1984. Recovery of fish from functional and haemolytic anaemia after bried exposure to a lethal concentration of nitrite. Aquaculture 43, 421–426. https://doi.org/10.1016/0044-8486(84)90250-3).

Scarano, G., Saroglia, M.G., Gray, R.H., Tibaldi, E., 1984. Hematological responses of sea bass *Dicentrarchus labrax* to sublethal nitrite exposures. Trans. Am. Fish. Soc. 113, 360–364 doi: 10.1577/1548-8659(1984)113 < 360:HROSBD > 2.0.CO;2.

Schram, E., Roques, J.A.C., van Kuijk, T., Abbink, W., van de Heul, J., de Vries, P., et al., 2014. The impact of elevated water ammonia and nitrate concentrations on physiology, growth and feed intake of pikeperch (*Sander lucioperca*). Aquaculture 420–421, 95–104. https://doi.org/10.1016/j.aquaculture.2013.10.027).

Seebacher, F., White, C.R., Franklin, C.E., 2014. Physiological plasticity increases resilience of ectothermic animals to climate change. Nat. Clim. Chang. 5, 61–66. https://doi.org/10.1038/nclimate2457).

Shukla, S., Sexena, A., 2018. Global status of nitrate contamination in groundwater: its occurrence, health impacts, and mitigation measure. In: Hossain, C.M. (Ed.), Handbook of Environmental Materials Management (Pp. 1-21). Springer International Publishing AG.

Sinclair, B.J., Ferguson, L.V., Salehipour-shirazi, G., MacMillan, H.A., 2013. Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. Integr. Comp. Biol. 53, 545–556. https://doi.org/10.1093/icb/ict004).

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., 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. https://doi.org/10.1016/j.marenvres.2012.04.003).

Stormer, J., Jensen, F.B., Rankin, J.C., 1996. Uptake of nitrite, nitrate, and bromide in rainbow trout, (*Oncorhynchus mykiss*): effects on ionic balance. Can. J. Fish. Aquat. Sci. 53, 1943–1950. https://doi.org/10.1139/cjfas-53-9-1943).

Svendsen, M.B.S., Bushnell, P.G., Steffensen, J.F., 2015. Design and setup of intermittent-flow respirometry system for aquatic organisms. J. Fish Biol. 88, 26–50. https://doi.org/10.1111/jfb.12797).

Todgham, A.E., Stillman, J.H., 2013. Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. Integr. Comp. Biol. 53, 539–544. https://doi.org/10.1093/icb/ict086).

Todgham, A.E., Schulte, P.M., Iwama, G.K., 2005. Cross-tolerance in the tidepool sculpin: the role of heat shock proteins. Physiol. Biochem. Zool. 78, 133–144. https://doi.org/10.1086/425205).

van Bussel, C.G.J., Schroeder, J.P., Wuertz, S., Schulz, C., 2012. The chronic effect of nitrate on production performance and health status of juvenile turbot (*Psetta maxima*). Aquaculture 326–329, 163–167. https://doi.org/10.1016/j.aquaculture.2011.11.019).

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., et al., 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol. Appl. 7, 737–750 doi:10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2).

Wells, R.G.M., Weber, R.E., 1990. The spleen in hypoxic and exercised rainbow trout. J. Exp. Biol. 150, 461–466.