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Published in:
Aquatic Toxicology

DOI:
10.1016/j.aquatox.2019.05.008

Publication date:
2019

Document version:
Accepted manuscript

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Citation for published version (APA):

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Download date: 14. Sep. 2023
Impact and tissue metabolism of nitrite at two acclimation temperatures in striped catfish (*Pangasianodon hypophthalmus*)

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Abstract
Elevated concentrations of nitrite develop occasionally in various aquatic habitats and aquaculture facilities, providing a potential danger for freshwater fish that take up nitrite via the gill chloride uptake mechanism. We studied the uptake, effects and metabolism of nitrite in blood, heart and skeletal muscle at two temperatures in striped catfish *Pangasianodon hypophthalmus*, a facultative air-breathing fish that is heavily cultivated in Southeast Asia. Exposure to 0.8 mM ambient nitrite increased blood [nitrite] and [methaemoglobin] (metHb) to high values at day 1, but values subsequently decreased towards controls at day 7. Blood [nitrite] and metHb content were unexpectedly higher at 27 °C (~1.2 mM; 69% at day 1) than at 33 °C (~0.9 mM; 55%), reflecting a lower nitrite uptake at the highest temperature, possibly via an increased reliance on air-breathing relatively to water-breathing with temperature increase. A large fraction of the nitrite taken up was effectively eliminated by being detoxified to nitrate. Further, erythrocyte metHb reductase activity was increased during nitrite exposure, efficiently reducing metHb to functional haemoglobin. The uptake of nitrite into white skeletal musculature (main part of the fish) was much lower than into heart tissue. While heart [nitrite] was close to blood plasma levels, muscle [nitrite] peaked at ~0.2 mM at day 1 and subsequently declined to ~0.05 mM at day 7, which is below levels reported in various commercial cured meat products. Nitrite was partly metabolized to iron-nitrosyl, S-nitroso and N-nitroso compounds. The increase in nitros(yl)ated compounds was marginal in skeletal muscle and more pronounced in heart tissue.

Key words: Methaemoglobin reductase; Nitrite; Nitric oxide; Nitros(yl)ation; Temperature effects
1. Introduction

Nitrite has long been regarded as dangerous and problematic when present at high concentrations. In humans, concerns have mainly addressed the risk of developing methaemoglobinaemia (Jaffé, 1981) or formation of potential carcinogenic N-nitrosamines via nitrite and nitrate (that can be reduced to nitrite in the oral cavity) in ingested food or drinking water (Sindelar and Milkowski, 2012). Nitrite is, however, also naturally present in organisms, where it is formed as an oxidative metabolite of the nitric oxide (NO) produced by NO synthases. Research over the past two decades has revealed that low levels of nitrite can have positive effects on e.g. cardiovascular function and enhance cytoprotection, which typically relate to the formation of nitric oxide from nitrite (Lundberg et al., 2008). Thus, low amounts of nitrite can be beneficial, and nitrite and nitrate may even be considered dietary nutrients (Bryan and Ivy, 2015). There is accordingly a delicate distinction between toxic effects of nitrite at high concentrations and positive effects at low concentrations.

Freshwater fish are at particular risk of nitrite toxicity. Elevated concentrations of nitrite can occasionally develop in both natural aquatic habitats and in aquaculture facilities, due to imbalance in bacterial nitrification and/or denitrification processes (Eddy and Williams, 1987; Jensen, 2003; Tomasso, 2012). As nitrite competes with chloride at the active Cl⁻ uptake mechanism in freshwater fish gills (Williams and Eddy, 1986), an elevated ambient [nitrite] can cause nitrite accumulation in blood plasma to concentrations far in excess of ambient levels (Margiocco et al., 1983). Nitrite oxidizes blood hemoglobin (Hb) to methemoglobin (metHb) and forces [metHb] up to levels that become critical for oxygen transport; but a series of other respiratory, cardiovascular, ion-regulatory and endocrine disturbances are also induced (Jensen, 2003). The uptake of nitrite into tissue cells varies between organ types (Margiocco et al., 1983) and appears associated with an excess NO production from nitrite and nitrosative stress (Jensen and Hansen, 2011). Thus, nitrite becomes partly metabolized into S-nitroso (SNO), N-nitroso (NNO) and iron-nitrosyl (FeNO) compounds (Jensen and Hansen, 2011; Jensen et al., 2015). Surplus nitrosylation of vital proteins is likely to induce a broad variety of cellular malfunctions (Calabrese et al., 2009; Foster et al., 2009).

However, the uptake and metabolism of nitrite across tissue types has been studied in few fish species. Knowledge on this topic is relevant in understanding the overall physiological impact of nitrite, but also in the evaluation of safe levels of nitrite and the extent to which dangerous metabolites develop during episodes of nitrite exposure in farmed fish intended for human consumption.

A number of facultative air-breathing fish species (including P. hypophthalmus) are cultured intensively throughout Southeast Asia under conditions where water [nitrite] sometimes increases. In contrast to the gradual increases in blood [nitrite] and [metHb] with time reported in most strictly
water-breathing fish during nitrite exposure (Jensen, 2003), these facultative air-breathers, capable of supplementing water-breathing with air-breathing, increase blood [nitrite] and [metHb] for a limited time, where after values gradually decline (Lefevre et al., 2011; 2012; Gam et al., 2017; 2018). These fish may be able to reduce nitrite uptake during exposure, but they also appear effective in detoxifying nitrite to the relatively non-toxic compound nitrate. Furthermore, the clown knifefish Chitala ornata was recently shown to upregulate erythrocyte metHb reductase activity during nitrite exposure, promoting the decline in blood metHb (Gam et al., 2017). It is pertinent to evaluate if an increase in red blood cell (RBC) metHb reductase activity also occurs in other fish species exposed to nitrite.

In the present study we exposed striped catfish *P. hypophthalmus*, an intensively cultured fish of high commercial value, to water with low (control) and high (0.8 mM) nitrite levels for the evaluation of nitrite uptake and its metabolism to other metabolites in blood, white muscle and heart tissues. We also tested whether RBC metHb reductase is increased by nitrite exposure. Our studies were conducted at two different acclimation temperatures to gain insight into temperature effects. The influence of temperature on nitrite uptake has only rarely been addressed (Jeberg and Jensen, 1994; Kroupová et al., 2006) but is important to examine in relation to natural temperature changes and global warming. The acclimation temperature of 27 °C used in the present study is the current mean temperature in Vietnam (Gasparrini et al., 2017) and the higher temperature of 33 °C is a possible predicted temperature by the end of the 21st century due to climate change (IPCC, 2014). *P. hypophthalmus* thrive well at 33 °C with increased growth rates compared to 27 °C (Phuong et al., 2017). We hypothesized that elevated temperature would increase nitrite uptake and turnover.

2. Materials and methods

2.1 Animals and experimental design

*P. hypophthalmus* weighing 11.9 ±0.3 g (Mean ±SE, N=96) were purchased from a hatchery in Can Tho City, Viet Nam and acclimated in 2 m³ outdoor tanks for at least 2 weeks prior to experimentation. The fish were maintained in well-aerated water at 27 °C on a natural photoperiod, and fed commercial pellets (30% crude protein, 3-5% crude lipid; Cargill, USA) twice daily. Feeding was withheld one day prior to experimentation. During this initial acclimation, 50% of the water was exchanged twice daily. Tank water composition was: oxygen > 6 mg/L, pH = 7.04 - 7.08, total ammonia < 0.009 mM, water [Cl-] = 0.2 mM.

The experimental design was a 2×2 factorial design with two acclimation temperatures (27 °C and 33 °C) and two nitrite levels (low and high) and therefore included 4 exposure conditions: (1) water with low [nitrite] (natural level) at 27 °C (27°C – control), (2) water with high [nitrite] (0.8 mM) at 27 °C (27°C–0.8 mM), (3) water with low [nitrite] at 33 °C (33°C – control) and (4)
water with 0.8 mM nitrite at 33 °C (33°C–0.8 mM). All fish were initially maintained at 27 °C in 200 L tanks with low [nitrite] for 2 days. To achieve the 33 °C exposure condition, the water temperature was raised from 27 °C to 33 °C at a rate of 2 °C per day, using a heater. The fish were maintained at 33 °C for a further 2 days before experimentation. The high nitrite concentration (0.8 mM) was achieved by adding the required volume of a 1 M NaNO₂ solution. Fish density was 30 individuals per 200 L tank (100 L water), and the experimental unit was the individual fish. The water nitrite concentration was analyzed twice a day, using the Griess reaction (Lefevre et al., 2011) and subsequently extra nitrite was added to maintain the concentration close to 0.8 mM during experiments. In control tanks a small increase in water [nitrite] occurred over the experimental period (cf. Results) despite the daily water exchange, but values remained in the low micromolar range.

2.2 Sampling and sample analysis

Eight fish were collected from each experimental tank at day 0, 1 and 7. The fish were individually netted and anesthetized with water containing 0.1 g/L benzocaine. A blood sample was taken from caudal vessels with a heparinized syringe. The fish were subsequently euthanized by severing the spinal cord. Samples of white muscle and heart ventricle were dissected out, briefly washed with a phosphate-buffered saline [50 mmol l⁻¹ phosphate-buffer, pH 7.8, 85 mmol l⁻¹ NaCl, 2.4 mmol l⁻¹ KCl, 10 mmol l⁻¹ N-ethylmaleimide (NEM) and 0.1 mmol l⁻¹ diethyleetriaminepentaacetic acid (DTPA)], rapidly dried on a filter paper, weighed and frozen in liquid nitrogen. The blood sample was divided into two parts; the first was used to measure haematocrit (Hct) and haemoglobin (Hb) derivatives and the second centrifuged to collect plasma that was frozen in liquid nitrogen for subsequent measurement of nitrite and its metabolites.

Hct was measured using a haematocrit centrifuge (Sigma 201m-Hettich). For analysis of Hb derivatives, 20 µL of blood was mixed with 1.2 ml weak phosphate buffer (0.01 M, pH 7.3), and the haemolysate centrifuged for 3 min at 20.000 g. The supernatant was transferred to a 1.5 mL cuvette and scanned in a spectrophotometer (Varian Cary 50 Spectrophotometer, Varian Inc.) from 480 to 700 nm in 0.5 nm steps. The concentrations of oxyhaemoglobin (oxyHb), deoxyhaemoglobin (deoxyHb), methaemoglobin (metHb) and nitrosylhaemoglobin (HbNO) were evaluated by spectral deconvolution (Jensen, 2007; Lefevre et al., 2011) using reference spectra of the four Hb derivatives prepared from P. hypophthalmus Hb.

For measurements of nitrite and its metabolites, the tissues were thawed in four times their mass of a 50 mmol l⁻¹ phosphate- buffer (pH 7.3) containing 10 mmol l⁻¹ NEM and 0.1 mmol l⁻¹ DTPA to stabilize S-nitrosothiols (Yang et al., 2003). The samples were homogenized and
centrifuged (6 min, 16,000 g, 5 °C) and the supernatants transferred to new Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C until analysis.

Nitrite and its metabolites were analyzed by reductive chemiluminescence, using a Sievers (Boulder, CO, USA) Nitric Oxide Analyzer (NOA, model 280i) and previously described procedures to distinguish between nitrite, S-nitroso (SNO) compounds and the sum of iron nitrosyl (FeNO) and N-nitroso (NNO) compounds (Hansen and Jensen, 2010; Jensen and Hansen, 2011). Plasma nitrate was measured spectrophotometrically (Varian Cary 50 Spectrophotometer, Varian Inc.) using the Griess reaction (Lefevre et al., 2011) after reducing NO$_3^-$ to NO$_2^-$ with vanadium (III) chloride (Miranda et al., 2001).

### 2.3 Erythrocyte MetHb reductase activity

Erythrocyte metHb reductase activity and its potential increase during nitrite exposure were examined in a separate experiment with the same four experimental conditions described above.

Two tanks were used per treatment, and 60 fish (10-15 g) were randomly sorted into each of the eight 200 L tanks, containing 100 L water each. During the experiment, water was aerated, changed twice daily, and the fish fasted. Samples were collected at day 0, 1 and 7. At each sampling time, blood was drawn as described above from twenty fish from each tank. Due to the small fish size, blood from ten fish was pooled to obtain sufficient blood for each assay replicate. The 20 fish sampled at each sampling time and two tanks per treatment, produced four replicates at each sampling time in all the four treatments.

The assay for determining metHb reductase activity in intact red blood cells (RBCs) was recently described in detail (Jensen and Nielsen, 2018) and was also used by Gam et al., (2017). To determine RBC metHb reductase activity in control fish (27°C-control and 33°C-control treatments), freshly drawn whole blood from control fish was centrifuged and the plasma removed. The RBCs were washed three times with cold ringer solution [NaCl, 120 mM; KH$_2$PO$_4$, 1.2 mM; MgSO$_4$.6 H$_2$O, 1 mM; CaCl$_2$.2H$_2$O, 2 mM; D-glucose, 6 mM; NaHCO$_3$, 20 mM; Albumin, 14 mg/mg (bovine serum albumin; Sigma-Aldrich)]. After washing, the RBCs were re-suspended in the same ringer to a Hct of approximately 20% and equilibrated for 45 min in a shaking glass tonometer to a humidified gas mixture of 99% air and 1% CO$_2$ delivered from a Wösthoff gas mixing pump (Wösthoff, Bochum, Germany). Temperatures were 27 °C and 33 °C as appropriate. After 45 min of equilibration, 250 µL RBC suspension was pipetted for measurements of Hct and Hb derivatives. The Hct value was used to calculate the volume of added 140 mM NaNO$_2$ stock solution necessary to produce an extracellular [NO$_2^-$] of 3 mM in the tonometers. Nitrite was added to elevate the metHb content of control RBCs. Following nitrite addition, 20 µL samples were drawn from the tonometer to follow the rise in metHb content with time, using spectral
deconvolution (cf. above), until a metHb content of 20% - 40% of total Hb was reached. This typically happened within 210 min. At this time, the RBC suspension was transferred from the tonometer into 1.5 mL Eppendorf tubes that were filled to the top and closed to maintain the equilibration. The Eppendorf tube was centrifuged and the supernatant removed and replaced with an equal volume of ringer (equilibrated to 99% air/1% CO₂), whereupon the cells were re-suspended. This washing of the RBCs was performed three times to ensure removal of nitrite from the RBC suspension (Jensen and Nielsen, 2018). The RBC suspension was then returned to the tonometer (time zero) for continued equilibration. Samples of RBC suspension were subsequently taken at specified times to follow the reduction of metHb with time (resulting from RBC metHb reductase activity). The logarithmic values of the measured metHb levels were plotted as a function of time, which resulted in a linear relationship. The rate constant \( k \) (min\(^{-1}\)) for erythrocyte metHb reduction was accordingly calculated from the slope \((k = -\text{slope/\log(e)})\) and used to characterize the RBC metHb reductase activity (Jensen and Nielsen, 2018).

In nitrite-exposed fish, blood metHb levels were elevated during exposure. Freshly drawn RBCs from exposed fish could therefore be immediately washed three times with nitrite-free ringer (to remove nitrite) and transferred to the tonometer equilibrated to 99% air and 1% CO₂ to follow the decline in metHb with time as described above.

2.4 Statistics

All data were analyzed using PASW statistics (SPSS 16.0). Statistical significance of all parameters were assessed by two-way ANOVA. In the metHb reductase activity experiments, the logarithm of metHb content was plotted against time and analyzed with linear regression to determine the slope used for calculation of the rate constant. Differences were considered significant at \( p < 0.05 \). Results are shown as means ± SE.

3. Results

MetHb levels in *P. hypophthalmus* were low in the two control groups (<0.5% of total Hb) throughout the experimental period, but was significantly increased by nitrite exposure at both 27 °C and 33 °C after 1 day (Fig. 1A). Interestingly, metHb rose to higher levels at 27 °C (68.9±1.3% of total Hb) than at 33 °C (55.3±2.6% of total Hb). Following the peak at day 1, metHb decreased to 5.9±1.1% and 5.2±1.8% at day 7. The changes in HbNO during nitrite exposure showed a similar pattern as that of metHb. At day 1, HbNO was significantly increased to 10.7±1.1% at 27 °C and 7.0±1.1% at 33 °C, where after levels reversed towards control levels at day 7 (Fig. 1B). Blood total Hb and Hct were constant in controls but decreased transiently and significantly at day 1 of nitrite exposure at both the experimental temperatures (Fig. 1C, 1D).
The water nitrite level was relatively stable at around 800 µM during exposure to elevated [nitrite] (Fig. 2A). In the control groups, water [nitrite] was low, but increased slightly during the 7 days experimental period in spite of daily water exchanges (Fig. 2A). Exposure to elevated [nitrite] caused a significant increase in plasma nitrite to values slightly above the ambient level (from 0.9 µM to 1184 µM at 27 °C and from 1.6 µM to 892 µM at 33 °C) at day 1 (Fig. 2B). Plasma nitrite subsequently decreased towards control values at day 7 (Fig. 2B). Interestingly, plasma [nitrite] in nitrite-exposed fish was higher at 27 °C than at 33 °C throughout the experiment. Control animals experienced only slight plasma [nitrite] elevation during the 7 day experiment (from around 1 µM to around 10 µM). The increase in plasma nitrite was associated with significant increases in the concentration of nitrite metabolites in plasma. Plasma nitrate increased significantly to the millimolar range during nitrite exposure at day 1, and continued to increase until experimental termination at day 7 (Fig.2C), reaching 3999 µM and 3585 µM in the 27 °C and 33 °C exposure groups, respectively. The sum of plasma [NO₂⁻] and [NO₃⁻], which can be taken as an indicator of total nitrite uptake, showed a major increase at day 1, but values then levelled off and showed only slight further increase to day 7 (Fig 2D). At day 1, plasma [SNO] and [FeNO + NNO] rose to low micromolar values that again were higher at 27 °C (5 µM and 0.7 µM) than at 33 °C (3.55 µM and 0.43 µM), after which their levels also decreased towards the control level at day 7 (Fig. 2E, Fig. 2F).

Nitrite was taken up into white muscle in exposure groups, producing a significantly higher muscle nitrite concentration at 27 °C (289 µM) compared to 33 °C (207 µM) on day 1 (Fig. 3A). The mean nitrite concentrations in muscle were lower than in plasma, and following the peak at day 1, the concentrations declined at day 7 (Fig. 3A). The increase of muscle nitrite caused small elevations in muscle SNO and muscle FeNO+NNO compounds (Fig. 3B, 3C). The levels were, however, low and similar to values attained in controls at day 7 (Fig. 3B, 3C).

Nitrite exposure caused a profound increase in [nitrite] in the heart ventricle, reaching mean values of 1119 µM at 27 °C and 701 µM at 33 °C on day 1, after which values decreased to 110 µM and 79 µM, respectively, on day 7 (Fig. 4A). In control fish, heart [nitrite] was slightly elevated from 1.5 µM to 9 µM at day 7 in parallel with the minor increase in plasma [nitrite] in these fish (cf. above). In general, heart [nitrite] was similar to but slightly lower than the plasma nitrite concentration. Nitrite exposure caused a small but significant increase in SNO compounds inside the heart tissue (Fig. 4B). Further, there was a major significant elevation of [FeNO+NNO] at day 1 of nitrite exposure, reaching values of 79.4 µM at 33 °C and 149 µM at 27 °C (Fig. 4C). The levels subsequently decreased at day 7 but stayed significantly elevated above controls.

To investigate relationships between plasma and tissue (white muscle and heart) nitrite concentrations, we made a double logarithmic plot of tissue nitrite concentrations in individual fish
from all four experimental groups against the corresponding plasma nitrite concentrations (Fig. 5). There was a highly significant correlation between log[NO$_2^-$]$\text{muscle}$ and log[NO$_2^-$]$\text{plasma}$, with the points from control and nitrite-exposed fish grouping around the same regression line at both temperatures (Fig. 5A). Thus the relationship could be described by a single equation that applied to all tested conditions: log[NO$_2^-$]$\text{muscle}$ = 0.754 × \text{log}[\text{NO}_2^-]_{\text{plasma}} + 0.006 (R^2 = 0.934; P < 0.001). There was also a single highly significant correlation between heart nitrite and plasma nitrite given by the equation: log[NO$_2^-$]$\text{heart}$ = 0.896 × \text{log}[\text{NO}_2^-]_{\text{plasma}} + 0.086 (R^2 = 0.931; P < 0.001); Fig. 5B). The slope of the regression line was considerably higher in the heart (0.896) than in white muscle (0.754), reflecting a higher increase of [NO$_2^-$]$\text{heart}$ than [NO$_2^-$]$\text{muscle}$ with rising [NO$_2^-$]$\text{plasma}$.

The sum of nitros(yl)ated metabolites ([SNO] + [FeNO+NNO]) showed a correlation with the tissue nitrite concentration in both white muscle (Fig. 6A) and the heart (Fig. 6B). The slope of the regression line in the double logarithmic plot was much higher for the heart than for white muscle, reflecting a larger metabolism of nitrite into nitros(yl)ated compounds in the heart.

The potential increase of erythrocyte metHb reductase activity during nitrite exposure was evaluated by determining the first order rate constants for metHb reduction in all four experimental groups (Fig. 7). The $k$ values for erythrocyte metHb reduction stayed constant with time in the control groups but increased with time during nitrite exposure. At 27 °C, $k$ increased from 0.011 min$^{-1}$ to 0.0164 min$^{-1}$ at day 7, while the increase was from 0.031 min$^{-1}$ to 0.0428 min$^{-1}$ at 33 °C. Thus, there was a significant increase of erythrocyte metHb reductase activity during nitrite exposure. The data furthermore provides insight into the general temperature sensitivity of RBC metHb reductase activity. Merging the $k$ values at day 0 from the two 27 °C groups (both in absence of added nitrite at this time point) and the $k$ values from the two 33 °C groups, allowed the calculation of $Q_{10}$ between 27 °C and 33 °C as $Q_{10} = (k_{33°C}/k_{27°C})^{(10/(33-27))}$, which was found to be 5.7, revealing a very strong temperature sensitivity.

4. Discussion

The general time course of plasma nitrite and metHb formation observed in this study closely resembles that seen in previous studies on facultative air-breathing fish, with an initial increase during the first day of nitrite exposure followed by a subsequent decline despite the continued exposure to ambient nitrite (Lefevre et al., 2011, 2012; Gam et al., 2017, 2018). MetHb and plasma nitrite in the present study peaked at day 1 but were only slightly elevated above the control values at day 7. Nitrite reacts with oxyHb to form nitrate and metHb, but functional Hb is reformed from metHb by metHb reductase activity inside the erythrocytes, so this series of reactions can be considered a detoxification mechanism if metHb reduction can keep pace with Hb oxidation (Doblander and Lackner, 1997; Jensen, 2003). It has previously been suggested that
metHb reductase activity might be increased during nitrite exposure in *P. hypophthalmus* (Lefevre *et al.*, 2011, 2012), and an upregulation was indeed later observed in the osteoglossiform air-breathing fish *C. ornata* (Gam *et al.*, 2017). The significant rise in *k* values for metHb reduction during nitrite exposure in the present study (Fig. 7) documents that metHb-reductase activity is also increased by nitrite exposure in *P. hypophthalmus*. Further, the rate constants (*k* values) for erythrocyte metHb-reduction in *P. hypophthalmus* at 27 °C before and after nitrite exposure are rather similar to those reported in *C. ornata* exposed to 1 mM nitrite at 25 °C, where *k* increased from 0.01 min⁻¹ before exposure to 0.019 min⁻¹ at day 6 days of nitrite exposure (Gam *et al.*, 2017). The rise in erythrocyte metHb reductase activity speeds up the reduction of metHb to functional Hb, which enables both a decline in [metHb] and a continued high detoxification of nitrite to nitrate via the oxyHb reaction, which helps to decrease [nitrite]. This is not, however, the full explanation for the decrease in nitrite concentrations between day 1 and day 7. The major increase in the sum of plasma [NO₂⁻] and [NO₃⁻] on day 1, followed by a levelling off of values between day 1 and day 7 (Fig. 2D), suggests that the total nitrite uptake from the environment could be reduced over the course of nitrite exposure, which enables a gradual decline in [nitrite] via its metabolism to other compounds (including nitrate). A further contributing factor could be increased excretion of nitrite and nitrate in the kidney, but this remains to be established in fish.

Interestingly, plasma [nitrite] and metHb elevations during the initial stages of exposure were more pronounced at 27 °C than at 33 °C. This result was unexpected, because our original hypothesis was that a temperature elevation would induce a general increase in branchial ion exchange rates, and hence more rapid nitrite uptake at 33 °C. An increased accumulation of nitrite at elevated temperature was previously observed in crayfish (Jeberg and Jensen, 1994) and common carp (Kroupová *et al.*, 2006). However, during nitrite exposure in *P. hypophthalmus*, [nitrite] increased to higher values at 27 °C than at 33 °C in both plasma, skeletal muscle and heart muscle (Fig. 2B, 3A, 4A). Similarly, plasma nitrate concentrations were higher at 27 °C than at 33 °C during nitrite exposure (Fig. 2C). A possible explanation for this would be the ability of this species to shift its mode of oxygen uptake. Data on the partitioning of oxygen uptake with temperature increase do not exist in *P. hypophthalmus*, but a common pattern among facultative air-breathing fish is an increased reliance on air-breathing relatively to water-breathing as water temperature increases. This pattern has been observed in several air-breathing fish (Horn and Riggs, 1973; Roth and Gerardus, 1973; Vivekanandan and Pandian, 1977; Patra *et al*., 1983; Geiger *et al*., 2000). Hence, it is conceivable that as *P. hypophthalmus* gives higher priority to oxygen uptake via the air-breathing organ at elevated temperature, the relative gill perfusion is reduced, hence reducing branchial nitrite uptake.
After nitrite is taken up into the plasma across the gill epithelium, it becomes distributed throughout the body and transported into tissues. The amount of nitrite entering tissue cells depends on tissue type. Thus, we observed a considerably higher level of nitrite in cardiac muscle (Fig. 4A) compared to skeletal muscle (Fig. 3A). The heart tissue may have higher perfusion rate than skeletal muscle, but both tissues are amply perfused to sustain metabolism, and cells in both tissues experience the same extracellular nitrite concentrations. Nitrite can be expected to show a tissue-specific steady-state equilibrium distribution between intracellular and extracellular compartments at any given time (Jensen and Rohde, 2010). The extracellular fluid, consisting of plasma (in capillaries) and interstitial fluid (bathing the cells), constitutes a similar percentage of total tissue water in the two tissues, and this percentage is low compared to the percentage of intracellular fluid. Tissue nitrite concentrations were measured on tissue homogenates and therefore comprise nitrite in both extracellular and intracellular fluid. However, as intracellular fluid dominates tissue water content, the reported values will largely reflect cellular values. Nitrite concentrations in the heart were close to plasma levels, while [nitrite] in skeletal muscle was considerably below plasma [nitrite]. This reduced level of nitrite in skeletal muscle compared to other tissues seems to be a general trend in nitrite-exposed fish (Margiocco et al., 1983; Jensen and Hansen, 2011; Jensen et al., 2015). There was a strong correlation between tissue [nitrite] and plasma [nitrite] in both the skeletal muscle and heart (Fig. 5), but the slope of the log[NO$_2^-$]$_{tissue}$ vs. log[NO$_2^-$]$_{plasma}$ relationship is much lower in skeletal muscle, reflecting a restricted rise in muscle [NO$_2^-$] as plasma [NO$_2^-$] increases. The slope of 0.754 in skeletal muscle in *P. hypophthalmus* (Fig. 5) is strikingly similar to that found in goldfish (0.76) (Jensen and Hansen, 2011), while the slope of 0.9 in the heart (Fig. 5) compares well with that reported for red blood cells in goldfish (0.92) (Jensen and Hansen, 2011). It may be speculated that low skeletal muscle [nitrite] relates to the fact that fish have a predominance of white musculature with relatively low myoglobin and mitochondria contents, because nitrite has been shown to increase to lower levels in white muscle compared to red muscle (with high myoglobin and mitochondria contents) during deep hypoxia in crucian carp (Hansen et al., 2016). Whether this difference also applies during nitrite exposure, however, remains to be studied.

Within tissue cells, nitrite is metabolized to a variety of compounds. Some cellular proteins mediate reduction of nitrite to NO (Lundberg et al., 2008); leading to iron-nitrosylation (FeNO) of heme proteins, and nitrite accumulation also causes increased nitrosation of cellular thiols (SNO) and amines (NNO). We observed only modest rises in [SNO] and [FeNO+NNO] in skeletal muscle (Fig. 3) but more pronounced elevations in the heart (Fig. 4), which compares well with observations in nitrite-exposed brown trout (Jensen et al., 2015) and goldfish (Jensen and Hansen, 2011). Log([SNO+FeNO+NNO]) was significantly correlated with tissue log[NO$_2^-$] in both tissues,
and the steeper slope in the heart reflects the more extensive cellular nitrosylation in this tissue at
elevated [nitrite] (Fig. 6). The elevation of cellular [SNO] supports the notion that nitrite
accumulation can induce nitrosative stress (Jensen and Hansen, 2011), where excess S-nitrosation
of various vital proteins induce physiological malfunctions (Calabrese et al., 2009; Foster et al.,
2009). Similarly, excess nitrosylation of heme proteins inhibits their function. In the heart, the high
[FeNO+NNO] in nitrite-exposed fish (Fig. 4C) is probably dominated by high [FeNO], with NO
bound to myoglobin and mitochondrial cytochromes (both present at high levels in cardiac muscle).
When cellular [nitrite] declined between day 1 and day 7 (Fig. 4A), cellular [FeNO+NNO] also
decreased (Fig. 4C), reflecting slow reversal of heme iron nitrosylation, whereas S-nitrosated
compounds stayed elevated (Fig. 4B).

From a food safety perspective it appears gratifying that the level of nitrite and its
metabolites are at their lowest in white muscle, the principal part of the fish used for human
consumption. Thus, occasional exposure of fish to low nitrite levels in aquaculture facilities would
not likely lead to critical concentrations in the main muscle mass. The current level of residual
nitrite in cured meat products such as cooked sausages, bacon and ham is around 7 ppm (mg/kg)
(Sindelar and Milkowski, 2012), which corresponds to some 150 µM (with a molar weight of nitrite
of 46 g/mol and 1 kg wet weight ~ 1 l). The muscle [nitrite] values observed in nitrite-exposed P.
hypophthalmus are slightly above this level at day 1 but reduced to well below this level at day 7
(Fig. 3A). Thus, the observed concentrations of nitrite in the muscle do not appear particularly
alarming, given that a relatively high exposure concentration was used, and that most natural
exposures would involve lower water [nitrite]. In humans, the daily consumption of nitrite is mainly
(>90%) due to reduction of salivary nitrate to nitrite by commensal bacteria in the oral cavity
(Lundberg et al., 2008) and only a small part is due to nitrite contained in meat and vegetables
(Sindelar and Milkowski, 2012). A source of concern regarding nitrite (and nitrate) intake in
humans has been the potential to produce carcinogenic N-nitrosamines. It is known that certain N-
nitrosamines are mutagenic and carcinogenic, but a firm causal link between nitrite or nitrate intake
and human cancers remains to be established (Bryan, 2006; Bryan and Ivy, 2015; Lundberg et al.,
2008). N-nitrosamines can be endogenously synthesized or contained in the diet. In the present
study we did observe a slight elevation of [FeNO+NNO] to about 1 µM in white muscle (Fig. 3C).
A significant fraction of this will be FeNO, but the analytical technique cannot discriminate
between FeNO and NNO. Future studies using alternative techniques are required to decipher
whether critical levels of N-nitrosamines are reached in muscles of nitrite-exposed fish.

The control k values for erythrocyte metHb reductase activity in P. hypophthalmus at 27 °C
(Fig. 7) are similar to values observed in clown knifefish (Gam et al., 2017), carp and rainbow trout
(Jensen and Nielsen, 2018) at 25 °C. The k values for erythrocyte metHb reduction exceed k for
autoxidation of fish haemoglobins (Jensen, 2001) by at least one order of magnitude, and the high
temperature sensitivity of metHb reductase activity (Fig. 7; Jensen and Nielsen, 2018) matches a
similarly high temperature sensitivity of hemoglobin autoxidation (Jensen, 2001). Thus, the RBCs
seem to have sufficient metHb reductase capacity to effectively combat Hb oxidation in most
situations. It is only when oxidizing agents (e.g. nitrite) increase to undue levels that elevated
metHb levels occur. Most water-breathing freshwater fish show a gradual increase in blood [NO\textsubscript{2}^-]
with time during nitrite exposure, which disturbs the normal balance between Hb oxidation and
reduction, eventually forcing metHb concentrations up to intolerable levels (Jensen, 2003).
Facultative air-breathing striped catfish and clown knifefish oppose this outcome partly via an
increase of RBC metHb reductase activity during nitrite exposure (Fig. 7; Gam \textit{et al.}, 2017). It
would be of interest to study to what extent water-breathing fish can upregulate erythrocyte metHb
reduction during oxidative stress.

\section*{Acknowledgements}
The research was funded by Interdisciplinary Project on Climate change in Tropical Aquaculture
(iAQUA), the Danish Ministry of Foreign Affairs (DANIDA).

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nitrite tolerance in the clown knifefish \textit{Chitala ornata} is linked to up-regulation of


Figure legends

Fig. 1. (A) Percentage methaemoglobin (metHb), (B) percentage nitrosylhaemoglobin (HbNO), (C) total hemoglobin concentration (as heme) and (D) haematocrit (Hct) in whole blood of *P. hypophthalmus* during exposure to low (~0 mM, control) and high (~0.8 mM) ambient [nitrite] at 27 °C and 33 °C. Values are means ± SE (N=8 at individual points). On individual days, a significant influence of temperature is indicated by asterisks (*), and a significant effect of nitrite concentration is shown by number sign (#). Interaction between nitrite and temperature factors is specified by plus sign (+).

Fig. 2. Concentration of (A) nitrite in ambient water, and plasma concentrations of (B) nitrite, (C) nitrate, (D) the sum of nitrite and nitrate, (E) S-nitroso (SNO) compounds and (F) the sum of iron-nitrosyl and N-nitroso (FeNO+NNO) compounds in *P. hypophthalmus* during exposure to low (~0 mM, control) and high (~0.8 mM) ambient [nitrite] at 27 °C and 33 °C. Other details are as in Fig. 1.

Fig. 3. Muscle concentrations of (A) nitrite, (B) SNO compounds and (C) FeNO+NNO compounds in *P. hypophthalmus* during exposure to low (~0 mM, control) and high (~0.8 mM) ambient [nitrite] at 27 °C and 33 °C. Other details are as in Fig. 1.

Fig. 4. Heart concentrations of (A) nitrite, (B) SNO compounds and (C) FeNO+NNO compounds in *P. hypophthalmus* during exposure to low (~0 mM, control) and high (~0.8 mM) ambient [nitrite] at 27 °C and 33 °C. Other details are as in Fig. 1.

Fig. 5. (A) Relationship between muscle [nitrite] ([NO$_2^-$]$_{\text{muscle}}$) and plasma [nitrite] ([NO$_2^-$]$_{\text{plasma}}$). The shown regression line includes individual points from the two control groups and the two nitrite exposure groups at temperatures 27 °C (black) and 33 °C (red). The regression equation is: log[NO$_2^-$]$_{\text{muscle}}$ = 0.754 × log[NO$_2^-$]$_{\text{plasma}}$ + 0.006 (R$^2$ =0.934; P<0.001). (B) Relationship between heart [nitrite] ([NO$_2^-$]$_{\text{heart}}$) and plasma [nitrite] ([NO$_2^-$]$_{\text{plasma}}$). The equation for this regression is log[NO$_2^-$]$_{\text{heart}}$ = 0.896 × log[NO$_2^-$]$_{\text{plasma}}$ + 0.086 (R$^2$ =0.931; P<0.001).

Fig. 6. (A) Relationship between the muscle concentration of nitros(yl)ated metabolites ([SNO+FeNO+NNO]$_{\text{muscle}}$) and muscle [nitrite] ([NO$_2^-$]$_{\text{muscle}}$). The regression includes individual points from the two control groups and the two nitrite exposure groups at temperatures 27 °C (black) and 33 °C (red) and has the equation: log([SNO] + [FeNO+NNO])$_{\text{muscle}}$ = 0.280 × log[NO$_2^-$]$_{\text{muscle}}$ - 0.665 (R$^2$ =0.348; P<0.001). (B) Relationship between the heart concentration of
nitrosylated metabolites ([SNO+FeNO+NNO]_heart) and nitrite ([NO₂⁻]_heart). The equation for this regression is: \[ \log([\text{SNO}] + [\text{FeNO}+\text{NNO}])_{\text{heart}} = 0.747 \times \log([\text{NO₂⁻}])_{\text{heart}} - 0.071 \] (\(R^2 = 0.895\); \(P<0.001\)).

Fig. 7. First-order rate constant \(k\) (min\(^{-1}\)) for erythrocyte metHb reduction in *P.hypophthalmus* from the four experimental groups. Values are means ± SE (\(N=4\) at individual points). On individual days, a significant influence of temperature is indicated by asterisks (*), and a significant effect of nitrite concentration is shown by number sign (#).
Fig. 1
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.

Fig. 7.