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RESEARCH ARTICLE

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

Frank B. Jensen*, Lucie Gerber, Marie N. Hansen and Steffen S. Madsen

ABSTRACT

Nitrite secures essential nitric oxide (NO) bioavailability in hypoxia at low endogenous concentrations, whereas it becomes toxic at high concentrations. We exposed brown trout to normoxic and hypoxic water in the absence and presence of added ambient nitrite to decipher the cellular metabolism and effects of nitrite at basal and elevated concentrations under different oxygen regimes. We also tested hypotheses concerning the influence of nitrite on branchial nitric oxide synthase (*NOS*), Na^+/K^+-ATPase (*nka*) and heat shock protein (*hsp70*) mRNA expression. Basal plasma and erythrocyte nitrite levels were higher in hypoxia than normoxia, suggesting increased NOS activity. Nitrite exposure strongly elevated nitrite concentrations in plasma, erythrocytes, heart tissue and white muscle, which was associated with an extensive metabolism of nitrite to nitrate and to iron-nitrosylated and *S*-nitrosated compounds. Nitrite uptake was slightly higher in hypoxia than normoxia, and high internal nitrite levels extensively converted blood hemoglobin to methemoglobin and nitrosylhemoglobin. Hypoxia increased inducible NOS (*iNOS*) mRNA levels in the gills, which was overruled by a strong inhibition of *iNOS* expression by nitrite in both normoxia and hypoxia, suggesting negative-feedback regulation of *iNOS* gene expression by nitrite. A similar inhibition was absent for neuronal *NOS*. Branchial NKA activity stayed unchanged, but mRNA levels of the *nkaα1a* subunit increased with hypoxia and nitrite, which may have countered an initial NKA inhibition. Nitrite also increased *hsp70* gene expression, probably contributing to the cytoprotective effects of nitrite at low concentrations. Nitrite displays a concentration-dependent switch between positive and negative effects similar to other signaling molecules.

KEY WORDS: Hypoxia, Na^+/K^+-ATPase, Nitric oxide, Nitrite, NOS, HSP70

INTRODUCTION

Nitrite is a double-edged sword in animal biology that exerts positive effects at low concentrations and harmful effects at high concentrations. Nitrite is generated endogenously as an oxidative metabolite of the signaling molecule nitric oxide (NO) produced by nitric oxide synthases (NOS), giving rise to nitrite concentrations at or below the micromolar range in blood and tissues of fish (Hansen and Jensen, 2010; Sandvik et al., 2012) and mammals (Bryan et al., 2005). The natural pool of nitrite functions as a reservoir of NO availability, from which NO can be regenerated under hypoxic conditions via a number of cellular proteins (including deoxygenated hemoglobin and myoglobin) that reduce nitrite to NO, allowing a continued NO production when NOS enzymes become compromised by a lack of O_2 (Lundberg et al., 2008; Jensen, 2009). This route of NO generation contributes to hypoxic vasodilation, regulation of mitochondrial respiration and cytoprotection (Lundberg et al., 2008) and is extensively exploited in hypoxia-tolerant fish during severe hypoxia (Hansen and Jensen, 2010; Sandvik et al., 2012).

Freshwater fish also take up nitrite actively across the gills, causing an excessive uptake in nitrite-contaminated water, which leads to toxic internal concentrations (Jensen, 2003). The toxicity and physiological disturbances induced by nitrite have been widely studied in fish because of the potential risk of nitrite built-up in both natural aquatic ecosystems and aquaculture systems (Lewis and Morris, 1986; Jensen, 2003). Nitrite is taken up in competition with chloride for the active Cl^--uptake mechanism in the gills of freshwater fish, and uptake rates vary with species-specific requirements for Cl^- (Williams and Eddy, 1986; Tomasso and Grosell, 2005) and ambient conditions, such as [Cl^-], temperature and CO_2 tensions (Jensen, 2003). As nitrite gradually accumulates in the blood during nitrite exposure, a severe methemoglobinemia develops, because nitrite-induced oxidation of functional hemoglobin (Hb) to methemoglobin (metHb) increasingly exceeds the capacity of erythrocyte metHb reductase to reduce metHb back to functional Hb. Certainly, methemoglobinemia is a central physiological problem during nitrite exposure, but additional cardiorespiratory, ion regulatory and endocrine disturbances are also induced (Jensen, 2003).

Recent studies have addressed the possibility that nitrite, when accumulated to high concentrations, causes an excess of NO generation that contributes to nitrite toxicity. Indeed, the enhanced nitrite reduction to NO during nitrite exposure is associated with elevated levels of nitrosyl hemoglobin (HbNO, with NO tightly bound to heme), which – together with the elevated metHb levels – contributes significantly to lowering of the blood O_2-carrying capacity (Jensen, 2007; Jensen and Hansen, 2011; Lefevre et al., 2011). Nitrite accumulation also induces iron-nitrosylation (FeNO) of other heme proteins as well as extensive nitrosation of cellular thiols (forming SNO) and amines (forming NNO), suggestive of nitrosative stress and malfunctioning of critical proteins (Jensen and Hansen, 2011). Such effects of nitrite exposure are likely to differ in normoxic and hypoxic environments, because hypoxia promotes NO formation from nitrite, and because hypoxia increases the respiratory surface area of the gills, which may increase the branchial ion permeability and hence the need for active ion uptake.

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List of symbols and abbreviations

EF1α	elongation factor 1α
FeNO	iron-nitrosyl
Hb	hemoglobin
HbNO	nitrosylhemoglobin
HSP70	heat shock protein 70
iNOS	inducible nitric oxide synthase
metHb	methemoglobin
NKA	Na^+/K^+-ATPase
NNO	*N*-nitroso compound
nNOS	neuronal nitric oxide synthase
P_{O_2}	oxygen tension
SNO	*S*-nitroso compound

This idea has so far only been investigated in goldfish, where surprisingly, nitrite uptake was found to be reduced rather than increased during hypoxia (Jensen and Hansen, 2011). This may be a special feature related to the extensive gill rearrangements that occur in goldfish (and crucian carp) during hypoxia (Nilsson, 2007), which is associated with a reduced rather than increased rate of active Cl^- uptake (Mitrovic et al., 2009). Thus, it is pertinent to investigate fish without such gill remodeling, such as trout, which rely on traditional lamellar recruitment (Booth, 1979; Soivio and Tuurala, 1981) for increasing gill surface area in hypoxia.

Nitrite intermingles with several physiological processes, and the study of these at both low and high nitrite concentrations can help to identify these mechanisms and their potential roles and consequences under both natural and toxic scenarios. In the present study, we examined effects and metabolism of nitrite at low (basal) and high (exposed) concentrations under both normoxic and hypoxic conditions, using a species – brown trout (*Salmo trutta* Linnaeus 1758) – that is comparatively intolerant of hypoxia. The aim was to gain new comparative knowledge on basal NO metabolite levels and on the uptake and metabolism of nitrite in blood and tissues under different oxygen regimes. We also tested specific new hypotheses that relate to the NO-generating power of nitrite. Thus, NO has been shown to inhibit branchial Na^+/K^+-ATPase (NKA) activity in brown trout (Tipsmark and Madsen, 2003), and we predicted that nitrite would exert a similar inhibition, and that this would become reflected in a reduced branchial NKA activity and increased *nka* mRNA expression. We also hypothesized the existence of crosstalk between nitrite and the NOS reaction, so that nitrite – as a natural product of the NOS reaction – exerts a negative-feedback inhibition on *NOS* gene expression. As fish lack an endothelial *NOS* gene, this idea was tested through measurements of the branchial mRNA expression of the two NOS isoforms that are present in fish (Andreakis et al., 2011): neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2). Finally, since it has been shown that nitrite upregulates gene expression of cytoprotective heat shock protein 70 (HSP70) in mammalian tissues (Bryan et al., 2005), we hypothesized that nitrite exposure would induce increased *hsp70* mRNA expression in fish.

RESULTS

Exposure to normoxia (P_{O_2}>140 mmHg), hypoxia (P_{O_2}=70 mmHg) or nitrite (1 mmol l^{-1}) in normoxic water for 24 h did not cause mortality, whereas 3 out of 8 fish died during 24 h combined exposure to nitrite and hypoxia. Total blood Hb did not differ significantly between the four experimental groups (Fig. 1A). MetHb was below 0.5% of total Hb in normoxic and hypoxic control fish, whereas it increased to 64% of total Hb after 24 h exposure to 1 mmol l^{-1} ambient nitrite under both normoxic and hypoxic conditions (Fig. 1B). Blood [HbNO] also increased during nitrite exposure, reaching mean values of 7 and 9% of total Hb in normoxic and hypoxic fish (Fig. 1C).

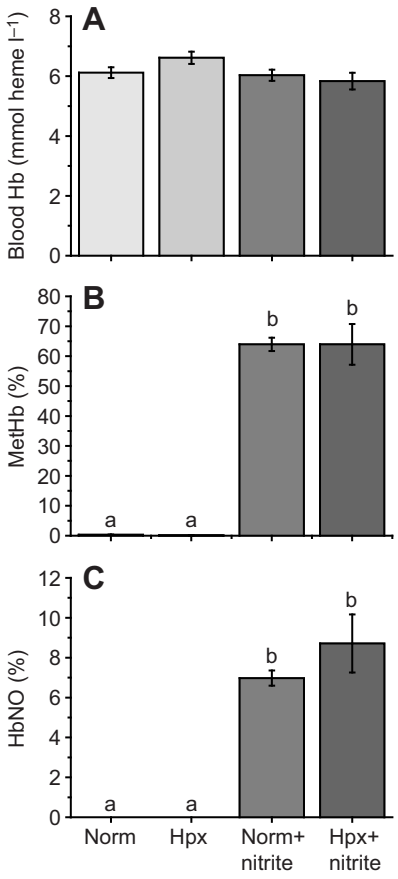

Fig. 1. Total hemoglobin, methemoglobin and nitrosylhemoglobin in brown trout under different oxygen and nitrite regimes. Total Hb concentration (A), metHb content (B) and HbNO content (C) in whole blood of brown trout in normoxic (Norm; P_{O2}>140 mmHg) and hypoxic (Hpx; 1 day at P_{O_2}=70 mmHg) water and after 1 day exposure to 1 mmol l^{-1} ambient nitrite in normoxic and hypoxic water. Values are means±s.e.m. (*N*=7 in normoxia; *N*=8 in hypoxia; *N*=8 in normoxia+nitrite; *N*=5 in hypoxia+nitrite). Different letters indicate a significant difference (P<0.05).

Basal plasma [nitrite] was 0.33 µmol l^{-1} in normoxic fish and increased slightly but significantly to 0.66 µmol l^{-1} after 24 h hypoxia (Fig. 2A). Exposure to nitrite for 24 h was associated with pronounced increases in plasma [nitrite] to values around 2.6 mmol l^{-1} (Fig. 2A). Thus, nitrite was accumulated to values above the ambient level (1 mmol l^{-1}), but the degree of accumulation was similar in normoxic and hypoxic fish. Plasma [nitrate] was 65 µmol l^{-1} in control fish and increased significantly to the millimolar range upon nitrite exposure, reaching higher values in hypoxic compared with normoxic fish (Fig. 2B).

Plasma [lactate] increased with nitrite exposure and with hypoxia exposure, reaching the highest values during combined hypoxia and nitrite exposure; but the statistics were not entirely conclusive (Fig. 2C).

The basal concentration of nitrite in red blood cells (RBCs) was higher in hypoxic than in normoxic fish (around 1 and 2 µmol l^{-1}, respectively) (Fig. 3A). Nitrite exposure was associated with profound increases in RBC [nitrite] to the millimolar range, with no significant difference in values between normoxic and hypoxic

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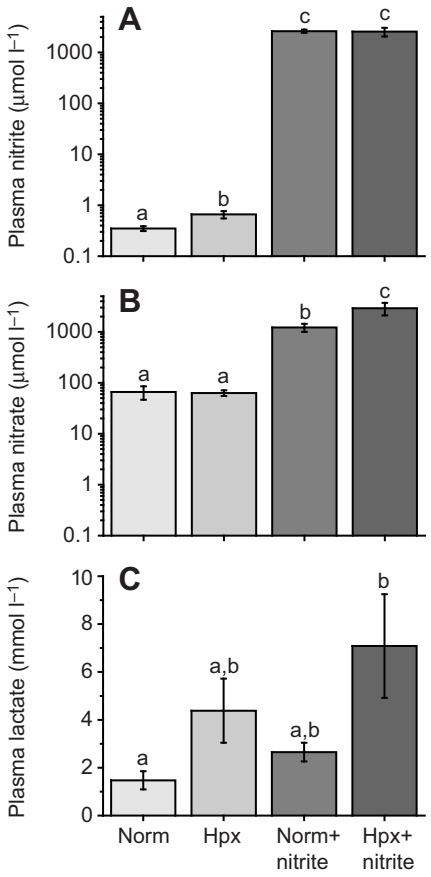

Fig. 2. Plasma nitrite, nitrate and lactate levels in brown trout under different oxygen and nitrite regimes. Plasma concentrations of nitrite (A), nitrate (B) and lactate (C) in brown trout under normoxic and hypoxic conditions and after 1-day exposure to 1 mmol l^{-1} ambient nitrite under normoxic and hypoxic conditions. Note the log scale for nitrite and nitrate panels. Other details are as in Fig. 1.

fish (Fig. 3A). The major rise in erythrocyte [nitrite] induced very large increases in erythrocyte SNO and HbNO from submicromolar towards millimolar values (Fig. 3B,C). Notably, erythrocyte SNO levels were significantly higher under hypoxic than normoxic conditions (Fig. 3B). The thiol compounds that were *S*-nitrosated include both proteins (mainly Hb in RBCs) and glutathione.

In the heart ventricle, nitrite was elevated from a basal level around 1 μmol l^{-1} to 1000-fold higher values in nitrite-exposed fish under both normoxia and hypoxia (Fig. 3D). This produced significant elevations of both SNO (Fig. 3E) and FeNO+NNO (Fig. 3F) in cardiac tissue.

White muscle tissue also experienced significant elevations of cellular [nitrite] (Fig. 3G) and cellular [SNO] (Fig. 3H) during nitrite exposure, but the absolute values reached were significantly lower than corresponding values in cardiac muscle.

The mRNA expression of *ef1α* in gill tissue did not change with either hypoxia or nitrite exposure, rendering *ef1α* an appropriate gene to which other gene expressions could be normalized. Gill *iNOS* mRNA levels were significantly elevated in hypoxic fish compared with normoxic controls, revealing increased *iNOS* gene expression after 24 h of hypoxia (Fig. 4A), assuming that protein expression follows the increase in transcription. Nitrite exposure, in contrast, was associated with strongly decreased *iNOS* mRNA levels under both normoxic and hypoxic conditions, suggesting a profound inhibition of *iNOS* gene expression by nitrite (Fig. 4A, note log scale). Changes in gill *nNOS* mRNA expression were more modest, with a tendency for slightly higher expression with hypoxia and nitrite than in normoxic controls (Fig. 4B).

Gill NKA activity did not vary significantly between the four groups (Fig. 5A), showing that the maximal enzymatic capacity was not influenced by 24 h of hypoxia or nitrite exposure. The mRNA expression of the *nkaα1a* subunit was, however, significantly elevated by hypoxia, and nitrite exposure induced a further significant increase (Fig. 5B), whereas expression of *nkaα1b* mRNA rose significantly only during normoxic nitrite exposure (Fig. 5C). The mRNA expression of gill *hsp70* was the same in normoxic and hypoxic fish, whereas nitrite exposure caused a significant elevation of *hsp70* mRNA levels under both normoxic and hypoxic conditions (Fig. 6).

DISCUSSION

Nitrite uptake and metabolism

Nitrite is naturally present in organisms at or below micromolar concentrations, because it is formed as an oxidative metabolite of NO produced by NOS enzymes. Plasma [nitrite] was 0.33 μmol l^{-1} in normoxic control fish (Fig. 2A), which compares with values in marine fish and mammals (Jensen, 2009) and is slightly lower than values seen in freshwater goldfish and crucian carp (Hansen and Jensen, 2010; Sandvik et al., 2012). The plasma concentration increased to 0.66 μmol l^{-1} in hypoxic brown trout, which contrasts with declines in plasma [nitrite] during hypoxia in flounder, goldfish and crucian carp (Jensen, 2009; Hansen and Jensen, 2010; Sandvik et al., 2012). While a decline in plasma [nitrite] under strong hypoxia can be explained by decreased NOS enzyme activity (because O_2 is a co-substrate of the reaction, and O_2 becomes limiting under strong hypoxia) and shift of nitrite from plasma to tissues (Hansen and Jensen, 2010; Sandvik et al., 2012), the more moderate hypoxia (in terms of absolute P_{O_2}) in the present experiments may have allowed an increased protein expression of NOS with subsequent increased NO and nitrite productions, as reported in rainbow trout (McNeill and Perry, 2006). Indeed, we observed an increased expression of *NOS* mRNA in the gills of hypoxic brown trout (Fig. 4), which may also have occurred in other tissues, and increased *NOS* mRNA expression is typically translated into increased protein levels (LeCras et al., 1996; McNeill and Perry, 2006; Thompson et al., 2009).

Freshwater fish take up nitrite across the gills, particularly when ambient nitrite rises to levels comparable to ambient [Cl^-], because nitrite and chloride competes for the same active branchial Cl^--uptake mechanism (Williams and Eddy, 1986; Jensen, 2003). During nitrite exposure, the plasma nitrite concentration rose well above the ambient level (Fig. 2). Plasma [nitrite] was, however, the same under normoxia and hypoxia (Fig. 2A). Plasma [nitrite] during nitrite exposure is a complex function of branchial uptake rates, transport between extracellular and intracellular compartments, tissue nitrite metabolism to other metabolites and kidney excretion. Cellular [nitrite] was substantially elevated in RBCs, heart and skeletal muscle (Fig. 3A,D,C), showing that nitrite taken up across the gills was transported into tissues. The levels attained in RBCs and heart ventricle did not differ significantly between normoxia and hypoxia (Fig. 3A,D), whereas nitrite increased to higher values under hypoxic than normoxic conditions in white skeletal muscle (Fig. 3G). The latter suggests an increased total body nitrite burden under hypoxic conditions, because white skeletal muscle constitutes some 50% of total fish mass. A substantial amount of nitrite taken up across the gills is internally metabolized and detoxified to nitrate

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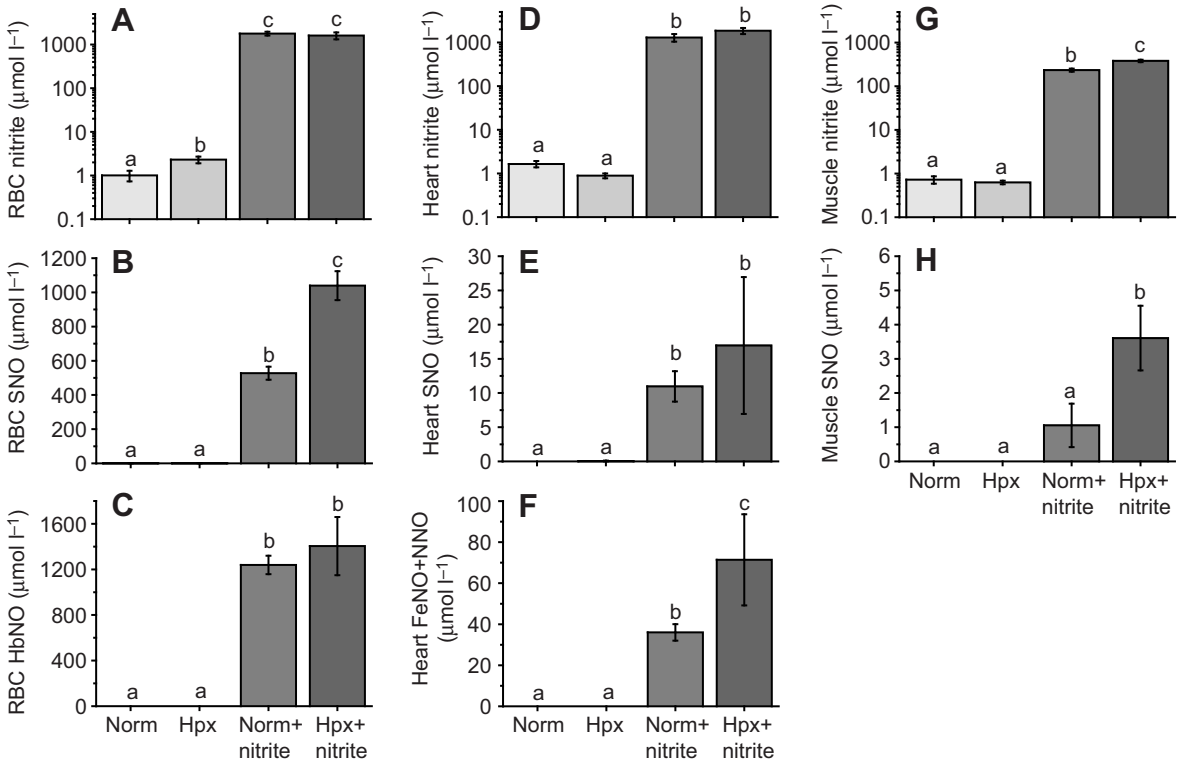

Fig. 3. Cellular levels of nitrite and nitros(yl)ated metabolites in brown trout. Values are given for red blood cells (A–C), heart ventricle (D–F) and white skeletal muscle (G,H) of brown trout exposed to normoxia and hypoxia in the absence and presence of ambient nitrite. Nitrite levels are shown in A, D and G (note log scale) and SNO levels are shown in B, E and H. Erythrocyte HbNO is shown in C and heart FeNO+NNO is shown in F. White muscle FeNO+NNO is not depicted because levels were below detection level with the available amount of sample. Other details are as in Fig. 1.

(e.g. in RBCs and liver), raising plasma nitrate levels during nitrite exposure (Doblander and Lackner, 1996; Stormer et al., 1996). This nitrate production should be taken into account when judging the magnitude of branchial nitrite uptake. The higher plasma nitrate levels during hypoxic compared with normoxic nitrite exposure (Fig. 2B) accordingly support a somewhat higher nitrite uptake during hypoxia than during normoxia. The same applies to the alternative metabolism of nitrite to SNO and FeNO compounds, as cellular SNO and FeNO metabolites were generally higher during hypoxia than normoxia (Fig. 3B–F). Taken together, the data therefore suggest a slightly higher nitrite uptake in hypoxic than normoxic brown trout, in support of the idea that hypoxia-induced lamellar recruitment increases the need for active ion uptake (cf. Introduction). The difference is, however, small and not comparable to the large difference in nitrite uptake between normoxic and hypoxic goldfish (Jensen and Hansen, 2011).

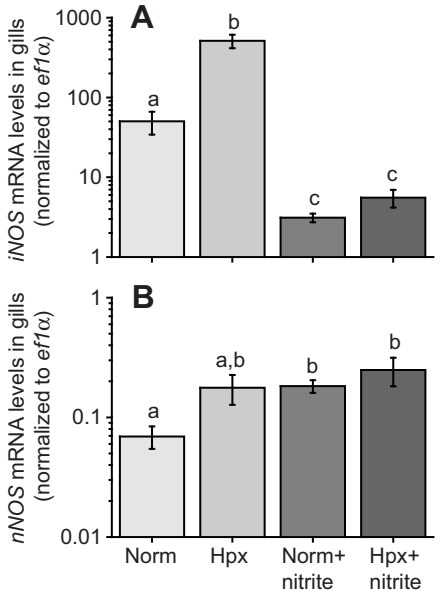

Fig. 4. Expression of inducible NOS and neural NOS in gills of brown trout. Levels of *iNOS* mRNA (A) and *nNOS* mRNA (B) under the four experimental conditions are revealed. Levels were normalized to the expression of *ef1α* (which did not change with treatment). Note log scale. Other details as in Fig. 1.

The extensive formation of cellular FeNO (via NO formation from nitrite), NNO and SNO (e.g. via formation of nitrosating N_2O_3) in nitrite-exposed brown trout (Fig. 3) is similar to observations in nitrite-exposed goldfish, supporting the view that a high nitrite concentration induces nitrosative stress with excessive nitros(yl)ation of critical proteins (Jensen and Hansen, 2011). The formation of FeNO+NNO and SNO compounds was largest in RBCs followed by heart and white muscle tissue, and it was typically augmented by hypoxia (Fig. 3).

Branchial expression of *iNOS* and *nNOS*

Hypoxia significantly increased gill *iNOS* mRNA levels and tended to increase *nNOS* mRNA levels (Fig. 4). This bears resemblance to increased *nNOS* mRNA and protein expression in the brain and posterior cardinal vein of hypoxic rainbow trout (McNeill and Perry, 2006), increased mRNA and protein expression of *iNOS* and *eNOS* in hypoxic rat lungs (LeCras et al., 1996) and an increased iNOS and unchanged nNOS expression in hypoxic fetal guinea pig hearts (Thompson et al., 2009). An upregulation of *iNOS* expression in

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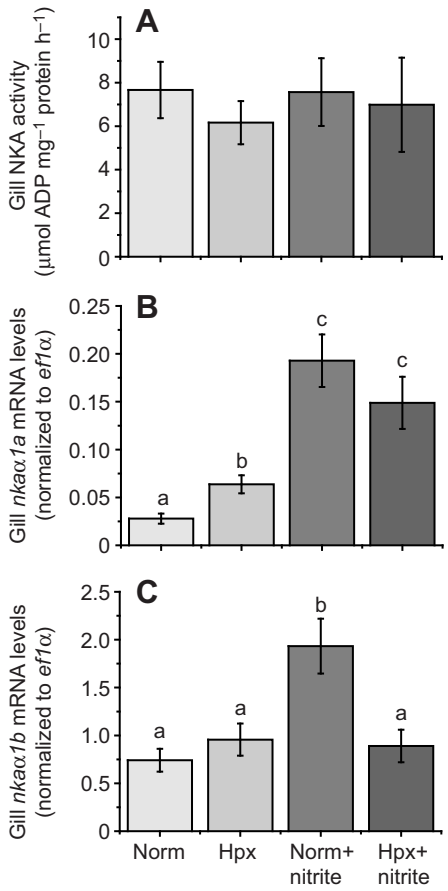

Fig. 5. Gill Na^+/K^+-ATPase activity and expression in brown trout. Enzymatic activity of NKA (A) and mRNA levels of *nkaα1a* (B) and *nkaα1b* (C) subunits in gill tissue of brown trout under the four experimental conditions. Other details as in Fig. 1.

hypoxia may be mediated by hypoxia inducible factor-1, but other possibilities also exist (Thompson et al., 2009; Robinson et al., 2011). The activity (NO production) of iNOS enzymes is mainly regulated at the transcriptional level (Aktan, 2004). Thus, as mentioned above, the increased NOS expression can increase NO production under moderate hypoxia, where the O_2 substrate is not reduced below levels that are critical for the NOS reaction to occur. A more severe degree of hypoxia was found to decrease *iNOS* mRNA expression in crucian carp gills, and the decrease was augmented by anoxia (Sandvik et al., 2012). It thus appears that the influence of hypoxia on *NOS* gene expression may depend on the degree of hypoxia and the species.

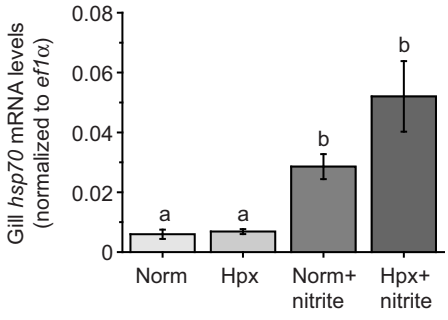

Fig. 6. Expression of heat shock protein 70 in gills of brown trout. Levels of *hsp70* mRNA in gill tissue under the four experimental conditions. Other details as in Fig. 1.

An exciting discovery in the present study was the profound decrease in *iNOS* mRNA levels induced by nitrite (Fig. 4A). This finding supports negative-feedback regulation of *iNOS* gene expression by a product of the NOS reaction (the NO metabolite nitrite), provided that translational efficiency remains unaltered. NO has been reported to inhibit *iNOS* gene expression in mammalian glial cells and hepatocytes by inhibiting the nuclear transcription factor NF-κB (Colasanti et al., 1995; Taylor et al., 1997; Park et al., 1997). It has furthermore been shown that *S*-nitrosation of conserved NF-κB cysteines inhibits gene transcription by decreasing binding of NF-κB to the *iNOS* promoter (Marshall and Stamler, 2001; Kelleher et al., 2007). Since SNO formation is generally increased during nitrite exposure (Fig. 3), it is conceivable that the profound nitrite-induced inhibition of *iNOS* mRNA expression could be triggered by *S*-nitrosation of NF-κB. The inhibition of *iNOS* gene transcription by nitrite was very potent and strongly overruled the induction of *iNOS* that was caused by hypoxia (Fig. 4A). A similar nitrite-induced inhibition was absent for branchial *nNOS* (Fig. 4B).

Branchial Na^+/K^+-ATPase activity

On the basis of a reported inhibitory effect of NO on gill NKA activity (Tipsmark and Madsen, 2003; Ebbesson et al., 2005) and the formation of NO from nitrite, particularly under hypoxic conditions, we anticipated that nitrite exposure would inhibit branchial NKA activity. Hypoxia per se can also decrease gill NKA activity in fish (Richards et al., 2007; Wood et al., 2007; Lundgreen et al., 2008; Hansen and Jensen, 2010). It was therefore unexpected that neither nitrite nor hypoxia changed NKA activity in the present experiments (Fig. 4). It is possible that water P_{O_2} in both normoxia (P_{O_2}>140 mmHg) and hypoxia (P_{O_2}=70 mmHg) were sufficiently high to avoid cellular hypoxia in the branchial cells, given their intimate contact with the water. This would limit both conversion of nitrite to NO (promoted by very low P_{O_2}) and any direct hypoxia effect. The relative short exposure time (24 h) could also be important. Earlier reports of a decreased branchial NKA activity during hypoxia used either stronger hypoxia and/or longer exposures (Richards et al., 2007; Wood et al., 2007; Lundgreen et al., 2008; Hansen and Jensen, 2010). Indeed, short-term (4 h) hypoxia at a water P_{O_2} of 80 mmHg did not change gill NKA activity in rainbow trout (Iftikar et al., 2010) as we show here for brown trout. Effects of nitrite exposure on NKA activity have previously been examined in two marine fish. In the sea bass *Lates calcarifer*, branchial NKA activity was reduced after exposure to 3.6 mmol l^{-1} ambient nitrite in normoxic seawater for 4 days (Woo and Chiu, 1997), whereas NKA activity increased after exposure to the same concentration for 7 days in silver sea bream *Sparus sarba* (Deane and Woo, 2007). The effects of nitrite accordingly appear to vary, which could be due, in part, to a compensatory upregulation of pump abundance following an inhibition of its activity.

We observed significantly higher mRNA levels of *nkaα1a* in brown trout gills with hypoxia and a further larger increase upon nitrite exposure (Fig. 5B). The increased mRNA expression of *nkaα1a*, which is the dominant NKA α1 isoform in freshwater fish (Richards et al., 2003), can be viewed as a compensatory transcriptional response that counteracts an inhibition of NKA. Thus, if the increased mRNA levels translated into increased protein abundance, an inhibition of NKA by hypoxia and nitrite would be alleviated and total NKA activity could be unchanged, as we actually observed.

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Branchial *hsp70* expression

Hypoxia can potentially induce HSP70 expression in fish (Roberts et al., 2010), but branchial *hsp70* mRNA levels were not elevated above basal levels at the present moderate degree of hypoxia. Nitrite exposure, in contrast, significantly elevated *hsp70* mRNA levels in gills under both normoxic and hypoxic conditions (Fig. 6). This finding corroborates recent reports of increased *hsp70* mRNA (Sun et al., 2014) and protein (Deane and Woo, 2007) expression in tissues of nitrite-exposed fish. Based on mammalian data it seems likely that nitrite can increase HSP70 protein expression at only moderately elevated nitrite concentrations (Bryan et al., 2005). This would contribute to the cytoprotective effect of moderately elevated nitrite concentrations that reduce tissue injury during ischemia–reperfusion events in mammals (Shiva et al., 2007; Chouchani et al., 2013). The cytoprotection acts, in part, through Fe-nitrosylation and *S*-nitrosation of complexes in the mitochondrial respiratory chain, which limits generation of reactive oxygen species (ROS) and oxidative protein damage (Shiva et al., 2007; Chouchani et al., 2013). The protection seems to be exploited by crucian carp and freshwater turtles that naturally endure cycles of anoxia and reoxygenation (Sandvik et al., 2012; Jensen et al., 2014). Induction of HSP70 assists cytoprotection by ensuring correct protein folding and repair of damaged protein. This would also limit damage in a toxicological setting, but high nitrite concentrations may lead to excessive nitrosative stress, as is the case with high NO concentrations (Thomas et al., 2008), which will overrule cytoprotective effects. This is supported by reports of ultrastructural tissue damage in fish where nitrite had accumulated to high (millimolar) concentrations (Arillo et al., 1984; Sun et al., 2014).

Heme-based reactions of nitrite in RBCs

Nitrite reacts with oxygenated Hb inside RBCs to form metHb and nitrate, and it reacts with deoxygenated Hb to form metHb and NO, with most of the generated NO subsequently binding to ferrous deoxygenated heme to produce HbNO or reacting with oxygenated Hb to produce metHb and nitrate (Jensen, 2009). Nitrite accumulation therefore leads to extensive metHb formation (Fig. 1), which overrides the capacity of erythrocyte metHb reductase to reduce metHb to functional Hb. Owing to the presence of deoxygenated Hb in venous blood, significant amounts of HbNO are also formed (Fig. 1), as first shown in nitrite-exposed zebrafish (Jensen, 2007) and subsequently, in nitrite-exposed goldfish and striped catfish (Jensen and Hansen, 2011; Lefevre et al., 2011). MetHb and HbNO are both non-functional with respect to O_2 transport, and their sum therefore means that the nitrite-exposed brown trout had a blood-O_2-carrying capacity that was less than 30% of normal (Fig. 1). This will decrease swimming performance and aerobic scope (Lefevre et al., 2011) and lower the tolerance towards hypoxia. Indeed, brown trout exposed to both hypoxia and nitrite were the most stressed fish, as reflected by lactate values (Fig. 1C) and the presence of mortality, which was absent in the other experimental groups.

Concluding remarks

Our results emphasize nitrite as an important dichotomous player in animal physiology. Nitrite is an integral part of NO homeostasis by being an oxidative metabolite of NOS-derived NO, a donor of NO under hypoxia and – as here shown – a negative-feedback inhibitor of *NOS* gene expression. At moderately elevated levels, the ability of nitrite to iron-nitrosylate and *S*-nitrosate proteins is involved in cytoprotection, which becomes aided by nitrite-induced induction of HSP70. However, at the high levels that develop in nitrite-exposed fish, nitro(sy)lation becomes excessive and switches into nitrosative stress, and blood Hb is extensively converted into non-functional metHb and HbNO. Such a concentration-dependent switch between positive and negative effects is, in principle, no different from that in other signaling molecules such as NO, H_2S and CO.

MATERIALS AND METHODS

Animals and experimental protocol

Male brown trout (*Salmo trutta*) of body mass 61.4±2.2 g (mean±s.e.m., *N*=31) were obtained from Funen Salmon Fish (Elsesminde, Odense, Denmark) and distributed among 4 identical aquaria with 80 l of fresh water (Odense tap water with $[Cl^-]$=1.3 mmol l^{-1} and $[HCO_3^-]$=4.8 mmol l^{-1}) with 7–8 fish in each aquarium. The fish were acclimated to 15°C and normoxic water (P_{O_2}>140 mmHg via air bubbling) under a 12 h:12 h light: dark cycle for 1 week. Forty liters of water was exchanged twice daily to avoid accumulation of waste products. Following acclimation, the fish were assigned to four different experimental conditions: (1) 24 h continued normoxia (normoxic control, *N*=7); (2) 24 h of hypoxia (hypoxic control, *N*=8); (3) 24 h of nitrite exposure at 1 mmol l^{-1} nitrite in normoxic water (normoxia+nitrite; *N*=8); and (4) 24 h of nitrite exposure at 1 mmol l^{-1} nitrite in hypoxic water (hypoxia+nitrite; *N*=8). Normoxia (P_{O_2}>140 mmHg) was obtained by bubbling with air, and hypoxia was obtained by bubbling with N_2. The level of hypoxia was P_{O_2} ~70 mmHg. Water P_{O_2} was measured and controlled with a Loligo OXY-REG oxygen analyzer and regulator system (Loligo Systems Aps, Tjele, Denmark), using a set point of 44% air saturation (equivalent to P_{O_2}=70 mmHg). Nitrite was added as $NaNO_2$, and the level of 1 mmol l^{-1} was confirmed by measurement via reductive chemiluminescence (cf. below).

Sampling of blood and tissues

Fish were individually netted and anesthetized in water with 100 mg l^{-1} MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, Steinheim, Germany). A blood sample was taken from the caudal vessels with a heparinized syringe, whereafter the fish was euthanized by cutting the spinal cord. Procedures were in accordance with Danish laws of animal experimentation. The blood was immediately processed, while, at the same time, gills (second and third gill arch on the left side), heart ventricle and white skeletal muscle were quickly dissected out. One gill arch (the second) was placed in SEI-buffer solution (300 mmol l^{-1} sucrose; 20 mmol l^{-1} Na_2EDTA; 50 mmol l^{-1} imidazole; pH 7.3) for later measurement of gill Na^+/K^+-ATPase activity, and the other gill arch (the third) was immediately frozen in liquid N_2 for later analysis of mRNA expression of selected genes. The ventricle and white muscle tissues were quickly washed in 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), 0.1 mmol l^{-1} diethylenetriaminepentaacetic acid (DTPA)], and then dried on a filter paper, weighed and frozen in liquid N_2.

Blood was transferred to a pre-weighed Eppendorf tube and subsamples were taken for measurements of hematocrit (Hct) and hemoglobin (Hb) derivatives. The remaining blood was centrifuged (2 min, 16,000 ***g***, 5°C), and the plasma was transferred to a new Eppendorf tube. A subsample of plasma was taken for lactate measurement, and the remaining plasma was frozen in liquid N_2. The tube containing the RBCs was weighed (to determine RBC mass) and instantly frozen in liquid N_2.

Measurements

For measurements of nitrite, nitrate, *S*-nitroso and iron-nitrosyl compounds, the samples of ventricle and white skeletal muscle 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***, 2°C), after which the supernatants were frozen in liquid nitrogen and stored at −80°C until measured. The RBC samples were thawed by adding nine times their mass of a nitrite/SNO preservation solution, consisting of 5 mmol l^{-1} $K_3[Fe(CN)_6]$, 10 mmol l^{-1} NEM, 0.1 mmol l^{-1} DTPA and 1% NP-40 (Yang et al., 2003; Hansen and Jensen, 2010). The hemolysate was

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Table 1. Target genes and sequences of primers used to investigate mRNA levels in the gills of brown trout

Target	Forward primer 5′→3′	Reverse primer 5′→3′	Accession no.
iNOS	CCCAGCACTCCAGTCATTCT	ATGTCGTGTAGCCGTTGTTG	AJ300555
nNOS	TGTAGGACGCATTCAGTGGT	ACATTCCATGAGCCGTTGTG	DQ640498
nkaα1a	CCCAGGATCACTCAATGTCAC	CCAAAGGCAAATGGGTTTAAT	AY319391
nkaα1b	CTGCTACATCTCAAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	AY319390
hsp70	CATCGACTTCTACACCTCCATCAC	CTGAAGAGGTCGGAACACATCTC	AJ632154
ef1α	GAGAACCATTGAGAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	AF321836

vortexed and centrifuged, and supernatants were immediately measured. Nitrite and its metabolites were assessed by reductive chemiluminescence, using a Sievers (Boulder, CO, USA) nitric oxide analyzer (model 280i) and previously described procedures to distinguish between [nitrate], [nitrite], [SNO] and [FeNO+NNO] (Yang et al., 2003; Hansen and Jensen, 2010; Jensen and Hansen, 2011).

Gill Na^+/K^+-ATPase (NKA) activity was determined by a semi-micro method (McCormick, 1993) using a microplate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA, USA). The enzyme activity was normalized to the protein content of the sample and expressed as μmol ADP mg^{-1} protein h^{-1}. Protein concentration was measured by the Lowry method (Lowry et al., 1951).

For analysis of Hb derivatives, an accurate amount of freshly drawn blood was hemolyzed in 1 ml of 20 mmol l^{-1} phosphate buffer with pH 7.3. Following centrifugation, a spectral scan was made on the hemolysate from 480 to 700 nm (Cecil CE2041, Cambridge, UK). The concentrations of oxyhemoglobin, methemoglobin, nitrosylhemoglobin and deoxyhemoglobin were evaluated by spectral deconvolution, using a least-squares curve-fitting procedure and reference spectra of the four Hb derivatives, as previously described (Jensen, 2007). Hematocrit was measured by centrifugation of blood (2 min at 13,700 ***g***) in glass capillaries. Plasma lactate was determined by the lactate dehydrogenase enzymatic method after deproteinization of plasma with 0.6 N perchloric acid.

mRNA expression of genes encoding iNOS, nNOS, NKA α1a, NKA α1b and HSP70 in gill tissue

Total RNA was isolated and extracted with TRIsure™ reagent (Bioline, London, UK) following the manufacturer's instructions. RNA concentration and purity was determined by measuring A260/A280. Total RNA (1 μg) was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and used in first-strand cDNA synthesis with a high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster, CA, USA) using random hexamers as a primer. Semi-quantitative real-time PCR analysis using SYBR Green detection was carried out on an Mx3000P instrument (Stratagene, La Jolla, CA, USA) using standard software settings. Reactions were carried out with 1 μl cDNA (50 ng total RNA), 6 μl forward/reverse primers mix (final concentration, 150 nmol l^{-1}), 10 μl LightCycler® 480 SYBR green I Master (Roche, Indianapolis, IN, USA) made up to a total volume of 20 μl with distilled water. Cycling conditions: 94°C for 15 s, 60°C for 60 s (*ef1α*, *nkaα1a*, *nkaα1b*, *hsp70*) or 64°C for 60 s (*iNOS*, *nNOS*) for 40 cycles. Melting curve analysis was carried out routinely with 30 s for each 1°C temperature interval from 55° to 95°C.

For each primer set, a cDNA four-fold dilution series of six points was generated in duplicate and analyzed by qPCR to determine amplification efficiency. The amplification efficiency for each primer set was used for calculation of relative copy numbers of the respective target gene. Normalized copy numbers of the respective target genes were calculated according to Pfaffl (2001): $(1+E_{\text{tar}})^{-\text{Cttar}})/(1+E_{ef1\alpha})^{-\text{Ct}ef1\alpha}$; where E is the amplification efficiency for the primer pairs, Ct is the cycle threshold value of the PCR products, tar is the target gene and *ef1α* was used as a normalization gene.

iNOS and *nNOS* primers were originally developed for rainbow trout (*Onchorynchus mykiss*) but proved to work well on the closely related brown trout. They were designed using Netprimer software (Premier Biosoft International, CA, USA) with standard settings and double checked using Primer3 software (v.0.4.0, Rozen and Skaletsky, 2000) based on *nNOS*, *iNOS* mRNA sequences from rainbow trout found in the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) GenBank. Primers for *hsp70* were designed from Atlantic salmon (*Salmo salar*) sequence as described by Takle et al. (2006). Primers for *nkaα1a* and *ef1α* were designed from respectively rainbow trout and Atlantic salmon sequences as described by Kiilerich et al. (2007). Primers for *nkaα1b* are identical to the *nka* α-subunit specific primers based on rainbow trout mRNA sequences described by Richards et al. (2003). The high degree of conservation of nucleotide sequence between salmon and trout permits the use of primers between these two species. All primers were tested for nonspecific product amplification and primer dimer formation using both melting curve analysis and 2.5% agarose gel verification. Primer sequences are listed in Table 1.

Statistics

All results are presented as means±s.e.m. Statistical differences between exposure groups were evaluated by one-way ANOVA followed by the Tukey *post hoc* means comparison test (Origin 8.5, OriginLab Corporation, Northampton, MA, USA). Differences between means were considered significant at $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.B.J. conceived the study; F.B.J., L.G., M.N.H. and S.S.M. designed and performed the experiments; F.B.J., L.G., M.N.H. and S.S.M. analyzed the data; F.B.J. wrote the paper; L.G., M.N.H. and S.S.M. edited the manuscript.

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