

## RESEARCH ARTICLE

# Nitric oxide metabolites during anoxia and reoxygenation in the anoxia-tolerant vertebrate *Trachemys scripta*

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**ABSTRACT**

Moderate elevations of nitrite and nitric oxide (NO) protect mammalian tissues against ischemia (anoxia)-reperfusion damage by inhibiting mitochondrial electron transport complexes and reducing the formation of reactive oxygen species (ROS) upon reoxygenation. Crucian carp appear to exploit this mechanism by upregulating nitrite and other nitrite/NO metabolites (*S*-nitroso and iron-nitrosyl compounds) in several tissues when exposed to anoxia. We investigated whether this is a common strategy amongst anoxia-tolerant vertebrates by evaluating NO metabolites in red-eared slider turtles during long-term (9 days) anoxia and subsequent reoxygenation at low temperature, a situation naturally encountered by turtles in ice-covered ponds. We also measured glutathione in selected tissues and assessed the impact of anoxia on electrolyte status. Anoxia induced major increases in [nitrite] in the heart, pectoral muscle and red blood cells, while [nitrite] was maintained unaltered in brain and liver. Concomitantly, the concentrations of *S*-nitroso and iron-nitrosyl compounds increased, showing that nitrite was used to produce NO and to *S*-nitrosate cellular molecules during anoxia. The changes were gradually reversed during reoxygenation (1 h and 24 h), testifying that the processes were reversible. The increased NO bioavailability occurred in the absence of NO synthase activity (due to global anoxia) and may involve mobilization of internal/external nitrite reservoirs. Our data support the theory that anoxic upregulation of nitrite and other NO metabolites could be a general cytoprotective strategy amongst anoxia-tolerant vertebrates. The possible mechanisms of nitrite-derived NO and *S*-nitrosation in protecting cells from destructive Ca<sup>2+</sup> influx during anoxia and in limiting ROS formation during reoxygenation are discussed.

**KEY WORDS:** Anoxia, Cytoprotection, Nitric oxide, Nitrite, Reoxygenation

**INTRODUCTION**

Episodes with suspended oxygen supply followed by reoxygenation represent a major challenge to animals. Most vertebrates only tolerate brief anoxia periods because of rapid cellular depletion of ATP and the subsequent failure of ion-transporting ATPases to uphold ionic gradients, hence leading to depolarization, cellular Ca<sup>2+</sup> overload and other destructive events that ultimately cause cell death in vital organs such as the brain or the heart (Lutz, 1992). Reoxygenation is also problematic, as it is associated with excessive production of reactive oxygen species (ROS) that damage cells through protein oxidation and lipid peroxidation (Lefter and Granger,

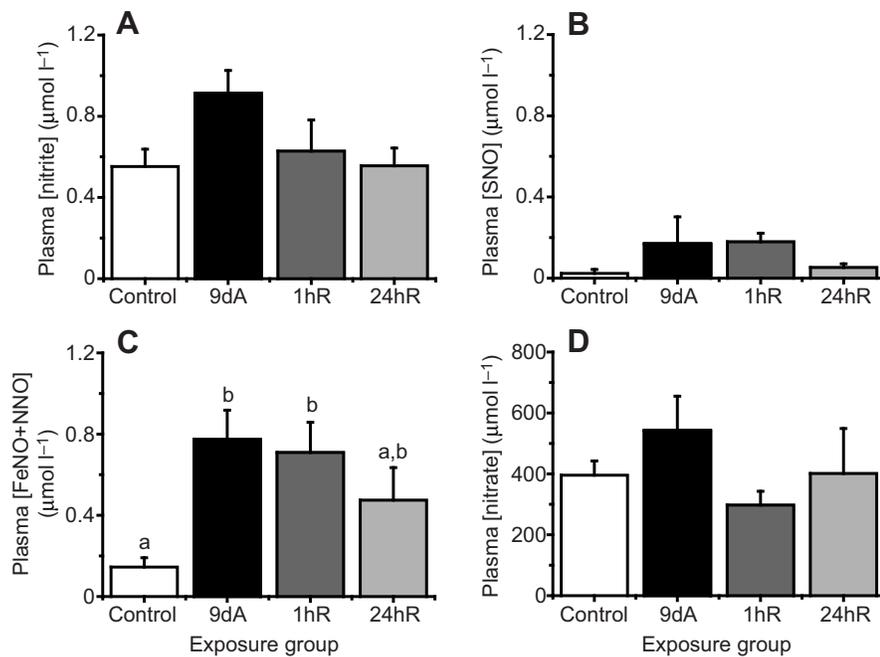
2000). The consequences of anoxia and reoxygenation are under intense study in both medical science and comparative physiology. Medical interest relates to the clinical importance of ischemia-reperfusion events and the search for therapeutic means to alleviate damage caused by myocardial infarction or stroke, while comparative physiologists are intrigued by the mechanisms that enable some animals to cope with extended periods of oxygen lack in their natural habitat. Anoxia-tolerant vertebrates such as crucian carp and some freshwater turtles are excellent models to reveal how evolution has solved the problems with anoxia, and the mechanism employed in these animals may also have medical application (Nilsson and Lutz, 2004).

Anoxia-tolerant turtles, such as the red-eared slider turtle [*Trachemys scripta elegans* (Wied-Neuwied 1839)], defend ATP levels during anoxia through metabolic depression, which includes downregulation of protein synthesis and a reduced cellular ion leakage that allows reduced ATP expenditure on ion pumping (Jackson, 2002; Milton and Prentice, 2007; Bickler and Buck, 2007; Stecyk et al., 2009). Also, red-eared slider turtles apparently go through cycles of anoxia and reoxygenation without the free radical damage seen during ischemia-reperfusion of tissues in mammals (Willmore and Storey, 1997a). Thus, the turtles have evolved effective defense mechanisms that may involve reduced ROS production and/or efficient means of scavenging ROS (Milton and Prentice, 2007; Milton et al., 2007). While some hypoxia-tolerant species enhance antioxidant enzymes (e.g. superoxide dismutase, catalase) during periods of oxygen lack, red-eared sliders actually show decreased activities of some antioxidant enzymes during anoxia, but the constitutive activities are high and may be sufficient to protect tissues from oxidation damage upon reoxygenation (Willmore and Storey, 1997a; Hermes-Lima and Zenteno-Savín, 2002). Levels of the antioxidant glutathione are also high, but may decrease in some organs during anoxia (Willmore and Storey, 1997b). There are, however, alternative means to protect against ROS formation when anoxia is succeeded by reoxygenation. One such possibility is moderate elevation of nitric oxide (NO) and some of its metabolites that, through inhibition of complexes in the mitochondrial respiratory chain, can limit ROS generation and tissue injury in mammalian ischemia/reperfusion models (Shiva et al., 2007a; Murillo et al., 2011; Chouchani et al., 2013). The involvement of NO and its metabolites in cytoprotection have not yet been explored in anoxia-tolerant turtles.

NO is a ubiquitous signaling molecule that under aerobic conditions is produced from the reaction of L-arginine with O<sub>2</sub> catalyzed by nitric oxide synthases (NOS). NO typically exerts its physiological influences through nitrosylation of heme groups, forming iron-nitrosyl (FeNO) proteins, or via *S*-nitrosation of cysteines, forming *S*-nitroso (SNO) compounds (Denninger and Marletta, 1999; Foster et al., 2009; Hill et al., 2010). Other products derived from NO production include *N*-nitroso (NNO) compounds (formed in reactions with amines), nitrite (formed in reaction with

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**Fig. 1. Plasma nitric oxide (NO) metabolites in red-eared slider turtles under normoxic conditions (control), after 9 days of anoxia (9dA), after 9 days anoxia with subsequent 1 h reoxygenation (1hR), and after 9 days of anoxia with subsequent 24 h reoxygenation (24hR).** The panels depict concentrations of (A) nitrite; (B) S-nitroso compounds (SNO); (C) iron-nitrosyl and N-nitroso compounds (FeNO+NNO); and (D) nitrate. Values are means + s.e.m. and  $N=7$  (control) or 6 (9dA, 1hR and 24hR) in each group. Different letters above bars indicate a significant difference between groups ( $P<0.05$ ).

O<sub>2</sub>) and nitrate (formed in reactions with oxygenated hemoglobin and myoglobin) (Umbrello et al., 2013). Recent research shows that endogenous nitrite represents a reservoir of NO activity that can be activated by a number of cellular proteins under O<sub>2</sub>-limiting conditions (Lundberg et al., 2008). Thus, nitrite can be reduced to NO by deoxygenated myoglobin/hemoglobin, xanthine oxidoreductase and other proteins, which supply an alternative pathway for NO formation, when NOS activity is compromised by oxygen shortage. Nitrate may similarly be recycled to NO, if it is first reduced to nitrite (Jansson et al., 2008; Lundberg et al., 2008). Furthermore, SNO compounds can donate NO activity by transnitrosation between low-molecular-weight SNOs and proteins or protein–protein transnitrosation (Nakamura and Lipton, 2013). In this way, many central NO metabolites contribute to the overall NO bioavailability, which could play an important role in animals under hypoxic and anoxic conditions.

We recently showed that hypoxia-tolerant fish have a profound capacity for defending intracellular NO metabolite levels during prolonged periods of hypoxia (Hansen and Jensen, 2010) and anoxia (Sandvik et al., 2012). Notably, in crucian carp, anoxia was associated with a dramatic increase of nitrite levels in heart tissue, which was paralleled by elevated concentrations of SNO and FeNO compounds, showing that nitrite was partially metabolized to NO (FeNO) and SNO (Sandvik et al., 2012). Given that experimental elevation of nitrite and NO alleviate injury to cardiac muscle during ischemia/reperfusion in mammals (Shiva et al., 2007a; Hengden-Cotta et al., 2008), it is likely that crucian carp upregulates nitrite and NO levels to protect the heart from anoxia/reoxygenation damage (Sandvik et al., 2012). To ascertain whether this is a general and evolutionary conserved cytoprotective strategy amongst anoxia-tolerant vertebrates, it is imperative to study other archetype anoxia-tolerant vertebrates, *viz.* freshwater turtles, some of which can survive anoxia for months at low temperature (Jackson, 2002).

There is only limited information on NO homeostasis in turtles. Circulating levels of NO metabolites are relatively high in *T. scripta* and increase during short-term anoxia and reoxygenation at room temperature (21°C) (Jacobsen et al., 2012), but tissue NO levels, which are central in relation to cytoprotection, remain unknown. In

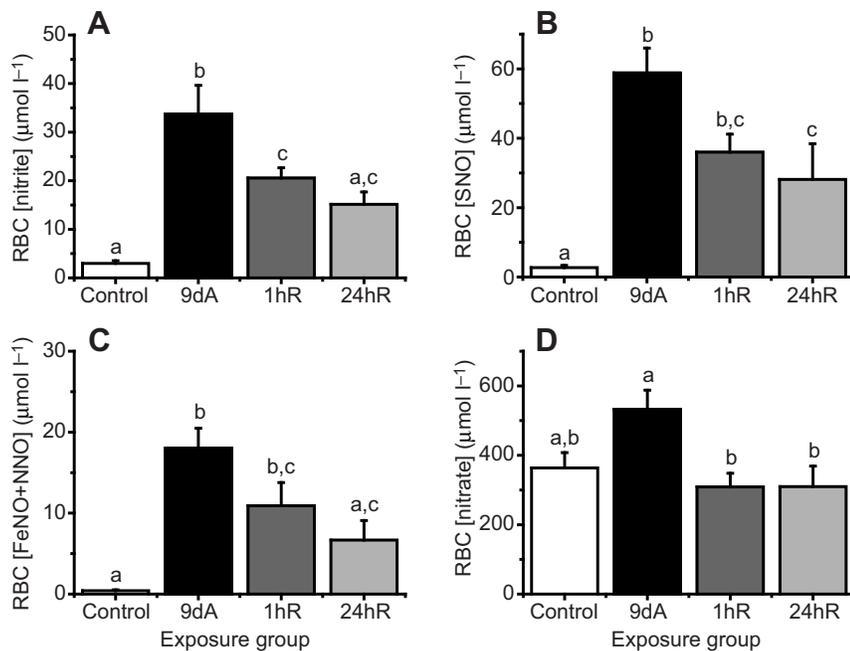
the present study we focus on NO metabolites in a number of different tissues (heart, brain, liver, skeletal muscle and blood) during long-term anoxia at low temperature to simulate the ecological relevant situation of winter dormancy in ice-covered ponds (Jackson and Ultsch, 2010). We hypothesized that anoxic red-eared slider turtles would increase NO bioavailability in the tissues to cope with anoxia and subsequent reoxygenation. To gain detailed insight into the reoxygenation phase, we included both an acute (1 h) and a longer-lasting (24 h) reoxygenation scheme. We also assessed glutathione in selected tissues and analyzed extracellular ionic composition to evaluate lactate load and acid-base-related consequences of anaerobiosis. Our results support a vivid participation of NO metabolites in the tolerance of red-eared sliders to anoxia and reoxygenation.

## RESULTS

Plasma nitrite was 0.55 µmol l<sup>-1</sup> in normoxic control animals and did not change significantly with anoxia or reoxygenation (Fig. 1A). Plasma [SNO] similarly did not vary significantly between groups (Fig. 1B), whereas plasma [FeNO+NNO] was significantly elevated after 9 days of anoxia in order to recover partially during 24 h of reoxygenation (Fig. 1C). Plasma nitrate was relatively high in normoxic control animals (~400 µmol l<sup>-1</sup>) and did not differ significantly among experimental groups (Fig. 1D).

In contrast to the modest changes in the plasma, there were large significant increases in [nitrite], [SNO] and [FeNO+NNO] inside the red blood cells (RBCs) after 9 days of anoxia (Fig. 2A–C). The intracellular NO metabolites increased to values considerably above values both in normoxic controls (Fig. 2) and in the extracellular compartment (Fig. 1). Reoxygenation reversed the changes and induced significant decreases in erythrocyte nitrite, SNO and FeNO+NNO concentrations, but values did not quite reach basal levels by 24 h reoxygenation (Fig. 2). Nitrate concentration tended to increase with anoxia ( $P=0.085$ ) and then decreased with reoxygenation (Fig. 2D).

In the heart ventricle, [nitrite] showed a significant fivefold increase from 0.54 µmol l<sup>-1</sup> in normoxic controls to 2.83 µmol l<sup>-1</sup> after 9 days of anoxia, which was succeeded by a gradual and partial recovery



**Fig. 2. Red blood cell (RBC) NO metabolites.**

Concentrations of nitrite (A), SNO (B), FeNO+NNO (C) and nitrate (D) in RBCs of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

towards control values during 24 h of reoxygenation (Fig. 3A). Ventricle [SNO] also showed a large increase with anoxia, followed by a decline in values during reoxygenation. This decrease in [SNO] was significant already by 1 h of reoxygenation (Fig. 3B). Heart [FeNO+NNO] similarly rose during anoxia and decreased during reoxygenation (Fig. 3C), whereas nitrate did not change significantly (Fig. 3D). Heart protein was constant around 50 mg g<sup>-1</sup> wet mass in all exposure groups. As a consequence, heart NO metabolites normalized to protein showed the same patterns of changes as for the absolute concentrations presented in Fig. 3 (not illustrated), revealing that the concentrations were not significantly influenced by water shifts between extracellular and intracellular compartments.

Brain nitrite and nitrate remained relative constant with treatment (Fig. 4A,C), while the concentration of nitros(yl)ated species (SNO, FeNO and NNO) in the brain increased significantly with anoxia and reoxygenation (Fig. 4B).

Liver [nitrite] was kept relatively constant at 0.8–1 µmol l<sup>-1</sup> in normoxic, anoxic and reoxygenated turtles (Fig. 5A). At the same time, there were major increases in liver [SNO] (Fig. 5B) and [FeNO+NNO] (Fig. 5C) during anoxia that reversed towards control values during subsequent reoxygenation. Liver [nitrate] increased with anoxia and decreased with reoxygenation (Fig. 5D).

Anoxia triggered a significant elevation of the nitrite concentration in pectoral muscle with subsequent partial recovery during reoxygenation (Fig. 6A). Concomitantly there were major anoxia-induced increases in muscle [SNO] and [FeNO+NNO] (Fig. 6B,C). The muscle [SNO] recovered towards control values already by 1 h of reoxygenation (Fig. 6B), while the decrease in [FeNO+NNO] during reoxygenation was slower (Fig. 6C). Muscle nitrate was not affected by treatment (Fig. 6D).

Plasma glutathione levels were in the low micromolar range in normoxic turtles and increased significantly with anoxia in order to recover with reoxygenation (Fig. 7A). Total glutathione was around 1 mmol l<sup>-1</sup> in the heart and some 2–3 mmol l<sup>-1</sup> in the liver (Fig. 7B,C) and did not change significantly with anoxia or reoxygenation.

Plasma lactate showed a pronounced significant increase from 1 mmol l<sup>-1</sup> to 51 mmol l<sup>-1</sup> during 9 days of anoxia, followed by a

gradual and significant recovery to 34.4 mmol l<sup>-1</sup> by 24 h reoxygenation (Fig. 8A). Lactate values were, however, considerably above controls at 24 h of recovery. Plasma chloride decreased significantly by some 10 mmol l<sup>-1</sup> during anoxia (Fig. 8A). Among cations, significant increases were observed in plasma K<sup>+</sup> (from 3.1 to 5.7 mmol l<sup>-1</sup>), Ca<sup>2+</sup> (from 2.2 to 7.7 mmol l<sup>-1</sup>) and Mg<sup>2+</sup> (from 1.6 to 3.1 mmol l<sup>-1</sup>) during anoxia, followed by incomplete recovery during reoxygenation (Fig. 8B). Plasma Na<sup>+</sup> showed a trend for an increase, but changes in Na<sup>+</sup> were not significant. Anoxia caused increases in the sum of measured anions (Fig. 8A) and the sum of measured cations (Fig. 8B), suggesting an increase in total osmolality. It was also evident that the sum of strong anions (Cl<sup>-</sup> and lactate) increased more than the sum of strong cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>) during anoxia (Fig. 8A,B). The strong ion difference (SID), which is a major independent variable in acid–base status (Stewart, 1983), was accordingly calculated as:

$$\text{SID} = ([\text{Na}^+] + [\text{K}^+] + 2[\text{Ca}^{2+}] + 2[\text{Mg}^{2+}]) - ([\text{Cl}^-] + [\text{lactate}]). \quad (1)$$

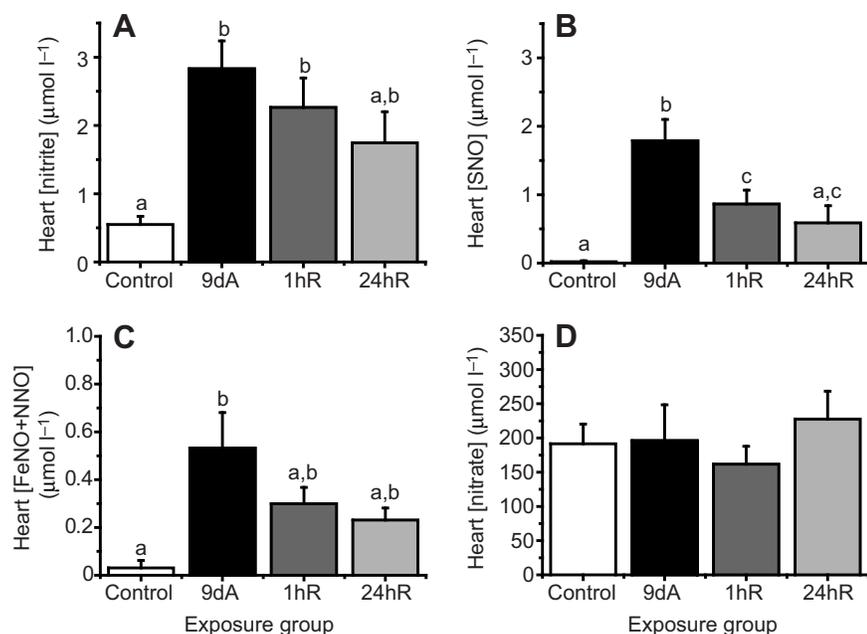
Anoxia induced a significant decrease in SID, with subsequent recovery during reoxygenation (Fig. 8C).

## DISCUSSION

The present study documents profound and dynamic changes in tissue NO metabolites that seem to be part of the adaptation strategy to anoxia and reoxygenation in the freshwater turtle *T. scripta elegans*. Nitrite was either increased or kept constant in anoxic tissues. In all the tissues, nitrite was used to produce NO, as evidenced by a general increase in iron-nitrosylated (FeNO) compounds. At the same time there was a universal elevation of cellular SNO compounds. This overall increase in NO bioavailability may protect the cells from destructive Ca<sup>2+</sup> influx during anoxia and limit ROS formation and ROS-induced damage during reoxygenation, as discussed below.

### Tissue NO metabolites in anoxia and reoxygenation

Nitrite was increased considerably above control levels in the anoxic turtle heart, and this was associated with major increases in [SNO] and [FeNO+NNO] (Fig. 3). Similar changes were recently reported in



**Fig. 3. Heart ventricle NO metabolites.**

Concentrations of nitrite (A), SNO (B), FeNO+NNO (C) and nitrate (D) in heart tissue of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). The values were determined on a wet mass basis and are shown as absolute concentrations in  $\mu\text{mol l}^{-1}$ , assuming a tissue density of  $1 \text{ kg l}^{-1}$ . Other details as in Fig. 1.

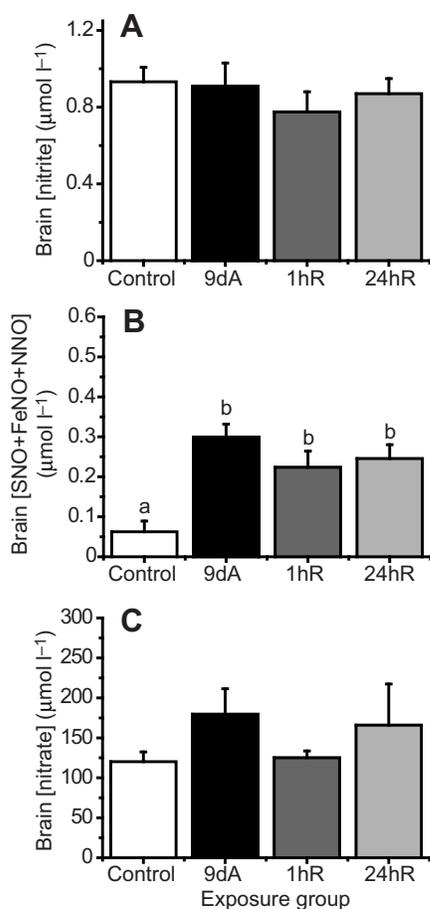
the hearts of anoxia-exposed crucian carp (Sandvik et al., 2012). Thus, it appears that an increase in NO bioavailability inside the heart constitutes a general mechanism for cardioprotection in anoxia-tolerant vertebrates. Many studies with mammalian models have shown that moderate elevations of nitrite reduce cell death and infarct size following myocardial ischemia and reperfusion (Webb et al., 2004; Duranski et al., 2005; Shiva et al., 2007a; Hendgen-Cotta et al., 2008). Such cytoprotection from elevated nitrite could be essential to preserve cardiac function in turtle and crucian carp during and after anoxia. Notably, in contrast to the experimental addition of nitrite that is required for cytoprotection in mammals, the freshwater turtle and crucian carp possess innate physiological mechanisms that elevate cardiac nitrite in anoxia. In the anoxic heart, nitrite is reduced to NO mainly by deoxygenated myoglobin, and much of the nitrite/NO-dependent cytoprotection is targeted at the mitochondria, involving Fe-nitrosylation of complex IV and *S*-nitrosation of complex I of the respiratory chain (Hendgen-Cotta et al., 2008; Murillo et al., 2011). Inhibition of complex I by *S*-nitrosation seems to play a central role in cytoprotection by limiting mitochondrial ROS generation and ROS-induced damage upon reoxygenation (Shiva et al., 2007a; Murillo et al., 2011). The key mechanism seems to be *S*-nitrosation of a conserved cysteine on the ND3 subunit of complex I, which slows the reactivation of mitochondria during early reoxygenation, thereby decreasing ROS production and oxidative damage (Chouchani et al., 2013). However, a number of other proteins may also be *S*-nitrosated and contribute to cardioprotection (Sun et al., 2007; Lima et al., 2009; Murphy et al., 2012). For instance, problems with cytosolic  $\text{Ca}^{2+}$  overload during anoxia and early reoxygenation may be alleviated by *S*-nitrosation of the L-type  $\text{Ca}^{2+}$  channel (which inhibits its activity and reduces cellular  $\text{Ca}^{2+}$  influx) and by *S*-nitrosation of the  $\text{Ca}^{2+}$  ATPase SERCA2a (which increases  $\text{Ca}^{2+}$  uptake into the sarcoplasmic reticulum) (Sun et al., 2007; Sun and Murphy, 2010). Furthermore, *S*-nitrosation of protein thiols provides general protection against ROS-induced irreversible oxidation of thiols, limiting the need for degradation and re-synthesis of damaged proteins after an anoxia/reoxygenation event (Sun and Murphy, 2010; Kohr et al., 2011).

The rise in cardiac SNO in anoxic turtles was succeeded by a decrease at 1 h of reoxygenation (Fig. 3B), which supports a

dynamic role of *S*-nitrosation and documents that the process is reversible, as required for an appropriate cardioprotective signal. Thus, while *S*-nitrosation of complex I would be beneficial by limiting ROS formation during early reoxygenation (Shiva et al., 2007a; Chouchani et al., 2013), the inhibition of complex I should gradually be reversed to allow oxidative phosphorylation to resume. The kinetics of these changes may be relatively slow in the turtles because of the low temperature and anoxia-induced hypometabolism.

In the brain (Fig. 4) and the liver (Fig. 5), nitrite stayed constant with anoxia, whereas nitros(y)lated species increased, enlarging the overall pool of NO metabolites. This resembles the situation in anoxic crucian carp (Sandvik et al., 2012). Thus, while there are organ-specific differences in individual NO metabolite changes, these are rather similar in the two anoxia-tolerant species. Conceivably, nitrite-linked NO and SNO formation provide cytoprotection in liver and brain – like in the heart – as supported by mammalian ischemia/reperfusion experiments addressing these organs (Duranski et al., 2005; Jung et al., 2006; Shiva et al., 2007a; Dezfulian et al., 2012). Turtle neurons have reduced membrane ion permeability ('channel arrest') in anoxia, which decreases the requirements for active ion pumping and thereby ATP expenditure (Nilsson and Lutz, 2004). An important target is the *N*-methyl-D-aspartate (NMDA) receptor, because downregulation of this cation channel in anoxic turtles is central for avoiding the massive  $\text{Ca}^{2+}$  influx that triggers neuronal death in anoxic mammalian brains (Buck and Bickler, 1998; Bickler and Buck, 2007). Interestingly, the NMDA receptor is preferentially inhibited by NO via *S*-nitrosation under low-oxygen conditions (Takahashi et al., 2007), suggesting that *S*-nitrosation via nitrite or nitrite-derived NO could contribute to NMDR receptor downregulation in the anoxic turtle brain.

Anoxia caused significant elevations of nitrite, SNO and FeNO in the pectoral muscle of the turtles (Fig. 6). This response differs from the decrease in nitrite and marginal change of SNO and FeNO in white skeletal muscle of hypoxic goldfish (Hansen and Jensen, 2010) or anoxic crucian carp (Sandvik et al., 2012). The pectoral muscle in turtles contains both white and red fibers, and it therefore seems that a higher muscle myoglobin and mitochondria content due to presence of red fibers is associated with higher nitrite levels,



**Fig. 4. Brain NO metabolites.** Concentrations of nitrite (A), SNO+FeNO+NNO (B) and nitrate (C) in brain tissue of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

higher SNO levels and higher NO formation from nitrite (FeNO) during anoxia. Deoxygenated myoglobin effectively reduces nitrite to NO (Shiva et al., 2007b; Helbo et al., 2013), but myoglobin may also be important in elevating muscle nitrite levels. We suggest that the negatively charged nitrite ion binds to positive charges on myoglobin, and that this binding increases during anoxia, because the net positive charge on myoglobin progressively increases as a result of its buffering of  $\text{H}^+$  from the anoxia-induced acidosis. Such binding of nitrite to cellular proteins would elevate total cellular nitrite while keeping the free cytosolic nitrite concentration low to promote influx of nitrite from the extracellular compartment (Hansen and Jensen, 2010). Binding of nitrite to myoglobin may also underlie the rise in cardiac nitrite during anoxia (Fig. 3A), while the rise in RBC nitrite (Fig. 2A) can be explained by similar nitrite binding to deoxygenated haemoglobin (Hb).

The rise in RBC nitrite in anoxic turtles was much higher than observed in anoxic crucian carp (Sandvik et al., 2012). This may be a consequence of the very large anaerobic lactic acid production in turtles, which titrates the Hb towards possession of more positive charges (potentially binding nitrite), whereas crucian carp convert lactate to ethanol, rendering their anaerobic metabolism acid-base neutral (Nilsson and Lutz, 2004; Bickler and Buck, 2007). Erythrocyte [SNO] and [FeNO+NNO] also increased considerably in anoxic turtles (Fig. 2), reflecting a high RBC thiol content and a relatively high nitrite-reductase capability of the deoxygenated Hb (Jacobsen et

al., 2012). Apart from being cytoprotective, the extraordinary large increases in RBC nitrite and SNO may play a role in hemostasis. Blood circulation is extremely slow in anoxic turtles at low temperature (heart rate  $<0.5 \text{ beats min}^{-1}$ ), which introduces the risk of blood clotting due to stagnant blood (Jackson, 2002). Endothelial NO inhibits platelet activation and aggregation in mammals (Wu and Thiagarajan, 1996). Assuming a similar inhibition in turtle thrombocytes, the high RBC levels of nitrite and SNO could deliver NO bioavailability to inhibit thrombosis formation in anoxia. This entails RBC escape of NO produced by deoxyHb-mediated nitrite reduction and/or transnitrosation processes across the membrane (Pawloski et al., 2001; Cosby et al., 2003; Jensen, 2009b). Platelet inhibition by nitrite-derived NO from deoxygenated erythrocytes was recently documented in human RBCs (Srihirun et al., 2012).

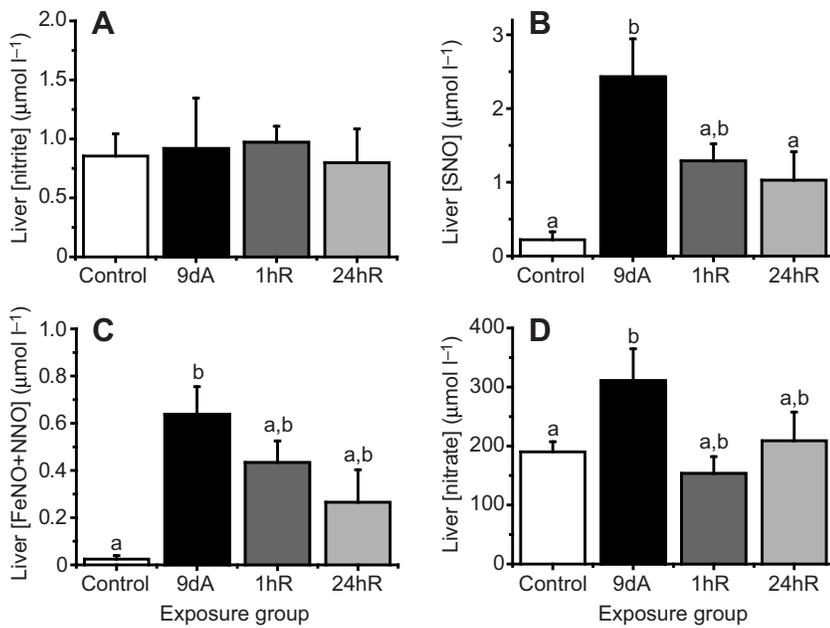
#### Where does tissue nitrite originate?

It is intriguing that the steady-state concentration of nitrite in tissues either increased (heart, pectoral muscle, RBCs) or remained constant (brain, liver) during anoxia, where nitrite formation from autoxidation of NOS-derived NO is halted because of a complete lack of oxygen, and where nitrite is continually used to produce NO (as shown by elevated FeNO) and SNO. A similar situation was observed in anoxic crucian carp (Sandvik et al., 2012) and hypoxic goldfish (Hansen and Jensen, 2010). In both of these fish species, plasma nitrite declined during hypoxia/anoxia, suggesting that nitrite was transferred from extracellular to intracellular compartments (Hansen and Jensen, 2010; Sandvik et al., 2012). In the anoxic turtle, plasma nitrite tended to increase (Fig. 1A). One possible explanation that is compatible with these findings is that nitrite originates from an internal or external reservoir and is carried in the blood to be taken up into the tissues. This would increase or decrease steady-state plasma nitrite, depending on uptake/release rates from the reservoir and tissue nitrite consumption rates. In the case of fish, the reservoir could be ambient nitrite that is taken up across the gills (Jensen, 2009a; Sandvik et al., 2012). Ambient nitrite could also play a role in the turtle, if it swallows water and takes up nitrite across the intestine. Additionally, we hypothesize that the shell contains reservoirs of nitrite and nitrate salts that are released to the blood upon acidification during anoxia, much in line with the release of calcium and magnesium carbonates used in buffering (see below). We are currently investigating these possibilities.

It is furthermore plausible that tissue nitrite could be supplemented through reduction of nitrate to nitrite (Lundberg et al., 2008). While the tissue nitrate measurements did not support this idea by showing a decrease in  $[\text{NO}_3^-]$  during anoxia, it should be remembered that maintenance or increase in tissue nitrite would require an only low micromolar change in nitrate, which is difficult to detect on the very high background concentration of nitrate in the turtles. Furthermore, tissue nitrate could be upheld through supply from reservoirs, as suggested for nitrite, compounding interpretations from concentration measurements. Nitrate reduction to nitrite therefore remains an option for further investigation.

#### Glutathione and protein thiols

Glutathione is an important cellular redox buffer and antioxidant, and high levels would provide protection against oxidative stress when anoxic tissues are reoxygenated (Deneke, 2000). Tissue levels of total glutathione in *T. scripta* are relatively high in comparison with other ectotherms, but were reported to decrease in some organs (including liver and heart) during 20 h anoxia at  $5^\circ\text{C}$  (Willmore and Storey, 1997b). Our glutathione concentrations in liver and heart are comparable with the previous reported values, but a decrease in



**Fig. 5. Liver NO metabolites.** Concentrations of nitrite (A), SNO (B), FeNO+NNO (C) and nitrate (D) in liver tissue of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

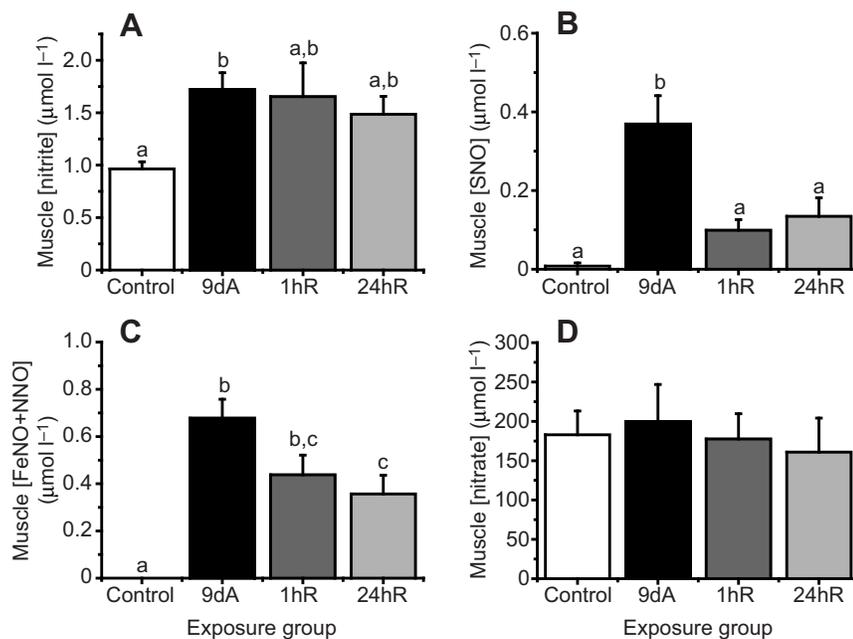
values was not observed following the longer (9 days) anoxia exposure used here (Fig. 7). While tissue glutathione concentrations are in the low millimolar range, plasma values are only in the micromolar range (Fig. 7). A significant increase in plasma glutathione was observed during anoxia (Fig. 7A), which can be ascribed to export from cells, possibly liver cells (Deneke, 2000).

The main antioxidant capacity of the blood resides inside the RBCs. *Trachemys scripta* was suggested to contain Hb with a high pool of reactive thiols with antioxidant capacity (Reischl et al., 2007), and we recently measured a total erythrocyte thiol concentration of 24  $\text{mmol l}^{-1}$  in *T. scripta* (Jacobsen et al., 2012), which is much higher than in most vertebrates but similar to another freshwater turtle, *Phrynops hilarii* (Reischl, 1986). Given that the erythrocyte glutathione concentration is  $\sim 2 \text{ mmol l}^{-1}$  (Reischl, 1986), it would seem that turtle Hb contains many thiols that might participate in quenching ROS produced inside the mitochondria-

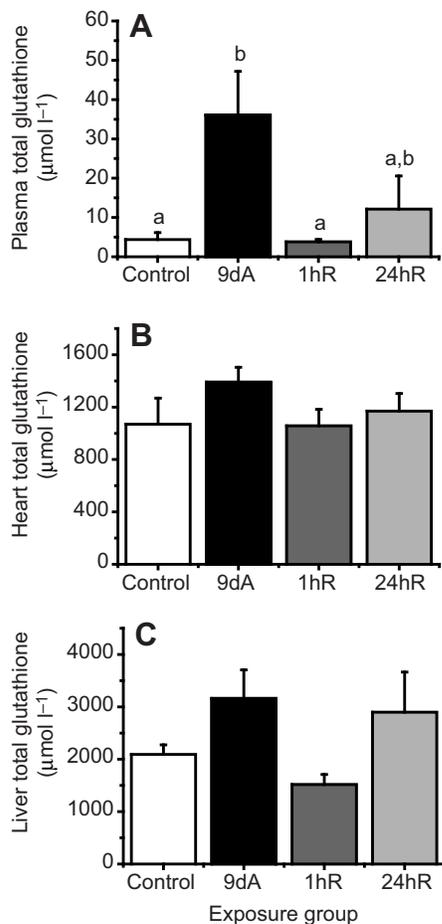
containing erythrocytes when they re-oxygenate. Protein surface thiols may actually generally dominate over glutathione in the protection against oxidative damage in tissue cells (Hansen et al., 2009; Requejo et al., 2010).

#### Electrolyte status

Anaerobiosis increased plasma lactate concentrations to 51  $\text{mmol l}^{-1}$  during 9 days of anoxia at 5°C (Fig. 8A), which matches extrapolation of time-dependent lactate changes during 7 days of anoxia in *T. scripta* (Warren and Jackson, 2007). The increases in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  during anoxia (Fig. 8) are also of the expected magnitude, and results from the release of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  carbonates from shell and skeleton to buffer the anaerobically produced lactic acid (Jackson, 2002; Warren and Jackson, 2007). The combined changes in strong anions ( $\text{Cl}^-$  and lactate) and strong cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) resulted in a significant decrease in SID



**Fig. 6. Skeletal muscle (pectoral muscle) NO metabolites.** Concentrations of nitrite (A), SNO (B), FeNO+NNO (C) and nitrate (D) in pectoral muscle of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

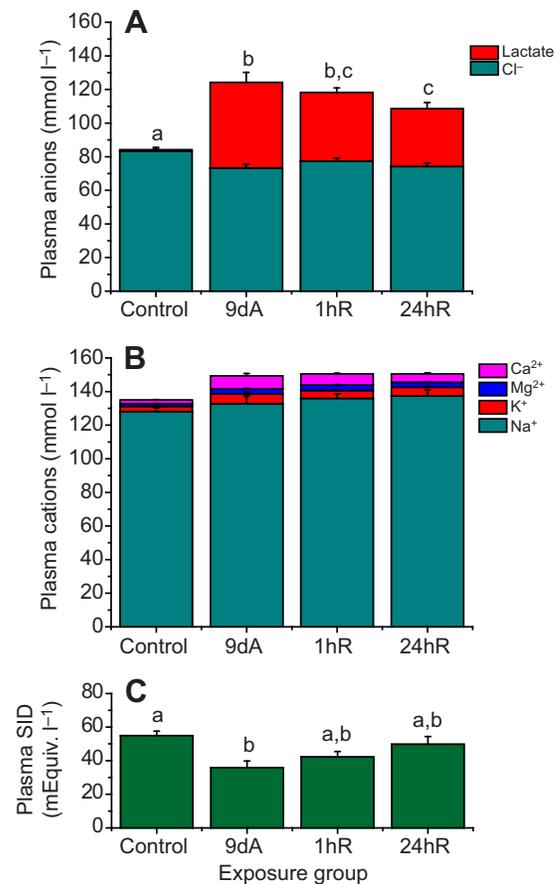


**Fig. 7. Glutathione concentrations.** Levels of total glutathione in plasma (A), heart (B) and liver (C) of red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

(Fig. 8C), effecting metabolic acidosis with associated decreases in plasma bicarbonate and the negative charge carried by plasma proteins and inorganic phosphate (Stewart, 1983; Fencil and Leith, 1993). While the total extracellular electrolyte concentration and osmolality increases during anoxia, the fractional water content in tissues (cardiac muscle, skeletal muscle and liver) remains stable as a result of effective cell volume regulation (Jackson and Heisler, 1983). This implies that the measured concentrations of NO metabolites were not influenced by water shifts between intracellular and extracellular compartments, as also supported by constancy in heart protein concentration during exposures (see Results).

### Concluding remarks

The findings that anoxia increases nitrite, SNO and FeNO concentrations in multiple tissues of both crucian carp (Sandvik et al., 2012) and red-eared sliders (present study) – two archetypical anoxia-tolerant vertebrates – suggest that this is a general and evolutionarily old mechanism involved in surviving anoxia and protecting tissues against injury during anoxia and subsequent reoxygenation. The elevation of nitrite to produce NO and S-nitrosate critical proteins may be particularly important in limiting ROS formation during reoxygenation and thus be central to the antioxidant strategy that prevents injury during anoxia and reoxygenation in anoxia-tolerant vertebrates. The increase in NO



**Fig. 8. Extracellular electrolyte status.** Plasma concentrations of anions (A; lactate and  $\text{Cl}^-$ ) and cations (B;  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and  $\text{Na}^+$ ) in red-eared slider turtles subjected to normoxia (control), anoxia (9dA) and anoxia with subsequent reoxygenation (1hR and 24hR). Values are stacked to reveal the sum of anions (A) and the sum of cations (B). The strong ion difference (SID) is shown in C. Values are means  $\pm$  s.e.m.; lowercase letters (as explained in the Fig. 1 legend) refer to lactate (A) and SID (C). Statistics for other electrolytes are mentioned in the Results.

bioavailability during anoxia, where ordinary NOS activity is halted, calls for future studies on the roles of internal and/or external nitrite reservoirs and cellular nitrate reduction to nitrite.

## MATERIALS AND METHODS

### Experimental animals

Red-eared sliders, *Trachemys scripta elegans*, with a body mass of  $395 \pm 14$  g (mean  $\pm$  s.e.m.,  $N=25$ ) were obtained from commercial suppliers and maintained in large aquaria (water temperature of  $25^\circ\text{C}$ ) with free access to dry platforms under infrared lamps for behavioral thermoregulation. The turtles were fed fish and mussels, but food was withheld for 2 weeks before temperature was reduced.

### Thermal acclimation and exposure to anoxia and reoxygenation

After 2 weeks of fasting, water temperature was lowered to  $20^\circ\text{C}$  for 1 week, followed by a further reduction to  $15^\circ\text{C}$  for an additional week. Then water temperature was reduced to  $10^\circ\text{C}$  for 2 weeks, followed by 3 days at  $5^\circ\text{C}$ . One group of normoxic turtles ( $N=7$ ) was then allowed continued access to air over the next 8 days, while 18 other turtles were subjected to anoxia for 9 days by submergence in anoxic water bubbled continuously with nitrogen. The anoxic aquarium was covered with a plastic lid and contained a metal mesh below the water surface, so that the turtles could not surface to breathe.

The turtles were unrestrained and free to move within the aquarium. After 9 days, six of the anoxic turtles were sampled (anoxic group). Another six anoxic turtles were allowed to breathe air for 1 h (1 h reoxygenation), while the remaining six anoxic turtles were allowed to breathe air for 24 h (24 h reoxygenation).

### Anaesthesia and sampling of blood and organs

The turtles were anaesthetized by an injection of 100 mg kg<sup>-1</sup> of the barbiturate Nembutal in the supravertebral venous sinus, which rendered the animals unresponsive within 5–15 min. The anoxic turtles had to be briefly removed from the water during the injection, but were immediately returned to the anoxic water while the anaesthesia took effect. When the turtles lost all responses to pinching of the legs as well as the corneal reflex, they were placed in a supine position on ice and a 6×4 cm portion of the plastron was quickly excised using a bone saw, so 2–3 ml blood could be sampled from the left aortic arch or by cardiac puncture into heparinized syringes. The blood was processed immediately, while samples of ventricle, pectoral muscle, liver and brain were excised.

Blood was transferred to a pre-weighed tube, and subsamples were taken for measurements of plasma lactate and plasma glutathione (see below). The remaining blood was centrifuged (2 min at 16,000 g and 5°C), and the plasma was transferred to a new tube and frozen in liquid nitrogen. The tube containing the RBCs was weighed (to determine RBC mass) and then frozen in liquid nitrogen. Each dissected organ/tissue was instantly washed in cold phosphate-buffered saline [50 mmol l<sup>-1</sup> phosphate buffer, pH 7.8; 85 mmol l<sup>-1</sup> NaCl; 2.4 mmol l<sup>-1</sup> KCl; 10 mmol l<sup>-1</sup> N-ethylmaleimide (NEM); 0.1 mmol l<sup>-1</sup> diethylethylaminepentaacetic acid (DTPA)], and then dried on a paper towel, weighed and frozen in liquid N<sub>2</sub>. The entire sampling procedure lasted less than 10 min. Procedures were performed in accordance with the laws of animal care and experimentation in Denmark.

### Measurements

For measurements of NO metabolites, the samples of ventricle, pectoral muscle, liver and brain tissues were thawed in four times their mass of a 50 mmol l<sup>-1</sup> phosphate buffer (pH 7.3), containing 10 mmol l<sup>-1</sup> NEM and 0.1 mmol l<sup>-1</sup> DTPA (Sigma-Aldrich, Steinheim, Germany) 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<sup>-1</sup> K<sub>3</sub>[Fe(CN)<sub>6</sub>], 10 mmol l<sup>-1</sup> NEM, 0.1 mmol l<sup>-1</sup> DTPA and 1% NP-40 (Yang et al., 2003; Hansen and Jensen, 2010). The hemolysate was vortexed and centrifuged (2 min, 16,000 g, 2°C), and supernatants were immediately measured.

NO metabolites were assessed by reductive chemiluminescence, using a Sievers Nitric Oxide Analyzer (model 280i, Boulder, CO, USA) and previously described procedures to distinguish between [nitrate], [nitrite], [SNO] and [FeNO+NNO] (Yang et al., 2003; Hansen and Jensen, 2010). Glutathione was measured in plasma, heart and liver, using a colorimetric detection kit according to the manufacturer's instructions (Arbor Assays, catalog number K006-H1, Ann Arbor, MI, USA). Protein was ascertained in heart homogenates by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Plasma lactate was assessed by the lactate dehydrogenase method after deproteinization of plasma with 0.6 mol l<sup>-1</sup> perchloric acid. Plasma chloride was measured by coulometric titration (Sherwood Scientific Chloride Analyzer 926S, Cambridge, UK), and plasma sodium, potassium, calcium and magnesium were determined by atomic absorption spectroscopy (Perkin Elmer AAnalyst 100, Norwalk, CT, USA).

### 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 financial interests.

### Author contributions

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

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