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1 **Nitric oxide metabolites during anoxia and reoxygenation in the anoxia-tolerant vertebrate,**
2 ***Trachemys scripta***
3
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18 **SUMMARY**

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

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INTRODUCTION

Episodes with suspended oxygen supply followed by reoxygenation represent a major challenge to animals. Most vertebrates only tolerate brief anoxia periods due to rapid cellular depletion of ATP and the subsequent failure of ion transporting ATPases to uphold ionic gradients, hence leading to depolarization, cellular Ca^{2+} 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 (Lefer 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 in 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 like 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*), defend ATP levels during anoxia through metabolic depression, which includes down-regulation 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 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-Savin, 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 and some of its metabolites that through

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70 inhibition of complexes in the mitochondrial respiratory chain can limit ROS generation and tissue
71 injury in mammalian ischemia/reperfusion models (Shiva et al., 2007a; Murillo et al., 2011;
72 Chouchani et al., 2013). The involvement of NO and its metabolites in cytoprotection have not yet
73 been explored in anoxia-tolerant turtles.

74 Nitric oxide (NO) is a ubiquitous signaling molecule that under aerobic conditions is produced
75 from the reaction of L-arginine with O_2 catalyzed by nitric oxide synthases (NOS). NO typically
76 exerts its physiological influences through nitrosylation of heme groups, forming iron-nitrosyl
77 (FeNO) proteins, or via S-nitrosation of cysteines, forming S-nitroso (SNO) compounds (Denninger
78 and Marletta, 1999; Foster et al., 2009; Hill et al., 2010). Other products derived from NO
79 production include N-nitroso (NNO) compounds (formed in reactions with amines), nitrite (formed
80 in reaction with O_2) and nitrate (formed in reactions with oxygenated hemoglobin and myoglobin)
81 (Umbrello et al., 2013). Recent research shows that endogenous nitrite represents a reservoir of NO
82 activity that can be activated by a number of cellular proteins under O_2-limiting conditions
83 (Lundberg et al., 2008). Thus, nitrite can be reduced to NO by deoxygenated
84 myoglobin/hemoglobin, xanthine oxidoreductase and other proteins, which supply an alternative
85 pathway for NO formation, when NOS activity is compromised by oxygen shortage. Nitrate may
86 similarly be recycled to NO, if it is first reduced to nitrite (Jansson et al., 2008; Lundberg et al.,
87 2008). Furthermore, SNO compounds can donate NO activity by transnitrosation between low-
88 molecular-weight SNOs and proteins or protein-protein transnitrosation (Nakamura and Lipton,
89 2013). In this way many central NO metabolites contribute to the overall NO bioavailability, which
90 could play an important role under hypoxic and anoxic conditions.

91 We recently showed that hypoxia-tolerant fish have profound capacity for defending intracellular
92 NO metabolite levels during prolonged periods of hypoxia (Hansen and Jensen, 2010) and anoxia
93 (Sandvik et al., 2012). Notably, in crucian carp, anoxia was associated with a dramatic increase of
94 nitrite levels in heart tissue, which was paralleled by elevated concentrations of SNO and FeNO
95 compounds, showing that nitrite was partially metabolized to NO (FeNO) and SNO (Sandvik et al.,
96 2012). Given that experimental elevation of nitrite and NO alleviate injury to cardiac muscle during
97 ischemia/reperfusion in mammals (Shiva et al., 2007; Hendgen-Cotta et al., 2008), it is likely that
98 crucian carp up-regulates nitrite and NO levels to protect the heart from anoxia/reoxygenation
99 damage (Sandvik et al., 2012). To ascertain whether this is a general and evolutionary conserved
100 cytoprotective strategy amongst anoxia-tolerant vertebrates, it is imperative to study other archetype

101 anoxia-tolerant vertebrates, *viz.* freshwater turtles, some of which can survive anoxia for months at
102 low temperature (Jackson, 2002).
103 There is only limited information on NO homeostasis in turtles. Circulating levels of NO
104 metabolites are relatively high in *T. scripta* and increase during short-term anoxia and
105 reoxygenation at room temperature (21 °C) (Jacobsen et al., 2012), but tissue NO levels, which are
106 central in relation to cytoprotection, remain unknown. In the present study we focus on NO
107 metabolites in a number of different tissues (heart, brain, liver, skeletal muscle and blood) during
108 long-term anoxia at low temperature to simulate the ecological relevant situation of winter
109 dormancy in ice-covered ponds (Jackson and Ultsch, 2010). We hypothesized that anoxic turtles
110 would increase NO bioavailability in the tissues to cope with anoxia and subsequent reoxygenation.
111 To gain detailed insight into the reoxygenation phase, we included both an acute (1 h) and longer-
112 lasting (24 h) reoxygenation scheme. We also assessed glutathione in selected tissues and analyzed
113 extracellular ionic composition to evaluate lactate load and acid-base-related consequences of
114 anaerobiosis. Our results support a vivid participation of nitric oxide metabolites in the tolerance of
115 red-eared sliders to anoxia and reoxygenation.
116
117 **MATERIALS AND METHODS**
118
119 *Experimental animals*
120 Red-eared sliders *Trachemys scripta* (Gray) with a body mass of 395 ± 14 g (mean ± s.e.m., N = 25)
121 were obtained from commercial suppliers and maintained in large aquaria (water temperature of 25
122 °C) with free access to dry platforms under infrared lamps for behavioral thermoregulation. The
123 turtles were fed fish and mussels, but food was withheld for two weeks before temperature was
124 reduced.
125
126 *Thermal acclimation and exposure to anoxia and reoxygenation*
127 After two weeks of fasting, water temperature was lowered to 20°C for one week, followed by a
128 further reduction to 15°C for an additional week. Then water temperature was reduced to 10°C for
129 two weeks, followed by three days at 5°C. One group of normoxic turtles (N=7) were then allowed
130 continued access to air over the next 8 days, while 18 other turtles were subjected to anoxia for 9
131 days by submergence in anoxic water bubbled continuously with nitrogen. The anoxic aquarium
132 was covered with a plastic lid and contained a metal mesh below the water surface, so that the

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133 turtles could not surface to breathe. The turtles were unrestrained and free to move within the
134 aquarium. After 9 days, 6 of the anoxic turtles were sampled (anoxic group). Another 6 anoxic
135 turtles were allowed to breathe air for 1 h (1 h reoxygenation), while the remaining 6 anoxic turtles
136 were allowed to breathe air for 24 h (24 h reoxygenation).
137
138 *Anaesthesia and sampling of blood and organs*
139 The turtles were anaesthetized by an injection of 100 mg $kg^{-1}$of the barbiturate Nembutal in the
140 supravertebral venous sinus, which rendered the animals unresponsive within 5-15 min. The anoxic
141 turtles had to be briefly removed from the water during the injection, but were immediately returned
142 to the anoxic water while the anesthesia took effect. When the turtles lost all responses to pinching
143 of the legs as well as the corneal reflex, they were placed in a supine position on ice and a 6 × 4 cm
144 portion of the plastron was quickly excised using a bone saw, so 2-3 ml blood could be sampled
145 from the left aortic arch or by cardiac puncture into heparinized syringes. The blood was processed
146 immediately, while samples of ventricle, pectoral muscle, liver and brain were excised.
147 Blood was transferred to a pre-weighed tube, and subsamples were taken for measurements of
148 plasma lactate and plasma glutathione (cf. below). The remaining blood was centrifuged (2 min at
149 16,000 ***g*** and 5°C), and the plasma was transferred to a new tube and frozen in liquid nitrogen. The
150 tube containing the red blood cells (RBCs) was weighed (to determine RBC mass) and then frozen
151 in liquid nitrogen. Each dissected organ/tissue was instantly washed in cold phosphate-buffered
152 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-
153 ethylmaleimide (NEM); 0.1 mmol l^{-1} diethyletriaminepentaacetic acid (DTPA)], and then dried on a
154 paper towel, weighed and frozen in liquid N_2. The entire sampling procedure lasted less than 10
155 min. Procedures were performed in accordance with the laws of animal care and experimentation in
156 Denmark.
157
158 *Measurements*
159 For measurements of NO metabolites, the samples of ventricle, pectoral muscle, liver and brain
160 tissues were thawed in four times their mass of a 50 mmol l^{-1} phosphate buffer (pH 7.3), containing
161 10 mmol l^{-1} NEM and 0.1 mmol l^{-1} DTPA (Sigma-Aldrich, Steinheim, Germany) to stabilize *S*-
162 nitrosothiols (Yang et al., 2003). The samples were homogenized and centrifuged (6 min, 16000 ***g***,
163 2°C), after which the supernatants were frozen in liquid nitrogen and stored at -80 °C until
164 measured. The RBCs samples were thawed by adding nine times their mass of a nitrite/SNO

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165 preservation solution, consisting of 5 mmol l^{-1} $K_3[Fe(CN)_6]$, 10 mmol l^{-1} NEM, 0,1 mmol l^{-1} DTPA
166 and 1% NP-40 (Yang et al., 2003; Hansen and Jensen, 2010). The hemolysate was vortexed and
167 centrifuged, and supernatants were immediately measured.
168 NO metabolites were assessed by reductive chemiluminescence, using a Sievers (Boulder, CO,
169 USA) Nitric Oxide Analyzer (model 280i) and previously described procedures to distinguish
170 between [nitrate], [nitrite], [SNO] and [FeNO + NNO] (Yang et al., 2003; Hansen and Jensen,
171 2010). Glutathione was measured in plasma, heart and liver, using a colorimetric detection kit
172 according to the manufactures instructions (Arbor Assays, catalog number K006-H1). Protein was
173 ascertained in heart homogenates by a NanoDrop 1000 spectrophotometer (Thermo Scientific,
174 Wilmington, DE, USA). Plasma lactate was assessed by the lactate dehydrogenase method after
175 deproteinization of plasma with 0.6 M perchloric acid. Plasma chloride was measured by
176 coulometric titration (Sherwood Scientific Chloride Analyzer 926S), and plasma sodium,
177 potassium, calcium and magnesium were determined by atomic absorption spectroscopy (Perkin
178 Elmer AAnalyst 100).
179
180 *Statistics*
181 All results are presented as means + s.e.m. Statistical differences between exposure groups were
182 evaluated by one-way ANOVA followed by the Tukey post hoc means comparison test (Origin 8.5,
183 OriginLab Corporation, Northampton, MA, USA). Differences between means were considered
184 significant at $P < 0.05$.
185
186 **RESULTS**
187 Plasma nitrite was 0.55 µM in normoxic control animals and did not change significantly with
188 anoxia or reoxygenation (Fig. 1A). Plasma [SNO] similarly did not vary significantly between
189 groups (Fig. 1B), whereas plasma [FeNO + NNO] was significantly elevated after 9 days of anoxia
190 in order to recover partially during 24 h of reoxygenation (Fig. 1C). Plasma nitrate was relatively
191 high in normoxic control animals (~400 µM) and did not differ significantly among experimental
192 groups (Fig. 1D).
193 In contrast to the modest changes in the plasma, there were large significant increases in [nitrite],
194 [SNO] and [FeNO + NNO] inside the RBCs after 9 days of anoxia (Fig. 2A,B,C). The intracellular
195 NO metabolites increased to values considerably above values both in normoxic controls (Fig. 2)
196 and in the extracellular compartment (Fig. 1). Reoxygenation reversed the changes and induced

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197 significant decreases in erythrocyte nitrite, SNO and FeNO+NNO, but values did not quite reach
198 basal levels by 24 h reoxygenation (Fig 2). Nitrate tended to increase with anoxia ($P = 0.085$) and
199 then decreased with reoxygenation (Fig. 2D).
200 In the heart ventricle, nitrite showed a significant five-fold increase from 0.54 µM in normoxic
201 controls to 2.83 µM after 9 days of anoxia, which was succeeded by a gradual and partial recovery
202 towards control values during 24 h of reoxygenation (Fig. 3A). Ventricle [SNO] also showed a
203 large increase with anoxia, followed by a decline in values during reoxygenation. This decrease in
204 [SNO] was significant already by 1 h of reoxygenation (Fig. 3B). Heart [FeNO + NNO] similarly
205 rose during anoxia and decreased during reoxygenation (Fig. 3C), whereas nitrate did not change
206 significantly (Fig. 3D). Heart protein was constant around 50 mg g^{-1}wetweight in all exposure
207 groups. As a consequence, heart NO metabolites normalized to protein showed the same patterns of
208 changes as for the absolute concentrations presented in Figure 3 (not illustrated), revealing that the
209 concentrations were not significantly influenced by water shifts between extracellular and
210 intracellular compartments.
211 Brain nitrite and nitrate remained relative constant with treatment (Fig. 4A,C), while the
212 concentration of nitros(yl)ated species (SNO+FeNO+NNO) in the brain increased significantly with
213 anoxia and reoxygenation (Fig. 4B).
214 Liver nitrite was kept relatively constant at 0.8-1 µM in normoxic, anoxic and reoxygenated
215 turtles (Fig. 5A). At the same time there were major increases in liver [SNO] (Fig. 5B) and
216 [FeNO+NNO] (Fig. 5C) during anoxia that reversed towards control values during subsequent
217 reoxygenation. Liver nitrate increased with anoxia and decreased with reoxygenation (Fig. 5D).
218 Anoxia triggered a significant elevation of the nitrite concentration in pectoral muscle with
219 subsequent partial recovery during reoxygenation (Fig. 6A). Concomitantly there were major
220 anoxia-induced increases in muscle [SNO] and [FeNO + NNO] (Fig. 6B,C). The muscle [SNO]
221 recovered towards control values already by 1 h of reoxygenation (Fig.6B), while the decrease in
222 [FeNO + NNO] during reoxygenation was slower (Fig. 6C). Muscle nitrate was not affected by
223 treatment (Fig. 6D).
224 Plasma glutathione levels were in the low micromolar range in normoxic turtles and increased
225 significantly with anoxia in order to recover with reoxygenation (Fig. 7A). Total glutathione was
226 around 1 mM in the heart and some 2-3 mM in the liver (Fig. 7B,C) and did not change
227 significantly with anoxia or reoxygenation.

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228 Plasma lactate showed a pronounced significant increase from 1 mM to 51 mM during 9 days of
229 anoxia, followed by a gradual and significant recovery to 34.4 mM by 24 h reoxygenation (Fig.
230 8A). Lactate values were, however, considerably above controls at 24 h of recovery. Plasma
231 chloride decreased significantly by some 10 mM during anoxia (Fig. 8A). Among cations,
232 significant increases were observed in plasma K^+ (from 3.1 to 5.7 mM), Ca^{2+} (from 2.2 to 7.7 mM)
233 and Mg^{2+} (from 1.6 to 3.1 mM) during anoxia followed by incomplete recovery during
234 reoxygenation (Fig. 8B). Plasma Na^+ showed a trend for an increase, but changes in Na^+ were not
235 significant. Anoxia caused increases in the sum of measured anions (Fig. 8A) and the sum of
236 measured cations (Fig. 8B), suggesting an increase in total osmolality. It was also evident that the
237 sum of strong anions (Cl^- and lactate) increased more than the sum of strong cations (Na^+, K^+, Ca^{2+}
238 and Mg^{2+}) during anoxia (Fig. 8A,B). The strong ion difference (SID), which is a major
239 independent variable in acid-base status (Stewart, 1983), was accordingly calculated as:
240
241 $$\text{SID} = ([Na^+] + [K^+] + 2[Ca^{2+}] + 2[Mg^{2+}]) - ([Cl^-] + [\text{lactate}])$$
242
243 Anoxia induced a significant decrease in SID with subsequent recovery during reoxygenation (Fig
244 8C).
245
246 **DISCUSSION**
247
248 The present study documents profound and dynamic changes in tissue NO metabolites that seems to
249 be part of the adaptation strategy to anoxia and reoxygenation in the freshwater turtle *T. scripta*.
250 Nitrite was either increased or kept constant in anoxic tissues. In all the tissues, nitrite was used to
251 produce NO, as evidenced by a general increase in iron-nitrosylated (FeNO) compounds. At the
252 same time there was a universal elevation of cellular *S*-nitroso (SNO) compounds. This overall
253 increase in NO bioavailability may protect the cells from destructive Ca^{2+} influx during anoxia and
254 limit ROS formation and ROS-induced damage during reoxygenation, as discussed below.
255
256 *Tissue NO metabolites in anoxia and reoxygenation*
257 Nitrite was increased considerably above control levels in the anoxic turtle heart, and this was
258 associated with major increases in [SNO] and [FeNO + NNO] (Fig. 3). Similar changes were
259 recently reported in the hearts of anoxia-exposed crucian carp (Sandvik et al., 2012). Thus, it

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260 appears that an increase in NO bioavailability inside the heart constitutes a general mechanism for
261 cardioprotection in anoxia-tolerant vertebrates. Many studies with mammalian models have shown
262 that moderate elevations of nitrite reduce cell death and infarct size following myocardial ischemia
263 and reperfusion (Webb et al., 2004; Duranski et al., 2005; Shiva et al., 2007a; Hendgen-Cotta et al.,
264 2008). Such cytoprotection from elevated nitrite could be essential to preserve cardiac function in
265 turtle and crucian carp during and after anoxia. Notably, in contrast to the experimental addition of
266 nitrite that is required for cytoprotection in mammals, the freshwater turtle and crucian carp possess
267 innate physiological mechanisms that elevate cardiac nitrite in anoxia. In the anoxic heart, nitrite is
268 reduced to NO mainly by deoxygenated myoglobin, and much of the nitrite/NO-dependent
269 cytoprotection is targeted at the mitochondria, involving Fe-nitrosylation of complex IV and S-
270 nitrosation of complex I of the respiratory chain (Hendgen-Cotta et al., 2008; Murillo et al., 2011).
271 Inhibition of complex I by S-nitrosation seems to play a central role by limiting mitochondrial ROS
272 generation and ROS-induced damage upon reoxygenation (Shiva et al., 2007a; Murillo et al., 2011).
273 The key mechanism seems to be S-nitrosation of a conserved cysteine on the ND3 subunit of
274 complex I, which slows the reactivation of mitochondria during early reoxygenation, thereby
275 decreasing ROS production and oxidative damage (Chouchani et al., 2013). However, a number of
276 other proteins may also be S-nitrosated and contribute to cardioprotection (Sun et al., 2007; Lima et
277 al., 2009; Murphy et al., 2012). For instance, problems with cytosolic Ca^{2+} overload during anoxia
278 and early reoxygenation may be alleviated by S-nitrosylation of the L-type Ca^{2+} channel (which
279 inhibits its activity and reduces cellular Ca^{2+} influx) and by S-nitrosylation of the Ca^{2+}ATPase
280 SERCA2a (which increases Ca^{2+} uptake into the sarcoplasmic reticulum) (Sun et al., 2007; Sun and
281 Murphy, 2010). Furthermore, S-nitrosation of protein thiols provide general protection against
282 ROS-induced irreversible oxidation of thiols, limiting the need for degradation and re-synthesis of
283 damaged proteins after an anoxia/reoxygenation event (Sun and Murphy, 2010; Kohr et al., 2011).
284 The rise in cardiac SNO in anoxic turtles was succeeded by a decrease at 1 h of reoxygenation
285 (Fig 3B), which supports a dynamic role of S-nitrosation and documents that the process is
286 reversible, as required for an appropriate cardioprotective signal. Thus, while S-nitrosation of
287 complex I would be beneficial by limiting ROS formation during early reoxygenation (Shiva et al.,
288 2007a; Chouchani et al., 2013), the inhibition of complex I should gradually be reversed to allow
289 oxidative phosphorylation to resume. The kinetics of these changes may be relatively slow in the
290 turtles due to the low temperature and anoxia-induced hypometabolism.

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291 In the brain (Fig. 4) and the liver (Fig. 5), nitrite stayed constant with anoxia, whereas
292 nitros(yl)ated species increased, enlarging the overall pool of NO metabolites. This resembles the
293 situation in anoxic crucian carp (Sandvik et al., 2012). Thus, while there are organ-specific
294 differences in individual NO metabolite changes, these are rather similar in the two anoxia-tolerant
295 species. Conceivably, nitrite-linked NO and SNO formation provide cytoprotection in liver and
296 brain – like in the heart – as supported by mammalian ischemia/reperfusion experiments addressing
297 these organs (Duranski et al., 2005; Jung et al., 2006; Shiva et al., 2007a; Dezfulian et al., 2012).
298 Turtle neurons have reduced membrane ion permeability ("channel arrest") in anoxia, which
299 decreases the requirements for active ion pumping and thereby ATP expenditure (Nilsson and Lutz,
300 2004). An important target is the *N*-methyl-D-aspartate (NMDA) receptor, because down-regulation
301 of this cation channel in anoxic turtles is central for avoiding the massive Ca^{2+} influx that triggers
302 neuronal death in anoxic mammalian brains (Buck and Bickler, 1998; Bickler and Buck, 2007).
303 Interestingly, the NMDA receptor is preferentially inhibited by NO via *S*-nitrosation under low-
304 oxygen conditions (Takahashi et al., 2007), suggesting that S-nitrosylation via nitrite or nitrite-
305 derived NO could contribute to NMDR receptor down-regulation in the anoxic turtle brain.
306 Anoxia caused significant elevations of nitrite, SNO and FeNO in the pectoral muscle of the
307 turtles (Fig. 6). This response differs from the decrease in nitrite and marginal change of SNO and
308 FeNO in white skeletal muscle of hypoxic goldfish (Hansen and Jensen, 2010) or anoxic crucian
309 carp (Sandvik et al., 2012). The pectoral muscle in turtles contains both white and red fibers, and it
310 therefore seems that a higher muscle myoglobin and mitochondria content due to presence of red
311 fibers is associated with higher nitrite levels, higher SNO levels and higher NO formation from
312 nitrite (FeNO) during anoxia. Deoxygenated myoglobin effectively reduces nitrite to NO (Shiva et
313 al., 2007b; Helbo et al., 2013), but myoglobin may also be important in elevating muscle nitrite
314 levels. We suggest that the negatively charged nitrite ion binds to positive charges on myoglobin,
315 and that this binding increases during anoxia, because the net positive charge on myoglobin
316 progressively increases due to its buffering of H^+ from the anoxia-induced acidosis. Such binding of
317 nitrite to cellular proteins would elevate total cellular nitrite while keeping the free cytosolic nitrite
318 concentration low to promote influx of nitrite from the extracellular compartment (Hansen and
319 Jensen, 2010). Binding of nitrite to myoglobin may also underlie the rise in cardiac nitrite during
320 anoxia (Fig. 3A), while the rise in RBC nitrite (Fig. 2A) can be explained by similar nitrite binding
321 to deoxygenated hemoglobin.

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322 The rise in RBC nitrite in anoxic turtles was much higher than observed in anoxic crucian carp
323 (Sandvik et a., 2012). This may be a consequence of the very large anaerobic lactic acid production
324 in turtles, which titrates the hemoglobin towards possession of more positive charges (potentially
325 binding nitrite), whereas crucian carp convert lactate to ethanol, rendering their anaerobic
326 metabolism acid-base neutral (Nilsson and Lutz, 2004; Bickler and Buck, 2007). Erythrocyte [SNO]
327 and [FeNO + NNO] also increased considerably in anoxic turtles (Fig. 2), reflecting a high RBC
328 thiol content and a relatively high nitrite-reductase capability of the deoxygenated Hb (Jacobsen et
329 al., 2012). Apart from being cytoprotective, the extraordinary large increases in RBC nitrite and
330 SNO may play a role in hemostasis. Blood circulation is extremely slow in anoxic turtles at low
331 temperature (heart rate < 0.5 min^{-1}), which introduces the risk of blood clotting due to stagnant
332 blood (Jackson, 2002). Endothelial NO inhibits platelet activation and aggregation in mammals (Wu
333 and Thiagarajan, 1996). Assuming a similar inhibition in turtle thrombocytes, the high RBC levels
334 of nitrite and SNO could deliver NO bioavailability to inhibit thrombosis formation in anoxia. This
335 entails RBC escape of NO produced by deoxyHb-mediated nitrite reduction and/or transnitrosation
336 processes across the membrane (Pawloski et al., 2001; Cosby et al., 2003; Jensen 2009b). Platelet
337 inhibition by nitrite-derived NO from deoxygenated erythrocytes was recently documented in
338 human RBCs (Srihirun et al., 2012).
339
340 *Where does tissue nitrite originate from?*
341 It is intriguing that the steady state concentration of nitrite in tissues either increased (heart, pectoral
342 muscle, RBCs) or remained constant (brain, liver) during anoxia, where nitrite formation from
343 autoxidation of NOS-derived NO is halted due to complete O_2 lack, and where nitrite is continually
344 used to produce NO (as shown by elevated FeNO) and SNO. A similar situation was observed in
345 anoxic crucian carp (Sandvik et al., 2012) and hypoxic goldfish (Hansen and Jensen, 2010). In both
346 of these fish species plasma nitrite declined during hypoxia/anoxia, suggesting that nitrite was
347 transferred from extracellular to intracellular compartments (Hansen and Jensen, 2010; Sandvik et
348 al., 2012). In the anoxic turtle, plasma nitrite tended to increase (Fig. 1A). One possible explanation
349 that is compatible with these findings is that nitrite originates from an internal or external reservoir
350 and is carried in the blood to be taken up into the tissues. This would increase or decrease steady
351 state plasma nitrite, depending on uptake/release rates from the reservoir and tissue nitrite
352 consumption rates. In the case of fish, the reservoir could be ambient nitrite that is taken up across
353 the gills (Jensen, 2009a; Sandvik et al., 2012). Ambient nitrite could also play a role in the turtle, if

354 it swallows water and take up nitrite across the intestine. Additionally, we hypothesize that the shell
355 contains reservoirs of nitrite and nitrate salts that are released to the blood upon acidification during
356 anoxia, much in line with the release of calcium and magnesium carbonates used in buffering (cf.
357 below). We are currently investigating these possibilities.
358 It is furthermore plausible that tissue nitrite could be supplemented through reduction of nitrate
359 to nitrite (Lundberg et al., 2008). While the tissue nitrate measurements did not support this idea by
360 showing a decrease in [NO_3^-] during anoxia, it should be remembered that maintenance or increase
361 in tissue nitrite would require an only low micromolar change in nitrate, which is difficult to detect
362 on the very high background concentration of nitrate in the turtles. Furthermore, tissue nitrate could
363 be upheld through supply from reservoirs, as suggested for nitrite, compounding interpretations
364 from concentration measurements. Nitrate reduction to nitrite therefore remains an option for
365 further investigation.
366
367 *Glutathione and protein thiols*
368 Glutathione is an important cellular redox buffer and antioxidant, and high levels would provide
369 protection against oxidative stress when anoxic tissues are reoxygenated (Deneke, 2000). Tissue
370 levels of total glutathione in *T. scripta* are relatively high in comparison with other ectotherms but
371 was reported to decrease in some organs (including liver and heart) during 20 h anoxia at 5 °C
372 (Willmore and Storey, 1997b). Our glutathione concentrations in liver and heart compares with the
373 previous reported values, but a decrease in values was not observed following the longer (9 days)
374 anoxia exposure here used (Fig. 7). While tissue glutathione concentrations are in the low
375 millimolar range, plasma values are only in the micromolar range (Fig. 7). A significant increase in
376 plasma glutathione was observed during anoxia (Fig. 7A), which can be ascribed to export from
377 cells, possibly liver cells (Deneke, 2000).
378 The main antioxidant capacity of the blood resides inside the RBCs. *T. scipta* was suggested to
379 contain Hb with a high pool of reactive thiols with antioxidant capacity (Reischl et al., 2007), and
380 we recently measured a total erythrocyte thiol concentration of 24 mM in *T. scripta* (Jacobsen et
381 al., 2012), which is much higher than in most vertebrates but similar to another freshwater turtle
382 *Phrynops hilarii* (Reischl, 1986). Given that erythrocyte glutathione is ~2 mM (Reischl, 1986), it
383 would seem that turtle Hb contains many thiols that might partake in quenching ROS produced
384 inside the mitochondria-containing erythrocytes when they re-oxygenate. Protein surface thiols may

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385 actually generally dominate over glutathione in the protection against oxidative damage in tissue
386 cells (Hansen et al., 2009; Requejo et al., 2010).
387
388 *Electrolyte status*
389 Anaerobiosis increased plasma lactate to 51 mM during 9 days of anoxia at 5°C (Fig. 8A), which
390 matches extrapolation of time-dependent lactate changes during 7 days of anoxia in *T. scripta*
391 (Warren and Jackson, 2007). The increases in Ca^{2+} and Mg^{2+} during anoxia (Fig. 8) are also of the
392 expected magnitude, and results from the release of Ca^{2+} and Mg^{2+} carbonates from shell and
393 skeleton to buffer the anaerobically-produced lactic acid (Jackson, 2002; Warren and Jackson,
394 2007). The combined changes in strong anions (Cl^- and lactate) and strong cations (Na^+, K^+, Ca^{2+}
395 and Mg^{2+}) resulted in a significant decrease in SID (Fig. 8C), effecting metabolic acidosis with
396 associated decreases in plasma bicarbonate and the negative charge carried by plasma proteins and
397 inorganic phosphate (Stewart, 1983; Fencl and Leith, 1993). While the total extracellular electrolyte
398 concentration and osmolality increases during anoxia, the fractional water content in tissues
399 (cardiac muscle, skeletal muscle and liver) remain stable due to effective cell volume regulation
400 (Jackson and Heisler, 1983). This implies that the measured concentrations of NO metabolites were
401 not influenced by water shifts between intracellular and extracellular compartments, as also
402 supported by constancy in heart protein concentration during exposures (cf. results).
403
404 *Concluding remarks*
405 The findings that anoxia increases nitrite, SNO and FeNO concentrations in multiple tissues of both
406 crucian carp (Sandvik et al., 2012) and red-eared sliders (present study) – two archetypical anoxia-
407 tolerant vertebrates – suggest that this is a general and evolutionary old mechanism involved in
408 surviving anoxia and protecting tissues against injury during anoxia and subsequent reoxygenation.
409 The elevation of nitrite to produce NO and S-nitrosate critical proteins may be particularly
410 important in limiting ROS formation during reoxygenation and thus be central to the anti-oxidant
411 strategy that prevents injury during anoxia and reoxygenation in anoxia-tolerant vertebrates. The
412 increase in NO bioavailability during anoxia, where ordinary NOS activity is halted, calls for future
413 studies on the roles of internal and/or external nitrite reservoirs and cellular nitrate reduction to
414 nitrite.
415
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419

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422

423 **Author contributions**
424 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.
425 performed the experiments; F.B.J. and M.N.H. analyzed the data; F.B.J. wrote the paper; M.N.H.,
426 G.M. and T.W. edited the manuscript.
427

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568 **Figure legends**

569 Fig. 1. Plasma nitric oxide (NO) metabolites in red-eared slider turtles under normoxic conditions
570 (control), after 9 days of anoxia (9dA), after 9 days anoxia with subsequent 1 h reoxygenation
571 (1hR), and after 9 days of anoxia with subsequent 24 h reoxygenation (24hR). The panels depict
572 concentrations of A: nitrite; B: S-nitroso compounds (SNO); C: iron-nitrosyl and N-nitroso
573 compounds (FeNO+NNO); D: nitrate. Values are means + s.e.m. and n = 7 (control) or 6 (9dA, 1hR
574 and 24hR) in each group. Different letters at bars indicate a significant difference between groups
575 ($P < 0.05$).

576

577 Fig. 2. Red blood cell (RBC) concentrations of nitrite (A), SNO (B), FeNO+NNO (C), and nitrate
578 (D) in red-eared slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia with
579 subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

580

581 Fig. 3. Heart ventricle concentrations of nitrite (A), SNO (B), FeNO+NNO (C), and nitrate (D) in
582 red-eared slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia with subsequent
583 reoxygenation (1hR and 24hR). The values were determined on a wet weight basis and are shown
584 as absolute concentrations in μmoll^{-1}, assuming a tissue density of 1kg l^{-1}. Other details as in Fig. 1.

585

586 Fig. 4. Brain concentrations of nitrite (A), SNO+FeNO+NNO (B), and nitrate (C) in red-eared
587 slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia with subsequent
588 reoxygenation (1hR and 24hR).

589

590 Fig. 5. Liver concentrations of nitrite (A), SNO (B), FeNO+NNO (C), and nitrate (D) in red-eared
591 slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia with subsequent
592 reoxygenation (1hR and 24hR). Other details as in Fig. 1.

593

594 Fig. 6. Skeletal muscle (pectoral muscle) concentrations of nitrite (A), SNO (B), FeNO+NNO (C),
595 and nitrate (D) in red-eared slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia
596 with subsequent reoxygenation (1hR and 24hR). Other details as in Fig. 1.

597

598 Fig 7. Glutathione concentrations in plasma (A), heart (B) and liver (C) of red-eared slider turtles
599 subjected to normoxia (control), anoxia (9dA), and anoxia with subsequent reoxygenation (1hR and
600 24hR).

601

602 Fig. 8. Plasma concentrations of anions (panel A; lactate and Cl^-) and cations (panel B; Ca^{2+}, Mg^{2+},
603 K^+ and Na^+) in red-eared slider turtles subjected to normoxia (control), anoxia (9dA), and anoxia
604 with subsequent reoxygenation (1hR and 24hR). Values are stacked to reveal the sum of anions (A)
605 and the sum of cations (B). The strong ion difference (SID) is shown in panel C. Values are means
606 + s.e.m. and the letter statistics (as explained in legend to Fig. 1) refer to lactate (panel A) and SID
607 (panel C). Statistics for other electrolytes are mentioned in the text.

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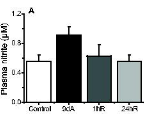
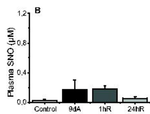

A
Plasma nitrite (µM)
1.2
0.8
0.4
0.0
Control
9dA
1hR
24hR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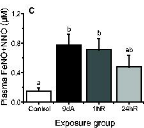
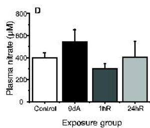
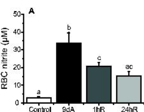
Plasma SNO (µM)
1,2
0,8
0,4
0,0
Control
9dA
1hR
24hR


C
Plasma FeNO+NNO (µM)
1.2
0.8
0.4
0.0
a
b
b
ab
Control
9dA
1hR
24hR
Exposure group


D
Plasma nitrate (µM)
800
600
400
200
0
Control
9dA
1hR
24hR
Exposure group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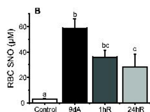
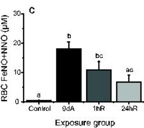
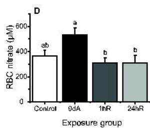
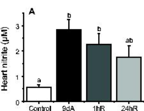
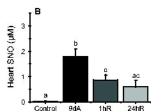
RBC nitrite (μM)
50
40
30
20
10
0
a
b
c
ac
Control
9dA
1hR
24hR

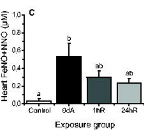

B
RBC SNO (μM)
60
40
20
0
a
b
bc
c
Control
9dA
1hR
24hR

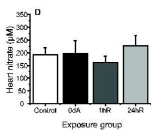

C
RBC FeNO+NNO (μM)
30
20
10
0
a
b
bc
ac
Control
9dA
1hR
24hR
Exposure group

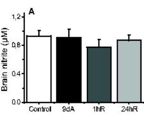

D
RBC nitrate (μM)
600
400
200
0
ab
a
b
b
Control
9dA
1hR
24hR
Exposure group

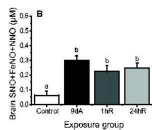

A
Heart nitrite (µM)
0
1
2
3
a
b
b
ab
Control
9dA
1hR
24hR

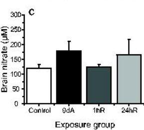

B
Heart SNO (µM)
0
1
2
3
a
b
c
ac
Control
9dA
1hR
24hR

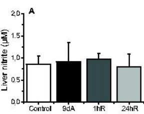

C
Heart FeNO+NNO (µM)
0,0
0,2
0,4
0,6
0,8
1,0
a
b
ab
ab
Control
9dA
1hR
24hR
Exposure group

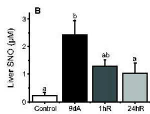

D
Heart nitrate (µM)
0
50
100
150
200
250
300
350
Control
9dA
1hR
24hR
Exposure group

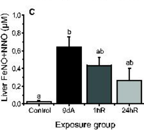

A
Brain nitrite (µM)
1,2
0,8
0,4
0,0
Control
9dA
1hR
24hR

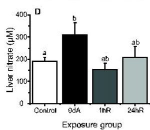

B
Brain SNO+FeNO+NNO (µM)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
a
b
b
b
Control
9dA
1hR
24hR
Exposure group

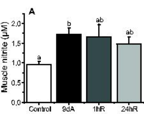

C
Brain nitrate (µM)
300
250
200
150
100
50
0
Control
9dA
1hR
24hR
Exposure group

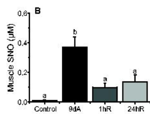

A
Liver nitrite (µM)
2,0
1.5
1,0
0.5
0,0
Control
9dA
1hR
24hR

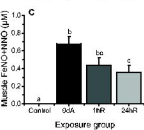

B
Liver SNO (µM)
3
2
1
0
a
b
ab
a
Control
9dA
1hR
24hR

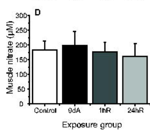

C
Liver FeNO+NNO (µM)
1,0
0,8
0,6
0,4
0,2
0,0
a
b
ab
ab
Control
9dA
1hR
24hR
Exposure group

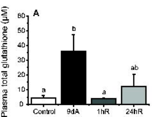
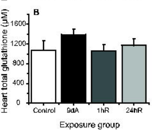
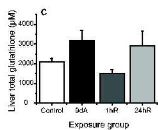

D
Liver nitrate (µM)
400
300
200
100
0
a
b
ab
ab
Control
9dA
1hR
24hR
Exposure group

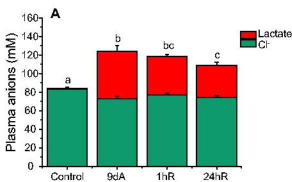

A
Muscle nitrite (μM)
2,0
1,5
1,0
0,5
0,0
a
b
ab
ab
Control
9dA
1hR
24hR

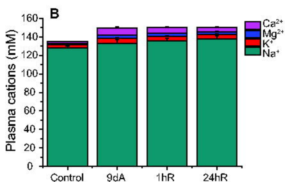

B
Muscle SNO (μM)
0,6
0,4
0,2
0,0
a
b
a
a
Control
9dA
1hR
24hR

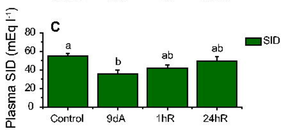

C
Muscle FeNO+NNO (μM)
1,0
0,8
0,6
0,4
0,2
0,0
a
b
bc
c
Control
9dA
1hR
24hR
Exposure group


D
Muscle nitrate (μM)
300
250
200
150
100
50
0
Control
9dA
1hR
24hR
Exposure group


A
Plasma total glutathione (μM)
0
10
20
30
40
50
60
a
b
a
ab
Control
9dA
1hR
24hR

B
Heart total glutathione (μM)
0
400
800
1200
1600
Control
9dA
1hR
24hR
Exposure group

C
Liver total glutathione (μM)
0
1000
2000
3000
4000
Control
9dA
1hR
24hR
Exposure group


A
Plasma anions (mM)
0
20
40
60
80
100
120
140
160
Lactate
Cl⁻
a
b
bc
c
Control
9dA
1hR
24hR


B
Plasma cations (mM)
0
20
40
60
80
100
120
140
160
Ca2+
Mg2+
K+
Na+
Control
9dA
1hR
24hR


C
Plasma SID (mEq l-1)
0
20
40
60
80
SID
a
b
ab
ab
Control
9dA
1hR
24hR
Exposure group