Comparative Biochemistry and Physiology, Part A 203 (2017) 1–8

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The metabolic consequences of repeated anoxic stress in the western painted turtle, *Chrysemys picta bellii*

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ARTICLE INFO

Article history:
Received 30 April 2016
Received in revised form 20 July 2016
Accepted 20 July 2016
Available online 27 July 2016

Keywords:
Citrate synthase
Cytochrome oxidase
FFA
Glycogen
Intermittent anoxia
Ions
NEFA
ROS

ABSTRACT

The painted turtle is known for its extreme tolerance to anoxia, but it is unknown whether previous experience with anoxic stress might alter physiological performance during or following a test bout of anoxia. Repeatedly subjecting 25°C-acclimated painted turtles to 2 h of anoxic stress every other day for 19 days (10 submergence bouts total) caused resting levels of liver glycogen to decrease by 17% and liver citrate synthase (CS) and cytochrome oxidase (COX) activities to increase by 33% and 112%, respectively. When the repeatedly submerged turtles were studied during a subsequent anoxic stress test, liver COX and CS activities decreased during anoxia to the same levels of naïve turtles, which were unchanged, and remained there throughout metabolic recovery. There were no effects of the repeated anoxia treatment on any of the other measured variables, which included lactate dehydrogenase and phosphofructokinase activities in liver, skeletal muscle, and ventricle, blood acid-base status, hemoglobin, hematocrit and plasma ion (Na, K, Ca, Mg, Cl) and metabolite concentrations (lactate, glucose, free-fatty acids), before, during, or after the anoxic stress test. We conclude that although painted turtles can show a physiological reaction to repeated anoxic stress, the changes appear to have no measurable effect on anaerobic physiological performance or ability to recover from anoxia.

1. Introduction

The anoxia-tolerant painted turtle, *Chrysemys picta*, can survive complete oxygen deprivation for >170 days when submerged in anoxic water at 3°C (Ultsch and Jackson, 1982) and for >30 h when submerged at 20°C (Johlin and Moreland, 1933). This extreme ability extends from this species' abilities to depress metabolism, utilize large tissue glycogen stores, especially in liver, and exploit the high buffering capacity of its extracellular fluid and shell (Jackson, 2002; Warren and Jackson, 2007b). Despite these important adaptations, major homeostatic disturbances still occur, including respiratory and lactic acidoses (Warren and Jackson, 2004, 2007a; Wasser et al., 1991), accumulations of extracellular K, Ca, Mg and inorganic phosphate (Herbert and Jackson, 1985; Warren and Jackson, 2007a), and depletions of tissue glycogen stores (Warren et al., 2006). Any enhancement of a turtle's anaerobic performance, such as preventing these deviations or more quickly restoring homeostasis, could prove advantageous to the turtle by allowing it to more quickly return to normal activities, such as foraging and finding mates.

Among other ectothermic and endothermic vertebrate species, there are numerous examples of enhanced anaerobic performance in response to prolonged or repeated reliance on anaerobic metabolism, such as chronic and intermittent hypoxia and repeated sprint exercise (Hoppeler and Vogt, 2001; Pinder and Burggren, 1983; Clanton and Klawitter, 2001; Barnett et al., 2004; Borowiec et al., 2015; Du et al., 2016). These studies have demonstrated that many species have plasticity that improves physiological performance, either during or following the insult. This can include increases in the blood-oxygen carrying capacity and changes in tissue metabolic enzyme activities (Hoppeler and Vogt, 2001; Pinder and Burggren, 1983; Clanton and Klawitter, 2001; Barnett et al., 2004; Borowiec et al., 2015).

There is evidence of seasonal variation in tissue metabolic enzyme activities (Olson, 1987; Olson and Crawford, 1989; Seebacher et al., 2004) and buffering capacity (Olson and Crawford, 1989), suggesting a potential for metabolic plasticity, but it is unknown whether the anoxia-tolerant painted turtle might also show responses to repeated anoxic stress that could improve anaerobic performance. To investigate this possibility, painted turtles were repeatedly subjected to anoxic stress at 25°C and the subsequent effects on the turtle's physiological performance during and following a test dive were studied. The specific parameters measured were (1) the baseline levels of tissue glycogen contents and metabolic enzyme activities, extracellular buffering, and blood-oxygen carrying capacity in resting turtles and (2) the metabolic,

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http://dx.doi.org/10.1016/j.cbpa.2016.07.012
1095-6433/

acid-base, and ionoregulatory responses during and after an anoxic stress bout.

2. Materials and methods

2.1. Animals

Adult western painted turtles, *Chrysemys picta bellii* Gray of both sexes, with a mean body mass of 391.0 g ($N = 64$; range 283.2–626.3 g) were obtained from Lemberger (Oshkosh, WI, USA) in September and maintained at Brown University in large tanks (56 × 55 × 208 cm, H × W × L) filled with water to 37 cm (~423 L) at 25°C. They were held under a 12:12 day: night photoperiod with access to heating lamps and a warming platform and were fed Turtle Brittle (Nasco, Fort Atkinson, WI, USA) *ad libitum* every other day until used in experiments. The experimental protocol used in this study was approved by the Brown University Institutional Animal Care and Use Committee (IACUC).

2.2. Repeated anoxic stress

Painted turtles ($N = 32$) were repeatedly submerged for 2 h at 25°C every 48 h over a 19 day period, for a total 10 cycles. During each dive cycle, the turtles were placed in a plastic box (14 × 38 × 79 cm, H × W × L), which was then flooded for the two-hour submergence period. The turtles were then removed from the box and placed back in their normal holding tanks at 25°C to recover and resume their normal activities. The next day, they were fed Turtle Brittle ad libitum. This dive protocol was chosen because it is known that painted turtles require 10–12 h to achieve full metabolic recovery from 2 h of anoxic stress at 25°C (Warren and Jackson, 2004) and to insure they had one full day to eat and bask between dive days. A second group of control turtles ($N = 32$), were treated in the same way except they were not placed in containers and submerged. The control turtles were fed on the same schedule as the repeatedly submerged turtles.

2.3. End-treatment sampling

Twenty-four hours after the final submergence bout, eight turtles from each group were randomly sampled to test the effects of the repeated anoxic stress treatment on resting tissue glycogen and metabolic enzyme activities, blood oxygen carrying capacity, and plasma ions. Following rapid decapitation and pithing, the plastron was quickly removed with a bone saw, and a 1 mL blood sample was drawn from the ventricle into a glass syringe previously flushed with 1000 IU mL^{-1} ammonium heparin to fill the air space in the tip. This blood sample was used for measurements of hematocrit, hemoglobin content, blood gases and pH. A second syringe without heparin was used to sample another 0.5 mL, which was used to analyze plasma Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, inorganic phosphate (Pi), lactate, glucose, and non-esterified free fatty acids (NEFA). Ventricle, liver and pectoralis muscle were quickly sampled and frozen in clamps cooled with liquid N_2 and stored at −75°C until analyzed for lactate, glycogen, and enzyme activities. Carapace was also sampled and stored at −20°C until analyzed for lactate and total CO_2 content. The details of these analytical procedures are described later.

2.4. Two-hour test submergence of cannulated turtles

The remaining 24 turtles in each treatment ($N = 48$ total, 24 submerged and 24 controls) were cannulated in the left subclavian artery one day after the End-Treatment turtles were sampled (48 h after the final submergence) according to previously published methods (Warren and Jackson, 2004). While the turtle was maintained under isoflurane anesthesia using a Hallowell Anesthesia Workstation and precision veterinary vaporizer (5% induction, 2% maintenance, 20–25 mL tidal volume, 10 breaths min^{-1}), a 2.5 cm hole was cut in the plastron through the left pectoral scute with a trephine and the vessel isolated and occlusively cannulated with an Intramedic PE90 catheter (Becton Dickinson, Sparks, MD, USA) that was flushed and filled with 20 IU mL^{-1} heparinized 0.8% NaCl saline and led out of a hole pierced in the skin of the neck. The hole in the plastron was plugged with an acrylic disc and sealed with dental acrylic (Bosworth Original Truliner, Stokie, IL, USA). The animals were ventilated with oxygen until recovered (1 − 3 h) and then placed in covered plastic containers (13 × 28 × 11 cm, W × L × H) at room temperature that contained 5–6 cm of water until readied for the experiment on the next day.

In order to prevent the turtles from becoming entangled in the cannula during the experiment, the animals were fixed to a large weight with adhesive tape and then placed in large aquarium (40 × 64 × 28 cm, W × L × H). Water was added to the tank just high enough to cover the carapace, while still giving the turtle easy access to air. The aquaria were maintained at 25°C throughout the experiment by a temperature controller. The cannulae were led out of the top of the covered aquaria so that blood samples could be drawn without disturbing the turtles.

After one day of acclimation to the setup, a 0.7 mL blood sample was drawn from the arterial catheters for measurements of blood-acid base status and a second 0.3 mL blood sample for analysis of plasma ions, lactate, glucose, and NEFA. After this point, eight of the cannulated turtles from both groups were euthanized with an overdose of Beuthanasia-D Special (Schering-Plough, Millsborough, DE, USA) via the arterial catheter. In the results section, these turtles are referred to as Post-surgery Controls. The tissues from these turtles were immediately sampled as described above for the End-Treatment group.

The remaining cannulated turtles from both groups ($N = 32$ total) were made anoxic by raising the water level in the aquarium to a level that prevented the turtles from reaching the surface to breathe. Arterial blood samples were obtained at 0.5, 1, 1.5, and 2 h from half of the turtles from each group ($N = 8$ per group) in order to quantify blood acid-base, ion, and metabolite status during the anoxic test submergence. After the final blood sample, these turtles were euthanized and sampled as described above. The data from this group were used to assess the effects of the repeated anoxic stress on anaerobic performance.

The remaining cannulated turtles from both groups ($N = 8$ per group) were allowed to recover by raising the turtles onto a platform that allowed them to breathe air again. Arterial blood samples were obtained from these animals at the end of the 2 h submergence period and at 1, 3, 6, 10 and 13 h into the recovery period. Immediately after the final sample, the turtles were euthanized and their tissues sampled as described above.

2.5. Tissue and metabolite analyses

2.5.1. Blood-gas analyses

Approximately 0.2 mL of the 0.7 mL blood sample was used to measure arterial Po_2 and Pco_2 using a Radiometer PHM73 pH/Blood gas monitor and BMS3 Mk2 Blood Microsystem (Radiometer, Copenhagen, Denmark) thermostatted to 25°C. The remainder (~0.5 mL) was immediately injected through a 4 cm segment of Intramedic® PE60 into the bottom of a 10 mm diameter round-bottom test tube containing an Orion pH electrode (8103BN, Thermo Orion, Beverly, MA, USA), also thermostatted to 25°C. This technique has been used in previous studies (Warren and Jackson, 2007a; Warren et al., 2006) and gives pH values similar to those using other methods. Plasma bicarbonate was calculated from the Henderson-Hasselbalch equation ($pH = pK \pm \log [HCO_3^-] / (\alpha CO_2 \times Pco_2)$) with pK′ 6.1295 (Nicol et al., 1983) and αCO_2 0.0404 (Severinghaus, 1965). Blood hemoglobin content was measured in End-Treatment turtles using a cyanomethemoglobin kit (Stanbio Laboratory, Boerne, TX, USA).

2.5.2. Plasma metabolites

Blood samples were centrifuged for 3 min at 9300 g and the plasma analyzed for lactate and glucose using an automated analyzer (2300

Stat, YSI, Yellow Springs, OH, USA), Ca^{2+} and Mg^{2+} using atomic absorption spectrophotometry (Perkin-Elmer, Boston, MA, USA), Na^{+} and K^{+} using flame photometry (IL 943, Instrumentation Laboratories, Lexington, MA, USA) and Cl^{-}, P_i, and non-esterified free fatty acids with colorimetric kits (Chloride Liquicolor, Stanbio Laboratory; Kit 670, Sigma, St Louis, MO; NEFA C, Wako, Richmond, VA).

2.5.3. Tissue metabolite analyses

Frozen samples of ventricle, liver, and pectoralis muscle (100–200 mg) were homogenized in 1 mL ice-cold 0.6 N perchloric acid using a Mini-Beadbeater 3110BX (Biospec Products, Bartlesville, OK, USA) with 1 mm glass beads for 4 min. A 150 μL sample of this homogenate was buffered with 75 μL 1 M $KHCO_3$, centrifuged for 3 min at 9300 g, and analyzed for glucose and lactate using standard kits (Glucose Assay Reagent, Sigma, St. Louis, MO, USA; Lactate Reagent, Trinity Biotech, St. Louis, MO, USA). A 50 μL sample of the homogenate was buffered with 25 μL 1 M $KHCO_3$ and analyzed for glycogen using the amyloglucosidase method (Keppler and Decker, 1974). Tissue glycogen content was calculated as the difference between the glucose content after the enzyme digestion and the free glucose content from the tissue homogenate.

2.5.4. Tissue enzyme activity assays

Frozen samples of ventricle, liver, and pectoralis muscle were analyzed for activities of phosphofructokinase (PFK), lactate dehydrogenase (LDH), citrate synthase (CS), and cytochrome C oxidase (COX) using the methods of (Seebacher et al., 2004). Unless otherwise noted, all reagents were acquired from Sigma-Aldrich (St. Louis, MO). Tissue samples (50–100 mg) were homogenized using an ice-cold glass homogenizer in 9 mL g^{-1} tissue of extraction buffer containing 50 mmol L^{-1} imidazole, 2 mmol L^{-1} magnesium sulfate, 5 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, and L mmol L^{-1} glutathione (added on the day of use) with final pH 7.5. All assays were carried out at 25°C in a 96-well quartz microplate, were read in a Spectramax Plus 384 plate reader, and analyzed using SOFTmax Pro software (Molecular Devices, Sunnyvale, CA).

LDH assays were carried out in a medium with final concentrations of the following: 75 mmol L^{-1} K_2HPO_4 25 mmol L^{-1} KH_2PO_4, 0.4 mmol L^{-1} sodium pyruvate, and 0.16 mmol L^{-1} NADH, pH 7.0. The absorbance at 340 nm for NADH (E = 6.22) was recorded during the reaction. Ventricle and pectoralis homogenates were diluted an additional 100× and liver by 10×.

PFK assays were carried out in a medium with final concentrations of the following: 75 mmol L^{-1} Tris/HCl, 7 mmol L^{-1} MgCl, 200 mmol L^{-1} KCl, 1 mmol L^{-1} KCN, 0.15 mmol L^{-1} NADH, 2 mmol L^{-1} AMP, 1 mmol L^{-1} ATP, 104 mmol L^{-1} fructose-6-phosphate, 10 IU mL^{-1} triose phosphate isomerase, 2 IU mL^{-1} glycerol-3-phosphate dehydrogenase, pH 8.0. The reaction was started by adding fructose-6-phosphate to the well containing the sample and the other reagents and recording the absorbance change at 340 nm for NADH. Ventricle and pectoralis homogenates were further diluted 10× while liver did not require additional diluting.

COX assays were carried out in a medium with final concentrations of the following: 64 mmol L^{-1} K_2HPO_4, 32 mmol L^{-1} KH_2PO_4, 0.05 mmol L^{-1} reduced cytochrome *c*, pH 7.5. Cytochrome C was reduced by adding a pinch of sodium hydrosulfide to 20 mL of the solution and aerating it until the absorbance at 550 nm was 9× greater than at 565 nm, about 20 min. The absorbance was recorded at 550 nm for cytochrome *c* (E = 19.1). All tissues were diluted an additional 5×.

The CS assays were carried out in a medium with final concentrations of the following: 100 mmol L^{-1} Tris/HCl, 0.1 mmol L^{-1} acetyl CoA, 0.1 mmol L^{-1} 5′5′ dithiobis(2-nitrobenzoic acid)(DTNB), 15 mmol L^{-1} oxaloacetate, pH 8.0. The oxaloacetate was added to start the reaction and the absorbance change recorded at 412 nm for DTNB (E = 13.6). Homogenates were further diluted 5× for liver and pectoralis and 10× for ventricle.

2.5.5. Shell CO_2 analysis

Bone carbon dioxide analyses were performed using a technique used previously (Warren and Jackson, 2005). Carapace was pulverized to a powder and a known amount (20–100 mg) was introduced into a flask containing 15 mL of 2 N HCl through which humidified nitrogen gas was passed (~240 mL min^{-1}). Carbon dioxide, generated as the result of HCO_3^-/CO_3^{2-} titration, was then carried by the nitrogen gas through a drying column (Drierite) and then through a CO_2 analyzer (AEI, model CD-3A, Pittsburgh, PA, USA), whose analog output was recorded and analyzed on a laptop computer (BIOPAC MP100, Goleta, CA). The volume of CO_2 generated was calculated by the following:

$$\text{Vol. } CO_2 \text{ generated} = (\text{Average } \%\ CO_2 \times \text{time averaged} \times N_2 \text{ flow rate})/100$$

All volumes were corrected to STPD and converted to mmoles CO_2 assuming the constant 22.26 mL per mmole CO_2 (Cameron, 1989).

2.6. Statistical analyses

Two-factor repeated measures ANOVA was used to determine whether any of the blood and plasma variables were affected by time or treatment during anoxia and recovery. One-factor ANOVA was used to determine whether tissue lactate concentrations differed between groups for each tissue. Student's *t*-tests were used to elucidate significant effects post-hoc and to compare means between treatments for all variables in the End-Treatment group. Statistical computations were carried out using JMP 5.0 (SAS Institute, Cary, NC).

3. Results

3.1. End-treatment animals

The resting levels of all measured parameters from both treatments are summarized in Tables 1 and 2 and Fig. 1A, C and D. In liver, anoxic stress at 25°C for 2 h each day, every other day for 19 days (10 bouts total), significantly increased resting activities of COX (Fig. 1A) and CS (Fig. 1C) and decreased glycogen content (Fig. 1E). None of these changes occurred in ventricle or pectoralis muscle, however (Table 1). The resting activities of PFK and LDH (Table 1), plasma ion concentrations

Table 1
Tissue enzyme activities and glycogen contents after 19 days of repeated anoxia.

	Control	Repeated anoxia
LDH activity		
Ventricle	334.7 ± 30.5	333.6 ± 29.6
Liver	39.1 ± 3.0	38.3 ± 4.0
Pectoralis	296.4 ± 21.2	282.3 ± 22.4
PFK activity		
Ventricle	38.1 ± 2.7	40.6 ± 2.1
Liver	7.5 ± 0.3	7.3 ± 0.7
Pectoralis	65.2 ± 4.9	70.7 ± 5.5
COX activity		
Ventricle	33.8 ± 3.7	27.4 ± 3.0
Liver	5.7 ± 0.9	12.1 ± 1.8*
Pectoralis	9.1 ± 1.8	8.5 ± 1.3
CS activity		
Ventricle	53.2 ± 5.5	49.3 ± 3.4
Liver	4.9 ± 0.4	6.5 ± 0.7*
Pectoralis	13.0 ± 1.0	16.5 ± 1.8
Glycogen content		
Ventricle	197.0 ± 13.6	212.1 ± 9.4
Liver	993.9 ± 23.4	824.3 ± 34.9*
Pectoralis	210.2 ± 10.9	233.8 ± 12.0

Values are mean ± s.e.m.
$N = 8$ per group.
Enzyme activities are expressed in units g^{-1} tissue.
Glycogen contents are expressed as glucosyl units (μmol g^{-1} tissue).
* Indicates a significant effect of repeated anoxia (*t*-test; $p < 0.05$).

Table 2
Hematological parameters after 19 days of repeated anoxia.

	Control	Repeated anoxia
Blood Po_2 (mm Hg)	48.5 ± 4.8	51.2 ± 4.2
Blood Pco_2 (mm Hg)	34.1 ± 1.8	39.7 ± 4.5
Blood pH	7.77 ± 0.02	7.73 ± 0.05
Blood HCO_3^- (mequ L^{-1})	56.4 ± 2.4	60.1 ± 3.6
Blood hemoglobin (g dL^{-1})	6.51 ± 0.32	6.60 ± 0.36
Hematocrit (%)	21.3 ± 1.4	21.1 ± 1.3
MCHC	0.31 ± 0.01	0.31 ± 0.01
Plasma lactate (mM)	2.66 ± 0.45	2.29 ± 0.54
Plasma glucose (mM)	3.11 ± 0.34	3.74 ± 0.37
Serum free fatty acids (μM)	96 ± 13	70 ± 4
Plasma Na^+ (mM)	135.5 ± 0.9	138.2 ± 1.8
Plasma K^+ (mM)	2.99 ± 0.14	3.47 ± 0.21
Plasma Ca^{2+} (mM)	2.3 ± 0.1	2.6 ± 0.1
Plasma Mg^{2+} (mM)	1.36 ± 0.06	1.43 ± 0.06
Plasma Cl^- (mM)	83.4 ± 3.2	87.0 ± 2.5
Plasma Pi (mM)	0.81 ± 0.05	0.80 ± 0.06

There were no significant differences between groups.
Values are mean ± s.e.m.
$N = 8$ per group.

(Table 2), and blood acid-base status (Table 2), including plasma HCO_3^- levels, were unaffected by the repeated anoxic stress treatment. There were also no differences in the resting levels of plasma lactate, glucose or FFA between the control and repeatedly submerged group (Table 2), although plasma FFA tended to be lower in repeatedly submerged group ($p = 0.0678$). Shell CO_2 contents also did not differ between the two groups (1.34 ± 0.03 and 1.32 ± 0.03 mmol g^{-1} dry powder in submerged and control turtles, respectively).

3.2. Test submergence - dive response and recovery

To determine whether the repeated anoxic stress and concomitant increases in liver CS and COX activities affected physiological performance during and following anoxic submergence, turtles that were and were not repeatedly treated with anoxic stress were cannulated, subjected to a two-hour test submergence and recovered for 13 h. A third of the cannulated turtles were sampled before, at the end of the anoxia, and at the end of recovery for determination of tissue enzyme activities and metabolite contents. There were no differences between the repeatedly submerged and control turtles for any of the parameters measured during or following the test dive. Consequently, the following sections describe the general pattern of change observed during 2 h of anoxic stress at 25°C in painted turtles.

3.2.1. Blood acid-base status

During the anoxic submergence, arterial Po_2 (Fig. 2) fell rapidly within 30 min and remained around 3 mm Hg for the remainder of the submergence bout. Arterial pH (Fig. 2) also fell rapidly, due mainly to respiratory acidosis, indicated by the increase in arterial Pco_2 with little change plasma HCO_3^-. After 30 min, arterial Pco_2 rose less steadily while pH continued to fall and HCO_3^- decreased, indicative of metabolic acidosis due to the accumulation of lactic acid (Fig. 2). The rates of lactate accumulation and HCO_3^- depletion began decreasing by 90 min, indicating the onset of metabolic depression.

Within 1 h of recovery, the turtles had fully compensated their metabolic acidosis with a respiratory alkalosis. Arterial Pco_2 and plasma HCO_3^- returned to resting by 3 h, while arterial Po_2 remained elevated for 6 h. By 10 h, plasma HCO_3^- slightly overshot the control values, but was not different by 13 h.

3.2.2. Lactate accumulation and metabolism

Plasma lactate (Fig. 2) increased during 2 h of anoxia and returned to control levels by 13 h. The rate of decrease was highest during the first 3 h, but fell steadily throughout the remaining recovery period (Fig. 3). Lactate also increased in pectoralis muscle, ventricle, liver and carapace during the anoxia (Table 3), with plasma accumulating the most (~20 mmol L^{-1}) and carapace the least (5–8 mmol L^{-1}). By the end of the 13 h recovery period, ventricle and liver had fully recovered

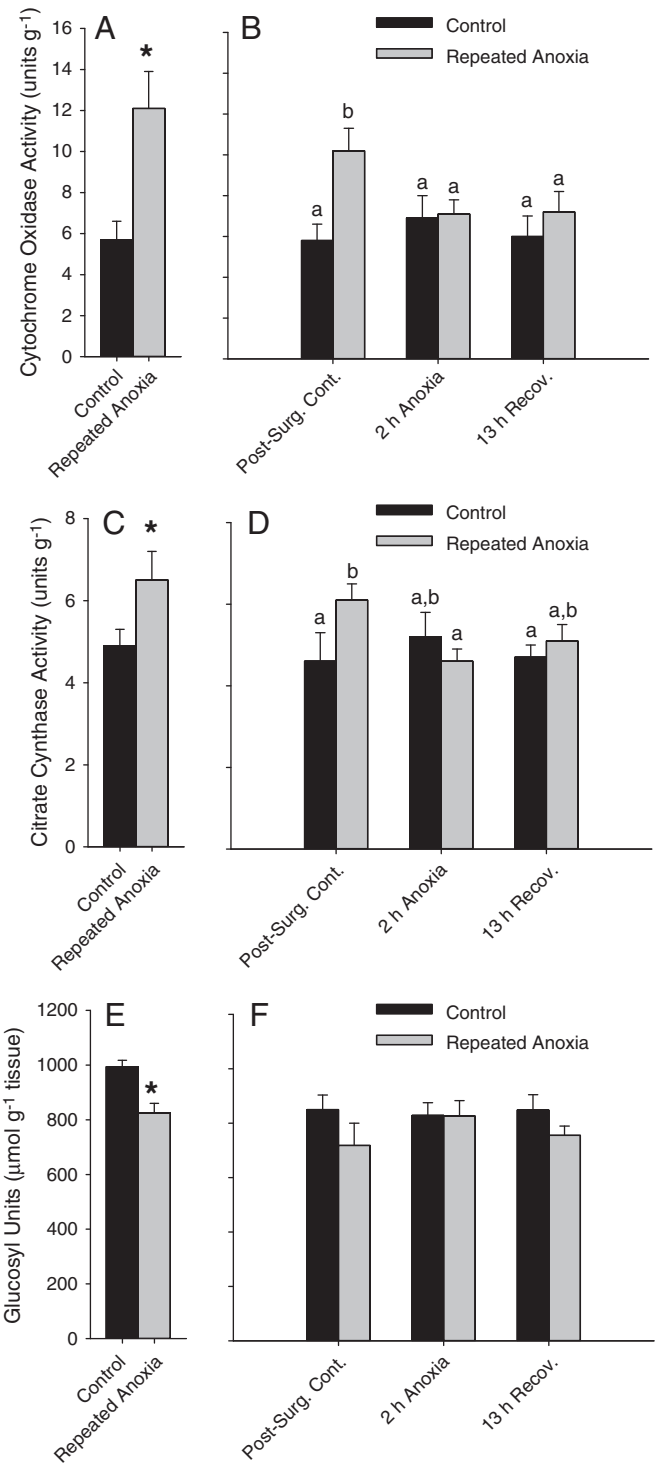

Fig. 1. Changes in liver metabolic properties after repeated anoxic stress and during and following the anoxic test dive. Mean ± s.e.m. cytochrome oxidase (COX; plates A and B), citrate synthase (CS; plates C and D), and glycogen (plates E and F). Plates A, C, and E show measurements from turtles at the end of the repeated anoxia treatment. * indicates a significant effect of repeated anoxia (*t*-test; $p < 0.05$). Plates B, D, and F show measurements from cannulated turtles before, during and after the 2 h anoxic test dive. Differing letters indicate significant differences between time-points for pooled data (two-way ANOVA followed by Student's *t*-test; $p < 0.05$). There was no interaction between treatment and time.

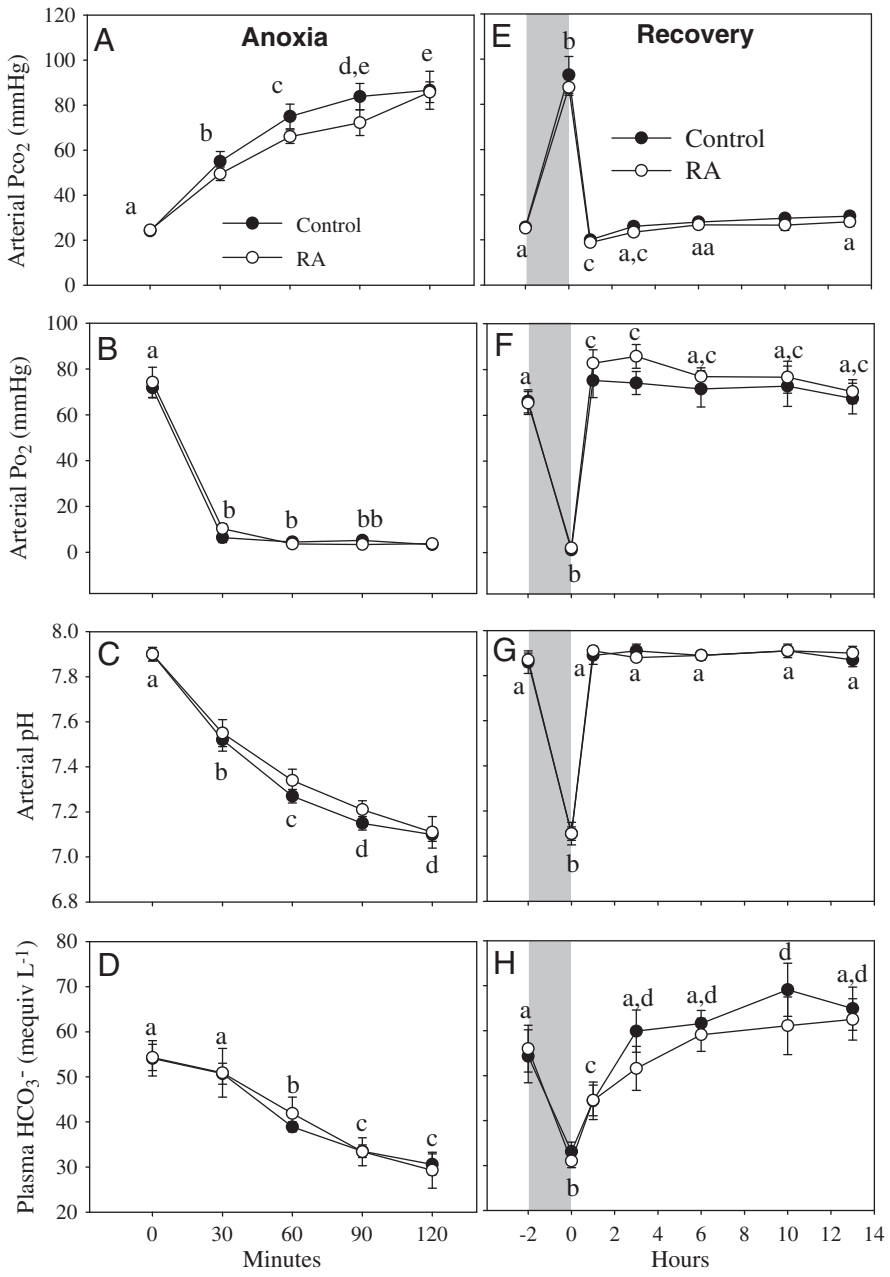

Fig. 2. Arterial blood-gas changes during and following the anoxic test dive. Mean ± s.e.m. arterial Pco_2, Po_2 and pH and plasma HCO_3^- during (plates A–D) and following (plates E–H) 2 h of anoxic submergence at 25°C. Repeated anoxia (RA) turtles were forcibly submerged for 2 h every other day for 19 days. Control turtles were treated identically without submergence. The grey bars in panels E–H indicate the submergence period. Differing letters indicate significant differences between time-points for pooled data. There was no interaction between treatment and time for any of these variables.

while carapace and pectoralis lactates remained slightly, but significantly, elevated.

3.2.3. Plasma glucose and FFA

Plasma glucose (Fig. 3) increased during the anoxia and remained elevated during recovery for 3 h, then declined thereafter but remained slightly, but significantly, elevated after 13 h of recovery. Serum FFA (Fig. 3) decreased significantly during the first 30 min of anoxia, increased during the subsequent 30 min, and then declined steadily thereafter. During recovery, FFA remained at these lower levels for 1 h, increased slightly for 2 h, then declined again until 10 h.

3.2.4. Tissue glycogen and glucose

Glycogen contents of liver and pectoralis were unaffected by anoxia and recovery in both treatments (Table 3, Fig. 1F). Ventricular glycogen was depleted by 50% after 2 h of anoxia, but was fully restored after 13 h of recovery. Free glucose contents of liver, ventricle and pectoralis were significantly elevated and returned to the control levels by 13 h of recovery (Table 3).

3.2.5. Tissue enzyme activities

The resting activities of liver COX and CS remained significantly elevated from controls in the repeatedly submerged turtles, even after the surgery and recovery day (Fig. 1B and D). However, during the test submergence, these activities decreased to the levels of controls and remained there throughout the 13 h of recovery. The test anoxic submergence did not affect liver COX or CS activities in the control turtles. COX and CS did not change in ventricle or pectoralis at any time, nor did the activities of LDH and PFK in any of the tissues sampled (Table 5).

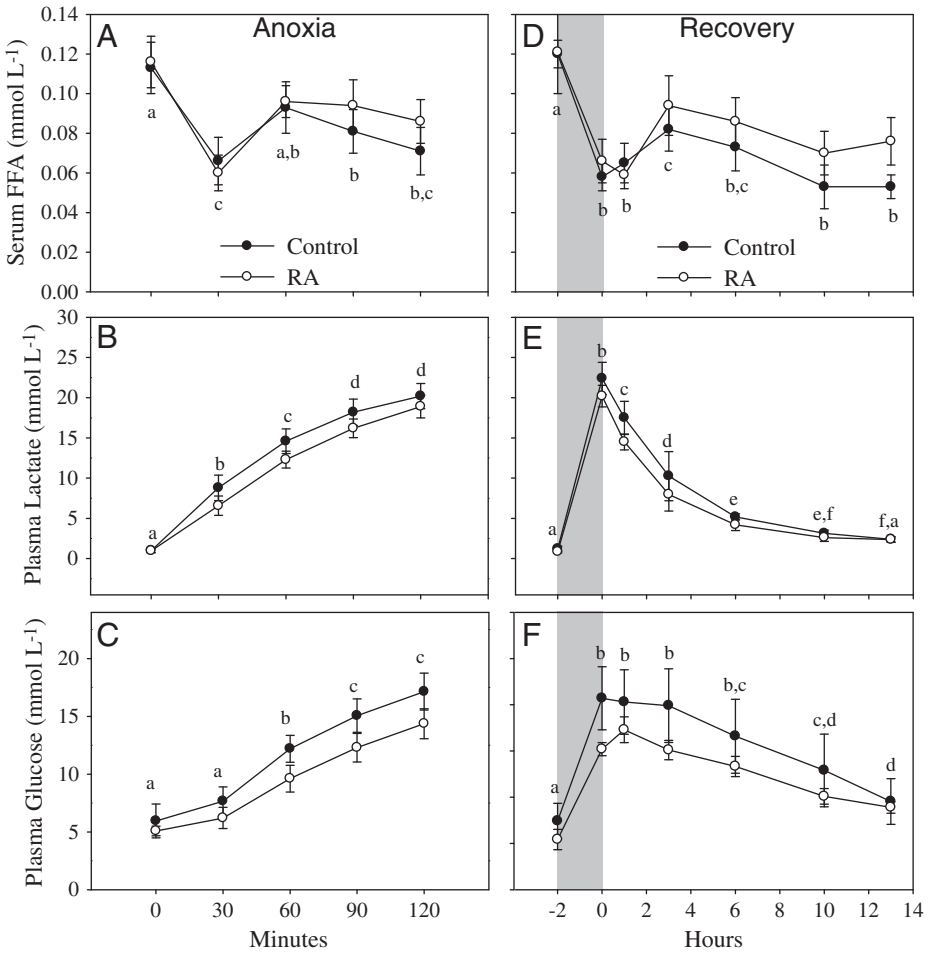

Fig. 3. Serum and plasma metabolite changes during the anoxic test dive. Mean ± s.e.m. serum FFA and plasma lactate and glucose during (plates A–C) and following (plates D–F) 2 h of anoxic submergence at 25°C. Repeated anoxia (RA) turtles were forcibly submerged for 2 h every other day for 19 days. Control turtles were treated identically without submergence. The grey bars in panels D–F indicate the submergence period. Differing letters indicate significant differences between time-points for pooled data. There was no interaction between treatment and time for any of these variables.

3.2.6. Plasma ion balance

These data are summarized in tables that can be downloaded as supplementary material. Plasma Na^+ and Cl^- did not change during anoxia or recovery while Ca^{2+} and Mg^{2+} increased slightly during the anoxia, indicating release of mineral buffers from the skeleton, and then quickly returned to control levels within 1 h of recovery. Plasma K^+ and Pi similarly increased during anoxia, but returned to resting by 3 h.

Table 3
Tissue glycogen, glucose and lactate contents before and after 2 h of anoxia and after 13 h of recovery.

	Post-surg. controls		2 h anoxia		13 h recovery	
	Control	RA	Control	RA	Control	RA
Glycogen						
Ventricle	187.5 ± 20.0[a]	197.7 ± 10.9	98.6 ± 8.6[b]	109.1 ± 17.3	163.1 ± 14.1[a]	191.3 ± 18.0
Liver	850.0 ± 53.3[a]	718.3 ± 81.7	828.9 ± 46.1[a]	825.6 ± 57.2	848.3 ± 56.3[a]	755.6 ± 33.8
Pectoralis	228.6 ± 15.3[a]	222.1 ± 26.3	164.1 ± 14.2[a]	209.1 ± 19.0	197.4 ± 20.7[a]	212.7 ± 14.6
Glucose						
Ventricle	5.0 ± 0.7[a]	6.3 ± 1.3	14.7 ± 1.5[b]	13.8 ± 1.3	7.7 ± 1.9[a]	7.6 ± 0.7
Liver	7.4 ± 0.7[a]	7.8 ± 1.1	25.1 ± 2.0[b]	20.4 ± 0.9	9.7 ± 1.7[a]	9.8 ± 1.1
Pectoralis	3.7 ± 0.9[a]	4.9 ± 1.1	12.9 ± 1.1[b]	10.4 ± 0.8	4.3 ± 0.8[a]	5.6 ± 0.8
Lactate						
Ventricle	1.79 ± 0.44[a]	1.58 ± 0.41	17.76 ± 1.64[b]	15.65 ± 1.29	2.44 ± 0.53[a]	2.32 ± 0.30
Liver	0.79 ± 0.19[a]	0.93 ± 0.11	9.72 ± 0.79[b]	9.21 ± 1.04	1.05 ± 0.13[a]	1.81 ± 0.82
Pectoralis	2.37 ± 0.86[a]	1.86 ± 0.27	16.10 ± 1.54[b]	15.05 ± 1.81	2.65 ± 0.53[a]	2.55 ± 0.71
Carapace	1.42 ± 0.34[a]	1.91 ± 0.24	7.42 ± 2.75[b]	5.86 ± 0.92	3.25 ± 0.37[c]	3.15 ± 0.63

Values are means ± s.e.m.
$N = 8$ per group.
Units are μmol g^{-1} tissue for glycogen (glucosyl units) and glucose and mmol g^{-1} tissue for lactate.
Differing letters indicate statistical differences between times. There was no time × treatment interaction.
Two-way ANOVA, Student's *t*-test, $p < 0.05$.

4. Discussion

Many vertebrates, including mammals (Clanton and Klawitter, 2001; Hoppeler and Vogt, 2001), amphibians (Pinder and Burggren, 1983), and fishes (Borowiec et al., 2015; Du et al., 2016) show adaptive metabolic and respiratory responses to long-term and intermittent or repeated hypoxia that lead to improved physiological performance under those conditions. The purpose of the present study was to determine if anoxia-tolerant painted turtles might also show responses to repeated anoxic stress that improve tolerance and/or recovery during a subsequent anoxic test submergence. Even though repeatedly submerging 25°C-acclimated painted turtles for 2 h every other day for 19 days increased the resting activities of liver citrate synthase (CS) and cytochrome oxidase (COX) and decreased liver glycogen content, the changes did not translate into an effect on the physiological performance of the turtle during or following a test submergence. Indeed, during the test submergence, the liver CS and COX activities of the repeatedly submerged turtles decreased to levels that were similar to those of the control turtles. There were also no effects of the repeated submergence treatment on blood oxygen transport, acid-base, or ionic properties, at rest or during the test submergence. The suppression of liver CS and COX activities during the test dive in the repeatedly submerged group suggests that low activities of these enzymes may be important for surviving and recovering from anoxic stress.

4.1. Metabolic responses to repeated anoxic stress

The most profound effects of the repeated anoxic stress were in the liver, where the resting activities of citrate synthase (CS) and cytochrome oxidase (COX) were increased and glycogen content decreased (Table 1). These changes have also been observed in response to intermittent hypoxia in the liver of adult killifish (Borowiec et al., 2015) and in the skeletal muscle of mammals (Clanton and Klawitter, 2001). In these studies, it has been postulated that increasing these enzymes could enhance tissue oxidative capacity, facilitating repayment of the "oxygen debt" that satisfies the energetic needs of gluconeogenesis and facilitates metabolic recovery. In turtles, the liver probably plays a more important role than muscle in lactate recovery metabolism during and after anoxia (Jackson et al., 1996), and so it, therefore, might be expected that an enhanced aerobic capacity of the liver after anoxia could support the energetic costs of gluconeogenesis. This does not appear to be the case, however, because, during the anoxic test submergence, the levels of both enzymes in the repeatedly submerged turtles decreased to the levels of those in the control turtles during the test submergence and remained there throughout recovery.

The function of suppressing hepatic COX and CS during anoxia is unclear, but it is notable because low oxidative enzyme activities should lead to an increase in the production of potentially injurious reactive oxygen species (ROS) during reperfusion (Li and Jackson, 2002; Turrens, 2004). A previous study of anoxic turtles (Willmore and Storey, 1997) showed that ROS levels were elevated in turtle liver after 20 h of anoxic submergence at 5°C, as indicated by a dramatic fall in the GSH:GSSH ratio (a marker for ROS production). Therefore, the turtle appears to utilize a strategy that uses the high antioxidant capacity of its tissues, rather than prevent ROS production altogether (Hermes-Lima and Zenteno-Silva, 2002).

The absence of any change in PFK and LDH activities in any tissue in response to the repeated anoxia treatment or during the anoxic test submergence differs from mammals, in which both are induced in response to sprint exercise in skeletal muscle (Ross and Leveritt, 2001) and to chronic hypoxia in heart (McClelland and Brooks, 2002). It is similar to adult killifish, however, which also show no change in muscle LDH activity with constant or intermittent hypoxia (Borowiec et al., 2015). A previous study of anoxic slider turtles also showed no changes in LDH in any tissue and a decrease in PFK in only liver and brain after 20 h of anoxic submergence at 7°C (Willmore et al., 2001). Thus, it would appear that any changes in tissue PFK and LDH function during anoxic stress in turtles should be by covalent modification (Xiong and Storey, 2012) (Brooks and Storey, 1989).

Large tissue glycogen stores are required to survive extended anoxia, and so the decrease in liver glycogen with repeated anoxic stress seems to decondition the turtles to survive anoxia. This could have been caused by reduced hepatic gluconeogenesis during recovery, which occurs with intermittent hypoxia in rats (Freminet et al., 1990), the accumulation of a small depletions over many dive and recovery cycles, or, given that liver CS and COX were also elevated in the repeatedly submerged turtles, the cell volume devoted to glycogen might have been displaced by a larger mitochondrial volume.

It is notable that this study provides the first measurements of plasma FFA in any reptile during or following a period of hypoxia or anoxia. Although FFA decreased overall during anoxia, the pattern of change was more complex, probably reflecting both changes in metabolic utilization (disappearance) and mobilization (appearance). This change was similar to that observed during hypoxia in carp and goldfish (Van den Thillart et al., 2002). In these species, lipolysis is inhibited by stimulation of beta-1 and alpha-2 adrenergic receptors and is believed to be an adaptive response to reduce accumulation of amphipathic molecules that damage cell membranes. Although decreased lipolysis could account for the decreased FFA during the submergence, muscle 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity decreases during anoxia in slider turtles (Willmore et al., 2001), suggesting that FFA utilization probably also decreases during anoxia in turtles.

4.2. Why don't turtles respond to repeated anoxia?

We hypothesized that painted turtles would, like other vertebrates, respond to repeated anoxic bouts by improving their metabolic and ionoregulatory performance during and following anoxia. Hypothetical improvements could have included a reduced anaerobic metabolic rate, faster metabolic and ionoregulatory recovery, and improved blood-oxygen carrying capacity. Although it is possible that the anoxic periods in the present study were either too short or infrequent to induce an adaptive response, our ability to detect changes in liver CS, COX, and glycogen and suggest that they were not. There was a slight difference in the procedure used for making the turtles anoxic in the two phases of the experiment, with the repeated anoxia occurring in unrestrained turtles placed in plastic containers and the test submergence in the turtles fixed to prevent entanglement with the catheter. However, both procedures have been used previously in studies of anoxic submergence at these temperatures (Herbert and Jackson 1985; Jackson et al. 1996; Warren et al. 2006) and the results produce similar changes in lactate production, and so we do not believe the stresses were dissimilar. Finally, answer may simply be that the physiological systems involved in tolerating and recovering from anoxic submergence stress in painted turtles are not plastic in response to repeated anoxic stress, but are, instead, constitutively adapted to survive extended periods of anoxia.

Acknowledgments

This study was supported by an NSF grant (IBN 01-10322) awarded to D.C.J.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2016.07.012.

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