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