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RESEARCH ARTICLE

Changes in the material properties of the shell during simulated aquatic hibernation in the anoxia-tolerant painted turtle

Dean T. Odegard[1], Michael A. Sonnenfelt[2], J. Gary Bledsoe[2], Sarah W. Keenan[1,*], Craig A. Hill[1,‡] and Daniel E. Warren[1,§]

ABSTRACT

Western painted turtles (*Chrysemys picta bellii*) tolerate anoxic submergence longer than any other tetrapod, surviving more than 170 days at 3°C. This ability is due, in part, to the shell and skeleton simultaneously releasing calcium and magnesium carbonates, and sequestering lactate and H^+ to prevent lethal decreases in body fluid pH. We evaluated the effects of anoxic submergence at 3°C on various material properties of painted turtle bone after 60, 130 and 167–170 days, and compared them with those of normoxic turtles held at the same temperature for the same time periods. To assess changes in the mechanical properties, beams (4×25 mm) were milled from the plastron and broken in a three-point flexural test. Bone mineral density, CO_2 concentration (a measure of total bone HCO_3^-/CO_3^{2-}) and elemental composition were measured using microcomputed tomography, HCO_3^-/CO_3^{2-} titration and inductively coupled plasma mass spectrometry (ICP-MS), respectively. Tissue mineral density of the sampled bone beams was not significantly altered by 167–170 days of aquatic overwintering in anoxic or normoxic water, but bone CO_2 and Mg were depleted in anoxic compared with normoxic turtles. At this time point, the plastron beams from anoxic turtles yielded at stresses that were significantly smaller and strains that were significantly greater than the plastron beams of normoxic turtles. When data from anoxic and normoxic turtles were pooled, plastron beams had a diminished elastic modulus after 167–170 days compared with those of control turtles sampled on day 1, indicating an effect of prolonged housing of the turtles in 3°C water without access to basking sites. There were no changes in the mechanical properties of the plastron beams at any of the earlier time points in either group. We conclude that anoxic hibernation can weaken the painted turtle's plastron, but likely only after durations that exceed what it might naturally experience. The duration of aquatic overwintering, regardless of oxygenation state, is likely to be an important factor determining the mechanical properties of the turtle shell during spring emergence.

KEY WORDS: Bone, Buffering, Carbonate, *Chrysemys picta bellii*, Metabolic acidosis, MicroCT

[1]Department of Biology, Saint Louis University, St Louis, MO 63103, USA. [2]Department of Biomedical Engineering, Saint Louis University, St Louis, MO 63103, USA.
*Present address: Department of Geology and Geological Engineering, South Dakota School of Mines & Technology, Rapid City, SD 57701, USA. ‡Present address: Department of Pediatrics, Washington University School of Medicine, St Louis, MO 63110, USA.

§Author for correspondence (Daniel.Warren@slu.edu)

D.E.W., 0000-0002-9043-7411

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INTRODUCTION

The stereotypical functions of mineralized tissues in animals are as sites for muscle attachment and for physical protection from predators. Less appreciated is their importance in ionic and acid–base homeostasis, the latter of which has come into sharper focus as a subject of study in recent years. Bone, in particular, has been shown to be important in buffering lactic acidosis during anoxia and exercise in a variety of ectothermic vertebrates including frogs (Warren and Jackson, 2005), lizards (Ruben and Bennett, 1981), caiman (Jackson et al., 2003) and turtles (Jackson, 2000).

The mechanisms underlying this ability have been especially well studied in several species of North American freshwater turtles that overwinter in ice-locked lakes and ponds. Despite having stable water temperatures around 3°C, these winter hibernacula can have low or undetectable oxygen levels (Crawford, 1991). Some aquatic turtles have evolved physiological adaptations to survive anoxia, which allow them to successfully overwinter under such conditions. The champion in this regard is the western painted turtle (*Chrysemys picta bellii*), which can survive 177 days of submergence in anoxic 3°C water (Ultsch and Jackson, 1982b). The physiological basis for this ability has been well described (Jackson, 1997; Jackson et al., 2000a; Jackson and Heisler, 1982, 1983; Reese et al., 2004; Ultsch, 1985; Ultsch and Jackson, 1982b) and includes (1) metabolic depression, (2) large glycogen stores and (3) utilization of mineralized tissues to defend body fluid pH. The large extracellular buffer capacity afforded by its shell allows this turtle species to accumulate lactate to concentrations that approach 200 mmol l^{-1} (Warburton and Jackson, 1995; Warren and Jackson, 2007).

Studies of bone from turtles and other reptilian or amphibian species have demonstrated that bone can buffer lactic acidosis through two mechanisms. In the first, carbonate (CO_3^{2-}) is released from Ca– or Mg–CO_3 complexes that are either loosely adsorbed to bone crystallite surfaces (Rey et al., 1989; Wilson et al., 2005) or derived from the bone mineral phase (i.e. from dissolution of the lattice-bound carbonate in carbonated apatite). During anoxic submergence in painted turtles, bone CO_2 levels decrease significantly (Warburton and Jackson, 1995), while plasma Ca and Mg increase (Jackson et al., 2000a,b; Jackson and Heisler, 1982; Warburton and Jackson, 1995). Plasma Ca and Mg have also been shown to increase after anoxia and exercise in frogs (Warren and Jackson, 2005) and during forced diving in caiman (Jackson et al., 2003). In the second mechanism, bone buffers lactic acidosis by sequestering lactate and a corresponding proton. Skeletal lactate sequestration has been documented in numerous species of freshwater turtles (Jackson, 1997; Jackson et al., 2000b, 2007; Davis and Jackson, 2007), during forced submergence in frogs (Warren and Jackson, 2005) and in the osteoderms of caiman during forced diving (Jackson et al., 2003).

However, it is unknown whether the mechanical properties of bone are affected by its use either as a source of mineral buffer or as

a site for sequestration of lactate. The potential for a trade-off between utilizing the bone as a buffer and its mechanical strength is particularly pertinent to the turtle, where agility has been largely sacrificed for the protection afforded by a shell. In theory, if a critical amount of mineral is lost from the shell, mechanical integrity could be diminished. Because the painted turtle possesses a large bone mass that also demonstrates demineralization under naturally anoxic conditions, it represents an ideal vertebrate model to ascertain when, if ever, such *in vivo* utilization of bone as a buffer can affect its mechanical properties.

In order to determine whether bone demineralization due to extreme lactic acidosis alters the strength, toughness and ductility of the plastron, we used a three-point flexural test to measure stress, strain and elastic modulus in beams cut from painted turtle plastron after anoxic or normoxic overwintering near the limit of their anoxic survival (the LT_{50} for the animals in this study was ∼167 days). We also employed microcomputed tomography (microCT) to determine whether lactic acid-induced demineralization causes detectable changes in bone density and morphology. To determine concentrations of relevant elements in bone and plasma, we used inductively coupled plasma mass spectrometry (ICP-MS). We used these data to correlate any changes in mechanical properties with the degree of demineralization. We found significant changes to the material properties of bone after both prolonged anoxic and normoxic submergence. We conclude that painted turtle shells may be structurally weakened following exposure to extended overwintering and acidosis in the wild.

MATERIALS AND METHODS

Experimental animals

Western painted turtles, *Chrysemys picta bellii* Gray 1830, were purchased from Niles Biological (Sacramento, CA, USA). These individuals had been collected by the supplier from a population in Minnesota, USA. They were housed in the indoor aquatics facility in the Department of Biology at Saint Louis University. Aquaria were filled with St Louis municipal water at 10–20°C under natural Minnesota photoperiods for 2–3 months before experiments began, during which they were fed Reptomin (Tetra, Blacksburg, VA, USA) three times each week *ad libitum*. The material for this experiment was generated over 2 years, and required 107 turtles (year 1: n=47, all male, plastron length 114.69±0.99 mm; year 2: n=60, 14 female, 46 male, plastron length 109.25±0.70 mm, mean±s.e.m.). After finding no significant differences in any metrics between groups of control turtles from each year, data from the two years were pooled for analysis. Each year, cold acclimation began in November; hibernation began in January and finished in June. All experiments were approved by the Saint Louis University Institutional Animal Care and Use Committee (IACUC protocol 2198).

Aquatic hibernation and tissue sampling

In each year, the turtles were separated into two identical aquaria (60×33×46 cm, l×w×d, filled with approximately 80 l of municipal water) that were housed within in a large deli refrigerator and acclimated to 3°C over a period of 8 days, where the temperature of the aquaria was lowered by 3°C on the first day and by 2°C each subsequent day until it reached 3°C. Minnesota photoperiods were maintained throughout acclimation. After 2 weeks, one aquarium (anoxic treatment, n=23 year 1 and n=25 year 2) was bubbled with nitrogen gas and the second aquarium (normoxic group, n=24 year 1 and n=35 year 2) was bubbled with air.

In the anoxic tank, turtles were prevented from accessing the surface by a plastic grate positioned about 8 cm below the water surface. The water was about 40 cm deep. In the normoxic (control) tank, the water was changed weekly, the water depth ranged between 20 and 40 cm, and the turtles had unobstructed access to the surface in order to breathe air. The day that nitrogen bubbling began, control turtles from the normoxic aquarium (n=9 in year 1 and n=8 in year 2) were killed by clamping their necks under water, followed by rapid decapitation and brain destruction. An additional turtle was sampled in year 1 because it was discovered that one had a plasma lactate concentration of 31 mmol l^{-1}, indicating a lactic acidosis at the start of the experiment. Because the purpose of this study was to determine how lactic acidosis affected the mechanical properties of the shell during anoxia, it was important that the control turtles all began the study with low lactate levels. Therefore, this one animal was excluded from the experiment.

Dissolved oxygen in the anoxic tank and temperature were monitored daily for the duration of the experiment (YSI DO200, Yellow Springs, OH, USA). After 60 days, turtles from the anoxic (year 1: n=8; year 2: n=8) and normoxic (year 1: n=8; year 2: n=8) groups were sampled. After 130 days, additional groups of anoxic (year 1: n=8; year 2: n=7) and normoxic (year 1: n=5; year 2: n=5) turtles from each aquarium were sampled. In year 1, three of the remaining seven anoxic turtles had died after 170 days, so the last four were sampled. In the year 2 experiment, five of the remaining 10 anoxic turtles had died after 165 days, so the last five were then sampled. Therefore, the longest anoxic time point in the pooled dataset will be considered 167 days (the weighted average of the two groups), which was used to estimate the LT_{50} for anoxic submergence in painted turtles. The remaining nine normoxic turtles from the year 2 experiment were sampled after 170 days. An uncharacterized, but previously reported (Ultsch, 1985) pathology, which presented as swelling and a whitish sloughing of the epithelium around the eyes and nares, caused the deaths of a few normoxic turtles each year (year 1: n=3; year 2: n=4), usually after about 100 days of submergence. These turtles and those found dead in the 167 day anoxic group (year 1: n=3; year 2: n=5) were subsequently excluded from all analyses. The sampling procedure included collection of plastron and blood (∼1 ml via cardiac puncture), and was carried out in a cold room at 3°C. The plastron was carefully cleaned of soft tissue and periosteum, and then flash-frozen in liquid nitrogen. A portion of the blood (∼0.3 ml) was injected into a thermostatically controlled (3°C) Radiometer BMS MKII (Radiometer, Copenhagen, Denmark) until the blood covered the tip of an Accumet pH Microprobe (Fisher Scientific, Waltham, MA, USA) placed at the bottom of the center well where injected blood flows after it exits the gas measurement cell. This method provided pH measurements directly comparable to those acquired in previous studies of painted turtles acclimated to 3°C (Jackson et al., 2000a). The remaining blood was centrifuged at 9600 ***g*** for 3 min, plasma was removed with a pipette and flash-frozen in an ethanol–dry ice slurry for subsequent analysis of lactate, glucose and ionic concentrations (see below). All tissues were stored at −80°C until further processing. Plastrons were stored individually in sealed plastic bags, and those bags were placed inside a larger sealed plastic bag containing approximately 500 ml water ice before being placed in the −80°C freezer.

Preparation of plastron beams for analyses

A band saw was used to cut beams (approximately 25×4 mm) from the frozen plastron for the various analyses used in this study. Sagittal cuts removed the lateral bridge regions and divided the plastron into left and right halves along the craniocaudal suture. A

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transverse cut along the hyohypoplastral suture further divided the plastron into quarters. Beams were fashioned from the regions immediately cranial and caudal to the hyohypoplastral suture, with their long axis in the mediolateral direction, such that the homogeneity of the thickness of each beam was maximized and the curvature of the beam was minimized. Up to four beams were made from each plastron (Fig. 1).

Determination of water and mineral content

Plastron beams were powdered in a liquid nitrogen-cooled Freezer/Mill (6770, SPEX, Metuchen, NJ, USA). An aliquot (approximately 25 mg) of wet powder was removed for later lactate analysis (see below), and the remaining wet powder was weighed in porcelain crucibles, dried at 80°C in an oven (L–C Oven, Lab-Line Instruments, Melrose Park, IL, USA) for 24 h and reweighed to determine water content. An aliquot of dry bone powder (approximately 25 mg) was removed for CO_2 analysis (see below). The remaining dry powder was ashed in a muffle furnace (F6000, Barnstead International, Dubuque, IA, USA) at 750°C for 24 h, after which the crucible and its contents were weighed to determine the ash fraction. Organic content (% wet mass) was deduced from total wet mass by subtracting the water content (% wet mass) and the ash content (% wet mass).

Lactate analyses of bone and plasma

Bone lactate was measured using a colorimetric enzymatic kit (Trinity Biotech, Bray, Co. Wicklow, Ireland) in a method similar to one previously published (Warren and Jackson, 2005). Briefly, 10–20 mg of powdered wet bone was incubated on ice in 5 volumes of ice-cold 0.6 mol l^{-1} HCl and vortexed every 15 min. After 2 h, it was centrifuged at 9600 ***g*** for 3 min. A subsample of the supernatant was added to the reagent, which generated a precipitate. This required the mixture to be centrifuged at 9600 ***g*** for an additional 3 min. The absorbance of that supernatant was measured using a spectrophotometer at 540 nm and lactate was calculated from a standard curve. Anoxic samples required dilution with deionized water to fall within the linear range of the assay. Plasma lactate was measured using a biochemistry analyzer (YSI 2300 Stat).

CO_2 analysis

Bone CO_2, a measure of the bone associated with HCO_3^- and CO_3^{2-}, was measured using the method of Warren and Jackson (2005). Briefly, an excess of 2 mol l^{-1} HCl was added to a closed flask containing 10–50 mg dry bone powder, through which nitrogen gas flowed at 700 ml min^{-1}. Titration of HCO_3^-/CO_3^{2-} in the bone powder caused release of CO_2, which was carried by the nitrogen stream through a desiccant column (silica gel, Fisher Scientific) and

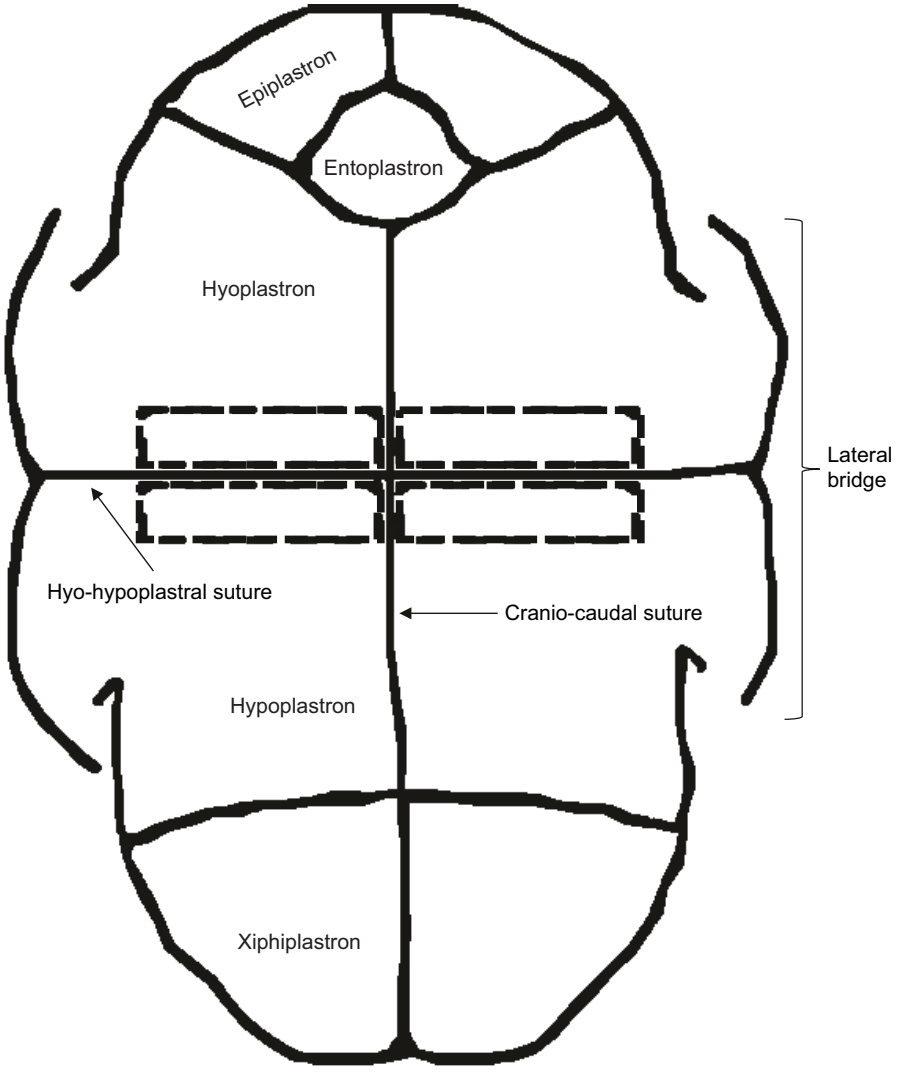

Fig. 1. Diagram of a turtle plastron showing the cut locations (indicated by dashed lines) of the beams used in the study.

through a CO_2 analyzer (CA-10, Sable Systems, Las Vegas, NV, USA), the analog output of which was digitized using an IX-404 (iWorx, Dover, NH) and recorded and analyzed using LabScribe (iWorx, Dover, NH, USA). The volume of CO_2 produced from the complete titration of the bone powder was calculated by multiplying the integral of the CO_2 elution curve by the flow rate. After correcting to standard temperature and pressure, dry (STPD), CO_2 volume was converted to mmol by assuming the constant 22.26 ml per mmol CO_2 (Cameron, 1989).

Elemental analyses of bone and plasma

Elemental compositions of plasma and ashed bone were measured using ICP-MS (7500ce, Agilent Technologies, Santa Clara, CA, USA), fed by an autosampler (ASX-520, Teledyne CETAC, Omaha, NE, USA). Samples (plasma, 50 µl; ashed bone, 10-25 mg) were digested in 5 ml 20% trace-metal grade nitric acid (Fisher Scientific) at 90°C until dissolved, filtered using 0.22 µm non-sterile PTFE filters (Midwest Scientific, Valley Park, MO, USA), and diluted with purified water (Milli-Q, Millipore Corporation, Billerica, MA, USA) to 2% nitric acid before analysis. When the amount of starting material permitted, samples were measured in duplicate and values were averaged. Here, we report data for Ca, Na, Mg, K and P. The detection limit for Ca, Na, Mg and K was 200 parts per billion; for P, it was 1 part per million.

MicroCT

To explore the possibility that the change in material properties observed was due to changes in mineral density or morphological changes, plastron beams were scanned (6 µm voxel size, 818 slices, high-resolution, energy 70 kVp, intensity 114 µA, integration time 300 ms) in a Scanco microCT 40 system (Scanco, Bassersdorf, Switzerland). To maximize similarity between the scanned bone and the region that was tested in three-point bending, new beams were cut from the hypoplastron immediately caudal to the beams broken in three-point bending. Beams were scanned from control animals (n=9), normoxic animals submerged for 170 days (n=5), and anoxic animals submerged for 167 days (n=6). Scans were transverse to the long axes of the beams, and included the middle 4.9 mm of each beam so that the center of the scan was where downward force would have been applied in three-point bending. Results were averaged over these slices. Tissue mineral density (TMD, calibrated to hydroxylapatite, HA) of the entire scanned region was determined using the manufacturer's 3D analysis tools. The threshold value was empirically determined (255/1000) to show reasonable binarization of bone structure.

For trabecular analysis, rectangular contours containing only trabecular bone were conservatively drawn over the diploë. The width of the contoured region in the coronal axis was always between 40% and 50% of the total trabecular region. Contours were hand-drawn every 40 slices (0.24 mm), and the contours in the remaining slices were morphed to fit using the manufacturer's contouring software. Bone volume fraction (bone volume divided by tissue volume, BV/TV), as well as trabecular number, thickness and separation were calculated using the manufacturer's 3D analysis tools. An identical contouring procedure was employed to analyze the TMD and BV/TV of the outer and inner layers of cortical bone. Images were constructed in 3D using publically available imaging software (OsiriX, Pixmeo, Geneva, Switzerland).

Mechanical testing

Immediately after being cut, beams were placed individually in ice-cold phosphate-buffered saline (PBS; maximum 30 min submergence) and transported to the grinder-polisher (Ecomet 6, Buehler, Lake Bluff, IL, USA), where they were lightly polished at 90 rpm on 320 grit paper under PBS irrigation until they were 4 mm in width (4.10±0.31 mm, mean±s.d.). Beam thickness varied depending on the turtle and was not altered by polishing (1.93±0.27 mm, mean±s.d., range 1.34–2.54 mm), and did not vary significantly between groups (P=0.445, one-way ANOVA, α=0.05). Beams were then broken in three-point bending (Criterion 42, MTS, Eden Prairie, MN, USA) using a custom-made fixture with rounded supports (1 mm diameter). The span length was 16 mm and loading rate was 0.1 mm s^{-1}. Force (N) and displacement (mm) were recorded by a load cell (MTS Model LSB.102, 100N) in the upper fixture at 50 Hz. Fracture was defined as the first drop in recorded force greater than 1 N. The width and thickness of the beams were measured twice each and averaged at the site of the break with calipers along the edges perpendicular to the coronal axis and the edges perpendicular to the craniocaudal axis, respectively. Force and displacement were converted to stress and strain, respectively, using R (http://www.R-project.org/) and the following beam theory equations (Hibbeler, 2014):

$$\text{Stress} = \frac{3 \times F \times L}{2 \times w \times t^2}. \quad (1)$$

$$\text{Strain} = \frac{6 \times d \times t}{L^2}, \quad (2)$$

where the units of stress are GPa, the units of strain are mm mm^{-1}, F is the applied force (kN), L is the breaking span (16 mm), w is the beam width (mm), t is the beam thickness (mm) and d is the displacement (mm). Elastic modulus was calculated as the slope of the linear-elastic region of the stress–strain curve. The yield point was determined using the 0.2% linear offset method (Hibbeler, 2014). Beams were excluded from analysis if they did not fracture cleanly or if they had non-linearity in their elastic deformation region. Preliminary measurements showed no difference in material properties across the four beam locations tested in this study. Whenever possible, two successful three-point bending tests from each animal were included in this study; in those cases, the results of the two tests were then averaged to give a more accurate value for all mechanical parameters.

Statistics

Differences between groups were tested by one-way analysis of variance (ANOVA), with Student–Newman–Keuls (SNK) *post hoc* multiple comparisons tests (α=0.05). If data did not meet the assumptions of the ANOVA, a natural logarithmic transformation was performed. If, after transformation, the data still violated the assumptions of ANOVA, a Kruskal–Wallis ANOVA on ranks was employed. All statistics were carried out in SigmaPlot 11 (SYSTAT Software, Inc., San Jose, CA, USA).

RESULTS

Compared with normoxic controls, anoxic turtles showed a progressive lactic acidosis characterized by decreases in blood pH and increases in plasma lactate (Fig. 2A,B). This coincided with an increase in bone lactate (Fig. 2C) and a depletion of bone carbonate (Fig. 3A).

The concentrations of Ca, P, Mg, K and Na in the plasma and in ashed bone, as measured by ICP-MS, are presented in Table 1. Large increases in plasma Ca, Mg and K concentrations were observed with anoxic submergence. Plasma Na did not change, however, and plasma P was below detectable levels (<5 mg l^{-1} or

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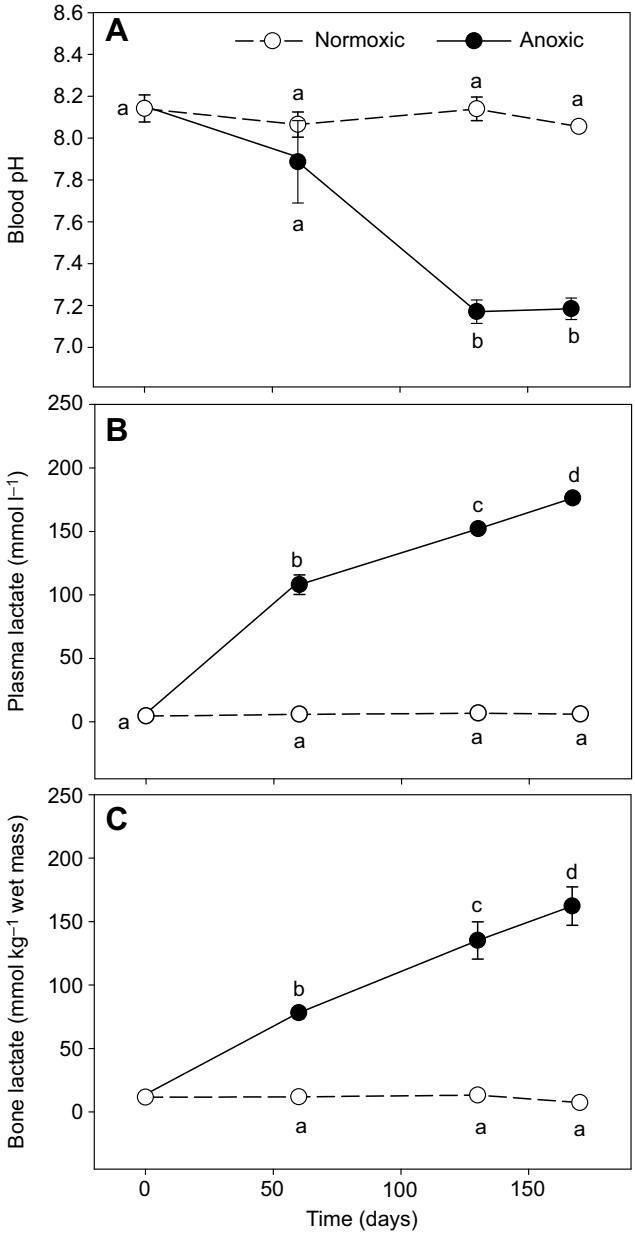

Fig. 2. Physiological changes during simulated anoxic and normoxic hibernation at 3°C. (A) Blood pH, (B) plasma lactate and (C) bone lactate. Values are means±s.e.m. Differing letters indicate significant differences between sampled groups (P<0.05, one-way ANOVA, SNK *post hoc*). See Table 1 for *n* values in each group.

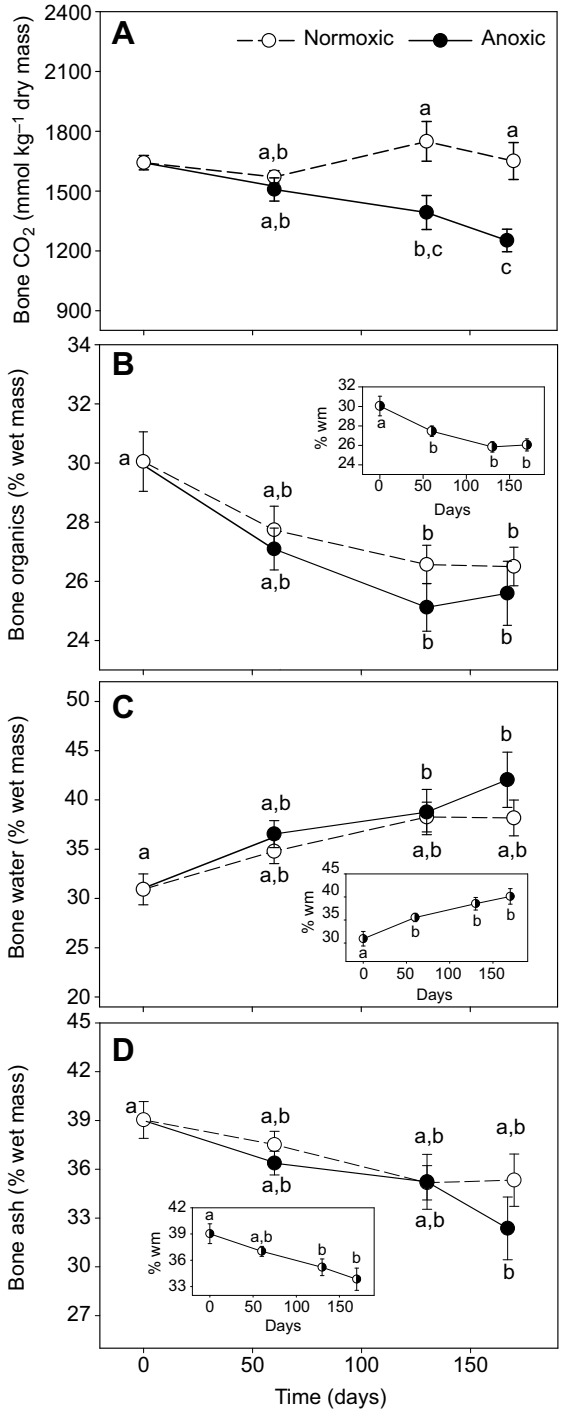

Fig. 3. Compositional changes of plastron beams during simulated aquatic hibernation. CO_2 (A), organics (B), water (C) and ash (D) content were measured in plastron beams. The insets in B–D show the pooled mean ±s.e.m. wet mass (wm) from all of the turtles at each time point in order to illustrate the effects of time on the measured variables. Differing letters indicate significant differences between means for a sampling group (P<0.05, one-way ANOVA, SNK *post hoc*). See Table 1 for *n* values in each group.

0.167 mmol l^{-1}) for all samples. In bone ash, Mg levels decreased significantly during anoxic submergence and increased significantly during normoxic submergence, but no other changes were detected.

During submergence, the bone organic content of both anoxic and normoxic hibernating turtles decreased, regardless of aeration state (Fig. 3B), while bone water content increased (Fig. 3C) and bone mineral content decreased (Fig. 3D).

MicroCT did not detect any significant changes to bone morphology in either trabecular or cortical bone between normoxic and anoxic treatments after 167–170 days of submergence (Fig. 4). In the inner cortical layer, TMD was lower after 167 days of anoxia compared with that of controls, but the difference was not significant (P=0.054, one-way ANOVA) (Fig. 4C). Across all treatments, TMD was higher in the outer table of cortical bone than in the inner table, and cortical bone always had a higher TMD than trabecular bone (Fig. 4C). In the outer cortical layer, BV/TV was significantly lower in the submerged groups (P=0.023, Kruskal–Wallis one-way ANOVA

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Table 1. Effects of simulated aquatic hibernation on plasma and shell elemental composition

	Days	Aeration	*N*	*n*	Ca	*n*	P	*n*	Mg	*n*	K	*n*	Na
Plasma	0	Normoxic	13	13	2.6±0.08^{A}	0	b.d.	13	2.2±0.04^{A,B}	13	2.1±0.11^{A,B}	13	104.5±2.6
	60	Normoxic	8	8	2.4±0.08^{A}	0	b.d.	6	1.9±0.03^{A}	8	1.8±0.11^{A}	8	91.7±2.8
	60	Anoxic	6	6	16.2±0.95^{C}	0	b.d.	6	8.6±0.32^{B,C}	6	3.9±0.29^{C}	6	105.7±5.2
	130	Normoxic	7	7	3.1±0.17^{B}	0	b.d.	7	2.3±0.18^{A,B}	7	2.5±0.20^{B}	7	99.1±14.1
	130	Anoxic	8	8	42.2±1.97^{D}	0	b.d.	8	14.8±1.14^{C}	8	7.2±0.67^{D}	8	105.2±11.7
	170	Normoxic	9	9	2.7±0.26^{A,B}	0	b.d.	8	2.4±0.18^{A,B}	9	2.6±0.27^{B}	9	87.7±6.1
	167	Anoxic	9	9	46.3±3.12^{D}	0	b.d.	9	14.1±0.92^{C}	9	7.1±0.68^{D}	9	83.1±6.4
Plastron	0	Normoxic	13	13	9885.3±678.3^{A,B}	13	4731.3±250.0^{A,C}	13	230.9±5.22^{A,B}	6	3.0±0.44^{A,B}	11	137.9±34.89
	60	Normoxic	7	7	7517.5±353.7^{A,C}	7	4014.2±149.6^{C}	7	207.9±11.53^{A,D}	4	2.4±0.45^{A}	7	155.2±24.46
	60	Anoxic	6	6	6909.9±344.3^{C}	6	3979.4±107.9^{C}	6	182.0±7.96^{D}	5	3.2±0.14^{A,B}	6	121.5±6.70
	130	Normoxic	8	8	10,447.1±734.2^{A,B}	8	5293.7±828.4^{A,B}	8	248.1±15.04^{B,C}	2	3.4±0.20^{A,B}	6	171.1±34.84
	130	Anoxic	8	8	10,502.1±627.1^{A,B}	7	5397.0±646.0^{A,B}	7	195.4±6.42^{D}	1	14.9^{C}	6	208.9±89.05
	170	Normoxic	9	9	11,855.0±1124.4^{B}	9	5781.0±383.4^{B}	9	277.1±16.32^{C}	5	5.4±1.05^{B}	6	104.3±26.86
	167	Anoxic	9	9	11,569.8±872.6^{B}	9	5558.5±293.6^{A,B}	9	177.1±8.02^{D}	2	3.5±0.57^{A,B}	5	81.1±15.66

Values are means±s.e.m. for plasma concentration (mmol l^{-1}) and shell (plastron) concentration (mmol kg^{-1} ash) of Ca, P, Mg, K and Na. Superscript letters show significant differences (one-way ANOVA, $P<0.05$). *N* designates the number of animals from which plasma or shell samples were measured; *n* indicates the number of those animals for which a given measurement was above detection. P levels were below detection (b.d.) for all plasma samples. Detection limits were 1 ppm for P and 200 ppb for all other elements.

on ranks) (Fig. 4D). The maximum stress (Fig. 5A) a plastron beam sustained before fracture decreased significantly after 167 days of anoxic submergence, as did the elastic modulus (Fig. 5B). The maximum strain (Fig. 5C) reached before fracture increased significantly after 170 days of submergence, regardless of aeration state. Toughness was unaffected by submergence time or aeration treatment (Fig. 5D).

DISCUSSION

Physiological responses to anoxia

The majority of our observations of the physiological response of the painted turtle to anoxic submergence at 3°C are consistent with previous studies (Jackson, 1997; Jackson et al., 2000a; Jackson and Heisler, 1983; Jackson and Ultsch, 1982; Ultsch and Jackson, 1982a, 1995; Warburton and Jackson, 1995). We observed a large decrease in blood pH during anoxic submergence, with a concurrent increase in plasma lactate. The increasing concentration of lactate in the shell mirrored that of lactate in the plasma, providing evidence for the sequestration of lactate in the shell. We found strong evidence that the shell contributes buffer during anoxia: bone CO_2 and bone Mg decreased significantly during anoxic submergence, implying that magnesium carbonates were released to help titrate the acid load. Correspondingly, we observed an increase in plasma Mg. Plasma concentrations of Ca and K were also significantly elevated after anoxic submergence. Given the abundance of calcium compared with magnesium in bone, calcium carbonate release likely contributes the majority of the buffering capacity (Jackson et al., 1999). The fact that no decrease in bone calcium was observed is likely a consequence of the vast amount of background calcium in bone.

Our values for the sodium content of ashed bone differed greatly from those of Jackson et al. (2000a). The mean and s.e.m. for their control turtles was 362.2±8.3 µmol g^{-1} ash; ours was 137.9±34.89 µmol g^{-1} ash. This disparity was present across all treatment groups: our values were typically about 36% of those measured by Jackson et al. (2000a). The reason for this difference is unclear, but could be related to methodological differences between the studies. In the present study, ICP-MS was used, while Jackson et al. (2000a) employed a flame photometer to measure sodium. It has been previously shown that high calcium concentrations can interfere with the measurement of sodium when flame photometry is used (Foster and Hume, 1959; Spector, 1955), while ICP-MS is less prone to such calcium interference. Jackson et al. (2000a) observed a 19% decrease in shell sodium during anoxic submergence, while we observed no change in shell sodium.

Changes to bone material properties

This study shows that long-term (~170 days) anoxic submergence decreases the strength and elastic modulus of the plastron of the painted turtle. To our knowledge, this is the first study that links metabolic acidosis to changes in the mechanical properties of bone in any ectotherm. The values for maximum stress and elastic modulus for the control animals in our study are slightly smaller (53±8 versus 151±42 MPa and 2.5±0.6 versus 5.0±2.4 GPa, respectively, mean±s.e.m.) than those of a study that used three-point bending to measure the material properties of plastron beams from similarly sized terrestrial and pond-dwelling turtles (Magwene and Socha, 2013). However, that study included plastron from red-eared sliders (*Trachemys scripta*), diamondback terrapin (*Malaclemys terrapin*) and box turtles (*Terrapene carolina*), and only 2 of the 17 animals whose plastron was measured in three-point bending were painted turtles. Variations to experimental protocol

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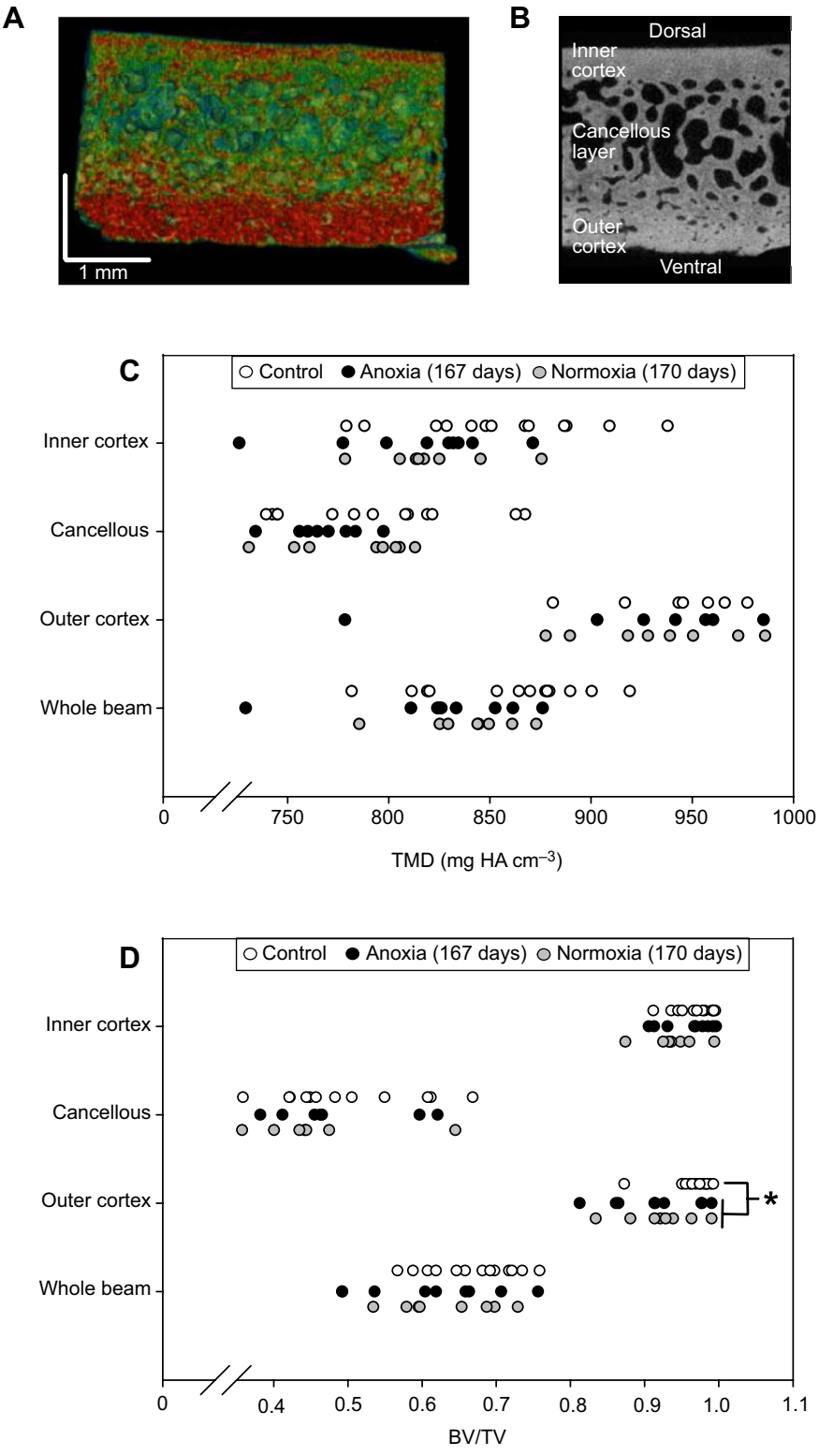

Fig. 4. Bone microcomputed tomography (microCT) analysis of bone beams milled from turtle plastron. (A) A color image showing the variation in bone density within the plastron beam. The densest bone is colored red and the less-dense bone is colored green. (B) A single monochromatic slice through the plastron beam with labeling of the different bone layers analyzed within the beam. (C) Tissue mineral density (TMD; calibrated to hydroxylapatite, HA) and (D) bone volume/tissue volume (BV/TV) before and after simulated overwintering either in anoxic (167 days) or in normoxic (170 days) water. At the whole-bone level, no changes were detected as a result of anoxic or normoxic submergence (BV/TV: P=0.206; TMD: P=0.139, one-way ANOVA). However, submergence caused a statistically significant decrease in the BV/TV of the outer cortical bone layer (*P=0.023, Kruskal–Wallis one-way ANOVA on ranks). There was a tendency for TMD to decrease in the inner cortical layer during the overwintering period, but the P-value did not reach significance (P=0.054, one-way ANOVA).

(e.g. strain rate or breaking span) could also cause disparities between studies (Magwene and Socha, 2013).

Three-point bending assumes that the bone being tested is homogeneous, isotropic and uniform in cross-section. By necessity, these assumptions are frequently violated in studies of the mechanical properties of mammalian long bones (Akhter et al., 2004; Silva et al., 2004; van Lenthe et al., 2008). Although turtle plastron is not homogeneous or isotropic, one relative advantage to testing it is that, unlike studies of long bone, beams can be fashioned that are consistently rectangular in cross-section, allowing for the mathematical assumptions of beam theory to be more closely upheld.

Aspect ratio, or the ratio of the thickness of the specimen to the span over which it is broken, is another critical variable in a bending test. A micro-fine element model of van Lenthe et al. (2008) calculated that elastic modulus, as derived from beam theory, is

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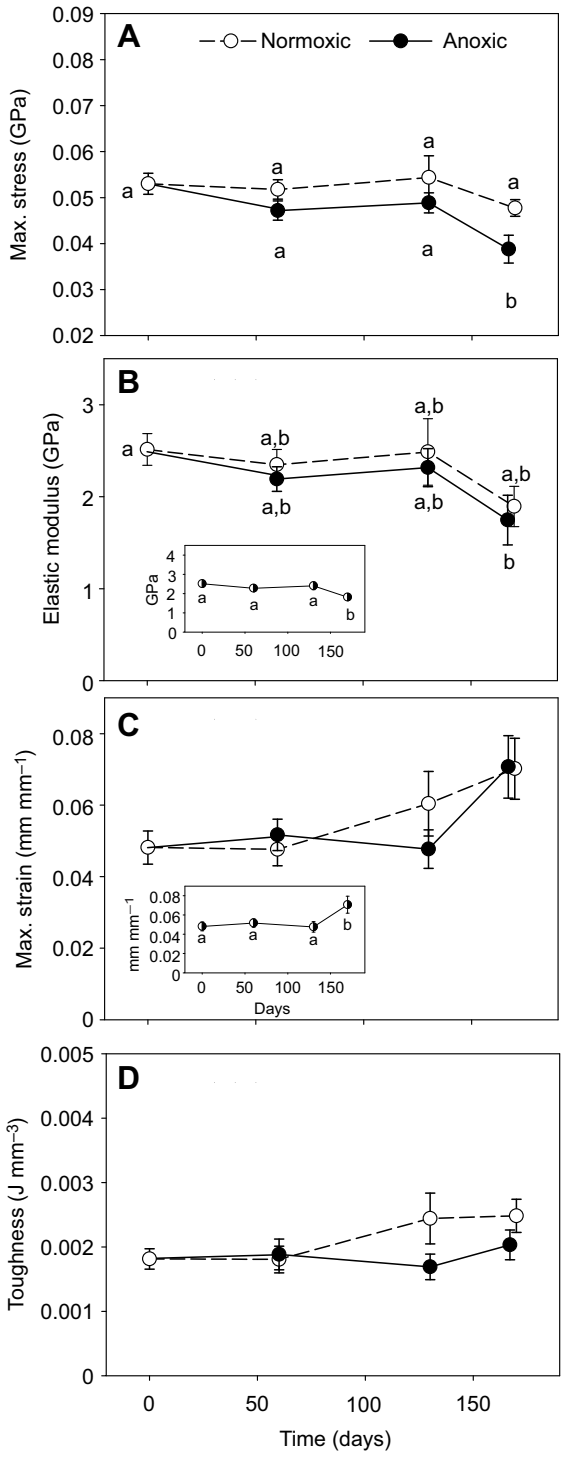

Fig. 5. Effects of simulated aquatic hibernation on the mechanical properties of turtle plastron beams. The measured variables included (A) maximum stress, (B) elastic modulus, (C) maximum strain and (D) toughness. The insets in B and C show the pooled means±s.e.m. from all of the turtles at each time point in order to illustrate the effects of time on the measured variables. Differing letters indicate significant differences between means for a sampling group (P<0.05, one-way ANOVA, SNK *post hoc*). See Table 1 for n values in each group.

underestimated by 29% when the aspect ratio is 5, and underestimation increases as the aspect ratio decreases. In our study, where the average beam thickness was 1.93 mm and the span length was 16 mm, the aspect ratio was about 8.3. While this is a slight improvement over murine models, where femoral cortical diameter is typically around 1.15 mm and breaking spans of 5–10 mm are common, it is far from the recommended aspect ratio for bending tests (17–20) (Turner and Burr, 1993; van Lenthe et al., 2008). In our study, the breaking span was constrained by the mediolateral width of the plastron, and likely contributed to our underestimation of the true elastic modulus.

Generally, three-point bending tests underestimate the elastic modulus of material because of deformation and shear stresses introduced where the beam rests on the supports; strain values are overestimated for the same reasons (Turner and Burr, 1993). This study did not employ strain gauges to control for this phenomenon. Measurements of the elastic modulus of rat tibia with strain gauge supplementation yielded values nearly 4-fold higher than those calculated from beam theory equations alone (Turner and Burr, 1993). Therefore, we believe the true intrinsic stiffness of painted turtle plastron to be greater than the values reported here; however, when relative comparisons are made using this method on similarly sized specimens, the measurements can be considered a normalized estimate of the bending moment (Currey, 1987). Although the conclusions of this study are not changed, caution should be taken when comparing values between studies.

In our study, the only significant changes in the mechanical properties of bone within each treatment occurred in turtles submerged in anoxic water after 167 days. However, when we pooled our data by submergence time (irrespective of the oxygenation state of the water), we found that elastic modulus was significantly decreased and maximum strain was significantly increased after 167–170 days (Fig. 5), but maximum stress and toughness did not change. This coincided with an increase in bone water and a decrease in bone ash after 167–170 days. While those trends were more pronounced in the anoxic group, by again pooling the data by time we detected an effect simply attributable to submergence (Fig. 3). The inverse relationship between these parameters is typically assumed to be obligatory (Biltz and Pellegrino, 1969), based on the observation that osteoid maintains a constant volume as it mineralizes (Elliott and Robinson, 1957; Hammett, 1925). However, in this case, the trend is in the opposite direction: anoxic turtle bone loses mineral and gains water. Although our microCT measurements did not indicate any geometric change to the plastron due to anoxia, we did detect a submergence-induced decrease in BV/TV in the outer cortical layer, which is consistent with the observation that long-term submergence causes turtle bone to lose mineral and gain water. Perplexingly, TMD in the plastron did not decrease significantly with submergence. The change in TMD was greatest in the inner cortical layer (855.6 versus 814.4 and 821.6 mg HA cm^{-3}, control versus 167 days anoxic and 170 days normoxic submergence, respectively; one-way ANOVA, P=0.054). It is worthy of note here that volumetric bone mineral density is preferred over TMD in cancellous bone due to problems with a partial volume effect in the microCT scan rendering software. We elected TMD (and thus slightly increased our risk of type II error) so that comparisons of mineral density could be more easily made across anatomical regions of the plastron.

Decreased bone ash is associated with decreases in strength and intrinsic stiffness (i.e. elastic modulus) (Currey, 1969, 1988; Zioupos et al., 1999), and because decreased bone ash typically corresponds to increased bone water, such specimens regularly show increases in ductility and toughness (Broz et al., 1993; Fernández-Seara et al., 2004). The mechanism by which water content affects bone material properties has historically been thought to involve the dramatic variation in the material properties

of collagen with its hydration state (Bembey et al., 2006b; Pathak et al., 2011; Sasaki and Enyo, 1995). More recent evidence suggests that molecular water occupies the channels of the apatite crystallites themselves, and contributes to material properties at the nanoscale (Armstrong and Mow, 1982; Bembey et al., 2006a; Pasteris et al., 2014; Samuel et al., 2014). Water in the organic phase is consistently associated with bone toughness (Gallant et al., 2014; Nyman et al., 2013, 2008), while water in the mineral phase is associated with both bone toughness and bone strength (Nyman et al., 2006). The propensity for water from the mineral phase to be exchanged *in vivo*, however, is not currently known.

This work has potential implications for our understanding of the interaction between the mechanical properties of bone and its utilization as a buffer in other species. It has been previously shown that bone can buffer acidosis in less heavily armored ectothermic vertebrates, including anurans (Warren and Jackson, 2005; Simkiss, 1968), crocodilians (Jackson et al., 2003) and squamates (Ruben and Bennett, 1981). The data from the present study suggest that utilizing bone for these purposes could have little or no effect on the mechanical and morphological properties of bone. Indeed, we have shown that bone must lose ∼23% of its carbonate before its strength is diminished (decreased maximum stress). It is important to emphasize bone carbonate content in the process, because there is little evidence to suggest that phosphate release from the bone is occurring (Jackson et al., 1999). If the released carbonate is derived from the mineral phase, then it must be from regions that contribute little to the overall strength of an individual plastron beam. There may also be implications for our understanding of dermal bone in the life history of Permo-Carboniferous amphibians, which are hypothesized to have used dermal bone as a buffer in the face of terrestrial acidosis (Janis et al., 2012). If the composition of that bone was similar to extant reptilian bone, then those early tetrapods likely used their bone as a buffer with little or no consequence to the mechanical properties of their dermal armor.

Relevance to painted turtle overwintering biology

This laboratory study has demonstrated the potential for a physiological trade-off between using the shell to buffer lactic acidosis and using it for physical protection post-emergence. In other words, if a painted turtle underwent anoxic submergence for 170 days, its shell would be weakened and would fail more easily. Whether this trade-off is relevant to wild painted turtles remains uncertain, however. Our survey of the literature revealed no studies to indicate that painted turtles routinely experience the 23–24 weeks of anoxic submergence required to weaken the plastron. Rollinson et al. (2008) reported ∼20 weeks of nearly anoxic water at the bottom of a turtle pond in Algonquin Provincial Park near Toronto, ON, Canada, and Taylor and Nol (1989) found anoxic conditions from only January–March at another location, also near Toronto. Crawford (1991) reported near-anoxic water for an average of 43 days in a turtle pond near Ann Arbor, MI, USA. Thus, under most relevant circumstances, the painted turtle can likely use its shell as a buffer without compromising its structural integrity. However, the changes to strain tolerance and elastic modulus after ∼26 weeks of submergence, regardless of oxygenation level, may be ecologically relevant. Ponds and lakes in Minnesota, where painted turtles are typically found, can become ice-locked as early as late October or early November and remain so for as many as 27 weeks (http://www.dnr.state.mn.us). Therefore, painted turtles experiencing the severest of winter conditions may begin their spring emergence with a shell that has an increased strain tolerance and decreased elastic modulus, which means that the plastron of these animals would be easier to physically deform without fracturing. These changes will not necessarily translate into increased vulnerability to predation because long submergences, by themselves, do not affect the maximum stress at which the plastron will fail. If, however, the submergence is combined with ∼170 days of lactic acidosis, the plastron will not be able to sustain the same maximum stress before fracturing and the turtle's primary defense against predation will be compromised.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.E.W.; Methodology: J.G.B., D.E.W.; Formal analysis: D.T.O., M.A.S.; Investigation: D.T.O., M.A.S., S.W.K., C.A.H., D.E.W.; Writing - original draft: D.T.O.; Writing - review & editing: D.T.O., M.A.S., J.G.B., S.W.K., C.A.H., D.E.W.; Supervision: D.E.W.; Project administration: D.E.W.; Funding acquisition: D.E.W.

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