Comparative shell buffering properties correlate with anoxia tolerance in freshwater turtles

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Jackson DC, Taylor SE, Asare VS, Villarnovo D, Gall JM, Reese SA. Comparative shell buffering properties correlate with anoxia tolerance in freshwater turtles. Am J Physiol Regul Integr Comp Physiol 292: R1008–R1015, 2007. First published September 28, 2006; doi:10.1152/ajpregu.00519.2006.—Freshwater turtles as a group are notably anoxia tolerant compared with other vertebrates, considerable differences are apparent when species are compared under similar conditions. A series of studies has compared the blood acid-base and ionic responses of four species of North American turtles that live in regions where prolonged periods of subfreezing conditions occur during the winter. Based on these studies, all experiments were conducted on turtles submerged at 3°C in N2-equilibrated water. The species were split into two groups: a more anoxia-tolerant group consisting of the snapping turtle, Chelydra serpentina (26), and the painted turtle, C. picta bellii (28); and a less anoxia-tolerant group consisting of the map turtle, Graptemys geographic (25), and the musk turtle, Sternotherus odoratus (30). A fifth species, the red-eared slider, Trachemys scripta (33), whose home range is generally not as far north as the other species, was studied in similar fashion recently and fell into the less anoxia-tolerant group. It was included in the present study because it is a particularly well-studied species and has often been used as a model organism for studying adaptations to anoxia.

The criterion for anoxia tolerance was the length of time a turtle was submerged at 3°C until blood pH fell from the control value of 7.8–8.0 to ~7.1, near the limit for survival at this temperature. For the more anoxia-tolerant species, the duration was 100–125 days, and for the less anoxia-tolerant species, the duration ranged from 25 to 50 days. It is important to emphasize that all of these species are very anoxia tolerant by general vertebrate standards, although the ecological rele-

THE SHELL OF A TURTLE, in addition to serving as a protective armor, also functions as its major mineral reservoir. Most of the body’s calcium, magnesium, phosphate, and sodium can be found in the shell and in the skeletal bone not associated with the shell. These structures also contain the bulk of the body’s CO2, primarily in the form of carbonate. Mineral exchange with the blood perfusing the shell is thought to be important in body fluid ionic homeostasis of the turtle, similar to the function of bone generally (6). In the Western painted turtle, Chrysemys picta bellii, the shell constitutes ~32% of the body mass of the animal (20), and the portion of the skeleton not incorporated into the shell adds another 5.5% (unpublished observations).

Recent studies have documented an important role for the turtle shell and skeleton in acid-base homeostasis, particularly during long-term submergence, when lactic acid production and accumulation subject the animal to severe acidic stress (14). The major subject for these studies has been the painted turtle, the most anoxia-tolerant tetrapod studied to date. In laboratory studies, this animal can survive and recover from at least 3 mo of anoxic submergence at 3°C (9). During long submergences at this temperature that simulate overwintering in ice-covered ponds, plasma lactate levels rise from the normal value of ~1 mM to as high as 150–200 mM (31), an acid load that far exceeds the normal buffering capacity of the body fluids (18). Supplemental buffering provided by the shell and skeleton in the form of calcium and magnesium carbonates (or bicarbonates) keeps the blood pH within a viable range. In addition, lactic acid enters the shell and skeleton and is buffered there. In extreme cases, >40% of the total body lactate is found in bone (14). The buffering within shell and skeleton also is thought to involve carbonate or bicarbonate as the buffer anion(s) (17).

Although turtles as a group are notably anoxia tolerant compared with other vertebrates, considerable differences are apparent when species are compared under similar conditions. A series of studies has compared the blood acid-base and ionic responses of four species of North American turtles that live in regions where prolonged periods of subfreezing conditions occur during the winter. Based on these studies, all experiments were conducted on turtles submerged at 3°C in N2-equilibrated water. The species were split into two groups: a more anoxia-tolerant group consisting of the snapping turtle, Chelydra serpentina (26), and the painted turtle, C. picta bellii (28); and a less anoxia-tolerant group consisting of the map turtle, Graptemys geographic (25), and the musk turtle, Sternotherus odoratus (30). A fifth species, the red-eared slider, Trachemys scripta (33), whose home range is generally not as far north as the other species, was studied in similar fashion recently and fell into the less anoxia-tolerant group. It was included in the present study because it is a particularly well-studied species and has often been used as a model organism for studying adaptations to anoxia.

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vance is that the so-called less anoxia-tolerant species would be less likely to survive in an overwintering site that became anoxic than the more tolerant species.

What ultimately limits how long a turtle can remain anoxic is not certain, but exhaustion of glycogen reserves and acidosis are probable factors. Acidosis at low temperature results primarily from anaerobically produced lactic acid so that prolongation of anoxic exposure can be accomplished either by slowing the rate of lactic acid production or by more effectively buffering lactic acid. Because shell and skeleton are crucial in lactic acid buffering, variation in the effectiveness of buffering could contribute to interspecific differences in anoxia tolerance. For example, the poorly mineralized shell of the softshell turtle, *Apalone spinifera*, has been suggested to be a factor in the poor performance of this species in anoxia (19, 27). Turtles we have designated in the present study as less anoxia tolerant, however, all have large, well-mineralized shells similar to those of the more tolerant species.

We hypothesized, therefore, that differences in buffering characteristics of the shells of turtles could explain some of the differences in anoxia tolerance. To test this hypothesis, we have compared shells from the five species in the following ways: 1) mineral composition, 2) total CO₂ concentrations, 3) volume of acid required to pH-stat a given mass of shell powder for a set period (an indication of functional buffering capacity), and 4) amount of lactate sequestered in shell samples incubated to equilibrium in a standard lactate solution. Our results reveal differences in shell buffering characteristics that support our hypothesis.

**MATERIALS AND METHODS**

**Animals**

Shells from the following five species were the primary objects of study: Western painted turtle (*C. picta bellii*), snapping turtle (*C. serpentina*), map turtle (*G. geographicala*), musk turtle (*S. odoratus*), and red-eared slider (*T. scripta*). Painted turtles, map turtles, and red-eared sliders were all obtained as live animals from commercial suppliers; painted turtles were collected in Minnesota; and map turtles were collected in Michigan. The red-eared sliders’ provenance is uncertain but is probably from commercial farms in Louisiana. The musk turtles were trapped in Alabama and supplied by Dr. Gordon R. Uiltsch of the University of Alabama, and although they are not from a northern population, northern and southern musk turtles have similar physiological responses to anoxic submergence (30). Snapping turtle shells (carapaces) were obtained from a licensed commercial collector in Michigan. Shell mineral and CO₂ analyses were also made on the following species of turtles: box turtle (Terrapene c. carolina), gopher tortoise (Gopherus polyphemus), loggerhead turtle (Caretta caretta), and Atlantic Ridley turtle (Lepidochelys kempii). The first two are terrestrial species, and the last two are marine turtles. The gopher tortoises and box turtles were collected under permit in Alabama, and shell samples from the marine turtles were collected from animals stranded on the beach at Cumberland Island, GA, and kindly provided by Carol Ruckdeschel of the Cumberland Island Museum.

Painted turtles (*n = 5–21* depending on measurement), red-eared sliders (*n = 6–9* depending on measurement), box turtles (*n = 4*), and gopher tortoises (*n = 5*) had all been control subjects in previous unrealted experiments, and following euthanasia by decapitation and pithing, the plastron was removed and cleaned of all soft tissue and stored at −20°C until used in this study. Musk turtles (*n = 8*) and map turtles (*n = 8*) were purchased specifically for this study, and these animals were killed by decapitation and pithing after body weights were obtained. The entire shell of each animal was removed and thoroughly cleaned of adherent soft tissue, and fresh weight was recorded. The shells were held at −20°C until studied. Snapping turtle carapaces (*n = 12*) were stored in the −20°C freezer after receipt until studied. The experimental protocol used in this study was approved by the Brown University Institutional Animal Care and Use Committee.

**Experimental Procedures**

**Shell total CO₂ concentration.** Extraction of CO₂ was accomplished by adding dried shell powder to a reaction flask containing a stirred solution of 2 N HCl. CO₂-free gas flow carried the evolved CO₂ to a drying column and then to a CO₂ gas analyzer (model CD-3A; AEI Technology, Pittsburgh, PA), the output of which was recorded on a laptop computer using a data acquisition system (MP100; BIOPAC, Goleta, CA) and analyzed with installed software (Acqknowledge; BIOPAC).

Pieces of plastron from painted turtles (*n = 21*), map turtles (*n = 7*), musk turtles (*n = 8*), red-eared sliders (*n = 9*), box turtles (*n = 5*), gopher tortoise (*n = 4*), or carapace from snapping turtles (*n = 12*), loggerhead turtles (*n = 24*), and Atlantic Ridley turtles (*n = 5*) were ground to powder in a Freezer Mill (Spex Certiprep model 6700), and the powder was oven-dried to constant weight. The dry powder was placed in a preweighed removable side arm of the reaction flask. The side arm connection was a ground glass joint that could be rotated from a position in which the powder remained in the side arm bulb to a position that allowed powder to pour into the solution where mixing with HCl occurred. This apparatus, described previously (17), permitted the mixture of the powder and the acid in a closed system with no loss of CO₂ to the environment. The CO₂ was rapidly evolved from the shell powder, and extraction was considered complete when the CO₂ trace on the computer returned to baseline (~6 min). The analysis program was used to integrate the curve over time and provide an average CO₂ deflection. The gas flow through the system was set by a rotameter (series 150; Linde Union Carbide, Somerset, NJ) and measured following each experiment using a volumeter (model 1056; Brooks Instruments, Hatfield, PA). The side arm with powder was weighed before and after CO₂ extraction to provide the mass of measured powder (0.2–0.4 g). Shell CO₂ concentration was calculated from these data plus measured barometric pressure and is expressed as mmol CO₂/g dry weight.

On the basis of an earlier in vitro study (17), we assumed that most of the evolved CO₂ derives from carbonate, but we cannot exclude the possibility that some CO₂ is also in the form of bicarbonate. In mammalian bone, it is estimated that one-third of total bone CO₂ consists of bicarbonate that is located in the hydration shell of the apatite crystal and that this is the readily accessible pool of bone CO₂ (5). For the balance of this report, however, we refer to the shell CO₂ as carbonate but with the understanding that this may not be strictly the case.

**Functional buffer capacity.** With the use of the same apparatus described above, weighed amounts of dry shell powder from each of the five species were added to a buffered solution at pH 7.0. The number of shells tested was as follows: painted turtles, 5; map turtles, 7; musk turtles, 8; snapping turtles, 12; and red-eared sliders, 9. The composition of the buffer solution was 120 mM NaCl, 20 mM PIPES buffer, titrated to pH 7.0 with 1 M NaOH. Approximately 2 g of powder were added to 15 ml of buffer solution. A pH electrode (Radiometer type pHC4006; Hach, Loveland, CO) placed in the solution interfaced with a pH stat system (Radiometer Titralab 854; Hach) that automatically titrated 1 M HCl solution into the reaction vessel to hold the solution pH at 7.0. At this pH, the solution is alkalized by calcium and magnesium carbonate released from the shell powder (17). The cumulative volume of HCl titrated was recorded at 15, 30, 45, 60, 90, 120, 150, and 180 min following addition of the powder. Titration volumes were then divided by added shell mass and expressed as ml HCl/g dry weight. The mass of added powder was determined as described above by weighing the side arm before and after the experiment. Samples of the buffered solution were

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taken before the powder was added and at the end of the experiment and were stored frozen at −20°C until analyzed for calcium and magnesium concentrations by atomic absorption spectrophotometry (model 280; Perkin-Elmer, Norwalk, CT). The solutions from the snapping turtle experiments were not tested for calcium and magnesium concentrations.

In two experiments, one on red-eared slider shell and one on painted turtle shell, titration was continued for 24 h to ascertain whether interspecific differences observed were due to the kinetics of the buffer release process. In addition, samples of carapace from three map turtles were compared with plastron values from the same animals.

### Shell lactate uptake.

Shell disks (0.7-cm diameter) were punched from plastron or carapace (snapping turtle only) with a hand punch (model 5 Jr; Roger Whitney of Rockford, Rockford, IL). Disks were incubated in a solution with the following composition: 100 mM NaCl, 30 mM PIPES buffer, and 40 mM L-lactic acid, titrated to pH 7.0 with 1 M NaOH. Incubations lasted 2 days, which on the basis of previous observations (15), is adequate for complete equilibration.

After incubation, disks were stored frozen at −20°C until analyzed for lactate concentration. Incubations were conducted in pairs: map turtles vs. snapping turtles, musk turtles vs. painted turtles, map turtles vs. painted turtles, and snapping turtles vs. red-eared sliders. The total number of disks incubated were painted turtle, 10; snapping turtle, 6; map turtle, 6; red-eared slider, 6; and musk turtle, 5. Samples of the incubation solution were taken before shell disks were added and after the incubation, and these samples were also analyzed for lactate.

To prepare for shell lactate analysis, disks were ground to powder using the Freezer Mill as described above. Lactate was extracted by incubating shell powder in 11 parts (vol/wt) 0.6 M perchloric acid for 2 h with vortexing every 15 min. After centrifugation, an aliquot of the supernatant was analyzed for lactate using a standard kit (lactate reagent 735-10; Trinity Biotech, St. Louis, MO). The solution samples were diluted 12-fold with deionized water and analyzed similarly.

### Shell mineral analysis.

Mineral analysis was carried out on the five principal species and on the same four additional species that were tested for shell CO2 concentration. The numbers of shells tested for each species are given in Table 1. In some species it was possible to determine shell mass as a fraction of body mass, percent water in the shell by drying fresh shell samples in an oven, and contributions of ash and organic matter to fresh shell weight. In other samples, such as the snapping turtle shell and the marine turtle shells, the uncertain past history made some of these determinations impossible.

### RESULTS

#### Shell Total CO2 Concentration

The values for shell CO2 concentration for the various turtles (Fig. 1) differed significantly according to one-way ANOVA (P < 0.001). Pairwise comparison using Tukey’s test revealed that the CO2 concentration of painted turtle shell (1.316 ± 0.020 mmol/g dry weight) was significantly greater than that of all other species tested and that red-eared slider shell CO2 (0.834 ± 0.035 mmol/g dry weight) was significantly less than that of all other species.

#### Functional Buffering Capacity

The rate of acid titration was high following the addition of powder to the pH 7.0 solution and gradually slowed over the

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### Table 1. Composition of shells of turtles

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Shell, % BM</th>
<th>Water, % shell ww</th>
<th>Organic, % shell ww</th>
<th>Ash, % shell ww</th>
<th>[Ca], μmol/g ash</th>
<th>[Mg], μmol/g ash</th>
<th>[Na], μmol/g ash</th>
<th>[K], μmol/g ash</th>
<th>[P], μmol/g ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Painted turtle, <em>Chrysemys picta</em></td>
<td>5</td>
<td>32.4±1.0abc</td>
<td>33.6±1.1bc</td>
<td>25.4±0.2a</td>
<td>41.0±1.0a</td>
<td>9420±116ab</td>
<td>228±6.7ab</td>
<td>362±8.4ab</td>
<td>10.7±1.4bc</td>
<td>4800±32a</td>
</tr>
<tr>
<td>Snapping turtle, <em>Chelydra serpentina</em></td>
<td>12</td>
<td>43.0±0.9a</td>
<td>28.4±0.3abc</td>
<td>28.6±0.9ab</td>
<td>9435±75ab</td>
<td>210±3.5ab</td>
<td>292±9.0ab</td>
<td>16.2±1.0d</td>
<td>5027±48abc</td>
<td></td>
</tr>
<tr>
<td>Musk turtle, <em>Sternotherus odoratus</em></td>
<td>8</td>
<td>30.3±0.5a</td>
<td>23.0±0.9b</td>
<td>46.5±0.7a</td>
<td>9612±169ab</td>
<td>198±8.5ab</td>
<td>279±5.9ab</td>
<td>7.6±0.8b</td>
<td>5393±120ab</td>
<td></td>
</tr>
<tr>
<td>Map turtle, <em>Graptemys geographica</em></td>
<td>6</td>
<td>28.6±0.5b</td>
<td>31.7±3.3abc</td>
<td>41.2±1.4abc</td>
<td>9720±90b</td>
<td>181±6.4b</td>
<td>292±6.7ab</td>
<td>13.2±0.5ab</td>
<td>5320±62ab</td>
<td></td>
</tr>
<tr>
<td>Red-eared slider, <em>Trachemys scripta</em></td>
<td>6</td>
<td>32.7±1.0a</td>
<td>38.5±3.3a</td>
<td>32.8±1.6bc</td>
<td>8640±197b</td>
<td>226±5.1ab</td>
<td>288±20.1ab</td>
<td>19.4±2.1bc</td>
<td>5233±36ab</td>
<td></td>
</tr>
<tr>
<td>Box turtle, <em>Terrapene c. carolina</em></td>
<td>4</td>
<td>41.3±1.4a</td>
<td>26.9±0.6b</td>
<td>28.8±2.1b</td>
<td>9567±158ab</td>
<td>215±8.8ab</td>
<td>340±7.5ab</td>
<td>16.0±1.7bc</td>
<td>5523±123ab</td>
<td></td>
</tr>
<tr>
<td>Gopher tortoise, <em>Gopherus polyphemus</em></td>
<td>5</td>
<td>18.1±0.2b</td>
<td>44.2±2.8abc</td>
<td>37.8±2.7a</td>
<td>9360±57ab</td>
<td>289±12.4ab</td>
<td>326±5.6ab</td>
<td>25.5±1.8c</td>
<td>5826±53b</td>
<td></td>
</tr>
<tr>
<td>Loggerhead turtle, <em>Caretta caretta</em></td>
<td>22</td>
<td>9193±45b</td>
<td>507±11.9abc</td>
<td>444±17.0b</td>
<td>24.0±1.1abc</td>
<td>5778±54abc</td>
<td>374±20ab</td>
<td>31.0±4.2ac</td>
<td>5685±110bc</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. BM, body mass; ww, wet weight. Brackets indicate concentration. n is number of animals. a,b,c,d Statistical differences (P < 0.05) are indicated by different letters for the same measurement. *Data are from Ref. 20.
course of 3 h. The cumulative acid titrated differed significantly for the different species \((P < 0.001)\) and generally conformed to their reported anoxia tolerance. Snapping turtle and painted turtle shells had significantly higher rates of titration than did musk turtles, map turtles, and red-eared sliders (Fig. 2). For this calculation, only seven of the musk turtles were included; the omitted turtle had a final cumulative titration that was nearly double the mean of the other seven. If this high value is included, the species still differ significantly \((P < 0.001)\), but the data fail to pass the normality and equal variance tests, and the painted turtle value is only significantly higher than that of the red-eared sliders.

Samples of map turtle carapace had lower total titration volumes \((\text{mean } P < 0.005) 0.297 \text{ ml/g dry weight}) than plastron samples from the same three turtles \((0.363 \text{ ml/g dry weight})\) or from all eight map turtles tested \((0.353 \text{ ml/g dry weight})\). No other carapace-plastron comparisons were made, so we cannot assume this difference applies to the other species. These differences were not statistically significant.

To determine whether the observed species differences were independent of the incubation time, two samples of shell powder, one painted turtle and one red-eared slider, were incubated with pH-stat titration for 24 h. The significant difference observed between these species at 3 h persisted after 24 h (Fig. 3).

Calcium and magnesium were released from the shell powder into the incubating solution. Final concentrations of calcium in the incubating solution ranged from 21.4 ± 0.9 mM in the painted turtle to 15.5 ± 0.9 mM in the red-eared slider. Magnesium concentrations ranged from 3.9 ± 0.2 mM in the painted turtle to 2.3 ± 0.15 mM in the map turtle. On a per gram shell powder basis, the final calcium content \((\text{mmol/g})\) differed significantly \((P < 0.05)\), although on a pairwise basis, the value for the painted turtle was only significantly greater than for the map turtle (Fig. 4). Magnesium values \((\text{mmol/g})\) also differed significantly \((P < 0.001)\), and values for map turtles were significantly less than for the other three species, and values for painted turtles were also significantly higher than for musk turtles. When the sum of calcium and magnesium release was tested, values for the painted turtle were significantly higher than for both the map turtle and the red-eared slider. The total volume of 1 N HCl titrated correlated with the combined release of calcium and magnesium (Fig. 5). After 24-h titration of painted turtle shell powder, calcium and magnesium concentrations were 95.4 and 11.5 mM, respectively, comparable values for the solution incubating red-eared slider shell powder for 24 h were 45.6 and 6.2 mM.

**Lactate Uptake Into Shell**

Shell disks accumulated lactate after 2 days of incubation in lactate solution (Fig. 6). The mean shell concentrations for all species \((\text{expressed in } \mu\text{mol/g wet weight})\) were at or above the final solution lactate concentrations \((\text{expressed in } \mu\text{mol/ml})\). Because the water content of the shells is only 30–40% of the shell mass \((\text{see below})\), the high shell concentrations indicate sequestration in a combined form other than simple solution in the aqueous phase. The accumulation was not the same in all turtles, however, and ANOVA revealed a significant difference among the species \((P < 0.001)\). Pairwise multiple comparison using the Student-Newman-Keuls method revealed that snapping turtle shells accumulated more lactate than any of the other four species \((P < 0.05)\) and that painted turtle shells...
accumulated more lactate than red-eared sliders or map turtles ($P < 0.05$).

Mineral Composition of Shells

The principal elements in turtle shell, as in bone generally, are calcium and phosphate, but the ratio of these (Ca/P) varied among the turtles (Table 1). Significant differences were confirmed by ANOVA for all variables tested as depicted in Table 1. As shown in Fig. 7, the variation in Ca/P correlated with the CO$_2$ concentrations of the shells. The more anoxia-tolerant species, painted turtles and snapping turtles, had high ratios and high CO$_2$ concentrations. The less anoxia-tolerant species had lower Ca/P ratios and CO$_2$ concentrations, but, except for the red-eared slider, they were still higher than those observed in the terrestrial and marine species.


discussion

The results of this study support the hypothesis that acid-base-relevant characteristics of the shells of turtles contribute to observed differences in long-term anoxia tolerance. By all criteria tested (shell CO$_2$ concentration, HCl titrated, calcium and magnesium release, and shell lactate uptake), more anoxia-tolerant species (painted turtle and snapping turtle) had higher values than less anoxia-tolerant species (musk turtle, map turtle, and red-eared slider). The painted turtle, the most anoxia-tolerant turtle, was significantly higher than all three less anoxia-tolerant species in all respects except for calcium
and magnesium release and lactate uptake compared with the musk turtle. It is important to note, however, that whereas these differences in shell buffering properties are significant, they cannot fully account for observed differences in responses to long-term submergence in anoxic water. In addition, our in vitro measurements are unable to reveal possible in vivo differences in shell blood or exchange properties among the various species.

Comparative measurements similar to the ones made in this study have not been made previously on the shells of turtles to our knowledge, but a study by Biltz and Pellegrino (2) did compare composition of long bones (femur and tibia) in a variety of vertebrates, including the snapping turtle, *C. serpentina*. These authors found that the snapping turtle had a bone CO$_2$ content and Ca/P ratio that were higher than any of the other vertebrates tested. The CO$_2$ concentration they found, converted to the units we used, was 1.0 mmol/g dry weight, somewhat lower than our value of 1.18 mmol/g dry weight, but their Ca/P ratio of 1.88 was identical to what we report.

The accessible CO$_2$ is phosphate or hydroxyl group. Substitution for phosphate in apatite crystal would also raise the Ca/P ratio. The accessible fraction as bicarbonate localized in the hydration shell of the separate-phase carbonate is associated with a calcium but not with a phosphate. The magnitude of this separate-phase carbonate fraction, therefore, proportionately increases the Ca/P ratio. Recent descriptions of bone CO$_2$ (5) identify the accessible fraction as bicarbonate localized in the hydration shell on the crystal surface and also indicate that within the apatite crystal, carbonate may be substituted for either a phosphate or hydroxyl group. Substitution for phosphate in apatite crystal would also raise the Ca/P ratio. The accessible CO$_2$ is the “separate” or elutable phase, and this fraction was more than three times as high in the turtle as in any of the other animals studied (2). Interestingly, the fish studied (*Lutjanus ayu*) had no accessible carbonate, consistent with the generally poor intrinsic extracellular buffering of teleosts (8).

There are two key differences between the study of Biltz and Pellegrino (2) and our study. First, these earlier workers studied cortical bone (femur and tibia), whereas we studied shell; however, in an earlier study on the painted turtle (16), femur and shell, including both carapace and plastron, were similar in mineral composition as well as in other respects, such as the uptake of lactate. Thus we believe that the long bone data of Biltz and Pellegrino can be directly compared with our shell data. The second difference between our study and that of these authors is that we looked at a variety of turtle species, whereas they looked at a single species. Moreover, the species they selected for study, snapping turtle, is a very anoxia-tolerant turtle (26). By studying this species and assuming that it was representative of turtles generally, they clearly biased their conclusion about turtles. Based on our results, data for turtles would not have been at all unusual compared with the non-chelonians had they selected a tortoise or a marine turtle for study. Nonetheless, Biltz and Pellegrino (2) observed striking differences among the animals studied but concluded that “the physiological and phylogenetic meaning of the species variations in bone composition are yet to be ascertained.” We suggest that the contribution of skeletal and shell carbonate to acid buffering may provide some of that “meaning.”

We hypothesize that all the interspecific differences in shell acid-base function we have observed can be attributed to variations in the amount of accessible carbonate in the turtles. It is unlikely that the crystal structure of shell was affected at the pH (7.0) we employed in this study. In earlier in vitro work, shell powder from the painted turtle was incubated under pH-stat conditions in solutions at various pH values down to 6.0, and we never observed an increase in solution phosphate, a clear indication that apatite was not affected. Assuming that the carbonate bound to bone crystal also was not affected at pH > 6.0, then only the carbonate in the hydration shell (5) is relevant to our observations. One of our observations is a little puzzling in this regard, however. The musk turtle’s total shell CO$_2$ concentration was almost the same as that of the snapping turtle, yet its buffering capacity was significantly less, suggesting either that the fraction of carbonate bound to the crystal structure may also be variable or that hydration shell carbonate is not all equally accessible.

Our data from the pH-stat experiments also indicate that most of the shell magnesium is associated with the accessible carbonate fraction. By the end of the 24-h pH-stat experiment on painted turtle shell powder, >50% of the total shell magnesium had been released into the solution, but only ~12% of the calcium had been released. This calculation is based on the millimoles of cations in the solution per gram of dry shell powder, divided by the total millimoles of cations in dry shell. Previous studies, in vivo (e.g., 21, 16) and in vitro (17), also found that relative to their total concentrations in shell, magnesium is released preferentially to calcium in response to acidosis. Shell calcium is ~50 times the concentration of shell magnesium, but the increase in plasma calcium during prolonged anoxia is only about three times the increase in plasma magnesium (16), and following in vitro incubation in acid medium, the increase in solution calcium is about five to six times the increase in magnesium (Ref. 17; Fig. 4).

Another factor contributing to differences in buffering capacity among turtles that also may contribute to observed differences in shell carbonate among turtles and between turtles and other vertebrae is plasma HCO$_3^-$ concentration. Freshwater turtles as a group, as described in a classic study by Smith (29), have unusually high HCO$_3^-$ concentrations in plasma and other extracellular fluids compared with other vertebrates, but within the group of turtles we studied, significant differences in HCO$_3^-$ concentrations have been observed. Based on measured values from the submergence studies that provide the background for the present study, the more anoxia-tolerant species have higher plasma HCO$_3^-$ concentrations than the less anoxia-tolerant species (Table 2). It is possible that the high plasma HCO$_3^-$ concentration in the painted turtle and

<table>
<thead>
<tr>
<th>Plasma [HCO$_3^-$] (mmol/l)</th>
<th>Δ[Lactate/Δtime (mmol·l$^{-1}$·day$^{-1}$)]</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Painted turtle</td>
<td>−50</td>
<td>3.2</td>
</tr>
<tr>
<td>Snapping turtle</td>
<td>−45−50</td>
<td>3.6</td>
</tr>
<tr>
<td>Map turtle</td>
<td>−36−44</td>
<td>4.7</td>
</tr>
<tr>
<td>Musk turtle</td>
<td>−31</td>
<td>6.0</td>
</tr>
<tr>
<td>Red-eared slider</td>
<td>−25−33</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Table 2. Published values of plasma [HCO$_3^-$] and in vivo rates of increase in plasma lactate concentrations in the five species studied

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Snapping turtle contributes to the high shell carbonate in these species, but this has not been experimentally tested.

The uptake of lactate by a turtle’s shell is a major factor contributing to the animal’s ability to buffer lactic acid. By the end of long periods of anoxic submergence at 3°C of the painted turtle, some 40–45% of the total body lactate resides in its shell (14). Much of this lactate is thought to be in combined form, because the concentrations of lactate observed are far greater than can be accounted for by simple solution in shell water. Our hypothesis is that lactate combines with calcium and that protons are buffered by carbonate in the hydration shell. Lactate is also sequestered by turtle skeletal bone (Ref. 16; unpublished observations), and uptake of lactate probably can occur in mineralized tissues generally in all organisms under suitable conditions (15, 22, 24, 32). As we have demonstrated in this study, interspecific differences also exist in how much lactate can be taken up by the turtle shell in vitro, and it is the more anoxia-tolerant turtles whose shells have higher CO2 levels that also accumulate more lactate. The differences observed, however, although significant, are not large, and it is not clear that bone CO2 concentration is an accurate predictor of lactate sequestration. The only comparisons of in vivo shell lactate in anoxic turtles have been made between the painted turtle (16) and the red-eared slider (33) after about the same duration of submergence at 3°C (50 and 44 days, respectively). The calculated fraction of total lactate sequestered within shell and skeleton was actually higher in the red-eared slider than in the painted turtle (33), despite the lower in vitro lactate uptake and lower shell CO2 concentration of the red-eared slider observed in the present study. A positive correlation between bone CO2 and in vitro lactate accumulation also was not supported by a recent study of the leopard frog, Rana pipiens (32). Although femur CO2 content of the leopard frog (0.53 mmol/g wet weight) was less than one-half the value measured in shells of four of the freshwater turtles in the present study, the frog’s bone nevertheless sequestered lactate during in vitro incubation at low pH almost as well as the turtles’ shells (i.e., bone lactate concentration in μmol/g wet weight ≡ solution lactate concentration in μmol/ml).

It is important to emphasize again that all freshwater turtles we studied can survive long periods of global anoxia and are therefore distinct in this respect from mammals and birds and from most other ectothermic vertebrates. Those turtles we designate as less anoxia tolerant are all very anoxia tolerant compared with other vertebrates. All of the turtle’s organ systems, including brain and heart, continue to function during anoxia and regain full function following anoxia. The special adaptive traits identified in turtle brain (1, 23) and heart (3, 11, 12, 13, 24), many of which were studied in the red-eared slider, are no doubt shared by all turtles to some extent. Even tropical turtles, which under natural circumstances are rarely, if ever, subjected to anoxia, survived experimental anoxic submergence for 6 h at 20°C (7).

However, whereas turtles as a group are resistant to anoxia, they differ in the length of time they can remain anoxic. It is likely, therefore, that it is the consequences of anoxia, rather than anoxia per se, that limit anoxic submergence. Even highly adapted facultative anaerobes like freshwater turtles progressively deplete fuel reserves and accumulate metabolic acid end products during submergence anoxia. How effectively a turtle can extend the time before these changes become irreversible and lethal is what must account for described differences in anoxia tolerance among various species. The lesser in vivo anoxia tolerance of the red-eared slider is perhaps better explained by more rapid onset of glycogen depletion and/or low pH (33) than by poorer anoxia tolerance directly.

A critical trait that contributes to the remarkable anoxia tolerance of freshwater turtles is metabolic depression, slowing both the rate of lactic acid production and glycogen depletion. This is a well-known response of turtles to anoxia (4, 13) but may also vary among species and help to explain the disparity in submergence times. The rate of anaerobic metabolism can be estimated by the rate of increase in plasma lactate (10), and the species in this study differ significantly in this regard (Table 2) based on the rate at which lactate concentration increased during the first 11 days of anoxic submergence at 3°C. Note that lactate builds much more slowly in the painted turtle and snapping turtle than in the map turtle and musk turtle, consistent with the more rapid decline in blood pH and shorter duration of submergence in the latter two species. In contrast is the slow rate of lactate accumulation observed in the red-eared slider, designated as less anoxia tolerant in this study. Based on the submergence study (33), the red-eared slider appeared to be limited by low extracellular HCO3− concentration, low presubmergence blood pH, and low glycogen reserves. The contribution of its shell to buffering, assessed both by release of calcium and magnesium and by uptake of lactate, was as good as or better than comparative data from the painted turtle (16), despite the poor showing of the red-eared slider in our in vitro analyses. The reason for this discrepancy is unclear.

Metabolic acidosis may be the major limiting factor in surviving submergence anoxia, and traits that slow the development of acidosis are therefore likely targets for adaptive mechanisms. Improved buffering mechanisms are also clearly critical for freshwater turtles as a group and confer a distinct advantage over other vertebrates. As discussed above, high extracellular HCO3− concentrations provide an effective first line of defense against acidosis; however, it is the buffering contribution of the turtle’s shell that enables it to endure circulating levels of lactate that are impossible for other animals. As demonstrated in this study, the buffering properties of turtle shells vary among species and likely help to account for some, but not all, of the reported differences in anoxia tolerance (3, 11, 12, 21, 34).

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