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# Lactate sequestration by osteoderms of the broad-nose caiman, *Caiman latirostris*, following capture and forced submergence

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## Summary

**Lactate accumulation in osteoderms of the broad-nose caiman, *Caiman latirostris*, was determined following capture and surgery and after a period of forced submergence and related to concurrent values in blood. Control samples of bone and blood were taken after recovery from surgery and before submergence. In addition, samples of osteoderm were incubated in a lactate solution to determine equilibrium concentration, and additional samples were analyzed for elemental and $CO_2$ concentrations. The composition of the osteoderms closely resembles that of typical vertebrate bone, with a high concentration of calcium and phosphate. Plasma and osteoderm lactate concentrations were both elevated following surgery and decreased significantly after 1 day of recovery. Submergence produced a typical lactate pattern in the plasma, with only a modest increase during the dive and then a sharp increase during recovery to a peak of 31.2±1.9 μmol ml$^{-1}$ after 1 h. When caimans were anesthetized 2 h after submergence, osteoderm lactate in the same animals was significantly increased to 14.8 μmol g$^{-1}$ wet mass. The ratio of the osteoderm:plasma lactate concentration after submergence was similar to the ratio observed in the incubated samples, suggesting that osteoderm lactate concentrations *in vivo* were equilibrated with circulating plasma levels. We conclude that caiman osteoderms sequester lactate during lactic acidosis and that the time course is fast enough to have benefit to these animals following normal anaerobic burst activity.**

Key words: acid–base, acidosis, bone buffering, bone minerals, crocodilian, *Caiman latirostris*, osteoderm, dermal bone, diving, lactic acid.

## Introduction

Bone is an important source of acid–base buffering in many organisms. The most familiar mechanism, the release of carbonates, is common to numerous organisms with calcified skeletons or exoskeletons, including vertebrates, in which the predominant mineral is hydroxyapatite (Irving and Chute, 1932), and invertebrates, in which the predominant mineral is calcium carbonate (DeFur et al., 1980). The carbonate is lost, in effect, with a cation that may be calcium, magnesium or sodium.

A second way that bone buffers extracellular acid, by the uptake and buffering of lactic acid within the bone itself, has recently been described in the freshwater turtle *Chrysemys picta bellii* (Jackson, 1997). This animal is capable of tolerating large burdens of lactic acid during prolonged anoxic submergence by exploiting the large buffering capacity of its shell and skeleton, which together account for 35–40% of the turtle's body mass. Besides releasing significant amounts of carbonate buffers into the extracellular fluid, the turtle also accumulates high concentrations of lactate within its shell. Experiments on powdered shell *in vitro* demonstrated that this mechanism is acid–base relevant and is equivalent to the movement into the bone of lactic acid. In extreme experimental cases, when circulating lactate is very high, the lactate uptake mechanism can account for 40–45% of the total lactic acid buffering in the body. When the buffer release mechanism is considered as well, the shell and bone account for 70–75% of the total buffering, indicating that the buffering functions of the shell are crucial for the turtle's long-term anoxic tolerance.

Although the buffer release mechanism is generally regarded to be a universal property of bone, the general occurrence of the lactate uptake mechanism is uncertain. Lactate uptake was recently demonstrated in the carapace of the crayfish *Austropotamobius pallipes* during emersion hypoxia and lactic acidosis (Jackson et al., 2001) and has also recently been observed in the shell of the pulmonate snail *Helix aspersa* following 24 h of anoxia (D.C.J., unpublished observations). These invertebrate exoskeletons, unlike vertebrate bone, are not vascularized and are composed of calcium carbonate.

The objective of the present study was to investigate the occurrence of lactate accumulation during hypoxic acidosis in the mineralized tissues of the broad-nose caiman *Caiman*

*latirostris*. A particular object of our study was the dermal bone, or osteoderms, of this animal. We hypothesized that this bone, which, like the turtle's shell, forms an overlying armor on the dorsal surface of the animal, although only partial in the case of the caiman, may serve as a site for lactic acid buffering. To test this, we collected and analyzed osteoderms and blood after capture and before and after a period of enforced submergence.

## Materials and methods

### *Animals*

We studied nine caimans, *Caiman latirostris* Daudin, with a body mass of 3.31±1.16 kg (mean ± S.D.). Prior to study, these animals, all born in captivity, were housed in outdoor pens (100 $m^2$) with a 1 m-deep water pool of ~10 $m^2$ and a dry surface provided with basking areas and plants for shade and shelter at the Jacarezário, UNESP, Rio Claro, Brazil. They were fed twice a week with fish, beef and chicken.

### *Capture and surgical preparation*

Caimans were captured with a noose from their holding tank and, after tying their mouths closed with an elastic band, were brought into the laboratory and restrained on an operating board with rubber bands. A damp cloth covered their heads. The femoral arterial was dissected out and catheterized occlusively under local anesthesia (lidocaine) with either PE 60 or PE 90 catheter tubing. The wound was closed and the catheter was secured by suturing to the skin on the dorsal surface. At the conclusion of surgery, a blood sample was taken and a single osteoderm was taken from the mid-dorsal region, also using local anesthesia. During this entire procedure, the animals remained perfectly still with no apparent reaction to any of the procedures. The blood was centrifuged, and plasma and the osteoderm were stored frozen for later lactate analysis. In some experiments, blood pH and plasma total $CO_2$ were measured immediately.

### *Experimental protocol*

The caiman was placed in a plastic container (50 cm×40 cm×70 cm) with a small amount of water and allowed to recover overnight. The next morning, a blood sample was taken with minimal disturbance to the animal and the caiman was then restrained and a second dermal bone sample taken with local anesthesia within about 5 min of capture. Blood pH and total plasma $CO_2$ were analyzed, and bone and remaining plasma were stored frozen for later analysis.

The following morning (2 days after surgery), a blood sample was taken and analyzed for pH, total $CO_2$, $P_{O_2}$ (partial pressure of oxygen) and hematocrit, and a plasma sample was saved for lactate analysis. The caiman was then submerged without access to air for 30–60 min. At the end of the submergence period, before the resumption of breathing, blood samples were taken and analyzed as described above. The water level was then lowered to permit breathing and additional blood samples were taken for analysis at 30 min, 60 min and 120 min of recovery. After the final blood sample, the caiman was killed with an overdose of anesthesia (thiopentothal), and multiple samples of dermal bone were collected from the mid-dorsal trunk region and from the dorsal surface of the neck. In addition, in six animals, a section of femur was also removed. One of the animals served as a control and was not submerged. After its final blood sample, it was anesthetized and bone samples from a minimally disturbed animal were obtained. All bone samples were immediately frozen and were analyzed later for lactate, total $CO_2$, % water, % ash and ash mineral composition ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ and inorganic P).

### *Blood analysis*

Blood pH and $P_{O_2}$ were measured using Radiometer BMS Mk 2 thermostatted near the animals' body temperatures of 26–28°C. Plasma total $CO_2$ concentration was measured with a Cameron chamber (Cameron, 1971). $P_{CO_2}$ was calculated using the Henderson–Hasselbalch equation. Hematocrit was measured in capillary tubes centrifuged (10 000 ***g***) for 6 min with a Fanem (São Carlos, Brazil) hematocrit centrifuge.

### *Bone sample preparation and analysis*

Bone samples (dermal bone and femur) were prepared by removing all skin, muscle and connective tissue with a razor blade. Samples for water content analysis were immediately weighed and dried to constant mass in an oven. Samples for determination of solute composition were re-stored frozen until analysis. In preparation for analysis, samples were ground to powder at liquid $N_2$ temperature using a Freezer Mill (Certiprep Spex 6700) and dried in an oven. For lactate analysis, weighed aliquot parts of dry powder were incubated at room temperature in 0.73 mol $l^{-1}$ trichloroacetic acid (10 ml $g^{-1}$) to extract the lactate. Lactate was analyzed in bone extracts and in plasma (with suitable dilution where necessary) using lactic oxidase/peroxidase colorimetric assay (Sigma Procedure 735). Absorbances were read at 540 nm using a spectrophotometer (Model Ultrospec 2000; Pharmacia Biotech, Cambridge, UK).

For total $CO_2$ determination, weighed aliquot parts of dermal bone powder (~0.4 g) were incubated, with vigorous stirring, in 15 ml of 2 mol $l^{-1}$ HCl solution. To prevent loss of $CO_2$, the powder was added to the acid by rotating a side bulb containing the powder as previously described (Jackson et al., 1999). The evolved $CO_2$ was taken up by a measured flow of $CO_2$-free gas through the incubation flask and, after drying with Drierite, into a $CO_2$ analyzer (Model CD-3A; Applied Electrochemistry, Pittsburgh, PA, USA). The output from the analyzer was recorded and processed on a computer using Biopac Acknowledge MP100 hardware and software (Santa Barbara, CA, USA). Results are expressed as mmol $CO_2$ $g^{-1}$ dry mass). The analyzer was calibrated with a precision gas, the composition of which was verified by analysis with a Scholander 0.5 cc analyzer (Scholander, 1947).

For mineral determination, weighed aliquot parts of dry dermal bone powder were ashed in a muffle furnace at 450°C. Given the known water content of the bone, the resultant mass

change permitted calculation of % ash mass and % organic mass of the original fresh bone. Ash samples were dissolved in 2 mol $l^{-1}$ HCl (12 ml $g^{-1}$), and sodium and potassium were analyzed on this dilution using a flame photometer (Model 943; Instrumentation Laboratory, Lexington, MA, USA). The solution was diluted further to 60-fold with deionized water, and magnesium was measured using an atomic absorption spectrophotometer (Model 280; Perkin-Elmer, Norwalk, CT, USA). Finally, the solution was diluted to 1800-fold and analyzed for calcium using the atomic absorption spectrophotometer and for inorganic phosphorus using the Fiske and SubbaRow method (Sigma kit 670-A) and a spectrophotometer (Model 601; Milton Roy Spectronic, Rochester, NY, USA).

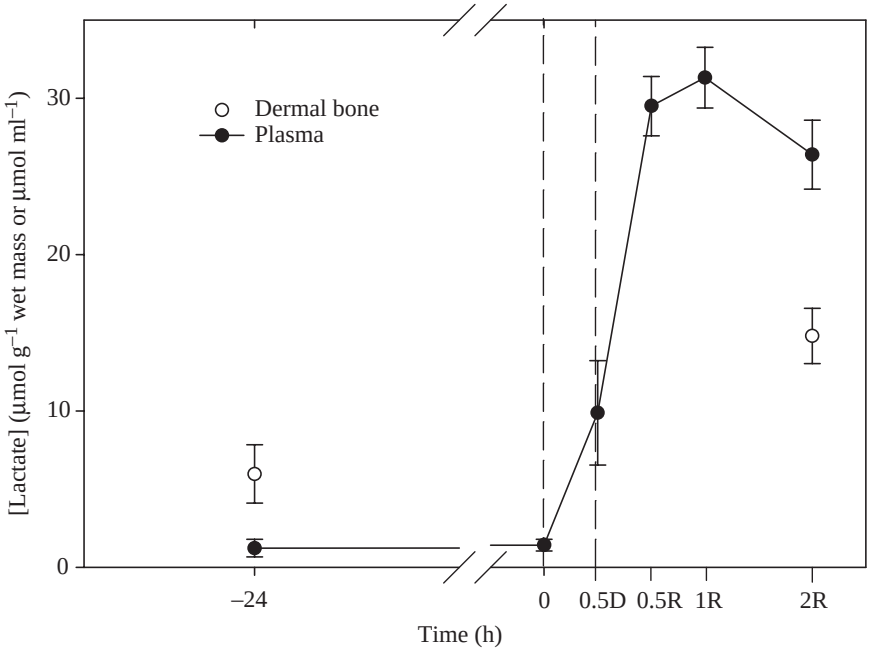


Fig. 1. Lactate concentration changes in plasma and dermal bone in the caiman. Samples were collected 1 day after surgery (–24 h) and then on the following day associated with a 0.5 h submergence (D) and 2 h recovery (R). Bone samples were taken immediately following surgery (not shown on graph), at –24 h and after 2 h recovery.

### In vitro *lactate uptake by dermal bone*

To test for equilibrated bone lactate uptake, four dermal bone samples from each of five caimans were first incubated overnight in buffered saline solution without lactate (125 mmol $l^{-1}$ NaCl, 10 mmol $l^{-1}$ $Na_2HPO_4$, titrated to pH 7.8 with 1.0 mol $l^{-1}$ NaOH). One sample from each animal was removed the following morning and stored frozen. The remaining samples were then placed in a high lactate solution (29 mmol $l^{-1}$ lactic acid, 95 mmol $l^{-1}$ NaCl, 10 mmol $l^{-1}$ $Na_2HPO_4$, titrated to pH 7.0 with NaOH). Additional samples from each animal were taken after 2 h, 8 h and 24 h incubation in this solution. During incubation, the bone pieces rested on a plastic screen submerged in the solution, which was continuously mixed with a magnetic stirrer and held at room temperature (~25°C). They were processed and analyzed for lactate concentration as described above. Based on the results of this trial, a second incubation of four bone samples, depleted of lactate as before, was carried out for 48 h and analyzed for lactate.

### *Statistics*

Values are expressed as means ± S.E.M. except where noted. Comparison between groups was made using *t*-tests, either paired *t*-tests for comparing control and recovery lactate levels in dermal bone or standard *t*-tests for comparing recovery lactate concentrations in dermal bone and femur. Blood gas and acid–base changes during and after submergence were tested by one-way analysis of variance (ANOVA) using the pre-dive values as controls. Differences were considered significant at $P<0.05$.

## Results

### *Plasma and bone lactate data*

Plasma lactate levels were elevated after surgery as a result of the struggling involved in capture. The mean value for seven animals was 16.3±2.8 μmol $ml^{-1}$. An eighth animal that was in a small tank and was captured with minimal stress had a post-surgical lactate of only 1.6 μmol $ml^{-1}$. The next day, mean plasma lactate from the same eight animals was 1.2±0.5 μmol $ml^{-1}$. Post-surgical lactate concentration of dermal bone for the same animals was 10.6±1.2 μmol $g^{-1}$ wet mass. On the following day, dermal bone lactate had decreased to 6.8±2.0 μmol $g^{-1}$ wet mass. A control animal that was given 2 days to recover and was anesthetized fully prior to sampling had a bone lactate concentration of 2.6 μmol $g^{-1}$ wet mass and a plasma value of 0.5 μmol $ml^{-1}$.

Because of the unpredictability of the response of the caimans to forced submergence, blood acid–base changes were not uniform in this study. One of the eight animals did not recover from submergence, although its lactate level was not elevated as much as that of four of the others, and no data from this animal are reported. In two other animals, plasma lactate levels did not rise above 9 μmol $ml^{-1}$ throughout the experiment and their dermal bone lactate levels were correspondingly low (mean, 8.0 μmol $g^{-1}$ wet mass). The remaining five animals responded similarly, with large increases in plasma lactate that reached peak values during the recovery period. The plasma and dermal bone lactate data from these five animals are shown in Fig. 1. Plasma lactate rose only moderately during submergence and then peaked at 31.2±1.9 μmol $ml^{-1}$ 1 h after the resumption of breathing. Bone lactate concentrations at the end of recovery were increased to 14.8 μmol $g^{-1}$ wet mass, which was significantly

Table 1. *Blood gas and acid–base data for three caimans submerged for 30 min and recovered for 120 min*

| | Pre-dive | 30 min dive | 30 min recovery | 60 min recovery | 120 min recovery |
|---|---|---|---|---|---|
| Blood pH | 7.580±0.032 | 7.134±0.129* | 6.990±0.048* | 7.021±0.047* | 7.119±0.027* |
| Blood $P_{O_2}$ (kPa) | 13.6±0.1 | 1.1±0.3* | 13.9±0.4 | 13.5±0.1 | 12.0±0.7 |
| Blood $P_{CO_2}$ (kPa) | 3.26±0.07 | 7.31±1.70 | 2.08±0.81 | 2.02±0.87 | 2.71±0.67 |
| Plasma $HCO_3^-$ (mmol $l^{-1}$) | 22.0±1.6 | 18.2±0.9 | 3.8±1.3* | 3.9±1.6* | 6.8±1.6* |
| Plasma lactate (mmol $l^{-1}$) | 1.9±0.4 | 12.1±3.5* | 29.6±1.6* | 29.4±0.4* | 23.9±0.8* |

Values are means ± S.E.M. for three animals.
*Significantly different from pre-dive value ($P$<0.05).

($P$<0.02) higher than the control values of the previous day. The increase after diving was also statistically significant if the two animals with low post-diving lactate are included in the analysis. Femur lactate was measured on four animals with high plasma lactate levels and on two animals with low plasma lactate levels. The mean value in the high lactate animals was 9.2±0.7 μmol $g^{-1}$ wet mass, which is significantly lower ($P$<0.02) than the dermal bone lactate from the same animals (13.0±0.9 μmol $g^{-1}$ wet mass). The femur lactate concentrations of the two animals with low plasma lactate were 1.9 μmol $g^{-1}$ wet mass and 1.2 μmol $g^{-1}$ wet mass.

### *Blood gas and acid–base data*

Of the five animals depicted in Fig. 1, we obtained complete blood data on only three. The results from these animals are summarized in Table 1. The results from the other two caimans were similar but incomplete. During the dive, the caimans developed a combined respiratory and lactic acidosis, but the fall in pH was minimized by the relatively small rise in lactate. Following the dive, lactate rose significantly, worsening the lactic acidosis, but $P_{CO_2}$ fell as the animals ventilated their lungs. Plasma [$HCO_3^-$] fell predictably as lactate rose during and following submergence, and, like lactate, recovered slightly by 120 min.

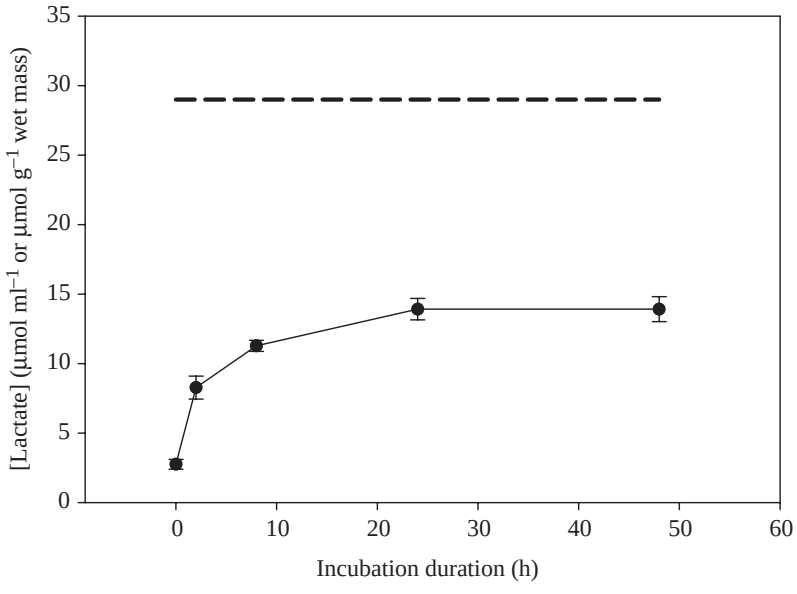


Fig. 2. Equilibration of individual osteoderms in a salt solution containing 29 meq. $l^{-1}$ lactate (shown by broken line). Values at each time point are means ± S.E.M. for 4–5 samples.

### In vitro *bone equilibration with lactate*

Dermal bone was almost 80% equilibrated with the bathing solution lactate at 8 h and was fully equilibrated by 24 h (Fig. 2). The final concentration of lactate in bone was 13.9 μmol $g^{-1}$ wet mass, approximately 50% of the solution lactate (29 mmol $l^{-1}$).

### *Dermal bone composition*

The composition of the osteoderms (Table 2) was generally similar to that of vertebrate bone, with the predominant components being calcium and phosphorus.

## Discussion

This study has demonstrated that lactate is sequestered into bone of the broad-nose caiman, *Caiman latirostris*, following capture from the holding pen and following a short period of forced submergence. The uptake, per g of bone, was greater in the osteoderms than in the femur. This confirms that the observed sequestration by turtle shell and bone is not peculiar to this animal but is very likely a property shared by vertebrate bone generally. In experiments in which turtles were submerged in anoxic water at 3°C for 4 months (Jackson et al., 2000), uptake was not significantly different between skeletal long bone (an endochondral bone) and shell (a largely dermal bone). The difference we observed in the caiman between osteoderm and femur may therefore be due to the short time course of the experiment and slower uptake by long bone. In the post-anoxic turtle, the lactate uptake mechanism plays a major role in storing and buffering the lactic acid produced during anoxia; at 3°C and 10°C, the shell contained over 40% of the total body lactate at the end of anoxic submergences lasting for 90 days and 9 days, respectively (Jackson, 1997). At 20°C, after 6 h anoxia, turtle shell held about 30% of the lactate.

Caiman bone mass is significantly less than in the

Table 2. *Composition of caiman dermal bone*

| $H_2O$ (% wet mass) | Ash (% wet mass) | Organic (% wet mass) | $[CO_2]$ (μmol $g^{-1}$ dry mass) | $[Na^+]$ (μmol $g^{-1}$ ash) | $[K^+]$ (μmol $g^{-1}$ ash) | $[Ca^{2+}]$ (μmol $g^{-1}$ ash) | $[Mg^{2+}]$ (μmol $g^{-1}$ ash) | $[P_i]$ (μmol $g^{-1}$ ash) |
|---|---|---|---|---|---|---|---|---|
| 30.5±3.8 | 40.2±1.6 | 29.3±1.6 | 0.93±0.05 | 420.6±16.3 | 29.0±5.3 | 10 026±646.5 | 204.0±13.0 | 5715±223.1 |
| (72, 9) | (10, 8) | (10, 8) | (6, 6) | (10, 8) | (10, 8) | (10, 8) | (10, 8) | (10, 8) |

Values are means ± S.D. Numbers in parentheses are *n*, *N* (number of samples analyzed, number of animals). Note that variance is expressed as S.D. based on *n*.

turtle. Based on allometric estimates (Calder, 1984), caiman skeleton is ~12% of its body mass and we estimate that osteoderms probably account for no more than 2% of body mass, although no thorough determination was attempted in this study. The smaller bone mass proportionately reduces the potential importance of the structure as a lactate repository, and we estimate, based on measured lactate concentrations in plasma and bone, that bone lactate accounted for only 5–10% of total lactate in the caiman. Even this relatively small amount, however, relieves the body fluids of an additional acid load and exploits the large buffering capacity of bone.

The caiman bone also had a relatively short time available for lactate exchange with the circulating blood. Assuming that blood flow to bone was limited during submergence, only the 2 h recovery period was available for lactate uptake. Nonetheless, this relatively short period was apparently long enough to permit full equilibration with the extracellular fluid. This is based on the *in vitro* incubation data, which demonstrate that osteoderm lactate concentration at equilibrium (expressed as μmol $g^{-1}$ wet mass) was about 50% of the bathing solution concentration (expressed as μmol $ml^{-1}$). This was the approximate ratio of bone to plasma lactate concentrations at the end of the recovery period. It should not be assumed that at equilibrium the concentrations expressed in these units will be equal. Measured water content of osteoderms was only 30% of bone mass, so equilibrium of dissolved lactate within only the aqueous phase would predict a much lower bone value when expressed per mass of wet bone. As in an earlier analysis of turtle shell and long bone (Jackson et al., 2000), we assume that much of the bone lactate exists in combination with calcium. A rapid equilibration between blood and osteoderms may also explain the relatively high bone lactate values in the 24 h pre-dive control samples. Because these samples were obtained after capturing and restraining the animals from their experimental tanks, circulating lactate levels probably increased, although we did not measure plasma lactate after this procedure to verify this.

The slow equilibration with lactate of osteoderms incubated *in vitro* can be explained by the absence of blood perfusion in the *in vitro* bone. In the animal, osteoderms are perfused by superficial arteries that run along their ventral surfaces, and a complex internal network of canals arising from these arteries indicates ‘a rich vascular supply in the living animal’ (Seidel, 1979). Presumably, this blood supply enabled lactic acid to rapidly enter these structures and exploit their buffering potential. The lower lactate in femur may be due to lesser perfusion of these bones and slower equilibration.

Our observation that lactate accumulates in a variety of mineralized tissues – vertebrate endochondrial and dermal bone, snail shell and crayfish carapace – suggests that a common chemical process is responsible. Our working hypothesis is that lactate equilibrates with the extracellular space of the mineralized structure by diffusion, which may or may not be facilitated by a carrier, and then further equilibrates with $Ca^{2+}$ to form a calcium–lactate complex as has previously been described in the plasma of turtles (Jackson and Heisler, 1982). Based on *in vitro* studies of turtle shell (Jackson et al., 1999), we also believe that $CaCO_3$ is the source of the $Ca^{2+}$ and that the carbonate fraction buffers the associated proton of lactic acid. Interestingly, the crayfish carapace, although largely composed of $CaCO_3$, does not accumulate lactate any more effectively than does vertebrate bone, despite the relatively small fraction of $Ca^{2+}$ associated with carbonate in the vertebrate bone.

The role of osteoderms in crocodilians as sources of buffering in acute acid–base disturbances adds an additional possible function for these structures. Seidel (1979) suggested heat exchange as a primary function, although he also recognized their use as protective armor and as anchors for insertions of epaxial muscles. The role in heat exchange would also be favored by the generous blood supply and would require that this flow could be altered to meet changing demands for heat loss or heat conservation.

The mineral composition of the osteoderms generally resembles that of vertebrate bone, with the predominant elements, calcium and phosphorus, in a ratio of 1.75. This is slightly above the ratio typical of mammalian hydroxyapatite (1.67) but may be due to a higher $CO_2$ content of dermal bone than has been reported for mammalian bone (Biltz and Pellegrino, 1969). $CO_2$ presumably exists in the bone as carbonate, associated primarily with calcium. This additional calcium can help account for the higher calcium:phosphorus ratio.

The blood data collected on the caimans conform with the classic diving reflex (Scholander, 1940) and specifically with studies on enforced submergence in another crocodilian, the American alligator *Alligator mississippiensis* (Andersen, 1961). A hallmark of the diving reflex is the retention of lactate in muscle until resumption of breathing, when a sharp increase in circulating lactate occurs. The increased lactate coincides with hyperventilation, which lowers blood $P_{CO_2}$ and limits the

lactic acid-induced acidemia. Our study exhibited these properties, although the duration of the diving period was short compared with earlier studies on the alligator due to the onset of struggling by the caimans. When voluntarily submerged and quiescent, these animals can remained submerged for much longer periods.

We believe that the responses we induced by capturing and submerging the caimans in this study are very relevant to the normal behavior of these animals, however. Caimans capture their prey and overcome them by a violent underwater struggle that must produce severe lactic acidosis. The contribution made by osteoderms and skeleton to buffer and sequester some of this lactate may contribute to their success in these struggles and to their recovery from them.

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