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The role of mineralized tissue in the buffering of lactic acid during anoxia and exercise in the leopard frog *Rana pipiens*

Daniel E. Warren* and Donald C. Jackson

Brown University, Department of Molecular Pharmacology, Physiology and Biotechnology, Box G, Providence, RI 02912, USA

*Author for correspondence (e-mail: Daniel_E_Warren@brown.edu)

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Summary

To evaluate the role of mineralized tissues of the leopard frog in buffering acid, we analyzed the composition of femur and auditory capsule, the latter of which encloses a portion of the endolymphatic lime sacs, and investigated the extent to which these tissues are involved in buffering lactic acid after 2.5 h of anoxia and 10–19 min of strenuous exercise at 15°C. We analyzed the following tissues for lactate: plasma, heart, liver, gastrocnemius muscle, femur, auditory capsule and carcass. Plasma [Ca^{2+}], [Mg^{2+}], [inorganic phosphate (P_i)], [Na^+] and [K^+] were also measured. Femur Ca^{2+}, P_i and CO_3^{2-} compositions were similar to bone in other vertebrates. Auditory capsule had significantly more $CaCO_3$ than femur. Lactate was significantly elevated in all tissues after anoxia and exercise, including femur and auditory capsule. Anoxia increased plasma [Ca^{2+}], [Mg^{2+}], [P_i] and [K^+] and had no effect on plasma [Na^+]. Exercise increased plasma [Mg^{2+}], [P_i] and [K^+] and had no effect on plasma [Ca^{2+}] or [Na^+]. The skeleton and endolymphatic lime sacs buffered 21% of the total lactate load during anoxia, and 9% after exercise. The exact contribution of the entire endolymphatic sac system to lactate buffering could not be determined in the present study. We conclude that the mineralized tissues function as buffers during anoxia and exercised induced lactic acidosis in amphibians.

Key words: anoxia, bone, buffering, Ca^{2+}, exercise, lactic acid, leopard frog, *Rana pipiens.*

Introduction

Exhaustive exercise and environmental hypoxia or anoxia lead to metabolic acidosis in amphibians, characterized by acidemia, decreased plasma bicarbonate and increased blood and tissue lactate concentrations (Boutilier et al., 1997; McDonald et al., 1980; Toews and Boutilier, 1986; Wegener and Krause, 1993). Traditionally, in these and most other vertebrates, plasma HCO_3^- is thought to be the principal extracellular buffer during metabolic acidosis. More recently, mineralized tissue has been shown to be important in buffering lactic acid during anoxic submergence in reptiles, specifically the skeleton and shell in painted turtles (Jackson, 2000) and skeleton and osteoderms in caiman (Jackson et al., 2003).

Reptile bone buffers lactic acid in several ways. It functions as a lactate ‘sink’ because it has low endogenous lactic acid production and, therefore, lactic acid produced by other tissues and distributed to the extracellular fluid can accumulate in the bone. When lactate accumulates to concentrations greater than calculated from the tissue water, the bone is said to sequester lactate, which is most likely by complexing to calcium in the bone. Vertebrate bone also contains significant quantities of calcium carbonate, which when liberated, chemically buffer protons generated by glycolysis. The resultant carbon dioxide from this chemical reaction diffuses into the surrounding water while the remaining calcium accumulates in the extracellular fluid and can reach high concentrations, especially at 3°C after 3 months of anoxic submergence in painted turtles (~100 mequiv l^{-1}; Jackson, 2002).

Anuran amphibians also possess significant calcium carbonate deposits in their endolymphatic system (Whiteside, 1922). Although restricted to the inner ear in most vertebrates, this system is large and extends down the length of the vertebral column in anuran amphibians. The exact function of the large endolymphatic system in frogs is not known. Proposed hypotheses include the protection of the spinal ganglia, as an endolymph reservoir when pressures in the auditory labyrinth are high, or as an aid in sound transmission (Simkiss, 1967). After he had observed that Ca^{2+} excretion increased during environmental hypercapnia, Simkiss (1968) proposed that the frog endolymphatic system helps maintain acid–base homeostasis by releasing $CaCO_3$. This hypothesis was further supported in a subsequent study (Tufts and Toews, 1985) in which hypercapnia increased plasma Ca^{2+} concentrations in toads. These investigators estimated that half of the observed compensatory HCO_3^- response must have

come from internal $CaCO_3$ stores because the levels could not be accounted for solely by uptake across the skin.

Our purpose was to determine whether the skeleton and endolymphatic system of the leopard frog *Rana pipiens* play roles in buffering lactic acid accumulated during anoxic submergence and strenuous exercise. There are no previous reports of lactate accumulation in mineralized tissue as a result of anoxia in an amphibian, or following exhaustive exercise in any vertebrate. Although the leopard frog is not likely to experience anoxia in its natural environment, it is very likely to accumulate extreme levels of lactic acid due to lesser degrees of oxygen lack during winter hibernation (Donohoe and Boutilier, 1999). Therefore, to study the frogs' response to anoxia is useful for determining a role for mineralized tissue responses in buffering lactic acid, which we assessed by measuring lactate accumulation in the mineralized tissues and comparing it to the lactate concentrations in other tissues of the frog. We assessed the skeletal contribution as sources of chemical buffering by measuring the concentrations of their major ionic constituents in plasma. Because it was easy to dissect rapidly, we sampled the entire frog auditory capsule, a part of the skull that encloses the membranous labyrinth and a portion of the endolymphatic sac (Fig. 1; Whiteside, 1922). The mineral compositions of femur and auditory capsule were analyzed for comparisons with bone of other vertebrates. We also incubated frog femur in 30 mmol l^{-1} lactate solutions at pH 8.0 and 7.3 for 6 and 24 h to ascertain the capacity for lactate uptake in frog bone.

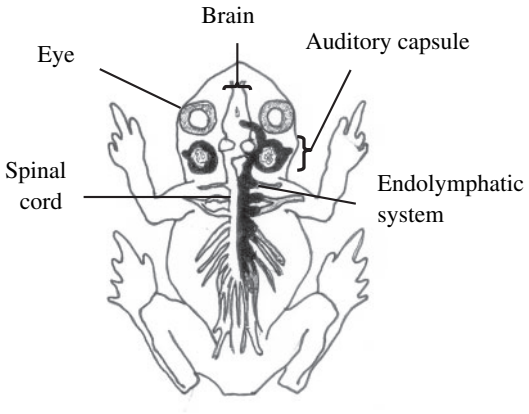

Fig. 1. Dorsal view of a frog depicting the location of the auditory capsule. The endolymphatic system (region shaded black) is omitted from the left side. (Modified from Whiteside, 1922.)

Materials and methods

Experimental animals

Leopard frogs *Rana pipiens* Schreber 1782 (body mass 26.9–101.9 g), were obtained from Lemberger (Oshkosh, WI, USA). Frogs used in the anoxia, exercise and mineral composition experiments were held at 15°C for 3–5 days until used in experiments. Frogs whose femurs were used in the bone incubation experiments were held at 6°C for 2–3 weeks until killed. The animals were held in Providence tapwater (Ca^{2+}=17.3 p.p.m., Mg^{2+}=0.6 p.p.m., Na^{+}=10.0 p.p.m., K^{+}=0.8 p.p.m.) and were not fed during the course of the experiments. All parts of this experiment were approved by the Brown University Institutional Animal Care and Use Committee (IACUC)

Determination of bone and auditory capsule Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and P_i concentrations

The ionic compositions of frog bone and auditory capsule were determined. Frogs were pithed and their femurs (N=1×5 animals) dissected from skeletal muscle. Auditory capsules (N=2×5 animals), formed by the prootic and exoccipital bones of the skull, were sampled by bisecting the skull mid-sagitally and dissecting them from surrounding muscle and the squamosal bone on the lateral edge of the skull. Femur and auditory capsules were dried for 2–3 days at 87°C and ground to a powder under liquid nitrogen (Spex 6700, Freezer Mill, Metuchen, NJ, USA). The powder was placed in porcelain vials and heated to 475°C for 2 days to burn away all organic material. The resultant ash was dissolved in 12 vol. 2 mol l^{-1} HCl and analyzed for Na^{+} and K^{+} using flame photometry (IL model 943; Lexington, MA, USA). The HCl was further diluted with 5 vol. of deionized water (60 vol. total) and analyzed for Mg^{2+}. A portion of this solution was further diluted with 30 vol. of deionized water (1800 vol. total) and analyzed for Ca^{2+} and inorganic phosphate (P_i; see below for the details of the analysis). Atomic absorption spectrophotometry (Perkin-Elmer model 280, Boston, MA, USA) was used to measure Ca^{2+} and Mg^{2+} levels and a standard kit for P_i (Kit 670 Sigma, St Louis, MO, USA).

Determination of bone and auditory capsule CO_3^{2-}

A known amount (0.02–0.1 g) of bone or auditory capsule powder (obtained as described above) was introduced into a flask containing 15 ml 2 mol l^{-1} HCl through which humidified nitrogen gas was passed (~240 ml min^{-1}; Jackson et al., 1999). Carbon dioxide, generated as the result of CO_3^{2-} titration, was then carried by the nitrogen gas through a drying column (Drierite, Xenia, OH, USA) and then through a CO_2 analyzer (AEI, model CD-3A, Pittsburgh, PA, USA). The output from the carbon dioxide analyzer was recorded on a laptop computer using a data acquisition system (BIOPAC MP100, Goleta, CA, USA) and analyzed using software (Acqknowledge, BIOPAC, Goleta, CA, USA). The volume of CO_2 generated was calculated by the following:

Vol. CO_2 generated =
(Average %CO_2 × Time averaged × N_2 flow rate) / 100

All volumes were corrected to STPD and converted to mmoles CO_2, assuming the constant 22.26 ml per mmole CO_2 (Cameron, 1989). One mole of CO_2 was assumed to be derived from one mole of CO_3^{2-}.

Anoxia and exercise experimental protocol

The anoxia and exercise experiments were carried out at

15°C. Frogs were randomly chosen as controls, anoxic or exercised.

Control frogs (*N*=6) were placed individually into darkened, 1-liter containers (diameter=11 cm, height=12 cm) containing 0.5 l aerated water. The next day, 5 ml of a buffered 0.1 g ml^{-1} MS222 solution (buffered to pH 7.0 with 1 mol l^{-1} NaOH) were added to one of the containers. After 20 min, the anesthetized animal was removed, pithed, weighed and sampled as described below. The remaining frogs were similarly sampled in turn. Control frogs were treated in this manner because preliminary experiments in which we pithed frogs without anesthesia showed significant lactate accumulation in their tissues and it was our intention to sample the tissues with tissue lactate contents as low as possible.

Anoxic frogs (*N*=6) were placed in a water-filled 15 cm×21 cm×43 cm (W×H×L) aquarium vigorously bubbled with nitrogen gas for at least 1 h prior, in order to displace any dissolved oxygen. A plastic mesh screen was placed just below the surface of the water to prevent access to air. An additional acrylic lid covered all but a small part of the water's surface to allow for nitrogen gas to escape. Vigorous bubbling of the nitrogen gas was continued throughout the submergence. After 2.5 h, the animals were removed, pithed, weighed and sampled as described below. Anoxic frogs were not anesthetized because the animals were quiescent, but still alive, by the end of anoxic submergence and did not struggle during the pithing. The quiescence has been observed in previous studies of anoxia in frogs (Wegener and Krause, 1993).

Exercised frogs (*N*=6) were placed in a 15 cm×21 cm×43 cm (W×H×L) aquarium with 2–3 cm deep water and chased with a metal rod until they were incapable of further burst swimming (10–19 min). The animals were removed, pithed, weighed and sampled as described below. Exercised frogs were not anesthetized because doing so would have required an additional 20 min after the exercise period ended and would have prevented us from examining the immediate post-exercise condition of the animal. The animals struggled little, if at all, during the pithing because they were exhausted from the exercise and so additional lactate production after the exercise period was unlikely.

Tissue sampling

All sampling was performed in a cold-room at 3°C to minimize changes in tissue metabolites during dissection. After pithing, a mid-line incision on the ventral side of the frog was made and blood (0.1–0.4 ml) was sampled *via* cardiac puncture and placed on ice. Heart, liver and gastrocnemius muscle were quickly sampled and flash-frozen in clamps cooled using liquid nitrogen. The femur (1 per animal) was cleared of skeletal muscle, its shaft cut lengthwise and the marrow removed. The bone fragments were quickly freeze clamped. Auditory capsules (2 per animal) were sampled by completely bisecting the skull mid-sagitally and cutting them free of surrounding muscle and bone (orbital and maxilla), and were freeze clamped. The remaining carcass was clamped and frozen by immersion in liquid nitrogen.

The blood samples were kept on ice for 1–2 h until they could be centrifuged for 3 min at 9300 ***g***. Previous experience in our laboratory has shown that the lactate and ionic composition of the plasma do not change when treated in this manner. The plasma was transferred to another vial and stored at –25°C until analyzed for lactate (Kit 735-10; Trinity Biotech, St Louis, MO, USA), Ca^{2+} and Mg^{2+} (atomic absorption spectrophotometry; Perkin-Elmer model 280), Na^{+} and K^{+} (flame photometry; IL model 946, Lexington, MA, USA) and P_i (Kit 670, Sigma). The frozen tissues were kept on dry ice for 1–2 h before they could be transferred to a deep freeze, where they were stored at –75°C until analyzed for lactate.

Lactate analyses of tissues and plasma

Frozen heart, liver and gastrocnemius muscle (~200 mg) were homogenized in 1 ml ice-cold 0.6 mol l^{-1} perchloric acid using a Mini-Beadbeater 3110BX (Biospec, Bartlesville, OK, USA) using 1 mm glass beads for 3 min. The frozen carcass was homogenized in 4 vol. of ice-cold 0.6 mol l^{-1} perchloric acid using a Virtis Super 30 homogenizer (Gardiner, NY, USA). Frozen femur and auditory capsules were ground to a powder under liquid nitrogen (Spex 6700, Freezer Mill). The powder was incubated in 5 vol. of ice-cold 0.6 mol l^{-1} perchloric acid for 2 h on ice, vortexing every 20 min. Samples of all tissue homogenates were centrifuged at 9300 ***g*** for 3 min. [Lactate] was measured in the resultant supernatants and plasma using an enzymatic assay (Kit 735-10, Trinity Biotech).

Bone incubations

Twelve leopard frogs were euthanized with intraperitoneal injections of Beuthanasia®-D Special (Schering-Plough, Millsborough, DE, USA) and their femurs removed, cleaned of soft tissue including marrow, and frozen at –25°C until used in the incubations. One femur from each of the 12 animals was incubated in a beaker containing a solution of 0.8% NaCl, 10 mmol l^{-1} TES and 30 mmol l^{-1} lactate at pH 8.0, and the other femurs in an identical solution at pH 7.3. The volume of solution in each beaker was 80 ml. Six femurs were sampled from each beaker after 6 h and 24 h, frozen in liquid nitrogen and stored at –25°C until analyzed for lactate as described above.

Statistical analyses

Differences in the composition of femur and auditory capsule were determined using *t*-tests. Two-way multivariate analysis of variance (MANOVA) was used to determine whether tissue type and treatment affected lactate concentration. Least-squares mean (LSM) tests were used to determine whether treatment affected the plasma concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and P_i, and whether time and pH affected lactate uptake into bone in the incubation experiment. Student's *t*-tests were used to elucidate differences revealed by the MANOVA and LSM tests. All statistical analyses were performed using JMP 4.0 (SAS Institute, Cary, NC, USA).

Table 1. *Composition of femur and auditory capsule of the leopard frog* Rana pipiens

Tissue	Water (%)	Organic (%)	Ash (%)	CO_3^{2-} (mmol kg^{-1} dry tissue)	Composition (mmol kg^{-1} ash)				
					P_i	Ca^{2+}	Mg^{2+}	Na^+	K^+
Femur	54.3±0.1*	25.5±0.1*	20.2±0.1*	533±24*	6192±44*	9900±99*	288±4*	483±17	36.7±4.1*
Auditory capsule	70.0±0.1	18.3±0.1	11.7±0.1	1398±156	5256±132	10764±105	256±6	453±42	62.4±6.3

Values are means ± S.E.M. (N=5 for each tissue).
*Significant difference between the two tissues (P<0.05, t-test).

Results

Femur and auditory capsule composition

The compositions of femur and auditory capsule are summarized in Table 1. All of the inorganic components, except for CO_3^{2-}, are expressed as mmol kg^{-1} ash. CO_3^{2-} was measured on dry bone powder and is expressed as mmol kg^{-1} dry tissue.

Auditory capsule contained more water and less organic matter than femur. Femur ash contained slightly more P_i and more Mg^{2+} than auditory capsule. There was no difference in the amount of Na^+ between the two tissues. Auditory capsule ash contained more Ca^{2+} and K^+ than femur ash. Dry auditory capsule contained almost twice the CO_3^{2-} of dry femur.

Lactate distribution in normoxic controls

Tissue lactate concentrations from anesthetized, but otherwise undisturbed, animals are shown in Fig. 2 and were low in all tissues examined (means ranged 0.6–1.8 mmol kg^{-1}).

Lactate distribution during anoxia

Anoxic submergence at 15°C for 2.5 h significantly increased lactate concentrations above control levels in all tissues, including femur and auditory capsule (Fig. 2). Femur and auditory capsule lactate concentrations (15.0 and 13.2 mmol kg^{-1}, respectively) were similar to those in carcass (13.5 mmol kg^{-1}). Gastrocnemius muscle accumulated the most lactate (24.7 mmol kg^{-1}) and liver the least (9.0 mmol kg^{-1}), both of which were significantly different from the other tissues. Plasma and heart accumulated similar lactate concentrations (20.4 and 18.4 mmol kg^{-1}, respectively), which were significantly greater than all other tissues except gastrocnemius muscle.

Lactate distribution during exhaustive exercise

The mean time to exhaustion was 13.1±1.6 min (range=10–19 min) at 15°C. Lactate concentrations were significantly elevated relative to controls in all tissues, including femur and auditory capsule (Fig. 2). Femur lactate concentrations were similar to those in liver and auditory capsule and were significantly lower than the other tissues. Auditory capsule lactate concentrations were significantly less than in plasma, gastrocnemius and heart, but similar to all other tissues. Gastrocnemius muscle and plasma accumulated the most lactate (13.9 and 14.0 mmol kg^{-1}, respectively) and femur and liver the least (5.3 and 4.6 mmol kg^{-1}, respectively). The lactate concentration in plasma did not differ from that in heart (10.6 mmol kg^{-1}).

Plasma ion changes during anoxia and exhaustive exercise

Plasma ion concentrations during anoxia and exhaustive exercise are summarized in Figs 3 and 4. Plasma $[Ca^{2+}]$ increased significantly relative to controls after 2.5 h anoxia, but was unaffected by exhaustive exercise. Plasma $[Mg^{2+}]$ increased in both anoxic and exercise groups by 35% and 17%, respectively, and was significantly different across all groups. Plasma $[P_i]$ and $[K^+]$ increased significantly in both anoxic and exercised frogs but did not differ between the two groups. Plasma $[Na^+]$ was unaffected by anoxia or exercise.

Bone incubations

The results of the bone incubations are presented in Fig. 5 and show that frog bone sequestered lactate at pH 8.0 and 7.3. There was no significant interaction between pH and time

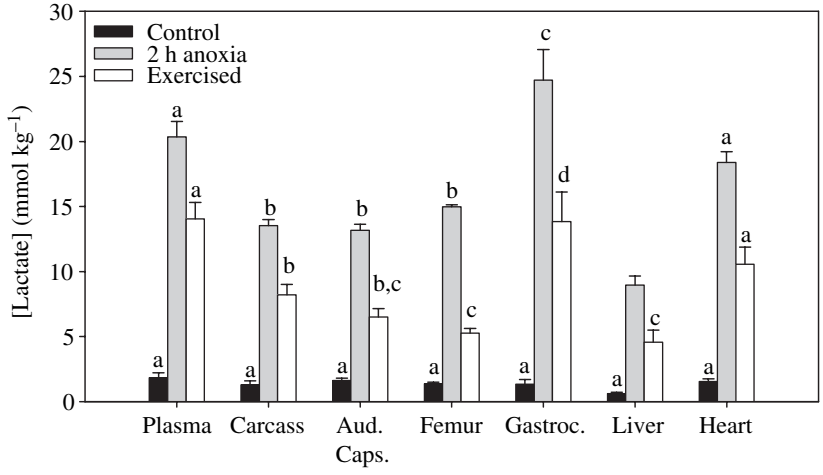

Fig. 2. Lactate concentration in plasma, carcass, auditory capsule (Aud. Caps.), femur, gastrocnemius muscle (Gastroc.), liver and heart in controls, after 2 h anoxia and after exhaustive exercise in leopard frogs at 15°C. Lactate concentrations after anoxia and exercise are significantly higher than controls for all tissues. Values are means ± S.E.M., N=5–6 per treatment for each tissue. Differences between tissues within a treatment are indicated by differing letters (two-way MANOVA, Student's *post-hoc t*-test)

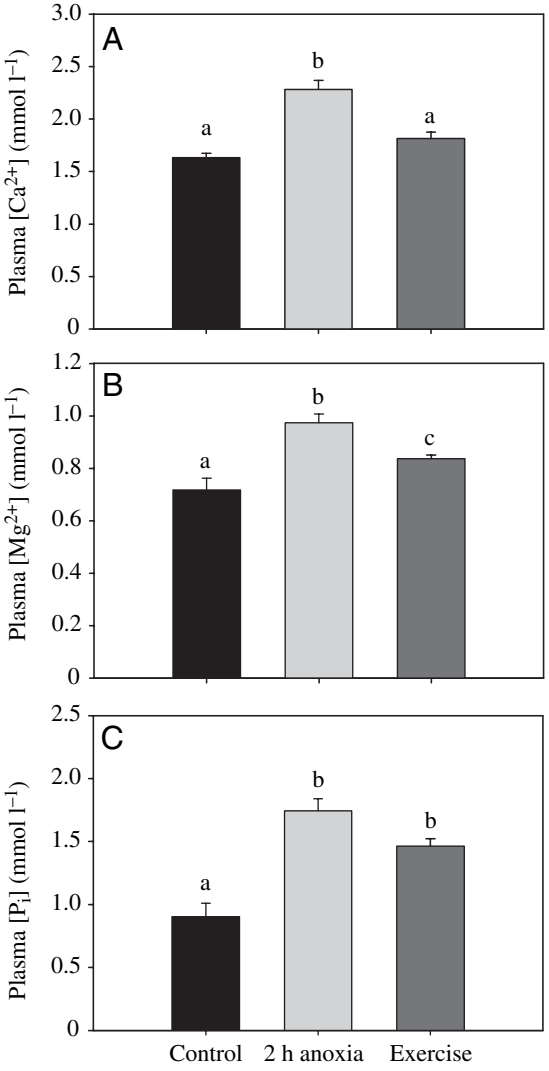

Fig. 3. Plasma concentrations of (A) Ca^{2+}, (B) Mg^{2+} and (C) P_i in controls, after 2 h anoxia and after exhaustive exercise in leopard frogs at 15°C. Values are means ± S.E.M., *N*=6 per treatment for each ion. Differences between treatments are indicated by differing letters (LSM, Student's *post-hoc t*-test).

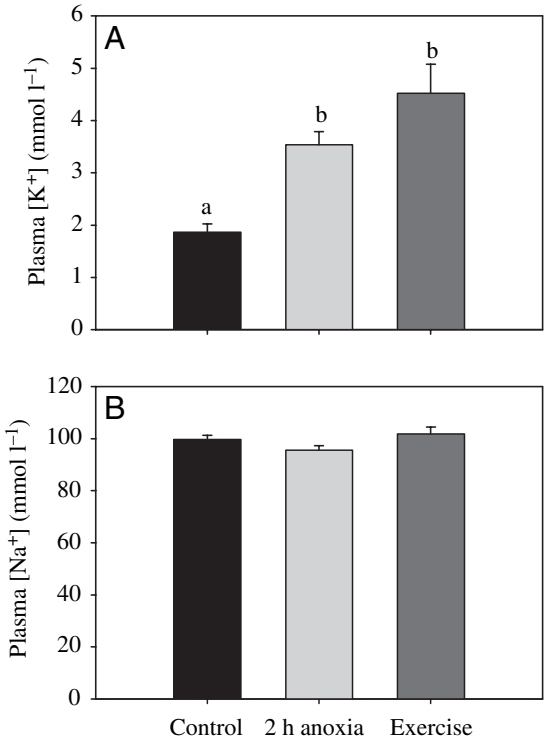

Fig. 4. Plasma concentrations of (A) K^+ and (B) Na^+ in controls, after 2 h anoxia and after exhaustive exercise in leopard frogs at 15°C. Values are means ± S.E.M., *N*=5 per treatment for each ion. Differences between treatments are indicated by differing letters (LSM, Student's *post-hoc t*-test).

(*P*=0.07), but time and pH, by themselves, had significant effects. Lactate concentrations of the solutions fell during incubation from 29.7 to 27.3 mmol l^{-1} at pH 7.3 and from 29.3 to 27.9 mmol l^{-1} at pH 8.0.

Discussion

This study extends previous observations on lactate uptake into bone during anoxia in other animals (Jackson, 2000; Jackson et al., 2003, 2001), but is the first report of uptake during anoxia in an amphibian and following a bout of exhaustive exercise in any vertebrate. We have demonstrated that the amphibian skeletal and endolymphatic systems accumulate lactate during both anoxic submergence and exhaustive exercise and can do so in as little as 10 min, the fastest time observed in any vertebrate examined. Based on changes in plasma $[Ca^{2+}]$, the skeleton and/or endolymphatic systems also functioned as sources of chemical buffer for the extracellular fluid during anoxic submergence, but not exercise, in our study. Our data further demonstrate that buffering of lactic acid is a general property of mineralized tissue.

The composition of frog bone in this study is similar to that in a previous analysis and to most other vertebrates (Biltz and Pellegrino, 1969). Frog femur contains less CO_3^{2-} than bone and shell of the extremely anoxia-tolerant painted turtle *Chrysemys picta bellii* (Jackson et al., 2000), and osteoderms of the broad-nosed caiman (Jackson et al., 2003). Auditory capsule has a $CaCO_3$ composition that is similar to that in the long bone and shell of the painted turtle *Chrysemys picta bellii* (Jackson et al., 2000) and greater than caiman osteoderm (Jackson et al., 2003).

The large $CaCO_3$ deposits in our analysis of auditory capsule are also consistent with the earliest descriptions of the endolymphatic lime deposits (Dempster, 1930; Whiteside, 1922) and indicate that we succeeded in sampling them with our protocol. This verification is important because (1) our sampling included the dermal bone encapsulating a portion of the endolymphatic lime sac, and (2) we used the same sampling technique in our lactate analyses after anoxia and exercise. The

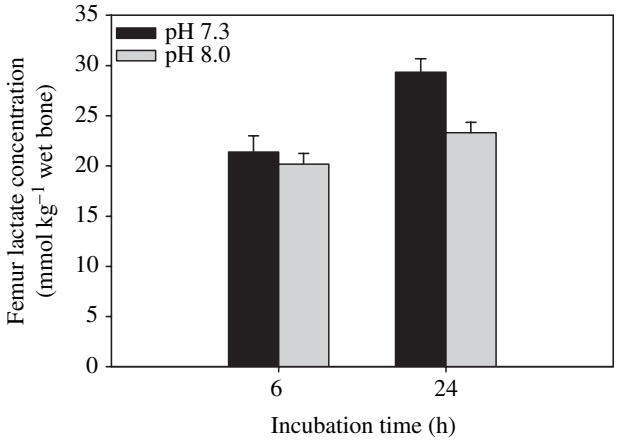

Fig. 5. Concentration of lactate (mmol kg^{-1} wet mass) in frog femurs incubated in 30 mmol l^{-1} lactate for 6 and 24 h at pH 8.0 or 7.3. Values are means ± S.E.M., N =6 per treatment at each time point. There was no significant time × pH interaction but there were separate pH and time effects (LSM, Student's *post-hoc t*-test).

sampled structure had a milky appearance that was consistent with earlier descriptions of the endolymphatic system.

Both exercise and anoxia increased lactate concentrations in all tissues examined to levels observed in previous studies (Andersen and Wang, 2003; Armentrout and Rose, 1971; Bennett and Licht, 1974; D'Eon et al., 1978; Donohoe and Boutilier, 1999; Hutchison and Turney, 1975; Warburton et al., 1989; Wasser et al., 1993, 1991; Wegener and Krause, 1993). With the exception of gastrocnemius and heart, plasma had a higher lactate concentration than the other tissues examined, as has also been observed in cold hypoxic frogs (Donohoe and Boutilier, 1999) and cold anoxic turtles (Jackson et al., 1996). High lactate levels in gastrocnemius and heart can be attributed to their intense activity during the exercise and during the initial period in the anoxia chamber. The frogs became quiescent about 30 min into the 2 h anoxia bout.

Higher extracellular than intracellular lactate concentrations have been reported during cold hypoxia in frogs by Donohoe and Boutilier (1999), who suggested that lactate is transported from locally anaerobic tissues, where it is produced, to locally aerobic tissues, where it can be oxidized or converted to glucose. In the anoxic frogs with no aerobic tissues, we propose that exporting lactate from the cells to the extracellular fluid (ECF) may better exploit the buffer capacity of the mineralized tissues, the skeleton and endolymphatic system.

Three pieces of evidence revealed by the present study suggest a potentially important role for the skeleton in buffering lactic acid after exercise and anoxia. First, the skeleton and auditory capsule accumulated significant amounts of lactate after both exercise and anoxia. The post-exercise lactate accumulation in frog bone is notable because it is the first of its kind to be reported and it occurred in as few as 10 min, a rate not seen before *in vivo* in any vertebrate. In the case of anoxia, the mineralized tissues accumulated more lactate than the liver and the lactate concentration in femur (15.0 mmol l^{-1}) was greater than what could be accounted for based on its water composition (54%) and the plasma lactate concentration (20.4 mmol l^{-1}), indicating that lactate was chemically bound to mineralized bone, most likely complexed with Ca^{2+}, as previously suggested to occur in painted turtle shell (Jackson, 2000). Although the post-exercise femurs and auditory capsules and the anoxic auditory capsules did not accumulate more lactate than predicted from their water contents, sequestration cannot be ruled out. This is especially true in the post-exercise femurs, which accumulated exactly what might be predicted from the water content and plasma [lactate]. The prediction is likely to be an overestimate because it assumes that all water is extracellular and in equilibrium with plasma.

Second, our *in vitro* incubation of frog femur demonstrates that significant sequestration of lactate is possible. These results reveal the accumulation that is possible at equilibrium, although the *in vivo* kinetics of exchange in these experiments did not permit full equilibration to occur. The *in vitro* bone lactate levels reached in the frog are similar to measurements on painted turtle (D.C.J., unpublished observation) and greater than published uptakes in caiman osteoderms (Jackson et al., 2003) and crayfish carapace (Jackson et al., 2001).

The third piece of evidence that frog bone contributes to the buffering of lactic acid is that plasma [Ca^{2+}] was elevated after anoxic submergence. We interpret this as the result of $CaCO_3$ release from mineralized tissues. It is unlikely that the changes in plasma [Ca^{2+}] were caused by hemoconcentration because plasma [Na^+] did not change. It is notable that an earlier study of anoxia in bullfrogs did not observe an increase in plasma [Ca^{2+}] (Warburton et al., 1989). However, the frogs in that study were paralyzed with succinylcholine, and lactate levels were only half of what we observed, suggesting that the severity of the acidosis may have been insufficient to demineralize bone.

The increase in plasma [P_i], during both anoxia and exercise is most likely derived from active muscle as a result of creatine phosphate hydrolysis during burst swimming and anoxic submergence (Wegener and Krause, 1993), rather than from bone. *In vitro* studies show that CO_3^{2-} and not P_i is the principle buffer anion released from both mouse calvariae (Bushinsky et al., 2002) and turtle shell powder (Jackson et al., 1999) when incubated in acid solutions.

The quantitative importance of the frog's skeleton in buffering lactic acidosis can be estimated by summing the fractions of the total lactate load that accumulated in the skeleton and that was buffered by $CaCO_3$ released from the skeleton and/or endolymphatic system. The basis for this calculation is the assumption that each mole of lactate accumulated in frog bone is accompanied by a proton, as is the case in turtle bone (Jackson et al., 1999). We assume that for each mole of Ca^{2+} released from the skeleton, a mole of CO_3^{2-}, which buffers 2 moles of H^+ derived from glycolyis, is also released. If a frog's wet skeletal mass is 16% of total body mass (D. E. Warren and D. C. Jackson, unpublished), then the percentage of the total lactate load contained within the

skeleton at the end of anoxia and exercise is 18% and 9%, respectively. Unfortunately, a similar calculation cannot be made for the endolymphatic system because we did not sample it entirely, although we assume it to be much smaller than the skeleton. If we assume that all the released Ca^{2+} distributes throughout the extracellular fluid and that the extracellular fluid volume is 26% of body mass (Thorson, 1964), then 3% of the total lactate load was buffered by CO_3^{2-} released from the skeletal or endolymphatic systems during anoxia. There is no evidence that the skeletal and endolymphatic systems release chemical buffers after exercise because plasma [Ca^{2+}] was not elevated. After summation, we estimate that 21% and 9% of the total lactate loads produced during anoxia and exercise, respectively, are buffered by mineralized tissue.

These estimates have several important implications that require more investigation. Although the quantitative contribution to lactic acid buffering is modest in exercise, the rapidity with which these mineralized tissues were recruited has not been observed previously. In addition, the amount and integrity of a frog's mineralized tissues may determine the severity of a lactic acidosis that an animal can tolerate. This might help to explain why frogs, although considered hypoxia tolerant, are anoxia intolerant. Under hypoxic conditions, lactic acid is produced in locally anaerobic tissue and moves into the extracellular fluid where it distributes to locally aerobic tissues to be oxidized, resulting in slower lactate accumulation and glycogen depletion rates (Donohoe and Boutilier, 1999). Although the extracellular distribution of lactate enables exploitation of the skeletal and endolymphatic buffers during anoxia, only a modest fraction of the lactate load is buffered, primarily because of the structures' small size relative to body mass. Therefore, only the lower rates of lactate accumulation that occur during hypoxia are sustainable while the higher rates during anoxia overwhelm the limited buffering capacity of the frog.

One intriguing conservation implication is that if mineralized tissues proved vulnerable to demineralization in an acidic environment, then the tolerance of frogs to anoxia or hypoxia and, possibly, anaerobic performance, could be compromised. Indeed, acidification of frog environments has been implicated as a cause of amphibian decline and low environmental pH has been shown to increase mortality rates in this species (Brodkin et al., 2003; Simon et al., 2002).

In conclusion, the leopard frog skeletal and endolymphatic systems contribute buffering during exercise by rapidly functioning as a sink for a small amount of the lactate load. Their buffering roles are more important during anoxia when these structures, especially bone, sequester a larger fraction of the lactate load as well as release chemical buffers. Future studies should investigate whether these systems are significant contributors to lactic acid buffering under the more ecologically relevant conditions of long-term hypoxia at cold temperatures, and determine how pH affects bone and lime sac demineralization in amphibians. Additional work should examine the relative contributions of skeleton and endolymphatic lime deposits to overall chemical buffer release. A better assessment of the size of the endolymphatic lime sacs is needed to determine their exact contribution to lactic acid buffering.

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